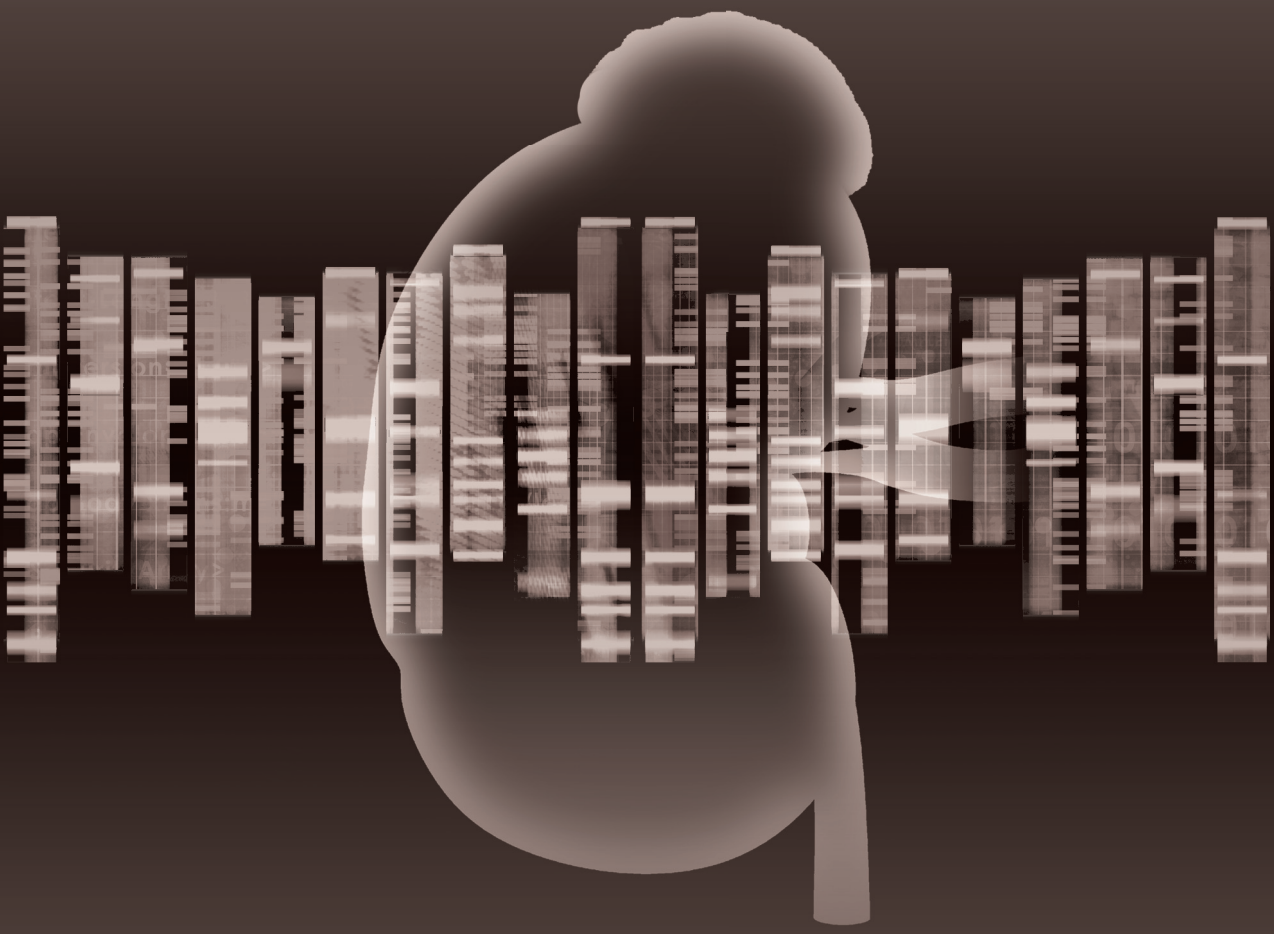
WILMS TUMORS:
GENOTYPES AND PHENOTYPES



Heidi Segers

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GENERAL INTRODUCTION

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1.1 WILMS TUMOR

Introduction

Wilms tumor, or nephroblastoma, represents about 90% of all pediatric renal tumors and about 7% of all pediatric malignancies (1). Most Wilms tumors are unilateral, although in 5-10% of the patients both kidneys are affected (2, 3). Wilms tumor typically occurs between the age of 2 and 4 years, and 90% of the patients are diagnosed before the age of 7 years (3-6). Above the age of 18 years, Wilms tumor is rare, representing less than 1% of all adult renal tumors (7).

Most pediatric Wilms tumor patients present with an asymptomatic abdominal mass, although abdominal pain, anorexia, vomiting, hematuria, fever and hypertension have frequently been described (2, 3, 8). Unusual presentations of Wilms tumor in children are acquired von Willebrand disease, sudden death due to pulmonary embolism, and Cushing syndrome (9-11).

Histology

Wilms tumor is an embryonal renal tumor with a classical triphasic histology with varying proportions of blastemal, epithelial and stromal cells recapitulating the fetal kidney (Figure 1) (12-14). However, frequently, only two or even one component predominate (2).

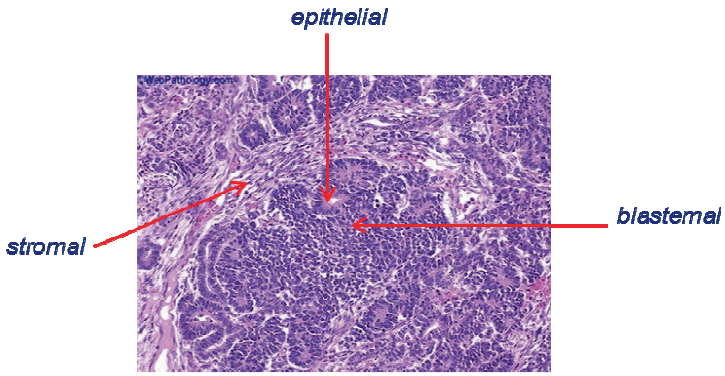


Figure 1: *Triphasic histology of Wilms tumor*

The homology with the developing kidney has led to the idea that Wilms tumor arises from metanephric blastemal cells during embryonal development (6) (Figure 2). These blastemal cells usually differentiate towards stromal components which give rise to the connective tissue as well as epithelial components that form the structural components of the mature nephron, such as glomeruli and tubuli (6, 15) (Figure 2A). Nephrogenic rests are foci of metanephric blastemal cells that persist after birth and are considered as potential Wilms tumor precursors (6) (Figure 2B). Some nephrogenic rests will proliferate to form hyperplastic rests and undergo malignant transformation to form a Wilms tumor, but the majority of nephrogenic rests become dormant or regress (6, 16) (Figure 2B). Nephrogenic rests are classified as perilobular (PLNR) or intralobar (ILNR) and both types may be sporadic or be part of a Wilms tumor predisposing syndrome (16, 17).

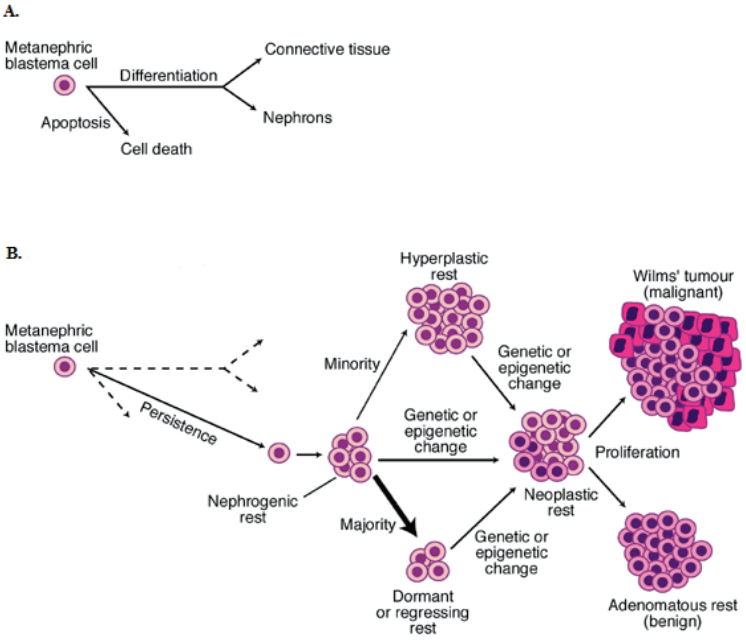
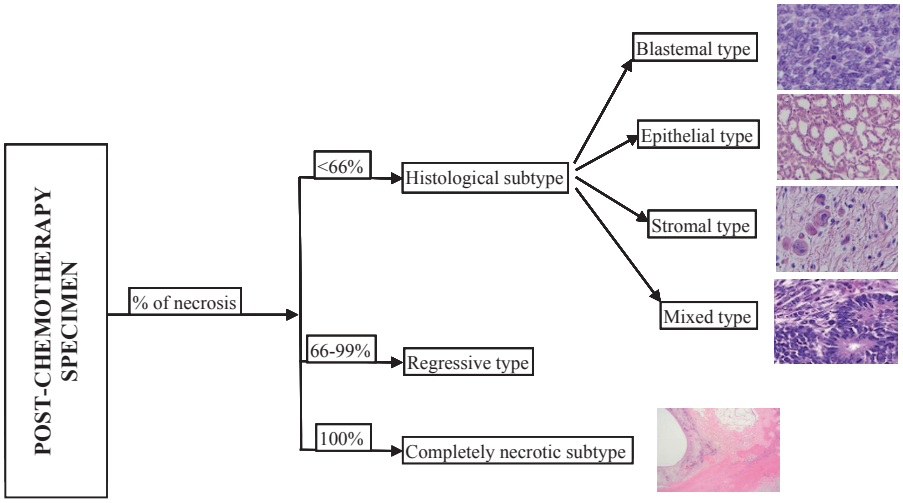


Figure 2: *Normal kidney (A) and Wilms tumor (B) development*
Brown KW, Malik KT. Expert reviews in molecular medicine. 2001;2001:1-16

The Renal Tumour Study Group of the International Society of Paediatric Oncology (SIOP) in Europe and the Children's Oncology Group (COG, formerly National Wilms Tumor Study group (NWTSG)) in North America have a different treatment approach, thereby inducing differences in histological classification (Table I) and in staging (Table II) (14, 18). The SIOP histological classification is based on pre-operative chemotherapy-induced changes, i.e. the percentage overall necrosis and the predominant cell type in the residual viable tumor, whereas the COG histological classification is based on the presence (unfavorable histology) or absence (favorable histology) of anaplasia without pre-operative chemotherapy (Table I). Anaplasia is defined by the presence of marked nuclear enlargement, hyperchromatic tumor cell nuclei, and multipolar mitotic figures (19-21). Currently, pathologists differentiate between focal and diffuse anaplasia, as it is of significant prognostic value (20, 21). Focal anaplasia is defined as anaplasia confined to a specific region of the primary intrarenal tumor without evidence of anaplasia elsewhere (20, 21). Diffuse anaplasia is diagnosed when anaplasia is present in more than one region of the primary tumor, or is found in any extrarenal or metastatic site, or in a random biopsy sample (20, 21). In the SIOP histological risk-classification, tumors showing complete response (100% necrosis) to pre-operative chemotherapy or cystic partially differentiated Wilms tumor are defined as low risk (Table I). Beside the long recognized high risk subgroup of diffuse anaplastic Wilms tumor, another high risk subgroup of 'blastemal type' Wilms tumor is defined, where the tumor shows less than two third of necrosis and blastemal cells represent more than two thirds of the viable components (Table I). All other histological subtypes are classified as intermediate risk (Table I).

Table I: *Histological risk-classification according to the current SIOP and COG criteria*

COG	
Favorable histology tumors	
	Includes classic triphasic Wilms tumor and all other non-anaplastic subtypes (if >2/3 of the tumor sample consists of one component, the tumor is assigned as a histological subtype)
Unfavorable (anaplastic) histology tumors	
	Diffuse anaplasia
	Focal anaplasia
SIOP (SIOP 2001 protocol)	
Low risk	
	-Completely necrotic Wilms tumor
	-Cystic partially differentiated nephroblastoma (CPDN)
Intermediate risk	
	All non-anaplastic and non-blastemal type Wilms tumors, i.e.:
	-Regressive type (>2/3 necrosis)
	-Epithelial type (>1/3 viable tumor, viable residue >2/3 epithelial and blastemal component <10%)
	-Stromal type (>1/3 viable tumor, viable residue >2/3 stromal and blastemal component <10%)
	-Mixed type (>1/3 viable tumor and no predominant cell type in viable residue)
	-Focal anaplasia
High risk	
	-Diffuse anaplasia
	-Blastemal type (>1/3 viable tumor and viable residue >2/3 blastemal)



Staging

The criteria for staging are based on the anatomical extent of the tumor and the presence of lymph node invasion or metastases, as well as on surgical factors such as the completeness of tumor resection and tumor spillage before or during surgery (Table II). The SIOP uses an upfront chemotherapy-based staging system, whereas the COG uses an upfront surgery-based staging system (Table II).

Table II: *Staging according to the current SIOP and COG criteria*

STAGE	SIOP	COG
Stage I	Tumor limited to the kidney, complete resection.	Tumor limited to kidney and completely excised. No penetration of the renal capsule or involvement of renal sinus vessels.
Stage II	Tumour extending outside the kidney but complete excision. Invasion beyond the capsule, perirenal/ perihilar. Invasion of regional lymph nodes. Invasion of extra renal vessels, or ureter.	Tumor extending beyond the kidney but completely excised. No residual tumor apparent at or beyond the margins of excision. Tumor thrombus in vessels outside the kidney is stage II if the thrombus is removed en bloc with the tumor.
Stage III	Incomplete excision, without hematogenous metastases. Peri-operative or pre-operative tumor rupture. Invasion of extra-regional nodes. Pre-operative biopsy.	Gross or microscopic residual tumor remaining postoperatively, including: • Inoperable tumor • Positive surgical margins • Diffuse tumor spillage or biopsy • Regional lymph node metastases • Transected tumor thrombus
Stage IV	Hematogenous metastases (lung, liver, bone, brain) or lymph node metastases outside the abdominal or pelvic cavities.	Hematogenous metastases (lung, liver, bone, brain) or lymph node metastases outside the abdominal or pelvic cavities.
Stage V	Bilateral renal tumors at diagnosis.	Bilateral renal tumors at diagnosis.

Prognostic factors

Tumor histology, stage, patient age at diagnosis and some biological factors are important prognostic factors which impact on treatment and outcome (22). As pre-operative chemotherapy alters histology and stage, these prognostic factors must be considered in the context of the therapy given.

Tumor histology

The most important prognostic histological feature is anaplasia. Both SIOP and COG classify tumors with diffuse anaplasia (~5% of cases) as 'high risk' due to its unfavorable outcome. Pathological assessment of the tumor after pre-operative chemotherapy provides an opportunity to test in vivo the chemosensitivity of the tumor and may serve as individual prognostic factor (14). Survival of a substantial proportion of blastema in the tumor has been identified as poor prognostic marker (14), while the presence of complete necrosis after pre-operative chemotherapy is associated with an excellent outcome (23). In addition, tumors in which epithelial or stromal components predominate after pre-operative chemotherapy appear to have a very favorable outcome (23, 24).

Stage

The second most important prognostic factor after tumor histology is tumor stage. Low stage predicts better outcome than high stage (25), although the prognostic significance of stage in localized tumors (stage I, II and III) has reduced because of the risk-adapted therapy they receive (26). Metastatic disease (stage IV) clearly identifies a group with a poorer outcome (26). Moreover, local stage of the tumor in metastatic patients is of prognostic importance (26). Stage V or bilateral disease is associated with outcomes even inferior to metastatic disease (25).

Age

Age at diagnosis younger than 2 years has been correlated with a better outcome (27, 28). Conversely, older age at diagnosis has been identified as an adverse factor (29). For adults with Wilms tumor, outcome is considerably worse compared to children, although better results are reported when treated with multimodal treatment plans adapted from pediatric treatment protocols (30-37). Multiple factors, including unfamiliarity of adult oncologists and pathologists with Wilms tumor, lack of standardized treatment and consequent delays in initiating appropriate risk-adapted therapy for this rare disease in adults, may contribute to the poor outcome. Therefore, we proposed a standardized approach for the management of adults with a Wilms tumor in this thesis.

Molecular markers

In the fifth National Wilms tumor study (NWTS-5), tumor-specific loss of heterozygosity (LOH) at chromosome 1p and 16q was associated with increased risk of relapse and death in patients with favorable histology Wilms tumors (38). The Children's Cancer and Leukemia Group (CCLG) of the United Kingdom (formerly UKCCSG) could only confirm LOH at 16q as an adverse risk factor in favorable histology Wilms tumors independent of treatment approach (immediate nephrectomy or pre-operative chemotherapy) (39).

Risk stratification

Histological subtype and stage at the moment of nephrectomy are the cornerstones of all Wilms tumor risk stratification systems. In the current COG risk stratification schema, patient age at diagnosis, tumor weight, LOH at 1p and 16q, and completeness of lung nodule response after 6 weeks of chemotherapy in case of metastatic disease supplement histology and stage in assigning risk for favorable histology Wilms tumor patients (25, 38, 40). The current SIOP risk stratification schema is based only on histology and stage after pre-operative chemotherapy, serving as an in-vivo test of treatment response and with the advantage to identify a novel high risk group at the moment of surgery, the blastemal subtype (14).

For relapsed Wilms tumors, three risk categories including standard risk, high risk and very high risk can be identified (41). Standard risk patients are defined as patients with favorable histology Wilms tumor relapsed after therapy with only vincristine and/or actinomycin D (41). High risk patients are favorable histology Wilms tumor patients relapsed after therapy with three or more chemotherapeutic drugs and/or radiotherapy (41). Very high risk patients are unfavorable of SIOP high risk Wilms tumor patients (41).

Treatment

The risk-adapted management of children with a Wilms tumor involves multimodal therapy including surgery, chemotherapy, and selectively radiotherapy. Historically, there are two main treatment approaches for children with a Wilms tumor both resulting in a similar outcome. The COG in North America recommends upfront surgery

with certain exceptions, whereas the SIOP in Europe advocates chemotherapy before nephrectomy. While the COG approach gives a better pathological view and accurate staging of the untreated tumor, the SIOP recommends pre-operative chemotherapy as it reduces the risk of tumor rupture from 15% to 3% during surgery thereby downstaging the tumor (42). As a result, the overall burden of therapy is lower in patients receiving pre-operative chemotherapy compared to patients treated with immediate nephrectomy (42). The more favorable tumor stage distribution and significant reduction in the overall burden of therapy together with reduced surgical complications after pre-operative chemotherapy were confirmed by a randomized comparison of these two approaches in the UKW3 trial performed by the CCLG (43). In the current SIOP 2001 study, Wilms tumor patients receive pre-operative chemotherapy followed by nephrectomy and postoperative treatment, existing of chemotherapy and selectively radiotherapy. Pre-operative chemotherapy exists of two drugs (vincristine and actinomycin D) for four weeks in case of localized disease, and three drugs (vincristine, actinomycin D and doxorubicin) for six weeks in case of metastatic disease. Bilateral disease patients receive at least eight weeks of pre-operative chemotherapy (two or three drugs depending on the response after four weeks of two drugs) before surgery. Nephron-sparing surgery is until now only advocated for patients with bilateral disease, whereas nephrectomy is the standard treatment in Wilms tumor patients with unilateral disease. Nephron-sparing surgery for unilateral Wilms tumor patients, with a risk for renal failure of less than 1% after nephrectomy (40), is only considered if the tumor is very small and can be resected with clean margins. Postoperative treatment is based on local stage and histology after surgery. Stage I, II and III Wilms tumor patients receive two drugs (vincristine and actinomycin D), except the 5% of stage I low risk patients, that are not advised postoperative treatment. Stage I high risk patients receive three drugs (vincristine, actinomycin D, and doxorubicin), whereas stage II and III high risk patients receive four drugs (etoposide, carboplatin, cyclophosphamide, and doxorubicin). Radiotherapy is only given to stage II diffuse anaplastic Wilms tumor patients and stage III intermediate and high risk patients. The intensity of the postoperative treatment of patients with metastases at presentation, including the decision about use of whole lung radiotherapy and further intensification of chemotherapy (inclusion

of carboplatin, cyclophosphamide and etoposide) is based on assessment of the metastatic response to chemotherapy combined with the histological risk group of the abdominal tumor.

In the current COG protocols, patients are commonly treated with immediate surgery followed by risk-adapted therapy based on histology, stage, and in certain favorable histology Wilms tumors also on patient age at diagnosis, tumor weight, LOH at 1p and 16q, and completeness of lung nodule response after 6 weeks of chemotherapy in case of metastatic disease. Generally, most patients with stage I and II favorable histology Wilms tumor are treated with two drugs (vincristine and actinomycin D). Most patients with stage III or IV favorable histology disease are treated with three drugs (vincristine, actinomycin D, and doxorubicin). Although the optimal regimen for anaplastic Wilms tumors has not been established, treatment requires more intensive therapy currently including the agents vincristine, doxorubicin, cyclophosphamide or ifosfamide, etoposide, and carboplatin. Radiotherapy is administered to individuals with advanced disease (stage III or IV).

A risk-stratified approach, based on initial treatment and prognostic factors of the tumor, is used for the treatment regimens of relapsed Wilms tumors with the principal aim to include chemotherapeutic drugs that are not used during primary chemotherapy (41, 44, 45). Several dose-intense chemotherapy regimens that variably include doxorubicin, cyclophosphamide or ifosfamide, etoposide, carboplatin and topotecan, are considered first treatment choice for relapsed Wilms tumors (41, 44-46). The effective use of high-dose chemotherapy with stem cell rescue for the treatment of relapsed Wilms tumor has been reported by several groups, although there is still a great deal of uncertainty concerning the efficacy and toxicity of high-dose chemotherapy compared to conventional chemotherapy (47). Until now, there is no good evidence on how to adequately administer surgery and radiotherapy at relapse (41).

Outcome

Over the years, the multimodal treatment strategy for Wilms tumors and the large multicenter randomized clinical trials conducted by international study groups have resulted in an improvement in survival from 30% in the 1930s to approximately 90%

nowadays. Despite this good outcome for the majority of patients, still approximately 10% of the patients with Wilms tumor will die due to refractory disease or following relapse. Approximately 15% of patients with favorable histology Wilms tumor and 50% of patients with anaplastic Wilms tumor experience relapse (38, 41). After relapse strong prognostic predictors of a worse outcome are anaplastic or SIOP high risk histology, and initial treatment with doxorubicin (41, 46, 48). Standard risk relapsed Wilms tumor patients have a relatively better prognosis with survival rates of 70-80% compared to the high risk relapsed patients with survival rates of 40-50% (41). Very high risk relapsed Wilms tumor patients frequently develop chemoresistent disease and have a very worse outcome with survival rates of only 10% (41). Novel therapeutic strategies will be necessary to cure these patients.

1.2 WILMS TUMOR GENETICS

Constitutional aberrations associated with Wilms tumors

Most Wilms tumors occur sporadic, whereas a genetic predisposition is described in 9%-17% of the Wilms tumor patients (49-51). The most common conditions that predispose to Wilms tumors are those associated with constitutional aberrations in the *WT1* gene (Table III) and those associated with overgrowth (Table IV) (50, 51).

Constitutional aberrations in the *WT1* gene are associated with a phenotypic range typified by various combinations of three main features: Wilms tumor, genitourinary abnormalities, and renal dysfunction. The *WT1* gene, located at chromosome 11p13, encodes a zinc finger protein that acts both as a transcription factor and an RNA binding protein, which plays a crucial role in the normal renal and gonadal development (52, 53). *WT1*-associated syndromes in Wilms tumor patients include WAGR syndrome (Wilms tumor, aniridia, genitourinary malformations and mental retardation) caused by a deletion of 11p13 including the *WT1* and *PAX6*-gene; Denys-Drash syndrome (DDS; mesangiosclerosis, Wilms tumor, and pseudohermaphroditism / genitourinary malformations) due to mutations in the *WT1* gene, which are mostly missense mutations in exon 8 or 9 (6, 50, 51, 54, 55); and Frasier syndrome (FS; gonadal dysgenesis, focal segmental glomerulosclerosis and gonadoblastoma) caused by

point mutations in the *WT1* intron 9 donor splice site (6, 50, 51, 54, 55). Although DDS is not typically associated with gonadoblastoma and FS not typically with Wilms tumor, there are several cases described in literature suggesting that DDS and FS represent two ends of a phenotypic range (51). Other *WT1*-associated phenotypes beside syndromes, i.e. either one or two of the three main features, have also been reported in patients with a constitutional *WT1* mutation (51). These patients mostly carry intragenic truncating *WT1* mutations (51).

Table III: *WT1-associated syndromes ~ Wilms tumor*

SYNDROME	CLINICAL FEATURES	GENETIC DEFECT
WAGR	Wilms tumor Aniridia Genitourinary malformations mental Retardation	deletion 11p13
Denys-Drash (DDS)	Diffuse mesangial glomerulosclerosis Genitourinary malformations Wilms tumor	*WT1* mutation, mostly missense mutation in exon 8 or 9
Frasier	Gonadal dysgenesis Focal segmental glomerulosclerosis Gonadoblastoma	*WT1* mutation, mostly point mutation intron 9 donor splice site
Other *WT1*-associated phenotypes	Genitourinary malformations	*WT1* mutation, mostly intragenic truncating mutations

In addition to Beckwith-Wiedemann syndrome (BWS), also other more rare overgrowth syndromes such as Perlman syndrome, Simpson–Golabi–Behmel syndrome and isolated hemihypertrophy are associated with an increased risk of Wilms tumor (51, 56-58). BWS is characterized by hemihypertrophy, macroglossia, macrosomia, neonatal hypoglycemia, abdominal wall defects and by its increased risk for Wilms tumor and other embryonal tumors (6, 50, 51, 54, 55, 59). BWS is due to epigenetic and genetic aberrations in the imprinted gene clusters on chromosome 11p15.5 (6, 50, 51, 54, 55, 59). Imprinted genes are genes whose expression is altered according to the parental origin of the allele. Locus 11p15.5 consists of two imprinted domains: domain 1 with imprinting center 1 (IC1) and domain 2 with IC2. IC1 regulates the expression of the paternally expressed insulin-like growth factor II (*IGF2)* and the maternally expressed *H19*, a non-coding RNA with tumor suppressor

properties (Figure 3a). IC2 regulates the expression of the maternally expressed genes *CDKN1C* and *KCNQ1,* and the paternally expressed gene *KCNQ10T1* or *LIT1* (2, 6, 50) (Figure 3a). In more than 80% of the patients with BWS a molecular aberration can be detected, i.e. loss of methylation at IC2 on the maternal allele (~50%) (Figure 3b), gain of methylation at IC1 on the maternal allele (~5%) (Figure 3c), paternal uniparental disomy of 11p15.5 (~20%) (Figure 3d), mutation of the maternal *CDKN1C* allele (~5%) (Figure 3e), and duplication, inversion, or translocation involving 11p15.5 (<1%).

Table IV: *Overgrowth syndromes ~ Wilms tumor*

SYNDROME	CLINICAL FEATURES	GENETIC DEFECT
Beckwith-Wiedemann (BWS)	Hemihypertrophy Macrosomia Neonatal hypoglycemia Macroglossia Abdominal wall defects Ear creases or pits High risk of embryonal tumors	Mutations and epigenetic events involving imprinted genes on chromosome 11p15.5
Perlman	Renal hamartomas Nephroblastomatosis Fetal gigantism	*DIS3L2* at chromosome 2q37.1
Simpsom-Golabi-Behmel	Postnatal overgrowth, coarse face, congenital heart defects, other congenital abnormalities	*GPC3* at chromosome Xq26
Isolated hemihypertrophy (IHH)	Isolated hemihypertrophy	Epigenetic events involving imprinted genes on chromosome 11p15.5 (20-35%) or unknown

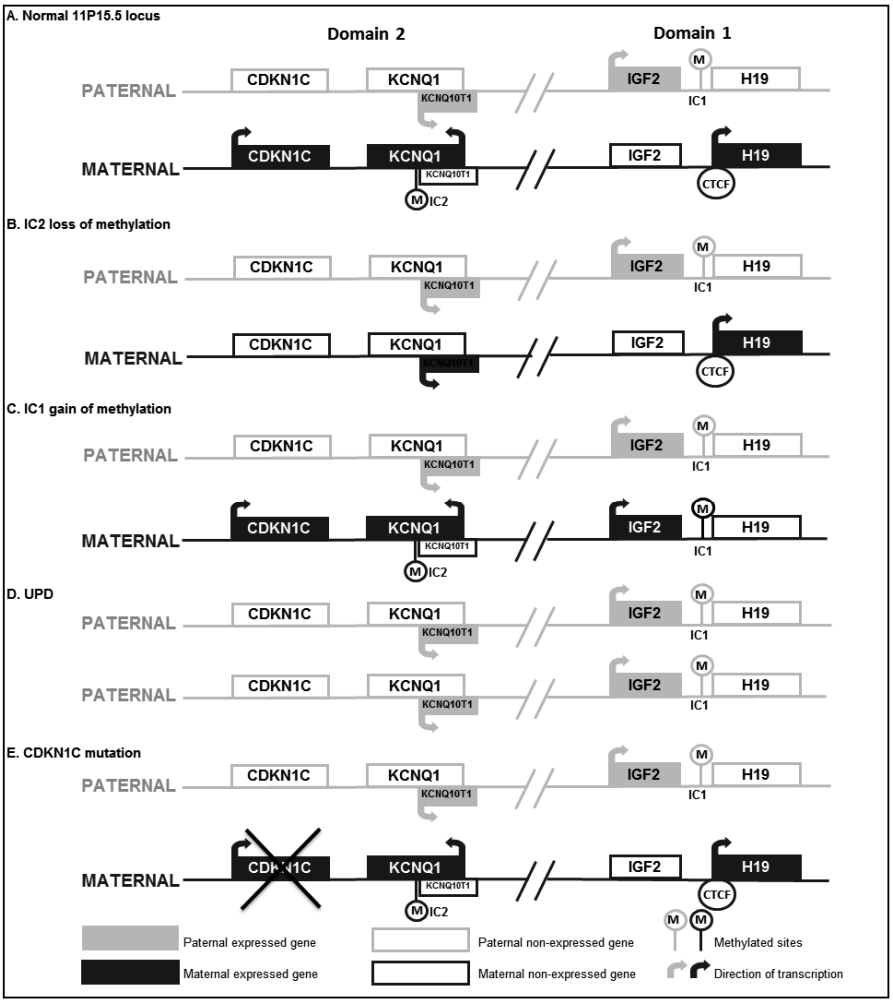


Figure 3: *11p15.5 cluster of imprinted genes and molecular mechanisms in BWS*
a. The 11p15.5 cluster of imprinted genes contains two imprinting centers (IC): IC1 and IC2.
b. Loss of methylation at IC2 on the maternal chromosome leads to transcription of the maternal *KCNQ10T1* or *LIT1* gene resulting in silencing of the maternal growth-inhibitory gene *CDKN1C* and *KCNQ1*. So, there is a decrease in growth suppression and thus a net increase in growth promoters.
c. Gain of methylation at IC1 on the maternal chromosome leads to silencing of the maternal *H19* gene and expression of the maternal *IGF2* resulting in an increase of growth promoters.
d. Uniparental disomy (UPD) refers to the situation in which both copies of a chromosome pair have originated from one parent. Paternal UPD results in two paternal copies of *IGF2* with overexpression of *IGF2* and loss of expression of *H19.*
e. Mutation of the maternal *CDKN1C* allele leads to a decrease in growth suppressors resulting in a net increase in growth promoters.

Constitutional *WT1* mutations and constitutional epigenetic changes of the imprinted gene clusters on chromosome 11p15 have also been described in Wilms tumor patients without phenotypic abnormalities (60, 61). Thus, the absence of a phenotypic abnormality does not exclude the presence of a constitutional *WT1* or locus 11p15 aberration.

In familial Wilms tumors, accounting for 1-2% of the Wilms tumor patients, there is usually no associated congenital abnormality or predisposition to other tumor types (6, 51, 54). Genetic linkage analyses have mapped familial Wilms tumor susceptibility genes on chromosome 17q12-21 (*FWT1*) and chromosome 19q13 (*FWT2*) (62, 63). Furthermore, Wilms tumor has been reported in patients with constitutional syndromes such as trisomy 18 and 13, and with certain tumor predisposition syndromes such as Bloom syndrome (associated with bi-allelic constitutional *BLM* mutation), Li–Fraumeni syndrome (associated with constitutional *TP53* mutation), and Fanconi anemia (51). Fanconi anemia (FA) is characterized by physical abnormalities, bone marrow failure, and increased risk of malignancy. Approximately 25-40% of individuals with FA have no physical abnormalities, and solid tumors may be the first manifestation of FA in these patients (64). Several reports have shown that bi-allelic mutations in two FA genes, *BRCA2/FANCD1* and *PALB2/FANCN,* may play a role in the etiology of Wilms tumors in FA patients (65, 66). Since *PALB2/FANCN* or *BRCA2/FANCD1* affected FA patients might not necessarily show an abnormal phenotype, we studied in this thesis the hypothesis that the FA diagnosis, caused by constitutional bi-allelic *PALB2/FANCN* or *BRCA2/FANCD1* mutations, can easily be missed in Wilms tumor patients without physical abnormalities (sporadic Wilms tumor patients).

Taken together, there are multiple constitutional aberrations that play a role in the etiology of Wilms tumors, but the exact frequency is not known especially not in children with an apparently sporadic Wilms tumor. In this thesis, we studied about the frequency of constitutional aberrations in childhood Wilms tumor patients.

Somatic aberrations associated with Wilms tumors

Apart from constitutional aberrations, also somatic aberrations in multiple genetic loci have been linked to Wilms tumorigenesis such as *WT1, WT2* or locus 11p15.5*, CTNNB1* (β-catenin) involved in the Wnt signaling pathway, *WTX* also involved in the

Wnt signaling pathway by controlling β-catenin activity, *TP53*, *FBXW7* and *MYCN*. Somatic mutations in *WT1, CTNNB1* and *WTX* which can occur as single aberration as well as in combination, only underlie the genetic basis in about one third of the Wilms tumors (67, 68). Genetic and epigenetic alterations at locus 11p15.5 have been found in approximately 70% of Wilms tumors as a somatic aberration (2, 6, 50, 69-73). Recently, Scott et al. analyzed how aberrations at *WT1*, *WTX*, *CTNNB1, TP53* and locus 11p15 interact in the development of Wilms tumors (74).

Only one study has been reported on the role of defects in the DNA mismatch repair system in the etiology of Wilms tumors; defects in DNA mismatch repair appeared important in 2% of the Wilms tumor cases (75).

Loss of heterozygosity (LOH) studies have implicated regions such as 1p, 4q, 7p, 11p, 11q, 14q, 16q and 22q in Wilms tumorigenesis, but all corresponding genes have not yet been identified (38, 76-78). LOH studies established the increased relapse and mortality risk of LOH at 1p and 16q in Wilms tumors (38, 39, 79, 80).

Conventional cytogenetic analyses revealed some tumor specific cytogenetic abnormalities such as gain of 1q, loss of 1p, 11q, 16q and 22q, and karyotype complexity as possible negative prognostic factors (38, 81-87). However, the frequency and the prognostic role of gain of 1q and other cytogenetic aberrations are not known in large cohorts of sporadic Wilms tumors and therefore we studied this in this thesis.

Taken together, Wilms tumor is genetically a heterogeneous and complex disease and the driving somatic genetic aberrations need to be further unraveled. In this thesis, we describe different studies about the role of somatic genetic aberrations in the development of childhood Wilms tumors.

1.3 AIM AND OUTLINE OF THIS THESIS

Aim

This thesis focuses on several genetic and clinical aspects of Wilms tumors. The general aim of this thesis is to explore constitutional and somatic genetic aberrations that may contribute to the pathogenesis of Wilms tumors and to reveal novel clinical insights.

Outline

Chapter 1 covers a general introduction of Wilms tumors. In **Chapter 2**, we described Cushing syndrome as a rare presenting symptom of childhood Wilms tumors. In **Chapter 3**, we show the frequency of constitutional *WT1* and locus 11p15 aberrations and concomitant phenotypes in a single center cohort of childhood Wilms tumor patients. **Chapter 4** describes the study on the occurrence of constitutional bi-allelic mutations of two Fanconi anemia genes, *PALB2/FANCN* and *BRCA2/FANCD1,* in an unselected cohort of Wilms tumor patients. In **Chapter 5**, we assessed the role of defects in the DNA mismatch repair system in the development of Wilms tumors. In **Chapter 6**, we describe the incidence, clinico-pathological associations, and prognostic significance of gain of 1q and other common cytogenetic aberrations in childhood Wilms tumors. In **Chapter 7**, we summarised clinical and outcome characteristics of adults with a Wilms tumor and provide recommendations based on an international consensus for the management of this rare disease in adults. **Chapter 8** comprises the general discussion, future perspectives and conclusions of this thesis.

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2

CUSHING SYNDROME AS A PRESENTING SYMPTOM OF RENAL TUMORS IN CHILDREN

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ABSTRACT

Cushing syndrome as the presenting symptom of a malignant renal tumor in children is rare. We report the first case of paraneoplastic Cushing syndrome due to a Wilms tumor, in which clinical and biological signs of hypercortisolism regressed during pre-operative chemotherapy. Additionally, we reviewed the literature on paraneoplastic Cushing syndrome secondary to pediatric renal tumors.

INTRODUCTION

Wilms tumor or nephroblastoma is the most frequent pediatric renal malignancy, representing about 85% of all pediatric renal tumors and about 7% of all pediatric malignancies (1). Most pediatric renal tumor patients present with an asymptomatic abdominal mass, although symptoms like abdominal pain, hematuria, hypertension, and fever have frequently been described (1, 2). Unusual presentations like acquired von Willebrand disease, sudden death due to pulmonary embolism, and Cushing syndrome have been described sporadic in pediatric Wilms tumors (2-10). Cushing syndrome results from prolonged glucocorticoid excess, which can be ACTH-dependent or –independent (11, 12). ACTH-dependent Cushing syndrome, accounting for about 80% of the cases of endogenous Cushing syndrome, is caused by a pituitary tumor (Cushing disease) or alternatively, but less often, by an ectopic source (11, 12). ACTH-dependent Cushing syndrome caused by ectopic adrenocorticotropic hormone (ACTH) and/or corticotrophin- releasing hormone (CRH) production, also called paraneoplastic Cushing syndrome, by a pediatric renal tumor was reported for the first time in 1974. Subsequently, only six cases have been reported (3-5, 7, 8, 10). In the current report, we describe a patient with a Wilms tumor and paraneoplastic Cushing syndrome who received pre-operative chemotherapy, resulting in a good response both for the tumor volume as for the endocrine status.

Case report

A 3-year-old previously healthy female presented with a 4-week history of abdominal distension, fatigue, weight gain (P90 percentile; length and weight in the past on P15 percentile), a cushingoid appearance, and hirsutism. Clinical examination revealed a palpable mass in the right upper abdomen and severe hypertension. Laboratory investigation showed normal potassium, glucose, and insulin-like growth factor-1 (IGF-1) levels and normal thyroid function. The late-night serum cortisol level, ACTH level, and the urinary free cortisol were all increased (Fig. 1). A high-dose intravenous dexamethasone suppression test (HDDST) (0.5 mg of dexamethasone per hour during 7 hr) revealed insufficient suppression of ACTH and cortisol (<50%), indicating an ACTH-dependent Cushing syndrome caused by ectopic ACTH and/or CRH production

(13). Radiological imaging revealed a mass of 7.7 cm x 9.0 cm x 6.3 cm (volume 233 ml) in the mid-upper pole of the right kidney with normal appearance of the adrenal glands and no evidence of pulmonary metastases. Based on the probable diagnosis of an ACTH-dependent Cushing syndrome, caused by ectopic production of ACTH and/or CRH by a renal tumor without metastases, pre-operative chemotherapy according to the SIOP (International Society of Pediatric Oncology) Wilms 2001 protocol (i.e., vincristine 1.5 mg/m2 weekly for 4 weeks and actinomycin D 45 mg/m2 at weeks 1 and 3) was started. This resulted in a very rapid decline of cortisol and ACTH serum levels (Fig. 1). Ten days after start of chemotherapy stressful events such as fever and vomiting started to occur, for which she received stress dosage hydrocortisone, followed by physiological suppletion with intermittent stress dosages during the period around surgery and radiotherapy and during episodes with fever and/or vomiting (Fig. 1). Consequently, the cortisol level at week 3 was elevated temporarily (701 nmol/L, normal 200–600 nmol/L) due to stress dosage of hydrocortisone (Fig. 1). After 4 weeks of chemotherapy, the tumor measured 6.5 cm x 6.1 cm x 4.3 cm (volume 91 ml) and the tumor resection was performed according to the SIOP 2001 guidelines. Pathology revealed a nephroblastoma with intermediate risk histology. It was classified as a stage III because of microscopic incomplete resection. Immunohistochemistry revealed no positive ACTH staining. CRH staining could not be performed on the tumor material. Postoperative treatment consisted of the SIOP 2001 protocol stage III intermediate risk including chemotherapy during 27 weeks (actinomycin D 45 mg/m2, vincristine 1.5 mg/m2, and doxorubicin with a cumulative dose of 250 mg/m2) combined with flank irradiation (15 Gy). Hydrocortisone suppletion therapy was tapered over a period of 12 weeks and stopped when the morning cortisol level was found to be adequate (>200 nmol/L) (Fig. 1).

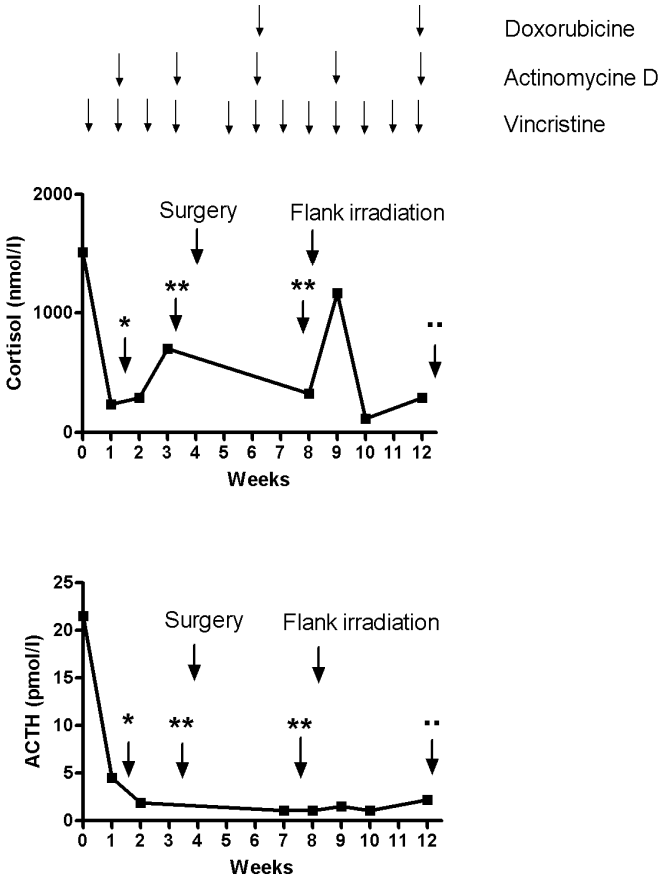

Figure 1: *Cortisol and ACTH levels.*
Cortisol (normal range 200–600 nmol/L) and ACTH levels (normal range <11 pmol/L) at diagnosis were markedly elevated and normalized very quickly after the first dose of chemotherapy. After week 12, chemotherapy was continued according to SIOP 2001 protocol stage III intermediate risk.
*Stress dosage hydrocortisone (HC) followed by physiological suppletion; **intermittent HC stress dosage; **..** stop HC suppletion.

DISCUSSION

Cushing syndrome as the presenting symptom of a renal tumor in children is rare (3-5, 7, 8, 10). Moreover, in reported cases, only the clinical course after direct surgery has been described (3-5, 7, 8, 10). We describe a patient with an ACTH- and/or CRH-producing Wilms tumor who responded well to pre-operative chemotherapy, not only with respect to the tumor volume but also to the endocrine status. In Europe, patients with renal tumors are treated according to SIOP 2001 protocol, which recommends pre-operative chemotherapy for a suspected nephroblastoma followed by tumor nephrectomy and risk-adapted postoperative chemotherapy, as it has been shown that chemotherapy before surgery results in downstaging and reduces the risk of tumor ruptures (14-16). Given the unconventional presentation, we discussed whether by exception our patient would benefit from up-front surgery as recommended by the COG RTWG (renal tumor working group) (14-16). Considering Wilms tumor the most probable diagnosis based on ultrasound and the absence of characteristics of other renal tumor types together with the fact that Cushing syndrome is not an ideal situation for surgery, we started pre-operative chemotherapy. The rapid decline of the ACTH and cortisol levels in combination with gradual regression of the cushingoid features and reduction of the tumor volume after pre-operative chemotherapy, supported the probable diagnosis of an ectopic ACTH- and/or CRH-producing Wilms tumor.

So far, only six cases of paraneoplastic Cushing syndrome in pediatric renal tumors have been described, that is, four ACTH-producing Wilms tumors, one ACTH-producing clear cell sarcoma (CCS) and recently one CRH-producing Wilms tumor (3-5, 7, 8, 10) (Table I). All six documented cases of ACTH-/CRH-producing pediatric renal tumor showed a rapid endocrine recovery after primary surgical removal of the tumor, which unmasked the tumor as producer of ectopic ACTH and/or CRH production (3-5, 7, 8, 10). Nevertheless, ACTH was not always found when the tumors were stained. Staining was positive in only two out of four tested patients. CRH positivity has been reported in one Wilms tumor patient. In our case, CRH nor corticotrophin releasing factor (CRF)-like staining and plasma CRH levels were available. The negative ACTH staining in our case may also have been due to the fact

that the pre-operative chemotherapy destroyed the ectopic ACTH-producing tumor cells consistent with the clinical response and hormone levels.

In adults, only two ACTH-producing renal tumors, both renal cell carcinoma, have been described (17, 18). Paraneoplastic Cushing syndrome in adults is more common in non-renal tumors, mainly in oat cell carcinomas of the lung and, less frequently, in endocrine carcinomas of foregut origin and neoplasms from neural crest tissue (5, 6, 19).

The response to chemotherapy in our case resulted in relatively low cortisol levels because the normal corticotroph cells had been suppressed by hypercortisolism due to the ACTH- and/or CRH producing Wilms tumor (11). Following earlier reports, we used glucocorticoid supplementation to avoid glucocorticoid withdrawal syndrome (11). This supplementation was tapered after the hypothalamic–pituitary–adrenal axis had recovered in week 12.

In conclusion, the endocrine and oncologic responses in this case with an ACTH- and/or CRH-producing Wilms tumor suggest that pre-operative chemotherapy can be considered for future patients in order to avoid complications during nephrectomy done at diagnosis.

TABLE I. *Reported patients with ACTH- and/or CRH-producing renal tumors in childhood*

Patient	References	Gender	Age	Histology	Stage	Treatment	ACTH staining on tumor material	CRH staining on tumor material	Outcome	Follow-up time
1	(3)	M	4 y 6 m	WT, nfs	III[a]	Surgery + postoperative chemotherapy (V + A) + RT	Positive	NA	Alive	1 y 6 m
2	(4)	F	5 y	WT, nfs	I[a]	Surgery + postoperative chemotherapy (V + A)	Positive	NA	Alive	1 y 8 m
3	(7)	F	7 y 6 m	WT, nfs	I	Surgery + postoperative chemotherapy (V + A)	NA	NA	NFS	NFS
4	(5)	F	7 y	Clear cell sarcoma	III[a]	Surgery + postoperative chemotherapy (V + A + Doxo) + RT	Negative	NA	Alive	NFS
5	(8)	M	2 y 8 m	WT, FH (non-anaplastic type)	II	Surgery + postoperative chemotherapy (V + A + Doxo)	NA	NA	Alive	1 y 4 m
6	(10)	M	4 y	WT, AH (focal anaplasia)	II	Surgery + postoperative chemotherapy + RT (flank)	Negative	Positive	Alive	3 y
7	This report (20)	F	3 y 5 m	WT, IR after pre-operative chemotherapy	III	According to SIOP 2001 protocol stage III IR[b]	Negative	NA	Alive	Still in treatment

WT, Wilms tumor; ACTH, adrenocorticotropic hormone; F, female; M, male; AH, anaplastic histology; FH, favorable histology; IR, intermediate risk; RT, radiotherapy; V, vincristine; A, actinomycin D; Doxo, doxorubicin; y, years; m, months; NA, not available; nfs, not further specified. [a] Derived from pathology and treatment data. [b] According to SIOP 2001 protocol pre-operative chemotherapy for localized disease followed by surgery and postoperative chemotherapy stage III IR.

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3

FREQUENCY OF WT1 AND 11P15 CONSTITUTIONAL ABERRATIONS AND PHENOTYPIC CORRELATION IN CHILDHOOD WILMS TUMOR PATIENTS

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ABSTRACT

Introduction

In 9-17% of Wilms tumor patients a predisposing syndrome is present, in particular *WT1*-associated syndromes and overgrowth syndromes. Constitutional *WT1* mutations or epigenetic changes on chromosome 11p15 have also been described in Wilms tumor patients without phenotypic abnormalities. Thus, the absence of phenotypic abnormalities does not exclude the presence of a genetic predisposition, suggesting that more Wilms tumor patients may have a constitutional abnormality. Therefore, we investigated the frequency of constitutional aberrations in combination with phenotype.

Patients and Methods

Clinical genetic assessment, as well as molecular analysis of *WT1* and locus 11p15 was offered to a single-center cohort of 109 childhood Wilms tumor patients.

Results

Twelve patients (11%) had a *WT1* aberration and eight patients (8%) had an 11p15 aberration. Of the 12 patients with a *WT1* aberration, four had WAGR syndrome, one had Denys-Drash syndrome, four had genitourinary anomalies without other syndromic features, and three had bilateral disease with stromal-predominant histology at young age without congenital anomalies. Of the eight patients with an 11p15 aberration, four had Beckwith-Wiedemann syndrome (BWS), two had minor features of BWS, and two had no stigmata of BWS or hemihypertrophy.

Conclusion

Constitutional *WT1* or 11p15 aberrations are frequent in Wilms tumor patients and careful clinical assessment can identify the majority of these patients. Therefore, we would recommend offering clinical genetic counseling to all Wilms tumor patients, as well as molecular analysis to patients with clinical signs of a syndrome or with features that may indicate a constitutional *WT1* or 11p15 aberration.

INTRODUCTION

Wilms tumor is the most common pediatric renal malignancy (1-3). In 9-17% of Wilms tumor patients a predisposing syndrome is present, in particular the *WT1*-associated syndromes and overgrowth syndromes (2-6). The most common *WT1*-associated syndromes in Wilms tumor patients include WAGR syndrome (Wilms tumor, aniridia, genitourinary malformations and mental retardation) caused by a deletion of 11p13 including the *WT1* and *PAX6*-gene; and Denys-Drash syndrome (DDS; mesangiosclerosis, Wilms tumor, and pseudohermaphroditism / genitourinary malformations) due to mutations in the *WT1* gene, which are mostly missense mutations in exon 8 or 9 (2-6). The most common overgrowth syndrome is Beckwith-Wiedemann syndrome (BWS) due to epigenetic and genetic aberrations in the imprinted gene clusters on chromosome 11p15 (2-7). BWS is characterized by hemihypertrophy, macroglossia, macrosomia, neonatal hypoglycemia, abdominal wall defects and increased risk for Wilms tumor and other embryonal tumors (2-7). Much less common overgrowth syndromes are Perlman syndrome and Simpson-Golabi-Behmel syndrome (8, 9).

Constitutional *WT1* mutations and constitutional epigenetic changes of the imprinted gene clusters on chromosome 11p15 have also been described in Wilms tumor patients without phenotypic abnormalities (10, 11). Thus, the absence of a phenotypic abnormality does not exclude the presence of a genetic predisposition. Therefore, the current study aimed to investigate the frequency of constitutional *WT1* and locus 11p15 aberrations in combination with phenotype. In order to achieve this, clinical genetic assessment in combination with molecular analysis of the *WT1*-gene and the 11p15 locus, was offered to a single-center cohort of childhood Wilms tumor patients and survivors.

PATIENTS AND METHODS

Patients

Survivors of childhood Wilms tumor diagnosed between January 1968 and January 2011 at Erasmus MC-Sophia Children's Hospital were offered clinical genetic assessment, genetic counseling and molecular analysis of the *WT1*-gene and the methylation status of the imprinted gene clusters on locus 11p15 after informed consent from the included patients and/or their parents.

Methods

Clinical assessment

Clinical information was retrieved from the medical records, including medical history and information about age of tumor-onset, tumor stage, and histological subtype. All patients were examined by the same clinical geneticist experienced in pediatric oncogenetics. Features of Wilms tumor associated syndromes like high birth weight, macroglossia, ear pits or creases, nevus flammeus, hemihypertrophy, genitourinary anomalies or other (congenital) anomalies like abdominal wall defects were noted and a three generation pedigree was constructed. Any phenotypic abnormality that may have been caused by the tumor or treatment, especially differences in the size of body parts as a result of radiotherapy or surgery, were not taken into account. If a different syndrome, unrelated to *WT1* or locus 11p15, was suspected, further (molecular) analyses were offered to the patient and/or his or her parents.

Molecular analysis

DNA was extracted from peripheral blood lymphocytes using standard methods. All coding exons including flanking intronic sequences of the *WT1* gene were amplified by PCR followed by direct sequencing using Bigdye v1.1 chemistry and an ABI3100 sequencer (Life Technologies, Carlsbad, CA, US). Sequences were analyzed using Codoncode Aligner (CodonCode Corporation, Dedham, MA, US). Multiplex ligation-dependent probe amplification (MLPA) was performed to detect deletions or duplications in the *WT1* gene, according to the manufacturer's instructions (MLPA kit P118, MRC Holland, Amsterdam, The Netherlands).

DNA methylation status at the *KCNQ10T1 (LIT1)* and *H19* loci was measured either by Southern Blot analysis as described by Bliek et al (samples analyzed before July 2007) (12), or methylation-specific high-resolution melting analysis (HRM-A) as described by Alders et al (samples analyzed since July 2007) (13). In case an aberrant methylation of both *KCNQ10T1* and *H19* was detected, STR markers D11S576, D11S4046, TH, D11S1318, D11S4088, D11S1288, D11S988, D11S1338 and D11S4149 were used to establish the presence of a paternal uniparental disomy (UPD). Primers were fluorescently labeled (6-FAM or HEX) and PCR products were run on an ABI310 or ABI3500 (Life Technologies). Results were analyzed using Genemapper software (Life Technologies). MLPA was performed to exclude a possible duplication or trisomy of the paternal allele, using the probemix of the kit ME030 (MRC Holland), but according to the normal copy number detection protocol.

Statistical analysis

Groups were compared using Mann–Whitney test or Fisher's exact test in cases of continuous or categorical data, respectively. $P < 0.05$ (two-sided) was considered statistically significant.

RESULTS

Frequency of WT1 and 11p15 constitutional aberrations in Wilms tumor patients and survivors

Of 184 Wilms tumor patients, 34 were not interested in genetic assessment, 10 refused molecular analysis after clinical genetic counseling, 19 had died, six had moved to another country, four did not visit the outpatient clinic and two were not asked as they were currently receiving treatment for a different disease. The evaluable population of 109 Wilms tumor patients was representative of the whole group for sex (51% male, 49% female), age at diagnosis (median age of 3.3 years; range 0.3-12.3 years) and stage (localized 83%, metastatic 11% and bilateral disease 6%). The median age at the time of the clinical genetic assessment was 11.6 years (range 0.2-49.2 years). A genetic or epigenetic aberration in the *WT1* gene or 11p15 locus was found in 20 (19%) of these 109 Wilms tumor patients analysed.

Constitutional WT1 aberrations and phenotype

There were 12 Wilms tumor patients with a *WT1* aberration (11%): four patients had a deletion of chromosome 11p13 including *WT1* and *PAX6* (4%) and eight patients had a *WT1* mutation (7%). In total, six distinct *WT1* mutations were identified in eight patients: one nonsense mutation (c.1084C>T / p.Arg362X) was identified in two patients and another nonsense mutation (c.901C>T / p.Arg301X) was also detected in two other patients (Table I). All mutations (3 nonsense, 2 small intragenic deletions and 1 splice site) were predicted to result in premature truncation of the WT1 protein (Table I). In six patients, the *WT1* mutation was de novo (Table I). This could not be confirmed in the other two patients with a *WT1* mutation, as there was no material of the parents available (Table I).

Compared to patients without a *WT1* mutation, patients with a *WT1* mutation were younger at diagnosis (median age 1.4 years; range 0.8-4.5 years versus 3.5 years; range 0.3-12.6 years; p<0.001), had more genitourinary abnormalities (50% versus 1%; p<0.001), had more frequent bilateral disease (25% versus 3%; p=0.02), and were more likely to have a stromal-predominant histology (58% versus 10%; p=0.001).

Five of the 12 patients carrying a constitutional *WT1* aberration were clinically diagnosed with a *WT1*-associated syndrome (5%): four with a deletion of chromosome 11p13 had WAGR and one with a nonsense mutation had DDS (Table I-II). The other seven patients could not be classified into a known syndrome and may be best classified as 'non-syndromic' *WT1*-associated Wilms tumor patients (6%). However, four of them had genitourinary abnormalities (cryptorchidism and/or hypospadias), whereas three had no aberrations on clinical examination (Table I-II). All these three patients without any phenotypic aberration on clinical examination did have bilateral Wilms tumors with stromal-predominant histology at young age (i.e. < 2 years) (Table I-II).

One patient without any phenotypic aberrations carried a variant in the *WT1* gene (p.Cys282Arg) of which the pathogenicity is uncertain (14, 15). This mutation was not included in the mutated group accordingly.

Table I: *Overview of childhood Wilms tumor patients and survivors with a constitutional* WT1 *or 11p15 defect*

Age Dx	G	Histology SIOP 2001***	St	Clinical Syndrome	Phenotypic signs observed clinically	Genetic aberration
8m	M	IR (stromal pred)°	V	-	-	WT1 mutation c.295delC / p.Gln99fs
10m	F	IR (stromal pred)°	V	-	Hip dysplasia	WT1 mutation c.895-2A>G / splicing defect
1y4m	F	IR (stromal pred)°	V	-	-	WT1 mutation c.901C>T / p.Arg301X
3y	M	IR (stromal pred)°	I	-	Cryptorchidism L, hypospadias	WT1 mutation c.901C>T / p.Arg301X
1y9m	M	IR (stromal pred)°	I	-	Cryptorchidism bilateral, hypospadias, double system L. Cheiloschisis, VSD.	WT1 mutation c.637C>T / p.Gln213X
4y6m	M	IR (mixed)°°	I	-	Cryptorchidism L, chronic progressive moderate RI based on FSGS. Syndactyly 2^{th}-3^{th} toes	WT1 mutation c.902_920del / p.Arg301fs
10m	M	IR (stromal pred)°	I	-	Cryptorchidism L, PFO	WT1 mutation c.1084C>T / p.Arg362X
1y	M	IR * °°	I	DDS	Cryptorchidism L, hypospadias, DMS with terminal RI, NBM	WT1 mutation c.1084C>T / p.Arg362X
1y11m	F	IR (stromal pred)	I	WAGR	Aniridia, bilateral congenital cataract and microphtalmos (Peters anomalia), PMR	Deletion chrom 11p13
1y1m	M	IR (regressive)	I	WAGR	Aniridia, cryptorchidism bilateral, PMR	Deletion chrom11p13
1y5m	F	IR (regressive)	I	WAGR	Aniridia, PMR	Deletion chrom 11p13
1y6m	F	IR **	I	WAGR	Aniridia, mild PMR	Deletion chrom 11p13 mosaicism
1y11m	F	IR (mixed)	I	-	Slight hypertrophy L feet and leg length discrepancy (L +1.5 cm)	Hypermethylation H19
1y2m	F	IR (regressive)	I	BWS	Macrosomia (>+2SD), neonatal hypoglycemia, macroglossia, umbilical hernia, dysplastic hypertrophic kidneys, asymmetric and leg length discrepancy L>R. NBM.	Hypermethylation H19
2y7m	F	IR (mixed)	I	BWS	Macrosomia (>+2SD), neonatal hypoglycemia, macroglossia, hyperplastic kidneys, hemihypertrophy R and leg length discrepancy (R +1.4 cm)	Hypermethylation H19
1y7m	M	Hyperplastic NR		BWS	Hemihypertrophy R feet and macroglossia. NBM.	Paternal UPD chrom 11p15 mosaicism
5y6m	M	LR (completely necrotic)	I	BWS	Macrosomia, neonatal hypoglycemia, macrosomia, macroglossia, hemihypertrophy R	Paternal UPD chrom 11p15 mosaicism
4y6m	F	IR (regressive)	II	-	Macrosomia (>+2SD) and neonatal hypoglycemia (1x)	Paternal UPD chrom 11p15 mosaicism
7y	F	HR (diffuse anaplasia)	I	-	-	Paternal UPD chrom 11p15 mosaicism
5y2m	M	LR (completely necrotic)	III	-	-	Hypomethylation LIT1

Abbreviations: BWS: Beckwith-Wiedemann syndrome, chrom: chromosome, DMS: diffuse mesangial glomerulosclerosis, Dx: diagnosis, G: gender, FSGS: focal segmental glomerulosclerosis, HR: high risk, F: female, M: male, IR: intermediate risk, L: left, LR: low risk, NBM: nephroblastomatosis, NR: nephrogenic rest, PFO: patent foramen ovale, PMR: psychomotor retardation, R: right, RI: renal insufficiency, St: stage, VSD: ventricular septal defect, WT: Wilms tumor.
-: No. ° de novo *WT1* mutations, °° from these 2 patients with a *WT1* mutation, there was no material of the parents available. * Classification according to SIOP9 and ** classification according to SIOP93-01, based on local pathology reports as no slides were available for panel revision. *** All other cases were classified according to SIOP 2001 classification (43).

Constitutional 11p15 aberrations and phenotype

Eight patients had a constitutional aberration at the imprinted gene clusters on chromosome 11p15 (8%): four with paternal UPD (4%), three with hypermethylation of *H19* (imprinting defect at imprinting center IC1) (3%), and one with hypomethylation of *KCNQ1OT1* (imprinting defect at IC2) (1%) (Table I). Four children (4%) were clinically diagnosed with BWS, of which one only after the diagnosis of the Wilms tumor (Table I-II). They all had clear phenotypic signs of BWS, and by molecular analysis two displayed UPD and two were found to have hypermethylation of *H19*. The other four 'non-syndromic' patients (4%) were diagnosed with 11p15 aberrations only after the diagnosis of Wilms tumor. Two of these patients only presented with minor phenotypic features: one with very subtle hypertrophy of the left feet and leg length discrepancy (hypermethylation *H19*) and one with high birth weight and one-time neonatal hypoglycaemia (paternal UPD) (Table I-II). The two other patients (2/109, 2%) had no clinical signs of BWS or other abnormalities (Table I-II). One had paternal UPD and the other had a hypomethylation of *KCNQ1OT1*. A possible caveat is that these two patients without clinical features of the Beckwith-Wiedemann spectrum were examined at adult age and may have lost some physical stigmata.

Hemihypertrophy was significantly more likely to be present in the group of patients with 11p15 aberration (62%) compared to the group without 11p15 aberration (6%) ($p<0.001$). Also high birth weight (>P97) was significantly more likely to be present in the group of patients with 11p15 aberration (57%) compared to the group without 11p15 aberration (5%) ($p=0.001$).

Table II: *Correlation (epi)genotype-phenotype in Wilms tumor patients*

PHENOTYPE \ GENOTYPE	*WT1*	LOCUS 11p15	NO ABERRATIONS WT1 / LOCUS 11p15
SYNDROMIC PATIENTS	**5 (5%)**	**4 (4%)**	**3 (3%)**
WAGR	4 (4%)	-	-
DDS	1 (1%)	-	-
BWS	-	4	-
Other (*)	-	-	3
NON SYNDROMIC PATIENTS	**7 (6%)**	**4 (4%)**	**86 (78%)**
FEATURES *	7 (6%)	2 (2%)	33 (30%)
- Young age at Dx (<2 y) #	5	1	18
- High BW (>P97)	-	1	4 (4/65)
- Neonatal hypoglycemia	-	1	-
- Macroglossia	-	-	-
- Hemihypertrophy	-	1	6
- Abdominal wall defects / umbilical hernia	-	-	2
	-	-	1
- Ear creases or pits	4	-	1
- GU-aberrations	3	-	3 (3/86)
- Stage V	3	-	5 (5/48)
- Stromal-predominant histology ##	-	2 (2%)	53 (48%)
**NO FEATURES ** **			
N (TOTAL) = 109	**12 (11%)**	**8 (8%)**	**89 (81%)**

Abbreviations: BW: birth weight, Dx: diagnosis, GU: genitourinary, y: years,
median (range), ## histology data according to classification of SIOP 2001 (43),
(*) other syndromes not related to constitutional *WT1* or 11p15 aberrations
* Features that may indicate a constitutional *WT1* or 11p15 aberration, but not a syndrome.
** No *WT1*-associated or overgrowth syndrome and no features that may indicate a constitutional *WT1* or 11p15 aberration.

Other syndromes in Wilms tumor patients and survivors without constitutional WT1 or 11p15 aberration

Eighty-nine Wilms tumor patients who were analyzed (81%) had no molecular aberration of *WT1* or 11p15. Three of these 89 Wilms tumor patients (3%) had another

syndrome. One patient was already diagnosed with Goldenhar syndrome before the onset of Wilms tumor. Two patients were diagnosed with Stickler syndrome, with one patient presenting with a confirmed pathogenic *COL2A1* mutation (Table IIIa).
There were 8 patients with a history of cancer at a young age (<50 years) in the family (first degree) (Table IIIb). One Wilms tumor patient had lost a brother to a rhabdoid tumor of the kidney (MRTK) and a primitive neuroectodermal tumor (PNET) of the brain at the age of 6 months. Mutation analysis of the *Snf5* gene showed no aberrations (16).
Thirty-three patients without a constitutional *WT1* or 11p15 aberration were, however, found to have one (27 patients) or two (6 patients) features that have been described in association with a constitutional *WT1* or 11p15 aberration (Table II).

Table IIIa: *Overview of patients with other clinical genetic syndromes*

Age Dx	G	Histology	St	Clinical syndrome	Phenotypic signs observed clinically	Genetic aberration
8y 10m	M	IR (regressive)	I	Goldenhar	Asymmetric face with hypoplastic chin and drooping mouth R, preauricular skin tags R, cleft lip L, congenital naevus R abdomen sharp bounded at the midline, congenital diaphragmatic hernia L.	-
11m	M	NA	I	Stickler	Severe myopia and nystagmus, hearing disabilities, typical face with flat midface with depressed nasal bridge, short nose with anteverted nares and long philtrum. PMR due to perinatal asphyxia.	-
2y 3m	M	NA	II	Stickler	Eye abnormalities: myopia, retinal detachment L and cataract R, facial abnormalities: broad nasal bridge, slight asymmetry and cleft soft palate, Perthes R with abduction contracture of hip. Dyslexia and concentration problems.	Col2A1 mutation c.3165+1G>T

Table IIIb: *Overview of patients with multiple malignancies or family history of malignancies at young age*

N	Specification of other morphological anomalies or diseases (N)
Multiple malignancies (n=1)	Alveolar RMS (11y) and Ewing sarcoma (14y)
Family history of malignancies at young age (<50 y) (n=8)	Sister with immature teratoma (1), sister with neuroblastoma at 13y (1), father with HL at 17y (1), brother with two different tumors (MRTK and PNET of the brain) at the age of 6 m and who died (1)*, mother with breast cancer at 48y (1), mother with melanoma at 26y (1), father of child with WT and hyperplasia had melanoma at 40y (1), father died of lung cancer < 50y (1)

Abbreviations: Dx: diagnosis, G: gender, F: female, M: male, IR: intermediate risk, L: left, NA: not available, R: right, RI: renal insufficiency, St: stage, WT: Wilms tumor, -: No.
HL: Hodgkin lymphoma, N: number, m: months, MRTK: malignant rhabdoid tumor of the kidney, PNET: primitive neuroectodermal tumor, RMS: rhabdomyosarcoma, y: years, *: no mutation of *Snf5* gene.

DISCUSSION

Frequency of constitutional WT1 or 11p15 aberrations in childhood Wilms tumor patients and survivors

Our study showed that 19% of Wilms tumor patients have a *WT1* or 11p15 aberration. This high frequency is in line with earlier publications describing a genetic predisposition in up to 17% of Wilms tumor patients (5, 17, 18). Similar to other studies, this number probably represents a slight overestimation, due to a selection bias, as patients with clear phenotypic signs suggestive of an underlying genetic disorder are more likely to agree to be referred for clinical genetic assessment.

Sensitivity of clinical evaluation in detecting constitutional WT1 or 11p15 aberration

The majority of patients with an underlying *WT1* or 11p15 aberration had a Wilms tumor predisposing syndrome (9%) or had features that may indicate an underlying constitutional *WT1* or 11p15 aberration (8%). Only 2% of the patients with an underlying *WT1* or 11p15 aberration had no features at all. This indicates that careful clinical evaluation may identify the majority of patients with a genetic predisposition in the *WT1* gene or 11p15 locus. Therefore, we would recommend offering routine

clinical genetic assessment and counseling to all Wilms tumor patients, as well as molecular analysis to patients with clinical signs of an underlying syndrome or with morphological or clinico-pathological features that may indicate a *WT1* or locus 11p15 aberration. The presence of a genetic predisposition is important information in terms of risk for future tumor for the patient, their siblings or their off-spring. However, we realise that a proportion of patients will not reveal a constitutional *WT1* or 11p15 aberration, as we set the threshold for molecular analysis at patients needing to have at least one clinical feature, as shown in table II (sensitivity 86%, specificity 59%). The cost effectiveness of genetic evaluation in Wilms tumor patients is currently difficult to estimate reliably. Further studies are warranted to investigate this in more detail.

WT1 genotype - phenotype

The well known association of constitutional *WT1* aberrations and genitourinary anomalies (hypospadias and cryptorchidism) in Wilms tumor patients (19-22) is underscored by our study and can be explained by the role of *WT1* in the normal genitourinary development (19-23). We also identified young age at diagnosis, bilateral disease and stromal-predominant histology in Wilms tumors treated with pre-operative chemotherapy as possible indicators of a constitutional *WT1* aberration. This is in line with previous studies (3, 10, 16, 20, 22, 24), although some studies could not establish an increased risk of carrying a constitutional *WT1* mutation for patients with bilateral tumors (10, 20, 25).

Our study also underscores some genotype-phenotype associations at *WT1* as previously described (6, 20, 21, 26, 27). Wilms tumor patients with genitourinary anomalies mostly carry constitutional mutations that delete the *WT1* gene or encode truncated proteins (6, 20, 21, 26, 27). All constitutional *WT1* mutations in our Wilms tumor patients with genitourinary anomalies are deletions of 11p13, and point mutations or small intragenic *WT1* deletions that result in a truncated protein including our only DDS patient. This DDS patient carried the *WT1* nonsense mutation c.1084C>T / p.Arg362X. This mutation has been described with a phenotypic spectrum varying from no aberrations, genitourinary anomalies only, to the typical DDS triad (28). This is in contrast to most DDS patients who carry constitutional

missense mutations resulting in a full-length abnormal WT1 protein (6, 20, 21, 26-28).

One patient without any phenotypic aberrations carried the *WT1* variant c.844T>C / p.Cys282Arg. This variant has been reported as a somatic mutation in acute myeloid leukemia (15), but has also been identified in 6/5376 individuals in the NHLBI GO Exome Sequencing Project (ESP) (http://evs.gs.washington.edu/EVS), and therefore probably represents a non pathogenic variant. This mutation was not included in the mutated group accordingly.

11p15 genotype - phenotype

In Wilms tumor patients carrying a constitutional 11p15 aberration, we identified two features which may point to a constitutional 11p15 aberration, specifically high birth weight (>P97) and hemihypertrophy. Scott et al. already identified constitutional 11p15 defects in 3% of 'non-syndromic' Wilms tumor patients with no or only subtle features of an overgrowth syndrome, but the researchers did not find clinical or histological features associated with a constitutional 11p15 aberration (11). This difference is possibly due to the fact that BWS patients were included in our cohort.

Our study also underscores the epigenotype-phenotype associations at 11p15 as previously published (7, 12, 29-31). Patients with an imprinting defect at the imprinted domain IC1 or with UPD have a significantly higher risk of neoplasia (+/-25%) than those with an IC2 defect (+/-5%) (7, 12, 29-31). The majority of our Wilms tumor patients with a constitutional 11p15 defect did in fact present with UPD or an imprinting defect at IC1. Only one Wilms tumor patient had an IC2 defect (hypomethylation of *KCNQ1OT1*), and this patient had no phenotypic features at all. To the best of our knowledge, this is the first 'non-syndromic' Wilms tumor patient with a hypomethylation of *KCNQ1OT1*.

Others syndromes and Wilms tumor

In the current study, 3% of Wilms tumor patients had other genetic syndromes: one had Goldenhar syndrome, and two had Stickler syndrome. Goldenhar syndrome is associated with several types of tumors, but as far as we know this is the first Goldenhar patient described with a Wilms tumor (32-40). Stickler syndrome is

a genetically heterogeneous disorder with mutations in several collagen genes. According to the current literature, there is no association between malignancies and Stickler syndrome (41, 42).

CONCLUSION

Constitutional *WT1* or 11p15 aberrations are frequent in Wilms tumors patients and careful clinical assessment can identify the majority of these patients. Therefore, we recommend offering routine clinical genetic assessment to detect morphological abnormalities and genetic counseling to all Wilms tumor patients. In addition, we would recommend offering molecular analysis to Wilms tumor patients with clinical signs of an underlying syndrome or with morphological or clinico-pathological features that may indicate a constitutional *WT1* or locus 11p15 aberration.

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4

FANCONI ANEMIA GENE MUTATIONS ARE NOT INVOLVED IN SPORADIC WILMS TUMOR

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ABSTRACT

Bi-allelic germline mutations of the Fanconi anemia (FA) genes, *PALB2/FANCN* and *BRCA2/FANCD1*, have been reported in a few Wilms tumor patients with an atypical FA phenotype. Therefore, we screened a random cohort of 47 Dutch Wilms tumor cases for germline mutations in these two FA-genes by DNA sequencing and Multiplex Ligation-dependent Probe Amplification. Although several cases appeared to carry missense variants, no bi-allelic pathogenic mutations were identified, indicating that bi-allelic mutations in these FA-genes do not contribute significantly to the occurrence of Wilms tumor.

INTRODUCTION

Wilms tumors, representing 7% of childhood malignancies, are known to be associated with congenital malformations as in Beckwith-Wiedemann syndrome (BWS), Denys-Drash syndrome, Frasier syndrome and WAGR syndrome (1, 2). Fanconi anemia (FA) is a heterogeneous autosomal recessive and X-linked disease with variable clinical features, including short stature, radial hypoplasia, thumb anomalies, hyper- and hypo-pigmentation, bone marrow failure and predisposition to cancers, most commonly acute myeloid leukemia and squamous cell carcinoma. FA has a high phenotypic variability, ranging from no dysmorphic features to multiple congenital malformations (3).

Bi-allelic (homozygous or compound heterozygous) germline mutations of the FA genes *PALB2/FANCN* and *BRCA2/FANCD1* give rise to a severe FA phenotype with childhood solid tumors (4-6). Twenty-two families have been described with bi-allelic *BRCA2/FANCD1* mutations including 26 cases with 35 childhood malignancies, including 7 Wilms tumors (4, 5). Most patients had pigmentation anomalies, such as café-au-lait spots, and short stature. Bi-allelic mutations in *PALB2/FANCN* have been described in 8 children, of which 3 developed a Wilms tumor (7, 8). All *PALB2/FANCN* patients suffered from growth retardation and a diversity of congenital malformations. In addition, *PALB2/FANCN* and *BRCA2/FANCD1* are known breast cancer susceptibility genes, in which mono-allelic (heterozygous) mutations are associated with a 2 fold increased risk of breast cancer in *PALB2/FANCN* and a 10-20 fold risk in *BRCA2/FANCD1* carriers (9).

Since FA is phenotypically highly variable, we hypothesized that sporadic Wilms tumor patients might carry germline bi-allelic *PALB2/FANCN* or *BRCA2/FANCD1* mutations. The clinical implications of missing the diagnosis of FA in Wilms tumor patients may be significant because of the hypersensitivity of these patients to chemotherapy and radiotherapy. We therefore decided to study the occurrence of bi-allelic germline *PALB2/FANCN* and *BRCA2/FANCD1* mutations in an unselected cohort of Wilms tumor patients.

METHODS

Patients

Whole peripheral blood DNA of a prospective cohort of 47 unselected Wilms tumor cases from 2 Dutch pediatric oncology centers (Emma Children's Hospital-AMC and Erasmus MC-Sophia Children's Hospital), 27 females, 20 males, median age 45 months (range 6-146 months). The patients were staged I (n=24), II (n=7), III (n=9), IV (n=5) and V (n=2). Patients were categorised as low risk (n=2), intermediated risk (n=40) and high risk (n=5) according to stage and histology (10). None had an apparent FA phenotype. Informed consent was obtained from all parents.

Sequencing and MLPA

The presence of germline mutations in *PALB2/FANCN* and *BRCA2/FAND1* was evaluated by direct sequencing. Sequencing was successful in all samples except for three cases in which one, four and six exons of *BRCA2/FANCD1* repeatedly did not show a result. Silent mutations, which were not present in splice acceptor/donor sites, are unlikely to be clinical relevant and therefore left out of the discussion. Pre-amplified DNA was analyzed for the presence of large rearrangements in the *PALB2/FANCN* gene using Multiplex Ligation-dependent Probe Amplification (MLPA), as previously described. MLPA is a rapid quantitative method for the detection of deletion/amplification of up to 40 specific DNA fragments in a single PCR reaction (11).

RESULTS

No truncating mutations of the *PALB2/FANCN* and *BRCA2/FANCD1* gene were identified in any of the 47 patients. In *PALB2/FANCN*, 9 different amino acid substitutions were identified of which 7 are known SNP's and were previously described in a large familial breast cancer study (12). Only three (Leu337Ser, Leu939Trp, Gly998Glu) amino acid substitutions were predicted to possibly affect the protein function by 2 or more programs (Table I).

Table I: *Missense variants identified in* PALB2/FANCN

PALB2/FANCN gDNA change	Protein change	SNP ID	Prediction programs				Clinical characteristics		
			Polyphen	SIFT	AGVGD	Mutation taster	Sample	Age (months)	WT stage and histology
c.629C>T	p.Pro210Leu	rs57605939	Benign	Affect protein function	Less likely (C0)	Presumably harmless	Rwt88	92	IV, regressive
c.721A>G	p.Asn241Asp	unknown	Benign	Tolerated	Less likely (C0)	Presumably harmless	Rwt71 Rwt123	10 88	V, stromal IV, regressive
c.1010T>C	p.Leu337Ser	rs45494092	Possibly damaging	Affect protein function	Less likely (C0)	Presumably harmless	*Rwt62* Rwt126	6 66	I, mixed I, regressive
c.2014G>C	p.Glu672Gln	rs45532440	Benign	Affect protein function	Less likely (C0)	Presumably harmless	*Rwt62*	6	I, mixed
c.2135C>T	p.Ala712Val	unknown	Benign	Tolerated	Less likely (C0)	Presumably harmless	Rwt44	18	III, mixed
c.2590C>T	p.Pro864Ser	rs45568339	Probably damaging	Tolerated	Less likely (C0)	Presumably harmless	Rwt137	28	I, mixed
c.2794G>A	p.Val932Met	rs45624036	Benign	Affect protein function	Less likely (C0)	Presumably harmless	Rwt106	48	III, stromal
c.2816T>G	p.Leu939Trp	rs45478192	Possibly damaging	Affect protein function	Likely (C55)	Disease potential unclear	Rwt47	21	IV , mixed
c.2993G>A	p.Gly998Glu	rs45551636	Probably damaging	Affect protein function	Less likely (C0)	Presumably disease causing	*Rwt62*	6	I, mixed

Nomenclature according to the human genome variation society (HGVS) recommendations (www.hgvs.org). Single Nucleotide Polymorphism (SNP) identification (ID) reference numbers (rs) are included if known. Pathogenicity of variants was predicted using 4 databases; Polymorphism Phenotyping (PolyPhen) prediction (http://genetics.bwh.harvard.edu/pph); Sorting Intolerant From Tolerant (SIFT) (http://blocks.fhcrc.org/sift/SIFT.html); Align GVGD (http://agvgd.iarc.fr) and Mutation Taster (http://neurocore.charite.de/MutationTaster/index.html). The samples marked in *italics* carry more than one variant.

Table II: Missense variants identified in *BRCA2/FAND1*

BRCA2/ FANCD1 gDNA change	Protein change	Prediction programs				BIC	Clinical characteristics		
		Polyphen	SIFT	AGVGD	Mutation taster		Sample	Age (months)	WT stage and histology
c.3499A>G	p.Ile1167Val	Benign	Tolerated	Less likely (C0)	Presumably harmless	not reported	Rwt130	9	I, mixed
c.5455C>T	p.Pro1819Ser	Probably damaging	Tolerated	Less likely (C0)	Presumably harmless	reported	Rwt 61	30	I, partially cystic differentiated
c.8187G>T	p.Lys2729Asn	Benign	Affect protein function	Less likely (C0)	Presumably harmless	reported	Rwt89	21	I, mixed

Nomenclature according to the human genome variation society (HGVS) recommendations (www.hgvs.org). Pathogenicity of variants was predicted using 4 databases; Polymorphism Phenotyping (PolyPhen) prediction (http://genetics.bwh.harvard.edu/pph); Sorting Intolerant From Tolerant (SIFT) (http://blocks.fhcrc.org/sift/SIFT.html); Align GVGD (http://agvgd.iarc.fr) and Mutation Taster (http://neurocore.charite.de/MutationTaster/index.html). No variant is a known Single Nucleotide Polymorphism (SNP), but two of the three variants are recurrently reported in the international Breast Cancer Information Core (BIC) database (http://research.nhgri.nih.gov/bic/.

In *BRCA2/FANCD1*, 3 missense variants were identified, of which the clinical importance of two is classified as "unknown" in the international Breast Cancer Information Core (BIC) database (http://research.nhgri.nih.gov/bic/), whereas the third one has not been reported (Table II).
No alterations in the *PALB2/FANCN* gene were identified by MLPA in 45 samples with available pre-amplified DNA.

DISCUSSION

Although the importance of FA genes in cancer predisposition is well described, the involvement of germline FA-gene mutations in solid childhood cancers has not been systematically investigated in a prospective study. Several reports have shown that bi-allelic mutations in two FA genes, *BRCA2/FANCD1* and *PALB2/FANCN,* may play a role in the etiology of Wilms tumors in patients with FA with an apparent FA phenotype (4, 7). Since *PALB2/FANCN* and *BRCA2/FANCD1* affected FA patients might not necessarily show an abnormal phenotype, the FA diagnosis can easily be missed in children with a seemingly sporadic Wilms tumor. Due to the hypersensitivity for cancer treatment causing higher morbidity and mortality in patients with FA, it is important to investigate a cohort of patients with sporadic Wilms tumor for *BRCA2/FANCD1* and *PALB2/FANCN* gene mutations. Moreover, since the occurrence of breast cancer in a child's family representing mono-allelic mutations might be unnoticed or unknown, we searched for subclinical FA cases in patients with sporadic Wilms tumors. The results of our unselected cohort revealed no bi-allelic pathogenic mutated cases, indicating that these mutations do not seem to play a major role in sporadic Wilms tumor. The chance of finding a bi-allelic mutated Wilms tumor patient was likely low due to the low prevalence of mutations in *BRCA2/FANCD1* and *PALB2/FANCN* in the population and our relatively small cohort.
Nevertheless, we did find several missense variants which may affect the function of the protein (Table I). The three missense variants in *PALB2/FANCN* that could affect the function of the protein have been previously described to occur with equal frequencies within cases and controls in a familial breast cancer study in which more

than 4000 alleles were screened (12). In addition, these three variants have a low confidence prediction and are therefore unlikely to be pathogenic. In our study, only 1 case carried two of these missense variants. Clinically, this child was not different from the other children in terms of the type of tumor, further the child had no congenital malformations. Interestingly, this child was only 6 months old at diagnosis of the Wilms tumor, an age at which germline mutations tend to occur more often than in older children with Wilms tumor.

BRCA2/FANCD1 mono-allelic missense variants were found in 3 cases. These variants did not result in major protein changes and are therefore unlikely to have any pathogenic effect. We did not observe any patient with more than one variant implicating that no bi-allelic mutation carrier was present in this cohort (Table II).

Although a higher incidence of Wilms tumors has not been reported in breast cancer families with mono-allelic *BRCA2/FANCD1* and *PALB2/FANCN* mutations, we cannot fully exclude a modifying effect of pathogenic mono-allelic mutations in the etiology of Wilms tumor (13).

In conclusion, germline bi-allelic mutations in both the *PALB2/FANCN* and *BRCA2/FANCD1* genes do not appear to play a major role in Wilms tumor development in Dutch patients. The role of mono-allelic missense *PALB2/FANCN* and *BRCA2/FANCD1* mutations remains to be determined.

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DEFECTS IN THE DNA MISMATCH REPAIR SYSTEM DO NOT CONTRIBUTE TO THE DEVELOPMENT OF CHILDHOOD WILMS TUMORS

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ABSTRACT

Background

Wilms tumor is the most common childhood renal malignancy. Most Wilms tumors occur sporadic, whereas a genetic predisposition is described in 9%-19% of the Wilms tumor patients. Beside constitutional aberrations, also somatic aberrations in multiple genetic loci such as *WT1, WT2* or locus 11p15.5*, CTNNB1*, *WTX*, *TP53*, *FBXW7* and *MYCN* have been linked to Wilms tumorigenesis. In sporadic Wilms tumors, however, the driving somatic genetic aberrations need to be further unraveled. Therefore, it is necessary to obtain more insight into other underlying mechanisms. Little is known about the role of defects in the DNA mismatch repair system in the etiology of Wilms tumors.

Materials and methods

To detect mismatch repair deficiency in a full cohort of Wilms tumor patients, we combined immunohistochemistry for the expression of mismatch repair proteins and microsatellite instability (MSI) analysis by a fluorescent multiplex PCR-based assay.

Results

Of the 121 Wilms tumor patients treated between 1987 and 2010 in our institution, 100 samples from 97 patients were available for analysis. Nuclear staining for MLH1, MSH2, MSH6 and PMS2 proteins was present in all 100 Wilms tumor samples. No pattern of MSI was found in any of the investigated 100 Wilms tumor samples.

Conclusion

The matching results of normal expression of the mismatch repair proteins detected by immunohistochemistry and the absence of MSI by DNA analysis in 100 Wilms tumor samples, lead us to conclude that defects in the DNA mismatch repair system do not play a significant role in the development of Wilms tumors.

INTRODUCTION

Wilms tumor represents approximately 90% of all childhood renal tumors (1). Most Wilms tumors occur sporadic, whereas a genetic predisposition is described in 9%-19% of the Wilms tumor patients (2-4). The most common conditions that predispose to Wilms tumors are those associated with constitutional aberrations in the *WT1* gene and those associated with overgrowth (3, 4). Beside constitutional aberrations, also somatic aberrations in multiple genetic loci such as *WT1, WT2* or locus 11p15.5 affecting the expression of *H19* and insulin-like growth factor 2 (*IGF2*)*, CTNNB1*, *WTX*, *TP53*, *FBXW7* and *MYCN,* have been linked to Wilms tumorigenesis (4-9). In sporadic Wilms tumors, however, the driving somatic genetic aberrations need to be further unraveled. Therefore, it is necessary to obtain more insight into other underlying mechanisms not only from a biological point of view but also for future therapeutic purposes.

Only scarce information is available on the contribution of defects in the DNA mismatch repair system to the etiology of Wilms tumors. A defective mismatch repair system makes cells more vulnerable to mutations resulting in an increased cancer risk (10, 11). A defective mismatch repair system may result from mutations or epigenetic alterations in one of the mismatch repair genes: *MLH1, MSH2, MSH6* and *PMS2* (10-15). These genes encode proteins that survey newly replicated DNA and repair mismatched nucleotides (10-15). If any of these proteins is inactive, mismatches preferentially occur in short repetitive DNA sequences, known as microsatellites (10-12, 14). The number of repeat units within a microsatellite in mismatch repair deficient cells deviates from patient matched normal cells and is called microsatellite instability (MSI) (10-12, 14). Hence, MSI is a phenotypic marker of a defective mismatch repair system (10-15).

Until now, only one study evaluated MSI in Wilms tumors. They found MSI in 2% of the Wilms tumors samples, suggesting that defects in DNA mismatch repair may contribute to the pathogenesis of Wilms tumors (16). However, this has never been confirmed in another Wilms tumor cohort and the underlying cause of MSI was not investigated. For that reason, we aimed to confirm the possible role of DNA mismatch repair defects in another Wilms tumor cohort and to elucidate whether

MSI in Wilms tumors resulted from a somatic *MLH1* promoter methylation or from a mostly inherited mutational inactivation of one of the mismatch repair genes. This is the first study on the evaluation of mismatch repair defects in Wilms tumor patients by the combined application of MSI analysis by a fluorescent multiplex PCR-based assay and immunohistochemistry for the expression of mismatch repair proteins.

MATERIALS AND METHODS

Materials

All included Wilms tumor patients were identified from a prospectively collected database that contains all pediatric (0-18 years) oncology patients in Erasmus MC - Sophia Children's Hospital. Extensive clinical information, histological data and tumor material were available for 121 Wilms tumor patients diagnosed between January 1987 and January 2010. For tumor DNA extraction and immunohistochemistry (IHC), we selected routine formalin-fixed and paraffin-embedded (FFPE) tissue blocks containing tumor, and, when possible, including all the three Wilms tumor components (stromal, epithelial and blastemal cells) with minimal admixture of necrosis or normal renal tissue. The molecular investigations with the tissues were performed according to the code for the proper secondary use of human tissue, established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org). Informed consent was obtained accordingly.

Methods

MSI-analysis

The pathology archive contained routine FFPE tumor-tissue samples from all patients. Twenty to 30 consecutive 4μm sections were cut from these FFPE tumor-tissue specimens and routinely mounted on microscopic glass slides. These sections were deparaffinised, and the last section of the series was routinely stained with hematoxylin and eosin (H&E). This H&E section was used as a reference for the isolated tissue parts. Although MSI can be reliably detected even when DNA is isolated from a tissue fragment composed of only 10% neoplastic cells, we preferred to isolate

tumor DNA from a tissue block with a high percentage of neoplastic cells. From 10 consecutive 4μm FFPE tumor-tissue sections, DNA was extracted by adding 300μl lysis buffer (10mM Tris/HCL pH 8.0, 1mM EDTA pH 8.0, 0.01% Tween 20) containing 5% Chelex 100 resin and 30μl proteinase K (2mg/ml). After overnight incubation at 56°C, proteinase K was inactivated at 100°C for 10 minutes. Next, dissolved DNA was separated from cell debris by centrifugation at maximum speed in a microcentrifuge for 5 minutes. The DNA-containing supernatant was then carefully transferred to another Eppendorf vial.

MSI analysis was performed with the MSI analysis system of Promega (Promega, Madison, WI, USA), a fluorescent multiplex PCR-based assay in which the PCR products are separated by capillary electrophoresis using an ABI PRISM 3130*x*/ genetic analyzer (Applied Biosystems, Foster City, CA, USA). PCR was performed in a total volume of 5μl that included 2μl of a 50-fold dilution of the isolated DNA solution and 3μl mastermix. The output data were analyzed with GeneMarker software (SoftGenetics, State College, PA USA) to determine the MSI status of the tumor samples. The Promega-kit includes fluorescently labelled primers for co-amplification of five quasi-monomorphic mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) and 2 pentanucleotide markers (Penta C and Penta D). To provide information on possible sample mix-up, we added the 2 pentanucleotide markers characterized by a high level of polymorphism. MSI analysis of isolated tumor DNA was compared to the microsatellite stable cell line K562 (9). If the result of one of the quasi-monomorphic mononucleotide repeat markers was doubtful, the tumor MSI analysis was repeated in combination with patient matched normal DNA.

Immunohistochemistry

For detection of mismatch repair protein expression, immunohistochemistry was used. For this, FFPE tissue sections (4μm) were deparaffinised with xylene and hydrated with descending grades of alcohol, and antigen was retrieved in 10mM Tris-EDTA buffer (pH 9.0) in a microwave oven for 45 minutes at 100ºC. We applied the primary antibodies anti-MLH1 (Pharmingen BD, Alphen aan den Rijn, the Netherlands; clone G168-728; dilution, 1:20), anti-MSH2 (Pharmingen BD; clone

G219-1129; dilution, 1:300), anti-MSH6 (Pharmingen BD; clone 44; dilution, 1:100), and anti-PMS2 (Pharmingen BD; clone A16-4; dilution, 1:50) for 1 hour at room temperature. After washing, immunoreactivity was visualized with the Envision kit (Dako, Glostrup, Denmark). Subsequently, the sections were counterstained with Mayer hematoxylin and evaluated under a light microscope. The immunostaining was scored as follows: negative when tumor cells showed no nuclear staining for the MMR protein indicated, but the nuclear expression of the mismatch repair protein was detected in the normal cells in the same tissue section, and positive when tumor cells showed nuclear staining for the mismatch repair protein indicated.

RESULTS

Clinical data

In our institution, 121 patients were diagnosed with a Wilms tumor between January 1987 and January 2010. From all these patients, tumor samples were obtained during nephrectomy. From six patients no tumor material was stored and 18 cases could not be analyzed because the available tumor tissue was necrotic. Five patients had a bilateral Wilms tumor. From three of these patients, material of both tumors was available. In total, 100 Wilms tumors samples from 97 patients (52 female) were available for mismatch repair analysis.

The mean age at diagnosis was 3.6 years (range 3 months - 12.2 years). Forty-seven patients had stage I disease, 16 stage II, 16 stage III, 13 stage IV and 5 stage V. Pathology revealed six Wilms tumors with diffuse anaplasia. Four patients were treated with immediate nephrectomy, all other 93 patients received pre-operative chemotherapy. All patients were treated according to the protocols of the SIOP.

MSI analysis

MSI analysis on isolated tumor DNA compared to a microsatellite stable cell line K562 was normal in 80 Wilms tumor samples (Figure 1A) (9). In 20 Wilms tumor cases, the result of one of the quasi-monomorphic mononucleotide repeat markers was doubtful. Therefore, we repeated the MSI analysis in 18 of these 20 tumor samples

with DNA extracted from normal renal tissue of the same patient as control. No MSI pattern was found in any of these 18 Wilms tumor samples (Figure 1B). Of the other two samples, there was no normal DNA material available. In one of these two cases there seemed to be heterozygous NR-21 microsatellite alleles and in the other sample the shift of BAT-25 was only one nucleotide, so this case also was classified as microsatellite stable according to the guidelines (9). Therefore, we concluded that MSI analysis showed no pattern of MSI in any of our 100 Wilms tumor samples.

Immunohistochemistry

Despite the fact that some Wilms tumor samples were old archival specimens which causes a low intensity of the nuclear labelling, immunohistochemical staining was successful in the samples. Nuclear staining for MLH1, MSH2, MSH6 and PMS2 proteins was present in all Wilms tumor samples as well as in the normal renal cells that served as internal control, if present on the slides, indicating that normal expression of the MLH1, MSH2, MSH6 and PMS2 proteins was present in all 100 Wilms tumor samples (Figure 2).

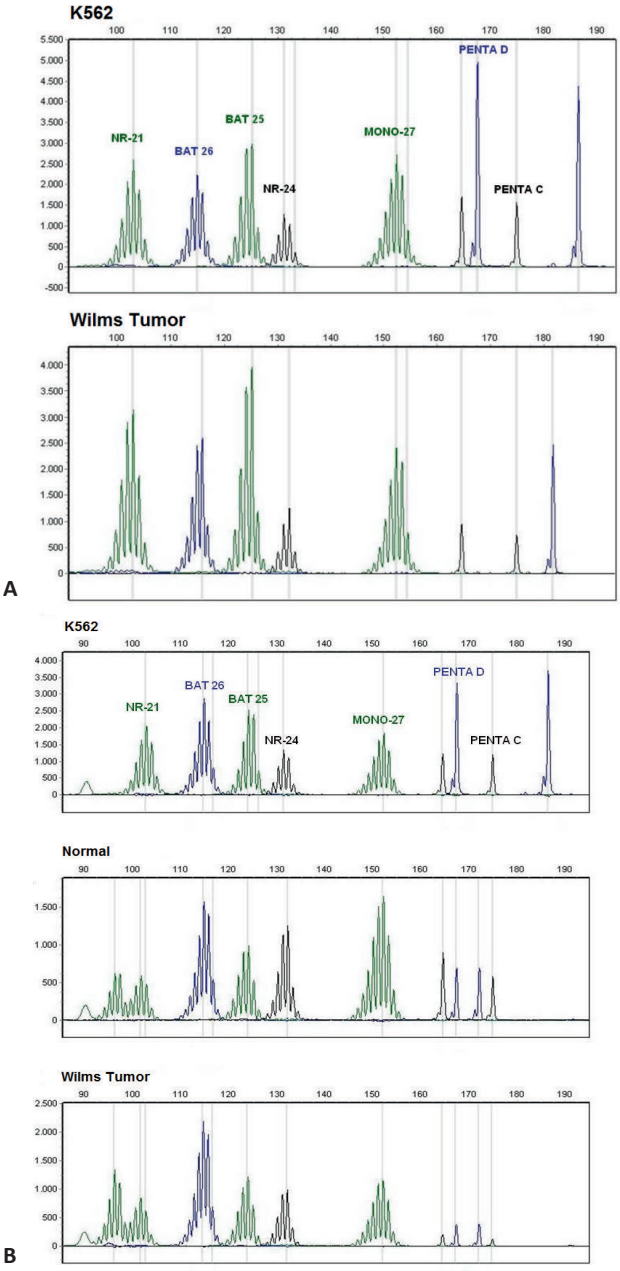

Figure 1: *MSI analysis by fluorescent multiplex PCR-based assay*
A. Normal MSI analysis on isolated Wilms tumor DNA compared to a microsatellite stable cell line K562.
B. Wilms tumor with one dubious quasi-monomorphic mononucleotide repeat marker NR-21 compared to the cell line K562, although compared to DNA extracted from normal renal tissue of the same patient, we saw that the NR-21 microsatellite alleles were heterozygous.

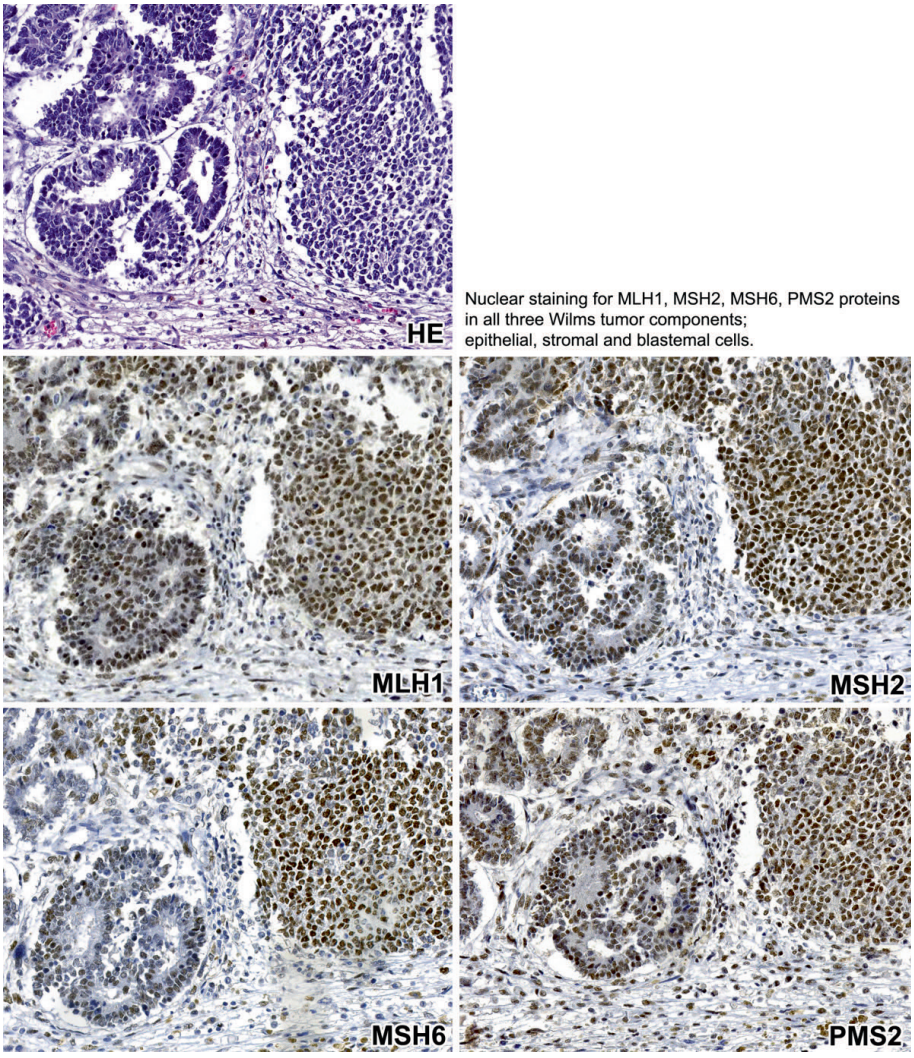

Figure 2: *Hematoxylin and eosin staining and MLH1, MSH2, MSH6 and PMS2 immunohistochemistry*

DISCUSSION

We did not identify any Wilms tumor case with MSI, so elucidating whether MSI in Wilms tumors resulted from a *MLH1* promoter methylation or a mutational inactivation of one of the mismatch repair genes was not relevant. Most sporadic other tumor types with MSI especially colon cancer result from a somatic *MLH1* promoter methylation event and not from a mutation in one of the mismatch repair genes (13, 15, 17-20). Inactivation of one of the mismatch repair genes by mutation is mostly inherited. Lynch syndrome, the most common hereditary colorectal cancer predisposing syndrome, is caused by a germline mutation in one of the mismatch repair genes with an autosomal dominant mode of inheritance (13, 15, 18, 20). Patients with Lynch syndrome have a high lifetime risk for colorectal cancer (20-70%), endometrial cancer (15-70%) and other extra-colonic cancers (<15%) (13, 15, 18, 20). Wilms tumors are not described in families with Lynch syndrome. But recently, one Wilms tumor patient with a defective mismatch repair system caused by compound heterozygosity for two *MLH1* mutations was described (21). Thus far, this is the only described Wilms tumor in a child diagnosed with the "constitutional mismatch repair-deficiency" (CMMR-D) syndrome, a childhood cancer syndrome caused by bi-allelic germline mutations in one of the mismatch repair genes (21, 22). CMMR-D syndrome is phenotypically characterized by multiple malignancies during childhood, mainly hematological malignancies, brain tumors and gastrointestinal tumors and often by clinical features of neurofibromatosis type 1 especially café-au-lait spots (21-24).

Mason and colleagues evaluated 96 Wilms tumor samples from the National Wilms Tumor Study Group (NWTSG) Biological Samples Bank by microsatellite analyses with up to 19 simple sequence repeat polymorphic genetic markers to screen for 16q LOH and two highly-sensitive MSI markers (BAT-26 and MLH1 exon 12) to screen for MSI (16, 25). In two of the 96 Wilms tumor samples, MSI was found in almost all the investigated markers including the two MSI markers, suggesting that defects in the mismatch repair system do contribute to the pathogenesis of a small minority of Wilms tumors (16). However, immunohistochemistry for the mismatch repair proteins expression was not done to substantiate these results. Moreover, neither mutation analysis of the mismatch repair genes nor a methylation assay of the *MLH1*

promoter was performed to define if the tumor was sporadically or hereditary (13, 15). Nevertheless, from Mason's study and our present study it is clear that defects in the mismatch repair system play no role or a very limited role in the development of Wilms tumors. However, it should be mentioned that the MSI pattern can vary in different tissues, as in endometrial carcinomas where the occurrence of only subtle shifts in the size of markers can lead to false negative MSI results (15, 21, 26). In addition, also in brain tumors of patients with CMMR-D syndrome, the extent of the MSI pattern can be different compared to colorectal cancers (21, 22, 24). Nevertheless, the combined analysis of mononucleotide microsatellites and mismatch repair protein immunohistochemistry in tumor samples is known to have a high sensitivity (>95%) for the detection of MSI (9, 15, 27). Moreover, in the present study all microdissected Wilms tumor tissue samples contained an adequate percentage (>10%) of neoplastic cells to detect MSI (9, 15).

A significant difference of both studies is the upfront treatment approach. In contrast to our study, in which almost all of the Wilms tumors were treated with chemotherapy before surgery (according to the SIOP protocols), in Mason's study Wilms tumors were treated according to the COG protocols, mainly with immediate nephrectomy. Van Lier and colleagues have already shown that there was no effect of pre-operative chemotherapy on the MSI status of rectal cancers (28). Our results, which match those from Mason's study, can confirm that chemotherapy also does not influence the MSI status of Wilms tumors and that surgical resection specimens obtained after chemotherapy can be used for MSI analysis.

Taken together, we conclude that mismatch repair deficiency does not play an important role in the development of Wilms tumors, indicating that future studies should be directed towards alternative mechanisms.

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6

GAIN OF 1Q IS A MARKER OF POOR PROGNOSIS IN WILMS TUMORS

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ABSTRACT

Introduction

Wilms tumor trials aim to better tailor treatment intensity to the risk of relapse and death. Currently, stage, histology, age (< or > 24 months) and combined loss of heterozygosity (LOH) at 1p and 16q in chemotherapy-naïve Wilms tumors, are the only risk factors used for treatment stratification. However, they predict only less than one third of all relapsing patients, implying that other factors are involved in treatment failure. Previous studies have associated 1q gain with adverse outcome. Therefore, we investigated the role of 1q gain and other common cytogenetic aberrations (CA) in Wilms tumors.

Materials and Methods

The prognostic role of 1q gain and other common CA was analyzed in the largest series of Wilms tumor karyotypes so far compiled and related to follow-up data from Wilms tumor patients treated in the UK.

Results

19% (64/331) had 1q gain. Gain of 1q was significantly associated with 16q loss ($p<0.001$) and 1p loss ($p<0.001$). In multivariate analysis taking account of age, tumor stage, anaplasia, and common CA (e.g. 1p loss and 16q loss), 1q gain was independently associated with adverse event free survival (EFS) (hazard ratio [HR] 2.45, $p=0.02$) and overall survival (HR 4.28, $p=0.004$). Loss of 14q was independently associated with an adverse EFS (HR 4.0, $p=0.04$).

Conclusion

Gain of 1q is a marker of poor prognosis in Wilms tumors, independent of high tumor stage and anaplasia which remain the overarching adverse prognostic factors. Confirmation in other studies is necessary, before future therapeutic studies can incorporate 1q gain into new risk stratification schema.

INTRODUCTION

Wilms tumor, the most common childhood renal tumor, has a high cure rate after treatment with chemotherapy, surgery and selectively, radiotherapy (1-11). The current treatment trials of the International Society of Paediatric Oncology (SIOP) and the Children's Oncology Group (COG, formerly National Wilms Tumor Study Group (NWTSG)), the two major international Wilms tumor study groups, report survival rates of approximately 90%. However, almost half of all survivors have currently been exposed to treatments with a risk of long term side effects and, for those that do relapse, about 50% will have a fatal outcome despite intensive second line treatment (11-13). There is therefore a clinical need to further improve patient selection for either more or less intensive first line therapy according to a more individualized prediction of their relapse risk. Currently, well established prognostic factors for risk-adapted treatment stratification include tumor stage and histology; in particular, diffuse anaplasia in pretreated (14, 15) as well as in chemotherapy-naïve tumors (16, 17) and blastemal type in pretreated tumors (15). In addition, the molecular markers loss of heterozygosity (LOH) at 1p and 16q have been identified as markers of poor prognosis in chemotherapy-naïve tumors and are now used for treatment stratification in COG studies (18). However, all these prognostic factors are present in the Wilms tumors of less than one third of the children who subsequently relapse or die (2, 3, 11). This indicates that other factors are involved in treatment failure, and underlines the need to identify novel prognostic markers for more effective treatment stratification.

Some previous studies have associated gain of 1q or overexpression of genes from 1q with an adverse outcome (2, 4, 19-21). In this study, we have further investigated the role of 1q gain as a biomarker in Wilms tumors. Since gain of 1q typically involves a large segment of the long arm of the chromosome, we took advantage of the availability of cytogenetic analysis by routine tumor karyotyping that has been performed in the majority of childhood cancer treatment centers in the United Kingdom (UK). We collected all available cytogenetic data from Wilms tumor patients treated in the UK to evaluate the adverse prognostic significance of 1q gain in a larger series than any previously reported. In addition, we wanted to clarify the cytogenetic mechanisms underlying 1q gain in Wilms tumors.

Apart from 1q gain, other cytogenetic abnormalities (CA) such as loss of 1p, 11q, or 16q, loss of chromosome 22 relative to the ploidy of the cell, and karyotype complexity have also been reported to be correlated with adverse outcome in Wilms tumors (2, 4, 18, 19, 21-24). A better understanding of such genetic outcome predictors could contribute to an improved stratification of current treatments. Therefore, as an extension of our previous study (21), we compiled the largest series of Wilms tumor karyotypes yet published, and we analyzed the possible clinico-pathological and outcome associations of the most common genetic features reputed to be of prognostic significance.

MATERIALS AND METHODS

Patients

As an extension of our previous national study (21), this study included 331 Wilms tumor patients treated at centres of the Children's Cancer and Leukaemia Group of the UK (CCLG) (formerly UK Children's Cancer Study Group=UKCCSG); 41 (12%) patients were enrolled in the clinical trial UKW2, 202 (61%) patients in the clinical trial UKW3, and 88 (27%) patients were included in the SIOP WT 2001 trial. In the UKW2 trial, most patients were treated with immediate nephrectomy (25), whereas in the UKW3 trial, patients were randomized to either pre-operative chemotherapy and delayed nephrectomy or to immediate surgery, with approximately half of the patients receiving one or other approach (7, 11). In the era of the SIOP WT 2001 trial (2001- onwards), the UK has altered its national standard for patients to be treated with pre-operative chemotherapy (26). Clinical data including age at diagnosis, sex, tumor stage, histology, treatment and outcome were collected. Histological subtype and staging were reviewed by the national CCLG pathologist (15, 27). All UKW2 Wilms tumor samples were classified into favorable (no anaplasia) and unfavorable (anaplasia) histology according to the criteria of Beckwith (28). This was also true for the UKW3 Wilms tumor samples treated with immediate or delayed nephrectomy. UKW3 Wilms tumor cases treated with pre-operative chemotherapy were later reclassified according to the SIOP 2001 classification by G. Vujanic (15). All SIOP

2001 Wilms tumor samples were classified according to the SIOP 2001 classification (15). For the analyses in this study, all cases were defined for histology as anaplastic (diffuse or focal) or non-anaplastic Wilms tumor regardless of the initial treatment modality (pre-operative chemotherapy or immediate nephrectomy).

Cytogenetic Analysis

Between the start of 1986 and the end of 2007, eighteen laboratories of the United Kingdom Cancer Cytogenetics Group (UKCCG) contributed karyotypes of Wilms tumor samples from nephrectomy or biopsy samples, taken prior to chemotherapy, of patients enrolled on clinical trials UKW2-3 or SIOP WT 2001. Cytogenetic methods varied slightly between the different laboratories, but typically involved collagenase disaggregation followed by short-term and/or long-term culture before harvesting by standard techniques and G-banding. All karyotypes were analyzed and described according to the International System for Human Cytogenetic Nomenclature (ISCN 2013) (29). Cytogenetic criteria for inclusion in the study were full analysis of at least ten metaphase cells for a normal karyotype, and at least two metaphases showing the same structural rearrangement or chromosome gain, or three metaphases showing the same chromosome loss for a clonal abnormality.

For the analysis of CA, numerical abnormalities were defined as the gain or loss of a whole chromosome relative to the ploidy of the cell. Structural aberrations were defined as aberrations resulting from breakpoints within at least one chromosome. For unbalanced structural abnormalities, the chromosomal regions of partial gain or loss were defined. For balanced translocations, we described only the breakpoint as - by definition - no chromosomal material was gained or lost at the resolution of conventional karyotyping. The presence of one or more numerical abnormalities together with two or more structural aberrations was considered as complex karyotype, consistent with our previously used definition (21). Abnormalities identified in incomplete (n=10) and composite karyotypes (n=32) were included. In addition, 42 of the abnormal karyotypes showed clonal sidelines: for these analyses, all CA in every sideline were included. Bilateral disease (stage V) was present in 29 patients: karyotypes from both tumors were available for only three of these – for the remaining 26 patients, karyotypes were available from only one tumor. All the CA of both karyotypes in these three stage V patients were amalgamated for analysis.

Statistical Analysis

The association of 1q gain and other frequent CA with age at diagnosis (<2 years, 2-4 years, >4 years) was tested by a Cochran-Armitage trend test. The trend test tends to be more powerful if the categories follow a certain ordering. The association of 1q gain and other frequent CA with stage (stage I, II, III, IV, and V) was analyzed using the standard χ^2-test, and with bilateral disease (stage V or no stage V = stage I-II-III-IV) and with histology (anaplastic or non-anaplastic) using the Fisher's exact test. The association of gain of 1q with loss of 16q, loss of 1p, or loss of 16q and 1p was also tested by the Fisher's Exact test.

Event free survival (EFS) was defined as the time from diagnosis to the first event or last follow-up. Events included refractory disease (coded as an event at day 0), first relapse, and death from any cause. Overall survival (OS) was defined as the time from diagnosis to death (from any cause) or to most recent follow-up. For EFS and OS analyses, patients who did not experience an event were censored at the time of last follow-up. All 29 stage V patients were excluded from the survival analyses, as in 26 of these cases the karyotype of the second tumor was unknown. Hazard ratios (HR), together with 95% confidence intervals (CI), for the effect of gain of 1q and other CA that had a prognostic value in previous studies or frequently occurred in Wilms tumors namely loss of 1p, loss of 11q, loss of 16q, loss of chromosome 22, complex karyotype, loss of 14q, loss of 4q, gain of chromosome 12, gain of chromosome 10, and gain of chromosome 8 and clinical characteristics (age at diagnosis, stage and histology) were estimated using Cox proportional hazard regression analysis. In order to evaluate confounding effect of the clinical characteristics and CA mentioned above, a multivariate model was also included. All variables were analyzed as categorical variables. These analyses were performed with SPSS Statistics version 17.0 (SPSS Inc. Chicago, IL, USA) and with the statistical environment R version 2.15.0 (30-3-2012) (30). All tests were two-tailed and a p-value less than 0.05 was considered significant.

RESULTS

Clinical characteristics and distribution of cytogenetic aberrations by clinically relevant parameters (age at diagnosis, stage, anaplasia)

Of the 331 included Wilms tumor cases, 209 (63%) showed clonal chromosome abnormalities, whereas a normal karyotype (46,XX or 46,XY) was present in the remainder (122/331, 37%). The median age at diagnosis of the 331 cases in the study was 2.8 years (range 0.1-14.2). Centrally reviewed data on tumor stage and histology were available for all but respectively 19 and 22 of the 331 cases. Stage distribution showed 33% stage I, 19% stage II, 21% stage III, 18% stage IV, and 9% stage V (Table 1). Anaplasia (diffuse or focal) was identified in 22 of 309 tumors (7%) (Table 1).

Patients with an abnormal karyotype Wilms tumor were older (median age 3.0 years; range 0.2-14.0) than patients with normal karyotype Wilms tumors (median age 2.1 years; range 0.1-11.9) ($p<0.001$ by Mann-Whitney U test). In addition, young children (age <2 years) were less likely to have 16q loss as compared to patients aged 2-4 years and older than 4 years (3% *vs* 20% *vs* 1%, $p<0.001$), as well as loss of chromosome 22 (2% *vs* 6% *vs* 11%, $p=0.01$), and isochromosome 1q (7% *vs* 40% *vs* 53%, $p=0.01$) (Table 1). Complex karyotype was associated with advanced disease (stage III and IV) ($p=0.002$) (Table 1).

Prevalence and underlying mechanisms of 1q gain

Of the 331 included Wilms tumor cases, 64 (19%) showed gain of 1q, the most frequently observed structural aberration. Among abnormal karyotypes, 31% (64/209) showed this feature. We found no difference in prevalence of 1q gain between Wilms tumors treated with immediate nephrectomy (n=26/119, 22%) or those treated with pre-operative chemotherapy (n=35/184, 19%) ($p=0.56$, Fisher's Exact test). There was no significant difference in age at diagnosis, stage, or anaplasia between the group with and without gain of 1q (Table 1).

Table 1: *Association of the most frequent cytogenetic aberrations with clinically relevant parameters*

	N (%)	Abnl ktype		1q+		1p-		16q-		11q-		cx		4q-		14q-		
		N (%)	p	N (%)	p	N (%)	p	N (%)	p	N (%)	p	N (%)	p	N (%)	p	N (%)	p	
All patients	331	209 (63)		64 (19)		21 (6)		42 (13)		21 (6)		53 (16)		14 (4)		14 (4)		
Age (yrs)			**<0.001**		0.07		0.12		**0.003**		*0.02*		*0.02*		0.97		0.5	*
0-2	113 (34)	54 (48)		15 (13)		4 (4)		3 (3)		3 (3)		12 (11)		5 (5)		5 (4)		
2-4	127 (38)	88 (69)		28 (22)		9 (7)		25 (20)		8 (6)		20 (16)		5 (4)		3 (2)		
>4	91 (28)	67 (74)		21 (23)		8 (9)		14 (15)		10 (11)		21 (23)		4 (4)		6 (7)		
Stage[a]	312	197 (63)	0.09	62 (20)	0.39	21 (7)	0.05	40 (13)	0.38	21 (7)	0.17	51 (17)	**0.002**	14 (4)	0.63	14 (5)	0.48	**
I	101 (33)	40 (39)		16 (16)		4 (4)		11 (11)		5 (5)		12 (12)		4 (4)		3 (3)		
II	60 (19)	47 (78)		12 (19)		3 (5)		11 (19)		3 (5)		5 (9)		3 (5)		3 (5)		
III	66 (21)	36 (55)		18 (27)		10 (15)		5 (8)		9 (14)		21 (32)		5 (8)		5 (8)		
IV	56 (18)	12 (27)		12 (22)		3 (6)		9 (16)		3 (5)		10 (18)		1 (2)		3 (5)		
V[b]	29 (9)	12 (41)		4 (14)		1 (3)		4 (14)		1 (3)		3 (10)		1 (4)		0 (0)		
Histology[c]	309	196 (63)	0.07	63 (20)	0.79	21 (7)	1.00	40 (13)	0.05	21 (7)	0.18	51 (17)	**<0.001**	14 (5)	**0.01**	14 (5)	**<0.001**	+
Anaplasia	22 (7)	5 (23)		5 (23)		1 (5)		6 (27)		3 (14)		11 (52)		4 (18)		6 (28)		

	N (%)	22-		8+		12+		t(1;16)		1q+ AND 16q-		i1q10		1q+ AND 1p-		1p- AND 16q-		
		N (%)	p	N (%)	p	N (%)	p	N (%)	p	N (%)	p	N (%)	p	N (%)	p	N (%)	p	
All patients	331	19 (6)		59 (18)		88 (27)		20 (6)		18 (6)		15 (5)		11 (3)		7 (2)		
Age (years)			**0.01**		0.14		0.06		0.21		0.33		**0.01**		0.07		0.24	*
0-2	113 (34)	2 (2)		24 (21)		26 (23)		3 (3)		2 (2)		1 (1)		1 (1)		1 (1)		
2-4	127 (38)	7 (6)		23 (18)		43 (34)		11 (10)		12 (9)		6 (5)		5 (4)		3 (2)		
>4	91 (28)	10 (11)		12 (13)		19 (21)		6 (7)		4 (4)		8 (9)		5 (5)		3 (3)		
Stage[a]	312	18 (6)	*0.03*	55 (18)	0.32	84 (27)	*0.02*	19 (6)	0.16	17 (5)	0.23	15 (5)	**0.01**	11 (4)	0.24	7 (2)	0.25	**
I	101 (33)	3 (3)		23 (23)		26 (26)		6 (6)		4 (4)		2 (2)		3 (3)		0 (0)		
II	60 (19)	1 (2)		12 (20)		23 (38)		5 (8)		3 (5)		0 (0)		0 (0)		3 (5)		
III	66 (21)	9 (14)		7 (11)		18 (27)		0 (0)		2 (3)		8 (12)		5 (8)		1 (2)		
IV	56 (18)	3 (5)		9 (16)		16 (29)		5 (9)		4 (7)		4 (7)		2 (4)		2 (4)		
V[b]	29 (9)	2 (7)		4 (14)		1 (3)		3 (10)		4 (14)		1 (3)		1 (3)		1 (3)		
Histology[c]	309	18 (6)	0.13	54 (18)	0.08	83 (27)	0.81	19 (6)	*0.03*	17 (5)	0.32	15 (5)	0.61	11 (4)	1.00	7 (2)	0.39	+
Anaplasia	22 (7)	3 (14)		7 (32)		5 (23)		4 (19)		2 (10)		0 (0)		0 (0)		1 (5)		

Association of the most frequent cytogenetic aberrations with clinically relevant parameters (age at diagnosis, stage, and anaplasia)
Abbreviations: Abnl ktype: abnormal karyotype, age: age in years at diagnosis, cx: complex karyotype, [a] all WT samples with available data about stage, [b] stage V or bilateral disease, [c] all WT samples with available data about histology namely anaplasia present or not. N (%): absolute number of patients with that CA (percentage of specific group, row). In **bold**: all proportions significantly different at the p< or = 0.01 level. In *italics*: all proportions significantly different at the p<0.05 level.
* trend test for age groups, ** χ^2 for stage, + Fisher's Exact test for anaplasia.

The major underlying mechanism of 1q gain was an unbalanced translocation (n=46). Among the unbalanced translocations, 16q was the most common partner site (n=17). There were 14 Wilms tumors with typical der(16)t(1q;16q) resulting in partial trisomy 1q and partial monosomy 16q. Breakpoints identified ranged from 1q10 to 1q21 and from 16q10 to 16q21. Isochromosome 1q was the other predominant form of 1q gain (n=15). A strong association was found between gain of 1q and loss of 1p and between gain of 1q and loss of 16q ($p<0.001$), reflecting respectively isochromosome 1q and the typical unbalanced translocation der(16)t(1q;16q) (Table 2).

Table 2: *Associations with 1q gain*

Cytogenetic aberrations	No 1q gain	1q gain	Fisher's exact test
1p loss	10	11	**p< 0.001**
No 1p loss	255	53	
16q loss	24	18	**p< 0.001**
No 16q loss	241	45	
Loss of 1p and 16q	4	3	p= 0.13
No loss of 1p and 16q	260	60	

Correlations between cytogenetic aberrations 1q gain, 1p loss and 16q loss determined by Fisher's exact test, with significant ($p<0.05$) values in **bold**.

Prevalence of other cytogenetic abnormalities

Overall, conventional karyotyping revealed CA in 63% of all Wilms tumor cases. Figure 1 shows that chromosome gains are more common than chromosome losses in Wilms tumors. Gain of chromosome 12 relative to the ploidy of the cell (n= 88/331, 27% of all cases) was the most frequently observed numerical CA in Wilms tumors (Figure 1). In addition, gains of chromosomes 8, 6, 7, 13 20, 18, 2, 3, 17, 10, and 9 were the other recurrent numerical CA (each seen in at least 5% of all cases). The most frequent chromosome loss was that of chromosome 22 (6%) (Figure 1).

Structural aberrations were collectively analyzed by chromosome arm, i.e. which partial chromosomal material was gained, lost or had a breakpoint of a balanced translocation (Figure 2). Chromosome arm 1q and 16q were the most frequently occurring structural CA (Figure 2). In addition to 1q gain and 16q loss, loss of 1p, 7p and 11q, and gain of 7q were the other recurrent structural CA (in at least 3% of all cases) (Figure 2).

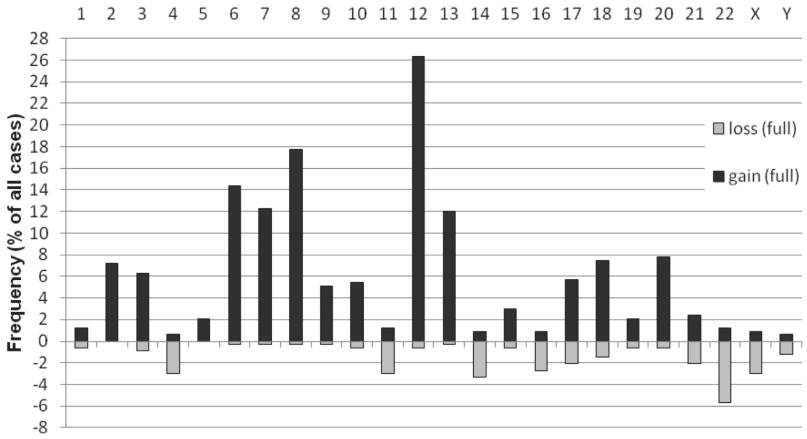

Figure 1: *Numerical aberrations: frequency (% of all cases) of gains and losses of full chromosomes*
Diagram showing frequency (percentage of all cases) of gains and losses of full chromosomes. Gains are shown on the positive Y-axis, losses are shown on the negative Y-axis. Chromosomes are on the X-axis.

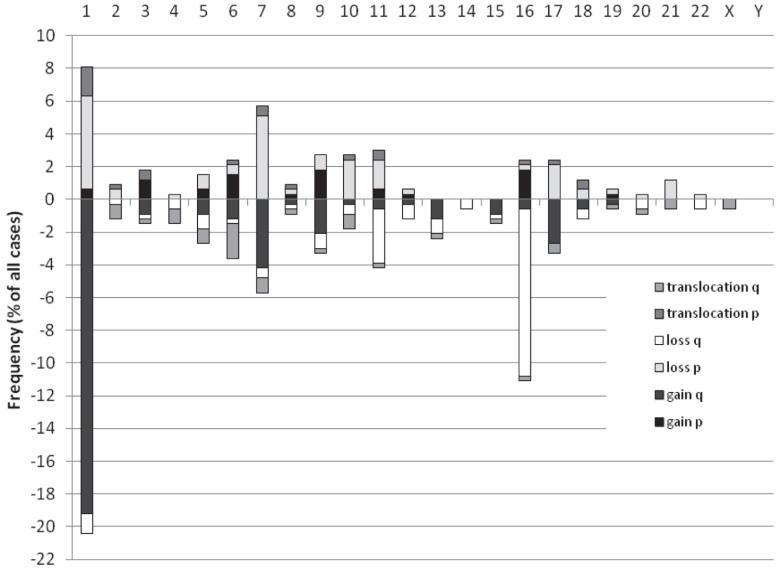

Figure 2: *Structural aberrations: frequency (% of all cases) of partial gains/losses and balanced translocations*
Diagram showing frequency (percentage of all cases) of only partial gains and losses and of translocation breakpoints. The short arms (p) of the chromosomes are shown in the positive Y-axis, the long arms (q) on the negative Y-axis. Lightest shades are used for losses, darkest shades are used for gains, medium shaded colors are used for translocations breakpoints. Chromosomes are on the X-axis.

Univariate analysis of the prognostic impact of 1q gain and the other most frequent cytogenetic aberrations and clinical parameters on survival

The median follow-up was 10 years. In all patients excluding stage V (n=29), 59 events (50 relapses, 2 disease progression, 7 deaths without relapse) and 36 deaths were observed. Five-years EFS of this group of patients was 80% (10-years EFS: 79%), and 5-year OS was 89% (10-years OS: 87%). Table 3 summarizes all results from univariate survival analyses of relevant clinical factors and the most frequent CA (for numerical CA in at least 5% of the cases, and for structural CA in at least 3% of the cases). Of the clinical factors, both advanced stage and anaplastic histology were strongly associated with both worse EFS and OS (Table 3). Of the cytogenetic aberrations, gain of 1q, loss of 16q, loss of chromosome 22 and loss of 14q were consistently negative prognostic factors for both EFS and OS (Table 3). For cases with complex karyotype and for cases with gain of 1q in combination with loss of 16q, a significant effect was evident only for EFS (Table 3). There was a trend for cases with complex karyotypes towards an adverse impact on OS (Table 3). The presence of gain of chromosome 10 was associated with a better EFS (Table 3). Neither loss of 1p nor loss of 1p in combination with loss of 16q were of prognostic importance (Table 3).

Multivariate analysis of the prognostic impact of 1q gain and the other most frequent cytogenetic aberrations and clinical parameters on survival

Table 4 summarizes the results of the multivariate survival analysis. Cox proportional hazards model for EFS and OS fitted on Wilms tumor patients from whom all prognostic variables (as described in our methods) were available (n=272) revealed gain of 1q as an independent negative prognostic factor for EFS (hazard ratio [HR] 2.45, 95% CI 1.17-5.15, p=0.02) and OS (HR 4.28, 95% CI 1.59-11.53, p=0.004) (Table 4). Loss of 14q was an independent negative prognostic factor for EFS (HR 4.0, 95% CI 1.05-15.32, p=0.04) (Table 4). The clinical parameters advanced disease (stage III and IV) (stage III; OS HR 4.55, 95% CI 1.42-14.6, p=0.01) (stage IV; EFS HR 3.63, 95% CI 1.62-8.13, p=0.002; OS HR 8.43, 95% CI 2.63-27.07, p<0.001) and anaplasia (OS HR 6.13, 95% CI 1.75-21.47, p=0.005) remained the most significant unfavorable prognostic predictors in these models (Table 4).

Table 3: *Results from univariate survival analysis*

		EFS (5y)					OS (5y)				
		N	**Events**	**HR**	**95% CI**	**p**	**N**	**Events**	**HR**	**95% CI**	**p**
Age (years)						0.33					0.31
0-2		89	14	1			89	8	1		
2-4		112	24	1.054	0.73-2.74		112	14	1.428	0.60-3.41	
>4		82	21	1.06	0.85-3.28		82	14	1.942	0.82-4.63	
Stage						**0.009**					**<0.001**
I		101	13	1			101	5	1		
II		60	9	1.215	0.52-2.84		60	4	1.367	0.37-5.09	
III		66	20	2.531	1.26-5.09		66	14	4.501	1.62-12.50	
IV		56	17	2.705	1.31-5.57		56	13	5.284	1.88-14.83	
Anaplasia	n	260	50	1		**<0.001**	260	30	1		**0.002**
	y	18	9	3.42	1.68-6.98		18	6	3.58	1.49-8.62	
CA											
1q+	n	224	41	1		**0.031**	224	24	1		0.05
	y	58	18	1.824	1.05-3.18		58	12	1.957	0.98-3.92	
1p-	n	262	54	1		0.72	262	33	1		0.83
	y	20	5	1.185	0.47-2.97		20	3	1.135	0.35-3.70	
11q-	n	263	56	1		0.49	263	33	1		0.79
	y	20	3	0.666	0.21-2.13		20	3	1.179	0.36-3.84	
16q-	n	245	44	1		**0.001**	245	27	1		**0.016**
	y	36	14	2.596	1.42-4.74		36	9	2.463	1.16-5.24	
22-	n	267	51	1		**<0.001**	267	30	1		**0.001**
	y	16	8	3.342	1.59-7.05		16	6	3.841	1.60-9.24	
cx	N	233	41	1		**0.004**	233	26	1		0.06
	y	48	17	2.255	1.28-3.97		48	10	1.982	0.96-4.11	
14q-	n	269	50	1		**<0.001**	269	30	1		**<0.001**
	y	14	9	4.58	2.24-9.35		14	6	4.374	1.82-10.52	
4q-	n	270	55	1		0.35	270	33	1		0.28
	y	13	4	1.647	0.60-4.54		13	3	1.9	0.58-6.21	

2+	n	261	58	1		0.06	261	36	1		0.07
	y	22	1	0.191	0.03-1.34		22	0	0.002	0-9999999	
3+	n	262	54	1		0.64	262	32	1		0.34
	y	21	5	1.247	0.50-3.12		21	4	1.647	0.58-4.66	
6+	n	240	51	1		0.73	240	32	1		0.46
	y	43	8	0.877	0.42-1.85		43	4	0.68	0.24-1.92	
7+	n	244	54	1		0.19	244	35	1		0.045
	y	39	5	0.548	0.22-1.37		39	1	0.168	0.02-1.22	
7p-	n	268	55	1		0.56	268	32	1		0.1
	y	15	4	1.355	0.49-3.74		15	4	2.364	0.84-6.69	
7q+	n	234	51	1		0.42	234	32	1		0.31
	y	49	8	0.735	0.35-1.55		49	4	0.59	0.21-1.67	
8+	n	232	52	1		0.18	232	32	1		0.24
	y	51	7	0.592	0.27-1.30		51	4	0.541	0.19-1.53	
9+	n	268	57	1		0.47	268	36	1		0.14
	y	15	2	0.601	0.15-2.46		15	0	0.002	0-9999999	
10+	n	265	59	1		**0.033**	265	36	1		0.11
	y	18	0	0.002	0-999999		18	0	0.002	0-9999999	
12+	n	200	48	1		0.05	200	29	1		0.16
	y	83	11	0.525	0.27-1.01		83	7	0.56	0.25-1.28	
13+	n	248	53	1		0.56	248	33	1		0.45
	y	35	6	0.776	0.33-1.81		35	3	0.635	0.20-2.07	
17+	n	266	57	1		0.41	266	34	1		0.93
	y	17	2	0.56	0.14-2.28		17	2	0.943	0.23-3.92	
18+	n	260	57	1		0.15	260	35	1		0.22
	y	23	2	0.369	0.09-1.51		23	1	0.31	0.04-2.26	
20+	n	259	54	1		0.95	259	34	1		0.52
	y	24	5	0.973	0.39-2.43		24	2	0.632	0.15-2.63	
t(1;16)	n	267	54	1		0.24	267	34	1		0.98
	y	16	5	1.715	0.69-4.29		16	2	1.015	0.24-4.23	

i1q10	n	269	54	1		0.14	269	32	1		0.07
	y	14	5	1.957	0.78-4.89		14	4	2.507	0.89-7.09	
1q+AND16q-	n	267	52	1		**0.011**	267	33	1		0.25
	y	13	6	2.869	1.23-6.69		13	3	1.974	0.61-6.44	
1p-AND16q-	n	274	56	1		0.52	274	35	1		0.86
	y	6	2	1.595	0.39-6.52		6	1	1.208	0.17-8.71	
1q+AND1p-	n	271	55	1		0.11	271	33	1		0.1
	y	10	4	2.222	0.81-6.13		10	3	2.632	0.81-8.56	

The relative risk (HR, together with 95% CI) of the 5-years probabilities of EFS and OS, for the effect of the most frequent characteristics, were estimated using a Cox proportional hazards model.

Abbreviations: N: number of patients, 95% CI: 95% confidence interval, CA: cytogenetic aberrations, cx: complex karyotype, HR: Hazard ratio, EFS: event free survival estimate (5 year), n: no, p: p-value from log-rank test, OS: overall survival estimate (5 year), y: yes. In **bold**: factors significant at p<0.05 level.

Table 4: *Results from multivariate survival analysis (Cox proportional hazards regression analysis)*

		EFS			OS		
		HR	**95% CI**	**p**	**HR**	**95% CI**	**p**
Age (years)							
0-2		1			1		
2-4		1.01	0.48-2.13	0.97	1.15	0.42-3.15	0.78
>4		0.75	0.34-1.66	0.47	0.85	0.3-2.4	0.76
Stage							
I		1			1		
II		1.23	0.49-3.12	0.66	1.64	0.4-6.75	0.5
III		2.29	1.01-5.21	0.05	4.55	1.42-14.6	**0.01**
IV		3.63	1.62-8.13	**0.002**	8.43	2.63-27.07	**<0.001**
Anaplasia	n	1			1		
	y	2.55	0.89-7.35	0.08	6.13	1.75-21.47	**0.005**
CA							
1q+	n	1			1		
	y	2.45	1.17-5.15	**0.02**	4.28	1.59-11.53	**0.004**
1p-	n	1			1		
	y	0.37	0.1-1.37	0.14	0.23	0.05-1.1	0.07
11q-	n	1			1		
	y	0.69	0.2-2.43	0.56	1.12	0.29-4.28	0.87
16q-	n	1			1		
	y	1.83	0.8-4.16	0.15	1.55	0.56-4.33	0.4
22-	n	1			1		
	y	2.56	0.95-6.87	0.06	2.64	0.79-8.86	0.12
cx	n	1			1		
	y	0.84	0.35-2.04	0.7	0.53	0.18-1.6	0.26
14q-	n	1			1		
	y	4	1.05-15.32	**0.04**	3.12	0.77-12.73	0.11
4q-	n	1			1		
	y	0.58	0.11-3.08	0.53	0.95	0.14-6.6	0.96
8+	n	1			1		
	y	1.36	0.52-3.59	0.53	1.08	0.3-3.84	0.91
10+	n	1			1		
	y	0	0-9999999	0.57	0	0-9999999	0.64
12+	n	1			1		
	y	0.53	0.22-1.24	0.14	0.44	0.14-1.38	0.16

Abbreviations: CA: cytogenetic aberrations, 95% CI: 95% confidence interval, cx: complex karyotype, HR: Hazard ratio, EFS: event free survival estimate (5 year), n: no, p: p-value from log-rank test, OS: overall survival estimate (5 year), y: yes. In **bold**: factors significant at p<0.05 level.

DISCUSSION

This analysis of the largest series of Wilms tumor karyotypes published to date revealed a high prevalence of 1q gain (19%) in Wilms tumors as well as an association with adverse outcome (4.3-fold increased risk of death), consistent with previous studies using different analytical approaches (2, 4, 19, 31-33). In addition, this analysis, as most others, showed that high tumor stage and anaplastic histology are always the overarching adverse factors for relapse or death in children with Wilms tumor. The combination of high prevalence and high relative risk of adverse outcome makes 1q gain a potentially strong biomarker for clinical application. The lack of association of 1q gain with age at diagnosis, stage and anaplasia indicates that 1q gain is a prognostic marker independent of these clinical characteristics, which was also confirmed by our multivariate analysis. In addition, there was no difference in prevalence of 1q gain between Wilms tumors treated either with immediate nephrectomy or pre-operative chemotherapy, suggesting that 1q gain is present throughout the tumor at diagnosis and not just in a chemoresistant subclone.

Fifty percent of the Wilms tumor cases with 1q gain were caused by an unbalanced translocation between chromosomes 1 and 16 (27%) mostly observed as der(16)t(1;16)(q10~q21;q10~q13) or by isochromosome 1q (23%). This can explain respectively the strong association between 1q gain and 16q loss and between 1q gain and 1p loss, similar to previous studies (4, 19). However, we could neither establish an adverse outcome of Wilms tumor patients with t(1;16) nor with isochromosome 1q. This is probably due to the small numbers in each subgroup. In addition, der(16)t(1;16) was responsible for 40% of all 16q loss cases, and isochromosome 1q for one-third of the 1p loss cases. We also could not confirm an independent prognostic value of loss of 16q and/or loss of 1p for survival, which possibly may be due to the small numbers in each subgroup. This is in contrast to previous studies that have revealed LOH at 16q and/or 1p as a significant predictor of tumor relapse and death (18, 34-37). Some of these studies have suggested that these genetic events may be surrogates for other events critical in Wilms tumorigenesis, in particular der(16)t(1;16) with simultaneous 16q loss and 1q gain or isochromosome 1 with 1p loss and 1q gain (18, 34, 35, 38, 39). We cannot exclude a small effect from

der(16)t(1;16) or isochromosome 1q being the underlying route to gain of 1q, but it may just be that only gain of 1q is the final mechanism that drives the adverse behavior. The important gene(s) on 1q that contribute to the adverse outcome are still unknown. Alternatively, gain of 1q may not be mechanistically so important but rather be the 'tip of the iceberg' indicating genomic instability in the tumor, not all of which will be evident on karyotyping. Studies using other more sensitive methods such as single nucleotide polymorphism (SNP) arrays that interrogate allelic imbalances as well as small regions of copy number variation may be necessary to further investigate this. It is therefore of interest that karyotype complexity, which is a reflection of genomic instability, was also an adverse factor for EFS, though not in the multivariate analysis. In addition, the association of karyotype complexity with advanced disease (stage III and IV) and anaplasia in our series may underscore that genomic instability also reflects more aggressiveness of Wilms tumors.

Since it appears that the most promising genetic targets for risk stratification in Wilms tumors are copy number abnormalities involving relatively large chromosome segments, the optimum technique to detect these abnormalities on a routine basis needs to be assessed. Classical cytogenetics, while highly informative, is considered labor-intensive and expensive. It can also be unreliable, with tissue culture failing to produce metaphase cells for karyotype analysis. Interphase FISH could be used to evaluate a set of the most common imbalances. Multiplex ligation-dependent probe amplification (MLPA) is an inexpensive approach to measure copy numbers at multiple loci and has been successfully applied to analysis of Wilms tumors (33) and neuroblastoma (40). While neither FISH nor MLPA give a fully pan-genomic overview of the tumor genome, array-based techniques do have this facility and both oligo-aCGH and SNP array approaches have been reported in Wilms tumor studies (2, 4, 24, 31, 41-44).

Recently, MLPA analysis of 226 NTWS-4 Wilms tumor samples also revealed 1q gain as a poor prognostic marker (33). Gain of 1q predicted a larger proportion (+/- 40%) of relapses, compared to LOH at 1p and 16q (+/- 9%) (18, 33). Large studies using MLPA are currently being performed in NWTS-5 (33) and SIOP 2001 Wilms tumor cases. If the adverse prognostic significance of 1q gain can be confirmed, future clinical trials can incorporate 1q gain into new risk stratification schema.

Overall, this study showed a normal karyotype in one third of all cases and a similar pattern of chromosomal aberrations to previous cytogenetic studies of Wilms tumors (23, 45, 46). Observing a normal karyotype with conventional karyotyping can be due to overgrowth of fibroblasts during culture. However, results from SNP array studies (41, 44) are consistent with 23-30% of Wilms tumors genuinely showing normal karyotypes in the malignant clone. Therefore, we accepted the normal karyotypes as representing the tumor clone, and included them in our statistical analyses. Gain of chromosomes relative to the ploidy of the cell was much more common than loss, with gain of chromosome 12 (27%) being the most common gain (19, 23, 32, 44-49)(50). As also previously described, loss of chromosome 22 (6%) was the most common loss in Wilms tumors, and was associated with an older age at diagnosis (> 4 years) (21, 22). Unlike previous studies (22, 23), we could not confirm an association with adverse outcome, although we cannot exclude a small effect due to the small numbers in this subgroup.

In a recent study, allelic loss of 4q and 14q were associated with anaplastic histology (24). Moreover, in a whole genome SNP array study, allelic imbalance at chromosome arm 14q was seen more often in relapsing tumors (2). Therefore, we decided to include these two less common CA (3.9% and respectively 3.6%) in our analyses. We could confirm an association between loss of 4q or 14q and anaplasia. Moreover, 14q loss was independently associated with adverse outcome in a multivariate analysis that took account of anaplasia. Hence, there may be additional features on 14q that drive this adverse tumor behavior, indicating that future studies should be directed towards possible causes of the adverse outcome associated with loss of 14q.

Taken together, this analysis of the largest series of Wilms tumor karyotype data strengthens the evidence for 1q gain as an adverse prognostic molecular marker in Wilms tumors, independently of high tumor stage and anaplastic histology which remain the overarching adverse factors for relapse or death. We also identified that loss of 14q was associated with an independent adverse EFS. If this adverse prognostic significance of 1q gain can be confirmed in other studies, 1q gain could be incorporated into new risk stratification schema of future therapeutic trials.

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MANAGEMENT OF ADULTS WITH WILMS TUMOR: RECOMMENDATIONS BASED ON INTERNATIONAL CONSENSUS

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ABSTRACT

Since Wilms tumor occurs rarely in adults, no standard treatment is available. Most adult patients will be diagnosed unexpectedly following nephrectomy for presumed renal cell carcinoma. Outcome for adults is inferior compared to children, although better results are reported when treated within pediatric trials. Multiple factors, including unfamiliarity of adult oncologists and pathologists with Wilms tumor, lack of standardized treatment and consequent delays in initiating appropriate risk-adapted therapy, may contribute to the poor outcome. A standardized approach for the management of adult Wilms tumors is proposed with the aim to limit treatment delay after surgery and encourage a uniform approach for this rare disease and thereby improve survival. These recommendations are based on discussions held with representatives of the renal tumor committees of the International Society of Paediatric Oncology (SIOP) or the Children's Oncology Group (COG), and has been updated with a review of more recently published institutional and trial experience of adults treated on pediatric protocols. They provide a critical evaluation of the current evidence for the management of adult Wilms tumors and propose details of how current pediatric therapeutic guidelines could be adapted for use in adults.

INTRODUCTION

Wilms tumor or nephroblastoma, an embryonal type of kidney cancer, is the most common renal malignancy in children over 6 months of age and represents approximately 85% of all pediatric renal tumors (1, 2). Currently, using a combination of surgery and chemotherapy for all children and additional radiotherapy for those with advanced stage disease and/or with unfavorable histology (in SIOP called 'high risk'), cure rates are high even for those with advanced stage disease (1, 2). In adults (≥19 years of age), Wilms tumor is extremely rare, representing less than 1% of all renal tumors (3-8). The diagnosis of Wilms tumor in adults is often unexpected following nephrectomy for presumed renal cell carcinoma (RCC), which is the most common (approximately 85%) adult renal cancer (9).

Due to the fact that Wilms tumor is exceedingly rare in adults, no standard therapy has been developed. Consequently, physicians faced with managing an adult with Wilms tumor develop an individualized treatment plan deduced from the experience in children, from numerous case reports and from a few relatively small series of adult cases (4-8, 10, 11) (Table I). For many years the literature has suggested that outcome for adults with Wilms tumor is considerably worse than that for pediatric patients (3-8, 10, 11). However, more recent data indicate the potential for improvement of outcome when adults are correctly diagnosed in time and treated according to (pediatric) protocols developed by the International Society of Paediatric Oncology (SIOP) or the Children's Oncology Group (COG) (4, 7). Still, despite these reports, the available information remains limited, making it difficult for surgeons and medical oncologists to manage an individual adult Wilms tumor patient. Because protocolized therapies in children and adolescents with Wilms tumor currently result in high survival rates (1, 2), pediatric oncologists are often be consulted by medical oncologists for the management of an adult Wilms tumor patient. This prompted several pediatric oncologists in Europe and North America with specific expertise in Wilms tumors to develop a consensus "best practice" guideline for the management of Wilms tumor in adults. The aim of this international consensus recommendation is to further improve outcome by shortening adjuvant treatment delay and by using standardized treatment. The target group of patients who will benefit from these

treatment recommendations are patients above the age of 19 years (or > 30 years in USA) who can't be included in open pediatric clinical trials.

METHODS

To provide a summary of the known literature about Wilms tumors in adults, a comprehensive search of the Pubmed database was performed for articles published until December 2010. Search-criteria relevant to adult Wilms tumor patients were used: adult, Wilms tumor, incidence, symptoms, management, treatment, outcome and toxicity. If not included initially, cross-references picked up during the review search were also selected. Only English articles were included.
The renal tumors committees SIOP and COG brought together those representatives of the major pediatric Wilms tumor treatment groups (AIEOP – Associazione Italiana Ematologia Oncologia Pediatrica, CCLG – Children's Cancer and Leukaemia group, COG Renal Tumor Committee, GPOH – Gesellschaft für Pädiatrische Onkologie und Hämatologie and SIOP-RTSG – SIOP Renal Tumor Study Group) with expertise in the treatment of adults with Wilms tumor, whether in international trial group protocols or on a national or institutional basis, to discuss how to adapt the pediatric therapeutic recommendations for use in adults. These discussions were wide-ranging, multidisciplinary and based on a review of the available literature as described above and personal experiences. Adult urologists/surgeons, medical oncologists and radiotherapists have reviewed the final recommendations to ensure their relevance for adult practice, which is where these patients present.

REVIEW OF THE LITERATURE

Epidemiology

Wilms tumor is the most common renal malignancy in children over 6 months of age with an incidence rate of 8 - 10 per million per year (1, 2, 6). The median age at diagnosis for children is 3-4 years and 90% are diagnosed before the age of 7 years

(1, 2, 6). In patients ≥ 16 years of age, it is a rarity with an incidence rate of less than 0.2 per million per year (3-8). A population-based European epidemiological study from EUROCARE (= European Cancer Registries study on cancer patients' survival and care) project, including data from 1983 to 1994 from 67 cancer registries that covered a combined population of 100 million in 22 European countries, showed that the median age at diagnosis for adult Wilms tumor patients (defined as >15 years of age) was 34 years (6) (Table 1). However, the range was wide with some patients even aged over 60 years of age (6).

Clinical presentation

While data on presenting symptoms in adults with Wilms tumor are very limited (5, 7), the few reported clinical presenting symptoms commonly consist of abdominal or flank pain, non-specific symptoms like malaise and weight loss, and less frequently hematuria and hypertension (5, 7). Although childhood Wilms tumors often are recognized on ultrasound, the radiological appearance of a Wilms tumor can be indistinguishable from the more common adult malignant renal neoplasm such as RCC (9, 12).

Genetics

A genetic predisposition is described in up to 10% of the pediatric Wilms tumor patients. The more common syndromes that predispose to Wilms tumor are those associated with germline mutations in the *WT1* gene which encodes a zinc finger nucleic acid-binding protein with multiple roles in gene regulation and development, and those associated with overgrowth typified by Beckwith-Wiedemann syndrome (1, 2). However, these syndromes seem not to be associated with onset of Wilms tumor in adulthood. Although, one case report of an adult Wilms tumor patient with a *WT1* mutation and one case report of an adult Wilms tumor with hypospadias and cryptorchidism are described (13, 14). Not only germline aberrations but also somatic mutations in several genes have been identified in pediatric Wilms tumors, like *WT1* mutations in ~ 15%, gain-of-function mutations in Wnt-signaling component CTNNB1 (β catenin) in ~ 15% usually in combination with *WT1* mutations, and *WTX* mutations in ~ 30% of sporadic pediatric Wilms tumors (15). *TP53* mutations occur mostly in the

relatively uncommon anaplastic Wilms tumor subtype (15). Epigenetic abnormalities at 11p15, affecting expression of *H19* and *IGF2*, are also found in pediatric Wilms tumors including those cases associated with Beckwith-Wiedemann syndrome (15). Recurrent large scale genomic changes, some associated with adverse outcome like 1p and 16q LOH, are also described in pediatric Wilms tumors. Moreover, 1p and 16q LOH are currently incorporated in the COG treatment protocols. A study of adults with Wilms tumor suggests a potential role for dysregulation of the Wnt-signaling pathway in adult Wilms tumors, as also demonstrated in some pediatric Wilms tumors (16). A further case showed isochromosome 7q as is sometimes seen in pediatric Wilms tumors (17). So far, the paucity of data available in adults makes it impossible to know whether Wilms tumors in adults and children are biologically comparable and thus similar tumor entities occurring in a different age group, as suggested by their morphological similarities.

Stage and histology

Available adult series report a higher incidence of advanced stage disease (stage III or IV) ranging from 45% till 70% (Table 1) compared with pediatric series where approximately one third of children are classified as stage III or IV after primary nephrectomy (4-8, 10, 11) (staging system in Supplementary table 2). So far, only one adult case has been reported with bilateral synchronous Wilms tumor, in contrast to the 5-7% incidence stage V disease described in the pediatric Wilms tumor population (10).

Histologically, Wilms tumor is typically described as “triphasic”, consisting of blastemal, epithelial and stromal elements (18-20). However, not infrequently, only two or even one component predominate (18, 19). There are no histological differences between Wilms tumors occurring in children and adults. However, the diagnosis of Wilms tumor is not that straightforward and can represent a diagnostic challenge not only for general pathologists who are usually not familiar with the histopathologic features and variants of these tumor, but also for pediatric pathologists. That this is also a challenge not only in adults but also in children with Wilms tumor is illustrated in the published SIOP experience: following central review by an expert pediatric pathologist, 15-20% of renal tumor cases were reclassified

(19). For that reason review of all cases by expert panels of pathologists is standard in pediatric Wilms tumor trials.

Treatment

A small number of reports are available to assess treatment and outcome in adults with Wilms tumors (4, 5, 7, 8, 10, 11) (Table 1). Most patients were treated with initial nephrectomy followed by stage adjusted chemotherapy with or without radiotherapy (4, 5, 7, 8, 10, 11) (Table 1). Only eight patients have been described who received pre-operative chemotherapy after biopsy prior to nephrectomy (7, 8) (Table 1). No cases receiving pre-operative radiotherapy have been described (4, 5, 7, 8, 10, 11) (Table 1). The fact that so many adults underwent immediate nephrectomy, even in Europe where surgery is usually recommended after pre-operative chemotherapy in children, is not surprising since immediate surgery is the most common approach for RCC. Patients after nephrectomy were generally treated with chemotherapy and/or radiotherapy, over the years more adapted according to stage and histology appropriate regimens used in pediatric trials (4, 5, 7, 8, 10, 11) (Table 1). In general, most patients with stage I favorable histology (FH) Wilms tumor received actinomycin D (ActD) and vincristine (VCR), those with stage II FH Wilms tumor received Act, VCR with or without DOX and/or radiotherapy and those with stage III and IV disease mostly received ActD, VCR and DOX in addition to radiation therapy. Available data indicate that 'exceptions' were made, with many patients treated more intensively than their stage equivalent pediatric counterparts (4, 5, 7, 8, 10, 11).

Survival / Prognosis

Available series suggest that outcome for adults with Wilms tumor has improved considerably due to the use of multimodality treatment protocols adapted from the pediatric treatment protocols (4, 5, 7, 8, 10, 11) (Table 1). The data clearly suggest that many adults with Wilms tumor, if treated appropriately, can expected to be cured, in particular if the tumor has not spread and/or is resected completely. In 1982, the NWTSG reported for the first time experience with 31 adult Wilms tumor patients (advanced stage 51.7%) with a 3-year OS of 24% compared with 74% in childhood patients at that time (11) (Table 1). In 1990, the second report from the

NWTSG showed an important improvement with a 3-year OS of 67% in 27 adults (median age 24 years, advanced stage 58%) by adopting the multimodal treatment protocols used in children (10) (Table 1). In 2004, the third report from the NWTSG showed a 5-year OS of 82.6% of 23 adult patients (median age 21.9 years, advanced stage 43%) treated similar to their childhood counterparts according to the NWTSG protocols (4) (Table 1). This improved outcome is similar to the reported outcome in a cohort of 27 German adult patients (median age 25.4 years, advanced stage 70%) treated according to the SIOP protocols (7) (Table 1).

Delay in starting adjuvant therapy after nephrectomy is common in adults and seems to effect outcome adversely (8). The pediatric Wilms tumor treatment protocols advise starting adjuvant therapy within 7 to 14 days after nephrectomy. The 17 adults with Wilms tumor reported by the Italian group started adjuvant therapy at a median of 59 days after surgery (8). The 10 patients who started treatment within 30 days of diagnosis had a 5-year event free survival (EFS) of 60% (+/- 15%) compared to 14.3% (+/- 13%) for the seven patients with a delay greater than 30 days ($p = 0.03$). Their OS was 80% (+/- 12%) versus 28.6% (+/- 17%) ($p = 0.05$), respectively (Spreafico, 'unpublished data'). No other factors than time to starting adjuvant therapy seemed to explain the worse survival in this group of patients with a delay > 30 days (8). By making a standardized approach available for this kind of tumor, we hope the time to start adjuvant therapy can be shortened and thereby will improve outcome.

Table 1: *Previous reports on characteristics and outcome of adult Wilms tumors*

Authors	Study Group	Period	N (F/M)	Median Age (range)	Stage						PA	Postoperative treatment				EFS	OS (5 y)	Mean follow-up
					I	II	III	IV	V	?		CT only	RT only	CT+RT	No			
Mitry 2004 (6)	Europe Eurocare*	1983-1994	133 69/64	34 y (15- >60)	Localized: 15 Regional extension: 15			14	-	89	NA	NA				NA	47,3% (relative)	5 y
Izawa 2008 (3)	Review**	1973-2006	128	26 y (15-73)	NA	NA	NA	NA	-	-	NA	43	-	77	7	NA	68%	4y 6m
Terenziani 2004 (8)	Italy CNR/AIEOP	1983-2001	17 (11/6)	17,5 y (16-29)	-	8	4	5(¨)	-	-	FH: 16 UH: 1	7	-	7	2	45%	62,4%	10y 11m
Kattan 1994 (5)	France	1973-1992	22 (14/8)	24 y (16-40)	4	8	3	7	-	-	FH: 21 UH: 1	6	1	15	-	41%	55% (100m)	8y 4m
Reinhard 2004 (7)	Germany SIOP 93-01/ GPOH	1994-2001	27 (12/15)	25,4 y (15-62) (#)	6	2	9 (““)	10	-	-	LR: - IR: 25 HR: 2 RCC:2***	13	-	14	-	57% (#)	83% (#)	4y (#)
Kalapurakal 2004 (4)	USA NWTS 4-5	1988-2001	23 (13/10)	21,9 y (16,3-51,3)	5	8	6	4	-	-	FH: 23 UH: -	10	-	13	-	77,3% ****	82,6% ****	5y 1m
Arrigo 1990 (10)	NWTS 2-3	1979-1987	27 (NA)	24 y (16-74)	6	5	4	11	1	-	FH: 23 UH: 4 (all st IV)	5	-	19	3	NA	67% (3y) (##)	2y 1m

Abbreviations:
NA: not available, SIOP: International Society of Pediatric Oncology, NWTS: National Wilms tumor studies, CNR-AIEOP: Consiglio Nazionale Ricerche/Asspcoazione Italiana Ematologia Oncologia Pediatrica, y: year(s), m: month(s), ref: references, CT: chemotherapy, RT: radiotherapy, LR: low risk, IR: intermediate risk, HR: high risk, PA: pathology/ histology, FH: favorable histology, UH: unfavourable histology, nephr: nephrectomy, st: stage.
* Epidemiologic study
** Review with collected data from 128 adult WT patients. 6 cases from their 2 Canadian centres which haven't been published before, the remaining 122 patients were from previous published case reports or case series including 3, 4, 5, and 6.
*** 2 patients with renal cell carcinoma (RCC) and Wilms tumor in the same kidney
**** Analyses based on data from this report combined with data from 22 FH Wilms tumors stage I-IV previously reported in 1990 by the NWTSG (Arrigo et al).
(“) Stage IV patient with diagnostic biopsy who died before nephrectomy. (““) Stage II N+ is considered stage III. ? unknown
(#): Calculated on the basis of 30 renal tumors (= 25 Wilms tumors, 2 Wilms tumors with RCC in the same kidney and 3 CCSK (clear cell sarcoma of the kidney)).
(##): Exclusion stage V patient.

Toxicity of treatment

A common late effect is cardiotoxicity due to anthracyclines (total cumulative dose, TCD > 300 mg/m2), which is more severe in combination with pulmonary irradiation. In addition, pulmonary irradiation can result in restrictive lung disease, whereas abdominal radiotherapy can cause fertility problems, growth abnormalities and impaired renal function. Renal dysfunction has been described after cyclophosphamide and carboplatin in adults, mostly as acute toxicity with a (slow) recovery (21-24). Neurotoxicity due to VCR as well as hepatotoxicity or veno-occlusive disease (=VOD) due to ActD are, like in children, also reported in adults (4, 7). In children, severe neurotoxicity due to VCR resulting in changing the dose or postpone a dose of VCR is described in 1.7% (25) and 2.4% (26) of a pediatric Wilms tumor study population. A small adult study by the German group reported that 13 of 27 (48%) adults suffered from severe (grade 3 to 4) neurotoxicity, resulting in treatment delay, dose reduction, or even discontinuation of treatment (40.7%) (7). In most patients however, the neuropathy was, at least partially, reversible (7). In children, the incidence of VOD ranges from 5 to 8% depending on the definition (27-29). If supportive management is initiated adequately and timely, it is reversible (27-29). There are no large adult series that described the incidence of VOD or severe hepatotoxicity after the administration of ActD. The GPOH reported hepatotoxicity in 1/30 (3%) adult renal tumor patients (27 Wilms tumor and three clear cell sarcoma of the kidney) and severe VOD in one patient (3%), which resolved without residual effects (7). The North American study reported 23 adult Wilms tumor patients of whom three (13%) died after treatment related liver toxicity, three to six months after treatment of ActD, in the absence of right flank irradiation (4).

EXPERT COMMENTARY: RECOMMENDATIONS FOR THE MANAGEMENT OF ADULT WILMS TUMOR

Diagnostics and staging

Based on the available literature on adult Wilms tumors combined with experience of the authors of this manuscript, standardized recommendations for diagnostic work-up, staging and treatment of adult Wilms tumor patients are proposed in the Supplementary tables 1 to 5. Patients that will benefit from these recommendations are adult Wilms tumor cases above the age of 19 years that are not eligible to be included in open pediatric trials. Our primary aim is to provide a "best practice" guideline, based on international consensus, thereby minimizing postoperative treatment delay and improving survival.

Once the diagnosis is suspected, we advise, in order to avoid delay in starting therapy, timely review by an expert pediatric pathologist thereby including immunohistochemistry and molecular biology studies to exclude more commonly found tumors in this age group (Supplementary table 3). Recommended staging investigations include a CT chest and abdomen, as this have become a standard approach in adults, to detect pulmonary and hepatic metastases and to assess tumor extension, vena cava inferior involvement and function of the contralateral kidney much more reliably than ultrasound in adults (Supplementary table 1). In the past, pediatric Wilms tumor staging was largely dependent on ultrasound and conventional chest X-ray. The clinical significance of small pulmonary nodules detectable only on CT scan remains controversial in pediatric practice, but could be assumed to represent metastatic (unless proven otherwise by histology) disease in adults, although adult studies are lacking. Bone, bone marrow and central nervous system (CNS) metastases are extremely rare in pediatric patients (30) and there are no data to suggest that this may be more common in adults with Wilms tumor. Accordingly, screening for these sites is not recommended in absence of suggestive symptoms. Moreover, in case of presence of these rare metastatic sites, reconsideration of the accuracy of the diagnosis is necessary. Sperm banking in males or ovarian preservation in females could be considered immediately before starting chemotherapy without any delay, especially regimens containing cyclophosphamide or carboplatin (31). Although, we

have to remark that ovarian preservation is not yet as successful to guarantee future offsprings as sperm banking.

Supplementary table 2 shows the proposed staging system for adult Wilms tumor patients after immediate nephrectomy. For tumor staging after pre-operative chemotherapy, all such tumors should be considered at least stage III (because they all have had a biopsy) and therefore not eligible to be treated with the minimal therapy used for stage I Wilms tumors (only VCR and ActD). Supplementary table 3A shows the histopathological classification system used after immediate nephrectomy. Two histological risk groups can be identified: favorable and unfavorable (focal and diffuse anaplasia) histology. In the rare instances when nephrectomy is performed after chemotherapy in adults with Wilms tumor, we recommend using the SIOP WT 2001 classification scheme (20) (Supplementary table 3B). We advise to consider patients with any anaplastic changes (diffuse or focal) high risk or unfavorable histology as no data are available in adults suggesting that focal anaplasia has a better outcome than diffuse anaplasia (32). For the very few adult patients receiving prenephrectomy chemotherapy, we recommend treating cases with blastemal predominant histology after pre-operative chemotherapy as like pediatric high risk cases (Supplementary table 4.3).

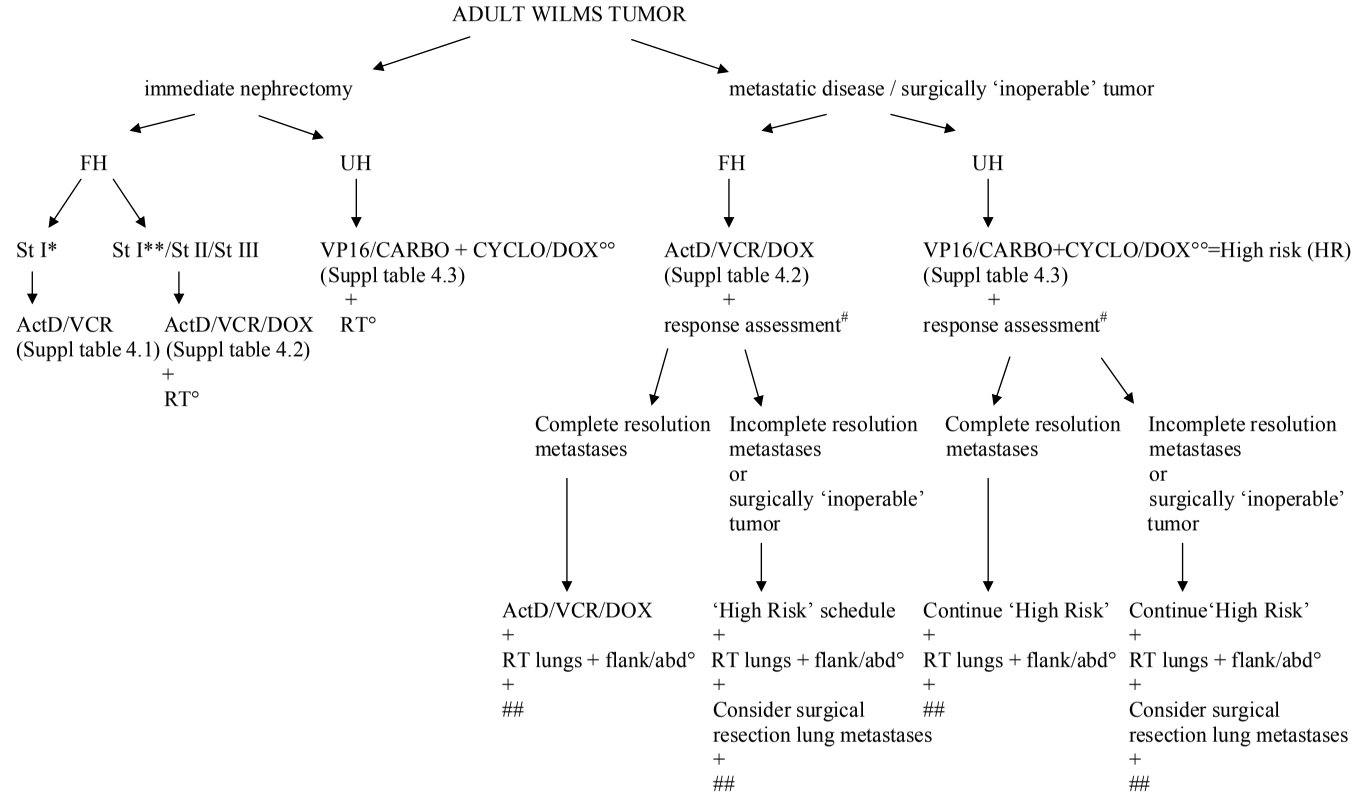
Treatment regimens

Wilms tumor is known to be a very chemosensitive tumor, with successive clinical trials aiming to improve risk stratification and reduce the burden of therapy. The backbone of pediatric chemotherapy regimens comprises VCR and ActD for low stage tumors, with DOX added in higher risk cases. Cyclophosphamide, etoposide, doxorubicin and carboplatin are used in the highest risk group.

The therapeutic recommendations for adult patients who can't be included in officially open pediatric trials are described in Figure 1 with detailed descriptions of the therapeutic regimens depicted in Supplementary tables 4.1-4.2-4.3. Although, we encourage including adults in pediatric trials where possible, like in the COG trial where cases up to 30 years are eligible for registration. Only non-advanced stage patients with optimal staging and short time interval from nephrectomy to commencement of chemotherapy are eligible for the minimum chemotherapy with

VCR and ActD, as in current pediatric SIOP and COG protocols. Although, children with stage II favorable histology Wilms tumor are treated without DOX in the current COG protocol, to improve survival, the consensus recommendation here is to include DOX, based on the lower survival rate and the average longer time from surgery to postoperative treatment in adult Wilms tumor patients. Moreover, VCR intensity is decreased in these guidelines as compared to current childhood protocols, as adults frequently develop severe neurological toxicities (7). However, these patients could always be critically discussed. In case of favorable histology by pediatric pathology review and rapid diagnosis with start of chemotherapy within 14 days after nephrectomy, treatment according to pediatric COG protocol stage II FH without DOX could be acceptable. Metastatic or 'inoperable' cases diagnosed by pre-operative biopsy should receive pre-operative chemotherapy based on histology (FH or UH) with postnephrectomy therapy based on stage, histology and response to pre-operative chemotherapy. In the very rare instances when the diagnosis of Wilms tumor is first made on a biopsy and the tumor looks amenable to immediate nephrectomy, we also recommend giving pre-operative chemotherapy. This is because the 'added value' of histological response can be incorporated into the risk stratification and because it reduces the risk of tumor rupture, makes surgery safer (in the pediatric setting) and hence should reduce the risk of needing either whole abdominal radiotherapy or boosts to areas of macroscopic residue.

Figure 1:


ADULT WILMS TUMOR
immediate nephrectomy
metastatic disease / surgically 'inoperable' tumor
FH
UH
FH
UH
St I*
St I**/St II/St III
VP16/CARBO + CYCLO/DOX°°
(Suppl table 4.3)
+
RT°
ActD/VCR/DOX
(Suppl table 4.2)
+
response assessment#
VP16/CARBO+CYCLO/DOX°°=High risk (HR)
(Suppl table 4.3)
+
response assessment#
ActD/VCR
(Suppl table 4.1)
ActD/VCR/DOX
(Suppl table 4.2)
+
RT°
Complete resolution metastases
Incomplete resolution metastases or surgically 'inoperable' tumor
Complete resolution metastases
Incomplete resolution metastases or surgically 'inoperable' tumor
ActD/VCR/DOX
+
RT lungs + flank/abd°
+
##
'High Risk' schedule
+
RT lungs + flank/abd°
+
Consider surgical resection lung metastases
+
##
Continue 'High Risk'
+
RT lungs + flank/abd°
+
##
Continue'High Risk'
+
RT lungs + flank/abd°
+
Consider surgical resection lung metastases
+
##

ActD: actinomycin D, CARBO: carboplatin, CT: computer tomography, CYCLO: cyclofosfamide, DOX: doxorubicin, VCR: vincristine, VP16: etoposide, RT: radiotherapy, st: stage, Suppl table: supplementary table.
* Only stage I FH if all four criteria are met i.e.
(1) stage and histology have been reviewed by a pediatric pathologist experienced in Wilms tumors
(2) histological examination and review has included at least one lymph node
(3) CT scan of the chest has excluded the presence of lung metastases
(4) chemotherapy can be started within 30 days of date of nephrectomy
** All other st I Wilms tumor cases
# Response assessment of metastases and operability of the primary tumor after 6 weeks by abdominal/pulmonary CT.
Delayed nephrectomy should be considered after 6 weeks of pre-operative chemotherapy if a nephtrectomy has not been performed at initial diagnosis. If the original diagnosis was made on biopsy only, then in the case of non-response or inadequate response to pre-operative chemotherapy, the histological diagnosis should be reviewed again and a further biopsy may be warranted if there is any uncertainty. Then in these cases where the Wilms tumor (histologically confirmed again) remains surgically 'inoperable', we advise to consider changing to the 'high risk' schedule or go on with this schedule and assessing operability again after 2 to 3 courses of chemotherapy.
° See Supplementary table 5
°° 'High risk' chemotherapy schedule

Surgical recommendations for nephrectomy are less detailed (Supplementary table 1) because in the majority of patients the diagnosis of Wilms tumor is made unexpectedly after nephrectomy for presumed RCC. An adult Wilms tumor patient, who has been treated surgically by anything other than an immediate open total nephrectomy with adequate lymph nodes sampling and timely pathology review, is not eligible for the pediatric style stage I therapy (only VCR and ActD). Although, we realize that open partial nephrectomy (OPN) has become the gold standard for a single small renal tumor in adults (33), and moreover that laparoscopic partial nephrectomy has emerged as a viable alternative to OPN in appropriately selected patients (33). Cases of partial nephrectomy with clear margins should be discussed individually with the national reference group for pediatric renal tumors. Laparoscopic nephrectomy should be considered as an experimental approach and needs to be treated with the three-drug chemotherapy schedule and radiotherapy. When the diagnosis of Wilms tumor has been made before nephrectomy, total nephrectomy is recommended according to adult nephrectomy guidelines for any renal cancer. We do emphasize the importance of adequate lymph nodes sampling in case of a Wilms tumor (Supplementary table 1).

Recommendations for radiotherapy are described in Supplementary Table 5. Radiotherapy, like also chemotherapy, should be planned to start ideally by day 14 postnephrectomy although starting by day 30 is acceptable.

Toxicity and tumor monitoring

Toxicity monitoring should comprise blood counts and liver function tests prior to each dose of ActD or DOX. Disproportionate thrombocytopenia and signs of hepatotoxicity will alert the physician to the possibility of VOD, a potential complication of ActD. Monitoring for impaired renal function (both glomerular and tubular) as well as possible cardiac stress/failure (especially in cases with lung irradiation in combination with DOX) or impaired lung function is recommended in patients bearing this risk.

During and after therapy, tumor monitoring by chest and abdominal imaging is recommended every 3 months for two years, since most of the relapses occur within two years of completion of therapy (1-5, 7, 8, 10).

Registration

We strongly recommend registration of each adult Wilms tumor patient within a pediatric renal tumor trial where possible according to each national regulatory situation. Although, we realize it is difficult to register patients who are treated at institutions where the pediatric trial is not open. This is a global problem and can really only be overcome by building a truly international database such as has been done successfully in the international pleuropulmonary blastoma registry. To achieve this, it requires a more specific endeavour by the academic community and funding.

FIVE-YEAR VIEW

Our aim is that these recommendations build the basis for collecting uniform and accurate data on clinical characteristics, treatment, outcome and toxicity in this rare group of adult patients with a pediatric cancer. Moreover, we hope that within five years, the many international efforts ongoing to address the problems of research and improvements in clinical practice for very rare diseases (eg RARECARE in Europe) might achieve the necessary regulatory framework approval processes that would make this feasible. So, registration of all adult Wilms tumor patients will be a fact. An international database with a coordinating center will then also facilitate the development of a biobank of adult Wilms tumors with the possibility for biology studies. The knowledge from these registry data, possibly in combination with data from biology studies, will help us to assess whether Wilms tumors in adults are any different than those occurring in children. Then, more evidence based guidelines for diagnosis and treatment of adult Wilms tumor patients with the aim to improve outcome and reduce toxicity can be developed in the future.

CONCLUSION

A standardized approach to diagnosis, staging and treatment of adults (≥19 years) with Wilms tumor is proposed based on available literature and international consensus from the field of pediatric Wilms tumor. Although we realize that with increasing age the tumor may reflect a more aggressive biological behavior and taken into account the risk of toxicity, we envisage that these consensus recommendations will facilitate decrease in postoperative treatment delay and thereby improvement in outcome.

KEY ISSUES

1. Consult a pediatric oncology colleague with experience in treatment of Wilms tumors as soon as histological diagnosis is suspected.
2. Pathological review by a pediatric pathologist expert in Wilms tumors (if Wilms tumor is suspected do not delay until other renal tumors are excluded by immunohistochemical and molecular studies).
3. Avoid delay in starting chemotherapy. Chemotherapy including radiotherapy if necessary should be planned to start ideally by day 14 postnephrectomy although starting till day 30 is acceptable.
4. Be alert for toxicity of VCR (neurotoxicity) and ActD (hepatic toxicity) in adults.
5. Register patients in pediatric renal tumor trials where possible according to each national regulatory situation.

Supplementary table 1: *Diagnostic and staging approach of adult Wilms tumor*

Pretreatment investigations
- History in combination with
    - personal history of congenital abnormalities or syndrome
    - family history of early onset cancer
- Clinical examination (in particular blood pressure and any asymmetries and/or genitourinary malformations)
- Laboratory investigations:
    - full blood and coagulation studies (to exclude acquired von Willebrand disease)
    - urea and creatinine, electrolytes, liver function tests
    - Urine analysis: dip stick for proteins
- Radiological investigations (staging)
- CT scan of chest and abdomen (where possible without introducing treatment delay otherwise chest X-ray and abdominal ultrasound)

Biopsy
- In case of pre-operative biopsy (either for reason of diagnostic difficulty or inoperability), we recommend:
→ percutaneous cutting needle biopsy (multiple core biopsies can be obtained without multiple puncture by using co-axial technique)
→ sufficient and adequate tissue for histological review, including full immunohistochemistry and molecular studies to exclude diagnoses such as synovial sarcoma and PNET.
- Any tumor requiring biopsy should be considered as stage III and therefore not eligible to be treated with the minimal therapy (only ActD and VCR) used for pediatric stage I Wilms tumors.

Surgery
- In case of nephrectomy following the diagnosis of Wilms tumor on biopsy, we recommend:
→ total nephrectomy according to adult nephrectomy guidelines for any renal cancer.
→ adequate sampling of renal perihilar lymph nodes by the surgeons, even if these appear macroscopically normal. While suspicious lymph nodes should be excised regardless of location, a formal lymph node dissection including a para-aortic lymph node clearance does not seem beneficial and is therefore not recommended.
→ delivery of the whole fresh nephrectomy specimen to a pathologist for assessment of integrity tumor capsule

- Delayed nephrectomy in case of 'inoperable' or metastatic disease:
→ consider after 6 weeks of pre-operative chemotherapy
→ In case of non-response or inadequate response to pre-operative chemotherapy: review of histological diagnosis is necessary and a further biopsy may be warranted if there is any uncertainty. If the diagnosis is confirmed again, we advise changing to the 'high risk' chemotherapy schedule or go on with this schedule and assessing operability again after 2 to 3 courses of chemotherapy.

- Any need for further surgery, whatever surgical approach was undertaken in an adult Wilms tumor patient, should always be discussed with the national pediatric surgeon from the pediatric Wilms tumor group.

Supplementary table 2: *Proposed adult staging system for Wilms tumor treated with immediate nephrectomy*

Stage I	Tumor confined to the kidney and completely resected. No penetration of the renal capsule or involvement of the renal sinus soft tissues and/or vessels. At least one lymph node has been examined histologically and found to be free of tumor.
Stage II	Tumor extends beyond the kidney but is completely resected (negative margins and lymph nodes). At least one of the following has occurred: - penetration of the renal capsule - invasion of the renal sinus soft tissues and/or vessels
Stage III	Gross or microscopic residual tumor remains postoperatively including: - inoperable tumor - positive surgical margins - tumor spillage - regional lymph node metastases - transected tumor thrombus - biopsy of tumor prior to removal
Stage IV	Hematogenous metastases or lymph node metastases outside the abdomen (lung, liver, bone, brain, etc.)
Stage V	Synchronous bilateral renal Wilms tumors

Supplementary table 3: *Histological risk groups in Wilms tumors according to initial treatment*

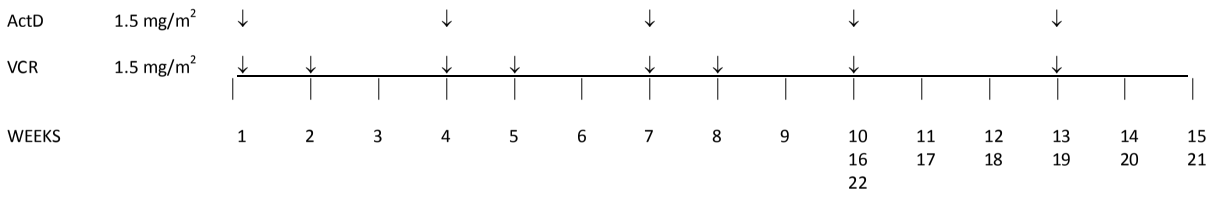
A. Cases treated with immediate nephrectomy
Favorable histology tumors
Includes classic triphasic Wilms tumor and all other non-anaplastic subtypes
Unfavorable (anaplastic) histology tumors
Diffuse anaplasia*
Focal anaplasia*
B. Cases treated with pre-operative chemotherapy prior to nephrectomy
Low risk
Completely necrotic
Intermediate risk
All non-anaplastic and non-blastemal type Wilms tumors, i.e**:**
-Regressive type (>2/3 necrosis)
-Epithelial type (>1/3 viable tumor, viable residue >2/3 epithelial and blastemal component <10%)
-Stromal type (>1/3 viable tumor, viable residue >2/3 stromal and blastemal component <10%)
-Mixed type (>1/3 viable tumor and no predominant cell type in viable residue)
High risk
Diffuse and focal anaplasia*
Blastemal type (>1/3 viable tumor and viable residue >2/3 blastemal)

*******The histological criteria for making a diagnosis of anaplastic Wilms tumor are the presence of all three criteria for anaplasia including:
a) presence of atypical tri/multipolar mitotic figures;
b) marked nuclear enlargement, with diameters at least three times those of adjacent cells and;
c) presence of hyperchromatic tumour cell nuclei.

Remark:
When an adult renal tumor has an embryonal appearance suggesting the diagnosis of Wilms tumor, it is suggested that the local pathologist consider ancillary studies such as immunohistochemistry for WT1. If no nuclear positivity is seen, immunohistochemistry for CD99 or molecular analysis for specific fusion transcripts characteristic of PNET, synovial sarcoma or desmoplastic small round cell tumor may be indicated. Occasionally an epithelial Wilms tumor may mimic papillary renal cell carcinoma (PRCC). PRCC is positive for cytokeratin 7 and epithelial membrane antigen, whereas epithelial Wilms tumor is at most focally positive for these markers. Thereafter, we recommend that the adult pathologist, in case of a now very suspected Wilms tumor, get in touch with a local pediatric pathologist who can contact the Wilms tumor review pathologist. It is recommended that all suspected adult Wilms tumor specimens should be sent for central review by a pediatric pathologist with expertise in Wilms tumor histopathology.

Supplementary table 4: THERAPEUTIC GUIDELINES

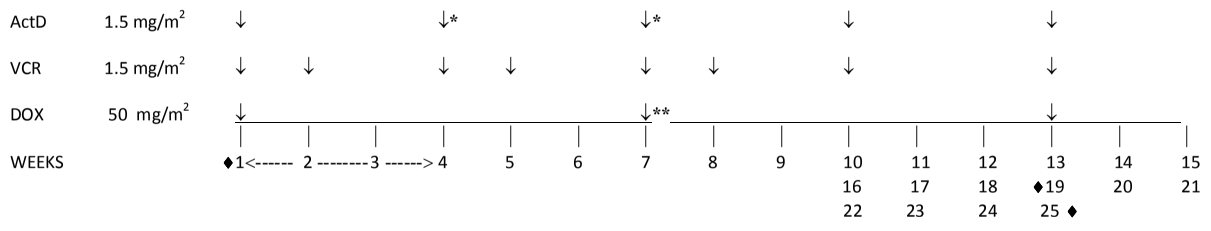
Table 4.1: *Stage I non-anaplastic (favorable) histology Wilms tumor after immediate nephrectomy*



ActD = actinomycin D = 1,5 mg/m^2 i.v. bolus injection (max 2 mg!). Weeks: 1, 4, 7, 10, 13, 16, 19, 22.
VCR = vincristine = 1.5 mg/m^2 i.v. bolus injection (max 2 mg!). Weeks: 1, 2, 4, 5, 7, 8, 10, 13, 16, 19, 22.

Administration of VCR is not count-dependent. Observe patient for signs of VCR neurotoxicity that may require dose reduction or omission.
Administration of ActD is count-dependent: each dose should only be given if absolute neutrophil count > 1.0 x 10^9/l and platelets > 100 x 10^9/l. If there is marked thrombocytopenia, check liver function tests and observe carefully for signs of hepatic veno-occlusive disease.

Table 4.2: *Stage II/III non-anaplastic histology Wilms tumor after immediate nephrectomy ('intensive AVA or three-drugs schedule')*



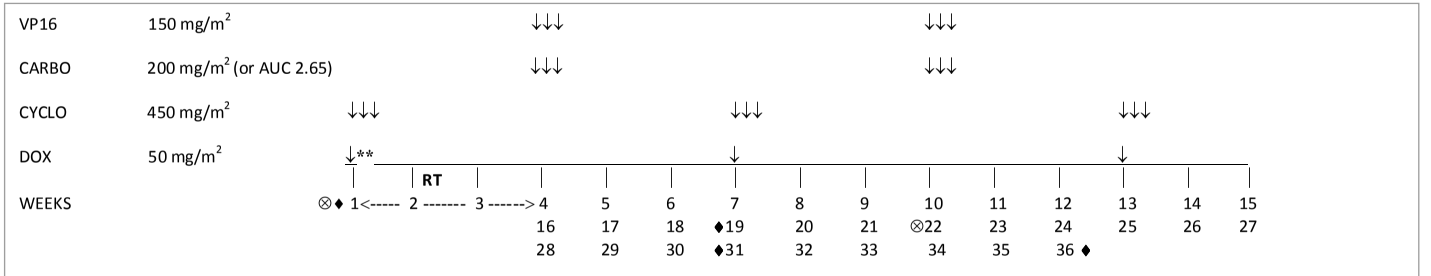
RT: Radiotherapy <----------->°
(°see Supplementary table 5)

♦ = Echocardiogram: at start of treatment, prior to week 19, and end of treatment.

ActD = actinomycin D = 1.5 mg/m^2 i.v. bolus injection (max 2 mg!). * omit until 14 days after the last fraction of radiotherapy. Weeks: 1, 4*, 7*, 10, 13, 16, 19, 22, 25
VCR = vincristine = 1.5 mg/m^2 i.v. bolus injection (max 2 mg!). Weeks: 1, 2, 4, 5, 7, 8, 10, 13, 16, 19, 22, 25
DOX = doxorubicin = 50 mg/m^2 i.v. infusion. ** omit until 7 days after last fraction of radiotherapy. Weeks: 1, 7**, 13, 19, 25

Administration of VCR is not dependent not on blood counts. Observe patient for signs of VCR neurotoxicity that may require dose reduction or omission.
Administration of DOX and ActD are blood count-dependent: each dose should only be given if absolute neutrophil count > 1.0 x 10^9/l and platelets > 100 x 10^9/l. If there is marked thrombocytopenia, check liver function tests and observe carefully for signs of hepatic veno-occlusive disease.

Table 4.3: *Anaplastic Histology Any Stage and Slowly Responding Stage IV non-anaplastic histology: High risk schedule*



RT: Radiotherapy <---------->°
(°see Supplementary table 5)

♦ = Echocardiogram*: at start of treatment, prior to week 19, week 31 and end of treatment.
⊗ = GFR (measure at every third course, or more frequently if there is evidence of renal dysfunction.)

VP16	= etoposide	= 150 mg/m^2/i.v./in 1 hour. Weeks: 4, 10, 16, 22, 28, 34.
CARBO	= carboplatin	= 200 mg/m^2/i.v./in 1 hour. Weeks: 4, 10, 16, 22, 28, 34.
CYCLO	= cyclophosphamide	= 450 mg/m^2/i.v./in 1 hour. Weeks: 1, 7, 13, 19, 25, 31.
DOX	= doxorubicin	= 50 mg/m^2/i.v./in 4-6 hours, just after the first CYCLO administration.**omit until 7 days after last fraction of radiotherapy. Weeks: 1, 7, 13, 19, 25, 31.

All courses are blood count dependent. If poor tolerance of chemotherapy or prolonged intervals between courses, then introduce GCSF 5 µg/kg/day, starting day 8 until neutrophil count ≥ 1.0 x 10^9/l for 2 consecutive days. If continued poor tolerance/slow count recovery, reduce doses of all drugs by 30% for subsequent courses.

Supplementary table 5: *Summary of radiotherapy recommendations*

Flank radiotherapy:
Total dose 15 Gy in 10 fractions should be given to all patients unless they have a stage I non-anaplastic Wilms tumor after immediate nephrectomy. A boost of up to 10 Gy should be considered for areas of macroscopic residual tumor.

Whole abdominal radiotherapy:
15 Gy in 10 fractions is recommended when there has been diffuse tumor spillage/rupture, either pre- or peri-operative.

Pulmonary irradiation:
All patients with pulmonary metastases at diagnosis should receive whole lung irradiation (12 Gy in 8 fractions) regardless they achieve complete remission to chemotherapy or following surgery.

Precautions in combination with chemotherapy:
Timing and dose of actinomycin D and doxorubicin should be altered to fit around the administration of radiotherapy, observing the following recommendations:
- Radiotherapy should not start until at least 7 days after a dose of actinomycin D/doxorubicin.
- Actinomycin D should not be resumed until 14 days after the last fraction of radiotherapy.
- Doxorubicin should not be resumed until 7 days after the last fraction of radiotherapy.
- Further omissions or dose reductions should be considered if there is a significant volume of liver within the radiotherapy field or if the patient experiences gut or liver toxicity during or immediately following the end of radiotherapy.

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8

SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

8.1 SUMMARY

Wilms tumor, the most common childhood renal malignancy, is genetically a heterogeneous and complex disease. There are several predisposing syndromes that are associated with an increased risk of developing a Wilms tumor, indicating a major role for genetic factors in Wilms tumorigenesis. Apart from constitutional chromosomal aberrations, several somatic molecular aberrations have been identified in the etiology of Wilms tumors in the last decades. However, the driving genetic aberrations that induce the development of this cancer type need to be further explored. About 90% of Wilms tumor patients currently survive, and approximately 10% of the patients with Wilms tumor die due to refractory disease or following relapse. Therefore, it is necessary to obtain more insight into other underlying mechanisms not only from a biological point of view but also for future therapeutic purposes. In this thesis, we studied several aspects of constitutional and somatic genetic changes underlying this disease.

In chapter 1, we gave an overview of the known clinical and genetic aspects of Wilms tumors. As the first aim of this thesis was to reveal novel clinical insights and phenotypes, we described the first case of paraneoplastic Cushing syndrome at presentation of Wilms tumor, in which clinical and biological signs of hypercortisolism regressed during pre-operative chemotherapy, and reviewed the literature on paraneoplastic Cushing syndrome secondary to other pediatric renal tumors (chapter 2).

A second aim of this thesis was to explore constitutional genetic aberrations that may contribute to the pathogenesis of Wilms tumors. There are multiple constitutional aberrations that play a role in the etiology of Wilms tumors, but the exact frequency is not known especially not in children with an apparently sporadic Wilms tumor. In chapter 3, we report on the frequency of constitutional *WT1* and locus 11p15 aberrations and concomitant phenotypes in our single centre cohort of 109 childhood Wilms tumor patients. We observed a high frequency of constitutional *WT1* (11%) and 11p15 (8%) aberrations in Wilms tumor patients and showed that thorough physical examination and clinical genetic assessment can identify the majority of these patients. We recommend to offer clinical genetic counseling to all Wilms tumor

patients, and advise to perform molecular-genetic analysis in patients with clinical signs of a syndrome or with features that may indicate a constitutional *WT1* or 11p15 aberration.

Apart from the common Wilms tumor predisposing syndromes, also other less common constitutional aberrations may play a role in the etiology of Wilms tumors. We investigated whether constitutional bi-allelic mutations in two Fanconi anemia genes, *PALB2/FANCN* and *BRCA2/FANCD1*, may play a role in the etiology of Wilms tumors, but this appeared not to be the case (chapter 4).

In chapter 5, we described the first study on mismatch repair defects in Wilms tumor patients by the combined application of microsatellite instability analysis by a fluorescent multiplex PCR-based assay and immunohistochemistry for the expression of mismatch repair proteins. A defective mismatch repair system makes cells more vulnerable to mutations resulting in an increased cancer risk. The matching results of normal expression of the mismatch repair proteins and the absence of microsatellite instability in 100 Wilms tumor samples, made us conclude that defects in the DNA mismatch repair appeared not to be important in the pathogenesis of Wilms tumors.

We further investigated the role of gain of 1q and other common cytogenetic aberrations, determined by conventional karyotyping, as prognostic markers in Wilms tumor patients in addition to the well-established prognostic factors tumor stage, histology, and combined loss of heterozygosity at 1p and 16q. This analysis of the largest series of Wilms tumor karyotypes (n=331) so far, only revealed 1q gain as an adverse prognostic molecular marker in Wilms tumors. Larger studies with multiplex ligation-dependent probe amplification (MLPA), a more feasible molecular method to perform on a routine basis in all Wilms tumors for risk-adapted treatment stratification, are currently performed by the COG and SIOP as integrated part of the trials. If this prognostic significance of 1q gain as a molecular marker can be confirmed in these studies, patients with tumor specific gain of 1q could benefit from stratification to more intensive treatment regimens (chapter 6).

Whereas Wilms tumors are the most common childhood renal malignancies, in adults it is extremely rare. Accordingly, no standard treatment is available. Outcome for adult Wilms tumor patients is considerably worse as compared to the survival in children, although better results are reported in adults when they receive

treatment according to adapted pediatric Wilms tumor protocols. Multiple factors, including unfamiliarity of adult oncologists and pathologists with Wilms tumor, lack of standardized treatment and consequent delays in initiating appropriate risk-adapted therapy for this rare disease in adults, may contribute to the poor outcome. Therefore, we proposed standardized recommendations based on an international consensus for the management of adults with a Wilms tumors (chapter 7).

8.2 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Advances in insight of genetic predisposition to Wilms tumor

A long time ago, genetic predisposition to Wilms tumor has been shown by the complete deletion of one *WT1* gene in children with WAGR (Wilms tumor, aniridia, genitourinary anomalies, and mental retardation) syndrome. However, it soon became clear that mutations in the *WT1* gene did not explain the majority of cases, and that different Wilms tumor predisposition genes must exist. Nowadays, as shown in the current thesis, a genetic predisposition seems present in 9-19% of all children who develop a Wilms tumor (1-5). This is the highest proportion seen in any childhood malignancy. Nevertheless, the exact prevalence is still not known in children with an apparently sporadic Wilms tumor.

We confirmed the high frequency of constitutional genetic aberrations in Wilms tumor patients, described in earlier publications (1-5). In addition, we showed that the majority of Wilms tumor patients with an underlying constitutional *WT1* or 11p15 aberration had clinical signs of an underlying Wilms tumor predisposing syndrome (9%) or had morphological or clinico-pathological features that may indicate an underlying constitutional *WT1* or 11p15 aberration (8%). Only 2% of the patients with an underlying *WT1* or 11p15 aberration had no features at all. This indicates that careful clinical and genetic assessment identifies the majority of patients with a genetic predisposition in the *WT1* gene or 11p15 locus, and moreover that only a minority of the patients with an underlying constitutional *WT1* or 11p15 aberration has no phenotypic abnormalities at all. This confirms findings from earlier studies (6, 7).

In a study of 47 unselected patients with sporadic Wilms tumor, we revealed no bi-allelic pathogenic mutations of *BRCA2/FANCD1* or *PALB2/FANCN*. So, we did not confirm our hypothesis that the diagnosis of Fanconi anemia could easily be missed in children with a sporadic Wilms tumor, as several reports have found bi-allelic mutations in these Fanconi anemia genes in Wilms tumor children with a normal phenotype (8, 9).

In our genotype-phenotype study, we also found that 3% of the Wilms tumor patients had a syndrome that had not been described before in Wilms tumor patients. For the

most common syndromes, i.e. *WT1*-associated and overgrowth conditions, and for some other less common syndromes, there is conclusive evidence of an increased risk of Wilms tumor (2, 3). However, this is only the case for a minority of the more than 50 constitutional aberrations that are associated with Wilms tumor, as in many their rarity precludes the possibility to study the association (2, 3).

If larger cohorts could be explored, this could possibly give rise to new Wilms tumor predisposition syndromes. Moreover, the exact prevalence of genetic aberrations underlying apparently sporadic Wilms tumors will be better defined. Large genome-wide association studies requiring international collaborations can contribute to this. The only genome-wide association study published so far described a number of new genetic aberrations associated with development of Wilms tumor, such as loci 2p24 and 11q14 (10). Ideally, besides clinical genetic examination of all Wilms tumor patients, genome wide screening using next-generation sequencing methods will be performed in every Wilms tumor patient on a large scale in the setting of international collaborations.

Due to the high prevalence of constitutional genetic aberrations in Wilms tumor patients and the knowledge that a small proportion of children with apparently sporadic Wilms tumor has a constitutional genetic change that has predisposed them to their tumor, we recommend routine clinical genetic assessment and counseling for all Wilms tumor patients, as well as molecular-genetic analysis to patients with clinical signs of an underlying syndrome or with morphological or clinico-pathological features that may indicate a *WT1* or locus 11p15 aberration. The recognition of a genetic predisposition may have implications in terms of risk for future tumors for the patient, their siblings and their off-spring (11, 12). This underscores that in the multidisciplinary approach of all children with Wilms tumor not only pediatric oncologists, pediatric surgeons, radiologists, pathologists, and radiation oncologists but also clinical geneticists are of utmost importance.

Ongoing and future research will result in a continued modification and elucidation of phenotypic groups and subgroups predisposed to Wilms tumor (3). This will further increase our insights into the molecular basis of these conditions and Wilms tumorigenesis, and will build a basis for the management and counseling of children with a possible Wilms tumor predisposition syndrome.

Advances in insight of somatic genetic aberrations

Wilms tumor is genetically a heterogeneous and complex disease. In the last decades, enormous progress has been made in our understanding of the molecular genetics of Wilms tumors. Somatic aberrations in multiple genetic loci have been linked to Wilms tumorigenesis such as *WT1, WT2* or locus 11p15.5*, CTNNB1* (β-catenin), *WTX*, *TP53, MYCN,* and *FBXW7* (Table I). Somatic *WT1* mutations have been identified in ~10-20% of sporadic Wilms tumor cases (2, 13). Many putative transcriptional targets of the WT1 protein are involved in cell growth, differentiation, and apoptosis (14). However, the biologically relevant precise targets that are involved in Wilms tumorigenesis remain to be determined (12, 13, 15-17). Consequently, until now there is not a drugable molecular target that has emerged for *WT1* mutated tumors (18). Somatic activating mutations of β-catenin (CTNNB1) have been identified in ~15% of Wilms tumors (13, 19), and frequently accompanies *WT1* mutations (12, 20-22). Somatic inactivating mutations of WTX, a protein that contributes to β-catenin degradation, have been detected in ~20% of Wilms tumors (13, 23). Both, β-catenin and WTX, are components of the Wnt signaling pathway, indicating that activation of this pathway is important in the development of a subset of Wilms tumors. Loss of heterozygosity (LOH) or loss of imprinting (LOI) at locus 11p15, both resulting in *IGF2* overexpression, have been found in ~70% of Wilms tumors as a somatic aberration (2, 24-30). *IGF2* overexpression seems to be a driver of Wilms tumorigenesis, as evidenced by increased risk of Wilms tumor in Beckwith-Wiedemann syndrome (BWS) patients with the molecular subtypes associated with overexpression of *IGF2* (2, 24-30). Hence, agents targeting the IGF1R pathway are attractive therapeutic targets for Wilms tumor (18).

Somatic mutations in the tumor suppressor gene *TP53*, which encodes the protein p53, have been reported in ~5% of sporadic Wilms tumors (31). *TP53* mutations mostly occur in Wilms tumors with anaplastic histology, which is associated with poor outcome (13, 32, 33). Recently, gain of MYCN oncogene was detected in 7-10% of Wilms tumors (34, 35). In addition, mutations in the *FBXW7* gene, which encodes a ubiquitin ligase component that targets several proto-oncogene products for ubiquitination and subsequently degradation, was recently identified in ~4% of sporadic Wilms tumors (34). FBXW7 mediates degradation of MYCN suggesting that a

common pathway is dysregulated by different mechanisms in various Wilms tumors. Only scarce information is available on the contribution of defects in the DNA mismatch repair system to the etiology of Wilms tumors. A defective mismatch repair system makes cells more vulnerable to mutations resulting in an increased cancer risk (36, 37). Our study (38) however confirms data from the COG (39), showing that DNA mismatch repair deficiency does not play an important role in the pathogenesis of Wilms tumors, indicating that future studies should be directed towards other alternative mechanisms.

Prognostic molecular markers in Wilms tumors

Until now, the two most powerful and widely used prognostic factors for risk-adapted treatment stratification in Wilms tumors are stage and histology (40-43). Despite the strength of these prognostic factors, many of the relapses occur without apparent unfavorable features. This urges to search for new molecular predictors of relapse. Several studies found that LOH at 16q, and to a lesser extent LOH at 1p, were predictors of relapse (44-46). The NWTS-5 trial confirmed the poor prognostic significance of combined LOH at 1p and 16q, and as a result current COG trials now treat patients with combined LOH at 1p and 16q in favorable histology Wilms tumors with more intensive regimens (47). However, LOH at 1p and 16q only detects 9% of the relapses (18). Moreover, the mechanism behind the prognostic role of LOH at 1p and 16q is unknown. It is possible that LOH at 1p and 16q is only a surrogate for a more important aberration such as gain of chromosome 1q (48, 49). It is therefore necessary to identify additional prognostic factors as, despite intensive research of the last decades, neither SIOP nor COG treatment approaches have yet been able to define a strong prognostic biomarker that is sufficiently sensitive or specific to identify the majority of patients with poor outcome.

We investigated the role of 1q gain and other common cytogenetic aberrations, determined by classical cytogenetics, as prognostic markers in Wilms tumor patients. Consistent with previous studies (18, 48, 50-54), we found a high prevalence of 1q gain (19%) in Wilms tumors as well as an association with adverse outcome (4.3-fold increased risk of death), making 1q gain a potentially strong biomarker for clinical application. We could not exclude a small effect from t(1;16) or isochromosome 1q

being the underlying route to gain of 1q, but gain of 1q was the strongest predictor of adverse outcome. The important gene(s) on 1q that contribute(s) to the adverse outcome are still unknown. Alternatively, gain of 1q may not be mechanistically so important but rather be the 'tip of the iceberg' indicating genomic instability in the tumor, not all of which will be evident on karyotyping. Studies using other more sensitive methods such as SNP arrays that interrogate allelic imbalances as well as small regions of copy number variation may be necessary to further investigate this. Large studies using multiplex ligation-dependent probe amplification (MLPA), a more feasible molecular method compared to classical cytogenetics, are currently being performed in NWTS-5 (18) and SIOP 2001 Wilms tumor series. If the adverse prognostic significance of 1q gain can be confirmed, future clinical trials can incorporate 1q gain into new risk stratification schema.

We also found an association between Wilms tumors with loss of 14q and anaplasia and between loss of 14q and adverse EFS. This is in line with a recent study of Williams et al. that revealed an association between allelic loss of 14q and anaplasia (35), and a whole genome SNP array study that showed an association with allelic imbalance at chromosome 14q and relapse in Wilms tumors (54) (Table I). In our study, loss of 14q was independently associated with adverse outcome in a multivariate analysis that took into account anaplasia. Hence, additional features on 14q drives this adverse tumor behavior, indicating that future studies should be directed towards possible causes of the adverse outcome associated with loss of 14q.

Nowadays, approximately 90% of all Wilms tumor patients will survive. Still 10% of the Wilms tumor patients will die due to refractory disease or following relapse, despite intensive second line therapies. Moreover, there are some subgroups, namely diffuse anaplasia and blastemal subtype after pre-operative chemotherapy, that are more resistant to current therapeutic agents. Therefore, it is necessary to look for new therapeutic targets. Telomerase is a reverse transcriptase that adds nucleotide repeats to telomeres, counteracting the progressive loss of DNA that occurs during replication (55, 56). The NWTS-5 trial showed that high telomerase RNA expression level is an adverse prognostic factor within the favorable histology Wilms tumor group (57). Telomerase inhibitors may therefore be attractive therapeutic agents in this subset of Wilms tumors (Table I).

Until now, it has been difficult to define a reproducible molecular signature predictive of poor outcome, despite many whole genome approaches performed by several groups (52, 53, 58-66). The expression levels of several genes involved in the retinoic acid (RA) signaling pathway were found to be associated with disease progression in several studies (66, 67) (Table I). Other analyses have highlighted alterations of the insulin-like growth factor signaling pathway as potential prognostic marker (58, 68, 69) (Table I). Both pathways are interesting as drugs against these pathways are already in clinical use (58). Also the expression of multiple genes involving apoptotic pathways (p53 and Bcl2), Wnt signaling pathway and epigenetic pathways, have been revealed to be important in relapse (58). In addition, *FRAP/mTOR* and CD40, also both associated with relapse, have been identified as potential therapeutic targets and are already used in clinical trials (58) (Table I). *FRAP1/mTOR*, a serine/threonine kinase involved in signal transduction, translation initiation, and elongation, has a known role in cancer (70-72). CD40, a member of the tumor necrosis factor family, induces anti-apoptotic genes including Bcl-2, early angiogenesis, and activation of certain cell proliferation signaling pathways (58, 73). Recently, a possible new stratification schema, based on gene expression, has been proposed by the COG (18, 69). They determined clinically significant subsets of favorable histology Wilms tumors, identified by gene expression patterns (18, 69).

In the future, integrative analyses of different genome-wide platforms such as whole genome sequencing, DNA methylation arrays, microRNA arrays, gene expression profiling and proteomic profiling, performed on a large scale with joined international forces, may point to novel involved and deregulated genes and signal transduction pathways. This can be of great help to further increase our insight into the driving processes in Wilms tumors and to identify new molecular stratification markers and drugable targets. However, Wilms tumors are heterogeneous tumors and molecular studies will thus always remain a challenge.

Table I: *Currently appreciated somatic molecular aberrations in Wilms tumors with respect to their clinical relevance*

Somatic molecular aberration	Association with	Prognostic significance SIOP	Prognostic significance COG	Potential drug target	Marker for treatment stratification in treatment protocols
WT1 mutation	Stromal histology (12) *CTNNB1* mutation (21, 22)	Stromal histology: good^ (82)	-	No	No
CTNNB1* mutation	Stromal histology (12) *WT1* mutation (21, 22)	-	-	Yes**	No
WTX* mutation	-	-	-	No	No
IGF2° overexpression	-	-	-	IGFIR inhibitors (18)	No
TP53 mutation	Diffuse and focal anaplasia (32, 33)	Anaplasia: poor+	Anaplasia: poor+	MDM2 inhibitor++ (18)	No+++
MYCN gain	Diffuse anaplasia (SIOP/COG) (34, 35)	-	-	No	No
FBXW7 mutation	Epithelial histology (SIOP) (34)	-	-	No	No
LOH 1p and 16q	-	-	FH: poor (18, 44, 47)	No	Yes (COG FH) (47)
Gain of 1q	Loss of 1p (48, 53) Loss of 16q (48, 53)	IR/HR: poor (*)	FH: poor (18)	No	Not yet
Loss of 14q	Diffuse anaplasia (COG) (35)		FH/UH: poor (↑ relapse) (54)	No	No
High telomerase activity	-	-	FH: poor (57)	Telomerase inhibitors (e.g. Imetelstat) (18)	No
Retinoic acid pathway°°	HR histology (SIOP) (83)	IR/HR: poor (↑ relapse) (66, 83)	FH: poor (64)	Retinoids (**) (64, 67, 83)	No
mTOR°°°	-	-	FH stage III: poor (↑ relapse) (58)	mTOR inhibitors (e.g. Rapamycin, Everolimus) (18, 58, 70-72)	No
CD40°°°	-	-	FH stage III: poor (↑ relapse) (58)	Anti-CD40 antibodies (58)	No

Abbreviations:
FH: favorable histology, HR: high risk (diffuse anaplasia and blastemal type), IR: intermediate risk, IGF2: insulin-like growth factor II, IGFIR: insulin-like growth factor I receptor, FH: favorable histology (no anaplasia), UH: unfavorable histology (diffuse or focal anaplasia), LOH: loss of heterozygosity, mTOR: mammalian target of Rapamycin, good: good outcome, poor: poor outcome, SIOP: Wilms tumor samples treated with pre-operative chemotherapy, COG: Wilms tumor samples treated with immediate nephrectomy, -: no (conclusive) data available, ^: stromal predominant histology is associated with a good outcome (82), *: Both, CTNNB1 (β-catenin) and WTX, are components of the Wnt signaling pathway, **: Disruption of the interaction of β-catenin with the transcription factors TCF/LEF by various compounds including flavonoids, PKF115-584, PKF118-310, CGP046090, FH535 and diaminoquinazolines (84), °: LOH/LOI of 11p15 leading to IGF2 overexpression, °°: deregulation of the retinoic acid pathway at different levels (67, 83), °°°: upregulation of this gene (58), $^{+}$: p53 mutations are described in the majority of anaplastic Wilms tumors, which are associated with a poor outcome, $^{++}$: works through activation of wild-type p53, $^{+++}$: anaplasia is a prognostic factor for risk-adapted treatment stratification in Wilms tumors in contrast to p53 mutation, (*): 1q gain in Wilms tumors treated in SIOP2001/GPOH trial C. Vokuhl et al (Abstract 8th International Conference on pediatric renal tumor biology, May 2013), (**): retinoids or retinoid therapy: classical retinoids ATRA (all-trans retinoic acid), 13*cis*- or 9*cis*-RA, and the synthetic retinoid fenretinide (4HPR) (83).

International collaboration

Progress has been made in understanding the molecular basis of Wilms tumor in the last decades. However, further research is necessary to further decrease the treatment intensity and its associated side effects for patients at low risk of relapse and to improve therapy efficacy for patients at high risk of relapse. As the poor risk groups contain small numbers, international collaborative research on this patient tailored approach is of utmost importance. The long-standing successes of the childhood cancer study groups such as SIOP and COG over the last decades may form a solid platform for these studies.

An example of the importance of international collaboration on small patient groups is our proposal of "best practice" guideline for the management of adults with Wilms tumor. Since Wilms tumor occurs rarely in adults, no standard treatment is available. Outcome for adults is inferior compared to children, although better results are reported when treated within pediatric trials (74-81). Multiple factors, including unfamiliarity of adult oncologists and pathologists with Wilms tumor, lack of standardized treatment and consequent delays in initiating appropriate risk-adapted therapy, may contribute to the poor outcome (74-81). Therefore, we proposed an international consensus how to manage adults with Wilms tumor. This resulted in a global recommendation with the aim to further improve outcome by using standardized treatment and by shortening adjuvant treatment delay. These recommendations will build the basis for collecting uniform and accurate data on clinical characteristics, treatment, outcome and toxicity in this rare group of adult patients with a pediatric cancer type. The knowledge obtained from this registry, in combination with data from biology studies, will help us to assess whether Wilms tumors in adults are any different than those occurring in children. The global approach is necessary to develop more evidence based guidelines for diagnosis and treatment of adult Wilms tumor patients in the future.

In addition, we have to realize that in children, the less frequently occurring non Wilms kidney tumors have the worst prognosis. Global cooperation between COG and SIOP may help to enhance outcome for these even more rare disease types, thereby benefitting from the aforementioned global task force.

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9

NEDERLANDSE SAMENVATTING

Wilms tumor, de meest voorkomende niertumor op de kinderleeftijd, is genetisch een heterogene en complexe ziekte. Er zijn verschillende predisponerende syndromen geassocieerd met een verhoogd risico op het ontwikkelen van een Wilms tumor. Dit wijst op een belangrijke rol van genetische factoren in het ontstaan van een Wilms tumor.

In de laatste decennia zijn er, naast aangeboren genetische afwijkingen, verschillende tumor specifieke of somatische genetische afwijkingen ontdekt, die een bijdrage hebben in het ontstaan van Wilms tumoren. Echter, de drijvende genetische afwijkingen die leiden tot de ontwikkeling van dit type kanker moeten verder worden onderzocht. Op dit ogenblik genezen ongeveer 90% van de patiënten met een Wilms tumor. Dit betekent echter dat nog steeds ongeveer 10% van de patiënten met een Wilms tumor overlijden als gevolg van therapieresistente ziekte of recidief. Daarom is het noodzakelijk om meer inzicht te krijgen in andere onderliggende mechanismen. In dit proefschrift bestudeerden we verschillende aspecten van aangeboren en tumor specifieke genetische afwijkingen die kunnen bijdragen aan het ontstaan van een Wilms tumor.

In hoofdstuk 1 geven we een overzicht van de gekende klinische en genetische aspecten van Wilms tumoren. Het onthullen van nieuwe klinische inzichten en fenotypes was een eerste doel van dit proefschrift. In hoofdstuk 2 beschrijven wij de eerste patiënt die zich presenteerde met een paraneoplastisch Cushing syndroom ten gevolge van een Wilms tumor, waarbij de klinische en biologische tekenen van hypercortisolisme afnamen tijdens preoperatieve chemotherapie. Daarnaast geven we een overzicht van de literatuur over paraneoplastisch Cushing syndroom ten gevolge van andere niertumoren op kinderleeftijd.

Een tweede doel van dit proefschrift was om aangeboren genetische afwijkingen die kunnen bijdragen aan de pathogenese van Wilms tumoren te onderzoeken. Er zijn verschillende aangeboren genetische afwijkingen die een rol spelen in het ontstaan van Wilms tumoren, maar de precieze frequentie is onbekend in het bijzonder bij kinderen zonder uitwendige afwijkingen met dus mogelijk een schijnbaar sporadische Wilms tumor. In hoofdstuk 3 beschrijven we de frequentie van aangeboren *WT1* en locus 11p15 afwijkingen in combinatie met de fenotypes van 109 kinderen met een Wilms tumor. Wij vonden een hoge frequentie van aangeboren *WT1* (11%) en 11p15

(8%) afwijkingen in Wilms tumor patiënten. Daarnaast konden we de meeste van deze patiënten met een onderliggende aangeboren *WT1* of 11p15 afwijking identificeren aan de hand van een grondig klinisch genetisch onderzoek. Daarom adviseren wij om alle Wilms tumor patiënten klinisch genetische counseling aan te bieden, met daarnaast moleculair-genetische analyse bij die patiënten die klinische kenmerken hebben die kunnen wijzen op een onderliggend syndroom of een onderliggende aangeboren *WT1* of 11p15 afwijking.

Naast de meest voorkomende Wilms tumor predisponerende syndromen, kunnen ook minder frequente aangeboren afwijkingen een rol spelen in het ontstaan van Wilms tumoren. Wij hebben onderzocht of aangeboren bi-allelische mutaties in twee Fanconi anemie genen, *PALB2/FANCN* en *BRCA2/FANCD1*, een rol kunnen spelen bij het ontstaan van een Wilms tumor, maar dit bleek niet het geval te zijn (hoofdstuk 4).

In hoofdstuk 5 beschrijven we de eerste studie over defecten in het DNA mismatch herstel mechanisme (of mismatch repair (MMR) systeem) in Wilms tumor patiënten door de gecombineerde toepassing van microsatelliet instabiliteit (MSI) analyse op basis van een fluorescentie multiplex PCR analyse en immunohistochemie voor de expressie van de MMR eiwitten. Een defect MMR systeem maakt cellen gevoeliger voor mutaties en dit leidt tot een verhoogd risico op kanker. Een normale expressie van de MMR eiwitten in combinatie met de afwezigheid van MSI in 100 Wilms tumor samples geven aan dat defecten in het DNA mismatch repair systeem geen belangrijke rol spelen in het ontstaan van Wilms tumoren.

We onderzochten ook de rol van gain of 1q en andere cytogenetische afwijkingen, bepaald met conventionele karyotypering, als prognostische factoren in Wilms tumor patiënten. Deze analyse van de tot nu toe grootste serie van Wilms tumor karyotypes (n = 331) weerhield 1q gain als een negatieve prognostische moleculaire marker in Wilms tumoren. Grotere studies met multiplex ligatie-afhankelijke probe amplificatie (MLPA), een meer haalbare moleculaire methode om standaard uit te voeren in alle Wilms tumoren, worden momenteel gedaan door de COG en de SIOP. Als de prognostische waarde van 1q gain kan worden bevestigd in deze studies, kunnen patiënten met gain of 1q in hun Wilms tumor in de toekomst behandeld worden met intensievere behandelschema's (hoofdstuk 6).

Terwijl Wilms tumor de meest voorkomende niertumor is op kinderleeftijd, is het bij volwassenen uiterst zeldzaam. Bijgevolg is er geen standaard behandeling beschikbaar. De overleving van volwassen met een Wilms tumor is aanzienlijk slechter vergeleken met de overleving van kinderen, hoewel betere resultaten worden beschreven bij volwassenen als ze behandeld worden volgens aangepaste pediatrische Wilms tumor protocollen. Meerdere factoren, waaronder onbekendheid van volwassen oncologen en pathologen met Wilms tumor en een gebrek aan een gestandaardiseerde behandeling en de daaruit voortvloeiende vertraging in het starten van een adequate risico-aangepaste therapie voor deze zeldzame ziekte bij volwassenen, kunnen bijdragen aan de slechte uitkomst. Daarom hebben wij gestandaardiseerde aanbevelingen voor de behandeling van volwassenen met een Wilms tumor voorgesteld op basis van een internationale consensus (hoofdstuk 7).

ABOUT THE AUTHOR

Curriculum vitae

PhD Portfolio

List of publications

Dankwoord / Thank you

CURRICULUM VITAE

Heidi Segers werd geboren te Maasmechelen (België) op 15 juli 1978. Na het behalen van haar diploma middelbaar onderwijs in 1996, afstudeerrichting Latijn-Grieks, startte ze met haar studie geneeskunde aan de Universiteit Hasselt te Diepenbeek. Na hier succesvol haar kandidaturen te doorlopen, studeerde zij verder aan de Katholieke Universiteit Leuven. Hier studeerde zij af als arts (cum laude) in juli 2003. Vervolgens begon zij aan deze universiteit aan de opleiding kindergeneeskunde. Ondertussen behaalde zij ook haar diploma acute geneeskunde. In augustus 2007 trok zij naar het Erasmus MC - Sophia Kinderziekenhuis te Rotterdam voor het laatste jaar van haar opleiding. Na het succesvol afronden van haar opleiding tot kinderarts in augustus 2008 kon zij hier, onder begeleiding van Prof. dr. R. Pieters, haar fellowship kinderhemato-oncologie aanvatten**.** Tijdens haar fellowship startte zij, onder begeleiding van Prof. dr. R. Pieters en Dr. M.M. van den Heuvel-Eibrink, een promotieonderzoek over de genetische en klinische aspecten van Wilms tumoren, dat resulteerde in het tot stand komen van dit proefschrift. Na haar benoeming tot kinderhemato-oncoloog in februari 2011, werkte zij nog in het Sophia Kinderziekenhuis tot augustus 2012.

Sinds september 2012 is zij werkzaam als kinderhemato-oncoloog in het Wilhelmina kinderziekenhuis te Utrecht. Daarnaast doet zij onderzoek naar het effect van profylactische toediening van immuunglobulines ter preventie van infecties bij kinderen met acute lymfatische leukemie (ALL). Zij is ook lid van de SIOP-RTSG (International Society of Paediatric Oncology – Renal Tumour Study Group), de ziektecommissie renale tumoren, de protocolcommissie ALL 11 en de taakgroep supportive care van de SKION (Stichting Kinderoncologie Nederland).

Zij is gehuwd met Rafaël Benats en ze verwachten hun eerste kindje.

PHD PORTOFOLIO

Summary of PhD training

Name PhD student:	Heidi Segers
Erasmus MC department:	Pediatric Oncology
Research school:	Molecular Medicine
PhD period:	Augustus 2008 – Augustus 2012
Promotor:	Prof.dr. R. Pieters
Supervisors:	Dr. M. M. van den Heuvel-Eibrink

1. PhD training

General Courses	**Year**
-Course of Molecular Diagnostics (Molmed)	2009
-Basic Introduction Course on SPSS	2009
-Biomedical English Writing and Communication	2011
-Biostatistical Methods (CCO2) (Netherlands Institute for Health Sciences (NIHES))	2011

Specific Courses

-ASPO 3th Postgraduate Course in Pediatric Oncology	2006
-ASPO 5th Postgraduate Course in Pediatric Oncology	2010
-Course Advanced Pediatric Life Support	2011

Seminars and Workshops

-Effective Presentation of Scientific Research	2009
-Annual Pediatric Research day, Erasmus MC, Rotterdam	2010
-Annual Pediatric Oncology Symposium, Erasmus MC, Rotterdam	2008-2012

-Weekly Pediatric Oncology Research Meetings, Erasmus MC, Rotterdam	2008-2012
-Yearly Meetings of the International Society of Pediatric Oncology, Renal Tumour Study Group (SIOP-RTSG)	2009-2012

Oral presentations

-Meeting Integraal Kanker Centrum Rotterdam (IKR), Rotterdam	2009
-SKION dagen, Utrecht	2010
-Management of adults with Wilms tumor: recommendations based on an international consensus. SIOP 2010, Boston, USA	2010
-Frequency of *WT1* and 11p15 constitutional aberrations and phenotypic correlation in childhood Wilms tumor patients. SIOP 2012, London, UK	2012
-Oral presentations at weekly Pediatric Oncology Research Meetings, Erasmus MC, Rotterdam	2008-2012

Poster presentations

-Uncommon Histiocytic Disorders: a case report of juvenile xanthogranuloma. Jahrestagung der Gesellschaft für Neonatologie und pädiatrische Intensivmedizin (GNPI), Hamburg, Germany	2007
-Cushing syndrome as presenting symptom of renal tumors in children. SIOP 2009, San Paulo, Brasil	2009
-Defects in the DNA mismatch repair do not contribute to the development of childhood Wilms tumors. SIOP 2012, London, UK	2012

International conferences

-Epiphamy conference, Aachen, Germany.	2009
-7th International Meeting on the Biology of Wilms tumors, Banff, Canada	2010
-42nd Congress of the International Society of Paediatric Oncology (SIOP), Boston, USA	2010
-44th Congress of the International Society of Paediatric Oncology (SIOP), London, UK	2012

2. Teaching

-Supervising 4th year Medical Student (Saskia Gooskens). "Clear cell sarcomas in children"	2009
-Seminar for Minor Students: "Renal Tumors in Children"	2010-2011

3. Other

-Management of the program for chemotherapy prescriptions, Chemotherapy committee, Sophia Children's Hospital, Rotterdam	2009-2010
-Visiting researcher, University College London (UCL), Institute of Child Health and Great Ormond Street Hospital for Children, London, UK (Supervision: Prof. Dr. K. Pritchard-Jones)	2011-2012

PUBLICATIONS

- **H. Segers,** V. Scharnhorst, J. Busari, M. Cnossen. *"Met de verkeerde in zee gaan": diagnostiek naar thalassemie.* Tijdschrift voor kindergeneeskunde, 2009;4:175-178.

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- M.A. Adank, **H. Segers**, S.E. van Mil, Y.M. van Helsdingen, N. Ameziane, A.M. van den Ouweland, A. Wagner, H. Meijers-Heijboer, M. Kool, J. De Kraker, Q. Waisfisz, M.M. van den Heuvel-Eibrink. *Fanconi anemia gene mutations are not involved in sporadic Wilms tumor.* Pediatric Blood & Cancer, 2010 Oct;55(4):742-4.

- **H. Segers**, M.M. van den Heuvel-Eibrink, M.J. Coppes, M. Aitchison, C. Bergeron, B. de Camargo, J.S. Dome, G. Gatta, N. Graf, P. Grundy, J.A. Kalapurakal, J. de Kraker, E.J. Perlman, H. Reinhard, F. Spreafico, G. Vujanic, A.B. Warwick, K. Pritchard-Jones, on behalf of the SIOP-RTSG and the COG-RTC. *Management of adults with Wilms tumor: recommendations based on international consensus*. Expert Review of Anticancer Therapy, 2011 Jul;11(7):1107-1115.

- G.A.M. Tytgat, **H. Segers**, M.M. van den Heuvel-Eibrink. *New insights in genetics and prognostic factors in Wilms tumour.* Treatment strategies – Paediatrics, 2012;2(2);55-61.

- **H. Segers**, R. Kersseboom, M. Alders, R. Pieters, A. Wagner, M.M. van den Heuvel-Eibrink. *Frequency of WT1 and 11p15 constitutional aberrations and phenotypic correlation in childhood Wilms tumour patients.* European Journal of Cancer, 2012 Nov;48(17):3249-56.

- **H. Segers**, M.M. van den Heuvel-Eibrink, R.R. de Krijger, R. Pieters, A. Wagner, W.N.M. Dinjens. *Defects in the DNA mismatch repair system do not contribute to the development of childhood Wilms tumors.* Pediatric & Developmental Pathology, 2013 Jan-Feb;16(1):14-9.

- **H. Segers**, M.M. van den Heuvel-Eibrink, R.D. Williams, H. van Tinteren, G. Vujanic, R. Pieters, K. Pritchard-Jones, N. Bown on behalf of the Children's Cancer and Leukaemia Group and the UK Cancer Cytogenetics Group. *Gain of 1q is a marker of poor prognosis in Wilms tumors.* Accepted in Genes, Chromosomes and Cancer, July 2013.

- K. Blijdorp, M.M. van den Heuvel-Eibrink, R. Pieters, S.M.F. Pluijm, A. Wagner, **H. Segers**, A.J. van der Lely, S.J.C.M.M. Neggers. *Final height and insulin-like growth factor-I in adult survivors of Wilms tumor.* Submitted European Journal of Endocrinology, July 2013.

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