WILMS TUMOR

Cover image: Disruption of the differentiation of renal progenitor cells may result in Wilms tumor formation. See page 157, chapter 10 for details.

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Edited by

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Foreword

I have been lucky and privileged to work with the Société Internationale d'Oncologie Pédiatrique (SIOP) panel of pathologists for 40 years. During this time, I have seen steady and remarkable progress and success in the treatment of Wilms' tumour, with an increase in survival rates from around 50 to more than 90%.

Max Wilms, a German pathologist and surgeon, hardly realized that his thesis on "Mischgeschwulste der Niere" ("Mixed tumours of the kidney"; 1899) would link his name to the most common renal tumour in children and also turn out to be an example of successful multimodal treatment. He collected nine, mostly large, tumours from children aged 11 weeks to 11 years, which were described as round cells or myosarcomas with a content of epithelial structures in which he saw the confusing similarity to the embryonic kidney. He suspected that the component he termed "round cell sarcoma" represented tumour stem cells with the potential for differentiating into mesenchyme and epithelium and proposed its origin from the "kidney blastema" and extensively discussed oncogenesis compared with renal embryology.

In the first half of the 20th century, surgical excision was the only treatment often with a fatal outcome partly due to large tumour size. An early attempt (1916) to treat an inoperable tumour had shown that X-rays could shrink a tumour and it gave initial success, but the method was not commonly used. Slowly, surgery improved and saved some children with small tumours. A general breakthrough in treatment came with advanced surgery together with irradiation and chemotherapy as reported by Sidney Farber and his group (1956), resulting in a 2-year survival rate of 81%. The next step came with the creation and contribution of two major groups, which gave an enormous impact on treatment success. The National Wilms' Tumor Study (NWTS) began in 1969, and through national and international collaboration, it collected a large number of patients and ran several clinical and randomized trials aiming to optimize treatment for various risk groups and possibly also to identify genetic risk factors. This has led to using loss of heterozygosity of 1p and 16q to stratify patients in the current Children's Oncology Group Wilms' Tumor risk stratification protocol. A cornerstone right from the beginning of NWTS clinical trials was the work of the iconic pathologist Bruce Beckwith, who firmly related histopathology to prognosis and identified tumours with favourable or unfavourable morphology. This classification is still valid for tumours without upfront treatment as NWTS never adopted this mode until recently in some clinical settings. Among his enormous contributions, the documentation and clinical importance of nephrogenic rests must also be mentioned.

In Europe, a small group of dedicated French doctors started a paediatric oncology club in 1961, which in 1969 transformed to SIOP. At first a bilingual society, but with the intention

of becoming international, it soon had members from all over Europe, and today, with members from all over the world, it warrants the English name International Society of Pediatric Oncology ("SIOP" still used reflecting the origins). It must also be mentioned that SIOP is dovetailed with United Kingdom Children's Cancer Study Group (UKCCSG) and the German organization, The Society for Paediatric Oncology and Haematology (GPOH). They share committee and panel members, as well as general rules for treatment and results, with a hub for statistics in Amsterdam although having different national offices.

From the first study in 1971, preoperative treatment was given to children >6 months to reduce operative rupture by shrinking and encapsulating the tumour and thereby lowering the stage level. Initially, radiation was used, but in a subsequent study, it was shown that preoperative two-drug chemotherapy was as efficient as radiotherapy. This became the standard in the SIOP protocol for the treatment of renal tumours in children. An added bonus was that the responsiveness to chemotherapy was revealed by reduced tumour volume, as well as the extent of regression seen at the pathological examination. Regressive changes were a challenge for us in the pathology panel, which I joined in 1973. The dilemma was how to assign risk group or grade tumours based on the amount of regression due to chemotherapy and to relate it to the different viable components. The main issue during the first studies was to register the amount of all these elements, which later led to the three-tier risk classification in SIOP 9301 trial and study. This was updated to "the revised SIOP working classification" used in the latest study (SIOP 2001) with the important change of placing the blastemic subtype in the high-risk group. Staging was also necessary to adapt to regression, a deviation from pure anatomical grounds. Compared with the straightforward grading and staging of nonpretreated tumours, there are quantitative histological threshold values, which sometimes are difficult to interpret and make high demands on local pathologists and also make access to reference pathology important. The SIOP risk classification, however, has shown to be of significant value for distinguishing between low-, intermediate-, and high-risk tumours. The guiding light for all these trials and studies was not only to titrate the optimal amount and type of chemotherapy and irradiation but also to lower the intensity or exclude components when possible in defined risk groups to reduce toxicity but retaining cure.

Over time there has been an increasing demand to find biomarkers for those tumours that are resistant to chemotherapy, markers which are not obvious with conventional histopathology. After recognition of the mutation in the *WT1* gene led to intensive molecular research, this field has expanded at a pace which is beyond keeping up with for an old histopathologist without at least one foot in molecular research. This new constellation of clinically active doctors will be evident in the present issue. It is noteworthy that this research now focuses on normal kidney embryology to relate it genetically to Wilms' tumour development, exactly what Max Wilms also was doing with the help of a light microscope more than a hundred years ago.

In this book, you will find selected topics that cover the most recent developments spiced with some new findings in pathology, clinical management, and biological research in Wilms' tumour based on what has been achieved by a long row of hardworking devoted individuals in the large international collaborative groups. I have been fortunate to work with, and meet, most of these extraordinary persons. Some sadly passed away, some retired but most still active and found among the authors here. Enjoy their important effort!

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Preface

Wilms' tumor (WT), also called nephroblastoma, is a rare kidney cancer that is usually diagnosed in children under the age of 6. WT arises from nephrogenic rests, which are undifferentiated embryonic tissues retained after birth. At molecular level, in a proportion of patients, WT has been shown to be the result of aberrations in *WT1* gene, located on chromosome 11p13. In addition to being a risk factor for WT, germ line *WT1* aberrations can cause renal and extrarenal developmental abnormalities and predispose to other malignancies. In the past two decades, there has been a considerable improvement in our understanding of WT and *WT1*. This book brings together recently uncovered basic and clinical aspects of the burgeoning WT and *WT1* research under three sections: epidemiology and clinical aspects, biology, and *WT1* gene aberrations in other malignancies.

Section I provides a comprehensive guide to the epidemiology, diagnosis, management, and treatment of WT. Chapter 1 describes the morphology and differential diagnosis of WT. It presents a clear view of the common histological components of WT. While stage and histological subtypes are well-known prognostic factors for WT, age at diagnosis is also an independent risk factor for recurrence. Chapter 2 elegantly summarizes the clinical relevance of age at presentation in WT management. Chapter 3 provides a comprehensive review of the histopathology, genetics, and molecular biology of WT. Also, this chapter discusses how these changes influence the prognosis and differential diagnosis. The clinical features and surgical management of WT is discussed in chapter 4. Especially, this chapter emphasizes the necessity of a multidisciplinary approach for the effective surgical management of WT.

Bilateral WT represents 4–7% of all WT, typically presenting at a younger age than unilateral WT. The major challenge in the treatment of bilateral WT is the preservation of renal function. Chapter 5 gives an overview of the current status of management of bilateral WT. Extrarenal WT is a rare entity, which usually occurs in the retroperitoneum or inguinal region. Chapter 6 presents a comprehensive review of the challenges in diagnosis, histopathology, staging, treatment, and prognosis of extrarenal WT. In chapter 7, the authors share their experience on the use of preoperative transcatheter arterial chemoembolization combined with systemic chemotherapy for the management of unilateral advanced WT. Treatment of advanced cancers that have metastasized to distant parts, irrespective of the cancer type, continues to be a challenge. Dendritic cell-based immunotherapy has been presented as a viable treatment option in many cancers. In chapter 8, the authors present autologous dendritic cell vaccines for the treatment of WT

as a possible future option. Chapter 9 addresses the problem of chronic kidney disease and renal function in WT survivors.

Section II covers the biological aspects of WT and *WT1* under three headings. WT displays morphological and molecular characteristics that resemble early stages of kidney development. Therefore, a study of molecular pathways relevant to normal kidney development may provide insights into the events that drive WT. Based on this rationale, chapter 10 gives an overview of the link between Wnt signaling, microRNA biogenesis, and β-catenin in regulating kidney differentiation. Chapter 11 focuses on the transcriptional regulation of the human thromboxane A2 receptor gene by WT1. The prostanoid thromboxane A2 is implicated in neoplastic diseases. In humans, TXA2 signals through the T-prostanoid (TP)α and TPβ isoforms of the TP receptor, two structurally related receptors transcriptionally regulated by distinct promoters, Prm1 and Prm3, respectively, within the TP gene (*TBXA2R*). A particular focus is placed on the role of WT1 in the regulation of TPα expression through Prm1 in megakaryoblastic and endothelial cells of vascular origin and in prostate and breast carcinoma cells. Chapter 12 gives a comprehensive review of the inflammatory microenvironment of human WT with a comprehensive picture of various immune cells and inflammatory markers.

Section III focuses on the role of *WT1* in cardiac development, prostate cancer, glioblastoma, and minimal residual disease. *WT1* has been identified as a crucial player in cardiac development. Absence of *WT1* leads to major cardiac malformations, including incomplete formation of coronary vasculature, resulting in embryonic lethality. Chapter 13 describes the diverse and unique roles of *WT1* during heart development and disease. *WT1* is expressed in prostate cancer (PC) epithelial cells and regulates PC critical genes. *WT1* promotes metastatic disease by enhancing motility of PC cells with low-migratory and metastatic potential. While the mechanisms are multifactorial, chapter 14 focuses on how *WT1* interacts with vascular endothelial growth factor (*VEGF*) and androgen receptor to promote prostate cancer progression and metastasis. While WT1 is widely considered as a tumor suppressor, it can also act as an oncogene in some cancers. For example, WT1 is overexpressed in most glioblastoma. Chapter 15 describes the functional role of WT1 in glioblastoma and how it regulates proliferation and apoptosis of glioblastoma cells. Finally, chapter 16 focuses on the role of *WT1* in minimal residual disease in acute myeloid leukemia (AML). *WT1* is overexpressed at mRNA level in 80–90% of AML cases, and there are reports of poor outcome for patients having *WT1* levels above reference thresholds at specific time points. This chapter gives a comprehensive review of the role of WT1 in AML, molecular markers to stratify high-risk AML patients, and interventional therapy based on *WT1* expression.

The intended audience of this book is students, basic scientists, and clinicians who are interested in the basic and/or clinical aspects of WT and *WT1*. It is our wish that this book would serve as an authoritative source for readers who want a comprehensive understanding of the development, progression, management, and treatment of WT.

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Section I

Epidemiology, Diagnostics, and Treatment

Chapter 1

Wilms' Tumour – Histology and Differential Diagnosis

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Abstract

Wilms' tumour (WT) is the most common paediatric renal tumour, which can present as a single nodule, as multifocal unilateral lesions or as bilateral tumours. Typically, WT comprises three histological components namely blastemal, epithelial and stromal. The proportion and the degree of maturation of these components vary significantly, making the histological appearance of each tumour unique. Classical triphasic WT rarely presents diagnostic difficulty for pathologists, but when only one component is present, especially in a small biopsy specimen, the differential diagnosis may

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include renal cell carcinoma, metanephric adenoma and hyperplastic nephrogenic rest for epithelial elements and clear cell sarcoma of the kidney, mesoblastic nephroma and synovial sarcoma for stromal elements. Pure blastemal-type WT may be difficult to distinguish from other embryonal 'small round blue cell tumours', including neuroblastoma, primitive neuroectodermal tumour/Ewing sarcoma, desmoplastic small round cell tumour and lymphoma. All the three components, though usually blastema, can become anaplastic, leading to the diagnosis of either focal or diffuse anaplasia. WT with diffuse anaplasia and WT with blastemal predominance (after preoperative chemotherapy) are regarded as high-risk tumours and require more aggressive treatment. Careful assessment of the tumour and the normal kidney is critical for accurate subtyping and staging of WT, which is the basis for post-operative treatment. In addition, the identification and correct interpretation of nephrogenic rests may affect prognosis and management. Histological distinction between WT and nephrogenic rest is not always possible based on morphology alone, and implementation of new molecular genetic tools may aid in this regard. Other molecular genetic signatures of WT, such as *P53* mutation and *MYCN* dysregulation, may provide future additional prognostic and therapeutic information.

Key words: Nephrogenic rest; Pathology; Wilms' tumour

Introduction

Renal tumours comprise 7–8% of all paediatric tumours in children under 15 years of age, and among those, Wilms' tumour (WT) or nephroblastoma is the most common neoplasm (1). The frequency of renal malignancies in childhood is listed in Table 1.

Table 1. Primary renal tumours in childhood

Tumour	Relative frequency (%)
Wilms' tumour	85
Mesoblastic nephroma	2–3
Clear cell sarcoma	3
Rhabdoid tumour	2
Renal cell carcinoma	5
Others	3

There are several reasons why making correct diagnosis of WT may be challenging for general or paediatric pathologists:

1. Rarity of the paediatric renal tumours results in lack of experience with these entities for most of the pathologists (2)
2. Presence of several subtypes of WT (morphological heterogeneity)
3. Morphological appearances may vary dramatically from case to case
4. Histological patterns of certain WT subtypes may appear initially similar to those of other rare paediatric renal tumours
5. Lack of sharp differential criteria distinguishing WT from nephrogenic rests (NRs), especially in limited biopsy material
6. Assessment of the tumour and determination of the local pathology stage are multi-step and time-consuming processes

Preoperative chemotherapy may create additional difficulty in precise tumour assessment because the criteria for tumour subtyping and risk-group stratification are different for treated and untreated cases (3–5).

Gross appearance

Macroscopically, WTs are usually large masses disconfigurating the renal contours, which can vary in size significantly. Multicentric tumours occur in 5%, and they are usually associated with NRs (6). Precaution should be taken for the cutting procedure because the cut surface of the tumour may expand from the surrounding pseudocapsule, making the microscopic assessment of tumour margins more difficult. Macroscopic appearance of the cut surface is heterogeneous in many cases, with areas of viable tumour, haemorrhage and necrosis, especially in pre-treated specimens. Viable tumour is usually solid, pale grey to slightly pink or yellow-grey with soft consistency. Some tumours are markedly cystic, and careful search for the presence of solid foci is required. To avoid artificial contamination by the tumour cells, it is important to sample the hilar margins, including vessels, if possible before the tumour is incised.

Histological features

Classical histological features of WT include a triphasic pattern of epithelial, stromal and blastemal components (Figure 1). The proportions of these components and their lines and degree of differentiation vary significantly, resulting in countless tumour appearances. Biphasic and monophasic variants are not uncommon. Preoperative chemotherapy, given to children treated according to the International Society of Paediatric Oncology (SIOP) protocol, may affect the original histology dramatically by reducing or enhancing certain elements or by inducing maturation (7, 8).

Blastema represents the least differentiated, and presumed most malignant, component and consists of small round blue cells with overlapping nuclei and brisk mitotic activity. Several histological

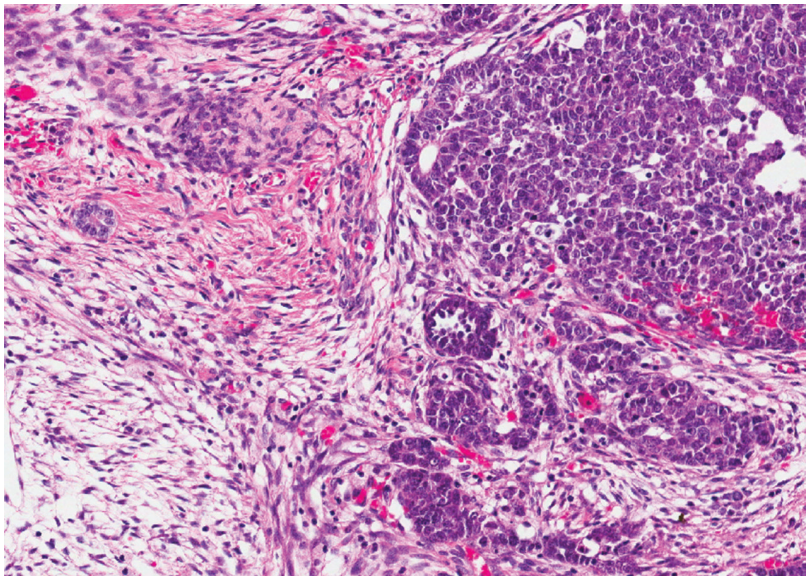

Figure 1. Wilms' tumour: mixed pattern with blastema, stroma and single epithelial structures.

patterns of blastema, including diffuse, serpentine, nodular and basaloid, have been described. The serpentine pattern of growth is characterised by broad bands of undifferentiated cells surrounded by fibromyxoid stroma. In the basaloid variant, nests or cords of blastema have a distinctive peripheral palisading of elongated cells with epithelial differentiation. All four above-mentioned patterns may be found in the same tumour and have no prognostic significance; however, their recognition in the histological slides can be helpful in differential diagnosis with other 'small round blue cell tumours' when the tumour is composed of the blastemal component only. It is worth noting that although WTs are mostly well circumscribed and surrounded by a pseudocapsule, which is used as one of the differential diagnostic criteria, blastemal-type WTs, usually with diffuse growth pattern, can show marked infiltrative growth with no pseudocapsule between the tumour and adjacent tissues. Primitive tubular epithelial structures sometimes present in the centre of blastemal nodules may morphologically mimic neuroblastoma-like areas with pseudorosettes. Vague epithelioid or spindle cell appearances are other possible histological features of blastema depending on the extent and pattern of early differentiation. There are no strict criteria to discriminate blastema from early epithelial differentiation (Figure 2) or stromal lineage, with almost all literature describing WT subtypes being based on subjective morphological criteria.

The epithelial component may demonstrate the whole spectrum of differentiation from early stages of tubular formation with primitive epithelial rosette-like structures to somewhat differentiating tubules or glomeruli-like structures, reflecting different stages of nephrogenesis. Squamous epithelial islands and mucinous epithelium are examples of heterologous differentiation within the epithelial component of WT.

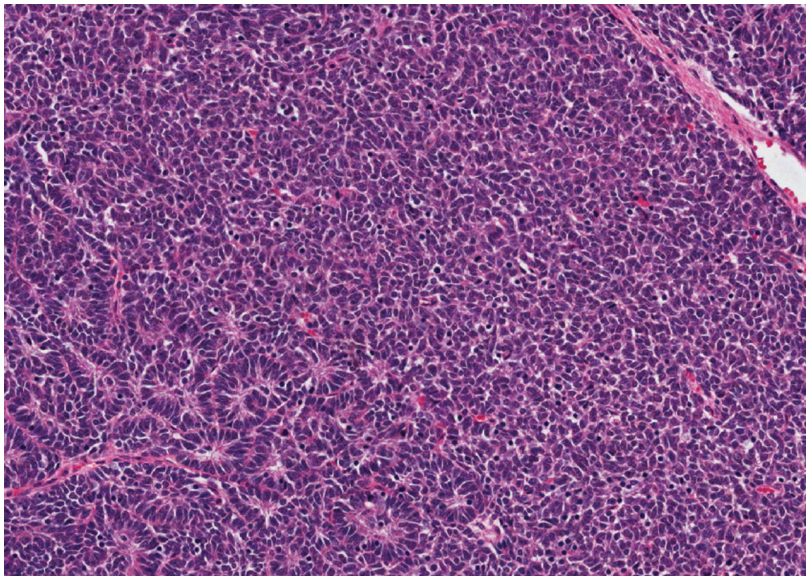

Figure 2. Blastemal-type Wilms' tumour with early epithelial differentiation.

The stromal component may include densely packed undifferentiated mesenchymal cells or loose cellular myxoid areas. The latter areas may be difficult to distinguish from non-tumorous stroma associated with chemotherapy-induced change (CIC). Heterologous differentiation of neoplastic stroma in the form of well-differentiated smooth or skeletal muscle cells, fat tissue, cartilage, bone and even glial tissue is present in some cases, especially in tumours that have undergone preoperative chemotherapy (Figure 3).

CIC includes areas of necrosis, haemorrhage and fibrosis of varying degree and areas with foamy and/or haemosiderin-laden macrophages. Primitive, highly proliferative blastemal component more readily responds to chemotherapy, leaving homogeneous eosinophilic areas where 'shadows' of pre-existing cells and structures may be seen. Mature epithelial and stromal components are often less sensitive to chemotherapy, and such tumours may show no significant response to pre-operative therapy in terms of tumour-size shrinkage. It is worth emphasising that the criteria and terminology used by the SIOP and National Wilms' Tumor Study/Children's Oncology Group (NWTS/COG) differ, so direct comparison of certain subtypes is not feasible. Histological assessment of tumour responsiveness to chemotherapy is important for risk-group stratification by the SIOP. For instance, completely necrotic WT is regarded as a low-risk tumour and requires less post-operative therapy than WTs from other groups. Further, stromal- or epithelial-type WTs are terms used by the SIOP for pre-treated tumours, whereas the NWTS/COG uses terms such as stromal or epithelial

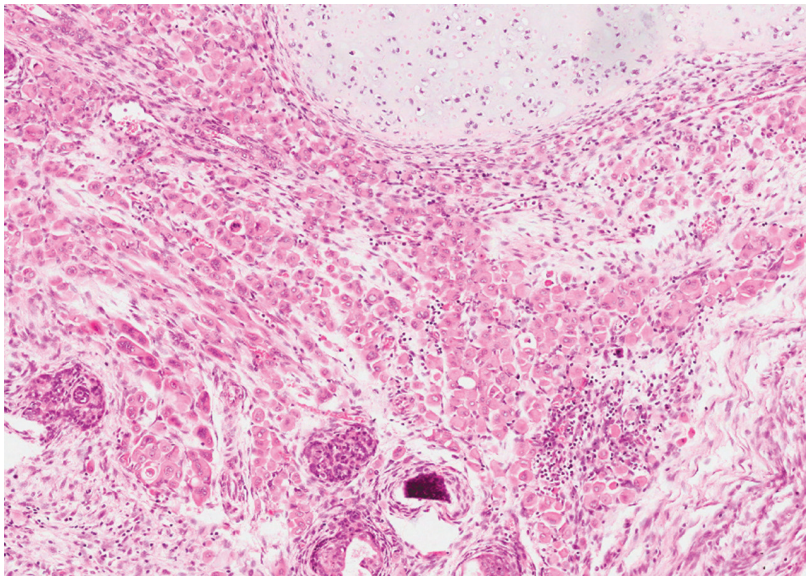

Figure 3. Preoperatively treated Wilms' tumour with prominent skeletal muscle differentiation and cartilage.

predominant WT (Table 2). In non-treated cases, stromal or epithelial predominant tumours may contain up to one-third of the blastemal component, whereas in pre-treated cases, the finding of >10% of blastema would result in the tumour being sub-classified as mixed type (9).

Anaplastic Wilms' tumours account for 5–8% of all WTs, and the majority of patients with anaplastic WT (Figure 4) are older than those with non-anaplastic WT. The criteria necessary for the diagnosis of anaplasia are the presence of large, atypical multipolar mitotic figures and significantly enlarged and hyperchromatic nuclei (10). These tumours are generally aneuploid. Anaplasia may be focal or diffuse. Focal anaplasia means that there is a localised and definitely completely excised area with anaplastic features. All other cases where anaplasia is found should be regarded as diffuse anaplasia. Diffuse anaplasia is regarded as the only unfavourable histological feature in WTs undergoing primary nephrectomy. Anaplasia is responsible for adverse outcome, especially in the cases with advanced tumour stage; thus, its recognition is essential for the prognosis and treatment. Because anaplasia is regarded as a chemo-resistant cell clone, it may be easier to detect it in pre-treated cases due to loss of other chemo-sensitive elements. Anaplastic tumours often express p53 on immunohistochemical staining and bear mutants in the *TP53* gene (11–14). *TP53* mutation has been shown to compromise patients' survival, overall and event-free, and therefore has the potential as an adverse prognostic factor combined with anaplastic

Table 2. Current SIOP classification of paediatric renal tumours

Pre-treated tumours*	**Primary nephrectomy tumours**
Low risk	Low risk
Mesoblastic nephroma	Mesoblastic nephroma
Cystic partially differentiated nephroblastoma	Cystic partially differentiated nephroblastoma
Completely necrotic nephroblastoma	
Intermediate risk	Intermediate risk
Nephroblastoma – epithelial type	Non-anaplastic nephroblastoma and its variants
Nephroblastoma – stromal type	Nephroblastoma – focal anaplasia type
Nephroblastoma – mixed type	
Nephroblastoma – regressive type	
Nephroblastoma – focal anaplasia type	
High risk	High risk
Nephroblastoma – blastemal type	Nephroblastoma – diffuse anaplasia type
Nephroblastoma – diffuse anaplasia type	Clear cell sarcoma of the kidney
Clear cell sarcoma of the kidney	Rhabdoid tumour of the kidney
Rhabdoid tumour of the kidney	

*The criteria for subclassifying pre-treated WTs are as follows: completely necrotic type shows no viable tumour elements. If more than 66% (two-thirds) of the tumour is non-viable (i.e., shows chemotherapy-induced changes), it is regarded as regressive type, irrespective of the presence of remaining viable tumour components. If viable tumour comprises more than one-third of the tumour mass, subtyping depends on the percentage of viable components: in mixed type, none of the components comprise more than 66% of the tumour; in epithelial (or stromal) type, in addition to having more than 66% of the tumour being composed of epithelial (or stromal) elements, the finding of only up to 10% of blastema is allowed (if the finding is more, then the tumour is subclassified as mixed type).

morphological features (15). Dysregulation of *MYCN* gene in WTs with anaplastic histology has also been reported to be involved in the development of tumours with adverse outcome (16).

Handling of the nephrectomy specimen

Core biopsies are done in some cases, and their main purpose is to confirm whether a tumour is a WT, in order to give appropriate pre-operative chemotherapy. If biopsies contain enough

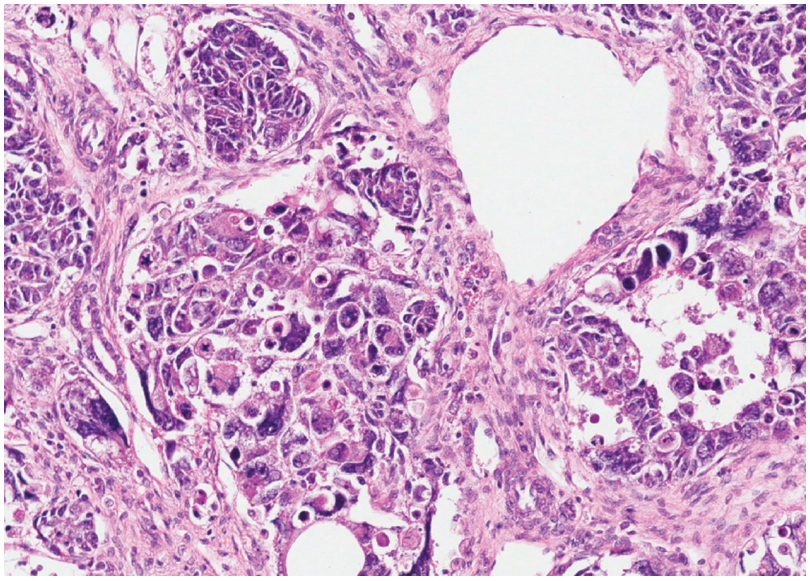

Figure 4. Wilms' tumour with anaplasia.

tissue for diagnostic purpose, some material should be kept frozen for molecular biology studies.

Immediately after surgery, the tumour should be delivered to the pathology department for appropriate handling of the specimen. Careful assessment of the surface and of the margins of renal vessels and the ureter and the assessment of the renal capsule for breaches are critical points for adequate staging (17). The nephrectomy specimen should be inked after photography and measurement. After opening (bivalving), tumour and normal renal tissues are taken for biological studies. Additional parallel slices are usually needed for a large tumour, but they should not compromise staging assessment of the fixed neoplasm. Careful mapping of the specimen, photographs and precise block guides are crucial in the staging assessment. At least one whole longitudinal slice of the tumour is sampled, with additional blocks taken from grossly different areas. When multicentric tumour is present, each nodule is sampled for histology and molecular biology study. Interface between the tumour and normal kidney as well as blocks containing renal and tumour capsule are always taken for histological examination. Evaluation of the renal sinus involvement is very important for the staging purpose; hence, this part of the specimen is a subject of thorough investigation especially when the tumour compromises the normal sinus architecture (18). The residual kidney is also sampled for possible presence of NRs. The hilar fat and all lymph nodes are sampled in search for possible metastases.

Current staging criteria for pre-treated and non-treated tumours are shown in Tables 3 and 4. The presence of tumour cells in the vessels within the tumour mass does not generally change the stage unless it is found in the vessels of the renal sinus. The finding of non-viable tumour and/or secondary inflammatory changes in the renal sinus or perirenal fat is not the criterion for stage II. However, the finding of non-viable tumour at the resection margins is currently regarded as a reason for stage III in the SIOP protocol (9). The presence of Tamm–Horsfall protein and mature tubules in lymph nodes is occasionally seen, but it should not be regarded as an evidence of metastatic disease (19). Recent studies have shown that there is considerable discrepancy in diagnosing and staging of these tumours between the institutional pathologists and the central pathology reviewers (around 20% of cases), so rapid central pathology review is being introduced and recommended in renal tumour trials (2, 20, 21).

Table 3. SIOP staging system

WT 2001 staging criteria for pre-operatively chemotherapy-treated tumour*
Stage I a. The tumour is limited to the kidney or surrounded with a fibrous pseudocapsule if outside the normal contours of the kidney. The renal capsule or pseudocapsule may be infiltrated by the tumour, but it does not reach the outer surface b. The tumour may be protruding ('bulging') into the pelvic system and 'dipping' into the ureter, but it is not infiltrating their walls c. The renal sinus (its vessels and soft tissues) is not involved d. Intrarenal vessels may be involved *Notes: Fine-needle aspiration or percutaneous core needle biopsy does not upstage the tumour, but the size of the needle gauge should be mentioned to the pathologist* *The presence of necrotic tumour or chemotherapy-induced change in the renal sinus and/or within the perirenal fat should not be regarded as a reason for upstaging the tumour, provided it is completely excised and does not reach the resection margins*
Stage II a. Viable tumour penetrates through the renal capsule and/or fibrous pseudocapsule into perirenal fat but is completely resected (resection margins 'clear') b. Viable tumour infiltrates the soft tissues and/or blood and/or lymphatic vessels of the renal sinus c. Viable tumour infiltrates the perirenal tissue, but it is completely resected d. Viable tumour infiltrates the renal pelvic or ureter's wall e. Viable tumour infiltrates adjacent organs or vena cava but is completely resected *Notes: Infiltration of the adrenal gland is not regarded as stage II if there is a (pseudo)capsule. Equally, tumour adherence to the liver is not regarded as stage II for which there should be a genuine infiltration of the liver parenchyma*

(*Continued*)

Table 3. (*Continued*)

WT 2001 staging criteria for pre-operatively chemotherapy-treated tumour*
Stage III a. Viable or non-viable tumour present at resection margins b. Any abdominal lymph nodes are involved c. Tumour rupture before or intraoperatively (irrespective of other criteria for staging) d. Tumour penetration through the peritoneal surface e. Tumour implants are found on the peritoneal surface f. Tumour thrombi present at resection margins of extra-renal vessels, transected or removed piecemeal by the surgeon g. The tumour has been surgically biopsied (wedge biopsy) prior to preoperative chemotherapy or surgery *Note: The presence of necrotic tumour or chemotherapy-induced changes in a lymph node or at the resection margins is regarded as a proof of previous tumour with microscopic residue, and therefore, the tumour is assigned stage III (because of the possibility that some viable tumour is left behind in the adjacent lymph node or beyond the resection margins)*
Stage IV a. Haematogenous metastases (lung, liver, bone, brain, etc.) or lymph node metastases outside the abdominopelvic region
Stage V a. Bilateral renal tumours at diagnosis. Each side should be substaged according to the above criteria

*Data from reference (9) with additional notes for stage II.

Nephrogenic rests and nephroblastomatosis

NRs are abnormal areas of embryonic tissue persisting beyond 36 weeks of development. They are found in 30–44% of kidneys with WT. The term 'nephroblastomatosis' was introduced in 1961 by Hou and Holman (22) in their description of a lesion composed of immature renal tissue in the kidney of a premature infant. Later, the term was adopted by Beckwith et al. (23)and Beckwith (24) who developed the theory of WT origin from NR.

There are two main types of NR – perilobar (PLNR) and intralobar (ILNR). The former is located at the periphery of the renal lobules and the latter in the central part of the lobe. ILNR is believed to arise earlier in the development when compared with PLNR, which may explain the higher frequency of heterologous elements in ILNR, such as striated muscle, fat, cartilage and bone. Depending on the stage of their development, both ILNR and PLNR might present with different morphological patterns. Beckwith suggested several histological types, including incipient (in newborns and young infants) or dormant (in older infants or children), regressing or sclerotic, obsolescent, and hyperplastic NR.

Table 4. COG staging system

WT staging criteria for non-treated tumours prior to operation*
Stage I a. Tumour limited to the kidney and completely resected b. Renal capsule intact c. The tumour was not ruptured or biopsied prior to removal d. Renal vein contains no tumour (intrarenal vessel involvement may be present) e. No residual tumour apparent beyond the margins of excision
Stage II a. Tumour extends beyond the kidney but is completely resected b. Regional extension of tumour (vascular invasion outside the renal parenchyma or within the renal sinus and/or capsular penetration with negative excision margin) c. Operative tumour spill confined to flank (no peritoneal contamination) d. Tumour biopsy (except fine-needle aspiration) prior to surgery
Stage III a. Non-haematogenous metastases confined to the abdomen (e.g., tumour in regional lymph nodes), including tumour implants on or penetrating the peritoneum b. Gross or microscopic tumour remains post-operative (tumour at the margins of resection) c. Tumour spill before or during surgery not confined to flank d. Piecemeal excision of the tumour (removal in >1 piece)
Stage IV a. Presence of haematogenous metastases or metastases to distant lymph nodes
Stage V a. Bilateral renal involvement at the time of initial diagnosis

*Data from reference (3).

The presence of multifocal NRs is defined as nephroblastomatosis. In the condition called diffuse hyperplastic perilobar nephroblastomatosis, which is often bilateral, a large portion of the cortical renal parenchyma is replaced with a thick 'crust' composed of proliferating nephroblastic tissue. It is important to distinguish NR from WT because their clinical management differs significantly. The usual differential diagnostic guides emphasise the criteria such as a lack of fibrous pseudocapsule in NR, which is almost always present in WT cases (Figures 5 and 6). This observation provides a useful tool for pathologists dealing with untreated nephrectomy specimens. However, for patients treated according to the SIOP protocol receiving pre-operative chemotherapy, a fibrous capsule may be present even around the foci of NRs. Conversely, blastemal-type WT may show no separation from the renal parenchyma by the pseudocapsule. In addition, because their microscopic features may be very similar, distinguishing WT from NR in limited needle biopsy material is virtually impossible. In such cases, it has been suggested to use the term

'nephroblastic process, consistent with either WT or NR' as optimal, with further radiologic–pathologic correlation being required (25). The main differential diagnostic criteria for NR and WT are summarised in Table 5, but one has to bear in mind that none of them is absolutely conclusive.

Another challenge for pathologists is to assess the local stage of the tumour in the presence of ILNR. Providing the frequent location of the ILNR next to the renal sinus or even in the sinus or in the calyceal wall can be misinterpreted as renal sinus invasion by the tumour, leading to upstaging and unnecessary more aggressive treatment.

There are no reliable immunohistochemical or molecular markers facilitating differential diagnosis of NR and WT. A recent study showed significant variability of methylation profiles in NRs and WTs and reported changes in the methylome to underlie NR formation and transformation to WTs in a subset of cases (26). These data have the potential for being implemented into the clinical differential diagnosis of these two lesions, but more extensive work is required.

Differential diagnosis

The diagnosis is usually straightforward in triphasic or even biphasic WTs, although their subclassification may be challenging (27). However, monophasic WTs may be very difficult

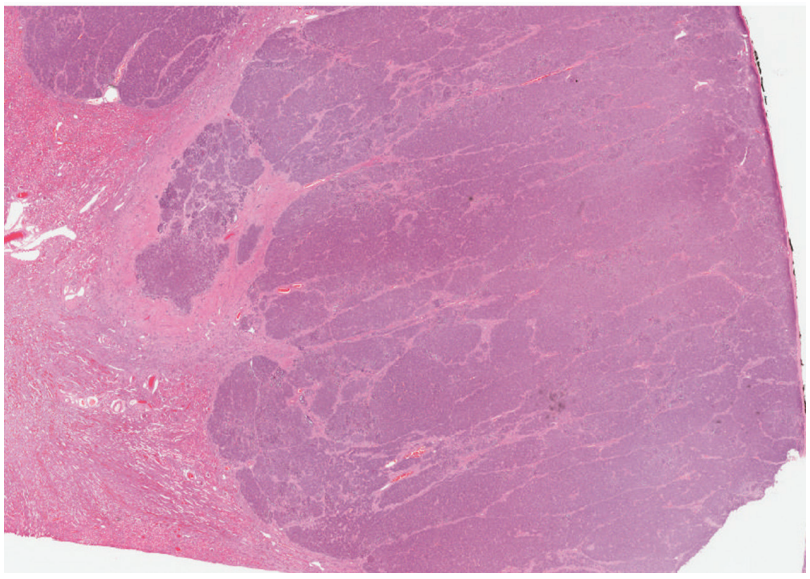

Figure 5. View of treated case of hyperplastic perilobar nephroblastomatosis. Partially developed fibrous capsule is seen.

Table 5. Features of NR and WT

WT	NR
Shape – spherical	Shape – oval
Fibrous capsule is present	No fibrous capsule*
Skeletal muscle differentiation is common	Skeletal muscle differentiation is uncommon
Usually solitary	Often multifocal

NR, nephrogenic rest; WT, Wilms' tumour.

*In untreated cases but in pre-treated cases, capsule may be present.

to separate from other renal tumours with similar histological features. Pure blastemal-type WTs have to be distinguished from other undifferentiated tumours, such as neuroblastoma, primitive neuroectodermal tumour/Ewing sarcoma of the kidney (28), desmoplastic small round cell tumour (29) and synovial sarcoma (30). It is particularly important to consider non-WTs in older patients (Table 5) and adults – WT in adults definitely exists, but many of the renal tumours that in the past were labelled as adult WTs proved to be some of the mentioned entities. In order to reach the correct diagnosis in such cases, it is critical to apply immunohistochemistry and molecular biology investigations looking for characteristic features. Although blastemal components may show focal CD99 positivity, it

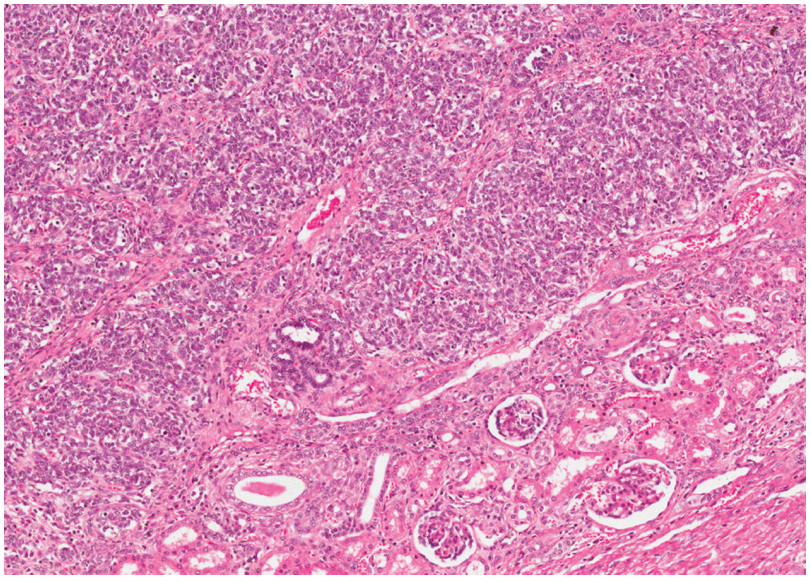

Figure 6. Hyperplastic perilobar nephroblastomatosis – direct interface with normal renal parenchyma with no pseudocapsule.

is usually not diffuse and membranous as in Ewing sarcoma of the kidney, where genetic studies also show characteristic translocations, with t(11;22)(q24;q12) being the most common (28). Desmoplastic small round cell tumour shares many immunohistochemical features with blastemal-type WT but is rare, and the diagnosis should only be made if genetic investigations demonstrate the *EWS-WT1* t(11;22)(q13;q12) translocation(29). Neuroblastoma usually shows elevated levels of catecholamines, and on histological examination, its cells reveal non-overlapping nuclei and coarse 'salt and pepper' chromatin. Both tumours may be positive for neuron-specific enolase and CD56, but WT1 marker is negative in neuroblastoma and NB84a marker is negative in WT. In the past, in rare cases, a rhabdoid tumour could be mistaken for a WT, but now it is simple to distinguish between them based on immunohistochemistry, with the lack of nuclear INI1 expression in rhabdoid tumour (31). Pure epithelial-type WT may be difficult to distinguish from metanephric adenoma, renal cell carcinoma and hyperplastic PLNR. Highly differentiated epithelial-type WT may be composed of small, well-differentiated and closely packed tubules similar to metanephric adenoma, but the latter can be diagnosed by the lack of capsule between the tumour and renal parenchyma and the absence of mitotic activity. The combination of CK7–, AMACR–, WT1+ and CD57+ has been shown as an immunohistochemical pattern of metanephric adenoma (32). Renal cell carcinomas in children associated with translocations show distinctive histological features, but papillary renal cell carcinoma (as seen in adults) may be more challenging to diagnose. Immunohistochemistry demonstrating the expression of markers such as CK7 and CD10 (33, 34) and cytogenetic findings may be very helpful (35).

In the differential diagnosis of pure stromal-type WTs, a clear cell sarcoma of the kidney and mesoblastic nephroma should be considered. In WTs treated with preoperative chemotherapy, the stroma may show a striking clear cell sarcoma-like appearance, and extensive sampling may be required in order to find the foci with other WT components.

WT with prominent cystic appearance has to be differentiated from cystic nephroma (CN) and cystic partially differentiated nephroblastoma(CPDN). CN and CPDN share some clinical-pathological features and were regarded as related lesions. They occur in young children, are well demarcated from the kidney and are composed of cysts only, with no solid nodules. Histologically, the only difference between these lesions is the finding of blastema in the septa of CPDN, whereas CN contain no blastema (36). However, despite clear similarities, recent studies showed that these lesions are not related at all and that CN shows DICER1 mutations that are never found in CPDN or WT (37). Still, both CN and CPDN are adequately treated with resection alone, with an excellent prognosis and no chemotherapy required, whereas cystic WTs, which are usually stage I and therefore having a fairly good

Table 6. Age and likely renal tumour*

Age (years)	Most common	Possible	Rare
Birth	MN	WT	RTK
<1	WT, MN	RTK, CCSK	
1–5	WT	CCSK	MN (<3 years), RTK
5–10	WT	CCSK, RCC	
11–15	WT, RCC	PNET	

*Data from reference (9).

CCSK, clear cell sarcoma of the kidney; MN, mesoblastic nephroma; PNET, primitive neuroectodermal tumour; RCC, renal cell carcinoma; RTK, rhabdoid tumour of the kidney; WT, Wilms' tumour.

prognosis, should be treated according to the current protocol for WT. Although CN is a benign neoplasm, its association with the malignant pleuropulmonary blastoma has been reported recently (38).

Awareness of the age distribution for paediatric kidney tumours (Table 6) might assist in their differential diagnosis (39–41).

Conclusion

Remarkable progress in classification, treatment and understanding of the pathology and molecular biology of WT has been made over recent decades. Because this tumour is rare, it still represents a diagnostic problem, and awareness of the potentially complex pathological features of this malignancy is required for the accurate diagnosis, subtyping and staging to allow appropriate treatment. Preoperative chemotherapy may affect histological and staging features, and diagnostic pathologists should be familiar with these when assessing such tumours. Adequate handling and sampling are essential prerequisites for correct diagnosis. Molecular biology markers are likely to play an even more important role in the tumour prognosis and differential diagnosis in future, but at present, pathological examination represents the gold standard for diagnosis, subtyping and prognosis.

Conflict of Interest

The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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Not relevant.

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Chapter 2

The Clinical Relevance of Age at Presentation in Nephroblastoma

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Abstract

The most important prognostic factors for Wilms tumor (WT) patients seem to be stage, histological subtype, and 1p/16q loss of heterozygosity (LOH) in chemotherapy-naive WTs.

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Over the last decade, age at diagnosis also was suggested to be an important risk factor for WT recurrence in Children's Oncology Group (COG), United Kingdom (UK), and International Society of Pediatric Oncology (SIOP) studies. Several studies have analyzed age as a prognostic factor; these studies revealed age <2 years as a favorable prognostic factor, while age >4 years has been described as an adverse prognostic factor. In adults (>18 years of age), WT represents less than 1% of all diagnosed renal tumors; therefore, diagnosis of WT in adults is often unexpected and poorly recognized, thereby inducing treatment delay with subsequent adverse outcome. One explanation for the higher risk of recurrence with increasing patient age is the higher frequency of anaplasia at higher age. Other suggested reasons are delay in diagnosis, advanced tumor stage at presentation, and intrinsically different biological behaviors. Whether age is really an *independent* risk factor, and whether age is a stronger prognostic factor than stage, histology, and LOH 1p/16q, needs to be further explored. This may provide some insight into whether older patients need to be treated more intensively, as is already advised for adult WT patients.

Key words: Age; Prognostic factor; Wilms tumor

Introduction

Wilms tumor (WT) is the most common type of childhood renal cancer. It affects approximately one child per 10,000 worldwide before the age of 15 years (1). The median age at diagnosis of WT is approximately 3.5 years (1). The two treatment approaches (European and North America) available for children with WT result in comparable overall survival rates, currently reaching 90%. The International Society of Pediatric Oncology (SIOP) in Europe advocates chemotherapy before nephrectomy, whereas the Children's Oncology Group (COG) in North America recommends immediate surgery (2).

The most important prognostic factors for WT patients seem to be stage, histological anaplastic subtype and blastemal subtype (the latter in chemotherapy-pretreated nephroblastoma cases only), and 1p/16q loss of heterozygosity (LOH) in chemotherapy-naive WTs (3–5). Tumor stage is the original prognostic factor for WT, while tumor histology is perhaps the most powerful prognostic factor for WT; (diffuse) anaplasia is associated with adverse outcome in both the COG and SIOP histologic classification systems, while the adverse prognostic effect of residual blastemal cells after pre-operative chemotherapy is only recognized in the SIOP classification system (4, 5). LOH of 1p/16q is found in around 5% of favorable-histology WTs, and it has been demonstrated to be significantly correlated with less favorable outcome (3).

Over the last decade, age at diagnosis also was suggested to be an important risk factor for WT recurrence in COG, UK (United Kingdom), and SIOP studies (6–9).

The clinical relevance of age

General

Cooperative studies have shown that increasing age is associated with an increased risk of recurrence of nonmetastatic WT (6, 7, 9–11). This is only partly explained by the fact that the occurrence of anaplasia increases with age (12); even in patients with favorable histology, older age seems to be associated with less favorable outcome. It still needs to be determined what the exact age threshold is at which outcome starts to deteriorate.

Infants

The "chemotherapy before surgery strategy" has been under debate internationally for years. SIOP protocols recommend to treat patients >6 months with preoperative chemotherapy; this has the clear evidence-based benefit of downstaging tumors, thereby sparing survivors the late effects of doxorubicin or radiotherapy (14). However, in young infants, the so-called non-WTs tend to occur up to a substantial proportion in the younger age group (13). This initiated a study on all renal tumors in infants (under the age of 7 months at presentation) on a global level, based on data in 750 children, treated in UK, COG, and SIOP protocols, showing that above 2 months of age at presentation, WT is the most common tumor type, while congenital mesoblastic nephroma occurred more often than WT under the age of 3 months at presentation (Figure 1) (13). In addition, the biologically more aggressive malignant rhabdoid tumor of the kidney has a high propensity in this young age group. This has forced

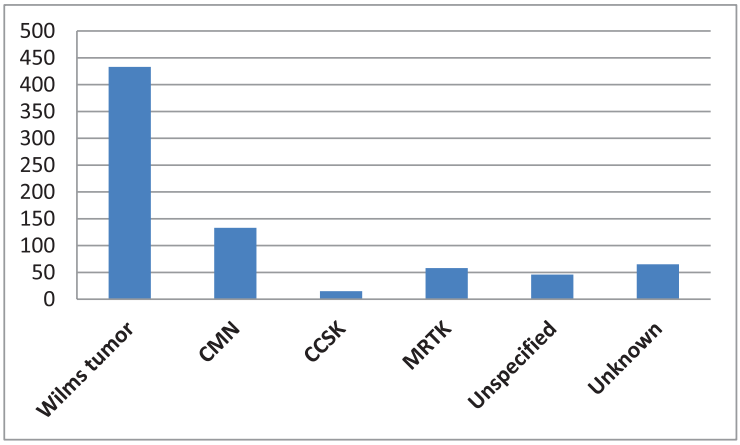

Figure 1. Distribution of renal tumors in children aged 7 months or less (13). CMN: congenital mesoblastic nephroma; CCSK: clear cell sarcoma of the kidney; MRTK: malignant rhabdoid tumor of the kidney.

an international recommendation to, even in the SIOP community, immediately perform surgery instead of pre-operative chemotherapy in these young children with renal tumors.

Survival rates for WT patients below the age of 7 months are very good (5-year overall survival 93.4%). In addition, the incidence of metastatic WT in this age group is very low (<1%) (13).

Children >6 months of age

Several studies have analyzed age as a prognostic factor (Table 1). These studies revealed age <2 years as a favorable prognostic factor, while age >4 years has been described as an adverse prognostic factor. One study specifically addressed the adverse outcome in teenagers (10–16 years of age) (15).

Currently, age is already incorporated into the risk stratification of COG studies (AREN0532); it is predicted that children under the age of 2 years with small tumors (<550 g) and stage I favorable-histology WTs can benefit from surgical treatment only (nephrectomy alone without adjuvant chemotherapy). As stage and histology are considered to be stronger prognostic markers, age is not used for risk stratification in the SIOP trials.

Adults

In adults (>18 years of age), WT represents less than 1% of all diagnosed renal tumors (17–22). The most common type of adult renal cancer is renal cell carcinoma (approximately 85%); therefore, diagnosis of WT in adults is often unexpected and poorly recognized, thereby inducing treatment delay with subsequent adverse outcome (23). This treatment delay, rather than more aggressive biology seems to determine the worse outcome in adults with WT as compared to in children (17–22, 24, 25). More recent data indicate the potential for improvement in adults when pediatric treatment approaches, including multimodality chemo- and radiotherapy adapted from the pediatric treatment protocols, are used (18, 19, 21, 22, 24, 25).

Multiple factors, including the unfamiliarity of adult oncologists with WT, lack of standardized treatment, delay in initiating the appropriate therapy and also a possible more biologically aggressive tumor type, may contribute to poor outcome (22).

This prompted several representatives of the renal tumor committees of the COG and the SIOP to develop, together with adult urologists, medical oncologists, and radiotherapists, a consensus "best practice" guideline for the management of WT in adults (26). The aim of this international consensus recommendation is to further improve outcome by shortening adjuvant treatment delay and by using standardized treatment (26).

Age in correlation with other prognostic factors

One explanation for the higher risk of recurrence with increasing patient age is the higher frequency of anaplasia at higher age. Anaplasia is only very rarely seen in WT diagnosed

Table 1. Age as a prognostic factor

Report	Study	Result	Multivariate analysis	Outcome measures
Green et al. (JCO 1993) (6)	NWTS1, NWTS2, NWTS3	Age <2 y as favorable prognostic factor in small (<550 g) stage I favorable-histology tumors	Not performed	4 y EFS
Pession et al. (EJC 2008) (16)	AIEOP 1989–1998	Age ≤2 y as favorable prognostic factor	Age *not* an independent prognostic factor	OS
Pritchard-Jones et al. (JCO 2003) (7)	UKW2, UKW3	Age >4 y as adverse prognostic factor in stage I favorable-histology tumors	Age an independent prognostic factor	4 y EFS and OS
Irtan et al. (EJC 2015) (10)	UKW3	Age >4 y as adverse prognostic factor	Age an independent prognostic factor	EFS
Shamberger et al. (Ann Surg 1999) (9)	NWTS4	Age >4 y as adverse prognostic factor	Age *not* an independent prognostic factor	EFS
Reinhard et al. (Oncol Rep 2008) (8)	SIOP-GPOH 1989–2003	Age >4 y as adverse prognostic factor	Age *not* an independent prognostic factor	OS
Breslow et al. (Cancer 1991) (11)	NWTS3	Age 0–23 m, 5.4% relapse; age 24–47 m, 9.5% relapse; age 48+ m, 16.3% relapse	Not performed	EFS
Popov et al. (Ped and Dev Pathology 2011) (15)	UKW3, SIOP 2001	Age 10–16 y as adverse prognostic factor	Not performed	5 y OS

NWTS: National Wilms Tumor Study; AIEOP: Associazione Italiana Ematologia ed Oncologia Pediatrica; UKW: United Kingdom Wilms Tumour Study; SIOP: International Society of Pediatric Oncology; GPOH: Gesellschaft fur Padiatrische Onkologie und Hamatologie; y: year; m: month; EFS: event-free survival; OS: overall survival.

during the first year of life and is also rare in the second year of life (12). Nevertheless, even in the group of patients with favorable histology, older age seems to be correlated with a higher risk of relapse and death, although prognostic factors such as stage or histology seem to be more powerful (7). Other suggested reasons for the adverse survival rates in older children are delay in diagnosis, advanced tumor stage at presentation, and intrinsically different biological behaviors (7).

While age has been described as an independent risk factor in two (UK) studies, it did not remain significant after multivariate analysis in other studies (Table 1). It is important to stress that studies reported are heterogeneous with respect to design, outcome measures, and treatment regimens. Whether age is really an *independent* risk factor, and whether age is a stronger prognostic factor than stage, histology, and LOH 1p/16q, needs to be further explored. This may provide some insight into whether older patients need to be treated more intensively, as is already advised for adult WT patients.

Conflict of Interest

The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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Chapter 3

Histopathological and Molecular Characteristics of Wilms Tumor

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Abstract

Diagnosis of malignant renal tumors does not mostly create difficulties. Although micrometastases may be encountered during postmortem examination, kidney is not a preferred organ for clinically detected metastases of malignant tumors. Therefore, almost all renal tumors in adults and children are primary tumors. When primary renal tumors are encountered, most of the cases pose a diagnostic simplicity. Indeed, diagnosis of malignant kidney tumors in children is Wilms tumor (WT) in 80–90% of the cases, while it is renal cell carcinoma in adults. In fact, a typical WT contains tissue components in three different morphologies. These are mesenchymal component resembling primitive fetal mesenchyme, epithelial component that reminds us fetal renal tubules and glomeruli, and blastomatous component consisting of clusters of blast

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cells that contributed to the coinage of the term "nephroblastoma." However, not all WTs are triphasic, and different tissue components in very restricted areas may be overlooked. Besides, immunohistochemical staining methods helpful in the differential diagnosis of other tumors are not much useful in WT. Embryonic development of kidney is a complex process in which different transcription factors, proto-oncogenes, and various types of growth factors are effective. WT can be considered a failure of this transition. A number of genes are involved in nephrogenesis, as well as in Wilms tumorigenesis. Recently, some of these genes are believed to be regulated by *HACE1*, *glypican 3* (GPC3), and six *WT genes*. The incidence of WT is 1:10,000 worldwide. Currently, high cure rates can be achieved, and multimodality treatment has resulted in a significant improvement in outcomes. In this chapter, histopathological features of WT, genetic and molecular modifications related to WT, the effects of these genetic abnormalities on prognosis, and clues for differential diagnosis were evaluated.

Key words: Anaplastic; Blastemal type; Differential diagnosis; Favorable histology

Introduction

Diagnosis of malignant renal tumors does not mostly create difficulties. Although micrometastases may be encountered during autopsy, kidney is not a preferred organ for clinically detected metastases of malignant tumors. Therefore, almost all renal tumors in adults and children are primary tumors (1–3). When primary renal tumors are encountered, most of the cases pose a diagnostic simplicity. Indeed, diagnosis of malignant kidney tumors in children is Wilms tumor (WT) in 80–90% of the cases, while it is renal cell carcinoma in most adults. In fact, a typical WT contains tissue components in three different morphologies. These components are mesenchymal component resembling primitive fetal mesenchyme, epithelial component that reminds us fetal renal tubules and glomeruli, and blastomatous component consisting of clusters of blast cells that contributed to the coinage of the term "nephroblastoma." However, not all WTs are triphasic, and different tissue components in very restricted areas may be overlooked. Immunohistochemical staining methods helpful in the differential diagnosis of other tumors are not much use in WT, such as clear cell sarcoma or even renal cell carcinoma subtypes or other even more rare renal tumors (1, 3, 4).

Embryonic development of kidney is a complex process in which different transcription factors, proto-oncogenes, and various types of growth factors are effective. WT can be considered a failure of this transition. A number of genes are involved in nephrogenesis, as well as in Wilms tumorigenesis. Recently, some of these genes are believed to be regulated by *HACE1*, *glypican 3* (GPC3), and six *WT genes*. In addition, several studies have demonstrated that Cav-1 interacts with multiple members of the EGF-R/RAS/ERK and PI3/AKT pathways to modify signaling activity (5, 6).

The incidence of WT, the most common primary malignant renal tumor of childhood, is 1:10,000 worldwide. Currently, high cure rates can be achieved, and multimodality treatment has resulted in a significant improvement in outcomes. Recent studies have revealed that several genetic abnormalities are associated with a worse prognosis in WT, even in those with localized stage and favorable histology (7, 8). In this chapter, histopathological features of WT, genetic and molecular modifications related to WT, the effects of these genetic abnormalities on prognosis, and clues for differential diagnosis were analyzed.

Pathogenesis of WT

Kidney development is a complex process, consisting of two distinct embryological origins, the nephrogenic (mesenchymal) and the ductogenic (ureteric) (9). Both development pathways are regulated by transcription factors, proto-oncogenes, polypeptide growth factors that act as signaling molecules, and their receptors (10, 11). WT is the direct result of maldevelopment of the embryonic kidney and has led to many fundamental insights such as the link between normal development and tumorigenesis. Understanding the normal kidney development has helped in our understanding and treatment of WT. The metanephric kidney develops from the intermediate mesoderm, and this structure gives rise to three cell types that will form the kidney. In conclusion, this structure consists of the epithelial nephric or Wolffian duct, Six2-positive mesenchymal cells that will form the nephrons, and Foxd1-positive cells that will give rise to the stromal cells (6). WT can be considered a failure of this transition. It arises from pluripotent renal precursors that undergo excessive proliferation resulting in undifferentiated stromal components, blastemal cells similar to the condensing mesenchyme, and primitive epithelial structures resembling comma and S-shaped bodies and glomeruli. The presence of associated nephrogenic rests consisting of foci of persistent embryonic remnant tissues that failed to mature to normal renal parenchyma further points toward impaired differentiation in early renal development (6, 9, 11–14). WT was one of the three types of cancer in which Knudson and Strong (15) based his two-hit model for tumor suppressor genes, and the loss of WT1 in a subset of WT cases remains an archetypal example of a classic tumor suppressor gene, as originally proposed (6). Since then, many variations in classifications and the genetics and mechanics of tumor suppressor genes have been found (16), and the biological basis of the multiple tumors that arise in genetically predisposed individuals may clearly involve genes other than WT1. A number of genes involved in nephrogenesis, especially in the mesenchymal to epithelial transition, have also been implicated in Wilms tumorigenesis (9, 17).

Common genetic abnormalities in WT

WT, or nephroblastoma which is currently the preferred term, is the most common pediatric renal cancer (6). The biology of WT illustrates some important aspects of childhood

neoplasms such as the relationship between malformation and neoplasia, the histological similarities between the organogenesis and oncogenesis, and the two-hit theory of recessive tumor suppressor genes (7). WT morphologically resemble embryonic kidneys with a disrupted architecture, associated with undifferentiated metanephric precursors (6–8). Previous studies demonstrated that the risk of WT is increased in at least three groups of congenital malformations associated with distinct chromosomal loci. Although WT arising in these malformations accounts for no more than 10% of cases, these syndromic tumors have provided important insight into the biology in this neoplasm (7).

The first disorder that is associated with WT is WAGR syndrome, characterized by WT, aniridia, genital anomalies, and mental retardation. Lifetime risk of developing WT in these patients is approximately 33%. Patients with WAGR syndrome carry germ line deletion of chromosome 11p13, and the first identified WT-associate gene, WT1, is located on this chromosome. WT1 deletion in WAGR syndrome represents a "first hit"; the development of WT in these individuals frequently correlates with the occurrence of the mutation in the second WT1 allele as the "second hit" (6–9). The second disorder, Denys–Drash syndrome (DDS), is characterized by gonadal dysgenesis and early-onset nephropathy based on glomerulosclerosis leading to renal failure. Lifetime risk of WT in patients with DDS is approximately 90%. These patients demonstrated germ line point mutations in the zinc finger region of the WT1 protein that affects its DNA-binding properties (7). However, bi-allelic inactivation of WT1 must be required for the development of the WT phenotype in DDS (13–17). The third disorder, Beckwith–Wiedemann syndrome (BWS), is characterized by organomegaly, macroglossia, hemihypertrophy, and omphalocele. BWS has served as a model for tumorigenesis by genomic imprinting. Genetic locus of BWS or WT2 gene is on the 11p15.5. Unlike WAGR syndrome or DDS, the genetic basis for BWS is considerably more heterogeneous, in that no single genetic region is involved in all cases. Recent genetic studies have also elucidated the role of beta-catenin in WT. Beta-catenin belongs to the WNT (wingless) signaling pathway. Gain-of-function mutations have been demonstrated in 10% of sporadic WT. Similarly, mutations and deletions of WT1 gene are less common in sporadic WT cases (7, 8, 17, 18).

Histopathological features of WT

WT recapitulates normal nephrogenic differentiation, but while normal developing nephrons are beautifully structured, nephrogenic structures in WTs are disorganized (6). Most WTs show triphasic patterns such as blastemal, epithelial, and stromal (Figure 1). Clinical investigations reveal that the outcome of children with WT is dependent on histology. The cure rate in these cases is close to 90% (12). Favorable histology is characterized by the presence of all three histological elements and the absence of diffuse anaplasia (12, 14, 19–22). In

WT cases that had been pretreated with chemotherapy before surgery, the blastemal type also has been well recognized now as an adverse prognostic subtype (23, 24). However, the histological features are not sufficient to predict the prognosis of WT, and some chromosomal mutations may play a role as adverse biological markers, even in those with localized (stage I and II) favorable histology WT (25–33).

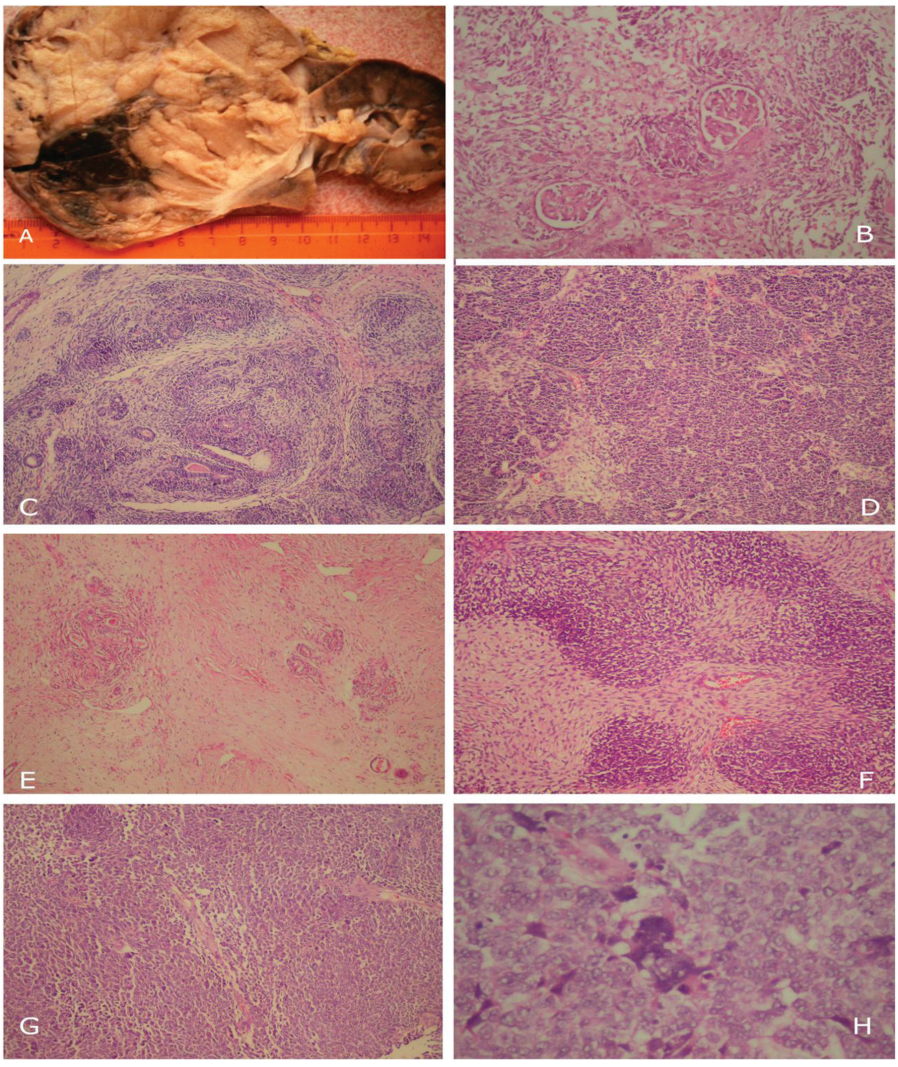

Figure 1. (A) Gross pathology of WT, (B) entrapped two normal glomeruli in a WT, (C, D) typical triphasic WTs, (E) differences after therapy, (F) blastemal and stromal areas in a WT, and (G, H) anaplastic WT.

The three histological components of WT have different proliferation potentials and different responses to therapy. In most reports, the lowest proliferation index was determined in the stromal component, and this component generally was not affected by chemotherapy. There were different results for the highest Ki-67 index in the literature. For example, the blastemal component had the highest proliferative activity in three studies, and the authors demonstrated that the surviving blastemal component after chemotherapy was a highly significant indicator of metastases and adverse outcome in WT (14, 30, 31). However, in two other studies, the highest Ki-67 index was determined in the epithelial component (22, 32). A fundamental difference in the behavior of normal versus tumor cells in culture is that normal cells divide for a limited number of times and exhibit cellular senescence, whereas tumor cells usually have the limitless proliferative capacity (14). The most prominent hypothesis is that the maintenance of telomere stability is required for the long-term proliferation of tumors. The tumor cells may escape from cellular senescence and become immortal by telomere maintenance. The simplest way of this maintenance is the activation of telomerase. Telomerase activity has been found in almost all tumors but not in adjacent normal cells (34–37). This activity was mainly evaluated with molecular studies, but it was also determined that the immunohistochemical staining pattern of TERT was correlated with telomerase activity (14, 34–38). The high telomerase reverse transcriptase (hTERT) staining was restricted to the nucleus in both normal telomerase-positive cells and cancer cells. The immunolocalization of hTERT in specimens of adult cancers revealed that the levels of telomerase activity mainly depended on the number of tumor cells with telomerase activity (14). Telomere maintenance is evident in virtually all types of malignant cells where either a telomerase-dependent or alternative lengthening of telomeres (ALT) mechanism exists. For this reason, effective strategies targeting telomere maintenance in cancer cells require telomerase inhibitors or ALT inhibitors (14, 34–38). The importance of telomerase activity is novel and potentially relevant in WT biology and progression because WT1 has been identified as a repressor of telomerase protein catalytic subunit promoter (36). In addition, functions of TERT other than telomere lengthening such as oncogenic transcriptional activation were reported (14). Although several genes such as HACE1, GPC3, and six WTs have been reported to involve in the pathogenesis of WT, they are not associated with specific histological features of WT (39–45).

Clues for differential diagnosis of WT

If a WT shows triphasic patterns, the diagnostic procedure is often not difficult. Wherein the case is of a monophasic pattern, differential diagnosis may be tiresome. In this condition, the main differential diagnosis of WT includes the so-called non-WT renal tumors,

that is, clear cell sarcomas of kidney, congenital mesoblastic nephroma, renal malignant rhabdoid tumors, neuroblastoma, and primitive neuroectodermal tumors (PNETs). In a pure epithelial tumor, metanephric adenoma should be considered for differential diagnosis. Especially, positivity of WT1 in metanephric adenoma creates the diagnostic difficulty in this tumor (1, 8, 17). Pure stromal WT is also rare, and in those cases, differential diagnosis includes the congenital mesoblastic nephroma. The age of cases is helpful for differential diagnosis, as most cases with mesoblastic nephroma occur in children younger than 6 months. In addition, WT with purely blastemal appearance after chemotherapy can be too hard to differentiate from neuroblastomas and PNETs (23, 24). Immunohistochemical stains provide limited benefit in the differential diagnosis of WT subtypes. Immunohistochemical profile of the various components of WT mirrors that of their counterparts in the developing kidney. For example, the blastematous elements show focal positivity for vimentin, the epithelial elements react for keratin and epithelial membrane antigen (EMA), and the mesenchymal elements show a heterogeneous reactivity according to the morphological appearances. Immunoreactivity for WT1 antigen is determined in the 90% of WTs, and it is the most useful marker for differential diagnosis. By contrary, positive immunoreactivity for TTF-1 is determined in 17% of WTs, and it represents a potential source of misdiagnosis (6, 7, 17). However, IHC can be very helpful in the conformation of non-WT subtypes.

Conclusion

In conclusion, WT that demonstrates monophasic appearance can be too hard to discriminate from other primary renal tumors, such as neuroblastoma, clear cell sarcoma, rhabdoid tumor, mesoblastic nephroma, or even sarcomatoid-type renal cell carcinoma (1, 3, 4). Apart from histology, genetic risk factors may aid in stratifying patients for future treatment.

Conflict of Interests

The author declares no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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Chapter 4

Wilms Tumor and Its Management in a Surgical Aspect

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Abstract

Nephroblastoma [Wilms tumor (WT)] is a rare, but the most common, primary renal tumor in children. WT is usually diagnosed between the ages of 1 and 5, with the most common diagnosis at the age of 3. While imaging (ultrasound, computed tomography, and magnetic resonance) can accurately predict up to 95% of WTs, they cannot predict the histologic subtypes and require tissue examination. Surgery is one of the cornerstones of WT treatment. Other aspects of management include chemotherapy and radiation therapy. The Societe Internationale D'oncologie Pediatrique (SIOP) advocates primary chemotherapy in patients less than 6 months of age, whereas the

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Children's Oncology Group (COG) recommends primary surgery in all cases except those considered not resectable by the surgeon. In this chapter, the surgical therapy of WT is reviewed.

Key words: Nephroblastoma; Pediatric oncology; Surgery for Wilms tumor

Introduction

Wilms tumor (WT), also called nephroblastoma, was first described by Thomas F. Rance in 1814 (1, 2). In 1899, Carl Max Wilhelm Wilms, a German surgeon and pathologist, gave a detailed histological description and since then the tumor bears his name (1, 3). The incidence of WT is 1:10000 in children under 15 (4). WT is the most common renal malignancy in children, and it represents 6% of all childhood cancers. It is also the second most common intra-abdominal cancer, and it is an embryonal malignancy of the kidney (5). About 75% of children are diagnosed before the age of 5, and the median age is 3.5 years (6).

WT is primarily a sporadic disease, but family history exists in 1–2% of cases (7). There are a number of syndromes associated with WT, including WAGR (WT, aniridia, genitourinary anomalies, and mental retardation), Denys-Drash syndrome (progressive renal disease, male pseudohermaphroditism, and WT), and Beckwith-Wiedemann syndrome (8). While WAGR syndrome is associated with large deletion on the WT1 chromosome located on 11p13, Denys-Drash syndrome has point mutations on WT1 (9, 10). Beckwith-Wiedemann syndrome is a syndrome characterized by macrosomia, macroglossia, omphalocele, and growth retardation, and hemihypertrophy is caused by a mutation located on 11p15, also known as the WT2 gene locus (11). Normal kidneys complete differentiation at the end of the 36th week of gestation; however, in about 1–2% of newborns, nephrogenic blastemal cells, also called nephrogenic rests, persist. WT is thought to arise from these nephrogenic rests (12). These nephrogenic rests have been detected in almost 35% of unilateral and 100% of bilateral WT patients (13). The prognosis of WT is closely related to its histology. An unfavorable prognosis occurs when the tumor consists of anaplastic cells; the prognosis is favorable otherwise. This anaplastic type represents 11.5% of all WTs and is responsible for 52% of the mortality rate (14). However, for pretreated patients, there exist three subgroups: low-, intermediate-, or high-risk tumor, according to the Stockholm working classification of renal tumors used by the Societe Internationale D'oncologie Pediatrique (SIOP) (15). WT is associated with mutations in various tumor suppressor genes (16). In the anaplastic type of WTs, p53 mutation is frequent (17).

Clinical presentation

WT is usually unilateral, and the mean age of presentation is 3.3 years. In 4 to 7% of cases, they are synchronous bilateral and appear at a younger age (mean age of 2.6 years) (18). The most common clinical presentation of WT is an abdominal mass. Over 90% of children are referred with an abdominal mass. Other common symptoms are abdominal pain, macroscopic hematuria, fever, and hypertension (19). Abdominal pain can be a symptom of a rupture or intratumoral hemorrhage. Macroscopic hematuria may occur when the tumor has extended to the collecting system (20). Nonspecific symptoms, such as microscopic hematuria, urinary disturbances, malaise, weight loss, and anemia, may be present at initial presentation. If spermatic veins are occluded, varicocele can occur (3) and a thorough examination of the abdomen is necessary. Varicocele is always an alarming symptom, but even more if it occurs on the right side [symptom of inferior vena cava (IVC) thrombosis] or occlusion of the right spermatic vein by the lower part of the tumor (right spermatic vein goes directly to IVC). During the physical examination, a firm, nontender mass in the abdomen is usually identified. Due to elevated renin levels, a follow-up of the blood pressure is very important in WT patients. The most common intravascular tumor extension sites are the renal vein, IVC, and atrium (21). Although the lung is the most common metastatic site, respiratory symptoms are not common. In summary, during examination, the associated anomalies should be considered, and the patient should be examined for aniridia, hemihypertrophy, and genitourinary anomalies.

Diagnosis

Ultrasonography (US) is the primary diagnostic tool for children suspected of having WT. US allows for measuring the tumor size, identifying its origin, establishing the relationship between vena cava and aorta, as well as possible IVC or renal vein. The second important diagnostic tool is computerized tomography (CT). CT is recommended for WT along with US (Figure 1). During interpretation of the CT, and also the US, the contralateral kidney and the liver should always be carefully examined. Magnetic resonance imaging (MRI) is also helpful in detecting the vascular involvement. MRI, although requires longer general anesthesia in the preschool age, offers the most accurate imaging of the kidney. It serves best when nephron-sparing resection is considered or in case of the need to distinguish between WT and nephroblastomatosis. A plain chest X-ray is a routine procedure for the evaluation of pulmonary metastases. A routine pulmonary CT is still controversial (22); however, many physicians prefer the thoracic CT because of its high sensitivity. The definite diagnoses of WT and its subtypes are made by histological evaluation. The preoperative laboratory tests that should be performed are the total blood count, renal and liver function tests, calcium level examination, and urinary examination. In rhabdoid types, the serum calcium increases (23).

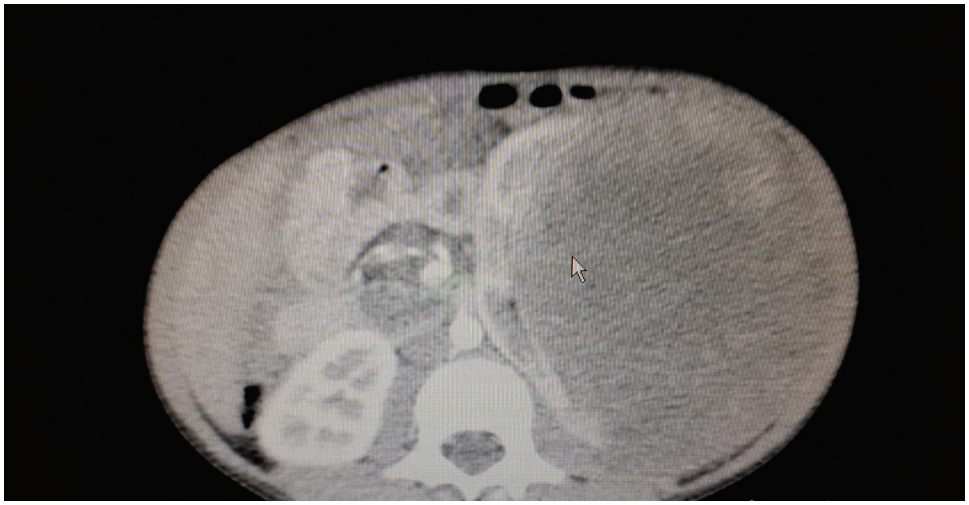

Figure 1. A CT scan of a left Wilms tumor.

In 8% of children with WTs, von Willebrand disease is a comorbidity (24). The routinely evaluated tumor markers include neuron-specific enolase, lactate dehydrogenase, alpha-fetoprotein, β-human chorionic gonadotropin, and ferritin. High-risk patients for the growth of WTs, such as patients with Beckwith-Wiedemann syndrome, should be routinely examined with an US (25). In addition, a 24-hour urine catecholamine test is essential to avoid misdiagnosing with neuroblastoma.

Staging

There are currently two major staging systems, the National Wilms Tumor Study (NWTS) and the SIOP, as summarized in Tables 1 and 2, respectively (26).

Surgical management

Surgery is the cornerstone for the treatment of WT. The Children's Oncology Group (COG) from North America, a group that conducted the NWTS trials, recommends surgery before chemotherapy, whereas SIOP in Europe suggests preoperative chemotherapy (26). As the SIOP group, the National Wilms Tumor Study Group (NWTSG) has concerns about performing a biopsy first because of the risk of tumor upstaging (27). The SIOP recommends preoperative chemotherapy to decrease the risk of intraoperative rupture, downstage the tumor, and to reduce the need for irradiation. The advantage of preoperative chemotherapy is the identification of chemoresistant high-risk blastemal predominant subtype that benefits from treatment intensification.

Table 1. The NWTS staging system*

Stages	Description
Stage 1	(a) Tumor is limited to the kidney and completely excised (b) The tumor is not ruptured before or during removal (c) The vessels of the renal sinus are not involved beyond 2 mm (d) There is no residual tumor apparent beyond the margins of excision
Stage 2	(a) Tumor extends beyond the kidney but is completely excised (b) No residual tumor is apparent at or beyond the margins of excision (c) Tumor thrombus in vessels outside the kidney is stage II if the thrombus is removed en bloc with the tumor
Stage 3	Residual nonhematogenous tumor is present and confined to abdomen (a) Lymph nodes in the renal hilum, the periaortic chains, or beyond are found to contain a tumor (b) Diffuse peritoneal contamination by the tumor (c) Implants are found on the peritoneal surfaces (d) Tumor extends beyond the surgical margins either microscopically or grossly (e) Tumor is not completely resectable because of local infiltration into vital structures
Stage 4	Hematogenous metastasis or lymph node metastasis
Stage 5	Bilateral renal involvement

*This system relies on surgical and pathological evaluation of a tumor in patients not submitted to the preoperative chemotherapy (4).

The management of WT requires a multidisciplinary approach with a pediatric radiologist, an oncologist, a surgeon, and a radiotherapist. First, the patient must be carefully evaluated using appropriate imaging techniques in the preoperative period to identify the origin of the tumor, its position in relation to the adjacent tissue, and vascular involvement. The bilaterality of the tumor, and presence of nephrogenic rests, must be evaluated. If the imaging is not suggestive of any bilateral lesion, there is no need to explore the contralateral kidney at surgery (28, 29). Among the results of WTSG-4, WT is detected in 7% of the patients whose preoperative abdominal CT of contralateral kidney is normal (30). An abdominal Doppler US is advised to check for possible thrombus in the renal vein and the IVC. In tumors with a renal vein and caval extension, it is advised to delay surgery and to start with chemotherapy (31). If the thrombus extends to the thoracic vena cava, an echocardiography should be performed. In case of the thrombus extending to the heart, the risk of pulmonary thrombosis produced by fragmented floating atrial/ventricular thrombus versus benefits of chemotherapy-induced regression must be carefully balanced. Some of these patients are cardiosurgical emergencies

Table 2. The SIOP staging system*

Stages	Description
Stage 1	(a) Tumor is limited to kidney and is completely resected (resection margins "clear") (b) The tumor may be protruding into the pelvic system and "dipping" into the ureter (but it is not infiltrating their walls) (c) The vessels of the renal sinus are not involved (d) Intrarenal vessel involvement may be present
Stage 2	(a) The tumor extends beyond kidney or penetrates through the renal capsule and/or fibrous pseudocapsule into perirenal fat but is completely resected (resection margins "clear") (b) The tumor infiltrates the renal sinus and/or invades blood and lymphatic vessels outside the renal parenchyma but is completely resected (c) The tumor infiltrates adjacent organs or vena cava but is completely resected
Stage 3	(a) Incomplete excision of the tumor, which extends beyond the resection margins (b) Any abdominal lymph nodes are involved (c) Tumor rupture before or intraoperatively (regardless of other criteria for staging) (d) The tumor has penetrated through the peritoneal surface (e) Tumor thrombi present at resection margins of vessels or ureter, transected or removed piecemeal by surgeon (f) The tumor has been surgically biopsied (wedge biopsy) prior to preoperative chemotherapy or surgery
Stage 4	Hematogenous metastases (lung, liver, bone, brain, etc.) or lymph node metastases outside the abdominopelvic region
Stage 5	Bilateral renal tumors at diagnosis

*This system relies on findings at postchemotherapy tumor nephrectomy and the microscopical examination of the whole sample.

and therefore an oncology-oriented pediatric surgeon and a cardiac surgeon are necessary, and the equipment must include the cardiopulmonary bypass device.

For unilateral WTs, a transperitoneal radical nephrectomy is the standard operation. Nephron-sparing surgery (NSS) is advocated only in selected cases of patients with solitary kidney or bilateral WTs (20).

Before the incision, rolled sterile pads should be placed under the patient in supine position. A nasogastric tube, a transient catheter, an arterial line, and a Foley catheter are placed in our center (Figure 2). A large transverse supraumbilical incision should be made, permitting the exploration of the entire abdomen and the contralateral kidney, if necessary (Figure 3). In patients with an intracardiac extension, where an upfront excision is not encouraged, a chevron incision or a combined transverse abdominal and sternal incision can be performed. Because the rupture of the tumor capsule and the spillage of the tumor increase with the tumor stage, and decrease survival, it is important to perform a suitably long incision to remove the tumor from the abdomen safely. At the beginning of the operation, the abdomen should be explored for any intra-abdominal implants. If deemed necessary based on preoperative imaging, the opening of Gerota fascia and careful exploration of the whole contra lateral kidney is recommended. Any suspicious areas should be biopsied. The peritoneum covering the tumor is opened as laterally as possible for easy closure of the peritoneum after the tumor resection (Figures 4 and 5). Most frequently, the best access to the right-sided WT is Kocher' maneuver, which offers a good exposition of the right renal vessels. For left-sided WTs, laterocolonal access is usually sufficient (Figure 6). The renal vein and the IVC should be palpated first to exclude thrombus. The nephrectomy should begin by ligating the renal artery to avoid thrombus embolism. Afterward, the renal vein should be ligated immediately (Figure 7). The ureter should be ligated as low as possible. Titanium clips are mostly used for the marking of the tumor area for possible further radiotherapy. An en bloc resection of the tumor without any tumor spillage is the most important aspect. Lymph node sampling is another important goal in WT surgery. Lymph node samples should be collected from the renal hilum, iliac, paracaval, or para-aortic areas for accurate staging. Spillage of the tumor or inadequate lymph node dissection results

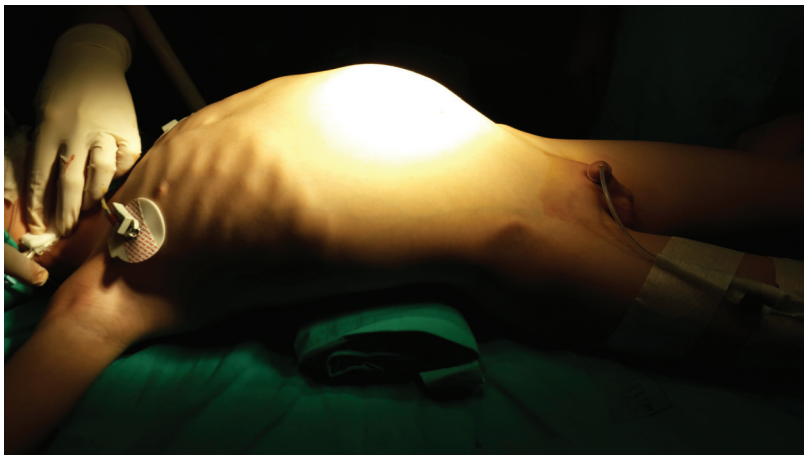

Figure 2. The position of the patient.

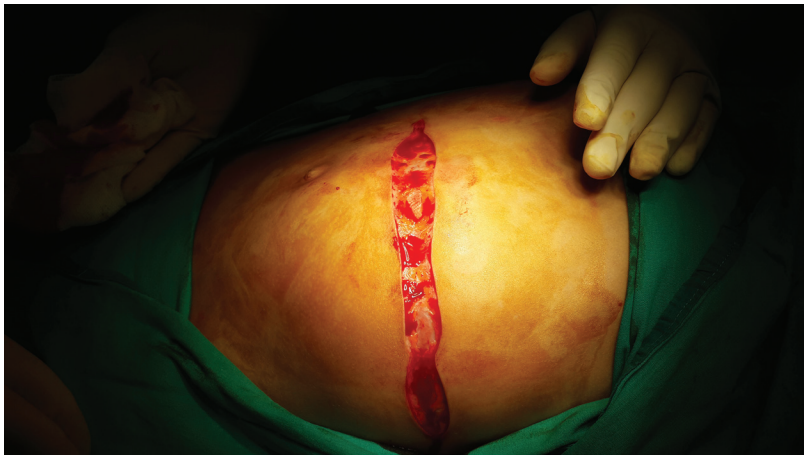

Figure 3. A large transverse supraumbilical incision, permitting the exploration of the entire abdomen and the contralateral kidney.

in incomplete chemotherapy and thus decreased survival rates. NWTS suggests seven regional nodes to get reliable information on lymph node involvement (32).

There is an emerging consensus on performing routine adrenalectomy during unilateral nephrectomy for WTs. One recent study proved that an adrenalectomy does not affect the 5-year survival rate, but it does increase the intraoperative spillage rates (33). Most surgeons leave the adrenal gland intact if it is not infiltrated by the tumor (34). In the study of van Waas et al. (35), it is stated that the removal of one adrenal gland does not result in clinical adrenal insufficiency.

Performing NSS by partial nephrectomy or enucleation in unilateral cases is also a matter of debate (36). We recommend this approach only in synchronous or metachronous bilateral cases or in solitary kidneys. Only less than 5% of all unilateral WTs are eligible for NSS because most of the tumors are locally advanced at the time of diagnosis (37). The surgical criteria for a partial resection are as follows: tumor is located in one pole and infiltrates approximately less than 1/3 of the kidney; no invasion of the renal vein; and the surgeon's experience in pediatric oncology (38). The SIOP WT 2001 trial reported 91 children (3%) with excellent survival rates in which NSS was performed (39). Minimally invasive nephrectomy can offer the same outcome as the classical laparotomic approach (40, 41).

For bilateral cases, the current approach is preoperative chemotherapy followed by bilateral NSS (42). Timing of surgery is important. Both COG/NWTSG and SIOP/RTSG recommend surgery after 9–12 weeks of chemotherapy. Where possible, both sides can be operated in the same session. In difficult cases, the easier side can be operated first. The more difficult

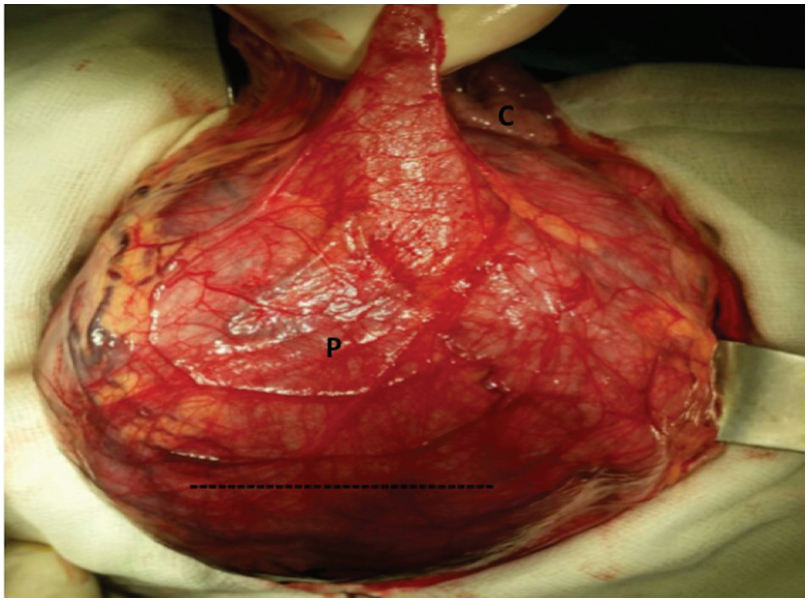

Figure 4. The peritoneal reflection covering a right Wilms tumor. P: peritoneum.

side can be operated after one or two courses of chemotherapy and stabilization of the renal function. The major goal of bilateral WT surgery is to achieve a cure by removing all tumoral renal tissue while preserving the maximum functioning kidney. Some authors suggest *in situ* topical cooling or perfusion with preservation solutions for meticulous dissection without the loss of renal function from ischemia. The ultrasonic scalpel is a useful tool in such resections (43). Millar et al. (44) reported 19 children with bilateral WT cases in which they achieved good results with appropriate chemotherapy and conservative NSS. They suggested a revision surgery if needed. Perioperative US is also a useful tool in NSS of bilateral cases for detecting the margins of normal renal tissue and the tumoral tissue.

Intracardiac extension of WT has been an important surgical challenge. First, an accurate preoperative radiological evaluation of the tumor and thrombus is necessary, and second, a multidisciplinary treatment plan by the cardiovascular surgeon and the pediatric surgeon is important. In general, upfront treatment with chemotherapy is advised. Then, surgical treatment can be considered as reported by SIOP group, which shows favorable results in patients who underwent surgery, including a cardiopulmonary bypass and hypothermia (45). Some surgeons have used this reported technique in pediatric WT cases (46–48).

Common surgical complications in primary nephrectomy patients with WT are ruptures, intestinal obstruction, bleeding, and surgical site infections. NWTSG conducted an

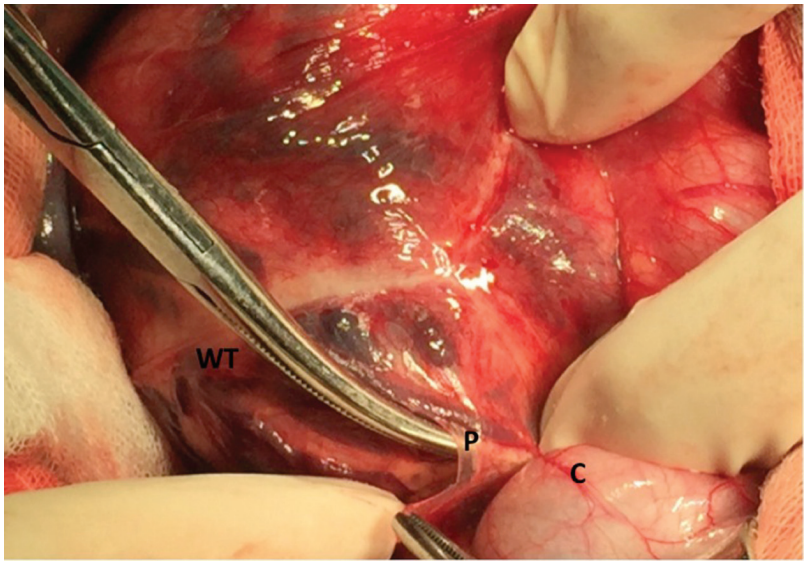

Figure 5. The lateral peritoneal reflection opened from an avascular area on the lateral side. WT: Wilms tumor, P: peritoneum, C: colon.

analysis on 3335 children who received primary nephrectomy for WT. They observed surgical complications in 12.7% of the patients. These complications were intestinal obstruction (5.1%), excessive bleeding (1.9%), surgical site infection (1.9%), and major vascular injuries (1.5%). Also, the risk of complications increased when the tumor size had exceeded 10 cm (49). The risk of tumor rupture was 15% in NWTSG for primary surgery and 3% for chemotherapy-pretreated cases in SIOP. This was replicated by a randomized study in UK (50). Godzinski (51) reported that not only the intraoperative tumor rupture but also other surgery-related complications became rare after the pretreatment. The rate of these complications did not exceed 8% in the pretreated patients. In selected cases, preoperative biopsy was considered. Tru-cut biopsy instead of a needle biopsy may be used to avoid upstaging of the tumor. Preoperative chemotherapy is advised in most bilateral cases, inoperable tumors, and tumors with an intracaval/cardiac extension (43).

Recurrent disease

About 10–15% of WT results in recurrence (52). Spillage occurs in almost every 10 unilateral nephrectomies and is correlated with right-side and larger tumors (53). The recent analysis of COG demonstrated that a relapse in the flank or abdominal site occurred in only 7.4% and 9.5%, respectively, of stage II WT patients with spill, whereas it occurred in 2.5% and 3%, respectively, for those without spill (54). According to SIOP trials, even though

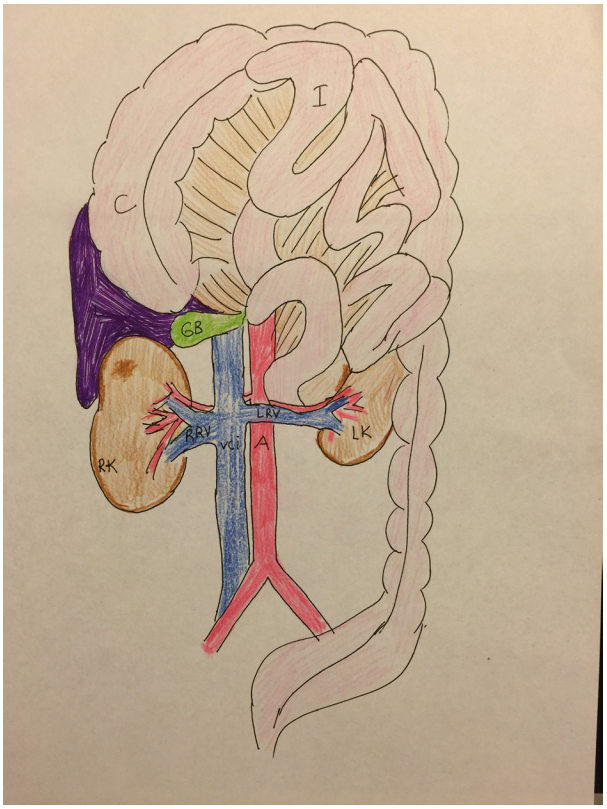

Figure 6. Image showing access to the right-sided WT in which the colon is reflected medially. C: Colon, I: ileum, GB: gall bladder, RK: right kidney, LK: left kidney, RRV: right renal vein, LRV: left renal vein, VCI: vena cava inferior, A: aorta.

the rupture frequency was much less in prechemotherapy group, event-free follow-up or overall survival rate was reported similarly (55). Drugs such as platinum compounds, ifosfamide, cyclophosphamide, etoposide (ICE), and their combinations are used in relapsed WT. Postrelapse survival rates of 50–60% have been reported with ICE chemotherapy (56). Survival rates also depend on the initial stage, initial treatment, metastatic burden, and the relapse-free interval (57). A complete resection of the recurrent lesion(s) has also been shown to be a favorable prognostic factor.

Conclusion

Whether chemotherapy is given preoperatively or not, surgery comprises the main part of the WT surgery. WT patients need a multimodal, multidisciplinary treatment with a close follow-up.

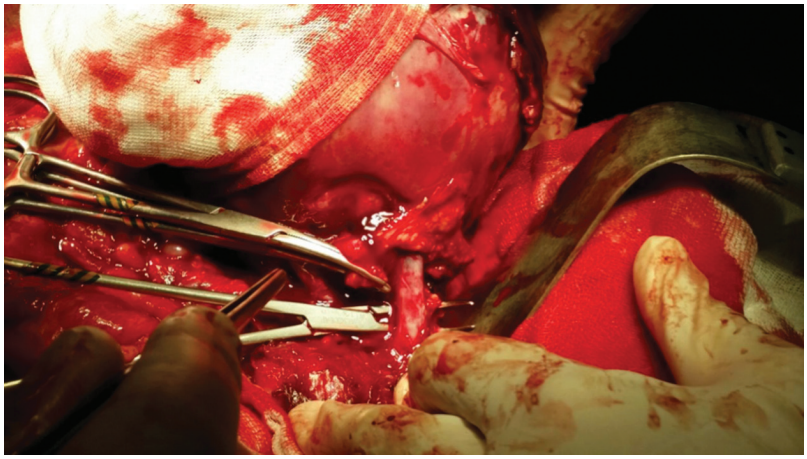

Figure 7. The ligation of the renal vein.

Conflict of Interest

The author declares no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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Chapter 5

Management of Bilateral Wilms Tumours

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Abstract

Synchronous bilateral Wilms tumours (BWTs) represent 4% to 7% of all Wilms tumours (WTs) and present at a younger age than unilateral WTs do. At least 10% of synchronous BWTs have unfavourable histology, and up to 22% are associated with genitourinary abnormalities, aniridia, WAGR (WT, aniridia, genitourinary anomalies, and retardation) syndrome, Denys–Drash syndrome, hemihypertrophy or one of the other overgrowth syndromes. The long-term disease-free survival rate of patients with unilateral WT is now approaching 90% and is around 70% for those with metastatic disease. For both synchronous and metachronous WTs, the prognosis is less favourable, with reported cure rates

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approaching 80% in the best centres but are often considerably less in resource-poor settings. Also, there is the potential for a reduced quality of life due to renal insufficiency and the possible need for renal transplantation. Thus, the major clinical challenge in BWTs is the preservation of functioning renal tissue, while achieving cure with the minimum of therapy-related morbidity. Although mortality is generally associated with progressive disease of anaplastic tumours, the emphasis of management has been increasingly placed on nephron-sparing surgical approaches in an attempt to reduce ultimate renal insufficiency. Chemotherapy followed by nephron-sparing surgery has been able, in most cases, to eradicate the tumour while preserving renal function. Radiotherapy has largely been avoided because of fear of long-term radiation injury to the residual functioning renal mass. Patient selection, appropriate pre- and post-operative chemotherapy, and skilful surgical techniques all contribute to excellent outcomes, where these are achievable.

Key words: Bilateral Wilms tumour; Nephroblastomatosis; Nephron-sparing surgery

Introduction

Nephroblastoma named after Max Wilms (1867–1918), a German surgeon, who published a monograph on 'Mixed tumours of the kidney' in 1899 while in Leipzig, working under the famous surgeon Professor Friedrich Trendelenburg, has become synonymous with the name "Wilms" (1). The tumour is an embryonal tumour derived from the metanephros containing epithelial, blastemal and stromal tissues. It is the third most frequently seen paediatric malignancy, is the most common renal tumour of childhood and is particularly seen in the under 5-year age group. It is associated with a number of 'overgrowth' syndromes mostly related to chromosome 11, as well as other genitourinary abnormalities.

Synchronous bilateral Wilms tumours (BWTs) make up 4% to 7% of all Wilms tumours (WTs) and present at a younger age than unilateral WTs (mean age, 2.6 vs ~3.3 years) (2, 3) do. Current results after the treatment of unilateral WT are excellent, even in some resource-limited settings, and protocols of management have been extensively interrogated on both sides of the Atlantic, in Children's Oncology Group (COG) and Société Internationale d'Oncologie Pediatrique (SIOP) trials. Both investigative groups have equivalent final outcomes with the 5-year overall survival in the region of 90% for favourable-histology unilateral WT, although there are differences between the groups in the approach to management, that is, in the use and timing of chemotherapy, surgery and radiotherapy. This has been emulated in some middle-income settings (4), but the outcome in patients with bilateral tumours is not as favourable, with the overall survival at 4 years varying between 81% for favourable histology and 55% for anaplastic histology in the National Wilms Tumor Study-5 trial (5, 6) and the overall 5-year survival being 85% in the SIOP-9 trial (7).

Predisposing factors

Nephrogenic rests are areas of embryonal metanephric tissue still present after the 36th week of life. Nephroblastomatosis refers to multiple or diffuse rests. While only a few will undergo clonal transformation into WTs, they are considered precursor lesions. Actively proliferating rests, termed hyperplastic rests, can cause the greatest diagnostic challenge and can appear radiologically and histologically similar to a WT (8). Rests, present on histological examination of resected tumours, increase the risk of a metachronous tumour developing in the other kidney, especially in the under 1-year age group. Diligence in follow-up and monitoring is thus required if nephroblastomatosis is present in a resected tumour. In a selected group of infants where unilateral tumours are well circumscribed and where nephroblastomatosis is identified pre- or intra-operatively, nephron-sparing surgery (NSS) may have a place. The NWTS group has recommended that this approach be assessed in a formal study. However, exclusion criteria for NSS such as those used in the SIOP 2001 protocol for unilateral WTs also need to be considered for BWT in order to stay oncologically safe (9). These are preoperative tumour rupture, abdominal lymph node metastases, tumour in the renal vein, multifocal tumour and infiltration into the renal pelvis (10). Nephrogenic rests may be seen in up to 90% of synchronous BWTs and 94% of metachronous BWTs (78% in our series) (11); about 70% of children with synchronous BWT in the NWTS series had multiple nephrogenic rests or nephroblastomatosis (12–14).

The prevalence of bilateral disease is higher among children with genetic predisposition syndromes such as WAGR (WT, aniridia, genitourinary anomalies and retardation), Beckwith–Wiedemann syndrome, hemihypertrophy and one of the overgrowth syndromes. These patients contribute to 22% of BWT series (15, 16).

Investigation

After investigation of history, blood pressure measurement, and physical examination looking specifically for signs of associated syndromes, all patients require routine testing of complete blood count, liver functions and renal functions and also urinalysis. Some centres advise coagulation studies because WT patients can develop acquired von Willebrand disease (17, 18).

Abdominal imaging includes a computed tomography (CT) scan with contrast or magnetic resonance imaging (MRI) (Figure 1a and b). Ultrasound scanning of the abdomen is often performed after clinical examination, which reveals a mass and guides more definitive imaging. In addition, ultrasound with Doppler may be the preferred method to assess intravascular extension of the tumour. MRI is of major value in the identification of nephrogenic rests. Nowadays metastatic workup includes a CT scan of the chest.

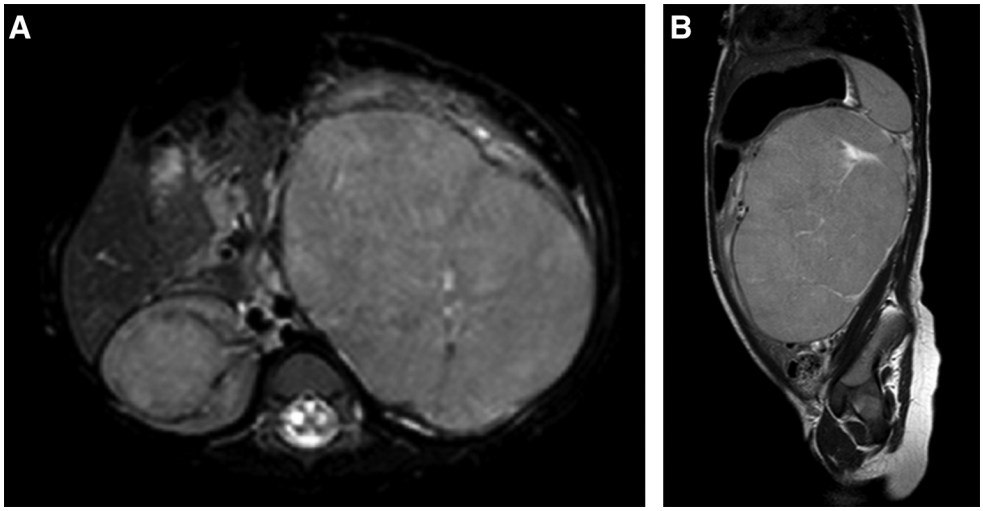

Figure 1. Axial (a) and sagittal (b) MRI scans of a 2-year-old child with bilateral Wilms tumours after 12 weeks of chemotherapy. Reprinted with permission from Red Cross War Memorial Children's Hospital Radiology Department.

There is only scarce evidence that an 18F-fluorodeoxyglucose (FDG)-positron emission tomography (PET)-CT may be helpful in patients with bilateral disease (19). WT is 18F-FDG avid; 18F-FDG-PET-CT imaging adds clinically applicable information to conventional imaging, and avid areas correspond to histologically active disease, but currently there is no evidence that it will discriminate WT from nephroblastomatosis.

Management

The aim of BWT management is to achieve cure with a minimum treatment-related morbidity, with the challenge in BWT being the preservation of adequate functioning renal tissue (20, 21). Renal tissue preservation may, however, be particularly difficult in cases with a delay in presentation and advanced local disease, in cases in which the chemotherapy response is poor, or in cases of a metachronous presentation where a contralateral nephrectomy has already been performed. Management may also be complicated by the presence of multiple nephrogenic rests or areas of nephroblastomatosis, as these are difficult to clinically distinguish from WTs (8, 22).

Management should commence with early clinical diagnosis, and for those presenting with ostensibly unilateral disease, a very close look at the contralateral kidney with biopsy of any suspicious areas is essential (23). Preoperative imaging is increasingly accurate (24) but should not be relied on due to some inconsistencies in imaging characteristics (8) and should not replace careful visualization of the contralateral kidney (25).

Preoperative chemotherapy in synchronous tumours is now the standard of care on both sides of the Atlantic. Previous protocols allowed for primary resection of tumours followed by chemotherapy. While the overall survival in a study by the United Kingdom Children's Cancer Study Group was similar in those undergoing primary surgery followed by chemotherapy and those treated with neoadjuvant chemotherapy followed by surgery, the incidence of renal failure was 20% in the initial surgery group versus 6% in the initial chemotherapy group (26).

With synchronous BWT, the traditional approach has been bilateral renal biopsies and staging of each kidney, followed by neoadjuvant chemotherapy and then renal salvage procedures (partial nephrectomy or tumourectomy) (14, 22). However, data from NWTS-4 and NWTS-5 have shown conclusively that a significant number of patients have unfavourable histology, which is revealed at the time of definitive surgery following chemotherapy but missed during initial biopsy.

Because anaplasia is difficult to detect (27) and bilateral childhood renal tumours are most likely to be WTs, initial biopsy is now controversial in patients with BWT. The current COG study of BWT and of patients with unilateral WT predisposed to the development of bilateral tumours attempts to avoid upfront biopsy but mandates biopsy after 6 weeks of three-drug chemotherapy (AREN0534, National Cancer Institute) (19, 28). The COG studies have shown a higher risk of local recurrence in patients who had tumour spillage or rupture, irrespective of the cause or extent of the soiling (with tumour biopsy being included as a cause of spillage). Thus, in the COG studies, all patients with tumour spillage, including biopsy, are considered stage III. When performing a biopsy, it is important to biopsy all lesions in both kidneys as discordant pathology occurs in up to 20% of cases with favourable histology on one side and anaplastic histology on the other (27).

In suspected BWT, where doubt exists due to atypical imaging or with a patient older than 10 years, an upfront biopsy may be indicated to rule out a non-Wilms tumour (AREN0534) even though it may not rule out anaplasia (28).

While most deaths occur because of progressive disease especially in the case of anaplastic tumours and usually in the first 2 years after diagnosis, emphasis has been increasingly placed on nephron-sparing surgical approaches to avoid subsequent renal insufficiency (15, 16, 29, 30). Nephron sparing is contraindicated in the face of diffuse anaplasia (3) and in cases of the Denys–Drash syndrome (31).

With metachronous presentation, the contralateral kidney has been removed and only the affected side remains. In this scenario, an attempt at NSS is indicated. Alternatively, should excision be performed, the patient may be free of disease but anephric, requiring renal transplantation. This has lifelong consequences of immune suppression and drug toxicity.

Chemotherapy

The current COG BWT study protocol is a response-based protocol. Neoadjuvant chemotherapy consists of three drugs (vincristine, dactinomycin and doxorubicin) given for 6 weeks, with a provision for a further 6 weeks if NSS is not feasible. Surgery is mandated at week 12. This protocol does not require pretreatment biopsies because bilateral tumours are very rarely clear cell sarcoma or rhabdoid tumours of the kidney. They are invariably WT, and therefore, biopsy does not change the therapy. In addition, anaplasia is difficult to diagnose, and biopsy may upstage the tumour and increase the chance of local recurrence.

SIOP (32) uses vincristine and dactinomycin for preoperative chemotherapy for 8 weeks, adding doxorubicin after 4 weeks only if there is a poor response. Despite adequate neoadjuvant chemotherapy, there is a subgroup of patients with BWT with progressive or non-responsive disease. This non-responsiveness may be due to anaplasia, and thus insensitivity to administered therapy, necrosis and rhabdomyomatous or mature stromal differentiation (33, 34). While the second group of patients do not respond radiologically to therapy, they have improved outcomes when compared with those with anaplasia. It is thus crucial to establish the exact histology, and therefore, all such patients are best served by surgery.

Post-operative chemotherapy is based on the histology of the surgical specimen and the stage and is chosen according to the kidney with the highest risk. Cyclophosphamide, etoposide and carboplatin are added for unfavourable histology.

Surgery

The surgery for BWTs needs a multidisciplinary approach for diagnosis and treatment, and BWT patients benefit from surgery in the national centres of excellence with the most experience in BWT.

The decision to operate on both kidneys at the outset or to do so sequentially depends on the size and site of the tumours and their response to therapy. In every case, assessment of differential renal function by way of radionucleotide scan is mandatory prior to surgery. In cases with small peripheral tumours, both sides can be approached at the same laparotomy. When the tumours are very large and/or centrally placed, we prefer to operate on the difficult side first so that we are aware of how much renal function remains before operating on the relatively easier side.

Traditionally, these tumours are resected via a transabdominal approach. More recently, a retroperitoneal approach has been advocated, citing earlier post-operative recovery and slightly less associated surgical morbidity with equivalent outcomes when compared to the usual transabdominal approach, but this approach is yet to be widely adopted (35).

Several innovations have facilitated the NSS. In situ topical cooling with vascular pedicle cross-clamping or ex situ perfusion with preservation solution and 'bench surgery', first described by Lilly and Starzl in 1975 (36), allows careful and extensive dissection and reconstruction in a bloodless field without the loss of renal function from ischaemia (37). Using in situ topical cooling results in a relatively short cross-clamp time and topical cooling with ice is sufficient to preserve the renal function in most cases. Bilateral extensive NSS with our technique of ice-dam cooling (Figure 2) without the need for bench surgery does allow for better preservation of renal function and avoids significant acute tubular necrosis in most cases. However, some surgeons have preferred not to use any form of cooling (38).

It is important to avoid traction or torsion of the renal artery to prevent spasm, intimal damage and vessel occlusion. Some surgeons have specifically avoided using a clamp that may injure the vessels and have used finger occlusion instead (38). Either way, prior to commencing the resection, the kidney should be fully mobilized on its vascular and ureteric pedicle. The resection line is then marked on the tumour mass, identifying the normal renal tissue which is to be preserved, prior to any cross-clamp in an attempt to minimize the ischaemic time. Considerable mobilization of the kidney is possible as the renal vessels usually display an increased diameter, due to the increased needs of the growing tumour prior to chemotherapy, and this allows for making surgical access relatively easy. At this point, the renal hilar and peri-aortic nodes are sampled to rule out lymphatic spread. The renal vein and inferior vena cava should be palpated for evidence of tumour extension.

Gerota's fascia is opened, and the perirenal fat is dissected off the renal surface, excluding the fat attached to the tumour mass. If the tumour is peripherally situated or is well localized to an upper or lower pole, a wedge or guillotine tumourectomy or partial nephrectomy is performed. For large tumours, the outline of the tumour to be resected is scored with diathermy on the renal mass prior to proceeding with the surgery (Figure 3). The capsule is peeled back to expose the adjacent renal parenchyma. Using either fine bipolar cautery or the ultrasonic scalpel in our centre to facilitate dissection, as both cut well in a wet environment, we resect the tumour while the residual part of the kidney to be preserved remains in ice (Figure 4). Fuchs has described three zones of technical repair, with the cortex lending itself to cautery haemostasis, the medulla to suture/ligation haemostasis and the pelvicalyceal system to fine absorbable suture repair (39). After the pelvi-calyceal repair, a double J stent can be placed to ensure better urine drainage and thus reduce the chance of a urine leak. The Cavitron ultrasonic surgical aspirator (Cavitron Surgical Systems, Stanford, CA) has also been used effectively by Ritchey and Coppes (37). Where possible, the residual renal parenchyma is 'folded' on itself with suture reconstitution of the renal capsule to achieve a near-normal post-operative appearance (Figure 5).

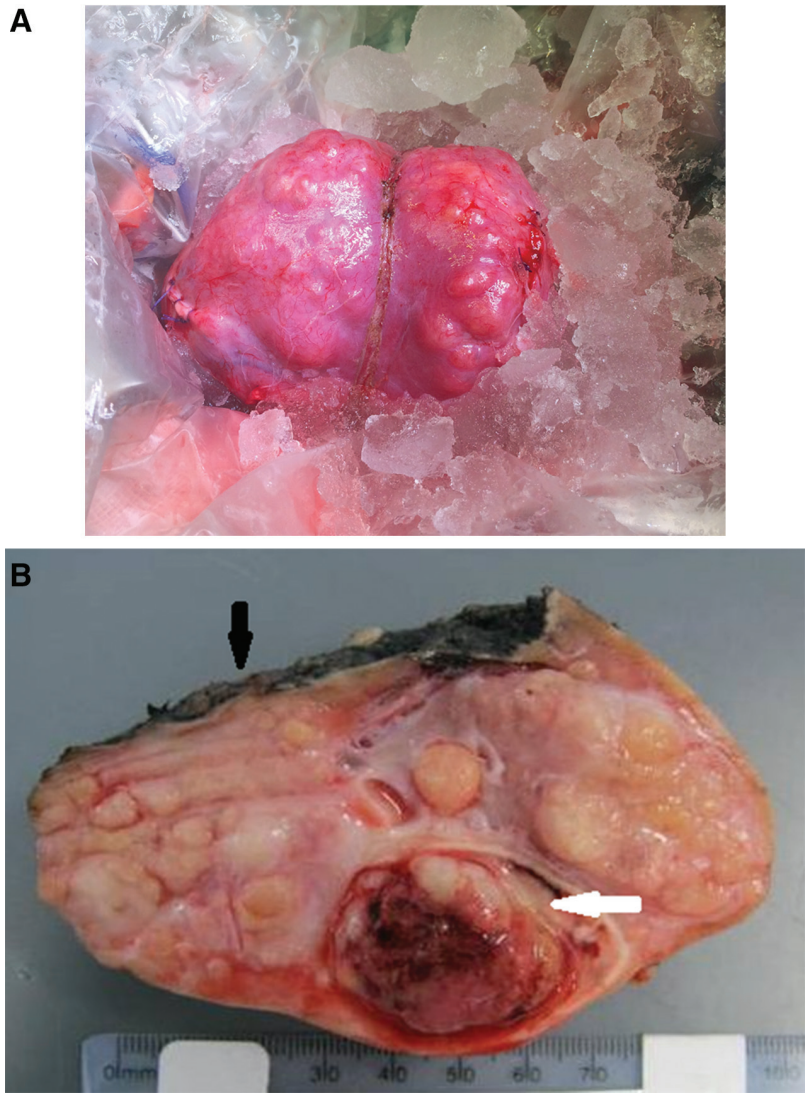

Figure 2. (a) Ice-bath kidney cooling in a 2-year-old child with bilateral nephroblastomatosis and an upper-pole Wilms tumour in the kidney. The score line indicating the line of dissection is clearly shown. (b) The resected specimen (cut surface shown by black arrow) shows the anaplastic Wilms tumour (white arrow) surrounded by nephrogenic rests. Reprinted with permission from Red Cross War Memorial Children's Hospital Surgery and Pathology Department.

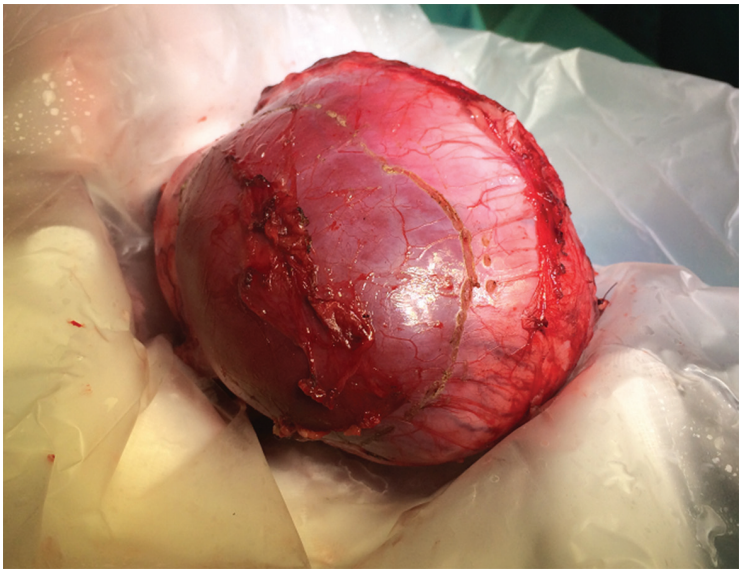

Figure 3. The left kidney of the child depicted in Figure 1. The kidney is cooled in an ice bath after dissection of the kidney to isolate the pedicle, and the outline of tumour to be resected is scored with diathermy on the renal mass. Reprinted with permission from Red Cross War Memorial Children's Hospital Surgery Department.

In exceptional circumstances, bench surgery with autotransplantation can be performed, as demonstrated in one of our cases (37, 40). Vessel re-anastomosis can usually be performed in the orthotopic position as the renal artery usually has an enlarged diameter; alternatively, the reconstructed residual kidney can be transplanted onto the iliac vessels. The disadvantage, however, is that it is very difficult to visually discriminate between tumour, nephroblastomatosis and normal renal tissue while performing ex vivo perfusion. In this situation, multiple frozen-section biopsies may be required. These are generally not satisfactory as these can also be difficult for the histopathologist to interpret. At the end of the procedure, a drain is inserted and a Foley catheter should remain in the bladder for a few days. The double J stent should be removed via cystoscopy in 4 to 6 weeks. If the surgical specimen reveals diffuse anaplasia and there is incomplete resection, additional surgery is indicated to ensure complete resection of the tumour (28). Successful bench surgery and subsequent contralateral tumourectomy were performed in one patient in our series. The patient then developed recurrence of tumour in that kidney, but he remains a long-term disease-free survivor with good renal function after a second resection.

Enucleation of the tumour by blunt dissection should only be considered for patients with favourable-histology WT. If anaplasia is present, enucleation is contraindicated as clear margins are mandated for anaplastic tumours (28).

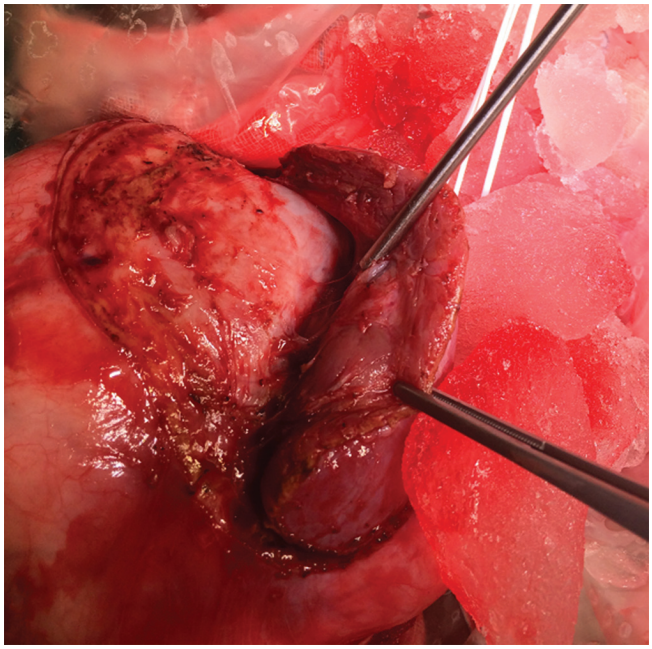

Figure 4. Dissection of the 'fillet' of normal renal tissue in the same child. Reprinted with permission from Red Cross War Memorial Children's Hospital Surgery Department.

Tumours not responding to chemotherapy and still not amenable to NSS may require nephrectomy. In this instance, the dissection plane is outside the Gerota's fascia. Unless the mass is in the upper pole or the adrenal is abutting the mass, the adrenal gland should be left in situ.

Resection of local recurrence in the residual kidney is possible, and it has shown some promising results (41). In our series, apart from the patient indicated above, there have been four other occasions where we have resected recurrences with good long-term results, two of which had tumour extending into the pelvi-calyceal system.

Radiotherapy

In BWT, radiotherapy use has decreased over the years; 57% of patients on NWTS-2 and NWTS-3 (12) received renal or renal-bed irradiation, while 42 (21.4%) of the 196 renal units registered on the renal salvage procedure arm of NWTS-4 were treated with radiotherapy (14). Radiation therapy is considered for abdominal stage III tumours or for stage II cases of anaplasia with involved margins at tumour resection. Its use could come at the cost of reduced renal function, particularly in young patients with the added toxicity of anthracycline chemotherapy (42). Fortunately, it has been shown that anthracyclines can be omitted

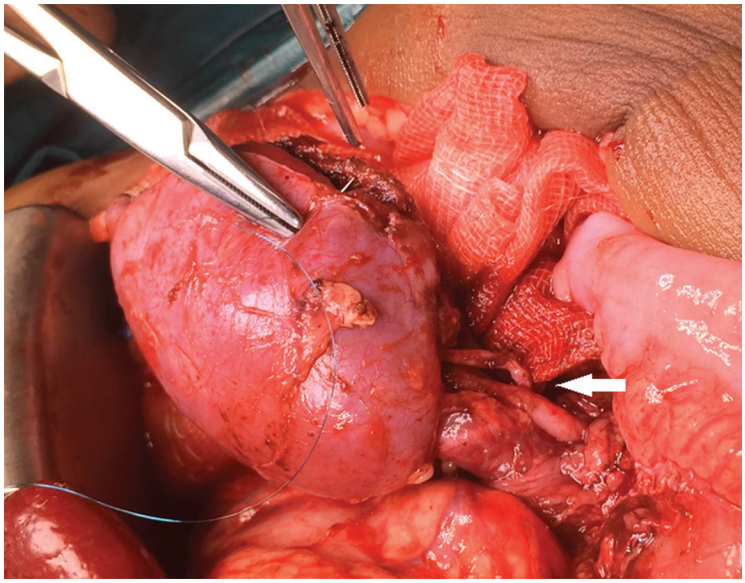

Figure 5. The residual kidney folded over to close the renal capsule with preserved renal artery and vein (arrow). Reprinted with permission from Red Cross War Memorial Children's Hospital Surgery Department.

in Intermediate risk stage III cases in SIOP; however, this needs to be determined in stage V cases (43). Other than cardiomyopathy and second malignant neoplasms, renal failure is the most common source of morbidity in BWT.

Prognosis

The variables identified for poor outcome have been unfavourable histology, advanced (local) stage and age over 3 years at diagnosis. Six of the seven patients in our series who presented with metastases died, although none had anaplastic histopathology (44). About 1% of unilateral WT will subsequently develop a contralateral tumour, with the risk for this tumour development being higher in children younger than 12 months who have nephrogenic rests at diagnosis (10).

Renal failure is an obvious concern in patients with BWT, the aetiology being multifactorial, and it can be due to the compounded effects of the inherent renal disease related to the patient's genetics, nephrotoxic chemotherapy, effects of radiotherapy and the loss of renal parenchyma. Risk factors for renal failure include Denys–Drash and other congenital syndromes, metachronous tumour and progressive disease in BWT with the need for bilateral nephrectomies and radiation nephritis, while the greatest risk factor is that of BWT (40, 45).

The risk of renal failure increases with the loss of more than 50% of renal mass. With the changing nature of treatment and the increasing efforts to preserve renal parenchyma, the rate of renal failure has decreased from 16.4% in NWTS-1 and NWTS-2 (1969–1979) to 9.9% in NWTS-3 (1979–1986) and 3.8% in NWTS-4 (1986–1998) (46). However, more recent long-term studies reflect a higher incidence of renal failure. The SIOP 9301 study reported a 14% rate of end-stage renal disease in 49 children treated from 1993 to 2001 (16). Dekkers et al. (47) showed that tumour nephrectomy, as well as radiotherapy, carries a higher risk of impaired renal function and hypertension. Nonetheless, the absence of significant renal impairment among most survivors is a proof of the success of NSS following initial chemotherapy. However, all BWT patients are eligible to be surveilled and to have systematic follow-up of blood pressure, urine protein and renal function.

In the few patients requiring bilateral nephrectomy, such as those with unresectable tumours or Denys–Drash syndrome, renal transplantation is usually performed between 1 and 2 years without the evidence of malignancy (34). The general consensus is to wait till at least 2 years of disease-free survival for a cadaver donor and 1 year for a living, related donor (34).

Conclusion

With appropriate patient selection and both pre- and post-operative chemotherapy and skilful surgical techniques, an excellent outcome can be achieved in most cases.

Conflict of Interest

The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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Chapter 6

Extrarenal Wilms' Tumor: Challenges in Diagnosis, Embryology, Treatment and Prognosis

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Abstract

Wilms' tumor is one of the most common childhood solid malignancies, which classically arises from primitive metanephric cells, but exceptionally it may arise in places other than kidneys. Extrarenal Wilms' tumor is a rare but challenging entity, considering its diagnosis,

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histopathology, staging, treatment, and prognosis. Diagnosis of extrarenal Wilms' tumor is always postsurgical, which may jeopardize treatment planning and consulting with parents in the first step. The histopathology of Wilms' tumor is very confusing. While most authors believe that it arises from primitive ectopic nephrogenic rests, teratoid Wilms' tumor leads to the debate whether this tumor is neoplastic or embryonic. Staging of extrarenal Wilms' tumor is also a challenge when we consider the National Wilms' Tumor Study (NWTS) recommendations; all these tumors should be considered as stage II or higher as they are beyond the renal capsule. This will mandate chemotherapy for all patients while most of the reported cases have a favorable histology, and long-term tumor-free survival has been reported even with exclusive surgery in some case reports. Although treatment strategies for extrarenal Wilms' tumor are the same as those for renal Wilms' tumor, different locations and neighboring organs may invoke special considerations and scenarios while planning for surgery and adjuvant therapies. Consulting with the parents is also a problem, considering the rarity of the disease and limited publications. In this chapter, we discuss all these topics in detail after a systematic review of extrarenal Wilms' tumor cases to date in order to provide a clear perspective for confronting this rare disease.

Key words: Extrarenal; Nephroblastoma; Pediatrics; Wilms' tumor

Introduction

Nephroblastoma or Wilms' tumor is one of the most common childhood malignancies, which accounts for almost 95% of renal malignancies in pediatrics. Extrarenal nephroblastoma is a rare entity, which was first described by Moyson et al. (1) in 1961. The estimated rate of occurrence of nephroblastoma outside the kidneys is almost 0.5 to 1% of all cases of Wilms' tumor. Extrarenal Wilms' tumor (ERWT) occurs mostly in childhood; however, it is also rarely reported in adults (2). Apart from the primary ERWT, nephroblastoma may be observed outside the kidneys in two other situations: metastatic disease and nephroblastoma arising in a teratoma; therefore, in the case of ERWT, it is mandatory to evaluate the kidneys for primary tumor preoperatively and search the whole specimen for any teratoid element postoperatively (3).

We reviewed all the reported childhood ERWTs (under 14 years), excluding those arising from teratomas (teratoid Wilms' tumor). The results are summarized in Table 1. Among 80 reported ERWT cases, more than 60% were younger than 4 years and a female predominance was observed while the female-to-male ratio was 3:2 (4).

The association of ERWT with a horseshoe kidney has been reported previously, and almost 7% of the reported ERWTs were found to be associated with the horseshoe kidney. Dysraphism is the second commonly found abnormality among ERWT patients (5).

Table 1. Review of the reported childhood ERWTs in literature

Author	Year	Gender	Age	ERWT location	Stage	Treatment	Follow-up
1. Moyson et al. (1)	1961	F	3	Mediastinal	II	Surgery + chemo	NA
2. Bhajkar et al. (6)	1964	M	2	Retroperitoneal	II	Surgery + chemo	NA
3. Edelstein et al. (7)	1965	M	3	Retroperitoneal	II	Surgery + chemo + RAD	2
4. Wu and Garcia (8)	1971	F	7	Pelvic	IV	Surgery + chemo + RAD	1
5. Thompson et al. (9)	1973	F	4	Inguinal	III	Surgery + chemo + RAD	2
6. Thompson et al. (9)	1973	M	3	Inguinal	IV	Surgery + chemo + RAD	0.5
7. Akhtar et al. (10)	1977	M	0.2	Inguinal	II	Surgery + chemo	1.5
8. Gaikwad et al. (11)	1977	M	0.2	Retroperitoneal	II	Surgery	0.5
9. Madanat et al. (12)	1978	F	9	Mediastinal	II	Surgery + chemo + RAD	3
10. Madanat et al. (12)	1978	M	0.3	Inguinal	NA	Surgery + chemo	2
11. McCauley et al. (13)	1979	F	4	Retroperitoneal	NA	Surgery + chemo + RAD	4
12. Aterman et al. (14)	1979	F	5	Retroperitoneal	NA	Surgery + chemo + RAD	0.7
13. Orlowski et al. (15)	1980	M	3.5	Paratesticular	II	Surgery	11
14. Fried et al. (16)	1980	M	3	Retroperitoneal	NA	Surgery + chemo	NA
15. Fernandes et al. (17)	1980	M	6	Retroperitoneal	III	Surgery + chemo + RAD	6
16. Johnson et al. (18)	1980	F	1	Retroperitoneal	NA	Surgery + chemo	1
17. Taylor et al. (19)	1980	M	0.5	Inguinal	NA	Surgery + chemo + RAD	0.5
18. Ho et al. (20)	1981	M	1.2	Paratesticular	I	Surgery	1
19. Bittencourt et al. (21)	1981	F	14	Female genital organs	III	Surgery + chemo + RAD	5.5

(*Continued*)

Table 1. Continued

Author	Year	Gender	Age	ERWT location	Stage	Treatment	Follow-up
20. Tamaro et al. (22)	1982	F	4	Retroperitoneal	NA	Surgery + chemo + RAD	1
21. Fernandes et al. (17)	1982	F	2	Retroperitoneal	II	Surgery + chemo	5
22. Adam et al. (23)	1983	M	10	Retroperitoneal	NA	NA	NA
23. Meng and Jagadeesan (24)	1983	M	3	Retroperitoneal	I	Surgery	1
24. Lüchtrath et al. (25)	1984	F	1.2	Inguinal	NA	Surgery + chemo	1.3
25. Bell et al. (26)	1985	F	13	Female genital organs	I	Surgery	9.5
26. Naito et al. (27)	1985	F	3	Retroperitoneal	NA	Surgery + chemo	2.3
27. Fernandes et al. (17)	1988	F	2	Retroperitoneal	II	Surgery + chemo	1
28. Lai et al. (28)	1888	F	3	Inguinal	II	Surgery + chemo	1.5
29. Narasimharao et al. (29)	1989	F	NA	Retroperitoneal	NA	NA	NA
30. Broecker et al. (30)	1989	F	0.9	Pelvic	II	Surgery + chemo	1
31. Fernandes et al. (17)	1989	M	6	Retroperitoneal	II	Surgery + chemo + RAD	7
32. Broecker et al. (30)	1989	F	2	Retroperitoneal	II	Surgery + chemo + RAD	7
33. Wakely et al. (31)	1989	F	4	Female genital organs	II	Surgery + chemo + RAD	6
34. Broecker et al. (30)	1989	F	2	Retroperitoneal	IV	Surgery + chemo	1.3
35. Wakely et al. (31)	1989	F	1.5	Retroperitoneal	II	Surgery + chemo + RAD	6
36. Strand et al. (32)	1990	M	12	Inguinal	III	Surgery + chemo	NA
37. Simha and Doctor (33)	1991	F	3	Inguinal	III	Surgery + chemo + RAD	NA

(*Continued*)

Table 1. Continued

Author	Year	Gender	Age	ERWT location	Stage	Treatment	Follow-up
38. Andrews et al. (34)	1992	F	NA	Lumbosacral	II	Surgery + chemo	1.4
39. Andrews et al. (34)	1992	M	NA	Retroperitoneal	II	Surgery + chemo	0.7
40. Andrews et al. (34)	1992	F	NA	Lumbosacral	II	Surgery + chemo	6.5
41. Andrews et al. (34)	1992	M	NA	Retroperitoneal	IV	Surgery + chemo + RAD	2
42. Andrews et al. (34)	1992	F	NA	Lumbosacral	III	Surgery + chemo + RAD	4
43. Andrews et al. (34)	1992	F	NA	Retroperitoneal	I	Surgery + chemo	3
44. Andrews et al. (34)	1992	M	NA	Retroperitoneal	II	Surgery + chemo	2
45. Andrews et al. (34)	1992	F	NA	Pelvic	II	Surgery + chemo	0.7
46. Suzuki et al. (35)	1993	M	2	Retroperitoneal	II	Surgery + chemo	NA
47. Rasheed et al. (36)	1993	M	3	Retroperitoneal	III	Surgery + chemo + RAD	7
48. Rasheed et al. (36)	1993	F	4	Retroperitoneal	III	Surgery + chemo + RAD	2
49. Fahner et al. (37)	1995	F	2.5	Lumbosacral	II	Surgery + chemo	1
50. Arkovitz et al. (38)	1996	M	3.5	Inguinal	III	Surgery + chemo + RAD	NA
51. López Cubillana et al. (39)	1997	F	2	Retroperitoneal	II	Surgery + chemo	3
52. Kapur et al. (40)	1998	F		Retroperitoneal	NA	Surgery + chemo	3
53. Kapur et al. (40)	1998	F	NA	Retroperitoneal	NA	Surgery + chemo	0.7
54. Benatar et al. (41)	1998	F	11	Female genital organs	II	Surgery + chemo	NA
55. Massarelli et al. (42)	1999	F	2	Female genital organs	III	Surgery + RAD	2.5

(*Continued*)

Table 1. Continued

Author	Year	Gender	Age	ERWT location	Stage	Treatment	Follow-up
56. Iraniha et al. (43)	1999	F	12	Female genital organs	II	Surgery + chemo	1
57. Govender et al. (44)	2000	M	4	Lumbosacral	III	Surgery + RAD	NA
58. Babin et al. (45)	2000	F	13	Female genital organs	III	Surgery + chemo + RAD	5
59. Arda et al. (46)	2001	F	5	Lumbosacral	III	Surgery + chemo + RAD	NA
60. Oner et al. (47)	2002	F	3.5	Female genital organs	II	Surgery + chemo	7
61. Yunus et al. (48)	2003	NA	NA	Lumbosacral	NA	NA	NA
62. Cojean et al. (49)	2003	M	0.2	Retroperitoneal	III	Surgery + chemo	NA
63. Ngan et al. (50)	2009	F	6	Retroperitoneal	II	Surgery	1
64. Cooke et al. (51)	2009	M	1.2	Inguinal	II	Surgery	2
65. Jeong et al. (52)	2011	M	9	Inguinal	III	Surgery + chemo + RAD	NA
66. Teerthanath (53)	2011	F	6	Retroperitoneal	II	Surgery + chemo	4
67. Chowhan (54)	2012	M	1.3	Retroperitoneal	II	Surgery + chemo	NA
68. Armanda et al. (2)	2012	F	0.1	Lumbosacral	I	Surgery + chemo	2
69. Yamamoto et al. (55)	2012	M	NA	Paratesticular	NA	NA	NA
70. Li et al. (56)	2012	F	2	Pelvic	III	Surgery + chemo + RAD	3
71. Marwah et al. (57)	2012	F	1.2	Retroperitoneal	II	Surgery + chemo	NA
72. Hiradfar et al. (58)	2012	F	9	Inguinal	II	Surgery + chemo	3
73. Baskaran (59)	2013	M	3	Retroperitoneal	II	Surgery	NA
74. Rojas et al. (60)	2013	M	2	Lumbosacral	II	Surgery + chemo	NA

(*Continued*)

Table 1. Continued

Author	Year	Gender	Age	ERWT location	Stage	Treatment	Follow-up
75. Morandi et al. (61)	2013	M	3	Paratesticular	I	Surgery	2
76. Goel et al. (62)	2014	NA	NA	Retroperitoneal	NA	Surgery	NA
77. Wu et al. (63)	2014	M	0.8	Retroperitoneal	II	Surgery	0.5
78. Wu et al. (63)	2014	M	1.5	Inguinal	II	Surgery	0.5
79. Al-Nsoor et al. (64)	2014	F	1.7	Retroperitoneal	II	Surgery	NA
80. Kumar et al. (5)	2015	F	7	Retroperitoneal	NA	Surgery	0.8

Age and follow-up time are in years.
NA, data not available; RAD, radiotherapy.

Embryogenesis

Embryonic nephrogenic tissue normally differentiates into metanephric blastema, which is considered as the precursor of nephrons and mesenchymal stroma. The pronephros is developed during the third gestational week in the cervical region, extending to the more caudal parts, the cloaca. The upper part of pronephros regresses, and in the caudal part, it gives rise to the mesonephric duct that persists in two lateral foci. While the mesonephric duct extends to the cloaca, it enters to the metanephric blastema that surrounds the ureteric bud to develop the kidneys.Therefore, the progenitors of renal tissue goes through a journey from the cranial to the caudal part of the embryo, and during the longitudinal growth of the fetus, the intrapelvic kidneys ascend up to their expected level in the flank.

It is believed that the Wilms' tumor is the result of a developmental abnormality in the metanephric blastema. Persistent metanephric tissue after the 36th week of gestation could be the precursor of nephroblastoma. Nephrogenic rests may be observed anywhere in the craniocaudal migration line of primitive mesonephros and metanephros cells (Figure 1).

The pathogenesis of ERWT remains elusive, and several theories are discussed about it. Some authors have suggested that ERWT may arise from teratomas although later classifications divided teratoid Wilms' tumor and a true ERWT into two different entities while a true primary ERWT lacks any teratogenic element.

The most widely accepted hypothesis for the pathogenesis of ERWT suggests that the ectopic nephrogenic rest develops into a nephroblastoma. It is well known that the persistent

intrarenal fetal nephrogenic blastemal tissue may undergo oncogenic mutation and develop nephroblastoma. Several reports pointed to the observation of ectopic nephrogenic rests, especially in inguinal or retroperitoneal and lumbosacral regions (63).

The hypothesis of ectopic blastematous cells explains the development of ERWT in the craniocaudal migration pathway of primitive metanephros cells. The observation of WT1 gene in 25% of ERWT supports the oncogenic mutation of nephrogenic rests causing ERWT (65). The question is how we can explain the relatively high prevalence of ERWT in the inguinal region. Some genital structures such as Gartner's duct, seminal vesicles, vas deferens, and epididymis are differentiated mesonephric ducts that could explain the presence of ectopic nephrogenic rests as a precursor of ERWT in the inguinal region. Currently, several evidences support the hypothesis of ectopic nephrogenic blastemal cells causing the ERWT, which help us in better understanding of Wilms' tumor pathogenesis. Early diagnosis of nephrogenic rests outside the kidneys as a precancerous tissue will mandate close observation and prompt intervention while facing any evidence of atypia or malignancy (66).

Pathology

A classic microscopic feature of Wilms' tumor consists of the triphasic pattern that includes mesenchymal, epithelial, and blastemal elements. Histologic diagnosis of ERWT is supported by the observation of classic triphasic histology in the absence of any teratoid component. The presence of heterotopic teratomatous elements in more than 50% of total microscopic field suggests the diagnosis of teratoid Wilms' tumor, which is a quite different entity in the pathogenesis and embryology with germ cell origin. While confronting an extrarenal nephroblastoma, whole specimen should be examined in multiple cuts to exclude the diagnosis of teratoid Wilms' tumor that accounts for half of the reported extrarenal nephroblastic malignancies (67).

As discussed previously, ectopic nephrogenic rest is believed to be the precursor of Wilms' tumor, and histologic discrimination between these two is always a challenge (68). The presence of disordered structures, atypical mitosis, and marked pleomorphisms indicate the presence of Wilms' tumor in contrast with proliferative nephrogenic rests without atypia (63).

Considering the histologic findings, ERWTs could be classified as favorable or unfavorable. The review of reported cases shows predominant favorable histology among ERWTs. Beckwith and Palmer (69) proposed the criteria for ERWT pathological diagnosis that include the documented classic triphasic Wilms' pattern outside kidneys in the absence of teratoid or anaplastic elements while both kidneys are tumor free in imaging (Figure 2).

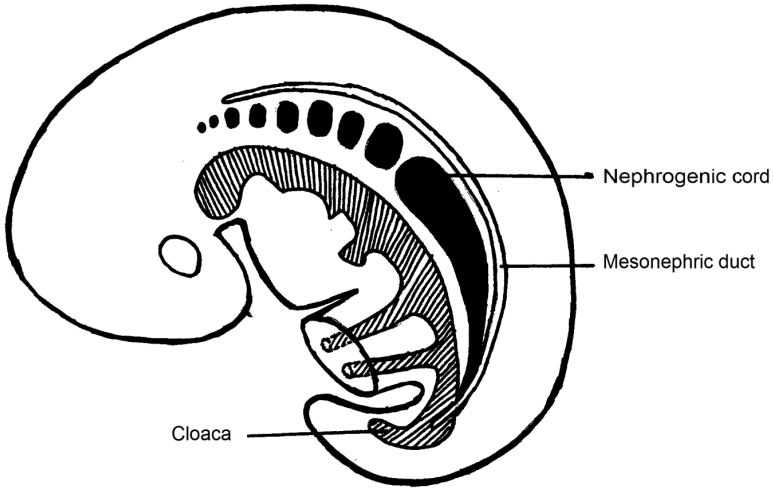

Figure 1. Schematic sagittal view of a 4-week-old embryo. Mesonephros consists of mesonephric duct and nephrogenic cord (Drawing by R. Shojaeian originally).

Clinical presentation and diagnosis

Clinical presentation of ERWTs depends on the location and stage of the tumor. As the symptoms are nonspecific and mostly due to the mass effect of tumor, diagnosis of ERWT often becomes apparent postoperatively. Common ERWT sites include retroperitoneum, inguinal area, lumbosacral and pelvic, female genital organs (uterus, cervix, vagina, and ovaries), mediastinum and chest wall, and spermatic cord and paratesticular region. Like classic Wilms' tumor, ERWT commonly manifests with asymptomatic mass or nonspecific symptoms, such as abdominal pain and discomfort, weight loss, and urologic or gynecologic symptoms. Symptoms related to the tumor site and size, such as inguinal mass that resembles hernia or lymphadenitis, vaginal bleeding or discharge, hematuria or dysuria, dyspnea, spinal cord compression signs, and even paraplegia (61, 3) may be observed.

Relation between ERWT and horseshoe kidney is suggested as we observed the coexistence of horseshoe kidney and ERWT in almost 13% of all previously reported cases. This may be explained by the higher chance of ectopic primitive renal tissue among patients with abnormal migration of nephrogenic cells, so the diagnosis of ERWT must be kept in mind while confronting with an abdominal mass in a patient with horseshoe kidney (59).

Ultrasound study is usually the first paraclinical step in the evaluation of an abdominal or pelvic mass. Further imaging such as computed tomography (CT) or magnetic resonance study may be needed in some cases. However, ERWT does not have a pathognomonic radiologic image, and the diagnosis of ERWT is almost always postoperative. After pathologic

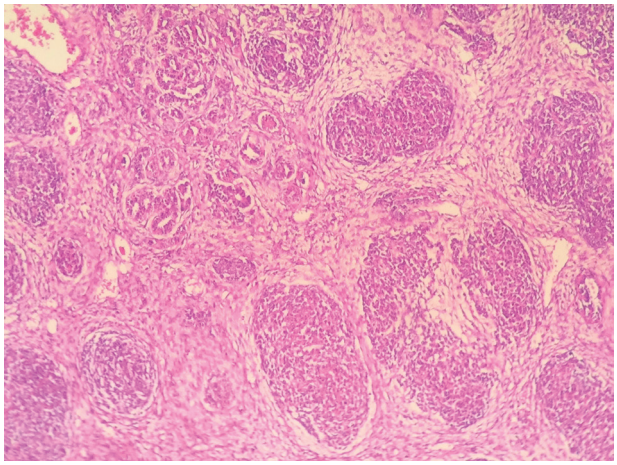

Figure 2. Microscopic evaluation of the specimen revealed composition of sheets, which were randomly arranged and tightly packed. Small blue cells were arranged in serpiginous aggregates (blastemal component), sharply circumscribed by focal spindling and intervening collagenous bundles apart from the surrounding stromal elements. There are also a few small tubules lined by primitive cuboidal cells and a small area of nephrogenic rest at the periphery. Pathological features suggested extrarenal Wilms' tumor (from Authors' personal archive).

confirmation of ERWT, both kidneys should be evaluated precisely with multislice spiral CT images to exclude any intrarenal tumor. CT scan with intravenous and oral contrast is often indicated and helpful to evaluate the tumor location and its resectability. Magnetic resonance imaging may also be helpful in paraspinal and thoracic tumors, especially with cord compression symptoms (64) (Figure 3). Recently, due to probable hazardous effects of CT scan especially in pediatric imaging, it is preferred to use MRI for evaluation of tumors in pediatric.

Tumor markers such as AFP (alpha-fetoprotein) and βHCG (beta-human chorionic gonadotropin) are useful to discriminate ERWT from other pediatric neoplasms such as the highly frequent germ cell tumors in childhood. Although a systemic search should be done for the tumor spread, ERWTs rarely metastasize. The most common sites of metastasis include lungs and liver. Three percent of the reported ERWT cases were metastatic based on our review.

Treatment

It is widely believed that National Wilms' Tumor Study (NWTS) system could be applied for ERWTs staging, while stage I definition should be modified as a localized tumor that could be completely excised with microscopic clear margins, no residue, and no tumor rupture during surgery; otherwise, there would not be any stage I ERWT as all tumors are located

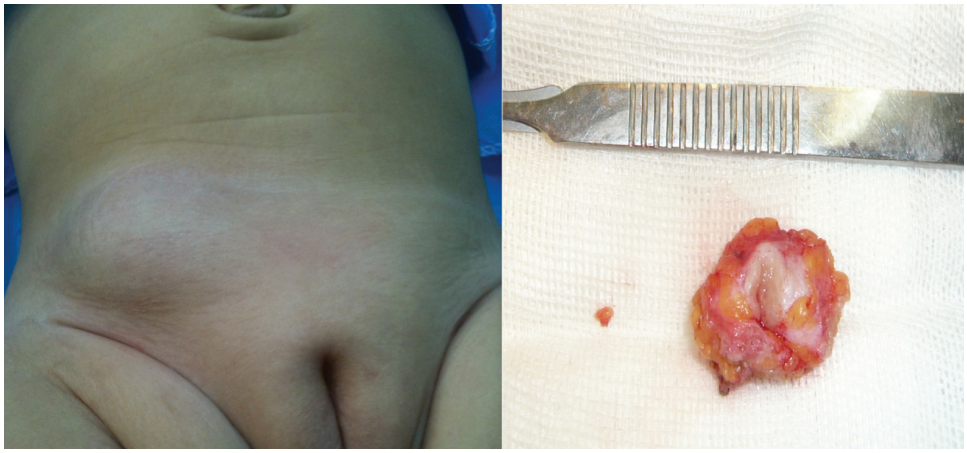

Figure 3. Left, Clinical manifestation of an inguinal ERWT. Right, Gross macroscopic view of inguinal ERWT (from Authors' personal archive).

beyond the borders of the kidneys. Although ERWT is uncommon, it should be kept in mind as a differential diagnosis of retroperitoneal or inguinal masses in childhood. Surgical approach depends on the location and surrounding structures. Rare cases of retroperitoneal or thoracic ERWT with intraspinal component and neurologic symptoms should be managed by multidisciplinary approach promptly to prevent irreversible neurologic sequels (64).

The role of intraoperative frozen section in an unidentified childhood mass or ERWTs has not been discussed clearly before and is not considered as a part of surgical principle, while total excision is the mainstay of treatment in most pediatric solid tumors when applicable (50).

Surgical excision remains the key step in the treatment of ERWT, especially when performed radically (70). Regional lymph node sampling is a part of the surgical principle as that for classic renal Wilms' tumor. Careful inspection of solid organs such as kidneys or liver and also peritoneum for tumor implants is recommended in abdominal ERWTs. Adjuvant chemotherapy is recommended for all ERWT cases postoperatively in spite of favorable histopathology in most of them. However, there are a few cases of successful treatment of stage I ERWTs with pure surgery.

Chemotherapy regimen is determined by histology and stage of the tumor, considering the NWTS protocols that consist of the administration of vincristine, actinomycin D, and doxorubicin. Regarding the current guidelines of NWTS, completely resected ERWTs with no evidence of tumor at or beyond the margins are considered as stage II and treated with vincristine and actinomycin D, while addition of doxorubicin will have benefits in stage III ERWTs.

Most ERWTs have favorable histology, but local recurrence is observed in about 11% of the reported cases, which is comparable with 15% predicted recurrence rate in classic renal Wilms' tumor with favorable histology. We found that 70% of ERWTs were in stage II and 23% in stage III, while distant metastasis was reported in 6% of patients. Two-year event-free survival of the reported ERWT cases was almost 85% and mortality rate was 5%, which are comparable with renal Wilms' tumor (60). Radiotherapy is reserved for unresectable tumors or for those with gross residue, recurrence, or metastasis (67). Bilateral ERWT has not been reported to date.

Conclusion

ERWT is considered a rare childhood malignancy with atypical presentations. The pathogenesis of ERWT becomes clearer by the popular theory, which suggests the heterotopic metanephric blastema as the precursor of ERWT while the diagnosis, staging, and treatment remain challenging. NWTS protocols are applied for ERWTs due to the rarity of the disease and lack of systematic data. We reviewed 87 reported childhood ERWT cases and observed favorable histology in most cases, which made the prognosis good and comparable to that of classic Wilms' tumor with the same stage and histology.

Conflict of Interests

The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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Chapter 7

Neoadjuvant Transcatheter Arterial Chemoembolization and Systemic Chemotherapy for the Treatment of Wilms Tumor

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Abstract

From 2003 to 2013, 55 patients (median age 3.3 years; 29 males, 26 females) with unresectable, metastatic, or diffuse anaplastic histology (AH) Wilms tumor were treated with neoadjuvant transcatheter arterial chemoembolization (TACE) and systemic chemotherapy. Characteristics

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of patients were maximal tumor diameter greater than 10 cm, involvement of periaortic lymph nodes, tumor thrombus in inferior vena cava/right atrium, distal metastasis, or diffuse AH. The chemoembolic emulsion for TACE consisted of pirarubicin, vindesine, and iodized oil. For the tumor with distal metastasis or diffuse AH, cisplatin was added in the chemoembolic emulsion. Intravenous chemotherapy with vindesine and actinomycin D was administered 2–3 weeks after TACE. For the patients with distal metastasis or diffuse AH Wilms tumor, intravenous chemotherapy consisted of ifosfamide and etoposide. Nephrectomy was performed 2–3 weeks after preoperative combination therapy. Surgical stage was assigned according to local operative findings in terms of the National Wilms Tumor Study (NWTS) Group combined with the pretherapeutic imaging to define metastatic disease. Postoperative treatment was based on tumor histology and surgical stage. All patients were followed up for 17–141 months (median: 82 months). No cardiotoxicity, renal insufficiency, and hepatic dysfunction after neoadjuvant TACE and systemic chemotherapy were found. Oral mucositis developed in 5 patients, grade I–II marrow suppression developed in 12 patients, and 19 patients became moderately febrile. In terms of response evaluation criteria in solid tumors, partial response (PR) in 34 (61.8%), stable disease (SD) in 19 (34.5%), and progressive disease (PD) in 2 (3.6%) patients were observed. Four of five patients had complete regression of inferior vena cava tumor thrombus. Atrial tumor thrombus retreated to inferior vena cava in one of two patients. Distant metastasis disappeared in four of six cases. Fifty patients (90.1%) underwent complete tumor resection. Tumor spillage occurred in 3 patients (5.5%). Two patients (3.6%) had microscopic residual disease. Surgical stages were stage II in 25, stage III in 24, and stage IV in 6 patients. On pathologic examination, tumor necrosis was >90% in 14 (25.5%), 50%–90% in 23 (41.8%), and <50% in 18 cases (32.7%). The 5-year event-free survival was 92.7%, and the overall survival was 94.5%. These preliminary results suggest that the use of neoadjuvant TACE and systemic chemotherapy may provide a promising choice in the treatment of unresectable, metastatic, or diffuse AH Wilms tumor in children. Further investigations are necessary.

Key words: Neoadjuvant therapy; Systemic chemotherapy; Transcatheter arterial chemoembolization; Wilms tumor

Introduction

Wilms tumor accounts for about 6% of all malignant tumors in children, and it is the most common malignant renal tumor of childhood. The outcome for patients with Wilms tumor has improved remarkably during the past decades owing to the use of adjuvant chemotherapy and neoadjuvant chemotherapy (1). However, the treatment of children with unresectable, metastatic, or diffuse anaplastic histology (AH) Wilms tumor remains a challenge (2–4). The unresectable criteria most commonly utilized are the tumor diameter greater than or equal to 10 cm, involvement of adjacent vital structures, and intracaval/atrial tumor extension. These

factors significantly increase the risk of surgical morbidity (4). Novel treatment strategies are needed to maximize survival and minimize long-term morbidity for these patients.

Preoperative embolization of the renal artery as a coadjuvant treatment in high-risk renal neoplasia has benefits for the subsequent nephrectomy (5–20). In an attempt to improve the outcome of patients with unresectable, metastatic, or diffuse AH Wilms tumor, we have performed preoperative transcatheter arterial chemoembolization (TACE) since 1995 (21–24). In previous studies, we found preoperative TACE combined with systemic chemotherapy could induce more massive necrosis of the tumor, further improving the complete resection rate of the tumor (25). In this phase II study, we examined the efficacy and safety of combined-modality neoadjuvant therapy using TACE and systemic chemotherapy as a first-line treatment for unresectable, metastatic, or diffuse AH Wilms tumor.

Patients and methods

From January 2003 to December 2013, 55 patients with unilateral unresectable, metastatic, or diffuse AH Wilms tumor were treated using preoperative TACE combined with systemic chemotherapy at our hospital.

All patients underwent abdominal computed tomography (CT), magnetic resonance imaging, ultrasound scan, and chest CT examination at admission. When metastasis in liver or lung was visible on CT, the patient was classified as stage IV disease. A core-needle biopsy for histologic diagnosis was performed before the treatment. Histology results were classified as unfavorable if diffuse anaplastic (AH) features were present and favorable (FH) if absent. This study was approved by the institutional ethics committee, and informed consent was obtained from the children's parents before enrollment. Characteristics of the patients were as follows: maximal tumor diameter greater than 10 cm, involvement of periaortic lymph nodes, tumor thrombus in inferior vena cava (IVC)/right atrium, distal metastasis based on the imaging studies, or tumor with diffuse AH according to the biopsy report (Table 1). Eligible patients were between 5 months and 11 years of age (median: 3.3 years), 29 boys and 26 girls. The right kidney was treated in 33 patients and the left in 22 patients. Patients with bilateral renal tumors, congenital mesoblastic nephroma, clear cell sarcoma of kidney, rhabdoid tumor of kidney, and renal cell carcinoma were excluded from this study.

The preoperative treatment consisted of alternating TACE and intravenous chemotherapy. Patients underwent TACE under intravenous and caudal epidural anesthesia. The femoral artery was catheterized using the Seldinger technique. A 5-F Pigtail catheter (Cook Vascular Incorporated, Pennsylvania) was introduced into the abdominal aorta to perform aortography and to define the tumor blood supply for the purpose of planning the chemoembolization (Figure 1A and B). The selective renal arterial catheterization and angiography of the

Table 1. Demographics and tumor characteristics of 55 patients with unresectable, metastatic, or diffuse AH Wilms tumor

Median age, y (range)	3.3 (0.5–11)
Sex, M:F ratio	29:26
Tumor side, right/left	33/22
Tumor characteristics on admission	
Greatest dimension >10 cm	29 (52.7%)
Involvement of periaortic lymph nodes	9 (16.4%)
Tumor thrombus in inferior vena cava/right atrium	7 (12.7%)
Distal metastasis	6 (10.9%)
Diffuse anaplastic histology	4 (7.3%)

involved kidney were performed using a 4-F or 5-F Cobra catheter (Cook Vascular Incorporated) (Figure 1C). The chemoembolic emulsion consisted of pirarubicin (Main Luck Pharmaceuticals Inc, Shenzhen, China) 40 mg/m^2; vindesine (Minsheng Pharmaceuticals Inc, Hangzhou, China) 3 mg/m^2; and iodized oil (Lipiodol; Guerbet, Aulnay-sous-Bois, France) 0.5 mL per tumor maximal diameter (in centimeters). The drugs were mixed, diluted in 120 mL of normal saline, and infused into the renal artery over 60 minutes. For the tumor with distal metastasis or diffuse AH, cisplatin 80 mg/m^2 was added in the chemoembolic emulsion. The catheter was removed after treatment. Intravenous hydration and alkalization were administered before, during, and after TACE. Postprocedure nausea and vomiting were treated with antiemetics.

Intravenous chemotherapy was administered 2–3 weeks after TACE. It consisted of vindesine 3 mg/m^2 (maximum 4 mg) weekly and actinomycin D 3 days × 15 μg/kg/d (maximum 500 μg) weeks 1 and 3. For the patients with distal metastasis or diffuse AH Wilms tumor, the intravenous chemotherapy consisted of ifosfamide 1,200 mg/m^2 and etoposide 100 mg/m^2 on days 2–4. For the patients younger than 1 year or with a body weight of <12 kg, the dosages of drugs for TACE and intravenous chemotherapy were reduced to two thirds.

Pirarubicin is a new anthracycline antibiotic with an antitumor efficacy similar to that of doxorubicin but less cardiotoxic because of its different pharmacodynamic properties (26). To minimize the side effects of anthracycline, we used pirarubicin instead of doxorubicin hydrochloride in this study. Vindesine is an analogue of the vinca alkaloids. Its spectrum of antitumor activity is similar to that of vincristine, but with milder neurotoxicity (27).

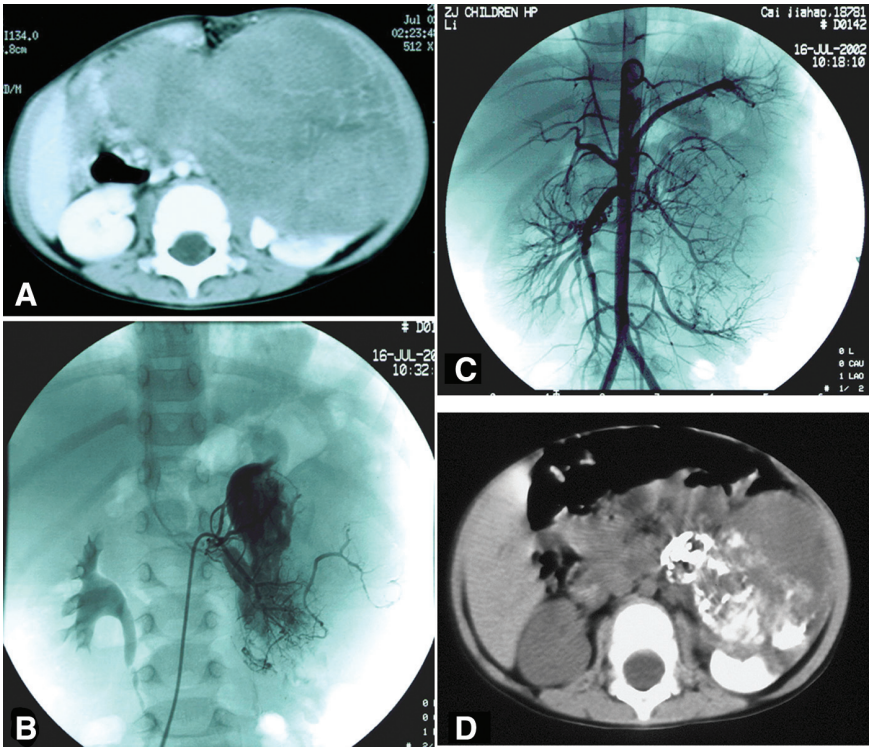

Figure 1. Preoperative TACE in a 2-year-old boy with left renal Wilms tumor. (A) Computed tomography (CT) finding left retroperitoneal huge tumor, invasion to the right side. (B) Aortography showing a large hypervascular lesion in the left kidney with abundant neovascularity. (C) Selective renal arterial catheterization and the chemoembolic emulsion infusing into the left renal artery. (D) Tumor volume is significantly reduced after alternating TACE and intravenous chemotherapy. Iodized oil deposits still visible within the tumor in the repeated CT scan before operation.

Nephrectomy was performed 2–3 weeks after preoperative TACE combined with systemic chemotherapy. Four patients underwent repeated preoperative combined treatment due to pulmonary metastasis. Tumor volumes were measured on ultrasound scans using the ellipsoid formula (length × thickness × depth × 0.523). The measurement was performed before preoperative therapy and repeated before surgery. Tumor volume reduction in comparison with the initial volume was calculated. Tumor response to treatment was defined according to the new response evaluation criteria in solid tumors [Response Evaluation Criteria In Solid Tumors (RECIST) 1.1] (28). Toxicity was scored according to the Children's Cancer Group toxicity grading system (29). After tumor resection, surgical specimens were microscopically examined for features of tumor, such as the surgical margin and necrosis, which was defined as complete if no viable cells were found in the tumor and nodules. Histopathologic

classification and surgical stage were assigned according to the National Wilms Tumor Study (NWTS) Group (30). Postoperative treatment using systemic chemotherapy and radiotherapy was based on tumor histology and its surgical stage. The treatment protocol was worked out according to the NWTS protocol modified by the Beijing Children's Hospital (31). Fifteen patients received radiotherapy after operation due to stage III or diffuse AH Wilms tumor. Postoperative follow-up was performed at the first month and then every 3–6 months after surgery, including physical, imaging (abdominal sonography and CT, chest x-ray, and electrocardiogram), and laboratory screening (blood and urine analysis, and liver and renal function tests). All patients were followed up until December 31, 2014.

Statistical methods

Standard methods were used for the analysis of censored and noncensored data. Event-free survival (EFS) time was defined as the time from the date of diagnosis to the first occurrence of progression, relapse, or death. Overall survival (OS) time was measured from the date of diagnosis to death or the patients being still alive on December 31, 2014. The Kaplan–Meier method was used to calculate EFS and OS rates, and the rates are presented as the rate ± SE. All analyses were carried out using the SPSS 16.0 statistical software system.

Response to treatment

No patients experienced death while preoperative treatment. No preoperative tumor rupture, drug-induced cardiotoxicity, renal insufficiency, or hepatic dysfunction were found in all of the patients. Oral mucositis developed in 5 (9.1%) patients, grade I–II marrow suppression developed in 12 (21.8%) patients, and 19 (34.5%) patients became moderately febrile after chemoembolization; this was controlled with symptomatic treatment (Table 2). Tumor volumes were significantly reduced after preoperative TACE and systemic chemotherapy. Iodized oil deposits were still visible within the tumor in the repeated CT scan before operation (Figure 1D). Color Doppler ultrasonography showed the abundant blood flow decreased significantly in the tumor after preoperative treatment (Figure 2A and B). Tumor volume was 488 mL (median) at diagnosis and 198 mL (median) before operation. In terms of new response evaluation criteria in solid tumors (RECIST 1.1) (28), partial response (PR) was observed in 34 (61.8%), stable disease (SD) in 19 (34.5%), and progressive disease (PD) in 2 (3.6%) patients after preoperative therapy (Table 2). Five patients had tumor invading the IVC. Four of them had complete regression of the IVC tumor thrombus before operation. One patient underwent vena caval thrombectomy during nephrectomy. Two cases additionally had extensive venothrombotic invasion to the right atrium at admission. The atrial tumor thrombus retreated to IVC in one patient who underwent radical nephrectomy and vena caval thrombectomy. The atrial tumor thrombus reduced obviously but not completely disappeared in another patient who underwent thrombectomy under cardiopulmonary bypass with deep

Table 2. Complications, tumor response, and outcome of 55 patients treated with preoperative TACE and systemic chemotherapy

Complications during preoperative treatment	
Death while treatment	0 (0.0%)
Cardiotoxicity	0 (0.0%)
Renal insufficiency	0 (0.0%)
Hepatic dysfunction	0 (0.0%)
Oral mucositis	5 (9.1%)
Grade I–II marrow suppression	12 (21.8%)
Moderate febrile	19 (34.5%)
Tumor volume, mL	
On admission	488 (292–804)
Before operation	198 (126–324)
Tumor response	
PR	34 (61.8%)
SD	19 (34.5%)
PD	2 (3.6%)
Inferior vena and atrial tumor thrombus disappeared	4/7 (57.1%)
Distal metastasis disappeared	4/6 (66.7%)
Complete tumor resection	50/55 (90.9%)
Rapture during operation	3/55 (5.5%)
Microscopic residual	2/55 (3.6%)
Tumor necrosis	
>90% tumor necrosis	14/55 (25.5%)
50–90% tumor necrosis	23/55 (41.8%)
<50% tumor necrosis	18/55 (32.7%)
Postoperative stage	
I	0 (0.0%)
II	25 (45.5%)
III	24 (43.6%)
IV	6 (10.9%)

(*Continued*)

Table 2. Continued

Histology	
FH	51 (92.7%)
AH	4 (7.3%)
Outcome	
EFS	92.7% (95% CI: 85.8–99.6%)
OS	94.5% (95% CI: 88.5–100%)

PR, partial response; SD, stable disease; PD, progressive disease; FH, favorable histology; AH, anaplastic histology; EFS, event-free survival; OS, overall survival.

hypothermia and circulatory arrest during nephrectomy. Histopathologic examination of the resected atrial thrombus showed epithelial and mesenchymal components. Six patients had distant metastasis on admission, including one case of liver metastasis and five cases of pulmonary metastasis. Distal metastasis disappeared in front of the renal tumor resection in four cases (Figure 3A–C) and during postoperative chemotherapy in two patients. Fifty patients (90.1%) underwent complete tumor resection after preoperative therapy. Tumor spillage occurred in 3 patients (5.5%). Two patients (3.6%) had microscopic residual disease. The overall distribution of patients in the series according to surgical staging was stage II in 25 (45.5%), stage III in 24 (43.6%), and stage IV in 6 (10.9%) patients (Table 2).

Histopathologic findings

Postoperative histopathology revealed FH Wilms tumor in 51 patients (92.7%) and diffuse AH in 4 patients (7.3%) (Table 2). Pathologic examination of the specimen found massive necrosis in the tumor and increased thickness of the fibrous envelope around the tumor (Figure 4A and B). Tumor necrosis was >90% in 14 (25.5%), 50%–90% in 23 (41.8%), and <50% in 18 cases (32.7%) (Table 2). Necrosis was visible not only in the main tumor but also in the metastases of periaortic lymph nodes (25). Iodized oil deposition in the para-aortic lymph nodes was observed in six cases. Postoperative histologic examination of these marked lymph nodes confirmed lymph node metastases with necrosis. This finding implies that the chemo-embolization agent flowed directly into the para-aortic lymph node metastases.

Outcomes

All patients were followed up until December 31, 2014. The median length of follow-up was 82 months (range: 17–141 months). Thirty-one patients had been followed up for more than 5 years. The 5-year EFS was 92.7% [95% confidence interval (CI): 85.8%–99.6%] and OS was 94.5% (95% CI: 88.5%–100%) (Table 2) (Figure 5A and B). Four patients relapsed.

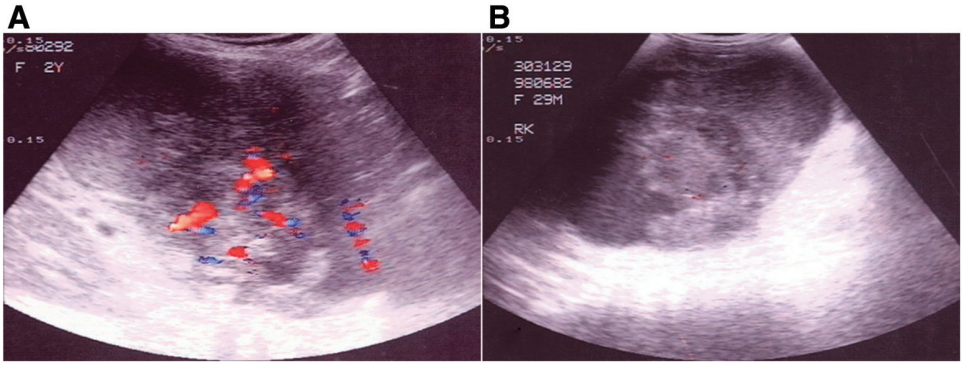

Figure 2. Color Doppler ultrasonography appearances of the left renal Wilms tumor in a 3-year-old boy. (A) Diffusely increased blood flow in the tumor before treatment. (B) Blood flow deceased significantly within the tumor after preoperative TACE and systemic chemotherapy.

One of them relapsed with pulmonary metastases 8 months after operation and was cured by chemotherapy. Three of them died. The first patient was a 6.5-year-old boy with a stage III FH Wilms tumor. He had a relapse involving the proximal tibia 1 year after operation that was unresponsive to treatment, and he died 20 months after presentation. The second patient, a 7-year-old boy with a stage II FH tumor, died 18 months after operation from liver metastatic disease. The third was an 8-year-old girl with a surgical stage III AH tumor and IVC thrombus invasion to the right atrium at admission. The tumor thrombus retreated to suprahepatic IVC after preoperative therapy, and nephrectomy with thrombus removal was performed. She relapsed with pulmonary and liver metastases 6 months after operation and subsequently died. Late effects of therapy were evaluated in the survivors. One patient had scoliosis caused by radiotherapy after operation. No case(s) of anthracycline cardiotoxicity, liver disease, hypertension, and renal dysfunction were documented in survivors. Puberty and growth disturbances were not observed in all patients. No new late cancers were detected on follow-up.

Discussion

Although the FH Wilms tumors showed excellent outcome, the survival rate for unresectable, metastatic, or diffuse AH Wilms tumor cases remains to be improved. Patients with unresectable or metastatic Wilms tumor fare worse than patients with localized and resectable tumors (32). The unresectable criteria commonly utilized are huge size of the tumor, involvement of adjacent vital structures, and intracaval/atrial tumor extension. These factors significantly increase the risk of surgical morbidity, principally hemorrhage, and tumor spillage during initial nephrectomy (4). Larger tumors are at higher risk of intraoperative

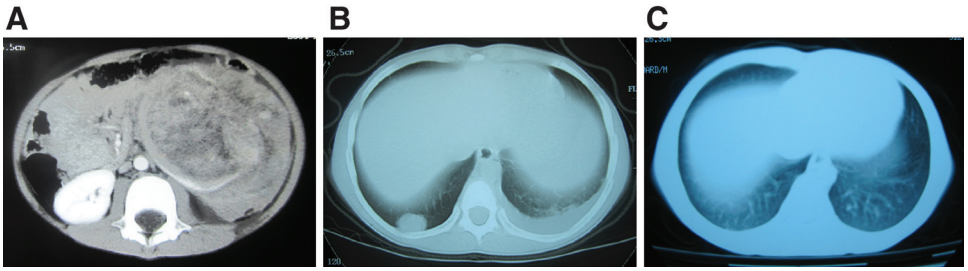

Figure 3. A 7-year-old boy had left Wilms tumor with pulmonary metastases. (A) Enhanced CT of the abdomen showing a huge mass in the left renal fossa. (B) Chest CT examination demonstrated right lung metastasis and left pleural effusion. (C) Pulmonary metastases disappeared after preoperative TACE and systemic chemotherapy.

tumor spillage (33). Primary nephrectomy for Wilms tumor diameter greater than or equal to 10 cm was also associated with an increased risk of surgical complications (4). The patients with stage III disease, diffuse AH, and tumor spillage during surgery also observed the relative risks of local recurrence and poor survival (34). Patients with diffuse anaplastic Wilms tumor, particularly stages III and IV, continue to have poor outcomes and may benefit from new treatment strategies (3, 30, 35).

The SIOP studies largely focus on the issue of preoperative chemotherapy to facilitate surgery of a shrunken tumor and to treat metastasis as early as possible. The duration of conventional preoperative chemotherapy is 4 or 8 weeks (2, 36–38). Preoperative chemotherapy is also used for the treatment of "inoperable" or "unresectable" Wilms tumor by NWTS and

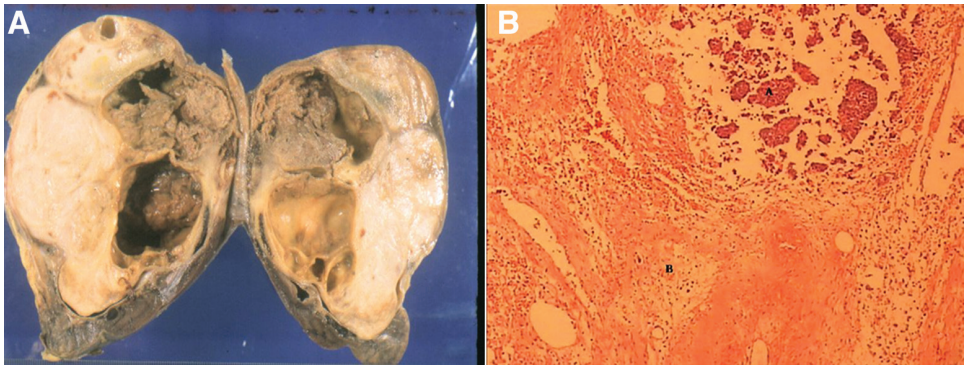

Figure 4. Pathologic examination of the surgical specimen. (A) Macroscopic examination of the specimen found massive necrosis and thickening of the tumor fibrous capsule. (B) Microscopically, extensive and homogenous necrosis was found in the tumor (hematoxylin and eosin stain, ×40).

United Kingdom Children's Cancer Study group in recent years (39, 40). However, some patients did not respond to conventional preoperative chemotherapy and died before the excision of the primary tumor. Ritchey et al. (4) reviewed 131 children in NWTS-3 who had received preoperative chemotherapy for unresectable tumors or were judged inoperable by imaging. Thirteen of them did not respond to chemotherapy, but the disease progressed. Eight children died before the removal of the primary tumor. Ora et al. (41) reported tumor progression during preoperative chemotherapy in 57 of 1,090 patients (5%) with localized Wilms tumors. Patients whose tumors do increase in size have poorer EFS and OS rates independently of stage distribution and histopathologic risk group.

Actinomycin D, vincristine, doxorubicin, ifosfamide, etoposide, and carboplatin are the commonly used drugs for more advanced and recalcitrant Wilms tumor (42). Actinomycin D and vincristine were used by the SIOP studies for patients with unilateral localized Wilms tumor and stage II or III with low-risk (LR) or intermediate-risk (IR) histology (43). Doxorubicin, cyclophosphamide, etoposide, and carboplatin are now the standard part of the treatment protocols for more advanced and recalcitrant cases (42, 44). NWTS-5 regimen for patients with stages II to IV diffuse AH Wilms tumor was treated with vincristine, doxorubicin, cyclophosphamide, and etoposide (3). Children's Cancer Group used ifosfamide, carboplatin, and etoposide in children with poor-risk relapsed Wilms tumor (45). Cisplatin is a well-known chemotherapeutic drug and is effective against various types of cancers. Combination therapies of cisplatin with other drugs have been highly considered to overcome drug resistance and to reduce toxicity (46). It is ideal to administer cisplatin intra-arterially because it has a very high affinity for tissue protein, which leads to the effective binding of cisplatin to the tumor tissue during its first pass (47, 48).

Almgard et al. (5) performed first embolization for the treatment of renal adenocarcinoma in 1973. Since then, renal artery embolization is increasingly being used for the treatment of advanced or unresectable renal tumors in adults. Clinical studies have shown that preoperative renal embolization significantly reduces blood loss during nephrectomy, especially in large hypervascular tumors (6–20). Renal artery embolization also has been used in the management of Wilms tumor in children (49–54). Although the value of preoperative embolization of Wilms tumor has been documented by many authors, opinions on its indication have differed, and its use in practice has remained relatively limited.

TACE has significantly contributed to the evolution of interventional radiology. TACE may effectively deliver highly concentrated doses of chemotherapy to the tumor bed. The merits of renal chemoembolization are based on the concept that the blood supply to tumor only comes from the renal artery. The anticancer drug and embolizing material are injected into the tumor-feeding artery, increasing the effect of the chemotherapy agents in the ischemic

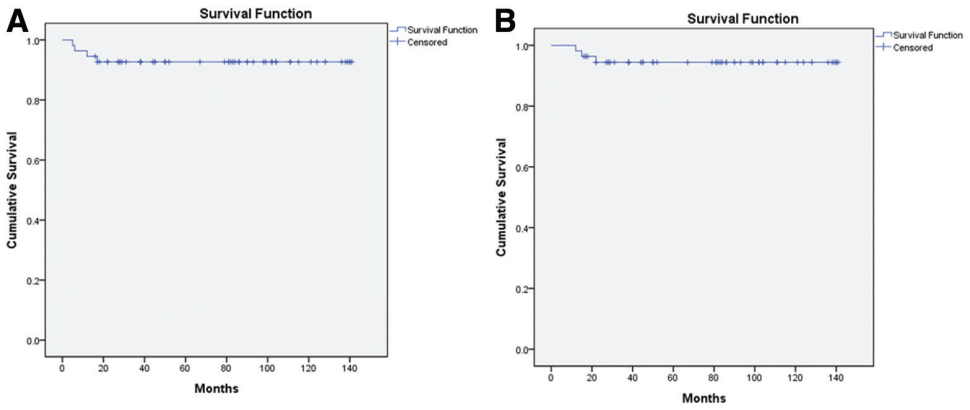

Figure 5. Event-free survival and overall survival rate by Kaplan–Meier estimates. (A) EFS, 92.7% (95% CI: 85.8–99.6%). (B) OS 94.5% (95% CI: 88.5–100%). EFS, event-free survival; OS, overall survival. (All the images in this chapter have not published previously and do not violate the copyright of the original publisher.)

tissue and reducing the risk of bleeding during surgery. In patients with extended renal carcinoma, survival was significantly higher after chemoembolization than after standard embolization of the renal artery (55–58). Animal experiments revealed that renal arterial chemoembolization can maintain high local concentrations of the anticancer drug, while maintaining low blood levels of the anticancer drug (59, 60).

We performed preoperative TACE for the treatment of advanced Wilms tumors since 1995 (21–24). The benefits of TACE for the treatment of advanced Wilms tumor are based on the concept that the anticancer drugs were directly injected into the tumor-feeding artery, increasing the effect of the chemotherapy agent within the tumor, while avoiding concomitant systemic toxicity. In our previous studies, we found preoperative chemoembolization combined with systemic chemotherapy for the treatment of advanced Wilms tumor showed a higher response rate than TACE alone (25). We used neoadjuvant TACE and systemic chemotherapy as for the treatment of unresectable, metastatic, or diffuse AH Wilms tumor since 2003. Our regimen is a platinum-based combination chemotherapy. The scientific rationale for the use of combination chemotherapy is to overcome drug resistance to individual agents. In addition to providing a broader range of coverage against naturally resistant tumor cells, combined chemotherapy may also prevent or delay the development of acquired resistance in initially responsive tumors and provide additive or synergistic cytotoxic effects. This regimen is also a multimodal combination therapy consisting of localized arterial chemotherapy, arterial embolization, and intravenous chemotherapy. This combination can induce more massive necrosis of tumor, eliminate the distant metastases, improve the complete resection rate of tumor, and achieve excellent survival rate.

Conclusion

The preliminary results in this phase II study suggest that the use of neoadjuvant TACE and systemic chemotherapy is well tolerated and may provide a promising choice in the treatment of unresectable, metastatic, or diffuse anaplastic Wilms tumor in children.

There were limitations in this study. Because of the small number of cases in this group and the short observation period, long-term effects warrant further investigation.

Conflict of Interest

The authors declare that they have no conflicts of interest with respect to research, authorship and/or publication of this book chapter.

Acknowledgments

This study was approved by our institutional review board; the need for informed consent for publication of data was waived. The authors thank all the children and their parents for allowing us to publish the data collected during this research project. The authors also thank the staff of the Department of Pediatric Surgery, Radiology, and Pathology for their cooperation.

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Chapter 8

Dendritic Cell-Based Cancer Immunotherapy Targeting Wilms' Tumor 1 for Pediatric Cancer

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Abstract

The treatment of advanced pediatric cancers that have metastasized to distant organs remains difficult. Investigations evaluating the potential treatment of these cancers using therapeutic vaccination with an active dendritic cell (DC)-based immunotherapy are also being conducted. This method induces an efficient immune response by the acquired immune system against tumor-associated antigens. Cancer vaccination therapies have been prepared using autologous monocyte-derived mature DCs exposed to granulocyte–macrophage colony-stimulating factor

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and interleukin-4, which are the molecules principally attributed to the presence of tumor-associated antigens. Wilms' tumor 1 (WT1), an attractive target antigen that has been widely detected in cancers including sarcoma and leukemia, has been shown to be the most potent tumor-associated antigen. DC-based immunotherapy targeting WT1 may have a potentially strong therapeutic activity against cancers. DC vaccines primed with human leukocyte antigen (HLA) class I-/II-restricted WT1 peptides (WT1-DC) are a feasible option. A 6-year-old girl with neuroblastoma and a 14-year-old girl with WT received autologous DC vaccination pulsed with a modified WT1 peptide compatible with HLA-A*24:02. The patients received 20 and 25 vaccines, respectively, and experienced no adverse effects aside from a grade 2 skin reaction at the injection site and a fever with tolerable elevation. WT1tetramer analysis after vaccination detected WT1-specific immune responses. This treatment strategy may be safe, tolerable, and even feasible for all patients who are refractory to treatment and for pediatric patients who have relapsed with neoplasms.

Key words: Cancer vaccination; Dendritic cells; Pediatric neoplasm; Tetramer analysis

Introduction

Despite significant advances in cancer therapeutics, including the introduction of immune checkpoint inhibitors (1–6), it remains extremely difficult to treat advanced cancers affecting multiple organs and involving distant metastases. Ralph Steinman, the Nobel Prize-winning scientist who discovered dendritic cells (DCs) in 1973 (7), experimentally immunized himself with DC vaccination therapy against his pancreatic cancer and survived for 4.5 years. The manufacturing technology used in the production of antigen-presenting cell (APC)-based immunotherapies involving active DCs, the immune system's most potent APCs, is currently under development as a means of therapeutic vaccination against cancer (8). DC-based immunotherapy not only appears to be associated with few adverse reactions but also has limited clinical effectiveness when assessed using conventional evaluation methods such as response evaluation criteria in solid tumors (9, 10). Due to a slow clinical response, a low response rate, and few differences in patient median survival time (MST), long-term cancer immunity results in a delayed separation of treated and untreated patient survival curves, with an eventual treatment advantage in prolonged overall survival (OS) (11, 12).

An *ex vivo* technique is being developed for DC-based cancer vaccination to promote strong induction of T cells against tumor antigens. Oil adjuvants for peptide vaccines act by locally accelerating the activation of lymphocytes (13). However, DCs have the potential antigen bioactivity and may be used as a suitable adjuvant (14–16). Human leukocyte antigen (HLA) molecules harbor cancer antigen peptides that promote DCs binding with receptors on $CD8^+$ killer and $CD4^+$ helper T cells, leading to an immune response against cancers (Figure 1). In contrast,

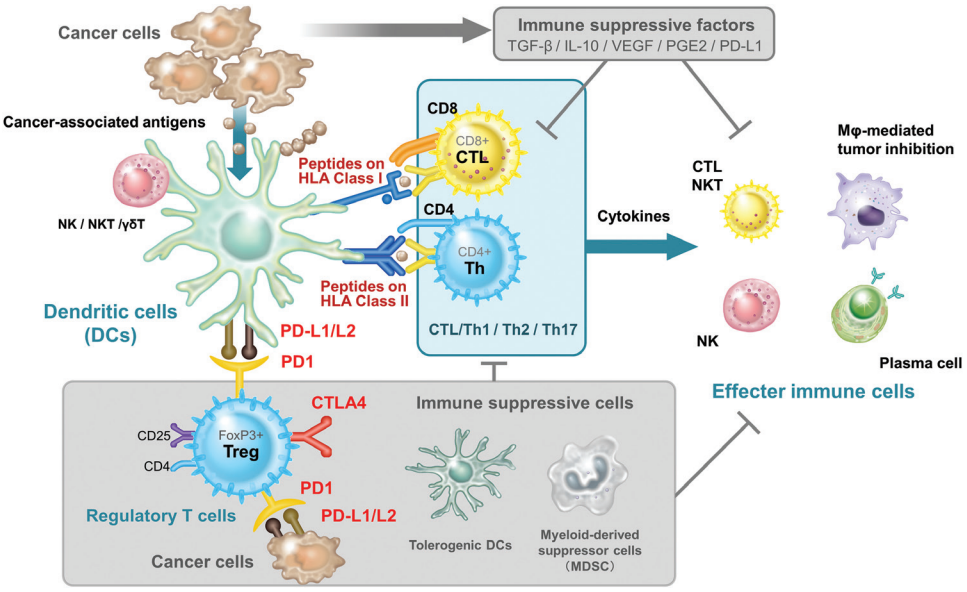

Figure 1. Dendritic cells and other immune cells in the cancer environment. Human leukocyte antigen (HLA) molecules harbor cancer antigen peptides, which induce DC binding with receptors on $CD8^+$ killer and $CD4^+$ helper T cells, leading to anticancer immune responses. In contrast, immune suppressor cells, such as regulatory T cells, tolerogenic DCs, and myeloid-derived suppressor cells, suppress autoreactive and cancer-derived mechanisms. (Original figure by Shimodaira S.)

immune suppressor cells, such as regulatory T cells, tolerogenic DCs, and myeloid-derived suppressor cells, suppress autoreactive and cancer-derived mechanisms (17–21). Immune suppressive factors are also stimulated by the presence of cancer cells. These factors are shown in Figure 1 and include transforming growth factor-β, interleukin (IL)-10, vascular endothelial growth factor, prostaglandin E2 (PGE2), and programmed death-ligand 1 (PD-L1) (22). The efficacy of DC vaccination can likely be attributed to the inhibition of these immune suppressors.

DCs are generated from peripheral monocytes following exposure to granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4. DCs expressing tumor-specific antigens have been used in active cancer immunotherapies (23, 24). The most common approach to DC vaccination is the preparation of autologous, mature, monocyte-derived DCs *ex vivo* with consequent, homogeneous, and functional DC generation. Cancer vaccination therapies are principally attributed to the presence of tumor-associated antigens using peptide, protein, tumor lysate, and RNA (25–29). Sipuleucel-T (Provenge®) is a US Food and Drug Administration-approved autologous DC-based immunotherapy for men with metastatic hormone-refractory prostate cancer, which provides a new treatment option for patients with this type

of cancer. Sipuleucel-T is manufactured by exposing an individual patient's affected blood cells to a recombinant fusion protein composed of a prostatic acid phosphatase fused to GM-CSF, enhancing immune cell activity against this type of cancer. The patient's own DC product is administered intravenously as part of a three-dose schedule, with approximately 2-week intervals between each dose. This regimen yields a survival benefit of 4.1 months in patients with hormone-resistant prostate cancer (30). According to the requirement for antigens, such as Wilms' tumor 1 (WT1), mucin 1, cell surface associated, human epidermal growth factor receptor 2, carcinoembryonic antigen, survivin, and prostate-specific antigen, WT1 was identified as the most potent cancer-associated antigen. WT1 has confirmed immunological and clinical effectiveness with respect to therapeutic functions, immunogenicity, specificity, and oncogenicity (31). HLA-restricted WT1 peptides were identified as being compatible with HLA-A*02:01- or HLA-A*02:06-restricted (126–134: RMFPNAPYL) and class II compatible with HLA-DRB1*04:05 (332–347: KRYFKLSHLQMHSRKH). The WT1 peptide was restricted to HLA-A*24:02 and modified $WT1_{235–243}$ peptide (CYTWNQMNL). Methionine (M), the second amino acid, was replaced with tyrosine (Y), which can induce cytotoxic T cells (CTLs) to be more effective than the wild-type peptide (32–35). The percentage results for HLA genotyping were as follows: genotypes of HLA-A*24:02 (60%), A*02:01 (20%), and A*02:06 (15%) and HLA class II genotypes of HLA-DRB1*04:05, DRB1*08:03, DRB1*15:01, DRB1*15:02, DPB1*05:01, or DPB1*09:01 (90%). Phase I clinical trials have been conducted with this regimen for various types of solid tumors and hematological malignancies (36–38). DC vaccines primed with HLA class I-/II-restricted WT1 peptides (WT1-DC) have been determined to be safe and feasible, with few adverse reactions reported by patients with advanced cancers, including lung, breast, stomach, biliary tract, pancreas, ovary, and even high-grade glioma (39–47). Clinical studies have indicated that the efficacy of DC vaccination may be enhanced by the off-target effects of chemotherapeutic drugs (39–44, 48) and chemoradiotherapy (47, 49, 50), suggesting a survival benefit in some patients. Different combinations with adjuvant chemotherapy and/or radiotherapy have been investigated, along with the periods required for adaptation. The development of combination therapy regimens, which could potentially include immune checkpoint inhibitors, should improve the outcomes of personalized therapy for patients with cancer (51). However, DC vaccination has been only rarely utilized to treat pediatric patients. There are a few reports describing its use in acute leukemia after allogeneic hematopoietic stem transplantation (52, 53). This article focuses on a pilot study evaluating autologous DC vaccination targeting WT1 in pediatric patients with neuroblastoma or WT.

Manufacture of a DC vaccine

Mature DCs (mDCs) were generated under Good Gene, Cell and Tissue Manufacturing Practice, conditions according to the "The Act on the Safety of Regenerative Medicine"

introduced in Japan on November 25, 2014 (54). Mononuclear cell-rich fractions (165 ml) were isolated from 4,000 ml of the patient's blood through apheresis using a COM.TEC® cell separator (Fresenius Kabi Japan K.K., Tokyo, Japan). Immature DCs were generated by culturing adherent cells in AIM-V® medium (Gibco, Gaithersburg, MD) containing GM-CSF (50 ng/ml; Gentaur, Brussels, Belgium) and IL-4 (50 ng/ml; R&D Systems Inc., Minneapolis, MN) in a CO_2 incubator equipped with a Cell Processing Isolator (H_2O_2-sterilizing system, Panasonic Corporation, Osaka, Japan) at the Shinshu University Hospital Cell Processing Center. After 5 days of culture, immature DCs were differentiated into mDCs by stimulation with OK-432 (10 μg/ml of streptococcal preparation; Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) and PGE2 (50 ng/ml; Daiichi Fine Chemical Co. Ltd., Toyama, Japan) for 24 h (55). The resulting mDCs were cryopreserved at −152°C or in the gas layer within a liquid nitrogen tank until the day of administration. Cell culture supernatants were collected for sterility testing at the time of mDC freezing. For each vaccination, an aliquot of frozen mDCs was thawed immediately prior to clinical use and primed with 100 μg/ml of good manufacturing practice-grade WT1 peptide (NeoMPS Inc., San Diego, CA) containing 1–2 KE of OK-432. WT1 peptides contained HLA-A*02:01- or A*02:06-restricted peptides (126–134: RMFPNAPYL), HLA-A*24:02-restricted modified WT1 peptides (CYTWNQML, residue 235–243), and/or class II peptide (332–347: KRYFKLSHLQMHSRKH) compatible with DRB1*04:05, DRB1*08:03, DRB1*15:01, DRB1*15:02, DPB1*05:01, or DPB1*09:01 (35, 43). One course of seven biweekly sessions was performed with 1–3 × 10^7 DCs with 1–2 KE of OK-432 intradermally injected at bilateral axillar and inguinal areas per session. For pediatric cases, the dose of adjuvant OK-432 was modified as 0.25–1.0 KE, and intradermal injection sits were selected at two points in either bilateral axillar or inguinal areas per session.

DC vaccine release criteria

The antigenic profiles of mDCs were determined using flow cytometry. mDCs were defined as $CD11c^+$, $CD14^-$, HLA^-DR^+, HLA^-ABC^+, $CD80^+$, $CD83^+$, $CD86^+$, $CD40^+$, and $CCR7^+$ cells (55). The criteria for DC vaccine administration were as follows: purity defined as >90% proportion of $CD11c^+$ $CD14^-$ $CD86^+$ HLA^-DR^+ >90% cells, >80% viability, mDC phenotype, negative for bacterial and fungal infection after 14 days, presence of endotoxin ≤0.05 EU/ml, and negative for mycoplasma (55).

DC vaccine study

Application and conditions for DC vaccine therapy

1. Adjuvant therapy after surgical resection or high risk of disease relapse
2. *De novo* cancer at an advanced stage or recurrent cancer after standard therapies

Indication for DC vaccine therapy and eligibility

1. Performance status: 0/1
2. No organ function abnormalities, no infectious diseases, no blood abnormalities, no bleeding tendency
3. Neither cardiovascular diseases nor respiratory disorders that would prevent blood apheresis
4. Tolerable to chemotherapy and radiotherapy as standard cancer treatments
5. Within 6 months of cancer diagnosis or recurrence, with cancer sensitivity to chemotherapy

Exclusion criteria

1. Requiring platelet or red blood cell transfusion or albumin infusion
2. Disseminated intravascular coagulation syndrome and deep vein thrombosis
3. An infectious disease such as viral hepatitis (following the standard of the Japanese Red Cross Blood Center)
4. Allergy to penicillin or OK-432
5. Steroid hormone therapy continuously administered for diseases other than the prevention of temporal chemotherapeutic drug allergy
6. Difficulty in arm vessel blood access for apheresis
7. A presumed length of survival period that would prevent seven sessions of one course at the outpatient clinic
8. No informed consent due to cancer
9. Inability to understand the risk and benefit of the DC vaccine therapy
10. Opposition to DC vaccine therapy
11. Pregnant or nursing women
12. Physician judgment that a patient is inappropriate for treatment

Evaluation of safety and effectiveness

1. In terms of safety evaluation, we evaluated (i) any allergic reaction after the intradermal injection of the DC vaccine (presence of reduced blood pressure, tachycardia, breathing difficulties, or rash) and (ii) local reactions, fever onset, nausea, vomiting, diarrhea, loss of appetite, ulcer of the mucosa, central nervous system damage, anemia, reduced white blood cells, reduced platelets, abnormal kidney function, and abnormal liver function either during or after the completion of treatment.
2. We assessed the cancerous lesions during the treatment course using various imaging techniques, such as computerized tomography, magnetic resonance imaging, and positron emission tomography, approximately 4 weeks after the completion of DC

vaccination. The DC vaccination study was conducted at Shinshu University Hospital and was approved by the Ethics Committee of Shinshu University School of Medicine (Approval Number 1199, December 2, 2008; Approval Number 2704, April 8, 2014).

Case report

Case 1: Neuroblastoma

A 6-year-old girl presented with adrenal gland neuroblastoma in December 2008 at the age of 4. Bone metastasis and bone marrow involvement were detected, resulting in a diagnosis of stage IV disease according to International Neuroblastoma Staging System (56). The patient underwent systemic chemotherapy according to the protocol of the Japanese Neuroblastoma Study Group, followed by surgical resection of the primary adrenal gland neuroblastoma. After intensive chemotherapy was administered in combination with thiotepa and melphalan, the patient subsequently underwent autologous hematopoietic stem cell transplantation (HSCT). The patient received 20 Gy of radiation therapy to the primary right adrenal gland area after recovery from myeloablation and achieved complete disease remission in December 2009. However, at the age of 6, she developed bone marrow relapse in June 2010 and was admitted for DC vaccination in combination with etoposide chemotherapy. Her HLA genotype was confirmed as HLA-A*24:02 compatible with modified WT1-235 peptide. One course (seven sessions, once every 3 weeks) of DC vaccination containing modified WT1-235 peptide (a total of 7.22×10^7 DCs; mean, 1.03×10^7 DCs per session) was administered from March to July 2011. DC vaccine-related toxicities were tolerable and included grade 2 skin reactions and pain at the injection sites along with grade 1 low-grade fever within 48 h of treatment. There were no ≥grade 3 adverse effects due to DC vaccination based on Common Terminology Criteria for Adverse Events (CTCAE), version 4.0 (http://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03_2010-06-14_QuickReference_5x7.pdf). The tumor markers (neuron-specific enolase, urinary vanillylmandelic acid, and homovanillic acid) were normalized in August 2011, and the magnetic resonance imaging indicated the lesion significantly reduced.

However, the increase of the recurrent tumor with multiple metastasis of bone marrow was detected by MRI and metaiodobenzylguanidine scintigraphy in November 2011. Temozolomide was added, and the DC vaccination was also continued after one course for an additional 15 sessions until October 2012. The patient died due to disease progression in August 2013. Progression-free survival and OS from diagnosis were 5 months after DC vaccination and 4 years and 8 months, respectively.

Case 2: Wilms' tumor

A 14-year-old girl presented with WT derived from her left kidney in January 2002 at the age of 4. Tumor cells were involved from inferior vena cava to the right atrium, with metastases to the

liver, lung, and iliac bone. She was diagnosed with stage IV disease according to the National Wilms' Tumor Study criteria, and pathological findings as favorable histology. She underwent systemic chemotherapy with the protocol of SIOP 93-01, followed by surgical resection of the primary left renal WT. After intensive chemotherapy according to the JWiTs DD-4A protocol of the Japan WT Study group was performed, the patient subsequently underwent radiation therapy targeting the primary left renal area and achieved complete disease remission in December 2002. However, she developed inferior vena cava relapse in November 2006. Although chemotherapy was started, effectiveness was few, and thereafter, localized radiotherapy was performed. She was admitted for DC vaccination at the age of 14. The HLA genotype was confirmed as HLA-A*24:02, which was compatible with the modified WT1-235 peptide. Two courses (seven sessions, once every 3 weeks) of DC vaccination containing modified WT1-235 peptide together with tumor lysate (a total of 31.46×10^7 DCs; mean, 2.25×10^7 DCs per session) were administered from November 2011 to August 2012. During DC vaccination, residual tumor cells extending from the inferior vena cava to the right atrium were surgically resected in March 2012. Despite the surgery, new tumor lesions were detected at the hepatic portal area in March 2013. DC vaccination at 1- to 3-month intervals was continued for a total of 11 additional sessions by November 2014. The patient died due to disease progression in May 2015. DC vaccination-related toxicities were tolerable and included grade 2 skin reactions and pain at the injection sites, along with grade 2 low-grade fever within 48 h of treatment. There were no ≥grade 3 adverse effects due to DC vaccination based on CTCAE ver.4.0. Disease-free survival during DC vaccination was achieved for 12 months, and OS since the time of initial diagnosis was 13 years and 4 months.

Immune monitoring with tetramer analysis

Freshly isolated peripheral blood mononuclear cells were stained with phycoerythrin (PE)-conjugated human immunodeficiency virus/HLA-A*24:02 tetramer as a negative control or with PE-conjugated WT1-modified peptide/HLA-A*24:02 tetramer (MBL; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). Other stains included allophycocyanin-conjugated anti-CD3 mAb and fluorescein isothiocyanate-conjugated anti-CD8 mAb prior to the analysis by flow cytometry (BD FACSCalibur™ and BD FACSCanto™ II) in Figure 2A. The presence of WT1 antigen-specific CTLs (WT1-CTLs) was defined according to the following criteria: (i) greater than 0.02% WT1-positive cells of all CD8+ T cells analyzing 50,000–10,000 lymphocytes with no evidence of false-positive cells and (ii) WT1-positive population clustered and not diffused as described (57). WT1-CTLs were determined either by WT1-peptide/HLA-A*24:02 tetramer analysis or by interferon (IFN)-γ-producing clones used in enzyme-linked immunosorbent spot (ELISPOT) assays after DC vaccination as a proof-of-concept analysis. Before DC vaccination in both cases, WT1-CTLs were detectable at levels above 0.02% as previously defined (57). After one course of DC vaccination, the immune monitoring assay demonstrated that WT1-CTLs consisted of 0.05% and 2.05% of the $CD8^+$ T-cell population in cases 1 and 2, respectively (Figure 2B

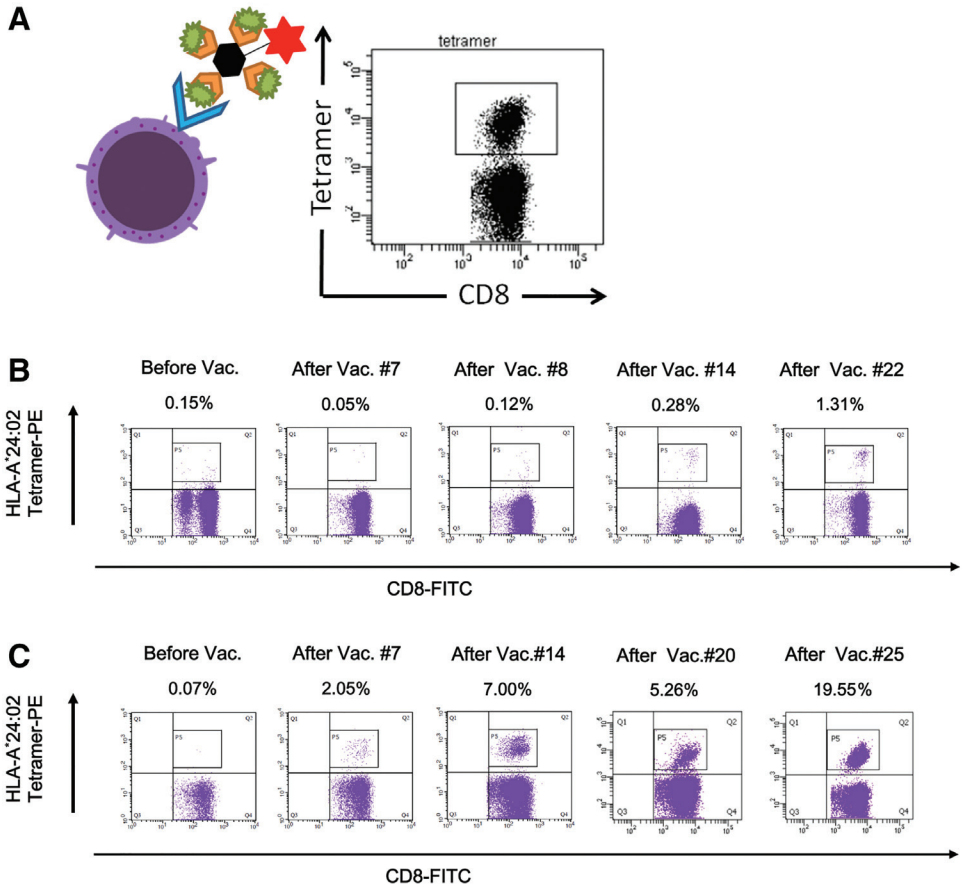

Figure 2. WT1 tetramer assay conducted during the course of DC vaccination. (A) PE-conjugated, WT1-modified peptide/HLA-A*24:02 tetramer was used to detect WT1-specific cytotoxic T cells. Before DC vaccination, WT1-CTLs were at detectable levels in both cases at a concentration of more than 0.02%. After one course of DC vaccination, the immune monitoring assay demonstrated that WT1-CTLs comprise 0.05% of $CD8^+$ T cells in case 1 (B) and 2.05% of $CD8^+$ T cells in case 2 (C). WT1-CTLs concentrations gradually increased after one course of DC vaccination. (Original figure by Shimodaira S.)

and C). WT1-specific T cells were markedly increased after one course during additional vaccination, contributing to the antitumor immune responses noted in our cases (Figure 2B and C).

DC vaccination technology for pediatric patients

Our preliminary study on pediatric patients has several limitations, such as the small sample size and a heterogeneous group of patients. However, DC vaccination targeting WT1 during a standard

therapy course may be both feasible and well tolerated for treating advanced neuroblastoma and WT. The findings also indicated that DC vaccination targeting WT1 generated immunogenicity. WT1-specific CTLs were detected at several levels at the time of initial vaccination, whereas they were distinctly increased after one course of additional vaccination and contributed to induction of the antitumor immune response in our cases. Spontaneous WT1-specific T-cell responses have been reported in acute myeloid leukemia patients (58). Therefore, one possible explanation for the immune response is that the WT1-specific T cells might have been spontaneously induced in these patients. WT1-specific T-cell responses were interestingly maximal at the last session of the course under their disease progression. It is possible that the response of WT1-specific CTLs might be merely boosted by tumor cell growth, although they were no longer able to control disease progression as described with a case of allogeneic DC vaccination targeting WT1 (53).

Case 1 with stage IV neuroblastoma, who relapsed 6 months, had the highest risk of death based on the time to first relapse (59). Our patient survived for 38 months after relapse under disease control with DC vaccination and low-dose etoposide, suggesting a survival benefit together with the maintenance of quality of life. WT1-DC vaccination would be helpful when selecting an optimal therapy for poor survival after neuroblastoma relapse. The WT in case 2 was classified as stage III very high risk for subsequent relapse among children with relapsed WTs (60). Despite the high-dose therapy, MST for very high-risk patients is less than 2 years. It is evident that an effect of the combined modality therapy including DC vaccination achieved the more than 8-year survival after the recurrence in this case, although the contribution of the WT1-DC vaccine to the patient's prolonged survival was unclear. As the number of WT1-CTLs was positively related to the WT1-specific IFN-γ production according to ELISPOT assays (57), the efficacy of DC vaccination would be presumed to be dependent on the number of WT-CTLs. However, the WT1-CTL response to neuroblastoma cells might be limited due to a lack of and downregulation of HLA-class I antigens in neuroblastoma and other renal cell cancers (61–63). Despite an increase in HLA-class I expression on neuroblastoma cells following exposure to IFN-γ (61), there is a concern regarding the attenuation of WT1 antigen in tumor cells during the course of WT1-DC vaccination.

A breakthrough in DC-based vaccine technology is required to achieve further improvement in its cancer treatment efficacy. An allogeneic DC vaccination targeting WT1 may be another potential strategy for patients with relapsed leukemia after HSCT. This strategy may be safe, tolerable, and even feasible for pediatric donors and patients with relapsed leukemia after HSCT as described (52, 53). A 15-year-old girl with acute lymphoblastic leukemia received allogeneic DC vaccination pulsed with WT1 peptide after her third HSCT. The vaccines were generated from her third HSCT donor, the patient's younger 12-year-old sister, who matched with HLA-A*24:02. The patient received 14 vaccine doses with no occurrence of graft-versus-host disease and no systemic adverse effects apart from a grade 2 local skin reaction at the injection site.

WT1-specific immune responses were detected postvaccination by both WT1 tetramer analysis and ELISPOT assays. The patient experienced 44 months of remission after the third HSCT with DC vaccinations, whereas she had been in remission for less than 14 months between her second and third HSCT. This finding suggests that WT1-specific DC vaccination contributed to the extended period of remission following the patient's third HSCT (53). One potential approach to overcome the phenomenon of tumor cells escaping immune detection is the generation of IFN-DCs from monocytes using GM-CSF and IFN-α. Mature forms of IFN-DCs would induce CTLs together with their strong adaptive antitumor effects, with natural killer cell activity independent of HLA-class I antigen expression (64). Another approach is the administration of granulocyte colony-stimulating factor (G-CSF), resulting in the upregulation of monocyte adhesion molecules. An evaluation of the hypothesis that acceleration of acquired cancer immunity using a G-CSF-primed WT1-DC vaccine is related to the type of cancer is ongoing.

The efficacy of DC vaccination may be enhanced by the off-target effects of chemotherapeutic drugs such as gemcitabine (2′,2′-difluorodeoxycytidine, GEM) and a combination of tegafur, gimeracil, and oteracil (48). It has also been reported that WT1 antigen expression in pancreatic cancer cell lines is increased by GEM treatment (65). Initial radiotherapy with additional chemotherapeutic drugs acting through their off-target effects may have accelerated the development of acquired cancer immunity and induced antigen-specific CTLs in patients receiving WT1-targeted DC vaccinations (47). Therefore, DC vaccines in combination with chemotherapy and radiotherapy should promote treatment efficacy against advanced disease. It is necessary to determine the best combinations of the DC vaccine with chemotherapeutic drugs for treating WT and pediatric neoplasms. Immune checkpoint inhibitors are rapidly being developed as chemotherapeutic agents (66). Further studies are required to evaluate whether effector memory T-cell numbers prior to vaccination and the exhaustion of markers for PD1-positive CTLs after DC vaccination influence the efficacy of DC vaccination. Targeted clinical trials could reveal the effectiveness of DC vaccine in combination with immune checkpoint inhibitors as cancer treatments in the near future. Predictive biomarkers for use with DC vaccination targeting WT1 are highly relevant to the personalized cancer therapy.

Conclusion

Our preliminary study suggests that DC vaccination targeting WT1 administered during the course of standard cancer therapies may be both feasible for and well tolerated by patients with neuroblastoma and WT. The study findings indicate that induction of acquired immunity by targeting WT1 was detected by immune monitoring with tetramer analysis during the course of DC vaccination, confirming the positive results for this proof-of-concept investigation in pediatric patients. The results also suggest that WT1-DC vaccination may prolong the survival of pediatric patients with neoplasms. In contrast, it was not clearly

determined whether there was an improvement in patient prognosis following WT1-DC vaccination because both patients died due to disease progression. Therefore, the efficacy and safety of DC vaccination should be determined by phase I/II prospective trials enrolling larger numbers of patients with pediatric neoplasms.

Conflicts of Interest

The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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Chapter 9

Chronic Kidney Disease in Wilms Tumour Survivors – What Do We Know Today?

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Abstract

Currently, the treatment of Wilms tumour (WT) is successful in approximately 90% of cases, and consists of chemotherapy, nephrectomy, and, in some cases, radiation therapy. All treatments have potential long-term influence on the function of solitary kidneys in WT survivors (WTS). Severe reduction in glomerular filtration rate occurs after nephrectomy. All patients who underwent surgical treatment for WT could be considered to have

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a risk of chronic kidney disease (CKD) because they lack a kidney. End-stage renal disease is rare (1.8% of National Wilms Tumour Study patients). Recent studies have revealed that patients with CKD have a greater risk of cardiovascular events and death. Most of the WTS have lower stages or no CKD. Regular biochemical studies and ultrasound examination at follow-up visits should be considered as indispensible elements of long-term care in uninephrectomized WTS. The evaluation of a single kidney function should be frequent, consisting of the assessment of estimated glomerular filtration rate (eGFR), assessment of albumin urine excretion, urine sediment analysis to detect abnormalities, ultrasound examination and measurements of blood pressure. According to Kidney Disease Improving Global Outcomes (KDIGO) recommendation and suggestions, GFR should be assessed using GFR-estimating equations that include serum creatinine and cystatin C concentrations. Cystatin C can be a more sensitive marker of kidney filtration function than creatinine, especially in diseases characterized by a mild decrease in glomerular filtration. This will facilitate the detection of early kidney impairment and assessment of the progression of CKD in WTS.

Key words: Chronic kidney disease; Renal function; Solitary kidney; Wilms tumour survivors

Introduction

Currently, the treatment of Wilms tumour (WT) is successful in approximately 90% of cases after chemotherapy, nephrectomy and, in some cases, radiation therapy (1). The number of survivors who have completed this treatment is increasing. All treatments can have potential long-term influence on the renal function of WT survivors (WTS) (2–4). A wide range of defects in kidney structure and function, from end-stage renal disease (ESRD) to varying degrees of chronic kidney disease (CKD), have been reported. CKD is associated with an increased risk of cardiovascular events, hospitalization and higher mortality (4, 5). The incidence and causes of renal dysfunctions vary depending on distinct clinical situations: sporadic (nonsyndromic) unilateral WT (UWT), sporadic bilateral WT (BWT) and WT arising in patients with genetic predisposition syndromes. All WTS are also considered to be at increased risk of acute kidney injury. Even mild deficiencies in the renal function may be associated with an increased risk of hypertension and cardiovascular disease.

End-stage renal disease in WT

ESRD, simply defined as the need for kidney replacement therapy, is very rare in WTS. Although observed mostly among patients who present with or develop BWT, it is also more frequent among children with syndromic WT. The latter include patients with microdeletion 11p13 syndrome (i.e., WAGR syndrome, MIM#194072: WT, Aniridia, Genitourinary

malformation, mental Retardation) and Denys–Drash syndrome (DDS, MIM#194080) (7). The 20-year cumulative risk of ESRD among WAGR syndrome and DDS patients was 43.3% [95% confidence interval (CI), 20.8–59.5%] and 82.7% (95% CI, 60.5–92.4%), respectively (8–10).

WT1 gene expression has a crucial role in normal kidney development. The disruption of the activity of WT1 protein may lead to fewer functional nephrons at birth, and histological studies of patients with WAGR syndrome indicate a reduction in the size of glomeruli that is presumably related to the *WT1* deletion. Reductions in nephron and podocyte number and mass could increase susceptibility to renal failure, particularly in patients with unilateral or partial bilateral nephrectomy. Reduced expression of WT1 in adult podocytes may reduce the GFR and eventually lead to glomerular sclerosis (9). Recently, Lipska et al. (11) evaluated genotype–phenotype associations in *WT1* glomerulopathy. The authors reported that diffuse mesangial sclerosis is largely specific for *WT1* disease, but focal segmental glomerulosclerosis was equally prevalent in *WT1*-positive and *WT1*-negative steroid-resistant nephrotic syndrome patients. According to the National Wilms Tumour Study (NWTS), the cumulative incidence of ESRD due to chronic renal failure (CRF) 20 years after WT diagnosis was 0.7% (9).

The low incidence of ESRD in WTS is also confirmed by European studies (12, 13). For ESRD due to progressive BWT, it was 4.0% at 3 years post-WT diagnosis in patients with synchronous BWT and 19.3% in patients with metachronous BWT. Lange et al. (9) concluded that metachronous BWT is associated with high rates of ESRD due to surgery for progressive WT. Carriers of germline *WT1* mutation had markedly increased risk of ESRD due to CRF, despite a low risk in non-*WT1* syndromic patients overall. Ritchey et al. (8) reported an incidence of renal failure of 0.25% among patients with UWT, with a median follow-up of 6 years from diagnosis (range: 2 months–22 years).

Analysis of NWTS trials performed by Grigoriev et al. (14) showed that ESRD was diagnosed in 173 patients among 9,162 individuals with WT treated between October 1969 and April 2002. The most common causes of ESRD were progressive BWT (55); DDS (27); WAGR syndrome (10); radiation nephritis (12); focal segmental glomerulosclerosis (18); CKD, aetiology unknown (16) and hypertension (7). Fifty-five patients whose ERSD resulted from progressive BWT experienced high early mortality from WT that limited their opportunity for transplant (47% at 5 years) and survival (44% at 10 years) compared with population controls. The remaining 118 patients, many of whom had *WT1*-associated congenital anomalies, had transplant (77% at 5 years) and survival (73% at 10 years) outcomes no worse than those for population controls. The risk of ESRD due to progressive BWT was largely confined to the first 3 years following the onset of bilateral disease, whereas the incidence rates of ESRD due to CKD continued to increase for 20–25 years from WT diagnosis (14).

It is known that patients with hereditary predisposition syndromes are at a higher risk of developing bilateral tumours (both synchronous and metachronous). Two main molecular subgroups are recognized: syndromes associated with germline *WT1* mutation and overgrowth syndromes associated with epigenetic alterations in chromosome 11p15 (15, 16). Germline *WT1* mutations are also associated with renal developmental abnormalities and are risk factors for renal dysfunction regardless of the occurrence of WT. The following syndromes are associated with *WT1* mutation: DDS, WAGR syndrome, Frasier syndrome (MIM#136680) and isolated WT (MIM#194070). The 20-year cumulative incidence of ESRD in patients with DDS and WAGR syndrome treated for WT can be as high as 80% and 90%, respectively (9). In patients with overgrowth syndromes, the most common being Beckwith–Wiedemann syndrome (BWS, MIM#130650), there does not seem to be a higher risk for renal dysfunction. Nevertheless, almost 20% of WT are bilateral in individuals with BWS (16).

Romao et al. (15) suggests that patients with hereditary predisposition syndromes who develop UWT should be treated with preoperative chemotherapy followed by nephron-sparing surgery (NSS), with the goal of preserving normal kidney function. The issue remains, however, strongly controversial. For instance, data presented by Lipska et al. (11) support pre-emptive/elective bilateral nephrectomy in patients with exonic germline *WT1* (i.e., Denys–Drash type) mutations (14). Using the international PodoNet cohort, Lipska et al. described the genotypic and phenotypic spectrum of *WT1*-associated kidney disease in 61 patients, the largest cohort of *WT1* nephropathy analyzed to date. Eighty-two percent of DDS patients needed kidney replacement therapy within 10 years of diagnosis. Among patients with exonic mutations who initially presented proteinuria of various degrees, 67% eventually developed WT, including 23% BWT. A total of 27 patients (44%), including 4 with intronic (Frasier type) mutations, underwent bilateral nephrectomy. Half of them, all with exonic mutations, underwent the surgery before their fifth birthday. Nephrectomy was performed electively before transplantation (n=18) due to BWT (n=5) or suspicious sonographic findings (n = 4) (11).

The development of renal dysfunction in survivors of BWT is a well-known complication. The philosophy of initial treatment with neoadjuvant chemotherapy for BWT is to avoid renal failure by maximal preservation of renal parenchyma (17). Bishop et al. (17) first reported a significant difference in the incidence of renal failure in NWTS patients with BWT (9% synchronous, 18% metachronous) versus unilateral involvement (1%). The primary cause of renal failure was bilateral nephrectomy for persistent or recurrent tumour. Within the NWTS 4, 23 out of 188 (12%) patients with bilateral disease followed from 1986 to 1994 developed ESRD (18). According to Lange et al. (9) who studied ESRD in non-*WT1*-syndromic patients treated by NWTS, the incidence of ESRD increased dramatically 20 years after diagnosis, reaching 3.1% for BWT. Non-*WT1*-syndromic BWTs have six times the risk of ESRD compared with unilateral ones.

The function of solitary kidney in WTS

The function of solitary kidney in WTS has been analyzed in several studies. Some investigators consider post-nephrectomy renal dysfunction as clinically insignificant (19, 20). In contrast, other investigators consider this renal dysfunction to be a harbinger of long-term consequences (21, 22). Romao et al. (15) were the first to draw attention to the need to develop risk stratification for renal dysfunction for unilateral nonsyndromic WT patients. Early detection of patients at risk may help tailor treatment (e.g., nephrectomy or NSS) and create focused monitoring protocols. As molecular biomarkers for both biological aggressiveness and multifocality are being discovered and incorporated into clinical practice, targeted interventions may be devised to improve the balance between cure and long-term morbidity.

Recently, Interiano et al. (23) evaluated the prevalence of hypertension and impaired renal function in a group of 75 long-term survivors of non-syndromic UWT (median length of follow-up, 19.6 years; range: 10.0–32.8 years) who were treated without nephrotoxic chemotherapy or ionizing radiation. Renal function was assessed by urinalysis and eGFR. Sixteen patients (21.3%) only had eGFR<90 ml/min/1.73 m2, no patient had an eGFR <60 ml/min/1.73 m2 and five patients (6.7%) had hypertension. At the time of last follow-up, no patient developed ESRD. The authors concluded that patients with UWT who were treated with unilateral radical nephrectomy without nephrotoxic chemotherapy or ionizing radiation appear to be at low risk of developing significant long-term renal dysfunction, but monitoring and counselling are important for early detection of subtle abnormalities. This group of WTS might be at an increased risk of adverse cardiovascular sequelae.

Hypertension is one of the components of the metabolic syndrome, which is indicated to be an important risk factor for developing cardiovascular diseases and type II diabetes mellitus. Van Waas et al. (24) reported that long-term adult survivors of childhood cancer are at increased risk of developing components of the metabolic syndrome. Their analysis of 500 adult survivors of childhood cancer provides information on the occurrence of components of the metabolic syndrome in long-term survivors of 11 types of childhood cancer. Systolic blood pressure was increased after the treatment of all types of malignancies, except for Langerhans cell histiocytosis and include WTS. It is unknown what determines the elevated blood pressure in survivors. Dysfunction of the endothelium has been hypothesized to be the initial step in the development of cardiovascular diseases. Chemotherapy agents like anthracyclines are known to damage the vascular endothelium. Additionally, radiotherapy could have a damaging effect on the endothelium.

Chronic kidney disease in WTS

According to the definition of the National Kidney Foundation (NKF), CKD is defined as abnormalities of kidney structure or function present for >3 months with implications for

health. CKD is either a kidney damage or an eGFR below 60 ml/min/1.73 m^2 for over 3 months (6). Kidney damage is defined as pathologic abnormalities or markers of damage, including abnormalities in blood or urine tests or imaging studies (6, 25,). Signs of kidney damage may indicate the risk of deterioration of kidney function in the future. Long-term monitoring of renal function in WTS will facilitate the identification of those with treatment-related impairment of function.WTS are at risk of deterioration of renal function and CKD because of the following: decreased number of nephrons – after nephrectomy, nephrotoxic side effects of chemotherapy (carboplatin, cisplatin, ifosfamide, cyclophosphamide) or radiation therapy – if solitary kidney was in the field of radiation (3, 4, 26, 27). According to Daw et al. (3), the most severe reduction in GFR, measured by 99Tc-DTPA (technetium-99m-diethylenetriamine pentaacetic acid) clearance, occurs after nephrectomy. Decreasing the number of nephrons causes a compensatory increase in the filtration of the remaining nephrons to maintain excretory demands. Subsequent glomerular hyperfiltration in the remaining kidney leads to further deterioration of viable nephrons. Over time, the number of viable nephrons may become insufficient, resulting in a further reduction in kidney function (28-30).

According to the Brenner theory, the reduced filtration surface area of the kidney resulting from an acquired deficit of glomeruli impairs the normal adjustment of blood pressure by pressure natriuresis (31). Therefore, patients with a solitary kidney reveal an increased risk of albuminuria, hypertension and CKD.CKD can be diagnosed in all WTS subjected to nephrectomy. According to Interiano et al. (23), the current guidelines do not recognize solitary kidney or unilateral nephrectomy as a structural abnormality, but further studies are needed to determine whether a lack of one kidney is a marker for CKD development. In our opinion, from the viewpoint of renal function and long-term survival among uninephrectomized WT patients, the above statement is neither certain nor obvious. There are few long-term studies that evaluate the renal function in adult WTS , and we do not have sufficient scientific evidence that confirms the validity of this thesis (4, 32-34). Certainly, solitary kidney is a risk factor for the progression of CKD.

Little is known about the renal function in adult WTS who underwent nephrectomy a long time ago. Patients with WT are mostly very young children, and the prevalence of severe renal dysfunction owing to multifactorial causes is likely to increase with longer follow-up and survival. Kern et al. (35) assessed the renal function in a group of 55 patients with non-syndromic UWT and reported that increasing time between surgery and the last known GFR follow-up was associated with decreased GFR. They concluded that longer follow-up may reveal that a clinically significant decline in the renal function occurs in the years following nephrectomy. Because of the potential for long-term renal insufficiency in children who undergo unilateral nephrectomy, some groups have advocated for NSS in patients with UWT to preserve renal parenchyma and function (32, 33). The use of NSS was judged by

these investigators as essential to reduce post-nephrectomy renal morbidity. NSS is infrequently performed in patients with UWT because the majority of cooperative group protocols recommend a radical nephrectomy (36). Partial nephrectomy for patients with UWT is a more controversial topic, particularly in the case of excellent oncologic outcomes with unilateral radical nephrectomy. The risk of local recurrence, need for therapy intensification, and unclear long-term renal function benefits have been the basis for debate against NSS in the treatment of unilateral non-syndromic WT (15). NSS is necessary in patients with BWT for the preservation of nephrons and renal function (17, 18).

Cozzi et al. (33) also examined and compared the renal function of 20 WTS treated with NSS, 40 WTS aged 2–30 years treated with nephrectomy, and 18 WTS aged 33–51 years treated with nephrectomy. While only 8% of NSS and 42% of nephrectomized young WTS presented mild-to-moderate renal function, this was 78% in the oldest nephrectomized WTS. The authors demonstrated a significant reduction in eGFR in the fifth decade of life in a group of WTS who underwent nephrectomy compared with patients during the third decade after surgery. Currently, the role of NSS in the treatment of unilateral nonsyndromic WT patients is discussed in the context of ensuring adequate local control and protection of renal function. So far, the standard of UWT treatment according to the Societe International de Oncologie Pediatrique (SIOP) and Children's Oncology Group protocols includes radical nephrectomy. Data from the SIOP 2001 study showed that NSS was only performed in 3% of patients with UWT. Wilde et al. (36) concluded that NSS as a new approach for UWT has now been shown to be safe in a small and highly selected group of patients, concordant with the intention of the SIOP 2001 protocol. The event-free and overall survival after NSS appeared to be as good as total nephrectomy with an equal local relapse rate as that of total nephrectomy. Despite excellent survival, the gain of nephrons needs to be weighed against the risk to induce stage III with intensified therapy. Studies on the renal function after NSS are based on relatively small patient groups. Larger prospective studies are needed to fully assess the gain of renal function and oncological outcome.

Previously, we have analyzed the prevalence of CKD in nephrectomized WTS in a group of 32 patients (children and adolescents). All participants had undergone unilateral nephrectomy and had been treated according to the chemotherapy protocols SIOP 9, SIOP 1992, SIOP 2001 between the years 1987 and 2008. Kidney damage was established by the assessment of GFR using 99 Tc-DTPA clearance, the Schwartz formula, the new Schwartz equation, Filler formula, serum cystatin C concentration, β2-microglobulin and albumin urine excretion, urine sediment and ultrasound examination. Blood pressure was measured. The mean values of GFR assessed with different methods, 99Tc-DTPA clearance, eGFR Schwartz, eGFR new Schwartz equation for children with CKD and eGFR Filler, were all well above 60 ml/min/1.73 m^2. Increased excretion of albumin (ACR >30 mg/g creatinine) and B-2-microglobuin (BCR >0.04 g/mol) was observed in 22% and 13% of patients, respectively.

Table. 1 Renal function in Wilms tumour survivors based on publish data in recent years

	Number of patients	Age at follow up mean±SD/ median (range)	Follow up duration [years] mean ±SD/median (range)	Stage	Treatment	eGFR * [ml/ min/1.73 m^2] mean±SD/ median (range)	Hypertension	Albuminuria or proteinuria	BCR**	Recommendations of monitoring		
Mavinkurve-Groothuis 2015 [44]	31	17.8±3.6	14.8±3.3	I-IV	SIOP	83±14 <60 – 4.2%	26%	-	-		No renal injury	eGFR<60 and/or treat-ment
										BP	1/y	2-4/y
										ACR***	1/y	2-4/y
										SCr****/ eGFR	1/5y	2-4/y
Schiavetti 2015 [43]	35	25	20±5.43	I-IV	SIOP	midly decreased G2, (84.5, 63-89) 22.9% CKD 8.6%	2.9%	A2 (30-300 mg/L), 14%	-	necessary		
Kern 2014 [36]	55	-	6.3 (median)	-	-	↓15%	-	-	-	necessary		
Spreafico 2014 [45]	15	18.3 (12.7-30.4)	13.3 (10.2-19.3)	I, II	VCR, ACTD	103.8 (89.2-166.7)	24 h ABPM***** normal	0.19 (0.02-13.2)	0.57 (0.2-1.0)	at least every 5 years		
Elli 2013 [46]	15	-	9.9 (2-21)	I-IV	NWTS	0%	24 h ABPM 20% 24 h SBP higher com-par to con-trol group ($p<0.05$)	Normal/0%	-	regular follow up should included BP monitoring, USG, ECHO, kidney function parameters		
Sanpakit 2013 [47]	30	-	4.8 (media)	I-IV	NWTS (ICE-9 patients)	119	30%	0%	-	necessary		

Dekkers 2013 [48]	85	27.9 (17.9-49.0)	24.4 (12.2-41.1)	I-IV	SIOP	60-90 51.8% 30-59 4,7% 15-29 0% <15 3.5%	31.4%	25%	-	necessary
Cozzi 2013 [34]	18	42.7±5.7	38.44±4.9	I-IV	VCR, ACTD, ADR	76.1±16.3 <90 – 78%	-	-	-	necessary
	42	15.8±8.0	11.38±7.8	I-IV	VCR, ACTD, ADR	95.1±18.5 <90 – 43%	-	-	-	
Stefanowicz 2011 [28]	32	12.2 (3.6-24.3)	7.75 (0.3-20.6)	I- IV	SIOP	94.28 (10.24)	6.3%	21.9%	12,5%	eGFR, ACR, BCR, urine sediment analyses, USG
Daw 2009 [3]	11	18.6 (12-25.6)	11.2 (7.3-12.8)	II-IV	ICE (ifosfamide, carboplatin, etoposide), nephrectomy	86 (10-169) <90 – 54% Renal transplantation 1 (with FSGS)	18%	18%	↑	necessary
Bailey 2002 [19]	40	14.31 (3.38-29.75)	8.8 (0.06-27.5)	I-IV	VCR, ACTD, ADR	100 (61-150) 60-90 25% 30-59 0% 15-29 0% <15 0%	0%	5%	-	necessary, not specified

*eGFR – estimated glomerular filtration rate.

**BCR – β2microglobulin/urine creatinine.

***ACR-albumin/urine creatinine.

****SCr – serum creatinine.

*****ABPM – ambulatory blood pressure monitoring.

Arterial hypertension, based on the mean values of systolic and diastolic blood pressures from three independent measurements, over the 95th centile was observed in 6.25% individuals (27).

Ultrasound examination of WTS provides an opportunity to detect signs of kidney damage. According to the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI), sonographic features associated with CKD include the following: nephrocalcinosis, stones, hydronephrosis, cysts, increased echogenicity of kidney, small 'hyperechoic kidney', large kidneys, size disparities and scars, and venous thrombosis or renal stenosis (25). Thus, ultrasound examination can be used to identify WTS with CKD and risk of deterioration of eGFR. In our previous study, sonographic signs of kidney damage, including hyperechoic rings around renal pyramids (37.5%), renal scars (9%), increased echogenicity of renal cortex (15.5%) and cysts (3%), were observed in 43% of WTS (27).

Hypertrophy of a solitary kidney [length or volume of kidney over 2SD (standard deviation of reference value)] in WTS was observed in 50–88% of individuals (9, 37, 38). Mean value of renal length was 128 ± 14% of the reference value. Mean value of renal volume was from 155±35% to 213% (39, 40). In one study, correlation between microalbuminuria and renal volume was observed (40). The parenchymal thickness/kidney length ratio correlated with the deterioration of renal function (cystatin C serum concentration) (38). Currently, in light of the definition of CKD for individuals with the risk of deterioration of kidney function, it is essential not only to assess GFR but also to establish the presence of structural and functional markers of kidney damage. A marker for CKD, which may be more sensitive to the detection of early renal impairment, is cystatin C. According to Kazama et al. (41), a cystatin C serum concentration greater than 0.98 mg/dl has a sensitivity of 88.5% and a specificity of 95.2% for detecting GFR below 80 ml/min/1.73 m2. Recently, Schiavetti et al. (42) evaluated the prevalence of and the possible risk factors for the renal impairment in 35 adult WTS by estimating GFR categories and CKD according to KDIGO guidelines from 2012 (6). Only eight (23%) survivors presented a mildly decreased eGFR, three survivors (9%) had CKD and one (3%) hypertension. Data on the renal function in WTS from recent reports are included in Table 1.

Conclusion

To conclude, in our opinion, the evaluation of a single kidney in WTS should be regular and consist of the assessment of eGFR using equations, albumin excretion, urine sediment analysis, ultrasound examination and blood pressure measurements. If we do not have progressive renal injury, this nephrological follow-up should be once per year. It is necessary to diagnose early renal impairment. According to KDIGO recommendations, CKD is defined as abnormalities of kidney structure or function, present for >3 months, with implications for health, and CKD is classified based on cause, GFR category and albuminuria category.

The assessment of eGFR and albumin excretion is necessary to determine the risk of CKD. For GFR evaluation, KDIGO recommends using serum creatinine and a GFR estimating equation for initial assessment and suggests using additional tests such as cystatin C for confirmatory testing in specific circumstances when eGFR based on serum creatinine is less accurate. In children, GFR can be estimated using Schwartz formula [41.3 × (height/serum creatinine)], where height is expressed in meters and serum creatinine in mg/dl (6).

Furthermore, according to Romao et al. (15), the discussion about renal dysfunction in WTS will evolve and receive more attention. At present, survival in patients with WT is very good. The number of survivors increases from year to year. It is necessary to agree on the standardized follow-up protocols and tools to measure renal dysfunction over time. A complete assessment of renal function in WTS should be simple, easy, generally available and, according to NKF recommendations, include estimated GFR, urine test with albuminuria, ultrasound examination and measurements of blood pressure. This will facilitate the assessment and detection of the progression of CKD in WTS.

Conflicts of Interest

The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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Section II

Biology

Chapter 10

Gene Expression in Wilms Tumor: Disturbance of the Wnt Signaling Pathway and MicroRNA Biogenesis

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Abstract

Wilms tumor (WT) originates from the metanephric blastemal cells that are unable to complete the mesenchymal–epithelial transition, resulting in a tumor with triphasic histology, including blastemal, epithelial, and stromal components. WT shows morphological and molecular characteristics that resemble the fetal kidney. Thus, the study of molecular pathways relevant to normal kidney differentiation provides insight into the events that drive

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Wilms tumorigenesis. The Wnt signaling pathway has been shown to be crucial for correct kidney differentiation. This pathway is activated by WNT proteins and consists of two highly connected main branches: the canonical (or β-catenin dependent) and the noncanonical (or β-catenin independent). Both branches are essential for controlling embryonic development and adult cell homeostasis. The activation of the canonical Wnt pathway leads to the nuclear accumulation of β-catenin, which acts as a coactivator for transcription factors. In the absence of WNT ligands, this pathway is inactivated by a destruction complex that phosphorylates β-catenin, leading to ubiquitination, proteasomal degradation, and the prevention of β-catenin accumulation in the nucleus. In this context, the expression and mutation analyses of genes involved in Wnt signaling pathways constitute an important approach for understanding WT etiology. Although the activation of the Wnt pathway is well understood in WT samples relative to normal kidney tissue or differentiated kidney cells, there is a remarkable variation among subgroups of WTs. Recently, five WT subgroups were identified, mainly through the use of gene expression data, and only two of them showed clear evidence of Wnt pathway activation, as measured by the presence of β-catenin in the nucleus. Interestingly, some of these subgroups exhibited recurrent germline or somatic mutations in genes involved in microRNA biogenesis, such as *DROSHA* and *DICER*. Here, we will review relevant findings regarding Wilms tumorigenesis as revealed by gene expression and mutation analyses, mainly in genes belonging to the Wnt signaling and microRNA biogenesis pathways.

Key words: β-catenin; microRNA biogenesis; Nephrogenesis; Wilms tumor; Wnt signaling pathway

Introduction

Wilms tumor (WT) is an embryonic tumor that is initiated from primitive renal cells that are incapable of completing kidney differentiation. The result is a tumor that recapitulates the earliest step of nephrogenesis and is morphologically and molecularly similar to the fetal kidney. As a consequence, WTs are composed of varying proportions of three morphologically distinct cell types: undifferentiated blastemal cells, epithelial cells ordered into primitive structures, and stromal cells (1). Accordingly, the blastemal component displays an expression profile similar to the earlier stages of kidney development (2).

Signal transduction pathways control signaling from the outside to the inside of a cell through interactions between proteins and cell surface receptors, triggering specific cellular processes, mainly via changes in gene expression. The precise control of gene activation or inactivation is crucial for correct kidney differentiation and function. Disturbances in this process through the mutation of genes that directly or indirectly control gene

expression result in the failure of precise kidney development and may in turn lead to renal disease in children, including renal agenesis, dysplasia, hypoplasia, and WT (3). In WT, mutations have been identified in tumor suppressor genes (TSG) and oncogenes (4) (mainly from the WNT signaling pathway) or in genes involved in microRNA (miRNA) biogenesis (5, 6). Thus, as carcinogenesis requires gene mutations, the morphological aspects of WT seem to be dependent on where and when mutations occur during the process of kidney differentiation. In this chapter, we present the current molecular and morphogenetic knowledge about nephrogenesis and WT, focusing on the Wnt signaling and miRNA biogenesis pathways.

Morphogenetic process of kidney development

Kidney development, also known as nephrogenesis, refers to the embryologic origins of this organ. Kidney morphogenesis begins at gastrulation, in the third week of gestation in humans, when the embryo exhibits the three germ layers: ectoderm, mesoderm, and endoderm. The intermediate mesoderm gradually forms the urogenital system, including the pronephros, mesonephros, and metanephros; the first two proceed to develop into transitory kidneys and the third differentiates into the mature and functional kidney (7). The metanephros originates through interactive signals in bidirectional communication between epithelial and mesenchymal cells that ultimately form the nephrons, the functional unit of the kidney. Thus, the entire process of the differentiation of the kidney, with its multifaceted functional structures, involves close interaction between epithelial and mesenchymal cells, in which the signal transduction pathway is imperative. Mesenchymal–epithelial transition (MET) is a crucial process operating during kidney differentiation (7), which comprises the transition from a multipolar or a spindle-shaped mesenchymal cell to a planar assembly of polarized cells known as epithelia. Epithelial cells are stationary and are characterized by apical–basal polarity, tight junctions, and the expression of cell–cell adhesion markers, such as E-cadherin (8), whereas mesenchymal cells do not form cell–cell contacts. Mesenchymal cells can invade through the extracellular matrix and express markers, such as vimentin, fibronectin, N-cadherin, basic helix-loop-helix transcription factor (TWIST), and zinc finger protein SNAI1 (SNAIL) (9).

The exact mechanism that triggers MET in kidney progenitor cells is not entirely known although it has been shown to depend on the silencing of specific genes (e.g., *Osr1* and *Six2*) (7). The morphological result of this process is the formation of a vesicle composed of the metanephric blastema, which further forms the comma-shaped body, followed by the S-shaped body, and then Bowman's capsule, finally culminating in the functional nephron (10). Next, we present some important aspects of WNT signaling, which is a key pathway in MET.

Wnt signaling pathway

The Wnt signaling pathway encompasses a variety of signaling cascades activated by the secreted WNT proteins with major involvement in nephrogenesis. The Wnt signaling pathway has been divided into two main branches: canonical and noncanonical. The canonical Wnt pathway (or the Wnt/β-catenin pathway) operates with the involvement of β-catenin, encoded by *CTNNB1*, whereas the noncanonical (or β-catenin independent) pathway does not involve β-catenin (11, 12). The noncanonical Wnt signaling pathway is mainly divided into the Wnt/calcium (Wnt/Ca^{2+}) and planar cell polarity (PCP) pathways. Despite several differences between the two branches, both are activated by the binding of a Wnt ligand to a Frizzled (FZD) family receptor (13, 14).

The canonical Wnt pathway is characterized by its intracellular mediator β-catenin and plays a crucial role in cell fate. In the canonical Wnt pathway, β-catenin can accumulate in the cytoplasm and either be directed to the membrane as a part of the cell–cell adhesion complex or be translocated into the nucleus, acting as a transcriptional coactivator of TCF/LEF family of transcription factors. Thus, β-catenin plays a dual role, either regulating the coordination of cell–cell adhesion (in the inactivated Wnt signaling pathway) or acting as a transcriptional cofactor when translocated to the nucleus (in the activated Wnt signaling pathway). Thus, the regulation of cytoplasmic levels of β-catenin by the APC/AXIN1 (adenomatous polyposis coli/) destruction complex (DC) represents a fundamental control step of the canonical Wnt pathway.

The DC is composed of AXIN1, PP2A, GSK3, CK1, Dishevelled (DSH), and APC and marks β-catenin for degradation by the proteasome through ubiquitination. The heterodimer formed by a Wnt ligand and an FDZ (frizzled) receptor (WNT–FDZ heterodimer) interacts with the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6), another cell surface protein, recruiting cytoplasmic AXIN1 and preventing the formation of APC/AXIN1. The WNT–FDZ heterodimer recruits and interacts with a series of cytoplasmic proteins to prevent the DC from ubiquitinating β-catenin and targeting it to the proteasome (11, 15). Conversely, in the absence of Wnt proteins, the DC phosphorylates β-catenin, which is further ubiquitinated by an E3 ubiquitin ligase (B-TrCP) and degraded in the proteasome (15). More recently, it was observed that the APC membrane recruitment protein 1 (AMER1) interacts with the APC/AXIN1 DC although its role is not yet completely understood (16). Additionally, controversies about β-catenin ubiquitination and degradation in the context of the AXIN1 complex and about the disassembly of the DC are noted in the literature (17).

In summary, in the canonical Wnt pathway, the binding of WNT proteins to FZD receptors suppresses β-catenin degradation, resulting in its cytoplasmic accumulation, followed by nuclear translocation, which finally releases the expression of certain genes involved in important cellular processes.

The noncanonical branch of the Wnt pathway resembles the canonical pathway only in its requirement for Wnt ligands [e.g., silberblick (WNT11) and pipetail (WNT5)], FZD receptors, and the cytoplasmic signal transduction molecule DSH. Upon binding of the noncanonical WNT proteins, complexes belonging to the PCP pathway are asymmetrically distributed in the proximal and distal cell membranes [reviewed in reference (18)]. The other downstream interactions remain unclear, but it is well established that the strabismus (STBM) and prickle (PK) proteins are involved, whereas AXIN1, GSK-3, and β-catenin are not. Despite some similarity between the vertebrate noncanonical Wnt pathway and the *Drosophila* PCP pathway, such as the involvement of DSH, no Wnt ligands are known to be involved in *Drosophila* PCP signaling.

Wnt signaling pathway in nephrogenesis

The Wnt/β-catenin pathway is one of the multiple signaling pathways that cooperates in the initiation and progression of MET (19). Several members of the Wnt family have been implicated in the induction of epithelial renal vesicles. WNT4 is required and sufficient for the transition of the metanephric mesenchyme to epithelial cells (20). *Wnt4* is also required for tubulogenesis, and it acts through a noncanonical Wnt pathway (21). WNT9B, which is secreted by cells from the ureteric bud, induces the expression of WNT4, and its loss can be rescued with WNT1, a putative canonical Wnt signaling activator (22). Similarly, WNT6 induces tubulogenesis by activating *WNT4* transcription, which leads to the expression of early markers of kidney tubulogenesis PAX2, PAX8, SFRP2, and E-cadherin genes (23).

Another key protein involved in nephrogenesis is *SIX2* (*Sine oculis homeobox 2 gene*), a transcription factor essential for maintaining the self-renewing and multipotent characteristics of nephron progenitor cells (24). *WNT4* is an upstream regulator of *SIX2*, and decreased expression of *SIX2* results in the commitment of the progenitor cells to undergo differentiation via MET (25).

Wnt/β-catenin signaling also regulates MET through the transcription repressor *SNAIL1*, which is downregulated during embryogenesis, and allows mesenchymal cells to differentiate into epithelia through MET (8, 26, 27).

Wilms tumors and the Wnt signaling pathways

Several studies have confirmed the activation of canonical Wnt signaling pathways in WT. The canonical Wnt signaling pathway, observed by nuclear positivity of β-catenin, is activated in approximately 15–25% of all WTs with a favorable histology (28).

Interestingly, nuclear accumulation of β-catenin in WTs is associated with a mutation in Wilms tumor 1 gene (*WT1*), which in turn is associated with WT cases that show stromal predominance,

where the nuclear positivity of the protein is largely confined to the mesenchymal cells (2, 28). However, the genetic and molecular mechanism that underlies these associations is not understood. The *WT1* is known as an inhibitor of Wnt/β-catenin signaling (29, 30). The antagonistic activities of WT1 and β-catenin probably arises because the two proteins bind to a common transcriptional coactivator, CREB-binding protein. Additionally, the WT1 protein is essential for MET and, hence, for normal embryonic kidney development (31), and genetic deletion and/or inactivating mutations in WT1 cause severe kidney disorders in mice (32).

APC is a tumor suppressor gene whose protein product is a component of the DC for β-catenin, thus its action negatively regulates the canonical Wnt signaling pathway, and this modulation appears to be essential in nephrogenesis. The APC protein exhibits distinct cellular localization during the differentiation process from the fetal kidney to the mature normal kidney. In earlier stages of kidney development, APC expression is nuclear; in later stages, it is cytoplasmic; and in intermediate stages, it is both nuclear and cytoplasmic. Interestingly, in WT samples, the localization of APC recapitulates that in the earliest stages of the fetal kidney, where APC expression is exclusively nuclear—a pattern that resembles the earliest stage in undifferentiated blastemal cells (2). Thus, the nucleocytoplasmic shuttling of APC may be critical in the context of the activation of the canonical Wnt pathway in WT, as well as in kidney development. Given that APC shuttles into and out of the nucleus (33), it is reasonable to speculate that the nuclear localization of APC might interfere with the export of β-catenin from the nucleus in Wnt-stimulated cells. In this context, nuclear APC positivity could be an indirect indication of Wnt signaling activation in WT (2). Although the involvement of the noncanonical Wnt pathway has been demonstrated in nephrogenesis (25) and cancer (34), few studies directly associate disturbances in this pathway and WT.

The noncanonical Wnt signaling pathway directly affects changes in the cytoskeleton, the PCP pathway, and the regulation of calcium release from the endoplasmic reticulum to control intracellular calcium levels via the Wnt/Ca^{2+} pathway.

PLCG2, a gene in the Wnt/Ca^{2+} pathway, is involved in the control of external calcium entry and in innate immune responses (35), and its expression is modulated during nephrogenesis (2). The mRNA and the protein levels of PLCG2 are reduced in kidney progenitor cells and increased in the mature kidney, where protein expression has been shown to be strongly positive in some cells of the nephron. Accordingly, the *PLCG2* expression pattern appears to be recapitulated in WT at the mRNA and protein levels, showing decreased or predominately negative expression, respectively, in WTs compared with differentiated kidneys (2).

WNT5A and *WNT5B*, which are members of the noncanonical Wnt signaling pathway, were also recently identified as being altered in WT. *WNT5A* is likely regulated by *PAX2*

and was found to be expressed at lower levels in WT than in the fetal kidney (36). The expression levels of *WNT5B* mRNA decrease during blastemal cell differentiation. *WNT5B* appears to be associated with the formation of cell polarity, as the WNT5B protein expression has only been observed after the renal vesicle formation, when cells are organizing to form kidney structures, such as glomeruli and tubules, where positivity is observed mainly in the apical cell membrane. Although the mRNA levels of *WNT5B* were observed to be elevated in WT (similar to the undifferentiated blastemal cells that give rise to WT) compared with the differentiated kidneys, the protein expression has been only detected in a minority of cases (37). The mechanism underlying WNT5A and WNT5B signaling remains to be elucidated.

These data provide strong evidence for the involvement of Wnt pathway-related genes in WT, whose pattern in the earliest stages of nephrogenesis is recapitulated in the tumor, marking the disruption of the complete differentiation of the kidney progenitor cells.

Wilms tumor and mutation repertoire

Mutations in *WT1*, a TSG, are present in approximately 20% of WTs. Other TSGs with inactivating mutations include *WTX*, which also occur in approximately 30% of cases (38). The WTX protein has been reported to negatively regulate the canonical WNT pathway, as part of the DC (39). Stabilizing mutations in *CTNNB1* (β-catenin), the major regulator of the canonical WNT pathway, are present in 15% of tumors. The well-known *TP53* gene has been found to be mutated in 5% of WT samples (4). Other genes that have been observed to be mutated in lower frequencies are *DIS3L2* (40), *FBXW7*, and *MYCN* (41). Together, mutations in these genes account for approximately 30% of WT samples.

Despite several lines of evidence supporting the overexpression of a number of downstream genes of the canonical WNT pathway in WT (42, 43), only two of the five currently known WT subtypes show clear evidence of canonical WNT pathway activation (44). These five subgroups (S1–S5) were defined by the hierarchical clustering analysis of expression data from genes of the canonical WT pathway. Only the subgroups, S1 and S2, showed evidence of strong WNT activation. Increased expression of LEF1 and FZD2 and decreased expression of CCND1 and JAG1 characterize the S1 subgroup. Interestingly, some samples from S2 subtype, lacking CTNNB1 or WTX mutations, also showed signals of strong Wnt activation. These findings suggest the presence of other mechanisms for canonical Wnt activation.

The repression of miRNA biogenesis through the inhibition of DROSHA and DICER1 expression impairs accurate kidney differentiation (45–47) and promotes tumorigenesis in several cell lines (48). Accordingly, mutations in genes involved in the miRNA biogenesis were recently identified in a higher proportion of WT samples and appear to be associated

with a predominant blastemal histology (5, 6, 49). Mutations in *DROSHA* are found in 12% of WTs, and a recurrent mutation (E1147K) has been shown to affect an RNase IIIb domain of the protein encoded (5, 6). The frequency of the E1147K mutation was further estimated in a validation set of 538 tumors being observed in 11% of the samples (49). If other genes in the miRNA biogenesis pathway are included, such as *DGCR8*, *TARBP2*, *XPO5*, and *DICER*, mutations in this pathway account for approximately 30% of WT samples.

The effect of the E1147K mutation in DROSHA is associated with a predominant reduction in the expression level of mature miRNAs (5, 6). miRNAs are critical regulators of gene expression, and consequently, the defective miRNA biogenesis observed in WT surely makes a crucial contribution to WT development. However, the effects of the deregulation of miRNAs on their target genes, particularly those assumed to be involved in the differentiation of the kidneys, have yet to be established.

Members of the cyclin gene family were recently described as being upregulated in blastemal-type WT samples, especially in samples with recurrent mutations in the SIX1/2 gene (50). This pattern may be an important underlying cause of the continued proliferation of the metanephric mesenchyme.

Interestingly, tumor samples with ectopic mesenchymal elements show upregulation of WNT-related genes, whereas tumors with epithelial elements do not. Moreover, samples from this "WNT-independent" subgroup often show perilobar nephrogenic rests (PLNRs), instead of intralobar nephrogenic rests, which may reflect the likely origin of these tumors in errors that occur later during kidney development (51). More recently, Walz et al. (49) showed a significant statistical association between the presence of PLNR and the mutations in miRNA processing genes. Further analysis may be necessary to clarify the consequences of this probable association.

Conclusion

The origins of WTs are closely related to the processes of kidney development. This is supported by the fact that genes involved in nephrogenesis are altered in WT (Figure 1). The involvement of WNT signaling pathway in kidney embryogenesis was demonstrated by alterations in several genes directly or indirectly. These alterations include mutations (*WT1*, *WTX*, *CTNNB1*, *TP53*, *DIS3L2*, *FBXW7*, and *MYCN*) or altered expression (*WNT5*, *APC*, and *PLCG2*) in WT. Additionally, mutations in genes from the miRNA biogenesis pathway (*DROSHA*, *DGCR8*, *TARBP2*, *XPO5*, and *DICER*) are found in a relatively high frequency in WT cases. Recently, it was demonstrated that one of these mutations affects the function of *DROSHA*, impairing the process of miRNA biogenesis. Currently, there is an urgent need to understand the possible interplay between the miRNA downregulation and the Wnt

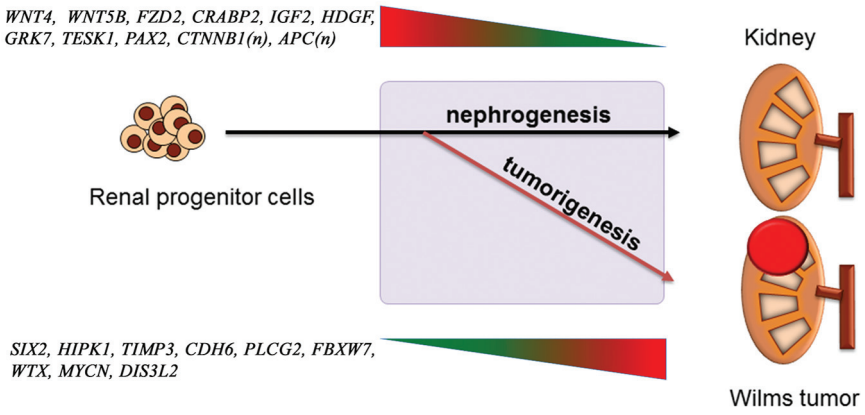

Figure 1. Disruption of differentiation of the precursor cells of the kidney may result in Wilms tumor formation. Several molecules are associated with nephrogenesis and tumorigenesis. The available evidence suggests that the delicate balance in the expression pattern of these molecules during embryogenesis is the determinant of nephrogenesis and that disturbance in the expression leads to tumorigenesis. The differential expression of selected molecules is depicted beside each triangle (red: higher, green: lower). n: nuclear positivity of protein.

signaling pathway. This knowledge may lead to new perspectives in the design of more effective anticancer therapies for WT.

Conflict of Interest

The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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Chapter 11

Transcriptional Regulation of the Human Thromboxane A_2 Receptor Gene by Wilms' Tumour (WT)1

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Abstract

The prostanoid thromboxane (TX)A_2 plays a fundamental role in vascular haemostasis and, more recently, is increasingly implicated in various neoplasms including in prostate, breast and bladder cancers, among others. In humans, TXA_2 signals through the TPα and TPβ isoforms of the T prostanoid receptor (TP), two structurally related receptors that display both common, over-lapping but also distinct, isoform-specific physiologic roles. Consistent with this, while TPα and TPβ are encoded by the same gene, the *TBXA2R*, they are

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differentially expressed due to their transcriptional regulation by distinct promoters where promoter (Prm) 1 regulates TPα expression and Prm3 regulates TPβ. While the clinical evidence for the role of the TXA_2-TP axis in neoplastic progression is increasing, few studies to date have investigated the role of the individual TPα/TPβ isoforms in human cancer or indeed in most other diseases in which the TXA_2-TP axis is implicated. Focusing on TPα, this review details the current understanding of the factors regulating its expression and transcriptional regulation through Prm1, including in prostate and breast cancers. Emphasis is placed on the *trans*-acting transcriptional regulators that bind to *cis*-elements within the core and upstream regulatory regions of Prm1 under basal conditions and in response to cellular differentiation. A particular focus is placed on the role of the tumour suppressor Wilms' tumour 1 in the regulation of TPα expression through Prm1 in megakaryoblastic cells of vascular origin and in prostate and breast carcinoma cells. Collectively, this review details current knowledge of the factors determining regulation of the TXA_2-TPα axis and thereby provides a genetic basis for understanding the role of TXA_2 in the progression of certain human cancers.

Key words: Cancer; Gene; Thromboxane receptor; Transcription; Wilms' tumour 1

Introduction

Thromboxane (TX)A_2, a prostanoid synthesized from arachidonic acid by the sequential enzymatic actions of cyclooxygenase (COX)-1/-2 and TXA synthase (TXS) predominantly in platelets and in monocytes/activated macrophages, plays an essential role in haemostasis regulating platelet aggregation and vascular tone (1). It also induces the constriction of various other types of smooth muscle (SM), including pulmonary, renal and prostate SM, and promotes vascular remodelling in response to endothelial injury contributing to neointimal hyperplasia and restenosis post-stenting (2–4). Accordingly, imbalances in the levels of TXA_2 or of its synthase (TXS) or of its receptor, the T prostanoid receptor (or, in short, the TP), are widely implicated in several cardiovascular, pulmonary, renal and prostate pathologies (1–3, 5). In humans and in other primates, the TP exists as two structurally related isoforms referred to as TPα and TPβ, which are identical for their N-terminal 328 amino acid residues but which differ exclusively in their intracellular C-terminal domains (6). While TPα and TPβ are encoded by the same TP gene, the *TBXA2R* (Figure 1), they are differentially expressed in several cell/tissue types being transcriptionally regulated by two different promoters referred to as Prm1 and Prm3, respectively, within the *TBXA2R* (7–10). Functionally, as members of the G-protein-coupled receptor (GPCR) superfamily, TPα and TPβ both primarily couple to Gαq-mediated phospholipase Cβ activation, raising intracellular calcium levels in response to inositol phosphate turnover, but also readily couple to Gα12-mediated RhoA and to extracellular signal-regulated protein kinase (ERK) activation (Figure 2) (6, 11–14). In contrast, TPα and TPβ undergo distinct mechanisms of agonist-induced

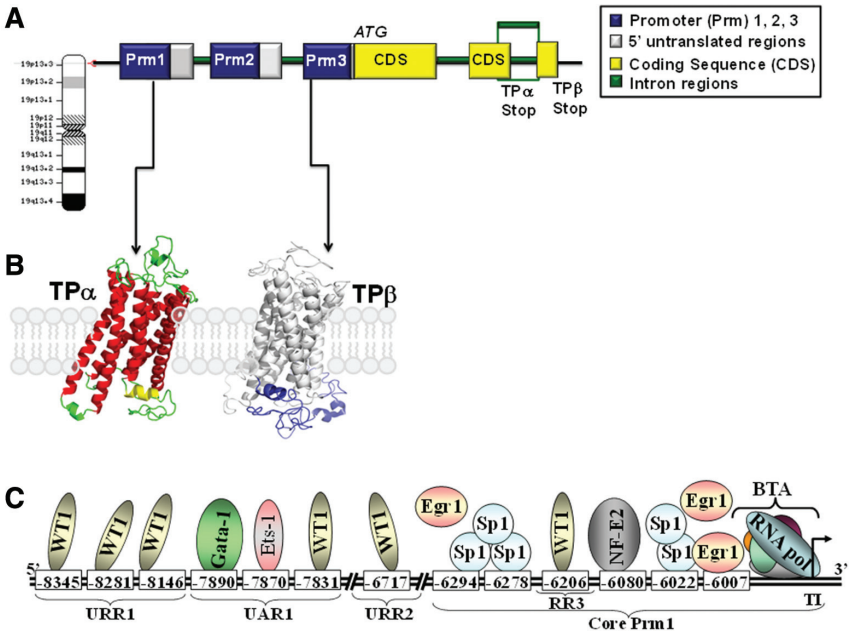

Figure 1. Structural organization of the *TBXA2R* gene. Panel A: In humans, a single *TBXA2R* gene, located on Chr19p13.3, encodes both the TPα and the TPβ isoforms of the T prostanoid receptor (the TP), where promoter (Prm) 1 exclusively regulates TPα and Prm3 regulates TPβ expression, respectively. The functional role of Prm2 is currently unclear but may contribute to the transcriptional regulation of TPα expression. Panel B: Structurally, TPα and TPβ are identical for their N-terminal 328 aa residues but differ exclusively in their intracellular C-terminal domains, where TPα (343 aa) and TPβ (407 aa) have 15 and 79 unique aa residues, respectively. Panel C: Schematic representation of Prm1 and the main *cis*-acting elements and *trans*-acting factors that regulate TPα expression through Prm1. Prm1 is located at -8500 to -5895 relative to the ATG translational initiation codon within the *TBXA2R*. Under basal or resting cell conditions, the Core Prm1 region, located between -6294 and -5895, is under the transcriptional regulation of Sp1, Egr1 and NF-E2 but also contains a repressor region (RR3) that is regulated by Wilms' tumour (WT) 1. Prm1 also contains several upstream repressor regions (URR) and upstream activator regions (UARs). The haematopoietic-specific factors Gata-1 and Ets-1 are the main *trans*-acting factors that bind and regulate UAR1 in mega-karyoblastic HEL92.1.7 cells and Ets-1/2 and Oct-1 regulate UAR2 (not shown). In contrast, WT1 is the main *trans*-acting factor that binds to multiple GC-enriched *cis*-elements within URR1, URR2 and RR3 to repress Prm1-directed TPα expression under basal/resting cellular conditions (e.g., in non-differentiated HEL cells).

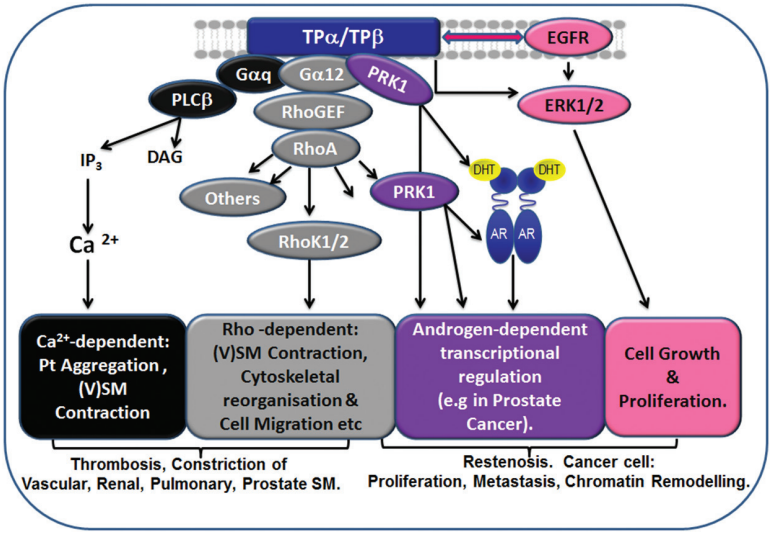

Figure 2. Summary of the main signalling cascades regulated by TPα and TPβ. 1 (Black): Both TPα and TPβ primarily couple to Gαq-mediated phospholipase (PL)Cβ activation, leading to the generation of IP_3 and mobilization of intracellular calcium (Ca^{2+}). Elevation in intracellular Ca^{2+} is the main signalling event that triggers TXA_2-induced platelet activation, including thrombosis, and constriction of various types of smooth muscle (SM), including vascular (V), renal, pulmonary and prostate SM. 2 (Grey): TPα and TPβ also couple to $G\alpha_{12}$-mediated activation of RhoGEF (Rho guanine nucleotide exchange factor), leading to activation of RhoA. Activated (GTP-bound) RhoA, in turn, interacts with a range of effector proteins including Rho kinase (K) 1/2, leading to Ca^{2+}-independent (V)SM contraction, to general reorganizations of the cellular cytoskeleton and to a host of events that promotes tumour cell migration and metastasis. In addition, activated RhoA can also interact and activate the effector protein kinase C-related kinase (PRK) 1. PRK1 can also interact with the activated (DHT-bound) androgen receptor (AR) which, in turn, can enhance AR-dependent transcriptional activation by promoting phosphorylation of histone H3 at Thr11 and, hence, androgen-induced chromatin remodelling. 3 (Purple): It has been recently discovered that TPα and TPβ can directly interact with and activate PRK1 which, in turn, can also phosphorylate histone H3 at Thr11 to augment androgen-induced chromatin remodelling in response to TPα/TPβ signalling. 4 (Pink): TPα and TPβ also lead to (i) activation of the extracellular signal-regulated protein kinase (ERK) 1/2 cascades (intermediate steps not shown), as well as to (ii) transactivation of the epidermal growth factor receptor (EGFR), both of which (i and ii) account for the ability of TXA_2 to promote cell proliferation and mitogenesis, including tumour progression. *Note*: The inflammatory or immune-modulatory roles of the TXA_2-TPα/TPβ axis are not shown but may also contribute to their role in certain cancers.

homologous (15, 16) and heterologous (17–19) desensitization to differentially regulate their intracellular signalling. Most notably, signalling by TPα, but not by TPβ, is completely desensitized/inhibited by the counter-regulatory *anti*-platelet and vasodilatory agents prostacyclin/prostaglandin I_2 and nitric oxide, which is mediated by direct protein kinase (PK) A and PKG phosphorylation of TPα at Ser^{329} and Ser^{331}, respectively, the very first residues within its unique carboxyl-terminal tail domain of TPα and divergent from those of TPβ (13, 18). The conclusion from those studies is that TPα is the TP isoform essential for haemostasis/thrombosis, while the role of TPβ in this pathophysiologic process remains unclear (13, 18). Hence, TPα and TPβ have both shared and unique patterns of expression and function to mediate the (patho)physiologic actions of the potent autocrine/paracrine mediator TXA_2 in human health and disease.

The role of thromboxane in cancer

In addition to its prominent role within the vasculature, there is growing evidence highlighting a central role for TXA_2 in human cancers (20, 21). In recent years, evidence supporting this hypothesis has been strengthened by several longitudinal studies showing the prophylactic benefits of long-term daily use of Aspirin in reducing the risk of many prevalent cancers, predominantly gastrointestinal but also breast, lung and prostate cancers (PCa), with numerous clinical trials completed or underway testing the benefits of Aspirin and other COX-1/-2 inhibitors in chemoprevention (22–29). While those longitudinal studies do not specify which COX-1/-2-derived prostanoid metabolite(s) is actually lowered by Aspirin to account for its prophylactic benefits in cancer risk reduction, recent reports strongly suggest that some/many of its *anti*-cancer effects may be due to its ability to inhibit TXA_2 generation, as stated a prostanoid more typically associated with thrombosis and cardiovascular disease (20, 21). Indeed, it has long been known that platelets, the main source of TXA_2 and key target of Aspirin, play a key role in cancer progression promoting cancer cell metastasis, immune evasion and extravasation (30). Furthermore, increased levels of TXA_2 and expression of its synthase and its T prostanoid receptor, the TP, occur in a number of prevalent cancers including, for example, strongly correlating with bladder (31), prostate (32, 33), colorectal (34, 35) and non-small-cell lung cancer (36). Mechanistically, the role of TXA_2 in neoplastic progression is at least partly explained by the ability of the TXA_2-TP axis to regulate key mitogenic/ERK- and RhoA-mediated signalling cascades that contribute to tumour development and metastasis (12, 14) and also by its ability to regulate local inflammation and immunity (37–42), including within the tumour (Figure 2; summary of TXA_2-TP signalling). Hence, aside from its regulation of ERK- and RhoA-mediated processes (Figure 2) (12, 14), TXA_2 is a potent proinflammatory and immune-modulatory agent being abundantly produced in monocytes/activated macrophages and promotes monocyte chemoattractant protein-1 expression in tumours, recruiting tumour-associated macrophages, and negatively

regulates the interaction between T-cells and dendritic cells, a process essential for adaptive/acquired immunity (38, 39, 43, 44). Moreover, TXA_2 is critical for early B-cell development, also with implications for its role in tumour-infiltrating B-cells (42, 45). Hence, due to its role in tumour growth and metastasis combined with its ability to regulate local inflammation and immunity, the TXA_2-TP axis can impact at multiple levels within the tumour environment.

Genome-wide association studies also reveal that certain single nucleotide polymorphisms within the TXS gene (the TBXAS1) may predispose individuals to breast cancer (46), while inhibition of TXS activity enhances apoptosis of lung carcinoma A549 cells *in vitro*, implicating a role for TXA_2 also in tumour cell survival (36). In the prostate, an increased expression of TXS and the TPα/TPβ isoforms directly correlate with the tumour Gleason score and pathologic stage (32, 33, 47), where expression of both TXS and the TP is mainly found in areas of perineural invasion, a recognized mechanism by which PCa cells invade the prostatic capsule and metastasize to other tissues (20, 32). Significantly in the context of PCa, through detailed mechanistic studies, we recently discovered that both the TPα and the TPβ isoforms directly interact with and regulate signalling by protein kinase C-related kinase/protein kinase novel (PRK/PKN) (48), a family of 3 AGC kinases that act immediately downstream of phosphatidylinositol 3′kinases, and are strongly, yet differentially, implicated in several cancers (49–51) and in B-cell development (52). Indeed, in addition to acting as Rho GTPase effectors, activation of the PRKs (e.g., PRK1) in response to androgen receptor (AR) signalling within the prostate catalyses phosphorylation of histone (H)3 at Thr11 (H3pThr11) which, in turn, serves as a specific epigenetic marker, and gatekeeper, of androgen-induced chromatin remodelling and transcriptional activation (48, 53–55). Hence, owing to their ability to regulate RhoA-/C-mediated responses, including metastatic processes, combined with their epigenetic priming of tumour cells, members of the PRK family are key chemotherapeutic targets particularly in castrate-resistant prostate cancer, the metastatic lethal form of PCa that occurs following androgen deprivation therapy (53, 55, 56).

Indeed, our research shows that TPα-/TPβ-mediated PRK1 activation not only leads to histone H3 threonine 11 phosphorylation in response to TXA_2 but can also cooperate with the AR to enhance the androgen-induced chromatin remodelling (H3pThr11) and transcriptional activation (48). Collectively, these studies raise the exciting possibility that TXA_2, through its ability to directly regulate PRK-induced H3pThr11, may be a strong epigenetic regulator, thereby adding to the range of possible mechanisms, whereby the Aspirin-target TXA_2 may influence the neoplastic growth. Added to this complexity, we recently established that the TPα and TPβ isoforms differentially associate with and regulate signalling by the other individual members of the PRKs (PRK1/PKNα, PRK2/PKNγ, PRK3/PKNβ) (57). Furthermore, consistent with our previous studies involving PRK1 (48), *si*RNA disruption

of PRK1 and PRK2, but not PRK3, expression eliminates TP-mediated cancer cell responses (proliferation, anchorage-independent growth, migration) and H3pThr11 phosphorylation in the prostate carcinoma PC-3 cell line (57). Identification of a direct, functional interaction of both TPα and TPβ with the PRKs provides yet another molecular link accounting for the role of TXA_2 in tumour progression, particularly in prostate and other cancers in which the TXA_2-TP and PRKs are increasingly implicated. Critically, as stated, it suggests that the TXA_2-TP axis may serve as an epigenetic regulator, adding to the range of possible mechanisms whereby the Aspirin-target TXA_2 may influence neoplastic growth.

Factors determining transcriptional regulation of TPα in platelet progenitor megakaryoblastic cells

Collectively, these and numerous other studies provide significant mechanistic insights into the role of TXA_2 and of the TPs (TPα/TPβ) in cancer progression. However, with only limited exceptions (20, 31), few of those studies investigated the roles of the individual TPα or TPβ isoforms or examined their transcriptional regulation in cancer. To address this and focussing on TPα, the predominant isoform expressed in most cell/tissue types (10), we recently examined its expression in prostate and breast cancer and identified a key role for the tumour suppressor gene product Wilms' tumour (WT)1 in its transcriptional regulation (58). Prior to presenting and discussing these findings, it is first relevant to review knowledge on the transcriptional regulation of TPα in the haematopoietic system where most data and insight is available.

As stated, while TPα and TPβ are encoded by the same *TBXA2R* gene (Figure 1), they are differentially expressed being regulated by distinct promoters, whereby promoter (Prm)1 exclusively regulates TPα expression and Prm3 regulates TPβ (7–9, 59, 60). Through initial studies carried out in the platelet progenitor megakaryoblastic human erythroleukaemia (HEL) 92.1.7 and K562 cell lineages (9, 60, 61), the transcription factors Sp1 (stimulating protein 1), early growth response 1 (Egr1) and NF-E2 were identified as the key *trans*-acting factors that bind to the 'core promoter region' of Prm1 to drive basal expression of TPα mRNA (9). In addition, several functional upstream activator regions (UARs; UAR1 and UAR2) and upstream repressor regions (URRs; URR1, URR2 and RR3, where repressor region 3 specifically lies within the core promoter; Figure 1) were identified within Prm1 (9). While GATA-1, Ets-1, Ets-2 and Oct-1 were identified as the main *trans*-acting transcription factors that regulate the UARs in the megakaryoblastic lineages, the tumour suppressor gene product WT1 was found to bind to several GC-enriched consensus *cis*-elements within the repressor regions (URR1, URR2 and RR3) to repress Prm1, maintaining TPα expression at relatively low levels when cells were cultured under basal conditions (60). However, following differentiation of the pluripotent megakaryoblastic cell lineages to the platelet phenotype, it was established that TPα expression was strongly upregulated, and this occurred

through a complex transcriptional mechanism involving coordinated: (i) *alleviation* of TPα/Prm1 repression by WT1 by displacement of its binding to its consensus GC-enriched *cis*-acting elements within the URRs of Prm1, (ii) *induction* of TPα expression by binding of the transcriptional activator Egr1 to the same *cis*-acting GC elements within Prm1, followed by (iii) *sustained upregulated expression* of TPα through binding of Sp1 also to the same *cis*-acting GC elements within Prm1 (Figure 3) (61). Hence, WT1 plays a central role in repressing TPα expression by binding to multiple *cis*-acting elements within the repressor regions (URR1, URR2 and RR3) of Prm1, maintaining TPα expression at low levels under basal/non-differentiated conditions. However, in response to cellular differentiation, WT1 repression is lifted in favour of sequential high-affinity binding of the transcriptional activators Egr1 followed by Sp1 to the same *cis*-acting GC elements within Prm1 to induce (by Egr1) and maintain (by Sp1) high levels of TPα expression following differentiation (Figure 3) (61).

In addition to its recognized role in normal and aberrant haematopoiesis (62), WT1 was initially described as a tumour suppressor in Wilms' tumour (WT), a rare form of renal cancer (63–65), but can also play an oncogenic role in certain cancers (66–69). Considering the recognized role of WT1 in WT of the kidney and in other cancers while also acting as a key transcriptional repressor/regulator of TPα expression in megakaryoblastic HEL and K562 cell lineages combined with the increasing awareness of the role of the TXA_2-TP axis in neoplastic progression, we recently investigated the possible regulation of TPα/Prm1 by WT1 in prostate and breast cancer, including in the model prostate PC3 and breast MCF-7 [a model oestrogen receptor/oestrogen receptor (ER)-positive breast cancer cell line] and MDA-MB-231 (a model oestrogen receptor/ER, progesterone receptor/PR and Her2/neu triple-negative breast cancer cell line) carcinoma cell lines, respectively. In brief and consistent with the findings in the megakaryoblastic lineages, it was established that WT1 can repress Prm1-directed TPα expression in both the prostate and the breast cancer lineages. Overall, as elaborated upon in detail later in this chapter, the study provided a comprehensive molecular analyses of the factors regulating the TPα expression through Prm1 in the prostate and breast and suggested that aberrant regulation by/or dysfunction of the tumour suppressors WT1, along with hypermethylated in cancer (HIC) 1, may account at least, in part, for the increased association of TXA_2/TP signalling with certain prostate and breast cancers and, potentially, in other cancers in which TXA_2, WT1 and/or HIC1 are implicated (58). The reader is referred to the original study for full details on the role of HIC1 in its regulation of TPα/Prm1 expression (58), while this communication will mainly focus on the role of WT1.

Role of Wilms' tumour 1 in regulating TPα expression in prostate and breast cancer

The WT1 gene encodes a zinc finger transcription factor critical for development of the genitourinary, haematopoietic and central nervous systems (62, 70). The finding that mutations

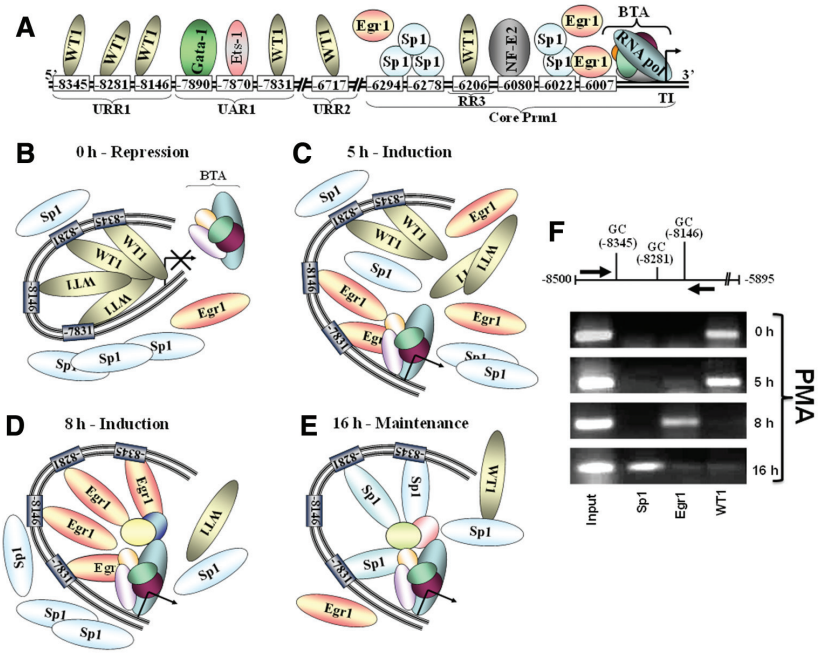

Figure 3. Proposed model for PMA-mediated increases in Prm1 activity. Panels A-E: Proposed model for PMA induction of Prm1/TPα mRNA transcriptional regulation in HEL92.1.7 and K562 megakaryoblastic cells. In resting cells, WT1 binds in a cooperative manner to multiple adjacent GC elements (at -8345, -8281, -8146 and -7831) within Prm1 to impair transcription initiation by the basal transcription apparatus (BTA) and thereby repressing TPα mRNA expression (Panels A and B; Repression). In response to exposure to PMA for ~5 h, the up-regulated expression of Egr1 results in increased high-affinity binding of Egr1 to Prm1, thereby activating Prm1-directed *TBXA2R* transcription to up-regulate TPα mRNA expression (Panel C; Induction). After exposure to PMA for ~8 h, a further, enhanced increase in Egr1 binding coincides with nuclear export and a resulting reduction in WT1 binding (Panel D; Induction). The decrease in WT1 binding results in de-repression of Prm1 and in a further increase in Egr1 binding, leading to a more pronounced transcriptional activation of Prm1 by the BTA (Panel D; Induction). Following the prolonged exposure to PMA for ~16 h, decreased Egr1 binding coincides with its rapid protein turnover (Panel E). PMA-mediated differentiation of cells can also lead to phosphorylation of Sp1 and/or its increased expression, enhancing its DNA-binding activity. Therefore, the increased affinity of Sp1 for Prm1, coinciding with the decreased Egr1 expression, facilitates binding of Sp1 to Prm1, thereby resulting in a sustained increase in Prm1 activity and TPα expression as the differentiation of HEL and K562 cells progresses toward the platelet phenotype (Panel E; Maintenance). Panel F: Representative chromatin immunoprecipitation (ChIP) analysis of Sp1, Egr1 and WT1 binding *in vivo* to Prm1 of the *TBXA2R* in HEL cells as a function of PMA-induced cellular differentiation. *Note*: Data presented in this figure were reproduced from our previous study (62), and the reader is referred to the original manuscript for the experimental details that led to the proposed model (panels B–E).

within the WT1 gene are a leading cause of the childhood renal cancer WT first led to the suggestion that it might serve as a tumour suppressor (64). However, the fact that non-mutated/wild-type WT1 is also abundantly expressed in a variety of cancers, including cancers of the breast (71), oesophagus (72) and pancreas (73), indicated that WT1 might also play an oncogenic role.

In addition to acting as a regulator of transcription, WT1 can also play a role in post-transcriptional regulation, including in RNA splicing (74, 75), and also in translation (76). Such diverse functions are likely due to fact that WT1 protein exists as multiple isoforms that arise owing to differential splicing and/or the use of multiple translational initiation sites within the WT1 gene (77). Of the most prevalent WT1 isoforms, the best characterized are the variants that differ due to the presence or absence (+/–) of exon 5 and +/–KTS (Lys-Thr-Ser) sequences (Figure 4). More specifically, differential splicing at these two sites yields four different isoforms, namely (+/+), (+/–), (–/+) and (–/–), each of which differ in respect of exon 5 and KTS sequences, respectively. While the -KTS isoforms can act as transcriptional repressors or activators, the +KTS isoforms do not readily bind DNA and, therefore, are less active in the process of transcription [reviewed in reference (62)]. WT1 has four Kruppel-like C_2H_2 fingers within its C-terminal region that share similarity with those of the aforementioned Egr1, another prominent member of the zinc finger family of transcription factors. While the zinc finger domain of WT1 can facilitate its DNA at the consensus Egr1 DNA-binding site (consensus sequence 5′GCG(G/T)GGGCG3′), the binding affinity of WT1 for the Egr1 consensus site is significantly less than that of Egr1 itself (78). In addition to binding to WT1 and/or Egr1 *cis*-acting elements, WT1 can also bind to another motif termed the Wilms' tumour element (consensus sequence, 5′GCGTGGGAGT3′) (79). Hence, depending on the cellular context and/or on the particular promoter, WT1 can therefore serve as a transcriptional repressor or activator. This is exemplified in the case of c-Myc where over-expression of WT1 in K562, a HEL cell line, and in breast cancer cells activates the c-Myc promoter (80) but WT1 represses the c-Myc promoter in HeLa cells (81). WT1 can also complex with other DNA-binding *trans*-acting co-factors, such as p53 (82), and with certain co-activators or co-repressors, such as CBP (83) or BASP1 (84), respectively, to regulate transcription. It is the identity of these co-factor-binding partners that determines whether WT1 serves as an activator or repressor during transcription. In the case of the *TBXA2R*, the overwhelming evidence is that WT1 predominantly acts as a transcriptional repressor by binding to multiple GC-enriched *cis*-acting elements within Prm1 to suppress TPα expression in pluripotent megakaryoblastic cells but that in response to cellular differentiation, this repression is lifted coinciding with the increased expression and binding of Egr1 to the same GC elements to induce TPα expression (Figure 3) (9, 60, 61). In turn, following Egr1-mediated induction, subsequent binding of the constitutive Sp1 to the same *cis*-elements within Prm1 maintains the expression of TPα at high levels in the fully

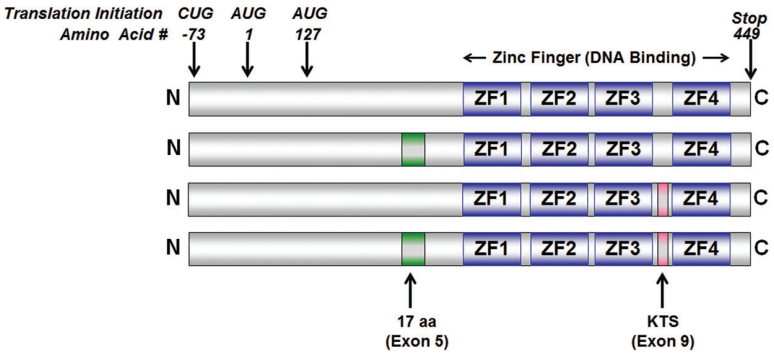

Figure 4. Schematic representation of Wilms' tumour (WT) 1 isoforms. WT1, encoded by the tumour suppressor gene WT1, can exist as multiple isoforms depending on the translational start site and the inclusion/exclusion of amino acids encoded by exon 5 (encodes a 17 aa sequence) or exon 9 [encodes the 3 aa sequence KTS (Lys-Thr-Ser) located between Zinc Finger (ZF) domains 3 and 4]. The best characterized isoforms are the ± exon 5 and the ± exon 9/KTS isoform variants, representing the four most abundantly expressed forms of WT1 with a molecular mass of 52–54 kDa. In addition, the initiation of translation can occur at an upstream CUG codon (-73), resulting in the generation of larger WT1 isoforms with a mass of 62–64 kDa or alternatively can occur at a downstream AUG codon (+127) giving rise to smaller WT1 isoforms of 32–34 kDa. WT1 has four Kruppel-like C_2H_2 zinc fingers, ZF1–ZF4, within its C-terminal DNA-binding domain that share significant identity with those of the early growth response (Egr)1, another member of the zinc finger family of transcription factors. While the -KTS isoforms of WT1 can either repress or activate transcription, the +KTS isoforms have a reduced ability to bind DNA and therefore are less transcriptionally active (62). The increased affinity of Egr1 relative to that of WT1 for binding to the same *cis*-acting elements accounts for why Egr1 can displace WT1 binding when progressing from transcriptional repression by WT1 to induction by Egr1 as exemplified by the coordinated regulation of Prm1/TPα expression during cell differentiation (Figure 3).

differentiated state (61). The known increased affinity of Egr1 relative to that of WT1 for binding to the same *cis*-acting elements accounts for why Egr1 can displace WT1 binding when progressing from transcriptional repression by WT1 to induction by Egr1 as exemplified by the coordinated regulation of TPα/Prm1 during cell differentiation (Figure 3) (61, 78).

In our recent studies investigating the expression and the transcriptional regulation of TPα through Prm1 in prostate and breast cancer, immunohistochemical analysis confirmed that the expression of TPα correlated with increasing prostate and breast tissue tumour grade (Figure 5), while stimulation of the prostate (PC3) and breast (MCF-7 and MDA-MB-231) carcinoma cell lines with the TXA_2 mimetic U46619 increased both cell proliferation and migration (58). Collectively, these data provided further evidence of a role for the TXA_2-TP signalling axis in prostate and breast cancer progression. In order to identify the factors

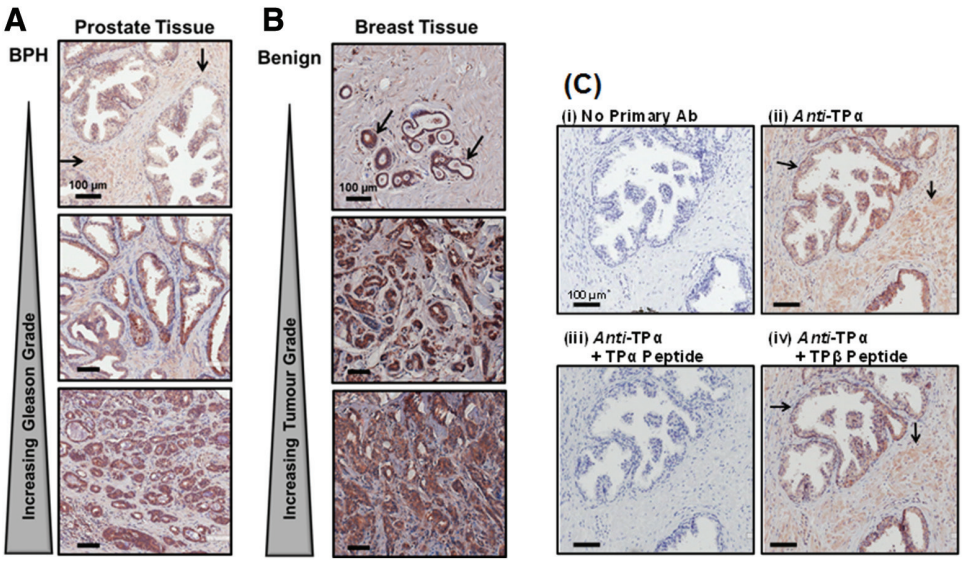

Figure 5. Analysis of TPα expression in the prostate and breast. Panels A and B: Immunohistochemical analysis of prostate and breast tissue (benign and increasing tumour grade) screened with an affinity-purified *anti*-TPα antibody (200× magnification; counterstained with haematoxylin). The increased TPα expression coincides with an increased prostate and breast cancer tumour grade. Panel C: Immunohistochemical analysis of full-face benign prostate tissue screened either (i) in the absence of a primary antibody or with the (ii) affinity-purified *anti*-TPα antibody. The specificity of the *anti*-TPα antibody was confirmed, whereby the (iii) immunogenic TPα peptide, (iv) but not a TPβ specific-peptide, competed out the *anti*-TPα immune-staining. The arrows in (ii) and (iv) indicate specific detection of TPα expression in the prostate glandular epithelial cells and in the fibromuscular stromal smooth muscle cells of the prostate tissue. All sections were counterstained with haematoxylin, and images shown were captured at 200× magnification. *Note*: Data presented in this figure were reproduced from our previous study (58).

regulating TPα in the prostate and breast through Prm1, genetic-based reporter analyses confirmed that the repressor regions, designated URR1, URR2 and RR3 and previously identified within Prm1, are functional in the prostate and breast carcinoma lineages. Furthermore, in each of the prostate and breast carcinoma lineages studied, over-expression of WT1 repressed TPα mRNA and Prm1-directed reporter gene expression, while chromatin immunoprecipitation analysis confirmed that WT1 binds *in vivo* to each of the consensus GC-enriched *cis*-elements within the repressor regions of Prm1. Furthermore, in the prostate and breast cellular systems, it was established that the tumour suppressor HIC1 represses TPα mRNA expression through its binding to a functional *cis*-element, referred to as the HIC1(b) element, within Prm1 in PC3 and MCF-7 cells, while a second HIC1 element,

referred to as HIC1(a), was identified within Prm1 through bioinformatics analyses but was not found to be functional (58).

Among the many cell-specific differences we observed in the transcriptional activity of Prm1 in the prostate and breast carcinoma-derived cell types, it was noteworthy that the UAR1/UAR2 repressor regions within Prm1, previously identified in the megakaryoblastic HEL92.1.7 and K562 cell lines (9) where they are regulated by GATA-1, Ets-1, Ets-2 and Oct-1, were not found to be functionally active in the prostate PC3 or breast MCF-7 lineages (Table 1). These observations pointed to clear cell-/tissue-specific differences in the regulation of TPα expression through Prm1 in the haematopoietic system *versus* prostate and/or breast tissues. Moreover, as summarized in Table 1, our analysis of Prm1 in the prostate and breast carcinoma lineages revealed additional cell-specific differences in the upstream regulatory regions. Included in this is a prostate-specific Novel URR identified in PC3 cells, a novel UAR1 in PC3 and MCF-7 cells and an additional Novel UAR2 in PC3, MCF-7 and MDA-MB-231 cells (58). Detailed bioinformatic analysis by us revealed a putative oestrogen response element (ERE) within the Novel URR (–7962 to –7859; Table 1), suggesting that this region might function as a binding site for the oestrogen and/or ARs (85). The finding that oestrogen production increases in men with age, mainly due to aromatase conversion of androgens to oestrogens, indicates that like androgens (86), oestrogens may also play a role in PC progression (87, 88). Whether the ERE within the Novel URR of Prm1 regulates the TPα expression in the prostate remains to be investigated.

With regard to the Novel UAR1 (–7504 to –6848; Table 1) identified in both the prostate PC3 and the ER-positive breast MCF-7 lines, but not in the triple-negative breast cancer MDA-MB-231 cell line, several putative *cis*-acting binding elements were identified through bioinformatics, including multiple EREs that lie in close proximity to each other. While remaining speculative, binding of the ER to some or all of these adjacent EREs may explain why the Novel UAR1 repressor region is functional in both the ER-positive PC3 and the MCF-7 lines but not active in the ER-negative MDA-MB-231 cell line.

In terms of the novel activator regions identified within Prm1 and confirmed to be transcriptionally active in all prostate and breast cell lineages examined, the Novel UAR2 located between –6648 and –6492 of Prm1 was of particular note (Table 1). Specifically, similar to that found in all other repressor regions (UAR1, UAR2 and RR3) within Prm1, putative binding elements for Egr1 and stimulating protein (Sp) 1 were also identified within this novel prostate-/breast-specific Novel UAR2 region of Prm1. As stated, our previous studies in differentiated megakaryoblastic HEL and K562 lineages established that both the inducible Egr1 and the constitutive Sp1 factors bind to multiple GC-consensus WT1 *cis*-elements within the UAR1, UAR2 and RR3 repressor regions of Prm1 to strongly up-regulate the TPα mRNA expression (9, 60, 61). Hence, it is indeed possible, if not likely, that the expression of TPα through Prm1 in the prostate and

Table 1. Activator and repressor regions within Prm1

		Cell type[1]			
Regulatory regions initially identified in megakaryoblastic lineages[2]					
Regulatory region[2]	**Position within Prm1[3]**	**HEL 92.1.7**	**PC3**	**MCF-7**	**MDA-MB-231**
URR1	–8500 to –7962	Yes	Yes	Yes	Yes
URR2	–6848 to –6648	Yes	Yes	Yes	Yes
RR3	–6258 to –6123	Yes	Yes	Yes	Yes
UAR1	–7962 to –7859	Yes	No	No	No
UAR2	–7859 to –7504	Yes	No	No	Yes
Core	–6320 to –5895	Yes	Yes	Yes	Yes
Novel regulatory regions identified in prostate or breast cancer cell lineages[1]					
Regulatory region[1]	**Position within Prm1**	**HEL 92.1.7**	**PC3**	**MCF-7**	**MDA-MB-231**
Novel URR	–7962 to –7859	No	Yes	No	No
Novel UAR1	–7504 to –6848	No	Yes	Yes	No
Novel UAR2	–6648 to –6492	No	Yes	Yes	Yes

[1]This table has been adapted from our previous study (58).

[2]From references (9, 60, 61).

[3]The nucleotide numbers given are relative to the translational initiation codon at +1 within the *TBXA2R*.

breast may be subject to a similar type of complex transcriptional regulation involving occupancy of the common/shared and Novel prostate-/breast-specific GC-enriched *cis*-acting elements that can act as consensus binding sites for WT1, Egr1 and/or Sp1, where temporal occupancy may possibly be determined by the (patho)physiologic setting. Clarity on this matter remains to be experimentally determined and is necessary to shed further light on the transcriptional regulation of TPα in both the normal and the malignant prostate/breast tissue and potentially in other tissues in which the TXA_2-TP axis is implicated. Critically, given that Egr1 serves as a master regulator in several key aspects of prostate and breast cancer progression (89, 90), investigation of the interplay between WT1 and Egr1 in the regulation of TPα expression through Prm1 within the *TBXA2R* as a function of tumour grade merits detailed investigation.

Conclusions and future perspectives

The prostanoid TXA_2 plays a central role in haemostasis and is widely implicated in a range of cardiovascular, renal, pulmonary and prostate diseases (1–3, 5). In humans, TXA_2 signals

through TPα and TPβ, two structurally related TP isoforms that display both common, overlapping and isoform-specific physiologic roles (6). TPα and TPβ are encoded by the same gene, the *TBXA2R*, but are differentially expressed being regulated by distinct promoters where Prm1 regulates TPα expression, mainly involving NF-E2, Sp1, GATA-1, Ets-1, WT-1/Egr1, and Prm3 regulates TPβ, involving cFos/cJun and Oct-1/-2 (7–9, 59, 60). While the clinical evidence for the role of the TXA_2-TP axis in neoplastic progression is increasing, few studies to date have investigated the role of the individual TPα/TPβ isoforms in human cancer or indeed in most other diseases in which TXA_2 is implicated (20, 31). Focussing on TPα, we investigated its expression and transcriptional regulation through Prm1 in prostate and breast cancer and established that the tumour suppressor protein WT1 plays a key role in regulating its expression in both tissue types. Critically, it was established that WT1 can repress TPα expression through binding to multiple GC-consensus *cis*-elements within previously recognized regulatory regions within Prm1, while several other prostate- and/or breast-specific novel regulatory regions were identified accounting for cell-/tissue-specific regulation of TPα. Taken together with previous studies in platelet progenitor megakaryoblastic lineages (9, 60, 61), the findings provide a strong genetic basis for understanding the diverse physiological roles played by TXA_2-TP axis within the vasculature and other systems, including potentially in the progression of certain human cancers. These studies may also provide a mechanistic basis accounting, at least in part, for the prophylactic benefits of Aspirin in reducing certain cancer risks by lowering the overall TXA_2 levels.

WT1 has recently emerged as an important target in immune-therapy approaches to treat certain cancers, and ongoing clinical trials involving the WT peptide 1 vaccination are proving positive in reducing tumour growth in breast and lung cancers, in leukaemia and, more recently, in glioblastoma (91–93). As the TXA_2-TP axis has been implicated in the development of prostate and breast cancer (20, 33, 47, 94, 95), the findings herein suggest that aberrant WT1 regulation of TPα expression may contribute to such cancers and potentially to WT itself. In addition, bearing in mind that the TPα and TPβ isoforms display a number of important functional similarities but also differences in terms of their signalling (6) and regulation including in certain cancers (96, 97), coupled with the fact that they are regulated by distinct promoters within the *TBXA2R* gene, it will be of considerable interest to investigate the expression and transcriptional regulation of TPβ in human cancers, in particular in prostate and breast cancer.

Abbreviations

ADT, androgen deprivation therapy; AR, androgen receptor; ChIP, chromatin immunoprecipitation; COX, cyclooxygenase/prostaglandin G2/H2 synthase; CRPC, castrate-resistant prostate cancer; C-tail, carboxyl-terminal tail; Egr1, early growth response 1; ER, oestrogen receptor; ERE, oestrogen response element; ERK, extracellular signal-regulated protein kinase; GPCR, G-protein-coupled receptor; HEL, human erythroleukaemia; H3Thr11, histone H3 threonine 11; IHC,

immunohistochemistry; PCa, prostate cancer; PDK-1, 3-phosphoinositide-dependent protein kinase-1; PG, prostaglandin; PI3'K, phosphatidylinositol 3'kinase; PK, protein kinase; PRK, protein kinase C-related kinase; RR, repressor region; SDM, site-directed mutagenesis; Sp1, stimulating protein 1; TP, thromboxane prostanoid receptor; TX, thromboxane; TXS, TXA synthase; UAR, upstream activator region; URR, upstream repressor region; WT1, Wilms' tumour 1.

Conflict of Interest

The author declares no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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Chapter 12

The Inflammatory Microenvironment in Wilms Tumors

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Abstract

For the past several decades, the role of inflammation in different types of tumors has been well defined. The significance of inflammation including the presence of various immune cells and inflammatory marker analysis of tumors helped the clinicians to use new treatment methods, which lead to high cure rates but failed to do so in some tumors due to lack of information about the tumor microenvironment. Although the importance of inflammation in various adult malignancies has been well defined, by contrast, Wilms tumor (WT), the most common childhood kidney cancer, which represents 6% of all pediatric tumors, has

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not been well studied. Nearly 75% of the WT cases have been noticed in children less than 5 years of age with a higher incidence at 2 to 3 years. Thus, very little is known about the inflammatory microenvironment in the development of WT. This inflammatory microenvironment may initiate oncogenic transformation, and in some instances, genetic and epigenetic modifications in tumor cells can also generate an inflammatory microenvironment that further supports tumor progression. Thus, the tumor microenvironment is highly dynamic, and linking the modulating factors and various inflammatory cells with tumor progression is of considerable interest. Although to some extent the currently used WT treatment methods such as surgical removal, chemotherapy, and radiation therapy are successful, the youngest children are at high risk for the irreversible adverse side effects. Thus, there is a need for alternative therapy/therapies exposing the child to the minimum possible adverse effects. This chapter gives a special focus on the inflammatory microenvironment of human WT with a comprehensive picture of various immune cells and other inflammatory markers. This may aid in the use of new therapeutic targets for the efficacious treatment of WT with the combination of currently adapted therapies or alone.

Key words: Cancer; Immune cells; Inflammation; Microenvironment; Wilms tumor

Introduction

Following the transformation of a normal cell to malignant or tumor cell, the inflammatory mediators promote the tumor growth, by inducing the proliferation and the evading immune surveillance. The unregulated inflammatory microenvironment plays a central role in the initiation and progression of tumor. In general, inflammation is initiated by the recruitment of a wide range of immune cells that affect malignant cells through the production of cytokines, chemokines, growth factors, prostaglandins, reactive oxygen and nitrogen species, proteases, and other bioactive molecules, which can act in an autocrine and/or paracrine manner (1). Altogether, this environment with various factors is known inflammatory tumor microenvironment. These inflammatory markers are very critical components to establish a link between inflammation and cancer although the activation of these inflammatory markers is influenced by various factors. This inflammatory microenvironment progresses the tumor cells with endowed immunosuppressive properties. Hence, the immune destruction property has now been proposed as one of the "hallmarks of cancer." Thus, the role of inflammation and inflammatory microenvironment in cancer is generally accepted and is an essential component of many tumors even though its relationship with inflammation has not been demonstrated (1–4). So far, the molecular mechanisms involved in establishing this inflammatory tumor microenvironment were not clearly understood and established. This may be due to multifaceted role of inflammatory markers/mediators, such as cytokines, chemokines, oncogenes, enzymes, transcription factors, and immune cells, in the tumor

microenvironment. Till date, studies are still going on to elucidate the complete link between the cancer and the inflammation. For the past one decade, studies using knockout animals have unraveled to some extent the molecular mechanisms that link inflammation and cancer in adult-onset cancers but not in pediatric cancers (5). These studies show that the inflammatory microenvironment is very important in tumor development. The inflammatory conditions may initiate or promote oncogenic transformation, or genetic and epigenetic changes in malignant cells can also generate an inflammatory microenvironment that further supports tumor progression (2). It is important to note that the acute inflammation regresses the tumor growth, whereas the chronic inflammation progresses the tumor. Thus, there is a need to be a balance between antitumor immunity and tumor-promoting immune activity within a tumor microenvironment that consists of tumor cells, stroma (including fibroblasts and endothelial cells), innate immune cells, and adaptive immune cells.

What is Wilms tumor and what are the various components of Wilms tumor?

Wilms tumor (WT) is the most common pediatric kidney cancer, which represents 6% of all pediatric tumors, and 9 out of 10 kidney cancers in children are WTs. Nearly 75% of the WT cases have been noticed in children less than 5 years of age with a higher incidence at 2 to 3 years. It is the most common cause of a renal mass in a child and more prevalent in the people of African descent (6, 7). WT is an undifferentiated mesodermal tumor, which consists of variable amount of embryonic renal elements, such as blastema, epithelium, and stroma (8, 9). The etiology of this childhood tumor is largely due to genetic alterations or mutations in the *WT1*, *CTNNB1*, and/or *WTX1* genes.

Most of the WTs are unilateral and most often involve only one tumor, but it has been observed that around 5% to 10% of children with WTs have more than one tumor in the same kidney. Only about 5% of children with WTs have bilateral disease. Most often, the size of the WT is much larger than that of the kidney before they were diagnosed and metastasized to other organs (10).

The mechanism/mechanisms of this pediatric cancer development at present is less clear, and the whole etiology of these diseases is also not completely understood. In general, pediatric cancers will not arise from epithelial tissues and will have different causative mechanisms than adult tumors. It is assumed that most of the childhood cancers arise as a result of inherited and/or acquired genetic events during embryogenesis (11).

Although in general the currently used WT treatment methods such as surgical removal, chemotherapy, and radiation therapy are successful, young children are still at high risk for the irreversible adverse side effects. In addition, a considerable number of patients relapse (20–25%), and part of these tumors resist to current therapies and progress (12, 13). Thus, the main

challenge is better stratification and development of novel therapeutic targets/approaches to eliminate or minimize these side effects and deficiencies. Such novel approaches critically depend on the in-depth understanding of the tumor microenvironment and on the mediating factors responsible for WT progression. Hence, this chapter focuses on the inflammatory microenvironment of human WT with a comprehensive picture of various immune cells and other inflammatory markers. This may aid in the advent of new therapeutic targets for the efficacious treatment of WT with the combination of currently adapted therapies or alone.

Types of Wilms tumors

Based on the histology, WTs are categorized into two major groups.

Unfavorable histology (anaplastic WTs)

In these tumors, the tumor cells vary widely, and the nuclei is very large and distorted. This is called anaplasia. The anaplastic tumors are very hard to cure. In preoperative chemotherapy, such as in the International Society of Paediatric Oncology (SIOP) settings, also cases with chemo-resistant blastemal subtype, are considered at high risk of relapse.

Favorable histology

These are nonanaplastic tumors. Interestingly, more than 9 out of 10 WTs have a favorable histology. This type of tumors can easily be cured (10).

What is known about kidney cancer and inflammation?

There are not many studies available to relate WT and inflammation with the complete analysis of WT inflammatory microenvironment. In a comparative analysis of adult tumors, Vakkila et al. (14) reported that human WTs were infiltrated with macrophages and to a very less extent with T lymphocytes. This study was incomplete because it was confined to one or two immune cell markers. The other two different groups independently observed cyclooxygenase-2 (COX-2) expression in human WT ubiquitously in all cases, independent of the type (15) and stage (16) of neoplasm. However, these studies were again restricted to only one inflammatory marker, COX-2. The coexpression of the hypoxia-inducible factor (HIF-1α and its one of the target genes, vascular endothelial growth factor (VEGF), was reported in human WTs. This finding suggested the possible role of hypoxic cascade driving the tumor angiogenesis, growth, and progression (17). In addition, very early studies on isolation and culturing of tumor-infiltrating leukocytes (TIL) with different doses of cytokines in human WT comparing with other pediatric tumors were also reported (18). But none of these studies was able to give a comprehensive view of tumor microenvironment in human WT. It is therefore critical and relevant to know the whole picture of tumor microenvironment, whereas its role in

Wilms tumorigenesis has not been widely explored. Because there was not much information available about the complete analysis of the inflammatory microenvironment, we recently reported a comprehensive overview of various inflammatory markers and immune cells (qualitative and quantitative) in human WTs by immunohistochemistry (19).

Molecular links between WT and inflammation

Although there are a plethora of publications to link inflammation and adult tumor development, only few studies are available to relate the molecular links between WT and inflammation. Some of the recent findings are summarized below.

Immune cell infiltration

Our qualitative and quantitative immunohistochemical examination of immune cells in WTs (19) revealed infiltration of both adaptive and innate immune cells in tumors, similar to that previously reported in five WT samples (14) in a comparative study with adult tumors. However, our examination of a larger panel of tumors revealed that the extent of infiltration varied among tumors and among different histologically distinct regions within the same tumor, and also there was a difference in the in the quantity and infiltration pattern of adaptive and innate immune cells. Interestingly, while adaptive immune cells (T cells and B cells) were mostly localized to tumor stroma, innate immune cells [e.g., tumor-associated macrophages (TAMs), tumor-infiltrating neutrophils (TINs), and mast cells (MCs)], were not only predominantly localized to tumor stroma but also present in all other regions of the tumor. This different spatial localization suggested that a similar spatial pattern of chemical mediators, including chemokines and cytokines and other inflammatory proteins, might exist, either as a cause or as an effect of the presence of immune cells, which have been demonstrated to be recruited by, and also, in some cases, produce such mediators. To assess this possibility, we analyzed the expression and the intratumor localization of COX-2, HIF-1α, p-Stat3, p-ERK1/2, and the angiogenic marker, VEGF, in human WTs.

The following section describes the role of adaptive immune cells.

T lymphocytes

Human WTs were highly infiltrated with CD3+ T cells when compared with control kidney tissues in our earlier study (19). These T lymphocytes were almost absent in control kidney sections. Strikingly, although tumor stroma has many of the T lymphocytes when compared with other regions such as epithelium and blastema, the peritumoral area adjacent to tumor islands also has a huge number of infiltrating T lymphocytes. The peritumoral infiltration of this mononuclear T lymphocytes was greater than intratumoral (in blastemal, epithelial, and stromal regions) area of the tumor. Thus, this mononuclear T-cell infiltration was detected intensely in peritumoral region of the tumor in most of the cases we analyzed.

B Lymphocytes

B lymphocytes (CD20+) were also scattered in intra- and peritumoral region of WT with complete absence in control kidney sections, suggesting that these mononuclear lymphocytes (both T and B lymphocytes) followed the same kind of inflammatory cell infiltration pattern (19). Again, the density of CD20+ B-cell infiltration tended to be higher in peritumoral area than in intratumoral stromal region of the WT. In some of the tumors, we found only very few or absent in the tumor stroma, with aggregated infiltration of B lymphocytes found in most of the tumor-adjacent regions.

The role of innate immune cells is described below.

Macrophages

Although the intratumoral regions have infiltrating CD68+ macrophages (CD68MØ) in human WT, the majority of these CD68MØ were mostly dispersed extensively in tumor stroma in our earlier reported study (19). In contrary to the T and B lymphocytes, the CD68MØ within the tumor islands were also present in blastemal and epithelial regions although they were sparse when compared with tumor stroma. These CD68MØ were mostly in direct contact with the adjacent tumor cells in the invasive front. Surprisingly, although there were peritumoral CD68MØ, they were not comparable with the very highly infiltrated intratumoral regions. This observation is absolutely opposite to the lymphocyte (both T and B) infiltration, which we observed earlier. Very clear staining either in the membrane or in the cytoplasm was observed, with no staining in the nucleus. The spatial uniformity of the macrophage infiltration and density in the intratumoral region was maintained. But some tumors showed considerably less CD68MØ infiltration in some areas.

Neutrophils

TINs were identified in the intratumoral region of human WT (19). These TINs were mostly concentrated in the blastemal or epithelial regions to a lesser extent in the tumor stroma. There is a huge remarkable difference in the density of these cells in these different regions of the tumor. Most of these TINs were either intraepithelial or intrablastemal or, to some extent, were in the stroma, which is adjacent to the differentiated epithelial tissue. Overall, these TINs followed the tumorocentric distribution, concentrating mostly in neoplastic area as a massive infiltrate and diminishing its number or density distant from the neoplasm in almost all the WT cases in the current study. This is also true with anaplastic histology tumors, but the size of the neutrophils was slightly bigger in these tumors. TINs were not detected in the normal kidney.

Mast cells

MCs have been identified in the tumor microenvironment of various human neoplasias; we first confirmed that the MCs also infiltrate human WT (19). The infiltrating MCs were

distributed mainly in the invasive area of most of the human WTs. MCs were found in very small groups around neoplastic cells in tumor stroma and also in the peritumor areas but were almost absent in other intratumoral areas such as blastema and epithelium.

Together with these, various immune cell infiltration clearly demonstrates that the tumor inflammatory microenvironment is also present in human WT.

Inflammatory mediators

The inflammatory mediators can induce genetic and epigenetic changes that result in aberrations in critical biochemical pathways responsible for maintaining the cellular homeostasis, which leads to progression of cancer (1, 3, 4, 20). These inflammatory mediators may be of many types, such as cytokines, chemokines, free radicals, prostaglandins, growth factors, and enzymes such as COX.

COX-2

Positive immunoreactivity for COX-2 protein was observed in the entire tumor sections stained with diffuse moderate-to-strong cytoplasmic expression in the blastemal and the epithelial components and with very intense staining in tumor stroma. The infiltrating immune cells and other cells such as fibroblasts in the stroma were immune reactive for COX-2 protein. However, some of the tumors with anaplastic histology showed strong nuclear localization COX-2. The staining pattern and the intensity varied from tumor to tumor. Normal kidney samples showed weak to moderate staining in the cytoplasm of tubular epithelial cells. However, very weak or no staining was observed in the renal interstitial cells or glomeruli. We also investigated the correlation of COX-2 expression with the other inflammatory markers such as HIF-1, Stat-3, and VEGF. In addition, two different groups independently observed COX-2 expression in human WT ubiquitously in all cases, independent of the type (15) and stage (16) of neoplasm. COX-2 expression has been reported in other kidney cancers (renal cell carcinoma) (21), but not in pediatric tumors. In addition, Lee et al. (22) reported that the inhibition of COX-2 by SC-236 disrupted the tumor vascular mural cell recruitment and survival signaling in an orthotopic xenograft model of human WT. And another group (22) reported that the use of the same COX-2 inhibitor reduced tumor metastasis and inflammatory signaling during the blockade of VEGF in orthotopic SKNEP1 model of pediatric cancer.

HIF-1α

Very prominent nuclear localization of the HIF-1α protein expression was noticed in most of the cases evaluated in blastema, stroma, and epithelium along with negative HIF-1α expression in matched control kidney slides as reported earlier (19). In addition, some tumor specimens showed cytoplasmic granular staining in the cell cytosol and membranous (only in blastema) expression in blastemal and stromal compartments. The immune cell infiltrate of

tumor stroma was immunoreactive for HIF-1α protein as observed for COX-2 expression. Thus, the stromal expression of HIF-1α resembles the COX-2 expression. HIF-1α overexpression was reported in a significant proportion of WTs (23). In their study, they found no significant association between the expression of HIF-1α and clinicopathological variables in WTs resected following chemotherapy. In addition, the coexpression of HIF-1α has been reported with the angiogenic marker VEGF (17).

VEGF

VEGF expression was observed in most of the specimens (19). Although majority of the tumors showed VEGF expression in the infiltrating immune cells, connective tissue, or fibroblasts in tumor stroma similar to COX-2 and HIF-1α expression, but the blastemal and epithelial cell components were also immunoreactive, to some extent, in some tumor specimens. In the normal kidney samples, VEGF expression was observed in the proximal and distal convoluted tubules. Rowe et al. (24) reported that the anti-VEGF antibody suppressed primary tumor growth and metastasis in experimental models of WT. And the combination of low-dose topotecan and anti-VEGF antibody therapy suppressed the tumor growth and metastasis in experimental WT mice more durably than either agent alone (25). The immunohistochemical expression of VEGF-C and VEGFR-2 in the stromal and epithelial components of WT was reported (26) and indicated a potent unfavorable risk factor and directed the use of antiangiogenic treatment strategies to control the tumor growth.

Phosphorylated-Stat3 (p-Stat3)

The p-Stat3 expression was predominantly confined to the nucleus with almost undetectable cytoplasmic staining in all WT cases evaluated (19). Immunoreactivity of p-STAT3 was not detected in the control kidney tissue. Majority of the tumors showed the expression of p-Stat3 in the infiltrating immune cells in the tumor stroma, as well as in blastemal region, and these were very little or absent in epithelial cells. In addition, p-STAT3-expressing cells were found in the peritumoral area adjacent to the tumor islands. Moreover, the positive cells in this peritumoral area were found to be with stronger expression of p-STAT3 in the nucleus. Significantly higher nuclear immunoreactivity for p-Stat3 was also found in tumors compared with normal kidney sections. Furthermore, the expression of p-STAT3 was positively correlated with the TAM, CD3+ T cells, B cells, and inflammatory markers such as COX-2, HIF-1α, and VEGF. Zhang et al. (27) reported that the p-STAT3 expression in WT may correlate with progression and predict unfavorable prognosis and a new therapeutic target for metastatic WTs.

Phosphorylated pERK1/2

The expression of pERK1/2 protein was detected with very diffuse in the cytoplasm and more prominent staining in the nucleus in most of the WT cases (19), but to a small

extent, we were also able to see the cytoplasmic expression in normal kidney. However, the expression of phospho-mitogen-activated protein kinases (MAPK-/ERK1/2)-positive nuclei was observed in both peritumoral and tumoral islands. In most of the tumor cases, the expression was localized in tumor stroma with some extent in blastemal cells. Epithelial cell component of the tumors was almost absent with either cytoplasmic or nuclear pERK1/2 expression. The stromal expression was similar to COX2, HIF-1α, and VEGF expressions. The correlation between the p-ERK expression and other immune cell markers was also assessed. Significantly higher expression of pERK1/2 was observed in tumors than in control kidneys. It has been observed that the Wt1 ablation and insulin growth factor-2 (IGF2) upregulation resulted in WTs with elevated ERK1/2 phosphorylation in mice (28).

Inducible nitric oxide synthase (iNOS)

Although there are not many studies available on the expression of iNOS in WTs, we observed the iNOS expression (19) in tumor stroma with intense nuclear or cytoplasmic staining in most of the cases and diffuse cytoplasmic staining in blastemal cells of the tumor. The inflammatory immune cells within the tumor stroma were highly immunoreactive for iNOS in some of the WT specimens. Surprisingly, immunoreactivity for iNOS was also detected in the peritumoral area of some of the tumor sections. However, none of the epithelial cells expressed iNOS. In addition, areas around the tumor with neovascularization showed positive staining for iNOS. However, no significant immunoreactivity for iNOS was detected in the control kidney sections.

Nitrotyrosine (NT)

In our observation (19), WTs showed NT expression in the cytoplasm of the inflammatory immune cells of tumor stroma, as well as with much diffused cytoplasmic staining in the blastemal and epithelial regions of the tumors. The NT expression was observed very rarely in the peritumoral region. The expression was mostly localized within the tumor.

Chemokines and cytokines

Chemokines play an important role in tumor development and metastasis. The expression or secretion of these chemokines in the tumor microenvironment of various cancers, including breast, ovarian, pancreatic, melanoma, lung cancers, etc, have been reported. The expression of chemokines and their receptors is altered in many malignancies, and it leads to aberrant chemokine receptor signaling. Although the chemokine expression has been reported in various cancers, there is not much information available in human WTs. However, the role of ELR-CXC chemokine family members CXCL2 and CXCL7 and their receptor CXCR2 was expressed at the earliest stages of metanephric development in the rat, and signaling through this receptor was required for the survival and maintenance of the undifferentiated metanephric mesenchyme (MM) (29).

Other markers expressed in WTs

CITED1

In general, CITED1 is expressed at high levels in the condensed metanephric mesenchyme (MM) surrounding ureteric bud (UB)tips, is downregulated temporally as these cells begin to differentiate into early epithelial structures, and is not expressed in differentiated elements of the adult kidney (30). WTs arise from the undifferentiated renal progenitor cells. CITED1, which is a transcriptional regulator, blocks the metanephric mesenchymal-to-epithelial transition and is expressed in the blastema of both the developing kidney and WTs. The overexpression of CITED1 in a human WT cell line significantly increased proliferation in vitro, and mutation of its functionally critical transactivation domain (DCR2) significantly reduced proliferation (31). CITED1 expression was observed in blastemal cell populations of both experimental rat nephroblastomas and human WTs, and that primary human WTs presenting with disseminated disease show the highest level of CITED1 expression (32). Rivera and Haber (33) reported the Cited1 expression in the undifferentiated MM cells of WTs, and its expression was primarily confined to the nucleus. These studies suggest CITED1 as a marker of primitive blastema in WTs, and its persistent expression and altered subcellular localization in the condensed MM might have a role in WT initiation and progression. And another possibility is that persistent expression of CITED1 in metanephric blastema may have adverse developmental role in the pathogenesis of WTs.

B7-H1

A membrane glycoprotein, B7-H1, has been reported to act as an important coregulator of antigen-specific T-cell-mediated immunity (34, 35). This is normally expressed by the macrophage lineage cells and is aberrantly expressed by multiple human malignancies (34–36). Interestingly, tumor B7-H1 has been observed to induce T-cell apoptosis or anergy, thereby downregulating the host antitumoral immunity (34). B7-H1 expression has been observed in WTs, and its expression correlated with tumor biology and is associated with an increased risk of recurrence in patients with favorable-histology tumors (36). Because B7-H1 is involved in T-cell apoptosis, its expression in WTs may be related to inflammation. Thus, B7-H1 expression may be used as a prognostic marker, which indicates the aggressive behavior for favorable-histology WT. In addition, B7-H1 may be used to distinguish patients with favorable-histology WT, who require aggressive treatment and unfavorable-histology tumors, and who are at very low risk for disease recurrence and death and to avoid unnecessary overtreatment (36).

CD44

CD44 is also a membrane glycoprotein like B7-H1 expressed in a variety of cells, including those of epithelial, mesenchymal, and hematopoietic origin (37). CD44 expression has been observed in three different isoforms, such as CD44s, CD44v5, and CD44v10. The expression

of CD44s has been observed in all three components of the WTs. Among the isoforms, overexpression of CD44v5 in blastemal cells of WT correlated with tumor stage, clinical progression, and tumor-related death (37). Therefore, CD44v5 expression may be used as a good prognostic marker in identifying WT patients with a high tendency for distant metastases. Several studies have indicated that the increased expression of the CD44 gene is associated with metastatic disease. Studies by various other groups also indicated that the expression of CD44 isoforms may be a good prognostic marker for various cancers (38–40).

Carbonic anhydrase IX (CAIX)

CAIX is a membrane glycoprotein, which plays an important role in the growth and survival of tumor cells under normoxic, as well as hypoxic, conditions (23, 41, 42). CAIX (mRNA and protein) expression has been reported to be upregulated in the untreated and treated WTs when compared with normal kidneys and WT precursor lesions (nephrogenic rests) (23). There was no correlation between CA expression and clinicopathological variables, including metastatic status in postchemotherapy-treated WTs. Cellular localization studies in untreated WTs suggest that CAIX and HIF-1α are regulated by hypoxic and nonhypoxic mechanisms.

Role of immune cell infiltration with COX-2 pathway components

The expression of the inflammatory markers such as COX-2, HIF-1, iNOS, p-ERK1/2, and VEGF was predominantly localized to tumor stroma similar to the expression of TAMs. The codistribution of major inflammatory marker COX-2 with TAM infiltration in the tumor stroma was observed in our study (19). This study suggests that the infiltration of inflammatory immune cells and the expression of inflammatory markers in the tumor stroma are related. This observation suggests a correlation between the infiltrating immune cells and the activated cytokines and chemokines. This TAM infiltration was further confirmed (F4/80 expression) in the mouse model of WT (19). TAM infiltration is known to be induced by COX-2 in the tumor microenvironment (43), especially in the tumor stroma, and TAMs can also induce the expression of COX-2 (44). We have reported earlier that the colocalization of COX-2 and TAMs in the tumor stroma (19) may activate each other in the tumor microenvironment. The mechanisms responsible for the abundant COX-2 expression in WTs are that the infiltrating immune cells themselves could be overexpressing COX-2, or tumor fibroblasts may be generating COX-2 in response to macrophage infiltration, or fetal mitogen IGF2 may induce COX2 by MEK/ERK pathway (45).

Cross talk between immune cells and other markers in WT microenvironment

As indicated earlier, TAMs are also involved in the production of proangiogenic factors, such as transforming growth factor β and VEGF (46, 47), and of immunosuppressive chemokines and cytokines, such as interleukin 10 and prostaglandin E2, which contribute to

tumor angiogenesis (46, 48, 49). Thus, the TAM infiltration might play a significant role in the increased VEGF expression and also in the vascularization of the tumors. The correlation and localization of TAMs in the tumor stroma with the expression of various inflammatory protein markers, such as COX-2, HIF-1, p-ERK1/2, iNOS, and NT, suggest a functional association of TAM infiltration with the overexpression of these markers and vice versa in WTs (19) and demonstrate the existence of a highly inflammatory microenvironment in this disease.

Possible mechanism/mechanisms responsible for COX-2 pathway activation

Reports from our laboratory indicated that p-ERK1/2 was induced in mice, which are engineered to overexpress IGF2 and along with ablation of Wt1 gene, and also in human WTs (28), suggesting a role for the ERK signaling in WT development. The robust expression of COX-2 and p-ERK1/2 in tumors may be a consequence of IGF2 overexpression in WTs because IGF2-mediated COX2 expression has been reported in other tumors (45). Thus, the upregulated COX-2 expression creates an inflammatory microenvironment in WTs, which may be mediated by the enhanced p-ERK signaling is depicted in Figure 1. In the tumor microenvironment, COX-2 can also activate the expression of HIF-1 through its enzymatic product prostaglandin E2 (45, 50). Furthermore, this upregulated expression of p-ERK1/2 stabilizes the HIF-1α protein by preventing its degradation via the blockage of prolyl hydroxylase activity, which regulates HIF-1 or activates HIF-1α protein (Figure 1). Spatially similar expression of COX-2 and HIF-1 was observed in WTs (19), suggesting the role of COX-2 in HIF-1 activation. COX-2 activation of HIF-1 can also occur through hypoxia (17, 51) or hypoxia-independent mechanisms (52), with the involvement of p-ERK1/2 (53). In addition, it has been reported that PGE2, the end product of COX-2 pathway, can also enhance HIF-1 transcriptional activity (51). HIF-1 can also directly upregulate the expression of COX-2 during hypoxia (54) and thus form a feedback loop to continually activate the COX-2 pathway (Figure 1). Hence, it may be assumed that IGF2 affects the inflammation, hyperproliferation, and angiogenesis in WTs by IGF2-induced Cox-2-mediated p-ERK1/2 pathway. Therefore, we speculate that COX-2 in this WT microenvironment may drive the inflammation and upregulate the aforementioned downstream targets.

Possible therapeutic targets in Wilms tumors

On the basis of the above evidences, it was found that WTs have highly inflammatory microenvironment, which further provides a link for the inflammatory etiology of cancer. The overexpression of different inflammatory markers provides a rationale for their use in the prevention and treatment of cancer. More specifically, our observation (19) strongly supports the therapeutic value of blocking COX-2 in WTs. The overexpression of inflammatory markers in tumors, in particular COX-2, has provided a rationale for their targeting in prevention and treatment of many cancers (55–59) by COX-2-specific inhibitors alone (60, 61) or in combination

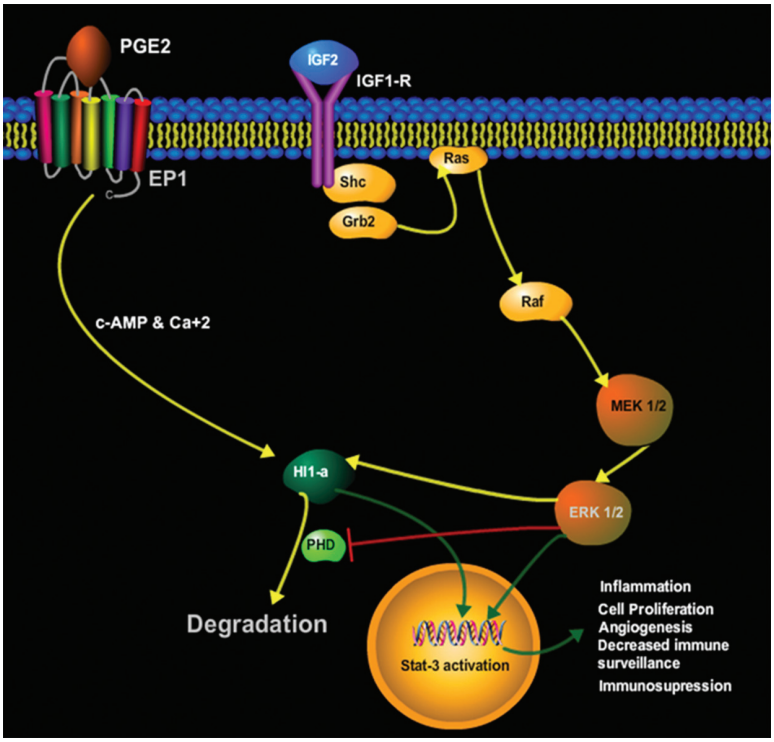

Figure 1. Possible mechanism/mechanisms responsible for COX-2 pathway activation: COX-2 activation of HIF-1 can also occur through hypoxia-dependent by PGE2 or hypoxia-independent mechanisms with the involvement of p-ERK1/2. HIF-1 can also directly upregulate the expression of COX-2 during hypoxia and thus form a feedback loop to continually activate the COX-2 pathway leading to activation. Hence, IGF2 affects the inflammation, hyperproliferation, and angiogenesis in WTs by IGF2-induced COX-2-mediated p-ERK1/2 pathway.

with other inhibitors (62). COX-2 inhibition may serve as an appropriate target for therapeutic intervention because all the downstream targets of COX-2 pathway components may be controlled or inhibited too. Also, COX-2 blockade may be effective in WT therapy owing to the inhibitory effect of COX-2 inhibitors in controlling the immune cell infiltration and tumor-promoting angiogenesis, thereby controlling tumor growth. Elucidating the molecular basis for the accumulation of the different inflammatory protein markers in tumors requires further in-depth study and warrants further investigation of this COX-2-mediated pathway.

Conclusions

The colocalization of TAMs in the tumor stroma along with COX-2 and its pathway components, such as HIF-1 and p-ERK1/2, suggests a functional association of TAM

infiltration with the overexpression of these markers and vice versa in WTs and demonstrate the existence of a highly inflammatory microenvironment in this cancer. The overexpression of inflammatory marker COX-2 has provided a rationale for their targeting COX-2 pathway using COX-2-specific inhibitors alone or in combination with other inhibitors, which may be effective in treating this childhood cancer.

Conflict of Interests

The author declares no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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Section III

WT1 Gene Aberrations in Other Malignancies

Chapter 13

WT1 in Cardiac Development and Disease

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Abstract

The heart is essential for realizing the distribution of oxygen and nutrients throughout the body. Therefore, the heart is the first organ to develop and is already functional in its most primitive structure during embryogenesis. Recent studies indicate that the transcription factor Wilms' tumor-1 (WT1) is important for many aspects of cardiac development. WT1 expression is first observed in the proepicardium, a group of progenitor cells that give rise to a mesothelial sheet covering the heart, the epicardium. WT1 expression in epicardial cells is required for their epithelial-to-mesenchymal transformation forming epicardium-derived cells that will contribute to the formation of coronary vessels and interstitial fibroblasts. Endothelial cells within the heart also express WT1, whereas the endothelial cells in other

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parts of the embryo do not. The endothelial expression of WT1 during cardiac development is likely to be important for vascular formation. After cardiac injury, WT1 is temporally upregulated in the epicardium and in the endothelial cells in the infarcted area and border zone, which points to a potential important role for WT1 in cardiac repair and regeneration. In this chapter, we describe the many faces of WT1 within the heart.

Key words: Cardiac development; Cardiac regeneration; Endothelial cells; Epicardium; Wilms' tumor-1

Introduction

The pleiotropic molecule Wilms' tumor-1 (WT1) is an transcription factor that was first discovered in renal tumors (1, 2). It contains four zinc-finger motifs at the C-terminus which are important for the binding of DNA to activate gene expression. Besides its function to activate the transcription of genes, WT1 is also involved in posttranscriptional processes (3). The expression of WT1 is essential during development of multiple organs, including kidneys, gonads, spleen, and the heart (4, 5). In the developing heart, WT1 is strongly expressed in the outer layer, i.e. the epicardium, and in the cardiac endothelial cells. After myocardial infarction this WT1 expression reemerges in both lineages. In this chapter, we describe the dynamic expression of WT1 during development and after cardiac injury. We focus on the multiple roles of WT1 including epithelial-to-mesenchymal transformation and angiogenesis in cardiac development, repair and regeneration.

WT1 in cardiac development

The expression of WT1 in the epicardium during early cardiac development

Knockout of *Wt1* in mice revealed that this transcription factor plays an essential role in cardiac development. In the absence of WT1, the vasculature of the heart is not formed, which disturbs proper formation of the heart and therefore results in prenatal death (3, 4). Already in the developing embryo, the heart is essential for the supply of oxygen and nutrients. Therefore, it is the first organ to develop and function during embryogenesis. The heart has a mesodermal origin and is formed through gastrulation. The earliest recognizable structure is the primitive heart tube, which is formed at embryonic day (E) 8 in mouse, corresponding with day 21 postfertilization in human. This hollow structure consists of two cardiac cell populations, namely cardiomyocytes on the outside and endothelial cells on the inside, which are separated by cardiac jelly (5) (Figure 1). The primitive tube elongates and undergoes rightward looping between E8.5 and E10.0 in mouse (days 23 and 28 in human) (Figure 1). Subsequent remodeling of the heart involves formation and expansion of the chambers, and formation of valves and septa, resulting in a septated four-chambered heart (6, 7)

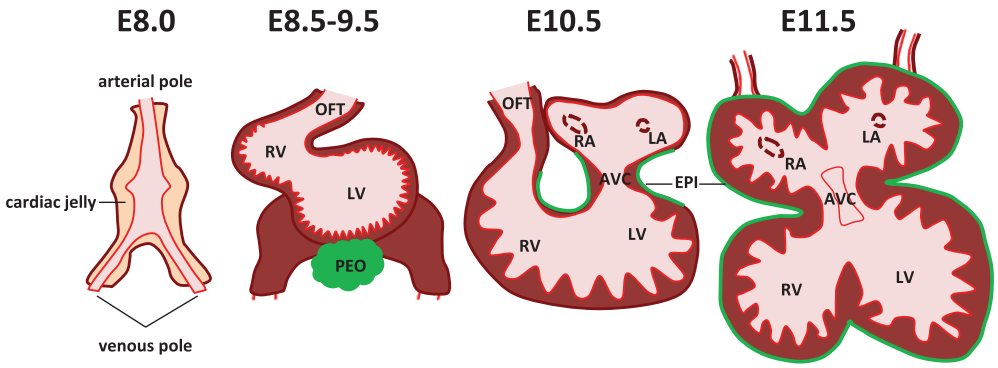

Figure 1. Early cardiac development and the expression of WT1. E8.0: The primitive tube consists of cardiomyocytes (brown) on the outside and endothelial cells (red) on the inside, which are separated by cardiac jelly. E8.5–E10.5: The primitive tube elongates and undergoes rightward looping. At the inflow tract of the developing heart, the proepicardium arises and the epicardium formation has initiated. E11.5: The four chambers of the heart can be recognized, and septa and valves are being formed. The expression of WT1 is indicated in green. AVC, atrioventricular cushion; EPI, epicardium; LA, left atrium; LV, left ventricle; OFT, outflow tract; PEO, proepicardium; RA, right atrium; RV, right ventricle.

(Figure 1). Even though the heart is already functional at an early stage of fetal development, the growth and maturation of the heart are not completed until after birth (8).

A third population of cardiac cells envelopes the heart during development, the epicardium. The cells forming the epicardium are derived from the proepicardium, a heterogeneous transient cluster of cells (9), located at the base of the inflow tract of the developing heart (Figure 1). The earliest expression of WT1 in the heart is found in cells of the proepicardium, at E9.5 (10–12) (Figure 1 and 2a). Proepicardial cells reach the bare heart tube by formation of a tissue bridge or by free-floating vesicles and spread over the myocardium covering the complete heart, forming the epicardium. Recently, we have shown that the covering of the myocardium with WT1-positive cells occurs in a dorsal to ventral pattern between E9.75 and E10.5. In addition, the epicardial covering of the right ventricle is delayed and less dense compared to the left ventricle. Complete covering of the myocardium with WT1-positive cells is established in the mouse by E12.5 (11) (Figure 1). During human cardiac development, complete covering of the myocardium is observed at week 5 (13). After enveloping the entire heart, the epicardium remains positive for WT1 both in mouse and in human during embryonic development (3, 10–18) (Figure 1 and Figure 2a).

After the formation of the epicardium, a subset of epicardial cells undergoes epithelial-to-mesenchymal transition (EMT), resulting in epicardium-derived cells (EPDCs) (19, 20)

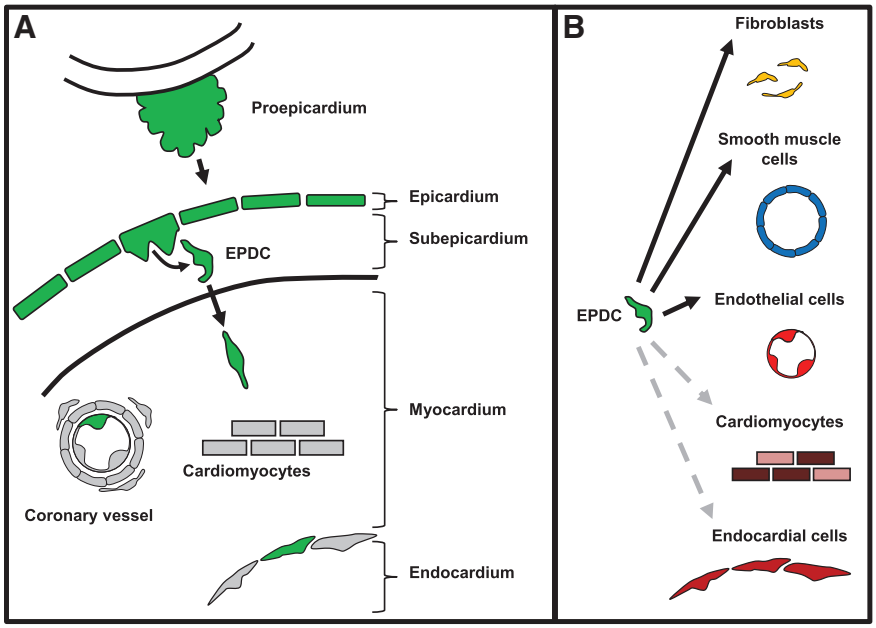

Figure 2. Expression of WT1 and the fate of epicardial cells. (a) The epicardium arises from the proepicardium. A subset of cells undergo EMT and migrate into the subepicardium and myocardium. WT1 (green) is expressed by cells in the proepicardium, epicardium, EPDC, endothelial cells, and endocardial cells. (b) EPDCs contribute to the formation of fibroblasts, smooth muscle cells and endothelial cells; the contribution to cardiomyocytes and endocardial cells is controversial. Cardiac fibroblasts can be subdivided into interstitial fibroblasts, perivascular fibroblast, and valvular interstitial cells.

(Figure 2a). Lineage-tracing studies have shown that EPDCs migrate into the myocardium and contribute to cardiac fibroblasts, endothelial and smooth muscle cells of the cardiac blood vessels, and cardiomyocytes (9, 12, 19, 21–23), although the contribution to the latter is still under debate (11, 14, 16, 24) (Figure 2b). The contribution of WT1-positive cells to endothelial cells however is minimal (12).

The role of WT1 in embryonic epithelial-to-mesenchymal transition

In addition to the essential role of WT1 in the formation of the epicardium (3, 4), several studies have shown that WT1 serves as a regulator of epicardial EMT. *Wt1*-knockout mice show a reduction of subepicardial mesenchyme (3). In addition, epicardial cells are unable to detach from the epicardium, and EPDCs do not migrate into the subepicardium (18, 25).

Epicardial EMT is regulated by WT1 via multiple genes and pathways. Knockdown of *Wt1* in epicardial cells reduced the expression of SNAIL and SLUG, whereas the

downstream target E-cadherin was upregulated. SNAIL and SLUG are key regulators of the EMT process and have an inhibitory effect on the expression of the epithelial marker E-cadherin (26). WT1 directly promotes EMT by enhancing the expression of SNAIL and inhibiting the expression of E-cadherin (25). Furthermore, WT1 was shown to be a positive upstream regulator of the Wnt pathway, which influences diverse aspects of cardiogenesis and is important for epicardial EMT (27). Knockdown of *Wt1* resulted in a decrease of the downstream effectors of the Wnt pathway, *Ctnnb1* and *Lef1* (18). WT1 directly regulates RALDH2, an enzyme involved in retinoic acid (RA) synthesis, and is expressed in the epicardium (18, 28, 29). RA signaling is essential during embryonic development, and RA deficiency has been shown to result in cardiac abnormalities, similar to the phenotype of *Wt1*-knockout mice (20, 30). In chicken, induction of RA signaling in EPDCs results in upregulation of WT1 (31), indicating a positive feedback loop between WT1 and RA.

Although most *in vivo* studies suggest an inducing role for WT1 in epicardial EMT, *in vitro* repression of WT1 induced the transformation of both human and mice cobblestone-like EPDCs into spindle-shaped cells, indicating a context-dependent and possibly, concentration-dependent function of WT1 (32, 33).

Expression of WT1 in cardiac endothelial cells

The classical consensus is that EPDCs lose their expression of WT1 during their migration from subepicardium into the myocardial layer (12, 34). We have recently shown that the expression of WT1 is not restricted to the epicardium and subepicardium but is also present in the myocardial layer during development from E12.5 onward in mice and from week 5 after fertilization in humans (11, 13, 14) (Figure 2a and Figure 3). This expression of WT1 starts at the epicardial side and expands toward the luminal site of the heart (13, 14). The myocardial wall of the heart consists of an outer condensed part, the compact layer, and an inner loosely arranged part, the trabecular layer. Before birth, the expression of WT1 within the myocardium in mice is restricted to the compact layer and expands into the trabeculae in the neonatal heart (14). In human, the trabecular expression of WT1 is already observed before birth (13). The cellular composition of the heart comprises cardiomyocytes, cardiac fibroblasts, smooth muscle cells, and endothelial cells (35). Interestingly, in the myocardial layer of the heart, we found the expression of WT1 in endothelial cells of both small capillaries and the larger coronary vessels in mice and human (13, 14) (Figure 2a and Figure 3). In mice, the expression of WT1 in endothelial cells is still present at neonatal stages but gradually decreases before adulthood (14). In human, the expression of WT1 in endothelial cells of at least the arteries decreases before birth (13). Another difference between mice and human is

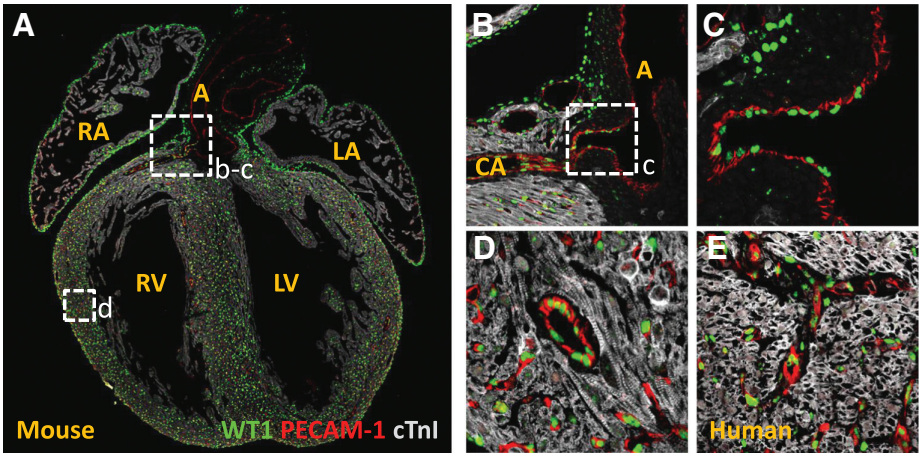

Figure 3. WT1 is expressed by cardiac endothelial cells during development. (a) Overview of the mouse heart at E17.5. WT1 is expressed in the epicardium and endothelial cells [coexpression of WT1 (green) and PECAM-1 (red)]. (b, c) Magnification of the connection between aorta and coronary vessel shows the specific cardiac expression of WT1 in endothelial cells. (d) WT1 is expressed in the vasculature of the heart during murine development. (e) WT1 is expressed in the vasculature of the heart during human development. A, aorta; CA, coronary artery; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle.

the widespread expression of WT1 in endocardial cells at early stages during human cardiogenesis (Figure 2a). The differences in WT1 expression between mouse and human can be explained by the difference in maturation time during pregnancy, as well as the differences in dimensions (36, 37).

WT1 in the infarcted heart

The epicardial expression of WT1 decreases after birth and remains at low levels during normal homeostasis (Figure 4a). The endothelial expression of WT1 in the adult heart is low and mostly observed in some capillaries and cardiac veins (14). In contrast to the quiescent appearance during adult physiological conditions, after myocardial infarction (MI) the epicardial and endothelial cells re-express WT1 (Figure 4d).

MI is the most common type of ischemic heart disease and the leading cause of death in the Western world (38, 39). During MI, coronary occlusion leads to a reduced supply of oxygen to the cardiac muscle, resulting in massive cell death of cardiomyocytes. The extracellular matrix (ECM) is degraded ensuring infiltration of inflammatory cells, which remove the cellular debris generated during acute cardiac injury (40, 41). The expression of WT1 in the epicardium is already upregulated 1 day after infarction and is induced throughout the entire

epicardial layer of the heart (42). The subepicardium thickens by resurgence of the EMT process and is most pronounced at the infarcted area (43) (Figure 4b). The re-expression of WT1 and the revival of epicardial EMT suggest that WT1 regains its fetal role after MI. In the subepicardium, WT1 is expressed in the αSMA-positive cells but not in the endothelial cells (44) (data not shown) (Figure 4d). Lineage tracing of epicardial cells indicated that the WT1-positive cells do not migrate into the infarcted area (44, 45). Remarkably, priming the mouse heart before MI with Thymosin beta-4 resulted in the migration of epicardial cells into the myocardium and functional differentiation into cardiomyocytes after MI (22).

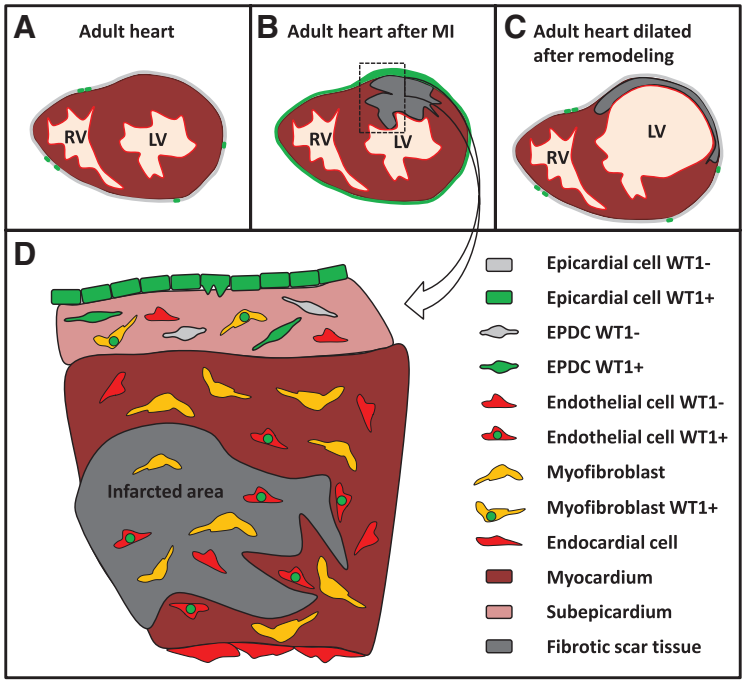

Figure 4. Expression of WT1 in the adult and injured heart. (a) The epicardial expression of WT1 is almost absent in the adult heart. (b) Myocardial infarction results in the loss of cardiomyocytes and the replacement by fibrotic scar tissue. After myocardial infarction, the epicardial expression of WT1 is reactivated in the entire heart. The thickening of the epicardium is most pronounced at the infarcted area. (c) Cardiac remodeling following myocardial infarction results in ventricular dilatation and impairment of cardiac pumping. (d) Myocardial infarction results in re-expression of WT1 in the epicardium, epicardial EMT, migration of EPDCs into the subepicardium, expression of WT1 in myofibroblasts of the subepicardium and in endothelial cells of the infarcted area and border zone. The expression of WT1 throughout adulthood and after injury is indicated in green. LV, left ventricle; RV, right ventricle.

In the infarcted area, an increase in granulation tissue is observed approximately 3 days after MI, which is characterized by the presence of interstitial fibroblasts, myofibroblasts, and forming blood vessels (12, 14, 43, 46, 47). As opposed to the expression in the subepicardium, upregulation of WT1 expression is present in endothelial cells and not in (myo) fibroblasts in the myocardial layer (14) (data not shown; Figure 4). Initially, upregulation of WT1 is observed in endothelial cells of the border zone and subsequently in the infarcted area, with a peak endothelial expression of WT1 at day 7 after MI (47). As time progresses, vessels in the infarcted area become more mature, fibrotic scar forms, and the expression of WT1 disappears (14). Interestingly, in the border zone, the expression of WT1 in endothelial cells is still detectable 4 weeks after MI, indicating that this region still undergoes active remodeling. The expression of WT1 in the epicardium gradual decreases after the first week to return to quiescent levels at 3 months after infarction (42) (Figure 4c). The remodeling and maturation of the fibrotic scar result in ventricular dilatation and impairment of cardiac functioning (48, 49) (Figure 4c).

The molecular mechanism causing WT1 reactivation in epicardial and endothelial cells is unclear. Inflammation might be a potential trigger for the activation of WT1 expression after MI. Proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are upregulated within the first few hours after injury (50). These cytokines are able to activate the transcription of NF-κB (51), which is highly present after MI (52–54). NF-κB upregulates WT1 expression (55), thereby potentially activating the epicardium after MI (Figure 5). The peak of NF-κB induction is found at day 3 after MI (56), the same day the first signs of angiogenesis, including the endothelial WT1 expression, are visible in the border zone (47). Interestingly, WT1 has an anti-inflammatory role because it inhibits the expression of inflammatory cytokines by stimulation of IL-10 (57) (Figure 5).

The upregulation of epicardial WT1 after injury might also be caused by soluble factors released by the myocardium within the pericardial fluid (PF). Injection of PF from MI patients into the pericardial cavity of mice induces the expression of WT1 in epicardial cells in the absence of infarction (58, 59). In addition, PF of patients affects gene expression in epicardial cells that are involved in EMT; among others, the expression of SNAIL and TWIST is stimulated (59). After MI, PF contains an increased number of exosomes, which are small extracellular microvesicles. These vesicles contain bioactive molecules and are important for intracellular communication and activation (60). Recent proteomic analysis by Foglio et al. (59) showed that clusterin is highly enriched in exosomes of PF of patients after MI. Clusterin is involved in EMT in prostate cancer (61), and administration of clusterin in the pericardial cavity induced EMT in epicardial cells (59).

The upregulation of endothelial WT1 after injury might be caused by hypoxia (Figure 5). Hypoxia is a well-known condition that induces vascular formation during development

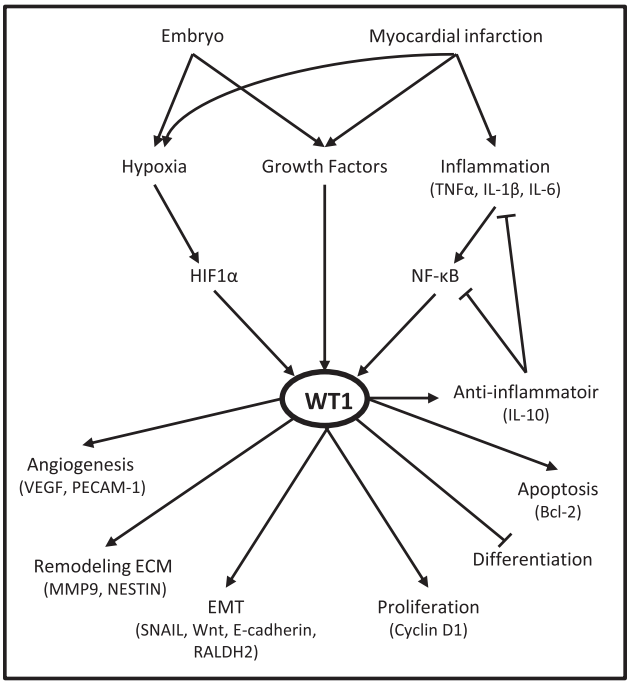

Figure 5. Working model of the regulation of WT1 and its target genes. The expression of WT1 is activated during embryonic development and in the adult heart after injury. Upregulation of WT1 is caused by hypoxia, growth factors, and inflammation. WT1 in turn is able to activate multiple genes that are important for the regulation of different processes, including EMT, angiogenesis, remodeling of the ECM.

and after MI via the hypoxia-inducible factor-1-alpha (HIF1α) (62). The expression of WT1 can be directly upregulated by hypoxia through the HIF1α-responsive elements in the WT1 promoter (63) (Figure 5). *In vitro* exposure of human endothelial cells to hypoxia increased the expression of WT1 (14, 64). Important for the response to hypoxia is that one of the downstream targets of WT1 is VEGF (65), one of the most potent angiogenic factors, both in embryonic vascular formation and in the growth of blood vessels after injury (66) (Figure 5).

The role of WT1 in endothelial cells

The expression of WT1 in endothelial cells is only found in the heart and not in other organs of the developing embryo (14) (Figure 3a–3c). The cardiac-specific expression of WT1 is supported by a recent study that identified the unique gene expression profiles of endothelial cells, isolated form different organs (67). In both human and mouse, the expression of WT1

in cardiac endothelial cells is significantly higher compared to noncardiac endothelial cells, confirming cardiac-specific expression of WT1 in endothelial cells (67). In fact, overexpression of WT1 was sufficient to differentiate endothelial cells into a more cardiac specialized population. The importance of WT1 for the development of blood vessels is highlighted by the re-expression in the cardiac vasculature after MI in mouse and after exposure of rats to hypoxia (14, 64). A study by Coosemans et al. (68) claimed the expression of WT1 in cardiac endothelial cells of patients that died after MI. Although the expression of WT1 in cardiac endothelial cells is unique during normal conditions, the expression is also present in endothelial cells in other organs in a pathological condition. WT1 is found in endothelial cells of the skin in patients with chronic dermatitis (69), and WT1 has been observed in endothelial cells in a wide variety of tumors (68–72).

It is unclear why under physiological conditions WT1 expression is found only in cardiac endothelial cells. In contrast to other organs, the heart has the unique feature that it is exposed to cyclic strain (73). It is known that mechanical forces during early development play an important role in cardiac morphology (74). In addition, cyclic strain is able to regulate the process of EMT (75). It is therefore tempting to speculate that stimulation of EMT by cyclic strain is regulated by an upregulation of the expression of WT1. Alternatively, WT1 might be induced by TGFβ, which is known to be upregulated by cyclic strain. TGFβ is able to upregulate WT1 expression via Par-4 (76–78). On the contrary, WT1 works as a negative feedback loop on TGFβ, by repressing its expression (79, 80).

At the very early stages of development, the fetal heart is predominantly dependent on glucose metabolism and shortly after birth the heart energy metabolism switches to fatty acid oxidation (81). Facilitating the uptake of fatty acids is a unique feature of cardiac endothelial cells (67, 82). WT1 expression is known to be essential for the cardiac endothelial fingerprint; therefore, WT1 might be important for the regulation of cardiac endothelial cell metabolism.

Patients with Denys–Drash syndrome (DDS), carrying partial-loss-of-function mutations in the *WT1* gene, develop glomerulosclerosis. In addition, the capillaries of the glomeruli show abnormal development. A cause of these malformations is found in a strong decrease in the expression of platelet endothelial cell adhesion molecule-1 (PECAM-1) in endothelial cell of the glomeruli (83). PECAM-1 is part of intercellular junctions and is present in mature vascular structures; additionally, its expression is upregulated during formation and remodeling of vascular networks (84, 85). WT1 is a positive regulator of PECAM-1 (71); this may explain the poor organization of capillaries in patients with DDS. Knockdown of *WT1* in human endothelial cells confirms the importance of WT1 in the formation of vascular networks, as these cells are unable to form proper networks (14, 64). The angiogenic role of WT1 is supported by a reduced sprouting capacity in an aortic ring angiogenesis

assay from mice lacking WT1 expression in endothelial cells. In addition, the same study revealed that the vessel density in matrigel plugs after subcutaneously injection, in mice lacking WT1 expression in endothelial cells, is significantly reduced compared to wild-type animals (71). Furthermore, deletion of WT1 in endothelial cells resulted in major reduction in cardiac vessel formation during mouse cardiac development supporting the presence of WT1 and the essential role of WT in cardiac endothelial cells (86).

Endothelial cells are anchored to a basement membrane that ensures structural and organizational stability. During vascular formation and remodeling, reorganization of the ECM is essential (87, 88). Remodeling of the ECM is the net result between synthesis and decomposition of the ECM (89). Degradation of the ECM is facilitated by an increase and activation of latent matrix metalloproteinases (MMPs). WT1 is able to directly upregulate the expression of MMP9 (70), thereby facilitating the degradation of ECM. The basement membrane is mostly made up of collagen IV, which is degraded by MMP9 (90). Interestingly, the study of Johnson et al. (91) showed that in the absence of MMP9 revascularization of infarcted tissue is strongly impaired, confirming that remodeling is essential for angiogenesis. The role of WT1 in remodeling is further strengthened by proteomic analysis in patients with DDS. Glomerular podocytes with WT1 mutations have a disturbed production of proteins forming the cytoskeleton (92). Furthermore, the expression of intermediate filament Nestin is regulated by WT1 (93). The expression of Nestin is increased in regenerating tissue and is believed to participate in cellular remodeling and angiogenesis (94–96). Coexpression of WT1 and Nestin was found in the epicardium and endothelial cells of the embryonic heart in mice (93) and in vascular endothelium of patients who died after MI (97, 98).

Changes in the cytoskeleton are also required for cells to adapt to a less differentiated phenotype, allowing them to proliferate and migrate. Nestin is present in proliferating progenitor cells and positively regulates proliferation and migration (96). Within the epicardium, a positive correlation was found between WT1 and proliferation (99). In addition, WT1 plays a role in regulating the cell cycle. *In vitro* studies knocking down WT1 in human endothelial cells show reduced proliferation and migration (14, 72). Proliferation of endothelial cells is directly regulated by WT1 via Cyclin D1 (14, 100), one of the many regulators of the cell cycle and present in the G1 phase (101). Interestingly, the expression of WT1 is upregulated in embryonic stem cells during embryonic body differentiation, a proliferative period for mesenchymal cells. Upon cellular differentiation, the expression of WT1 was reduced (25). The positive role of WT1 on migration might be the result of direct repression of the promoter of Cxcl10, an inhibitory chemokine preventing angiogenesis (17).

Finally, WT1 is known to play a role in apoptosis. This was already noticed in 1993 in *Wt1*-knockout mice; embryonic tissue of the kidney showed more cell death compared to wild-type littermates (4). Over the last years, it has become clear that WT1 can directly

regulate genes involved in apoptosis; however, it depends on the cellular context if WT1 has a pro- or anti-apoptotic effect (102). Future research is required to investigate the role of WT1 in apoptosis of endothelial cells; potentially WT1 protects the forming and maturing blood vessels against cell death.

Together these observations suggest a role for WT1 in the remodeling, proliferation, and migration of cardiac endothelial cells and the formation of a proper vascular network (Figure 5). Stress factors such as hypoxia and inflammation are likely to play a role in the activation of WT1 both during cardiac development and in the response after injury (Figure 5). Future research, focusing on the molecular mechanisms, can hopefully reveal all pathways by which the angiogenic function of WT1 can be explained.

Clinical perspective

Restoring the cardiac blood flow is the most important treatment of ischemic cardiomyopathy at this moment. To improve cardiac output, the cardiac wall consisting of cardiomyocytes, fibroblasts, endothelial and smooth muscle cells, has to be rebuild. Transplantation of cardiac stem cells after infarction improved the function (103–105); however, difficulties in acquisition of human tissue and *in vitro* expansion of cells limit the clinical applicability. An interesting approach would be to take advantage of the properties of WT1-expressing cells. The differentiation potential of WT1-expressing epicardial cells during development into the vasculature, fibroblasts and cardiomyocytes has positioned the epicardium as a promising target (20, 106, 107). WT1-expressing stem cell-like cells are residing in the epicardium (58, 108). In addition, Chong and colleagues (109) showed that cardiac colony-forming units originate from WT1-positive cells within the epicardium. Finally, activation of WT1 expression within the epicardium after injury revives the fetal differentiation potential in the epicardial cells. Facilitating the differentiation of these WT1-expressing cells towards cardiomyocytes, fibroblasts, endothelial and smooth muscles cells could provide a great tool to improve cardiac regeneration after injury. In addition, the indicated role of WT1 in the endothelial cells during development and injury has positioned the WT1-expressing endothelial cells as a potential target for improving angiogenesis in the diseased area.

A recent study indicates a role for the epicardium in autonomic modulation during early development. Within the initial stages of epicardial formation, WT1 in epicardial cells is coexpressed with the neuronal markers TUBB3 and NCAM (15). Interestingly, the expression of WT1 was also found in the ventral region of the neural tube, as well as the roof of the 4th ventricle of the brain, supporting the neuronal phenotype of the epicardium. Dysfunctioning of the cardiac autonomic nervous system plays a role in the pathogenesis of arrhythmias (110) and hypertension (111) and is involved in disease progression in heart failure (112). Understanding the mechanistic role of WT1 in the formation of the cANS

might help to unravel processes that govern normal cANS development and opens possibilities for treatment after cardiac injury.

WT1 is assoaciated with major generation processes during cardiac development like formation of epicardium, cardiac vasculature, valves, cANS, and also myocardial wall maturation, but also with major regeneration processes during cardiac repair like scar formation and angiogenesis. More knowledge on the upregulation of WT1 in cardiac cells and their subsequent response can contribute to the development and improvement of therapeutic strategies for cardiac repair, and thereby restoring a functional contractile cardiac wall.

Conflict of Interest

The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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Chapter 14

Functional Role of WT1 in Prostate Cancer

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Abstract

Although initial discoveries of Wilms tumor 1 (WT1) expression in extrarenal disease generated controversy, we and others have examined WT1 expression in non-Wilms cancers and have demonstrated that the WT1(A) isoform, lacking the lysine-threonine-serine tripeptide (KTS) insertion, transcriptionally regulates the expression of growth control genes in other cancer types. Here, we review our evidence that WT1 is expressed in prostate cancer (PC) epithelial cells and regulates PC critical genes. That WT1 may promote metastatic disease is consistent with previous findings that WT1 suppressed E-cadherin and enhanced motility of PC cells with low migratory and metastatic potential. Recent findings led us to ask

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whether WT1 acts as an angiogenic switch in PC. Although vascular endothelial growth factor (VEGF) is regulated at several levels and by a number of different factors, a mechanistic understanding of WT1-mediated transcriptional regulation in PC cells was previously lacking. Here, we discuss the evidence of WT1- and androgen receptor (AR)-binding sites in the VEGF promoter and show the potential for cooperation between hormone and WT1. These findings revealed that in AR-intact PC cells, WT1 was sufficient to upregulate VEGF transcription, and WT1 expression enhanced the hormone activation of VEGF expression. This notion that WT1 can activate an angiogenic switch in PC cells, to enhance tumor growth and progression to metastatic disease, is consistent with our understanding of the oncogenic nature of WT1 overexpression in inappropriate tissues or at inappropriate times. The potential for WT1 to promote both tumor angiogenesis and PC cell migration suggests that WT1 regulates genes that promote PC progression to lethal metastatic disease. Therapies targeting WT1 in PC may reduce metastatic spread and increase overall survival.

Key words: AR; E-cadherin; Prostate cancer; Transcription; VEGF; WT1

Introduction

The WT1 gene is a member of the early growth response gene I (EGR-1) family of transcription factors containing four Kruppel-like zinc fingers in the carboxyl terminus that bind nucleic acids (both DNA and RNA). The functions of the Wilms tumor 1 (WT1) protein are isoform-specific and reflect its structural domains (1). The four major isoforms of WT1 are formed by alternative splicing at two sites resulting in the inclusion or exclusion of (1) exon V and/or (2) a lysine-threonine-serine tripeptide (KTS) in exon 9 that alters the relative orientation of the 3rd and 4th zinc fingers and affects the DNA-binding structure. The isoform WT1(A), which lack both exon V and the KTS tripeptide, binds DNA and functions as a transcription factor, while isoform WT1(D) contains both elements and can function as a post-transcriptional regulator in certain contexts. Additional, less common isoforms initiate from internal or upstream start sites. Three of the four Cys_2-His_2 zinc fingers (2, 3)of the (-)KTS isoforms are involved in binding a common G-rich DNA consensus sequence, GNGNGGGNG, as well as the related Egr-1 recognition elements (4). The importance of the zinc finger domain for DNA binding is underscored by congenital syndromes associated with naturally occurring WT1 mutations, such as the Denys–Drash syndrome and Frasier syndrome, characterized by urogenital anomalies and elevated risk of Wilms tumor or gonadoblastoma, respectively (5, 6). While controversy exists over the ability of mutant forms of WT1 to bind DNA, it is possible that protein interaction sites remaining within the mutant WT1 protein could play a direct role in these anomalies (7). Indeed, controversy exists over the role of normal cytoplasmic WT1 protein, with some evidence supporting a shuttle function, as WT1 contains cytoplasmic and nuclear localization signals, as well as a nuclear export signal (8). The activity of

the cytoplasmic form may be related to phosphorylation status, as phospho-WT1 is thought to be retained in the cytoplasm (9, 10). Alternatively, as both +/- KTS isoforms have been identified in polysomes and bound to polyA RNPs (11), a post-transcriptional function has been suggested. Interestingly, one example of post-transcriptional regulation of vascular endothelial growth factor (VEGF) by WT1 involved transcriptional regulation of a splicing factor kinase that, in turn, altered VEGF splicing in podocytes (12). Recent evidence indicating the association of WT1 protein with histone and chromatin modifying enzymes also suggests an epigenetic function for WT1 [reviewed in reference (1)], mediated, in part, by WT1 recruitment of DNA methyltransferase DNMT1 and polycomb group protein enhancer of zeste homolog 2 (EZH2) (13) and CREB-binding protein (CBP), a histone acetyltransferase (14). Additionally, the evidence of epigenetic regulation of WT1 expression by lncRNA in acute leukemia (15) suggests that WT1 is intimately involved in both direct transcriptional and indirect epigenetic regulation. Thus, study of WT1 as a regulator of gene expression in key developmental processes, such as hematopoiesis, continues to be relevant.

Developmental expression of WT1

WT1 expression in the developmental processes was initially viewed as growth suppressive and necessary for cell differentiation, consistent with its earliest descriptions as a tumor suppressor gene (TSG). Within the developing kidney and genitourinary system, the timing of WT1 expression is exquisitely controlled, and once kidney development occurs, WT1 expression is tightly restricted to podocytes (16). WT1 is an essential regulator of nephrogenesis (17–19) and is expressed in both normal podocytes and in some Wilms tumors (18, 20). In addition to the kidney, WT1 is normally expressed in many other organs (6), including hematopoietic tissues such as the spleen, fetal liver, bone marrow, and lymph nodes, gonads, and peripheral nervous system (3, 21–25). However, its role is ambiguous depending on the organ involved and whether epithelial or mesenchymal differentiation occurs. For instance, in the normal development of the kidneys and the urogenital system, WT1 is needed to induce mesenchymal–epithelial transition (MET) leading to the formation of nephrons (26)and kidneys (16). In MET, the mesenchymal cells undergo multiple morphological changes associated with differentiation into epithelial cells and condensation into structures forming the organ. WT1 expression accompanies the opposite developmental role, epithelial to mesenchymal transition (EMT), in the developing heart where epithelial cells transform into motile mesenchymal cells that contribute to the organ's cellular structure and generate important signals (27). Furthermore, it has been demonstrated that WT1 is required for cardiovascular progenitor cell formation through the upregulation of Snail and downregulation of E-cadherin, two of the major molecules involved in EMT (28). Although WT1 has been proposed to regulate EMT by repressing E-cadherin; more recently, WT1 has been linked to the regulation of epicardial EMT through the β-catenin and retinoic acid signaling pathways (29). Interestingly, it has

been found that WT1 transcriptionally activates Snail with partial maintenance of E-cadherin, and WT1 is associated with epithelial characteristics in kidney cells and in clear cell renal cell carcinoma (31). Thus, in these examples, WT1 induces an epithelial–mesenchymal hybrid transition defined by Snail upregulation with E-cadherin maintenance, a tumor cell differentiation state in which cancer cells retain both mesenchymal and epithelial features that may contribute to tumor cell plasticity and tumor progression (30). Similarly in prostate cancer (PC), a partial EMT with features of both epithelial and mesenchymal cells has been observed (31). The transformation of metanephric mesenchyme to epithelial cells within the condensing glomeruli also is similar to the metastatic process of cancer cells, whereby motile cancer cells, after extravasation, must revert back to their epithelial state to survive at the metastatic site (32). Because WT1 is required for normal MET within the developing kidney, it seems plausible that it may also play a role in the metastatic MET process. Yet, little is known about the requirement for WT1 expression during the metastatic process.

WT1 expression in non-Wilms cancer

CD34+ hematopoietic progenitor cells express WT1, but like metanephric mesenchyme, once hematopoietic progenitors become lineage-committed then expression of WT1 is highly restricted within a small subpopulation of cells [reviewed in reference (33)]. Increased expression appears to persist in cancer cells, and WT1 expression in tumor tissue exceeds that of the normal cell counterparts. This dysregulated expression was regarded as an indicator of a potential growth-promoting effect of WT1 and led to the controversy over whether WT1 was truly a TSG as originally identified in Wilms tumor or whether WT1 was actually an oncogene-driving cancer cell proliferation and blocking differentiation, as observed in leukemia cell lines (25). As evidence accumulates on different tumor types that overexpress WT1 relative to their normal counterparts, it is clear that WT1 has a dichotomous role in cancer, and indeed, WT1 has been referred to as a chameleon [reviewed in references (33) and (25)]. Within the hematopoietic system, it is clear that WT1 can behave as a survival gene, enhancing cell viability, but also can induce quiescence, depending on the differentiation state of the leukemia cells involved [reviewed in reference (33)]. Many studies have shown elevated *WT1* expression in diverse cancer types, including leukemia (34–37), breast (38–40), Ewing sarcoma (41), ovarian (42), mesothelioma, and pulmonary adenocarcinomas (43). Additionally, *WT1* is being investigated as a potential prognostic marker for both leukemia and breast cancer (39, 44).

Expression and potential role of WT1 in PC

WT1 is expressed mainly during development, and it plays an important role in adrenogonadal development and sex determination [reviewed in reference (45)] via its regulation of SRY (46), so its expression in hormone-responsive tumors such as breast, ovarian, and

prostate was not unexpected. We and others initially identified WT1 mRNA in cultured PC cells (47–51)and then WT1 mRNA and protein in PC tissues (49). Because the prostate is a complex tissue and PC is a heterogeneous disease, we used laser capture microdissection (LCM) to isolate distinct cell-type populations from epithelial and stromal tissues in PC and identified *WT1* among the nearly 500 genes whose expression was significantly different between epithelial and stromal PC cells (49). Results of microarray analysis are posted at NCBI (Geo #GSE 20758). This differential expression of *WT1* in PC epithelial cells was validated by quantitative real-time polymerase chain reaction(PCR) and relevance confirmed by analysis of additional frozen tumor tissue biopsies and tissue microarray (TMA) sections (49). This cell-specific expression suggests a potential role for *WT1* in PC, likely involving the acquisition of characteristics necessary for metastatic growth of PC.

Metastatic disease is associated with a marked increase in the risk of mortality among PC patients. Ninety-nine percent of patients who develop primary PC are expected to live at least 5 years after diagnosis (52). Ninety-eight percent are alive after 10 years, and 94% live for at least 15 years if the disease remains localized. By contrast, patients with metastatic disease at diagnosis have a 5-year survival rate of only 28% (52). The process of metastasis requires that cancer cells acquire characteristics of enhanced motility and invasiveness. That WT1 may be involved in PC metastasis was suggested by immunohistochemical analysis of PC TMAs, demonstrating that WT1 protein was more often expressed in high Gleason grade PC epithelial cells than that in low grade, and it was not observed in non-neoplastic prostate tissue (49). Others have also suggested that WT1 could serve as a marker for PC progression (53). While Devilard et al. (53) demonstrated the expression of *WT1* by microarray analysis in a hormone-refractory LuCaP xenograft PC progression model, our results provide the most complete evidence of elevated *WT1* mRNA and protein in prostate tumors, and our study was the first to identify *WT1* expression in LCM human prostate epithelial tissue (49). We confirmed the relevance of the microarray analysis of LCM-captured tissue RNA by real-time PCR quantifying *WT1* expression in 20 additional sets of paired tumor and non-neoplastic tissues. *WT1* mRNA levels were elevated in 70% of invasive-stage T3 tumors examined when compared to the adjacent non-neoplastic tissue. Similarly, in three of four established PC cell lines, *WT1* expression was also significantly higher than the nontumorigenic, immortalized prostate epithelial cell line RWPE-1 (49). Further analysis of WT1 protein in formalin-fixed, paraffin-embedded TMAs identified WT1 expression in 65% of tumor samples (of Gleason grade 6–10) and, importantly, the absence of expression in non-neoplastic and benign prostatic hyperplasia (BPH) samples. WT1 expression in high-grade PC may indicate that WT1-responsive pathways promote the slow progression of latent PC to aggressive, hormone-refractory PC. Two possible mechanisms whereby WT1 expression in prostate could enhance metastatic tumor growth warrant discussion.

WT1 target genes relevant in PC

The transcriptionally active isoform of the Wilms tumor gene, WT1(A), regulates a large family of genes involved in growth control, sex determination, and genitourinary development [for reviews see references (6, 16, 54)]. We and others have demonstrated that WT1 regulates important PC pathways – both growth-promoting pathways, e.g., insulin-like growth factor axis (55, 56)and androgen signaling via androgen receptor (AR) (46, 50), and growth suppressing/apoptotic pathways via Bcl-2 (57–61). Recently, WT1 has been shown to control differentiation of epicardial cells by repressing E-cadherin expression, thereby inducing mesenchymal transformation (EMT) resulting in vascular endothelial cells, smooth muscle cells, and cardiomyocytes in the heart (28). WT1 could similarly facilitate the metastatic progression of PC cells by inducing EMT, which is marked by loss of epithelial markers such as E-cadherin and gain of mesenchymal markers such as N-cadherin. WT1 could also enhance metastatic tumor growth by inducing expression of the angiogenic regulatory gene, VEGF. Together, these gene regulatory functions could promote acquisition of the lethal metastatic phenotype of PC.

WT1 suppression of E-cadherin promotes cell motility

Initial studies in NIH-3T3 cells, in which it was demonstrated that E-cadherin is a WT1 target gene (62), and studies in cardiac epithelial cells have established the role of WT1 in E-cadherin regulation (28). E-cadherin is a transmembrane protein that mediates epithelial cell–cell interactions in the adherent junctions of the plasma membrane (63) through homophilic protein–protein interactions (64). Downregulation of E-cadherin results in increased invasiveness of distinct types of cancer, such as gastric (65, 66), breast (67), ovary (68, 69), endometrial (70), thyroid (71), hepatocellular carcinoma (72), oral (73), and pancreatic (74), and has been well documented in prostate adenocarcinoma (75–77). In PC, E-cadherin expression has been shown to be reduced by activation of AKT signaling (78), by high expression of transcription factors such as Snail (79, 80), Slug (81), Twist (82) and WT1 (48), and by hypermethylation of the E-cadherin promoter (83). The loss of this important cell adhesion molecule is a critical early event in invasion and metastasis that leads to the conversion from a stationary to a migratory cell phenotype (84). When cancer cells acquire motility and invasiveness, they exhibit marked morphological changes, lose epithelial features, and acquire a more mesenchymal phenotype (EMT) (85, 86). Interestingly, androgen exposure has been reported to increase levels of Snail, decrease levels of E-cadherin and β-catenin, and induce expression of the mesenchymal marker N-cadherin in PC cells (87). TGF-β also has been implicated in induction of EMT in PC through activation of SMAD3 (88) and promotion of PARP4 nuclear localization with the subsequent increase of Snail, vimentin and N-cadherin, and decrease of E-cadherin (89).

While initial experiments associated growth suppression and characteristics of epithelial differentiation, including upregulation of E-cadherin, with stable expression of WT1 in NIH 3T3 cells

(62), more recent studies in cardiac epithelial cells showed that WT1 transcriptionally repressed E-cadherin expression both directly and indirectly by the upregulation of Snail (28). Furthermore, it has been demonstrated that WT1 expression promotes metastasis and invasion in non-small-cell lung carcinoma patients through the suppression of E-cadherin (90). In the context of PC, we observed that WT1 expression was inversely related to E-cadherin expression in several PC cell lines, and, importantly, WT1 expression correlated with migratory potential (48). Mechanistic studies showed that WT1 could bind to the E-cadherin promoter *in vivo* and decrease the E-cadherin promoter activity through a novel-binding site located at -146 bp upstream from the transcription start site. Additionally, overexpression of WT1 in LNCaP cells decreased E-cadherin mRNA expression (2-fold, $p \leq 0.05$). Although LNCaP cells have low migratory potential as measured in migration chamber assays, forced expression of WT1 not only suppressed E-cadherin but also enhanced LNCaP cell migration 3-fold compared to control vector-transfected cells ($p \leq 0.001$). Moreover, silencing WT1 in PC3 cells, which exhibit higher WT1 expression and greater migratory potential, reduced their motility in migration chambers by 50% compared to scrambled control-transfected cells ($p \leq 0.01$). This strong inhibition of motility was confirmed in wound-healing assays showing a 4.4× reduction in the motility of siWT1 RNA-transfected PC3 cells compared to controls ($p \leq 0.001$) (48). Our study, the first to undertake a complete analysis of the effect of WT1 on E-cadherin expression and motility in PC cells, thus demonstrated that WT1 binding decreased activity of the E-cadherin promoter in the presence of WT1 and that repression of E-cadherin expression led to an increase in cell migration (Figure 1). Suppression of E-cadherin expression and enhancement of motility are both associated with EMT.

WT1 may contribute to tumor angiogenesis via regulation of VEGF

We have demonstrated that, in addition to enhancing PC migration by suppressing E-cadherin expression, WT1 also upregulates VEGF, thereby potentially promoting tumor angiogenesis and metastasis. VEGF is a mitogen secreted by tumor cells that is essential for tumor angiogenesis and is necessary for tumor growth beyond 1–3 mm^3 in volume (91). VEGF regulation is complex and occurs at both transcriptional and post-transcriptional levels (92, 93). While the VEGF promoter lacks a TATA-binding site, it contains a GC-rich core promoter region and additional distal enhancer sites including hypoxia response elements that bind hypoxia-inducible factor (HIF1)-alpha.

Coexpression of WT1 and VEGF

WT1 was previously shown to play a role in neovascularization in the proliferative response of coronary vasculature to regional ischemia (94). In vascular cells, WT1 expression was associated with an increase in proangiogenic molecules such as VEGF (95). Similarly, both VEGF and WT1 are elevated in some PC cells (96), consistent with its ability to regulate growth control pathways important in PC (46, 50, 55–61). Additionally, WT1

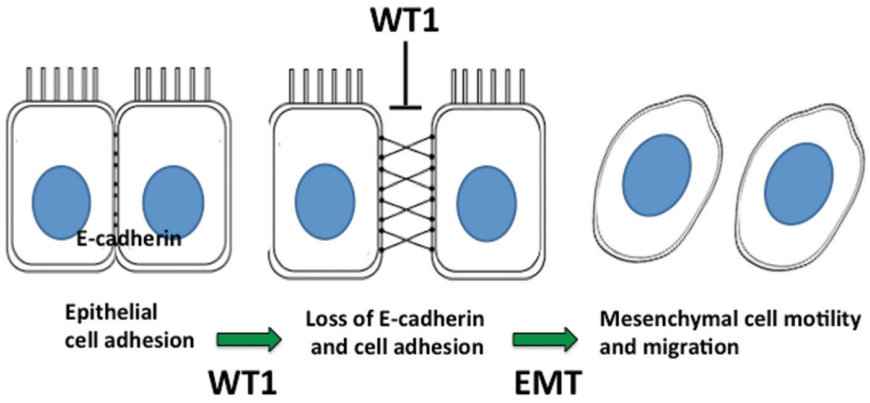

Figure 1. Proposed mechanism for WT1 regulation of motility of prostate cancer cells. WT1 transcriptionally represses E-cadherin, which would lead to loss of cell adhesion and promote prostate cancer cell migration, potentially enhancing the epithelial to mesenchymal transition (EMT) of PC cells expressing WT1.

and VEGF are coexpressed in both normal podocytes and some Wilms tumors (18, 20, 97). These findings of coordinate expression led to suggestions that WT1 plays an important regulatory role in developmental and tumor angiogenesis (20, 51, 98). For all these reasons, it seemed likely that VEGF was a physiologically relevant target of WT1 regulation in the prostate. In Ewing sarcoma cell lines, knockdown of WT1 expression using WT1-specific shRNA downregulated VEGF mRNA expression and decreased angiogenic activity (99). Conversely, overexpression of WT1 upregulated VEGF mRNA and increased angiogenic activity (99). Additionally, WT1 bound to the promoter of VEGF and increased promoter activity in response to hypoxia in Ewing sarcoma cells (100). Together, these results demonstrated that WT1 could directly regulate VEGF expression in Ewing sarcoma cells.

Regulation of VEGF by WT1 in prostate cancer

We assessed the WT1-mediated regulation of VEGF in PC cells. WT1-binding sites predicted by *in silico* analysis of the VEGF proximal promoter (101, 102) were demonstrated functional by reporter assays and protein binding *in vitro* and *in vivo* using electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assays, respectively. The latter result indicated the ability of WT1 protein to bind to native chromatin in LNCaP PC cells (101) and is consistent with results of luciferase reporter assays, showing that WT1 upregulates the VEGF promoter (102) in LNCaP cells. One of the functional binding sites identified initially as an Egr1-binding site was verified to bind WT1 by ChIP analysis in LNCaP cells (101). Site-directed mutagenesis of the proximal VEGF promoter construct V411 (Figure 2A)

was used to determine if this site was necessary for WT1-mediated transcriptional activation of the VEGF promoter. Cotransfection of a green fluorescent protein (GFP)-tagged WT1 expression construct (103) and mutant reporter into LNCaP cells revealed that disruption of this site significantly decreased the ability of WT1 to upregulate the proximal VEGF promoter (Figure 2B). We then asked whether this same pattern of regulation was occurring in other hormone-responsive PC cell lines. Testing two other hormone-responsive PC cell lines, CWR22Rv1 and C4-2, we found that WT1 regulated the VEGF proximal promoter (Figure 2C and 2D) similarly in all three cell lines. Thus, the data showed that WT1 bound and activated the VEGF proximal promoter in several PC cell lines.

The enhanced expression of VEGF mRNA in WT1-transfected LNCaP cells confirmed the *in vitro* promoter activation studies. Although overexpression of WT1 increased VEGF mRNA levels, the converse was not true (data not shown). Knockdown of WT1 expression in LNCaP cells using siRNA did not significantly affect VEGF mRNA levels. Together, these results indicate that WT1 is sufficient to upregulate VEGF expression, but not necessary, suggesting that other transcription factors (possibly SP1) play a role in the androgen activation of VEGF (104). Additionally, WT1-mediated regulation of VEGF appears to be cell specific as transfection of hormone-insensitive PC3 cells did not enhance VEGF promoter activity, and WT1 appears to repress the VEGF promoter in embryonic kidney HEK293 cells (102).

Combined androgen and WT1 activation of VEGF expression in hormone-responsive PC

Although hormone responsive (105–108), the VEGF promoter lacks canonical AR or estrogen receptor (ER)-binding sites. VEGF regulation by estrogen in endometrial and breast cancer cells involves interactions of ER-α and Sp1 (or Sp3) with GC boxes in the core promoter region of VEGF (–66 to –47 bases from start site) (108, 109). VEGF mRNA levels were significantly induced in ZR-75 breast cancer cells treated with estradiol, and the intact GC-rich core VEGF promoter region (–66 to –47) was required for such activation. The relevance of Sp1 and Sp3 in estradiol regulation of VEGF in breast cancer was suggested by binding assays *in vitro* (by EMSA) and *in vivo* (by ChIP). Similarly, multiple groups have shown that androgen treatment of human fetal fibroblasts and LNCaP cells significantly increases VEGF mRNA expression levels (110–112, 102). Additionally, VEGF protein levels have been demonstrated to be upregulated after the treatment of LNCaP cells with hormone (106), and the androgen antagonist flutamide blocked this upregulation (113). The mechanism of androgen-mediated regulation of VEGF expression, however, is less well understood.

In examining the mechanism of androgen-mediated regulation of VEGF expression, we identified AR/GC sites within the VEGF GC-rich core. Based on our earlier *in silico* analyses of the VEGF promoter (101) and the discovery that site-directed mutation of three AR half-sites did not eliminate hormone activation of the VEGF promoter (104), we hypothesized that

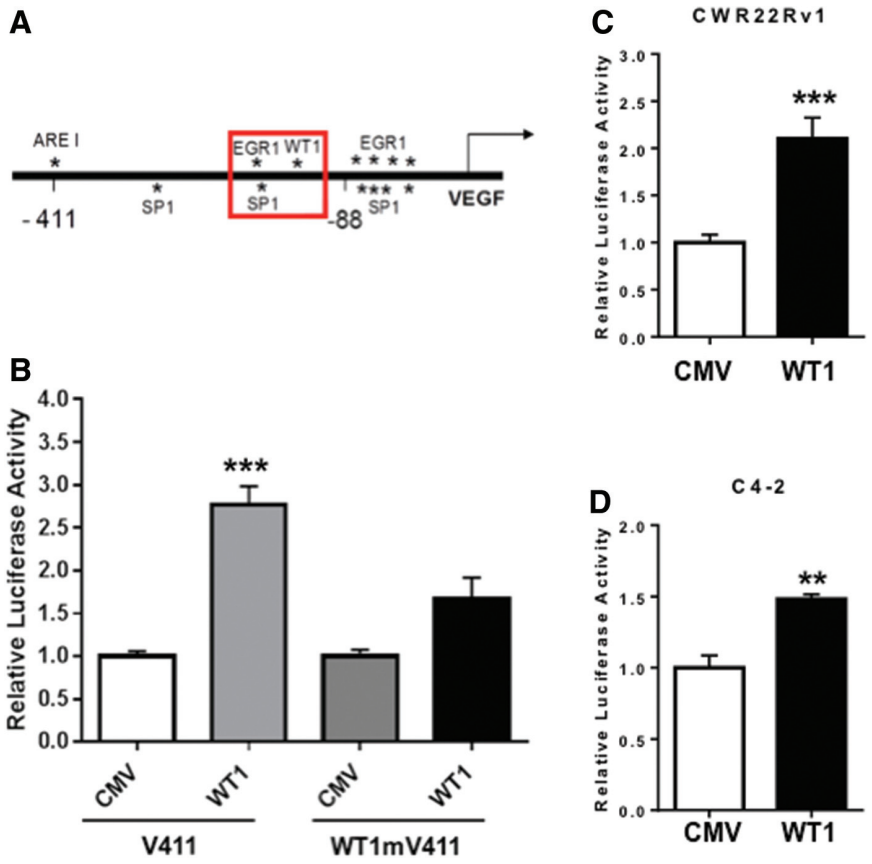

Figure 2. WT1 activates the VEGF promoter in prostate cancer cells via a WT1-binding site within the proximal VEGF promoter. (A) Site-directed mutagenesis of a predicted WT1-binding site (red box) was performed on the VEGF proximal promoter construct V411. (B) LNCaP cells were cotransfected with GFP-WT1 or the empty CMV expression construct, pcDNA3.1, along with 250 ng of either the wild-type V411(left) or the mutant (right) reporter constructs. (C) Cotransfection ofCWR22Rv1 and (D) C42 prostate cancer cells with V411 and CMV or WT1 expression construct as described above. Luciferase activity was normalized to protein concentration, and values represent average normalized luciferase activity (+SEM) relative to empty vector control. Experiments were repeated three times in triplicate, and statistical significance was determined by Student's t-test (**p< 0.01, ***p< 0.001).

WT1 might regulate the hormone-responsive VEGF promoter. Thus, we asked whether AR might bind at other sites via interaction with other zinc finger transcription factors (ZFTFs), such as SP1, EGR-1, or WT1. We hypothesized that if AR–ZFTF interactions were important mediators of androgen response, then cognate-binding sites should be located within the

promoter regions of hormone-responsive genes expressed in PC (101, 114). As expected, nonclassical AR half-sites were identified adjacent to WT1/EGR1/Sp1 sites in 8 of 11 promoters analyzed including VEGF (114). Binding at one of the three predicted nonclassical androgen receptor element half-sites (ARE-I) in the VEGF promoter region was tested by ChIP analysis of hormone-treated, WT1-transfected LNCaP cells (114). Endogenous AR and Sp1 proteins, along with exogenous WT1, were immunoprecipitated from native chromatin of these hormone-treated cells, indicating that the predicted WT1, Sp1, and AR sites in the VEGF proximal promoter region were functional and suggesting that the three factors may bind individually or as a complex. Based on these *in silico* predictions, we proposed three alternative models for AR-mediated regulation of VEGF promoter activity. The models differ primarily in the manner that AR binds the VEGF promoter (Figure 3). The first model proposes that AR binds to AREs as a dimer (Figure 3, model i), in the classical way that AR binds to many androgen-responsive genes, such as prostate-specific antigen (PSA). However, there have also been reports that noncanonical monomeric ARE half-sites are important (115–117). Thus, the second proposed model (Figure 3, model ii) shows AR monomers binding to an ARE half-site and bridged to WT1 (or other ZFTF, such as Sp1 or Egr1) binding sites by cofactors (marked as?), such as CBP or SRC-1; alternatively, AR dimers may bind to half-site ARE and bridge to WT1-binding site. Because AR is known to interact with Sp1, Egr1, and potentially WT1, the third and final model (Figure 3, model iii) proposes that AR is not bound to an ARE-binding site but is tethered via a ZFTF, which is bound to the G-rich VEGF promoter at either Egr1-/WT1-binding sites or GC boxes (SP1-/Sp3-binding sites).

To test the model for WT1 AR interaction, we examined the WT1 site within 200 bp of the ARE site to determine whether WT1 would modulate the hormone response of the proximal VEGF promoter. Cells were serum-starved to deplete androgens, cotransfected with the VEGF proximal promoter and either WT1 or empty vector control, then treated with 5 nM R1881, an androgen analog, or vehicle control dimethyl sulfoxide (DMSO) (Figure 4A). Luciferase assays confirmed that either hormone or WT1 alone increased VEGF transcription 3- to 4-fold compared to cytomegalovirus (CMV) empty vector, vehicle control. However, the combination of WT1 and 5nM R1881 activated this reporter construct more than 12-fold (Figure 4A), suggesting that their interaction strongly enhanced hormone response. This strong upregulation suggested that WT1 and AR may form a complex in the nucleus and bind the G-rich and the AR half-site (similar to Figure 3, model ii). Nuclear lysates from WT1-transfected LNCaP cells grown in full serum (containing endogenous hormone) were co-immunoprecipitated with WT1 and AR antibodies. Immunoblot analysis revealed that complexes precipitated by antibodies specific for WT1 also contained AR protein (Figure 4B). Conversely, AR-immunoprecipitated complexes contained low levels of WT1 protein (data not shown). Together, these results indicate that WT1 may interact with AR to enhance androgen induction VEGF expression in PC cells.

Surprisingly, the GC-rich VEGF core promoter (–88 to +51), which lacks AR half-sites, but contains multiple EGR-1/WT1/Sp1 overlapping sites, also demonstrated a hormone response. Consequently, the third model we tested (Figure 3, model iii) proposes that AR is not bound to an ARE but is tethered via SP1, which is bound to GC boxes in the VEGF core promoter. Because estrogen regulation of the VEGF core promoter has been shown to require Sp1 sites in breast cancer cells (109), we asked whether androgen might regulate VEGF in a similar fashion in PC cells. Sp1-associated binding of AR to novel-binding sites in the VEGF promoter was demonstrated *in vivo* by ChIP analysis in LNCaP cells (101, 104, 114). AR and Sp1 formed a nuclear complex and were shown to bind to the VEGF core promoter in hormone-treated CWR22Rv1 PC cells (104). Suppression of Sp1 binding in the VEGF core promoter by mutation of a specific Sp1-binding site abrogated VEGF promoter activation by androgen. Additionally, treatment with mithramycin A, which blocks access of proteins to GC-rich DNA, significantly reduced Sp1 binding and VEGF expression. Together, these results indicated that another mechanism of androgen-mediated induction of VEGF expression in PC cells involved interaction of AR with a specific, critical Sp1-binding site in the VEGF core promoter region (104) similar to that described here for WT1 interaction at the proximal promoter region. Overall hormone activation of the VEGF promoter region is enhanced by interaction of AR with transcription factor-binding partners in PC cells.

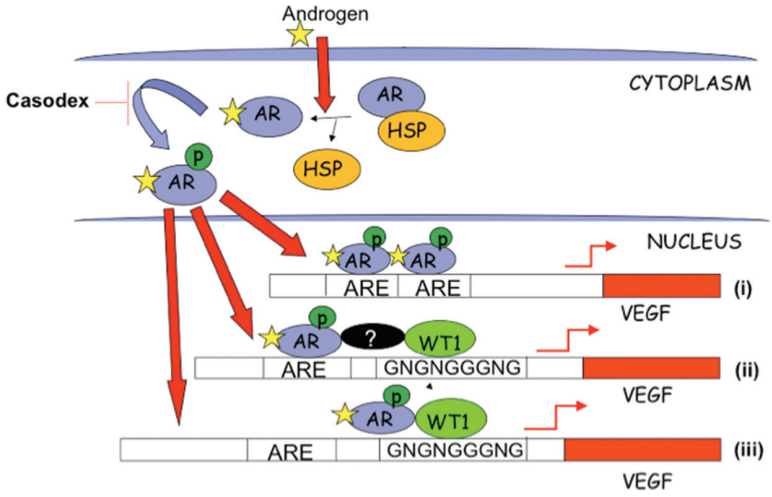

Figure 3. Proposed models of androgen regulation of VEGF in prostate cancer. Three potential ways that androgen is proposed to bind AR and regulate VEGF in prostate cancer: (i) AR binding to androgen response elements (AREs) as a dimer, (ii) monomeric AR binding to half-site ARE and bridged by unknown factor (?) to WT1 at its binding site, or (iii) AR tethering to WT1 at WT1-binding site, but not bound to ARE.

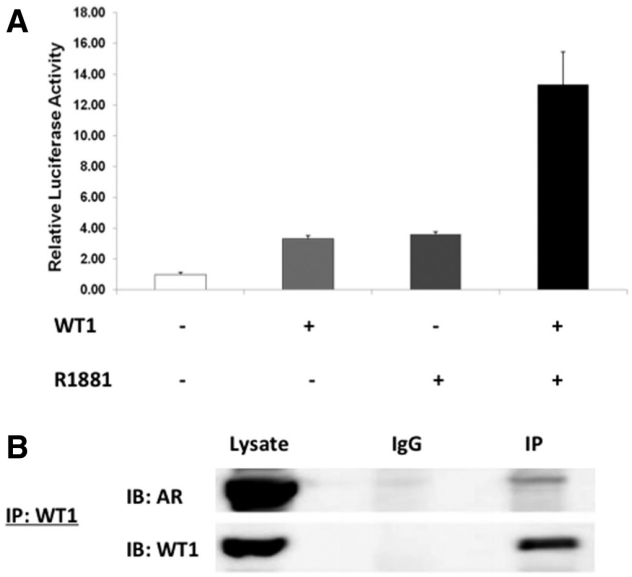

Figure 4. (A) WT1 and AR interact to activate the VEGF proximal promoter. (A) Serum-starved LNCaP cells were cotransfected as described above with V411 reporter and either GFP-WT1 or pcDNA3.1 vector control DNA. Transfected cells were treated with 0 or 5 nM R1881 in media containing 10% charcoal-stripped FCS. Values shown represent mean normalized luciferase activity (and SEM) relative to pCDNA3.1 empty vector control in the absence of hormone treatment (white). Each experiment was performed in triplicate and repeated twice. (B) The interaction of AR and WT1 in transfected LNCaP cells was demonstrated by coimmunoprecipitation of nuclear protein lysates by either WT1 Ab (Epitomics) or serum IgG. Immunoprecipitated (IP) proteins were electrophoresed and immunoblotted (IB) with either AR (top) or WT1 (bottom) antibodies. Left lanes show nuclear lysates, middle and right lanes show proteins immunoprecipitated by IgG or WT1 Ab, respectively.

Conclusion

Here, we review evidence that WT1 is expressed in PC epithelial cells and transcriptionally regulates PC critical genes. The relevance of WT1 to PC has been shown by finding that WT1 mRNA and protein are more often expressed in high-grade, invasive PC than low-grade localized tumors and that WT1 is not expressed in BPH or non-neoplastic prostate tissue (49). The identification of potential WT1-binding sites in the regulatory sequences of cancer-critical genes expressed in PC epithelial cells, together with the demonstration of WT1 protein bound to these gene promoters in native chromatin of transfected LNCaP cells, supported the notion that elevated WT1 expression in prostate epithelial cells affects transcriptional modulation of homeostatic genes important for PC (101). That WT1 may

promote metastatic disease is consistent with previous findings that WT1 suppressed E-cadherin, thereby increasing motility and metastatic potential of PC cells (48). The fact that WT1 transcriptionally upregulated VEGF expression and enhanced hormone induction of VEGF (102) suggested that WT1 could activate an angiogenic switch in PC cells. Taken together, the potential for WT1 to promote tumor angiogenesis and PC cell migration would suggest that WT1 regulates genes that enhance tumor growth and promote progression to lethal metastatic disease. These functions of WT1 are consistent with an oncogenic, not a tumor-suppressive, role and suggest that WT1 expression might serve as a marker for PC progression (53). Furthermore, therapies targeting WT1 in PC may block metastatic spread and increase the overall survival.

Conflict of Interest

The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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Chapter 15

Functional Roles of Wilms' Tumor 1 (WT1) in Malignant Brain Tumors

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Abstract

The pleiotropic transcription factor, Wilms' tumor 1 (WT1), is expressed in the majority of glioblastomas, the most common malignant brain tumors of adulthood. Despite intensive treatment, including surgery and chemoradiotherapy, the prognosis for patients with glioblastoma remains very poor. Encouragingly, immunotherapy targeting WT1 has proven to be effective in recurrent glioblastoma, suggesting that

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this approach may be an important new treatment modality for the disease. However, WT1 appears to function as a context-dependent tumor suppressor or oncogene, and the functional roles of WT1 in the pathogenesis of glioblastoma, and other types of brain tumors, have not been extensively studied. With this in mind, we briefly review WT1 expression data for a range of different brain tumor classes and address the role of WT1 in the regulation of proliferation and apoptosis in glioblastoma. We generated WT1 knockdown glioblastoma cells by using shRNA-expressing lentivirus. Proliferation was reduced and apoptosis increased in WT1 knockdown glioblastoma cells compared with control cells *in vitro*. Consistent with these data, when WT1 knockdown glioblastoma cells or control glioblastoma cells were intracranially injected into the immunodeficient mice, tumor growth was significantly reduced in WT1 knockdown cells compared with that in control cells. Thus, WT1 is an oncogene that regulates cell proliferation and apoptosis in glioblastoma.

Key words: Ependymoma; Glioblastoma; Medulloblastoma; Meningioma; Oligodendroglioma

Introduction

Wilms' tumor 1 (*WT1*) is a pleiotropic transcription factor expressed in various types of hematological malignancies and solid tumors (1–9). *WT1* was first defined as a tumor suppressor gene (10–15). However, accumulating evidence suggests that the *WT1* can act as an oncogene in some contexts. For example, the growth of a range of different WT1-expressing cancer cell types is inhibited by *WT1* antisense oligomer (16, 17) and *WT1*-specific shRNA (18). Furthermore, overexpression of WT1 promotes cell growth (19–21), migration, and invasion (22). Overexpression of WT1 also inhibits apoptosis (23) and induces tumorigenicity in leukemia (24). However, the functional roles of WT1 in the pathogenesis of malignant brain tumors have not been extensively studied.

Glioblastoma is one of the most common malignant brain tumors. Despite intensive treatment, including surgery, radiation, and chemotherapy, the prognosis is still very poor, and the median survival is only 12–15 months (25). Improved treatments are urgently required to improve the prognosis of glioblastoma patients, and various therapies have been tested or are in development. In this regard, WT1 peptide vaccine immunotherapy has proven to be effective in recurrent glioblastoma (26), suggesting that WT1 is a valid therapeutic target in glioblastoma.

We briefly review the available WT1 expression data for a range of different brain tumor classes and address the involvement of WT1 in the regulation of proliferation and apoptosis in glioblastoma cells. In addition, we also investigated whether WT1 is involved in glioblastoma tumorigenicity in an intracranial xenograft model.

WT1 expression in malignant brain tumors

Glioma

Glioma is one of the most common types of malignant brain tumor. According to the WHO classification of central nervous system tumors, glioma is divided into four different grades depending on the malignant potential. Immunohistochemical analyses demonstrated that WT1 is expressed in many gliomas (5, 26–29) (Figure 1) and expression is variable depending on the tumor grade. In less-aggressive grade II glioma and diffuse astrocytoma, WT1 expression was lower than that in grade IV glioblastoma. Furthermore, Rauscher et al. (29) reported that WT1 expression is a prognostic marker of WHO grade II and III tumors, and WT1 expression is reduced in recurrent tumors.

Oligodendroglioma

Oligodendroglioma is a type of glioma that is thought to originate from brain oligodendrocytes. It occurs primarily in adults (9.4% of all primary brain and central nervous system

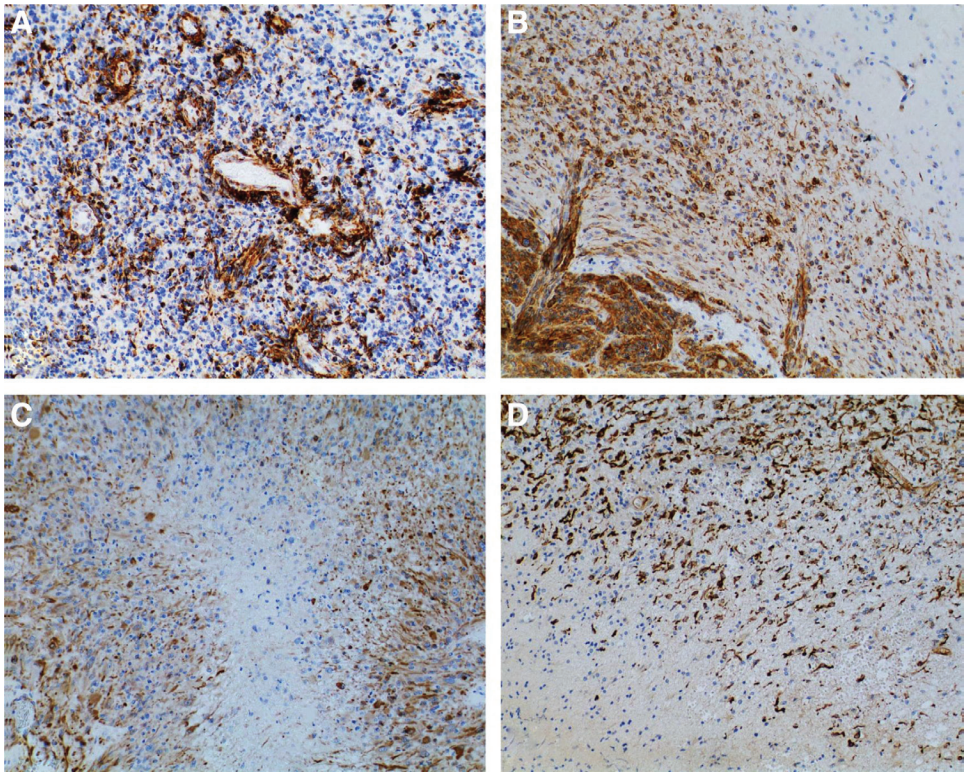

Figure 1. (A)–(D) Immunohistochemical analysis of WT1 expression in glioblastomas. Many glioblastomas express WT1 by immunohistochemistry.

tumors) but also affect children (4% of all primary brain tumors). WT1 is also expressed in oligodendrogliomas, and its expression is elevated in higher grade tumors (29).

Medulloblastoma

Medulloblastoma is the most common type of malignant pediatric brain tumor. A previous report showed that WT1 transcripts were detectable in five of nine primary medulloblastoma tumors (30), but in a separate study, WT1 expression was not detectable by immunohistochemistry (27). It is unclear whether this discrepancy is related to the heterogeneous WT1 expression levels in the four distinct molecular subtypes of medulloblastoma that have been described (31–33). Additional detailed studies are required to address the levels and the significance of WT1 expression in the pathogenesis of the various medulloblastoma subtypes.

Ependymoma

Ependymoma is a neuroepithelial malignancy of the central nervous system, which occurs in both children and adults. Although Idowu et al. (34) and Yeung et al. (35) reported that WT1 is expressed in ependymoma by immunohistochemistry, the significance of WT1 expression in ependymoma pathogenesis remains to be determined.

Meningioma

Meningioma is a common and predominantly benign intracranial tumor, which is classified as grade I according to the WHO classification of central nervous system tumors. Less than 10 percent of meningioma cases are classified as malignant WHO grade II or III tumors. The expression of WT1 in meningioma is controversial. Singh et al. (36) reported that WT1 is not expressed in WHO grade I meningiomas by immunohistochemistry, while Iwami et al. (37) showed that at the mRNA level, WT1 is expressed in many meningioma samples irrespective of WHO grade. Furthermore, Iwami et al. (37) reported that WT1 could be a therapeutic target for skull base meningioma.

In summary, WT1 is expressed in many different classes of intracranial tumors, including gliomas, oligodendrogliomas, ependymomas, and meningiomas. However, in most cases, the significance of WT1 expression in the pathogenesis of brain tumor remains unclear. In part, this is related to the fact that WT1 expression will need to be assessed in many more representatives of the various histological and/or molecular brain tumor subtypes to generate the statistically robust conclusions. At present, there are more than 100 histological subtypes of brain tumors according to the WHO classification, many of which are rare, and of which only a specific subset predominates in children, and WT1 expression data are limited or not available. In addition, consensus will be required to determine the WT1 detection method that is most appropriate for the comparison of data across laboratories.

The available data suggest that the expression of WT1 in some major brain tumor classes, most notably glioma, is likely to play an important role in tumor initiation and progression.

Based on this, the impetus is provided to assess WT1 expression in all brain tumor types to determine the validity of WT1 as a therapeutic target across the brain tumor spectrum.

Functional roles of WT1 in glioblastoma pathogenesis

Most studies investigating the functional roles of WT1 in the pathogenesis of malignant brain tumors have focused on glioblastoma. In other types of brain tumors, the functional roles of WT1 have not been assessed. Previous reports suggest that WT1 is involved in driving cell proliferation (38) and inhibiting apoptosis (18, 38) in glioblastoma. In an earlier study conducted in our laboratory (38), we transduced two glioblastoma cell lines, U87MG and U251, with lentivirus carrying WT1 shRNA to address the effect of WT1 knockdown on cell proliferation. We found that cell proliferation was significantly reduced (Figure 2A and 2B), suggesting that WT1 is involved in proliferation of glioblastoma cells. We also examined the effect of WT1 on glioblastoma progression *in vivo* by transducing U87MG cells with WT1 shRNA or control shRNA followed by intracranial injection into the immunodeficient Rag2$^{-/-}$ gamma c$^{-/-}$ mice. There was a significant difference in survival between the mice injected with U87MG cells transduced with WT1 shRNA and those injected with control U87MG cells. We also found that all the mice inoculated with U87MG cells transduced with control shRNA died of glioblastoma within 40 days, whereas none of the mice injected with WT1-shRNA-treated U87MG cells died of glioblastoma by the same time point (Figure 2C). These data demonstrated that *WT1* knockdown significantly inhibited glioblastoma growth *in vivo*.

Immunohistochemical analysis of formalin-fixed, paraffin-embedded tumor sections from mice inoculated with U87MG cells transduced with WT1 shRNA or control shRNA revealed that the Ki67 proliferation index was higher in control tumors compared with those transduced with WT1 shRNA (Figure 2D). Consistent with the *in vitro* data, these findings also suggest that WT1 drives cell proliferation and tumor formation *in vivo.*

We also investigated the differences in mRNA expression of selected apoptosis-related genes in U87MG and U251 cells transduced with WT1 shRNA or control shRNA by real-time PCR. We found that apoptosis-related genes such as *MAP3K5*, *PIK3CA*, and *p53* were upregulated in both U87MG and U251 WT1 knockdown cells compared with those in control cells. Extending these findings, we examined the differences in apoptosis between glioblastoma cells transduced with WT1 shRNA and control shRNA using an Annexin-V-Fluos kit. We found that the number of apoptotic cells was higher in U87MG and U251 WT1 knockdown cells compared with that in U87MG cells transduced with control shRNA (Figure 3A and 3B). These results showed that apoptosis was promoted in both U87MG and U251 WT1 knockdown cells, suggesting that WT1 drives glioblastoma tumorigenicity by regulating apoptosis.

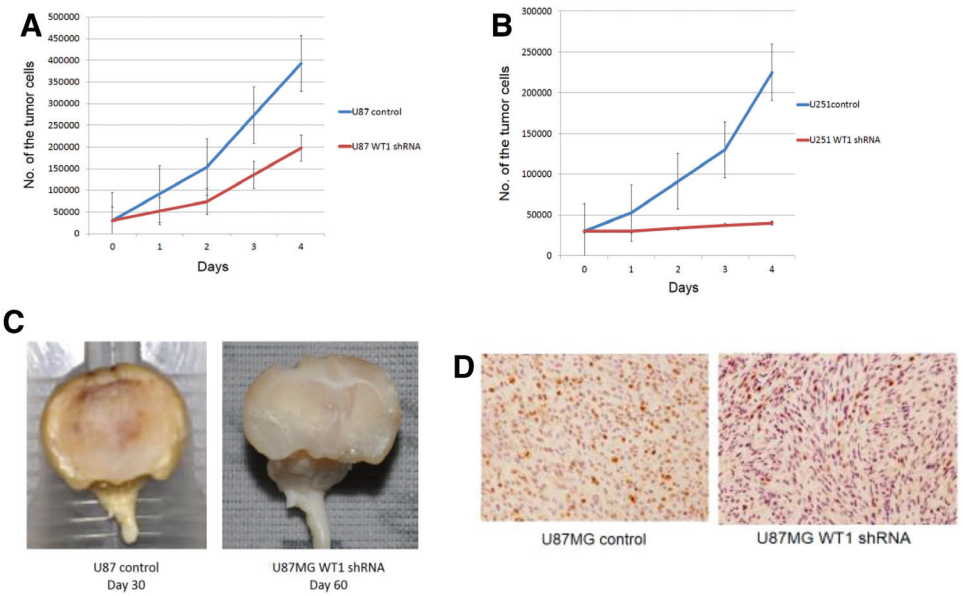

Figure 2. WT1 is involved in glioblastoma tumorigenicity *in vivo*. (A) Cell proliferation rate of U87MG cells transduced with control shRNA and WT1 shRNA. (B) Cell proliferation rate of U251 cells transduced with control shRNA and WT1 shRNA. (C) All the mice injected with U87MG cells transduced with control shRNA died of glioblastoma within 40 days after tumor cell xenograft, whereas none of the mice injected with U87MG cells transduced with WT1 shRNA succumbed by 40 days' post-transplant. (D) Immunohistochemical staining for the Ki67 proliferation marker in tumor samples from mice injected with U87MG cells transduced with WT1 shRNA or control shRNA.

In addition to our report, roles of WT1 in glioblastoma pathogenesis have been described in several studies (5, 39–41). Oji et al. (5) reported that the growth of U87MG cells was inhibited by a WT1 antisense oligomer consistent with WT1 being involved in the regulation of cell proliferation in glioblastoma. Tatsumi et al. (18) found that WT1 inhibited apoptosis in the A172 glioblastoma cell line. Clark et al. (39) reported that WT1 regulated cell proliferation in U251 cells and that U251 WT1 knockdown cells showed significantly lower tumorigenicity in a subcutaneous nude mouse model. However, contrasting data were presented in several other studies using different glioblastoma cell lines. Chidambaram et al. (40) reported that silencing of WT1 reduced the invasiveness of U1242 glioblastoma cells but had no effect on the U1242 cell proliferation *in vitro.* Clark et al. (39) found that transduction of T98G cells with WT1 shRNA had no effect on apoptosis compared with those transduced with control shRNA *in vitro.* These results suggest that the regulation of cell proliferation and apoptosis

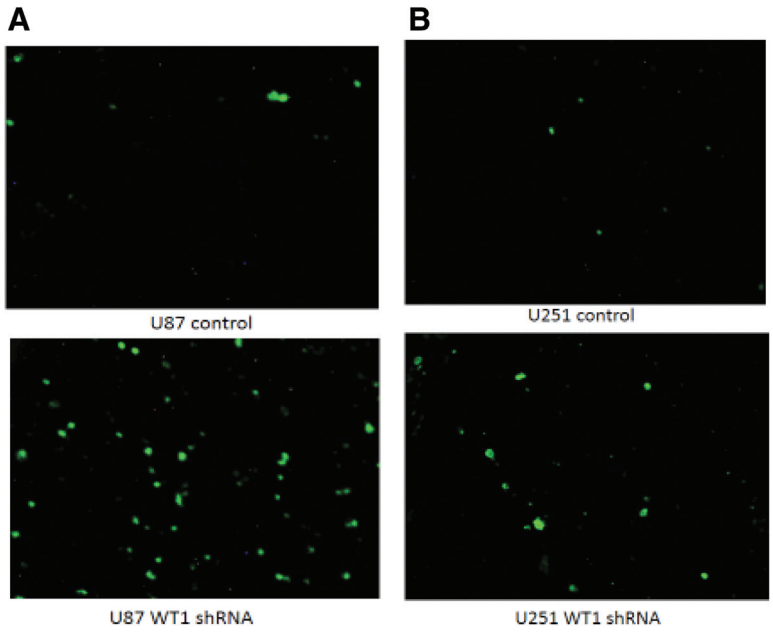

Figure 3. WT1 regulates apoptosis in glioblastoma cells. (A) The number of apoptotic cells per field and representative pictures of Annexin V-positive U87MG cells following transduction with control shRNA or WT1 shRNA. (B) The number of apoptotic cells per field and representative pictures of Annexin V-positive U251 cells following transduction with control shRNA or WT1 shRNA.

by WT1 *in vitro* is dependent on the specific glioblastoma cell line being studied, potentially reflecting subtype-specific molecular characteristics.

In summary, the functional roles of WT1 in glioblastoma pathogenesis remain controversial although the weight of evidence is consistent, with WT1 being involved in regulating cell proliferation and apoptosis in at least some glioblastoma. Clearly, more work is required to comprehensively address the functional roles of WT1 in the initiation and progression of glioblastoma and the many other types of malignant brain tumor that have not yet been adequately studied.

Immunotherapy targeting WT1 peptide for malignant glioma

Cancer vaccination is one of the immunotherapeutic strategies that have been developed to target many solid tumors. Recently, a large number of tumor-associated antigens, including WT1, were identified and assessed as potential candidates as cancer vaccines. Tumor antigen epitopes associated with human leukocyte antigen (HLA) class I molecules were

recognized by cytotoxic T lymphocytes, providing a potential mechanism for direct tumor cell killing. Furthermore, recent studies reported that systemic immunotherapy can induce an antitumor response within the immunologically privileged brain. These findings suggest that the peptide-based cancer immunotherapy could be a potent therapeutic strategy for the treatment of malignant brain tumors.

Izumoto et al. (26) carried out a phase II clinical trial of WT1 peptide vaccine immunotherapy for recurrent glioblastomas and found that the approach was effective in this context. To improve the efficacy of the treatment, we have been assessing a possible combination of WT1 peptide vaccine immunotherapy with temozolomide, a standard chemotherapeutic agent for newly diagnosed malignant glioma patients (42). We found that the combination therapy was tolerable without serious side effects. We are now moving on to the phase II clinical trial of combination WT1 peptide vaccine immunotherapy and temozolomide chemotherapy for newly diagnosed glioblastoma. This represents one example of combined immunotherapy with chemotherapeutic or other immunotherapeutic modalities, such as antiangiogenic agents or checkpoint inhibitors. Although WT1 peptide vaccine immunotherapy for malignant brain tumors has promise, more extensive studies are needed to determine the clinical efficacy of this approach.

Conclusions

WT1 expression in malignant brain tumors varies depending on the tumor type. WT1 functions as an oncogene in at least some glioblastomas, in part, by regulating cell proliferation and apoptosis. Overall, these findings suggest that WT1 is a valid molecular target for the treatment of glioblastoma and potentially a range of other malignant brain tumors.

Conflict of Interest

The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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Not relevant.

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Chapter 16

Wilms' Tumor Gene (WT1) Expression and Minimal Residual Disease in Acute Myeloid Leukemia

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Abstract

The identification of minimal residual disease (MRD) has led to substantial improvements in early recognition of the recurrence of acute myeloid leukemia (AML). Flow cytometry (FC), real-time quantitative polymerase chain reaction (RQ-PCR) and fluorescence in situ hybridization are useful methods for the detection of MRD in AML patients although molecular monitoring of leukemia-specific rearranged (RUNX1-RUNX1T1 and CBFB-MYH11) or mutated genetic (NPM1, CEBPA) sequences represents the most sensitive methodology.

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Besides, more than 50% of all AML patients lack one of these specific sequences, so it is crucial to identify molecular targets applicable for the majority of patients. WT1 is overexpressed at the mRNA level in 80–90% of AML cases at diagnosis in both peripheral blood and bone marrow, and is detectable in a consistent low range in normal donors. These features have led to its adoption for MRD detection using RQ-PCR. A European LeukemiaNet Study found the magnitude of WT1 log reduction after induction chemotherapy to be an independent predictor of relapse. Other studies showed a poorer outcome in patients having WT1 levels above reference thresholds at specific time points. WT1 expression was compared with other modalities of MRD assessment, such as RQ-PCR of specific fusion genes and FC, but no differences in terms of predictive value emerged. Finally, some authors translated the use of WT1 in the clinic giving donor lymphocytes infusions to patients with increasing WT1-mRNA levels after allogeneic stem cell transplantation and obtaining an improvement of survival in this subset. Data collected on WT1 expression over the past years provided evidence for the use of this molecular marker to stratify high-risk AML patients. It can also be used as a marker for early interventional therapy, but further studies are needed to demonstrate it.

Key words: Acute myeloid leukemia; Allogeneic stem cell transplantation; Minimal residual disease; Multiparameter flow cytometry; WT1 expression

Introduction

WT1 is an important regulatory molecule involved in cell growth and development. The presence of zinc fingers in the C-terminal half of the protein confers WT1 the role of a potent transcriptional factor, including important genes for cellular growth and metabolism among the targets (1). It has been found that WT1 can either enhance or repress the expression of specific target genes, depending on the levels of WT1 expression, the isoforms, the location of the transcriptional start site, and the cell type in which the experiment was performed (2, 3). In human hematopoietic cells, WT1 appears to behave as a tumor suppressor gene as the overexpression of WT1 in early human bone marrow (BM) cells leads to growth arrest and reduced colony formation. Indeed, in normal human BM, WT1 is expressed at extremely low levels and is confined to the primitive CD34+ population of cells (4, 5). Besides, WT1 is highly overexpressed in the BM or peripheral blood (PB) of a variety of leukemias, and these evidences support the role of WT1 as an oncogene in this subset (6, 7). Increased levels of WT1 expression can be found in both acute lymphoblastic and acute myeloid leukemia (AML) although more frequently in AML (frequencies varying from 73% to 91%) (8–10). Following the discovery of overexpression of WT1, there has been growing evidence that the WT1 expression levels may have a prognostic role in AML. In 139 de novo AML, Bergmann et al. (11) observed that the probability of the 3-year overall survival (OS) was 59%

in patients with low WT1 levels compared to 21% in patients with high levels. Similarly, Galimberti et al. (12) showed a higher probability of disease progression in AML patients presenting high WT1 levels, and recently, Nomdedeu et al. (13) also confirmed the prognostic role of high WT1 levels at diagnosis in a larger study population. However, these data are in contrast with results reported by others where WT1 levels did not correlate with the outcome (8–10, 14), thus suggesting a controversial role for WT1 expression at presentation. On the contrary, a greater agreement was found among groups that have used WT1 levels as a marker of minimal residual disease (MRD) in AML remission BMs (less than 5% of blast cells). In particular, WT1 expression has been shown to predict disease progression in AML patients treated with conventional chemotherapy (8–10, 15–17) and patients undergone allogeneic stem cell transplantation (allo-SCT) (18–22). Furthermore, when WT1 expression was compared with widely used techniques in monitoring MRD such as multiparameter flow cytometry (MFC) (23) or specific molecular targets such as fusion genes transcripts (PML-RARa, AML-ETO1, and CBFb-MYH11), comparable sensitivities were found in predicting the relapse in AML. Thus, we addressed our review on the main papers that focused on the predictive role of WT1 expression as an MRD marker in AML patients, as well as results from comparison between WT1 and other methodologies in monitoring MRD.

WT1 as a minimal residual disease marker after conventional chemotherapy

Many studies have shown that the assessment of MRD may prove useful to better stratify high-risk patients and address treatment intensity in AML (Table 1). The most sensitive method for this strategy involves the detection of fusion genes derived from chromosome translocations, such as PML-RARa, AML-ETO1, and CBFb-MYH11 (24, 25), and more recently gene mutations such as NPM1 (26, 27). Besides, more than 50% of AML lack known genetic lesions or clonality markers suitable for MRD monitoring. Thus, alternative markers for MRD are highly sought, and WT1 gene has been suggested as a candidate. Nondisease-specific genes should be abnormally high expressed in malignant cells when compared with normal controls to be used as an MRD marker. Cilloni et al. (8) first showed that the number of WT1 copies in 71 AML BMs and 14PB was 27,669 (ranges: 1,081–121,086) and 10,244 × 10^4 (ranges: 758–86,140) copies of Abelson gene (ABL) mRNA, respectively. Conversely, WT1 levels were extremely low in normal samples: median number of WT1 copies was 78 (range: 3–180) and 4 (1–22) × 10^4 ABL in BM and PB samples, respectively. Second, in order to assess the significance of the WT1 expression for the detection of MRD, the authors monitored WT1 levels in 10 AML patients characterized by the presence of fusion gene transcripts (CBFb-MYH11 and AML1-ETO);a good parallelism between sequential WT1 and fusion transcripts values was found: some patients who remained in complete remission (CR) (28) constantly showed WT1 values within the normal range, while patients who experienced a relapse showed a conversion to WT1 levels above the normal range in concomitance with fusion

Table 1. WT1 expression after conventional chemotherapy

Authors	MRD (cutoff)	LOG reduction	Time of assessment	Main results
Weisser et al. (16)*	0.4%	</≥2 Log	16–60 vs 61–120 vs 121–180 days after start of therapy	Within 61–120 and 121–180 days, levels ≤0.4%, and ≥2 log reduction were associated with improved EFS and OS
Cilloni et al. (9)*	250 × 10^4 ABL copies	</≥ 2 Log	Postinduction/ postconsolidation	WT1 transcript reduction ≥2 log after induction, and WT1 levels more than 250 × 10^4 ABL copies after consolidation predicted a significantly increased risk of relapse
Nomdedeu et al. (13)*	170 and 100× 10^4 ABL copies	-	Postinduction/ postintensification	WT1 levels greater than 170 copies after induction and 100 copies after intensification identified patients with the highest probability to relapse and die
Lapillone et al. (29)†	50 × 10^4 ABL copies	-	Postinduction	WT1 > 50× 10^4 ABL copies after induction is an independent prognostic risk factor of relapse and death
Rossi et al. (38)*	77 × 10^4 ABL copies	</≥ 1.96Log	Postinduction/ postconsolidation	Only postinduction MRD ≥ 77× 10^4 ABL copies and log reduction ≤1.96 predicted a shorter DFS and OS

*Study performed on adults.

†Study performed on children.

transcript increasing although patients were still in CR. Indeed, the quantitative assessment of WT1 transcript allows to distinguish between normal and pathological samples, as well as increasing WT1 levels above the normal range can be prognostically significant during the follow-up of patients. Weisser et al. (16), some years later, confirmed a significant correlation between WT1 levels and fusion genes (96%, median r = 0.996) in a similar study population. The authors also showed that more than 2 log reduction of WT1 levels within 61 and 180 days from the start of chemotherapy was associated with a significantly improved OS and event-free survival (EFS). Comparable results were published by the European LeukemiaNet (ELN) study group (9). In order to standardize the WT1 assay, Cilloni et al. (9)

first undertook a systematic evaluation of nine published and "in-house" real-time quantitative polymerase chain reaction (PCR) assays in a quality control study involving 11 ELN laboratories. Then, the selected ELN WT1 assay was applied to samples from 129 follow-up patients, and a significantly increased risk of relapse was found in patients achieving less than 2 log reduction in WT1 transcripts after induction therapy ($p = 0.004$). This study suggests that application of a standardized WT1 assay early during the patients' therapy could potentially be used to refine risk stratification in AML and decisions on the role of allogeneic transplant in first morphological CR. Recently, in a large study population of AML (n = 584), Nomdedeu et al. (13) defined three different prognostic groups after induction and intensification on the basis of WT1 levels. Patients having more than 170 copies after induction and more than 100 copies after intensification showed the highest probability to relapse and the lowest to OS. On pediatric AML also, similar results were obtained when WT1 was investigated in AML. Lapillone et al. (29) observed that WT1 higher than 50×10^4 ABL copies after induction was an independent prognostic risk factor of relapse ($p = 0.002$) and death ($p = 0.02$) in pediatric AML. Published results conferred to WT1 an important role in monitoring MRD and stratifying patients with AML, similarly to results obtained by MFC (30–37). When the techniques were compared, a different role was addressed to each one on the basis of the timing of assessment and quantification of MRD or log reduction. Generally, our group and others showed that detection of MRD by WT1 expression and MFC had comparable prognostic value and technical performance described in terms of sensitivity (sens), specificity (spec), predictive value (PV), and likelihood ratio (LR) (23). Besides, when we compared log reduction with MRD measured after conventional chemotherapy by both WT1 expression and MFC, important differences between the two methodologies were found (38). Log reduction and MRD well predicted the outcome at both timing of assessment according as both methodologies, but WT1 log reduction after induction (spec 84.2%, sens 46.2%, LR+ 2.92, LR- 0.64) identified the relapse better than the MRD (spec 57.7%, sens 84.2%, LR+ 1.99, LR- 0.27) and opposite results were true after consolidation for MFC (spec 80.8%, sens 57.9%, LR+ 3.01, LR- 0.52 vs spec 73.1%, sens 63.2%, LR+ 2.35, LR- 0.50 for MRD and log reduction, respectively), thus confirming what was previously published about either WT1 or MFC singularly.

WT1 as minimal residual disease marker in allogeneic stem cell transplantation

Allo-SCT represents the only effective therapy for high-risk patients with AML in first or subsequent CR. Nevertheless, relapse remains a crucial issue in this setting, and new methods able to prevent it are needed (39). Cytogenetics and response after induction therapy were uniformly recognized as predictors of relapse, but there is a growing evidence that quantification of MRD is also a powerful, independent predictor of prognosis (Table 2). Ogawa et al. (18) studied the impact of WT1 levels after allo-SCT on the relapse and the capability to prevent it

Table 2. WT1 expression in allogeneic stem cell transplantation

Authors	MRD (cutoff)	Time of assessment	Intervention MRD-based	Main results
Ogawa et al. (18)*	10^{-4}–10^{-2}	Post-transplant	Immune interventions (discontinuation of immunosuppressive therapy or DLI)	Probability to relapse within 40 days was significantly associated with WT1 expression levels. Among high-risk patients, a significantly longer doubling time of WT1 levels in patients who underwent preemptive measures
Zhao et al. (19)*	0.60%	Pre- and post-transplant (+120 days)	Immune interventions (DLI, tapering of immunosuppressive therapy) when WT1 levels were >0.60%	Greater than 0.60% after transplant has been shown as an independent risk factor for DFS and OS. High-risk patients who received immune interventions displayed a longer OS
Pozzi et al. (20)*	100×10^4 ABL copies	Pre- and post-transplant	DLI if MRD > 180×10^4 ABL copies	Post-transplant WT1 expression was the strongest predictor of relapse. Patients with increasing WT1 levels received DLI and showed an improved OS
Rossi et al. (43)*	138×10^4 ABL copies	Pre- and post-transplant (+30 days)	–	A shorter DFS was found in patients having high levels (≥138 copies) of WT1 at day +30 from transplant. The combination of MFC and WT1 may be preferred for preemptive immune interventions

*Study performed on adults.

by preemptive therapeutic measures in patients with leukemias [AML, acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML)]. First, the authors showed that the probability of relapse that occurred within 40 days significantly increased according to the increase in *WT1* expression levels (100% for 1.0×10^{-2} to 5.0×10^{-2}, 44.4% for 4.0×10^{-3} to 1.0×10^{-2}, 10.2% for 4.0×10^{-4} to 4.0×10^{-3}, and 0.8% for $<4.0 \times 10^{-4}$). Then, among high-risk patients, they found a significantly longer doubling time of WT1 levels in patients who underwent the discontinuation of immunosuppressive therapy or donor lymphocyte infusions (DLIs). In conclusion, they stated that WT1 was a very useful marker to predict and manage the relapse following the allo-SCT. Similar data were reported by Zhao et al. (19), who investigated the

prognostic significance of WT1 expression in a large study population (n = 138) of AL (AML, ALL) patients following allo-SCT. After measuring MRD by WT1 levels at designed time points, the authors showed that WT1 levels ≥0.60% before allo-SCT indicated higher rates of relapse post-transplant. Similarly, WT1 levels ≥0.60% at median time of +120 days from transplant was associated with lower DFS and OS. Besides, 20 patients showing high levels of WT1 expression received modified DLI, and a median of 0.22% of WT1 levels was observed after intervention. Indeed, patients showing a recurrence trend after allo-SCT, did not experience it due to interventions MRD –based. Recently, Pozzi et al. (20) also confirmed that AML patients in CR before transplant and with a median expression of WT1 >100 × 104 ABL had a higher relapse risk (53% vs 26%) and a lower 5-year survival (36% vs 62%) when compared with patients who had less than this cutoff. Similar results were obtained when the threshold of WT1 ⩽100 copies was considered at 30 days after allo-SCT. Thirty-eight patients achieving a CR but exceeding 180 × 104 ABL copies post-transplant were eligible for immune intervention by DLI: 17 patients received DLI and 21 did not. The interval between MRD positivity and relapse was significantly longer in patients receiving DLI. These studies clearly defined the predictive effect of WT1 expression on relapse in AML patients who underwent allo-SCT. In particular, post-transplant WT1 expression was the strongest predictor of outcome in multivariate analysis and was found to be a useful marker to select patients for preemptive immune intervention (DLI, tapering of immunosuppressive therapy). Comparable data were reported in a smaller number of patients monitored before and after transplant (21, 22). However, discordant results on prognosis were obtained when MFC and WT1 levels were compared (40–42). In our recent paper, we investigated technical performance of MRD detected by the two techniques at different time points, before and after transplant. At day +30 post-transplant, we recommended to study MRD by either or both methods, as it had a strong predictive role. Although post-transplant WT1 measurement is a valuable and essential marker for MRD monitoring also in our series, the combination of MFC and WT1 may be preferred to a single one when further treatments should be administered to prevent the relapse. In fact, double-positive MRD after allo-SCT correlated with a higher probability to experience a recurrence, based on higher product between specificity and sensitivity (43).

Conclusions

The relapse remains the main cause of treatment failure and death in AML. Although more than 80% of patients achieves a CR after conventional chemotherapy, a significant number of them experiences a recurrence disease (44). Indeed, more stringent criteria of response than CR are needed. The monitoring of leukemia-specific gene mutation by PCR represents the gold standard method to stratify patients on the basis of the risk to relapse. Unfortunately, more than 50% of AML cases lack one of these specific genes, and new genes to detect MRD are desirable. WT1 is a transcriptional factor, which has found an important role in acute

leukemias as MRD marker. To date, all published papers have confirmed the prognostic value of WT1 levels in AML patients achieving a CR after chemotherapy or allo-SCT. Indeed, despite the controversial role of WT1 expression at the presentation of disease, WT1 levels higher than the given thresholds in AML remission BM predicted the risk of relapse and death. The main concerns grown on this technique referred to cutoff that should be used and the influence of regenerating BM on quantification of the number of WT1 copies. Although WT1 assay has been standardized by ELN, methods to determine the positive threshold of MRD differentiate from one to another study group, with values ranging from 50 to 250 × 10^4 ABL. Further, WT1 transcript values were not univocally normalized with respect to the number of ABL.. On the contrary, regenerating CD34+ cells may be WT1 levels, affecting the sensitivity (45). According to the better sensitivity of 2log reduction compared to MRD and the amply demonstrated prognostic value of this cutoff after induction chemotherapy, the log reduction of copy number may overcome these pitfalls. Finally, post-transplant MRD positive by WT1 is a strong predictor of outcome, and it has been found that WT1 levels may be useful for preemptive immune intervention after transplant. Besides, the low product between sensitivity and specificity for WT1 expression suggests using another method such as MFC to detect MRD and decide for further treatments in case of double positivity.

Conflict of Interest

The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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