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Identification and Management of High Risk Wilms Tumors

Proefschrift

ter verkrijging van de graad van doctor aan de
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INTRODUCTION

Pediatric renal tumors comprise approximately 5% of malignancies in children under 15 years old and 3.6% of malignancies in children under 20 years old (1). Among 9731 patients registered with the National Wilms Tumor Study Group (NWTSG) (1969-2002), Wilms tumor composed the vast majority of childhood renal tumors (92%), followed by clear cell sarcoma of the kidney (3.4%), congenital mesoblastic nephroma (1.7%), malignant rhabdoid tumor (1.6%), and rare miscellaneous neoplasms including primitive neuroectodermal tumor (PNET), synovial sarcoma, neuroblastoma, and cystic nephroma (1.1%). Although not historically included on NWTSG studies, renal cell carcinoma accounts for 8% of renal tumors in children age 0-19 years according to data from the Surveillance, Epidemiology, and End Results (SEER) program (1).

The study of Wilms tumor has had significant impact on the field of oncology. Tenets of cancer biology, such as Knudson's two-hit model, tumor suppressor genes, and alteration of genomic imprinting as a cancer initiator, were pioneered in Wilms tumor. Wilms tumor also provided a paradigm for multidisciplinary treatment approaches and the conduct of cooperative group studies. Through the efforts of the NWTSG, International Society of Pediatric Oncology (SIOP), United Kingdom Children's Cancer Study Group (UKCCSG) and others, the overall durable survival rate for Wilms tumor now approximates 90%. This remarkable feat has been achieved while reducing radiation and anthracycline exposure in most patients.

Despite this success, it is premature to declare victory in the battle against Wilms tumor. A significant minority of patients do not fare well, including those with anaplastic histology, blastemal-predominant histology (among patients treated up-front chemotherapy), bilateral disease, and favorable histology with loss of heterozygosity (LOH) at chromosomes 1p and 16q. Together, these groups comprise 15-20% of Wilms tumor patients. An additional 10-15% of patients with favorable histology Wilms tumor experience recurrence without clear risk factors. Taken together, 25-30% of Wilms tumor patients have disease that is challenging to treat and resistant to first-line therapy.

The research contained in this PhD thesis focuses on the identification of patients with high-risk Wilms tumor and strategies to improve clinical outcome. The first two articles

describe research that identified telomerase expression level as a prognostic marker for favorable histology Wilms tumor. The studies indicate that high telomerase RNA expression may identify a group of patients who warrant additional therapy. The third article presents a large institution's experience with recurrent Wilms tumor and documents the significantly improved salvage rate that has occurred since the 1980s. The fourth article describes the results of the fifth National Wilms Tumor Study's treatment regimens for anplastic histology Wilms tumor. The fifth article describes the genetic and clinical characterization of Wilms tumor xenografts that were developed for pre-clinical testing of novel agents. The final article presents the results of a phase II study to evaluate the anti-tumor activity of topotecan, one of the agents predicted in the xenograft screens to be active against Wilms tumor.

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CHAPTER 1

High telomerase reverse transcriptase (*hTERT*) mRNA level correlates with tumor recurrence in patients with favorable histology Wilms tumor

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ABSTRACT

Telomerase is a reverse transcriptase that maintains chromosome ends, compensating for the progressive loss of DNA that occurs during replication. High telomerase enzyme activity is an unfavorable prognostic feature for several types of cancers. We investigated whether telomerase level predicts outcome for patients with the pediatric renal malignancy Wilms tumor. In a case-cohort study of 78 patients with favorable histology Wilms tumor, we compared tumor telomerase levels in patients with and without eventual recurrence. Three measures of telomerase were employed: 1) telomerase enzyme activity, 2) expression of hTR, the RNA component of telomerase, and 3) mRNA expression of *hTERT*, the gene that encodes the catalytic component of the enzyme. Of the evaluable samples, 81% had detectable telomerase activity, 97% had detectable *hTERT* transcript, and 100% had detectable hTR. Weak correlations were observed between telomerase activity and hTR level ($r=0.34$, $p=0.02$) and between telomerase activity and *hTERT* mRNA level ($r=0.32$, $p=0.04$). Of the variables assessed, only *hTERT* mRNA expression correlated with outcome. The median *hTERT* mRNA level in tumors with recurrence was higher than that in tumors without recurrence (1.42 units versus 0.97 units, $p=0.023$, Wilcoxon). Univariate analysis of *hTERT* mRNA level as a continuous variable suggested that each unit increase in *hTERT* mRNA level increased the risk of recurrence (RR) by a factor of 1.66 (95% confidence interval (CI) 1.2-2.3, $p<0.005$). Compared to tumors with *hTERT* mRNA levels of 0-1 units, tumors with *hTERT* mRNA levels of 1-2 units had a RR of 2.72 (95% CI 0.91-8.13, $p=0.074$) and tumors with *hTERT* mRNA levels >2 units had a RR of 6.40 (95% CI 1.49-27.67, $p=0.013$). Multivariate analysis of *hTERT* mRNA level as a predictor of recurrence, which adjusted for tumor stage and age at diagnosis, revealed a RR of 1.48 (95% CI 0.9 to 2.6, $p=0.16$). Measurement of *hTERT* mRNA level may therefore enable clinicians to identify a population of patients at high risk for recurrence, and to adjust their therapy accordingly. A larger study will be necessary to determine whether *hTERT* expression is an independent prognostic indicator. Further biological investigation is warranted to discern whether the link between high *hTERT* expression and unfavorable prognosis is causative or correlative.

Introduction

Telomerase is a specialized reverse transcriptase that adds nucleotide repeats to telomeres, counteracting the progressive loss of DNA that occurs during replication and maintaining karyotypic stability. Because telomerase is present in approximately 85-95% of cancer specimens, but absent in most normal tissue (1, 2), it has become a focus of active clinical investigation. Studies have demonstrated that the presence of telomerase activity can be used to distinguish malignant from normal tissue in various organs including the prostate (3), thyroid (4), cervix (5-7), and breast (8). Additionally, studies of neuroblastoma (9, 10), gastric cancer (11, 12), breast cancer (13), acute myelogenous leukemia (14), chronic lymphocytic leukemia (15), and meningioma (16) have revealed that high telomerase activity is associated with tumor recurrence or poor therapeutic outcome.

The renal malignancy Wilms tumor, the fourth most common cancer of childhood (17), is broadly classified into two histologic subtypes, favorable and anaplastic (18). Approximately 85-90% of patients with favorable histology tumors are cured with relatively light treatment, whereas only 50-60% of patients with anaplastic tumors are cured, despite aggressive therapy. Because histologic classification and staging fail to detect a subset of patients at high risk of recurrence, it would be beneficial to establish other prognostic markers for this disease. Based on the promising findings in other malignancies, we sought to survey telomerase expression in Wilms tumor and to determine whether telomerase level correlates with clinical outcome.

In a pilot study of 35 Wilms tumors, we observed a trend towards higher telomerase activity level in tumors with advanced stage disease and anaplastic histology (unpublished observation). We also found that tumors with low telomerase activity had a significantly lower relapse rate than tumors with high telomerase activity (unpublished observation). To confirm these findings, we designed a case-cohort study to compare telomerase levels in tumors that eventually recurred to levels in tumors that never recurred. In this study, we targeted patients with favorable histology disease because the therapy for this group would be amenable to intensification, if justified by an unfavorable prognostic feature. Moreover, patients with favorable histology disease constitute over

90% of the Wilms tumor population. We evaluated levels of three measures of telomerase by semi-quantitative methods: 1) telomerase enzyme activity, 2) expression of hTR, the RNA component of telomerase, and 3) mRNA expression of *hTERT*, the gene that encodes the catalytic component of the enzyme. Additionally, we ascertained whether DNA ploidy and proliferative index correlate with telomerase level and patient outcome.

Materials and Methods

Sample selection and tissue acquisition

A case-cohort design was employed to optimize our ability to compare biological differences in tumor samples from patients with and without relapsed disease. A cohort of 523 cases of favorable histology Wilms tumor was defined from patients enrolled on the National Wilms Tumor Studies (NWTS) 4 and 5 between 1988 and 1996 who were treated according to protocol and had samples submitted to the National Wilms Tumor Study Group (NWTSG) tumor bank. Patients were treated similarly according to stage. Following the methods of Prentice (19), an approximate 10% sample (60 patients) was selected from the identified cohort and defined as the "subcohort." To this group were added 39 cases from the initial cohort who were known to have relapsed as of March, 1997. Because tissue from several patients was depleted from the tumor bank, the final analysis was performed on 90 tumor samples from 88 patients. Two patients with bilateral disease had tissue from more than one tumor available; only the highest values for telomerase level, DNA ploidy, and S-phase fraction were used in the outcome analysis. The study was conducted in a blinded fashion; the assays were performed without knowledge of the patient characteristics, including outcome, corresponding to the tumor samples.

Wilms tumor specimens, which were snap frozen in liquid nitrogen, were obtained through the Cooperative Human Tissue Network. Because personal identifiers were not furnished and there was no risk of violation of patient confidentiality, formal review for this study was waived by the Johns Hopkins Hospital Joint Committee on Clinical Investigation. A frozen section of each sample was obtained for hematoxylin and eosin (H&E) staining to confirm the presence of viable tumor. From the cut edge of each specimen, an approximately 50 mg slice of tumor was removed with a clean scalpel and divided into two aliquots, one for the telomerase enzyme activity assay and one for RNA

isolation. Additional tissue was later taken from the original cut surface for DNA content and S-phase fraction analysis. Tissue from two normal adult kidneys in our tumor bank was also evaluated.

Determination of Telomerase Enzyme Activity

Telomerase enzyme activity determination was performed using a commercial telomeric repeat amplification protocol (TRAP) assay, according to the manufacturer's recommendations (TRAPeze, Oncor, Gaithersberg, MD). Tissue lysates were prepared in CHAPS lysis buffer as previously described (20) and 4 μg of protein were used for each 50 μl TRAP assay. In order to decrease primer-dimerization, hot-start reaction conditions were used (21). Paired samples were inactivated by preincubation with RNAse A (Boehringer Mannheim, Indianapolis, IN). A 30 minute extension step was performed at room temperature, followed by a 2-step PCR reaction with the following conditions: (94°C/30sec, 57°C/30sec) x 27 cycles. The linearity of the TRAP assay under these conditions was confirmed using a representative Wilms tumor sample. Reaction products were loaded on 10% nondenaturing polyacrylamide gel in 0.5X TBE (22) and products were visualized with a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Densitometry was performed with IPLabGel software (Signal Analytics, Vienna, VA). Quantitation was performed according to the TRAPeze kit protocol, with "telomeric products generated" (TPG) units calculated as described, except that final values were not multiplied by a factor of 100. Samples were considered to have detectable telomerase activity if they produced a characteristic telomeric repeat ladder that was extinguished by the addition of RNAse A. All of the reactions were repeated several months apart to ensure reproducibility of the assay over time.

Determination of hTR and hTERT mRNA levels

Expression levels of hTR and *hTERT* mRNA were determined by reverse transcriptase polymerase chain reactions (RT-PCR). Total RNA was isolated from approximately 25 mg of tissue using the Tri-Reagent protocol (Molecular Research Center, Inc., Cincinnati, OH). RNA was treated with DNAse 1 and quantified by UV spectrophotometry (22). 2 μg of RNA were used for each 50 μl RT reaction, which was run with pDN6 random primers and MMLV-RT (Gibco BRL, Gaithersberg, MD). For hTR detection, 2.5 μl of a 1:10 dilution of RT product, corresponding to an RNA input of 10 ng, was PCR-amplified using primers RF:

5'-ACCCTAACTGAGAAGGGCGTAG-3' and RR: 5'-GTTTGCTCTAGAATGAACGGTG-3', kindly donated by Dr. N. Kim (Geron Corporation), yielding a 122 bp fragment corresponding to nucleotides 143-264 of the *hTR* gene (Genbank accession # U86046). To control for differences in RNA quantity, as well as for differences in the PCR reaction, a 158 bp fragment of the human acidic ribosomal phosphoprotein PO housekeeping gene (*36B4*, Genbank accession # M17885) was coamplified with the hTR fragment in a one-tube reaction (36B4F: 5'-GATTGGCT

ACCCAACTGTTGCA-3' and 36B4R 5'-CAGGGGCAGCAGCCACAAAGGC-3'). Each 25 µl reaction contained 1x PCR Buffer (Perkin Elmer, Foster City, CA), 2.5mM $MgCl_2$, 2 µM of primers RR and RF, 0.5 µM of primers 36B4F and 36B4R, 320 µM dNTPs, and 0.5 U Taq polymerase. The reaction mixtures were thermal cycled as follows: (94°C/1 min., 62°C/1 min., 72°C/1 min.) x 25 cycles; (72°C/5 min) x 1 cycle. The linearity of the hTR and *36B4* reactions under these conditions was validated using RNA derived from the MCF-7 breast cancer cell line. Products were resolved on 2% agarose gels in TBE buffer and stained with ethidium bromide. Gels were imaged on a gel documentation system (UVP, Upland, CA) and densitometry was performed using IPLab Gel software. The corrected values for hTR were calculated by dividing the hTR level by the *36B4* level. For *hTERT* mRNA detection, 2.5 µl of a 1:10 dilution of RT product was amplified using primers MS113: 5'-AGAGTGTCTGGAGCAAGTTGC-3' and MS114: 5'-CGTAGTCCATGTTCACAATCG-3', yielding a 183 bp fragment corresponding to nucleotides 1789-1971 of *hTERT* cDNA (Genbank accession # AF018167). Because this primer set spans intron 4 of the *hTERT* gene, contaminating genomic DNA was not a factor in our analysis. The primer set does not encompass any regions reported to be involved in alternative splicing of the *hTERT* gene (23). Each 25 µl reaction contained 1x PCR Buffer (60mM Tris-HCl (pH 8.5), 15 mM ammonium sulfate, 2.5mM $MgCl_2$), 1 µM of each primer, 320 µM dNTPs, 2.5 µCi α-^{32}PdCTP, and 0.5 U Taq polymerase. Cycling conditions were as follows: (94°C/45 sec., 60°C/45 sec, 72°C/2 min) x 32 cycles; (72°C/5 min) x 1 cycle. A quantitative control using the primers for the *36B4* gene was performed, but in this case, a separate tube was required because of the difference in levels of *hTERT* and *36B4* transcripts. For the *36B*4 amplification, the reaction conditions described for the hTR reaction above were used, except that 2.5 µCi α-^{32}PdCTP was added to each assay and that only 20 cycles were performed. The linearity of the *hTERT* and *36B4* reactions under these conditions was validated using RNA isolated from

MCF-7 cells. Additionally, all tumor samples were run at three different dilutions of RNA input to ensure that each individual sample was in the linear range of detection for the PCR reaction. Both *hTERT* and *36B4* amplification products were loaded into a single lane of a 10% polyacrylamide gel and fractionated by electrophoresis at 350 volts for 2 hours. Images were visualized with a Phosphorimager screen and quantitated with Multi-Analyst (BioRAD, Hercules, CA) software. Corrected *hTERT* mRNA levels were obtained by dividing the *hTERT* level by the *36B4* level.

Flow Cytometric Determination of DNA ploidy and S-phase fraction

Samples containing 10^6 cells were centrifuged and resuspended in 1ml of propidium iodide staining solution (0.05 mg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100). Immediately prior to analysis by flow cytometry, each sample was treated at room temperature with DNAse-free RNAse (Calbiochem, San Diego, CA) at a final concentration of 0.0005 mg/ml for thirty minutes and filtered through 40 μm nylon mesh. Fluorescence at wavelength 563-607 nm emitted from propidium iodide-DNA complexes was measured from approximately 20,000 cells with a FACScan flow cytometer (Becton Dickinson Immunocytometry, San Jose, CA). The percentages of cells within the G1, S, and G2/M phases of the cell cycle were determined by analysis with the computer program Mod Fit (Verity Software House, Topsham, ME).

Statistical Analysis

Associations between biological variables were measured with Pearson correlation coefficients and p-values were determined by linear regression. Because of the right skewness of the distribution of telomerase activity, this variable was transformed by taking its natural logarithm. The value 0.1 was added to all records to avoid an infinite logarithm for those samples with a telomerase activity of zero. Outcome analysis was based on the relative risk regression model of Cox (24). Regression coefficients were estimated much as if complete cohort data were available, and they have the same interpretation. Standard errors of the coefficients, however, were adjusted by the robust method of Barlow (25), to account for the fact that only a fraction (<10%) of patients who did not relapse were included in the analysis. Both univariate and multivariate analysis, accounting for age at diagnosis

and tumor stage, were performed. Levels of clinical and biological parameters for patients with and without recurrence were compared with the Wilcoxon rank sum test.

Results

Sample selection and patient characteristics

Prior to performing the biological analyses, frozen sections of the selected Wilms tumor samples were stained with hematoxylin and eosin to confirm the presence of malignant cells. Histologic confirmation is necessary to minimize false-negative results for telomerase activity (2). Of the 90 samples originally selected by the statisticians, 10 had no evidence of malignant tissue or were completely necrotic. Of the remaining 80 tumors, representing 78 patients (two patients with bilateral disease had more than one tumor analyzed), the median fraction of non-necrotic malignant cells in the section was 80%. Of the 78 patients studied, 34 had recurrent disease. Four of the cases with recurrence were originally selected in the subcohort group, but were later added to the case group once relapse was documented. The median follow-up times for the original subcohort group and for the group of patients without recurrence were 2.6 years and 2.9 years, respectively. Most Wilms tumor recurrences occur within two years of diagnosis. The clinical characteristics of the analyzed patients are described in Table 1.

Linearity of the TRAP, hTR, and hTERT assays

Conditions for the TRAP, *hTERT*, and hTR reactions were optimized before performing assays on the patient samples. All three assays were in the linear range of detection for the amount of protein or RNA used and for the number of PCR cycles selected (Figure 1).

Telomerase activity, hTR, and hTERT levels

Samples were deemed to have evaluable levels of telomerase activity, hTR, and *hTERT* transcript if their corresponding internal PCR controls or housekeeping genes amplified correctly. Of the evaluable samples, 62/77 (81%) had detectable telomerase activity, 75/77 (97%) had detectable *hTERT* transcript, and 80/80 (100%) had detectable hTR. The median values of telomerase activity, *hTERT* mRNA, and hTR, in corrected units, were 0.37 (range, 0-15.29), 1.24 (range, 0-4.64), and 0.77 (range, 0.17-2.39), respectively. Two normal adult kidney samples were negative for telomerase activity and *hTERT* mRNA, but expressed

hTR at levels comparable to tumor tissue. In the tumor samples, there was a 78.4% concordance between the detection of telomerase activity and *hTERT* expression (Table 2). Most discordant cases had undetectable telomerase activity, but detectable *hTERT* transcript; only two cases had detectable telomerase activity in the setting of undetectable *hTERT* transcript.

Table 1.

Patient characteristics and univariate regression analysis of risk of recurrence.

Variable	Relapsed Cases	Controls	Relative Risk	95% confidence interval	p value
Gender					
Male	20	20	1.0		
Female	14	24	0.66	(0.27, 1.61)	0.36
Stage					
I	4	13	1.0		
II	14	11	5.35	(1.39, 20.6)	0.015
III	6	12	1.77	(0.42, 7.37)	0.43
IV and V	10	8	4.58	(1.09, 19.4)	0.038
Age at diagnosis (yrs.)					
0-2	6	14	1.0		
2-4	8	17	1.25	(0.35, 4.39)	0.73
4+	20	13	3.44	(1.07, 10.9)	0.037
Biological variables					
hTERT mRNA level			1.66	1.2 to 2.3	<0.005
log (telomerase+0.1)			1.15	0.8 to 1.7	0.46
hTR level			1.31	0.5 to 3.4	0.51
DNA ploidy>1			.757	0.3 to 2.0	0.56
S phase fraction			.996	0.9 to 1.0	0.81

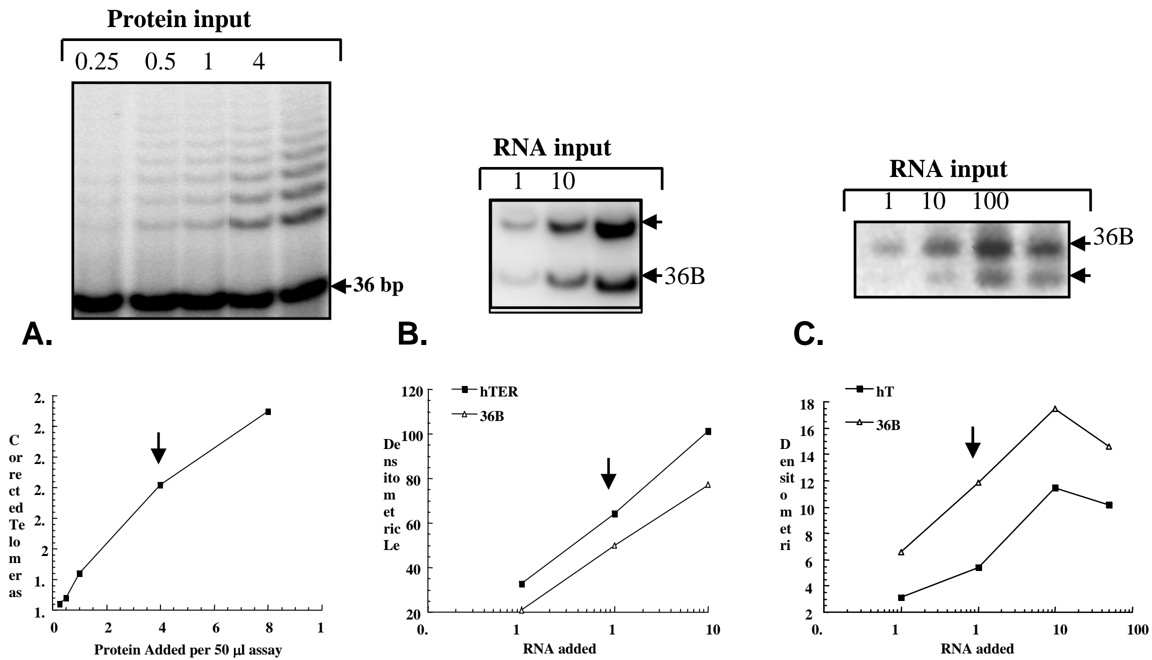

Figure 1. Validation of the semi-quantitative nature of the TRAP, hTR, and *hTERT* assays. Arrows above the curves indicate the amount of protein or RNA that was used for the assays of patient samples. *A*, TRAP assay of a representative Wilms tumor sample showing a linear relationship between the amount of protein added and the corrected telomerase activity. Reaction products were separated on a 10% nondenaturing polyacrylamide gel and visualized using a phosphorimager (Molecular Dynamics). The 36 base pair band at the bottom of the gel represents an internal PCR control. *B*, RT-PCR assay amplifying *hTERT* and *36B4* transcripts from RNA isolated from the MCF-7 breast cancer cell line. Reactions were performed in separate tubes because of the vast difference between transcript levels of *hTERT* and *36B4*, but products were separated on a single10% nondenaturing polyacrylamide gel and visualized using a phosphorimager. *C*, RT-PCR assay amplifying hTR and *36B4* transcripts. Reactions were performed in a single tube and products were separated on a 2% agarose gel and stained with ethidium bromide.

Table 2

Concordance between presence of telomerase activity and *hTERT* expression in tumor tissue

	hTERT mRNA	
Telomerase Activity	+	-
+	58	2
-	14	0

DNA Ploidy and S-Phase fraction

Seventy-nine of 80 tumor samples were evaluable for DNA content determination by flow cytometry. A DNA index of 1.0 is indicative of diploid DNA content. The median value for DNA index was 1.0, with a range from 0.93 -1.87. Sixty-nine of 80 samples yielded evaluable results for S-phase fraction. The median value was 21.4%, with a range from 4.2-53.8%.

Relationships between biological and clinical variables

The randomly chosen subcohort, but not the selected relapsed cases, was used in the correlation analyses because it represents an unbiased sampling of the Wilms tumor patient population. Weak correlations were observed between telomerase activity and hTR level (r=0.34, p=0.02) and between telomerase activity and *hTERT* mRNA level (r=0.32, p=0.04) (Figure 2). The correlation analysis of telomerase activity and hTR level included two outlying data points with high values that appeared to influence the analysis (Figure 2). When these points were omitted, the correlation between hTR and telomerase activity persisted (r=0.36, p=0.02).

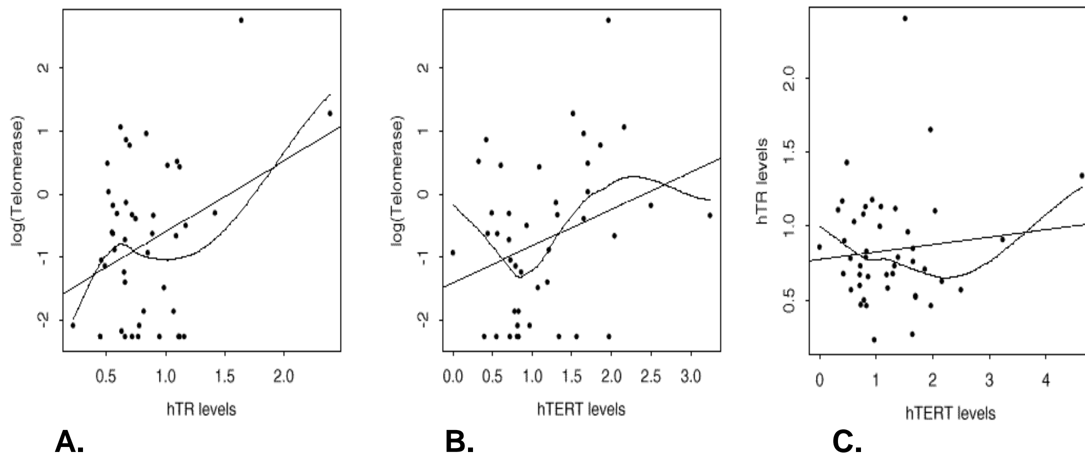

Figure 2. Correlation between telomerase activity, *hTERT* mRNA level, and hTR level in evaluable tumors from the randomly selected subcohort (n=48). Associations were measured with Pearson correlation coefficients and p-values were determined by linear regression. *A,* Correlation between log (telomerase+0.1) and hTR level (r=0.34, p=0.02). *B*, Correlation between log (telomerase+0.1) and *hTERT* mRNA level (r=0.32, p=0.04). *C,* Correlation between hTR level and *hTERT* mRNA level (r=0.11, p=0.48).

One concern regarding the telomerase activity analysis was the potential for false-negative results due to enzyme inactivation or inhibition. Correlation analyses were therefore repeated omitting samples with zero telomerase activity. In such analyses, the correlations between telomerase activity and hTR level ($r=0.49$, $p=0.006$) and between telomerase activity and *hTERT* mRNA level ($r=0.40$, $p=0.04$) strengthened. No relationships between hTR and *hTERT* mRNA level ($r=0.11$, $p=0.48$) or between any of the telomerase measurements and DNA content or proliferative index emerged (data not shown). Regarding clinical variables, no relationships between telomerase activity, hTR, or *hTERT* mRNA level and age at diagnosis or tumor stage were detected (data not shown).

Outcome analysis

Median values of the biological variables for patients with and without recurrence are listed in Table 2. Of the assessed parameters, only levels of *hTERT* transcript were significantly different between the relapsed and non-relapsed groups (median 1.42 versus 0.97, $p=0.023$, Wilcoxon). The median age at diagnosis was also significantly higher in patients with recurrence than in patients without recurrence (56.5 months versus 29.5 months, $p=0.003$).

Relative risks of recurrence (RR) were determined by Cox regression analysis. Of the five biological parameters measured, only *hTERT* expression level correlated with outcome. Univariate analysis of *hTERT* mRNA as a continuous variable suggested that each unit increase in *hTERT* mRNA level increased the RR by a factor of 1.66 (95% CI 1.2-2.3, $p<0.005$) (Table 1). Likewise, an analysis based on the grouping of *hTERT* transcript levels into three categories revealed a strong association between relapse and high *hTERT* mRNA level (Table 4).

The results of the outcome analysis were unchanged when two data points with outlying hTR and *hTERT* mRNA levels were omitted from consideration (data not shown). Relative risks of recurrence were also determined for clinical variables. Compared to diagnosis before the age of 2 years, diagnosis after the age of four years was associated with a RR of 3.44 (95% CI 1.07-10.9, $p=0.037$).

Table 3

Median values of clinical and biological variables for patients with and without recurrence.

Variable	Median (Interquartile range)	p-value (Wilcoxon)
Telomerase activity		
Relapse	0.38 (0.15 to 1.43)	0.36
No Relapse	0.40 (0.02 to 0.73)	
hTERT mRNA level		
Relapse	1.42 (1.03 to 2.00)	0.0225
No Relapse	0.97 (0.71 to 1.64)	
hTR level		
Relapse	0.81 (0.68 to 0.98)	0.56
No Relapse	0.71 (0.57 to 1.03)	
DNA ploidy		
Relapse	1.00 (1.00 to 1.15)	0.44
No Relapse	1.00 (1.00 to 1.12)	
S-phase %		
Relapse	21.25 (18.57 to 28.93)	0.40
No Relapse	20.90 (11.60 to 31.20)	
Age at diagnosis (months)		
Relapse	56.5 (31.0 to 84.0)	0.0034
No Relapse	29.5 (21.0 to 56.0)	

Compared to children with stage I disease, patients with stage II disease had a RR of 5.35 (95% CI 1.39-20.6, p=0.015) and patients with stages IV and V disease had a RR of 4.58 (95% CI 1.09-19.4), P=0.038) (Table 1). The high RR for stage II disease reflects, in part, the relatively high rate of recurrence of stage II patients in the NWTS-4 study (26).

Table 4

Unvariate analysis of risk of recurrence by *hTERT* mRNA level

hTERT mRNA level (corrected units)	Relapse		Relative Risk	95% confidence interval	p value
	No	Yes			
0-1	21	7	1.0		
1-2	16	18	2.72	(0.91, 8.13)	0.074
2+	4	9	6.40	(1.49, 27.67)	0.013
Test for trend					0.014

This finding is possibly related to undertreatment of patients with Stage II disease with peritoneal spillage of tumor (27). The multivariate analysis of *hTERT* mRNA level as a predictor of relapse, which adjusted for age at diagnosis and tumor stage, revealed a RR of 1.48 (95% CI 0.86-2.56, p=0.16) for each unit increase in *hTERT* mRNA level (Table 5). Although the RR of increasing *hTERT* expression persisted, statistical significance was lost.

Table 5

Multivariate regression analysis of relapse-free survival

Variable	Relative Risk	95% confidence interval	p value
hTERT mRNA level	1.48	0.9 to 2.6	0.16
Age at diagnosis	1.03	1.0 to 1.1	0.04
Stage II	10.7	0.98 to 118.2	0.05
Stage III	.931	0.1 to 8.3	0.95
Stage IV and V	4.768	0.48 to 47.3	0.18

Discussion

In this study, we explored the prognostic utility of telomerase in favorable histology Wilms tumor. Telomerase levels were measured in patients with and without eventual recurrence by measuring telomerase enzyme activity, expression of hTR (the RNA component of the

telomerase complex), and mRNA expression of *hTERT* (the gene that encodes the catalytic component of the enzyme). We employed a case-cohort design, which compared the features of relapsed cases to a random sampling of the overall Wilms tumor population, to allow for the study of clinical variables as risk factors in their own right. By contrast, a case-control study, in which relapsed cases are matched by clinical variable to controls, precludes such an analysis. Of the biological variables assessed, only *hTERT* mRNA expression correlated with outcome. Univariate analysis of *hTERT* mRNA as a continuous variable suggested that each unit increase in *hTERT* level increased the risk of recurrence (RR) by a factor of 1.66 (95% confidence interval (CI) 1.2-2.3, $p<0.005$). Measurement of *hTERT* mRNA may therefore enable clinicians to identify a population of patients at high risk for recurrence, and to adjust their therapy accordingly. A caveat to this finding is that although the elevated risk of high *hTERT* mRNA expression persisted in the multivariate analysis, statistical significance was lost. It is possible that high *hTERT* mRNA expression is not independent of patient age and tumor stage, but it is also possible that statistical uncertainty was introduced because of insufficient sample size. Of note, no relationship between *hTERT* mRNA level and age at diagnosis or tumor stage was detected. Further studies will be necessary to determine the true clinical utility of measuring tumor *hTERT* transcript levels in patients with Wilms tumor.

We did not detect a correlation between telomerase enzyme activity or hTR level and patient outcome. It was surprising that telomerase activity was not prognostic because it was this measurement that correlated with outcome in our pilot study and in the neuroblastoma studies. We attribute this disparity in findings to limitations of the TRAP assay, which measures telomerase activity. Although this assay has internal controls for PCR inhibition and spurious telomerase activity, it does not control for enzyme inhibition by tissue inhibitors, degradation of the RNA template, or enzyme inactivation with heat or time. The latter two issues may be especially problematic in multi-center studies in which tissue preservation technique is not uniform. By contrast, the *hTERT* RT-PCR assay accounts for RNA degradation with the amplification of a housekeeping gene. Moreover, because the RNA is purified, tissue inhibitors of the PCR reaction are inconsequential. It is possible that measuring *hTERT* mRNA expression, rather than telomerase activity, would increase the prognostic value of telomerase in other tumor types. The lack of association between hTR

level and tumor recurrence was not surprising because it is known that hTR is constitutively expressed in both normal and malignant tissue (28-33). Nevertheless, we report a weak positive correlation between hTR and telomerase activity, and other studies have indicated that hTR is upregulated during tumorigenesis (29, 34, 35). Although telomerase activity and hTR expression are clearly related, the overlap between hTR levels in normal and malignant tissue appears to limit the utility of hTR as a tumor marker.

Several studies have generated enthusiasm for the utility of telomerase as a prognostic indicator for human cancer. The relationship between high telomerase activity level and adverse clinical outcome was first suggested in an analysis of untreated neuroblastoma, which demonstrated that advanced stage disease, amplified *MYCN*, and poor survival were associated with high telomerase enzyme activity (9, 10). Strikingly, metastatic neuroblastoma classified as Stage 4S, a subtype that usually regresses spontaneously, had low or undetectable activity (9, 10, 36). High telomerase activity was later associated with unfavorable outcome in gastric cancer (11, 12), breast cancer (13), acute myelogenous leukemia (14, 37), chronic lymphocytic leukemia (15), and meningioma, but other reports have questioned these findings (38-40). It is unclear whether the conflicting results are due to differences in assay methodology, patient population, tumor type, tumor stage, or other unrecognized factors.

It is not immediately apparent how high levels of telomerase could contribute to tumor progression once the threshold of activation has been breached. One possibility relates to the telomere hypothesis of aging, which asserts that telomere length is a biological clock that regulates the number of divisions a cell can achieve. In the absence of telomerase, telomeres erode to a point at which signals are given for a cell to undergo senescence or apoptosis (reviewed in (41)). Based on this hypothesis, tumors without telomerase would be predicted to have a limited life span, as exemplified by stage 4S neuroblastoma (9, 10, 36). Most tumors, however, possess measurable telomerase activity. It is noteworthy that low levels of telomerase activity are not sufficient to arrest telomeric shortening, as demonstrated in hematopoietic stem cells (42, 43). If this observation applies to cancer cells, tumors with high telomerase activity may have a proliferative advantage over those with low telomerase activity. Hence, clinical outcome may be poorer in patients with tumors with high telomerase activity. A second reason that high telomerase level may correlate with poor

prognosis is that in addition to maintaining telomeres, telomerase appears to function as a chromosome healing enzyme (44-46). In this capacity, excess telomerase may mediate resistance to DNA damaging therapy. In support of this postulate, inhibition of telomerase in glioblastoma cells resulted in an increased sensitivity to the DNA-damaging agent cisplatin (47). Finally, it is possible that high telomerase activity represents a surrogate marker for an advanced malignant state. In this case, even if telomerase does not contribute to tumor proliferative capacity or resistance to therapy, it could still be a useful clinical tool.

Our data indicate that telomerase activity and *hTERT* transcript levels do not correlate with proliferative index in Wilms tumor. This contrasts with studies that indicate that telomerase activation is tightly linked to cellular division in normal (48-50) and malignant (13) tissue. The coupling is not absolute, however, as demonstrated by the lack of telomerase activity in cultured fibroblasts prior to crisis (41) and in hyperplastic conditions such as uterine fibroids and benign prostatic hypertrophy (3). Moreover, telomerase activity did not correlate with proliferative index in reports of acute myelogenous leukemia (14), breast cancer (51, 52), and gastric carcinoma (12). Although telomerase activity is clearly linked with proliferation in some cell types, certain tumors appear to upregulate telomerase independent of proliferation.

The relationship between cellular DNA content and telomerase level remains to be determined. Our study, which did not reveal a relationship between DNA ploidy and telomerase level, is consistent with reports of renal cell carcinoma (38) and breast cancer (51). By contrast, other studies of breast cancer (13), breast ductal carcinoma *in situ* (52), and gastric cancer (12) revealed a positive correlation between DNA index and telomerase activity level. A number of factors, including differences in assay methodology, patient population, tumor type, and tumor stage, can be invoked to explain the lack of consistency amongst studies.

In conclusion, our findings indicate that tumor *hTERT* mRNA expression level correlates with outcome in patients with favorable histology Wilms tumor. A larger study will be necessary to determine whether *hTERT* mRNA expression is predictive of outcome independent of patient age and tumor stage. If so, determination of *hTERT* mRNA level may be a valuable clinical tool for stratifying patients with favorable histology Wilms tumor

into risk-appropriate treatment groups. Further biological studies are warranted to discern whether the link between high *hTERT* expression and unfavorable prognosis is causative or correlative. Such studies will lend insight into the value of telomerase inhibition as a therapeutic modality for cancer.

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CHAPTER 2

High Telomerase RNA Expression Level is an Adverse Prognostic Factor for Favorable Histology Wilms Tumor

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ABSTRACT

Purpose

A primary objective of the fifth National Wilms Tumor Study (NWTS-5) was to identify prognostic indicators for patients with favorable histology (FH) Wilms tumor (WT). The prognostic significance of telomerase expression level in primary tumor samples was assessed.

Patients and Methods

A case-cohort study was conducted involving 291 NWTS-5 registrants. Telomerase activity was measured using the telomeric repeat amplification protocol (TRAP). Expression levels of *TERT* mRNA (encoding the telomerase catalytic component) and TERC/hTR (the telomerase RNA template) were measured using quantitative real-time PCR.

Results

After excluding samples due to lack of viable tumor, RNA degradation, or insufficient clinical information, 244 patients remained for the final analysis: 96 with relapse and 148 without relapse. Univariate analysis revealed a positive correlation between relative risk of relapse (RR) and levels of *TERT* mRNA and TERC expression. For each doubling in *TERT* mRNA and TERC level, the RR increased by a factor of 1.16 (95% CI 1.04-1.29, p=0.01) and 1.35 (95% CI 1.11-1.64, p=0.003), respectively. The third of patients whose tumors had the highest TERC expression level had a RR of 2.06 (95% CI 1.14-3.70, p=0.02) compared to those with the lowest level. TERC expression level remained a significant prognostic indicator in a multivariate analysis adjusting for *TERT* mRNA, tumor stage and patient age. TRAP level did not correlate with RR of relapse. Telomerase expression levels were not predictive of overall survival.

Conclusion

Telomerase RNA expression level may provide a clinically useful adjunct to the current risk classification schema for FH WT.

Introduction

Modern treatment approaches for favorable histology Wilms tumor have yielded cure rates of approximately 90%.[1] The current challenge for the cooperative groups is to limit therapy, and its associated toxicities, for patients at low risk of relapse while intensifying therapy, and improving cure rates, for patients at high risk of relapse.[2] Towards this end, the primary aim of the fifth National Wilms Tumor Study (NWTS-5) was to evaluate novel biological prognostic factors for Wilms tumor.

Telomerase is a specialized reverse transcriptase that adds nucleotide repeats to telomeres, counteracting the progressive loss of DNA that occurs during replication. The enzyme, which plays a key role in cellular immortalization, is minimally composed of a catalytic subunit (TERT), and an RNA subunit (TERC/hTR), which provides the template for nucleotide repeat generation.[3-5] Because telomerase is expressed in approximately 85-95% of cancer specimens, but absent in most normal tissue [6;7], it has been proposed as tumor marker and therapeutic target. Moreover, the presence of telomerase expression has emerged as a predictor of adverse outcome in a variety of adult and pediatric malignancies.[8]

In a pilot study of 78 Wilms tumor samples of favorable histology, we demonstrated a positive correlation between expression level of *TERT* mRNA and risk of relapse.[9] Univariate analysis of *TERT* mRNA level as a continuous variable suggested that each unit increase in *TERT* mRNA level increased the risk of relapse by a factor of 1.66 (95% confidence interval (CI) 1.2-2.3, $p<0.005$). This study did not detect an association between levels of telomerase enzyme activity or TERC and patient outcome, but was limited by its relatively small sample size. In the present study, we sought to further evaluate whether telomerase expression level provides an independent prognostic indicator for favorable histology Wilms tumor.

Patients and Methods

Patients

A stratified sampling design was employed to optimize our ability to compare biological differences in primary tumor samples from patients with and without relapsed or progressive disease. Patients were selected from NWTS-5, which was a multi-institutional therapeutic and biology study of pediatric renal tumors that was open to accrual from August, 1995 through May, 2002. Participating institutions obtained local Institutional Review Board (IRB) approval to activate the study and patients provided written informed consent to be treated on the study and to bank frozen tumor, serum, and urine for biological studies. Additionally, the St. Jude Children's Research Hospital IRB approved the laboratory component of this specific study. Patients were treated similarly according to stage, as previously described.[10]

A cohort of 1013 patients with favorable histology Wilms tumor was defined from patients who registered on NWTS-5 before July 1st 1999 and had pre-treatment tumor tissues available in the biologic specimens bank. Following the design of Prentice [11], an approximate 20% random sample, known as the "subcohort," was selected from the identified cohort. To this group, all patients not already included who relapsed or had progressive disease prior to August 2001 were added. A total of 291 patients (195 subcohort and 96 relapsed cases outside of the subcohort) were selected in this manner. Because the subcohort was randomly selected, it contained patients with tumor relapse or progression. A first event of metachronous Wilms tumor in the contralateral kidney was not considered as a relapse for this study because the occurrence of metachronous Wilms tumor was not thought to relate biologically to telomerase expression in the primary tumor. One subcohort patient who died of infection in the absence of disease was treated as "censored" at the time of this event.

Tissue processing and analysis

Snap-frozen primary tumor tissue from the selected 291 Wilms tumor cases and 16 adjacent normal kidney specimens were obtained through the Cooperative Human Tissue Network (CHTN). A frozen section of each sample was stained with hematoxylin and eosin (H&E) to

confirm the presence of at least 80% viable tumor. Approximately 50 mg of tumor was divided into two aliquots, one for protein isolation and one for RNA isolation.

Molecular assays of telomerase expression

Three distinct measures of telomerase expression were undertaken: telomerase enzyme activity, mRNA expression of *TERT*, and expression of TERC. Telomerase enzyme activity determination was performed using the TeloTAGGG-telomerase PCR-ELISA telomeric repeat amplification protocol (TRAP) assay (Roche, Indianapolis, IN), which was quantitative through a broad range of telomerase activity levels and reproducible. Tissue lysates were prepared in CHAPS lysis buffer as previously described [12] and 4 μg of protein were used for each 50 μl TRAP assay. The levels of telomerase activity that we report represent the optical density (OD) readings from tumor samples relative to those of a standard 293 cell protein extract, as specified in the manufacturer's instructions. Because TRAP activity can degrade with heat and time, we assessed whether alkaline phosphatase activity can serve as a quality control measure for enzymatic activity, as previously suggested. [13] Alkaline phosphatase activity was stable for up to 24 hours in five Wilms tumor samples maintained at room temperature, whereas TRAP activity showed a gradual degradation. Therefore, measurement of alkaline phosphatase activity was not considered to be a suitable control for TRAP activity degradation in Wilms tumor.

Expression levels of TERC and *TERT* mRNA were determined by quantitative real-time reverse transcriptase polymerase chain reactions (RT-PCR) [14-16]. Total RNA was isolated from approximately 25 mg of tissue using the Tri-Reagent protocol (Molecular Research Center, Cincinnati, OH). RNA was quantified by UV spectrophotometry and 2 μg of RNA were used for each 50 μl reverse transcriptase reaction, which was run with pDN6 random primers and M-MLV reverse transcriptase (Invitrogen, Grand Island, NY). Two μl of cDNA product were used for each 50 μl real-time PCR reaction. To detect *TERT* mRNA expression, we used previously reported primer-probe sets (forward: CGGAAGAGTGTCTGGAGCAA; reverse:GGATGAAGCGGAGTCTGGA; probe: 6FAM-TTGCAAAGCATTGGAATCAGACAGCACT-TAMRA).[16] To measure the α+β+ TERT splice form, we generated a novel primer-probe set using Primer Express software (Applied Biosystems, Foster City, CA) (forward: CTTTGTCAAGGTGGATGTG ; reverse: TACGGCTGGAGGTCTGT; probe: 6FAM-ACACCATCCCCCAGGACAGGCT

C-TAMRA). Likewise, for TERC expression, we designed and optimized a novel primer-probe set (forward: TGAGCTGTGGGACGTGCA; reverse: CCACCAACAGGAAAGCG AA; probe: 6FAM-CCAGGACTCGGCTCACACATGCA-TAMRA) The increase in fluorescence intensity resulting from degradation of the probe, as measured by the ABI PRISM 7900 Sequence Detector (Applied Biosystems), was proportional to the amount of PCR product accumulated. The fractional cycle number (C_t) at which the amount of fluorescence reached a defined threshold was taken as a measure of the quantity of target sequence initially present. For quantification, each reaction was normalized to a standard curve (RNA isolated from SY5Y neuroblastoma cells). Each sample was analyzed in triplicate and the reported results represent the mean of the three assays.

For each sample, we assessed expression levels of the housekeeping genes glyceraldehyde phosphate dehydrogenase (*GAPDH*) and *RNAse P* using commercially available primer-probe sets (Applied Biosystems) and *36B4* using a primer-probe set that we optimized (forward:GGCGACCTGGAAGTCCAACT; reverse: CCATCAGCACCACAGCCTTC; probe:VIC-ATCTGCTGCATCTGCTTGGAGCCCA-TAMRA). We found that levels of all three housekeeping genes were significantly lower in normal kidney samples compared to tumor samples, alerting us to the possibility that levels of the housekeeping genes varied according to tissue biology, thus defeating the purpose of such controls. Therefore, we used expression of GAPDH only to exclude samples with very low RNA content; we did not incorporate GAPDH into the final calculations of TERC and *TERT* mRNA expression.

The molecular assays were conducted in a blinded fashion; the assays were performed without knowledge of the patient characteristics, including outcome, corresponding to the tumor samples. To ensure the reproducibility of the measurements of telomerase activity, *TERT* mRNA, and TERC over time, approximately 10% of the samples were randomly selected for a repeat assay that was conducted several months from the original assays. All three assays were highly reproducible over time, with correlation coefficients (r) between values for the first and second assays greater than 0.9 (data not shown).

Statistical Analysis

A sampling weight for non-relapsed patients was first determined so that the relapse-free survival curve for all patients included in the statistical analyses matched the curve for

comparable NWTS-5 patients. Multiplying this weight by the number of subcohort patients whose tissues were successfully assayed for telomerase expression yielded an effective main cohort sample size of 771. Relative risks (RR) of relapse were estimated by a Cox regression analysis that was adjusted using the sampling weight to account for the case-cohort design.[17;18] Very similar estimates of RR were obtained using the original method of analysis proposed by Prentice [11], which does not require specification of a sampling weight. Kaplan-Meier [19] estimates of the relapse-free survival curves within patient subgroups defined by telomerase expression levels were also weighted.

Results

Sample selection

Of the 291 patients selected for this study, 35 whose tumors contained less than 80% viable tumor on H&E stain were excluded from the analysis. Nine patients with viable tumor were further excluded due to RNA degradation, as determined by very low expression of the housekeeping gene GAPDH. Among the 247 patients remaining, of whom 164 had been sampled for the subcohort, 96 had tumor relapse or progression (henceforth referred to as relapse), 148 did not have relapse, and 3 had missing or incomplete outcome data.. One subcohort patient who did not relapse had ample tissue available to evaluate telomerase enzyme activity, but not *TERT* mRNA or TERC expression. Hence, the final analysis of telomerase expression as a prognostic marker was conducted on 243 or 244 patients, depending on which variables were analyzed.

Telomerase expression in Wilms tumor versus adjacent normal kidney

Three distinct measures of telomerase expression were undertaken: telomerase enzyme activity (henceforth referred to as "TRAP activity"), mRNA expression of *TERT*, and expression of TERC. We first evaluated whether telomerase is differentially expressed between Wilms tumors and adjacent normal kidneys. Of the 164 Wilms tumor samples for subcohort patients in which TRAP level was assessed, 157 (95.7%) had detectable TRAP activity. Of the 163 Wilms tumor samples in which *TERT* mRNA and TERC expression were evaluated, 161 (98.8%) had detectable levels of *TERT* mRNA and 163 (100%) had detectable levels of TERC. Of the 16 adjacent normal kidneys, four had detectable *TERT* mRNA, 16 had detectable TERC, and two had detectable TRAP activity.

The few normal kidneys that had detectable *TERT* mRNA and TRAP activity had very low levels of expression; mean levels of *TERT* mRNA and TRAP activity were significantly higher in Wilms tumor compared to normal kidneys (Table 1).

Table 1

Mean level of telomerase expression (± standard error of the mean) in Wilms tumors and adjacent normal kidneys

	Normal kidney (n = 16)	Wilms tumor (n = 164)	P-value (t-test)
TERT mRNA	0.05 (±0.01)	5.98 (±0.67)	0.006
TERC	3.80 (±1.13)	12.65 (±1.03)	0.009
TRAP activity	0.05 (±0.01)	1.06 (±0.04)	0.001

In analyzing the raw data for *TERT* mRNA and TERC expression, we found that the mean values greatly exceeded the median values, suggesting that the distributions of *TERT* mRNA and TERC levels were highly skewed to the right. To eliminate this asymmetry, and to give the relative risk coefficients a desired interpretation in terms of doubling of the assay levels, the statistical analyses used base 2 log-transformed *TERT* mRNA and TERC levels. This resulted in covariates that were more normally distributed although skewed slightly to the left.

Based on the biological relationship between *TERT* mRNA, TERC, and TRAP activity, we expected that these three measurements would correlate with each other. As shown in Figure 1, there were indeed correlations between log2 (*TERT* mRNA) and TRAP activity, log2 (TERC) and TRAP activity, and log2 (*TERT*) and log2 (TERC), although the correlations were not strong.

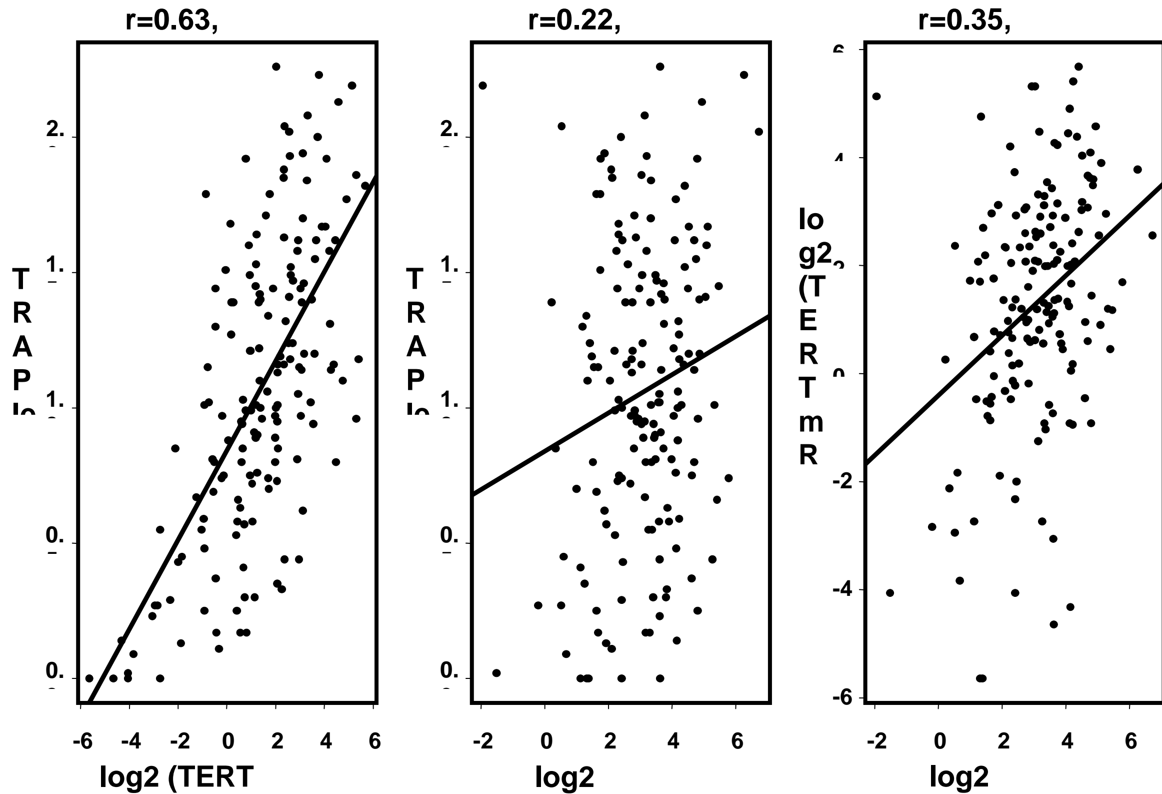

Figure 1. Correlation between measures of telomerase expression in favorable histology Wilms tumor.

Association between telomerase expression and risk of relapse

Table 2 shows the results from case-cohort analyses of the relative risk of relapse using a single regression variable. Among the demographic and biological variables, tumor stage, log-transformed *TERT* level, and log-transformed TERC level were significant prognostic factors for tumor relapse. Treating *TERT* and TERC expression as continuous variables, each doubling of *TERT* mRNA and TERC level increased the relative risk of relapse (RR) by a factor of 1.16 (95% CI 1.04-1.29, p=0.01) and 1.35 (95% CI 1.11-1.64, p=0.003), respectively. TRAP activity level did not correlate with the risk of relapse.

To evaluate the risk of relapse in the tumors with the highest *TERT* mRNA or TERC expression, we further categorized log-transformed *TERT* mRNA and TERC levels into three approximately equal groups representing low, medium, and high expression levels. In this analysis, the relative risk associated with a high *TERT* mRNA level was 1.77 (95% CI 0.96-3.23) compared to the low level (p=0.07).

Table 2

Baseline characteristics of cases and controls, with relative risks (RR) of relapse estimated from univariate case-cohort analyses.

	No. of relapsed cases*	No. of controls*	RR	95% C.I.	P-value
Sex					
Male	40	64	1.0		
Female	56	84	1.10	(0.68-1.80)	0.70
Stage					
I	11	45	1.0		
II	37	53	2.78	(1.30-5.94)	0.01
III	25	37	2.55	(1.14-5.68)	0.02
IV & V	23	13	5.65	(2.41-13.2)	0.0001
Age at diagnosis (yr)					
0-2	26	58	1.0		
2-4	24	43	1.25	(0.65-2.39)	0.51
4+	46	47	2.00	(1.12-3.55)	0.02
Log2 (*TERT* mRNA)					
Low (-5.40-0.87)	25	55	1.0		
Medium (0.87-2.42)	34	47	1.56	(0.85-2.85)	0.15
High (2.42+)	37	45	1.77	(0.96-3.23)	0.07
Log2 (TERC)					
Low (-2-2.72)	27	54	1.0		
Medium (2.72-3.87)	27	53	1.07	(0.58-1.98)	0.83
High (3.87+)	42	40	2.06	(1.14-3.70)	0.02
Continuous biological variables					
TRAP activity			1.44	(0.90-2.31)	0.13
Log2 (*TERT* mRNA)			1.16	(1.04-1.29)	0.01
Log2 (TERC)			1.35	(1.11-1.64)	0.003

The relative risk associated with a high TERC level was 2.06 (95% CI 1.14-3.70) compared to the low level (p=0.02) (Table 2). Weighted estimates of the relapse-free survival curves by TERC tertile are shown in Figure 2. Patients with the highest TERC levels had double

the relapse risk compared to patients with the lowest TERC levels (4 year RFS: 78.7% *v* 89.4%). Combining the variables of *TERT* mRNA and TERC expression did not enhance the association with risk of relapse beyond that observed with TERC expression alone.

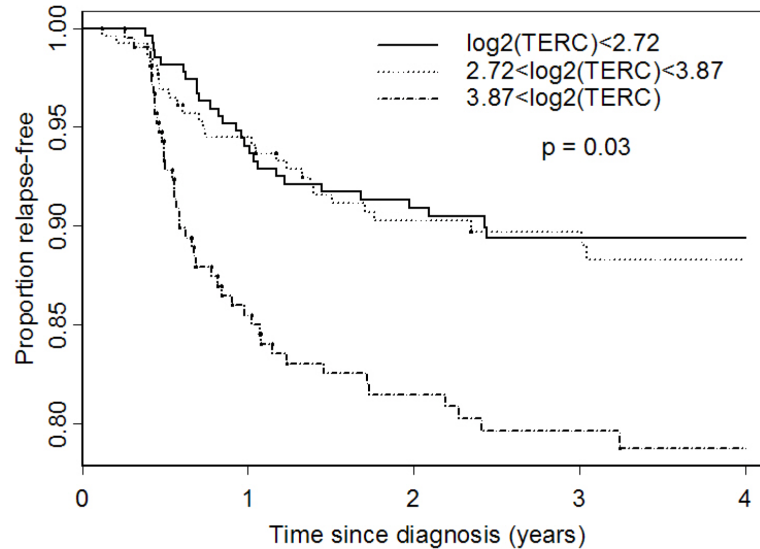

Figure 2. Relapse-free survival estimates according to level of log2 (TERC) expression.

Levels of *TERT* mRNA and TERC expression were not predictive of overall survival, though the relatively small number of deaths precluded accurate assessment of the survival outcome.

We next evaluated telomerase expression level as a prognostic indicator in the context of other known prognostic factors. Patient age and tumor stage were not significantly associated with any measures of telomerase expression (data not shown). A case-cohort analysis of the separate effects of *TERT* mRNA level and TERC level after adjustment for age at diagnosis, tumor stage, and each other revealed that age, high stage, and TERC level all had statistically significant effects (Table 3). After adjustment for the other variables, each doubling of the TERC level increased the RR by a factor of 1.30 (95% CI 1.06-1.60; P=0.007).

Table 3

Relative risks (RR) of relapse estimated by multivariate case-cohort analysis. All RR are adjusted for the remaining variables shown.

		RR	95% C.I.	P-value
Log2 (TERC)		1.30	(1.06-1.60)	0.007
Log2 (*TERT* mRNA)		1.07	(0.94-1.23)	0.33
Age at diagnosis (yr)		1.11	(1.01-1.21)	0.04
Stage				
	I	1.0		
	II	2.14	(0.94-4.88)	0.07
	III	1.93	(0.79-4.68)	0.15
	IV & V	4.25	(1.71-10.55)	0.002

Expression of TERT mRNA alternative splice forms in Wilms tumor

It was recently recognized that human cells generate multiple alternative splice forms of *TERT* mRNA. A total of seven alternative splicing sites (four insertions and three deletions) have been identified.[20-24] Deletions α, β, and γ and insertions 1 and 2 are predicted to abrogate telomerase catalytic activity because they interfere with the reverse transcriptase (RT) domains (Figure 3A). Insertions 3 and 4 occur distal to the RT domains and are not predicted to alter the catalytic function of the protein (although this has not been tested formally). Alternative splicing of *TERT* mRNA is responsible for telomerase repression after week 15 during the development of the fetal kidney.[22] Because Wilms tumors arise from embryonal kidney, we surmised that alternative splicing of *TERT* mRNA may regulate telomerase activity in Wilms tumor.

Using a primer set that encompasses the region of the α and β deletions (Figure 3A), we evaluated the *TERT* mRNA alternative splicing patterns in 30 Wilms tumors. As demonstrated in Figure 3B, the α+β- splice form, predicted to encode a catalytically inactive protein, was the predominant splice variant. The ratio of the full length/active α+β+ variant to the inactive α+β- variant was variable from tumor to tumor.

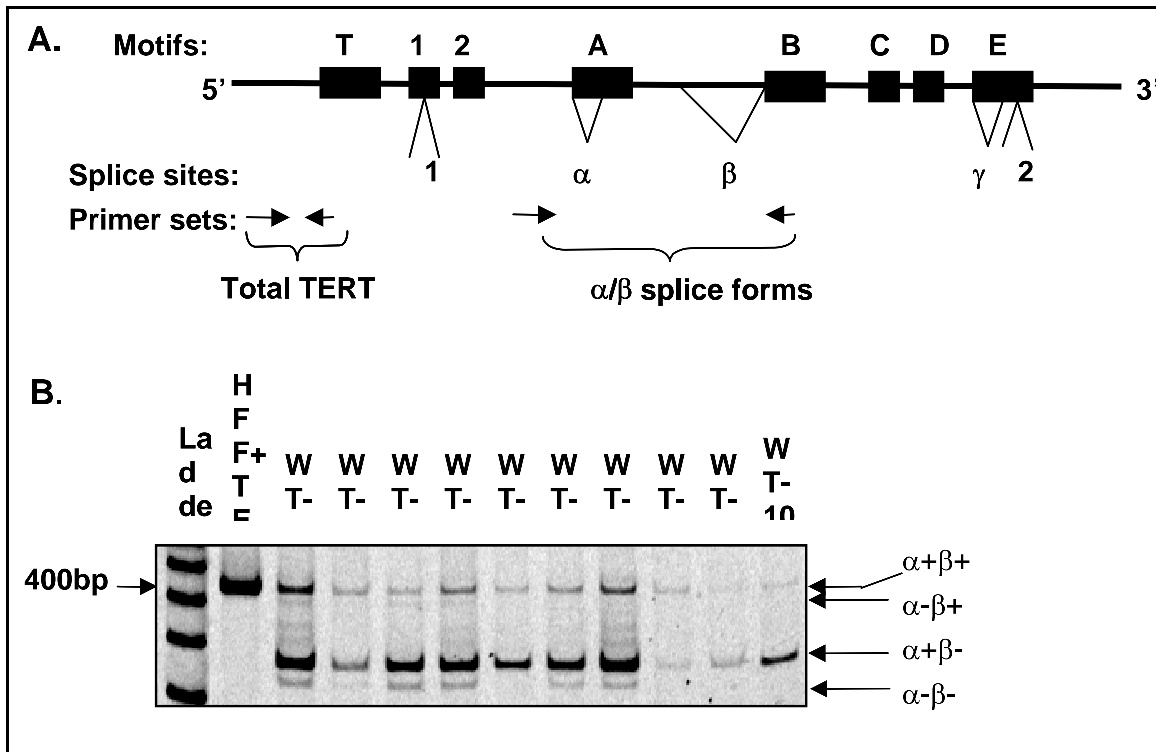

Figure 3. **(A)** Schematic diagram of *TERT* mRNA. The telomerase (T) and reverse transcriptase (1,2, A-E) motifs are shown. Alternative splice forms predicted to encode inactive protein are illustrated. **(B)** RT-PCR reaction demonstrating four alternative splice forms involving the α and β splice forms in Wilms tumors. HFF+TERT represents human foreskin fibroblasts with ectopic TERT expression.

The primer set that was utilized to assess the prognostic significance of *TERT* mRNA expression level was directed against a region of cDNA that amplifies all *TERT* mRNA splice forms, both active and inactive (Figure 3A). We therefore designed a real-time PCR primer-probe set to specifically measure levels of the "active" α+β+ splice form. There was strong correlation between levels of total *TERT* mRNA and levels of the α+β+ splice form (r=0.836, p<0.0001). Levels of the α+β+ *TERT* mRNA splice form were no more predictive of relapse than were levels of total *TERT* mRNA (data not shown).

Discussion

This study demonstrated a positive correlation between risk of recurrence in patients with favorable histology Wilms tumor and tumor expression level of telomerase RNA (TERC and, to a lesser extent, *TERT* mRNA). The third of patients with the highest TERC expression level had twice the risk of relapse compared to those with the lowest TERC expression level. TERC expression level remained a significant predictor of relapse after adjustment for the known prognostic factors of tumor age and patient stage. These results indicate that measurement of TERC expression may be a useful adjunct to the current risk classification schema for favorable histology Wilms tumor.

Our findings add to a growing body of evidence that high telomerase expression level is associated with unfavorable outcome in human cancer.[8] In pediatric cancer, detectable or high level of telomerase expression has been associated with unfavorable prognosis in neuroblastoma [25-28], hepatoblastoma [29], osteosarcoma [30], and AML [31]. The biological basis for this correlation is poorly understood. The simplest explanation is that tumors with low or absent telomerase expression are unable to maintain telomeres and therefore have limited proliferative potential.[32] It is also possible that excess telomerase expression may mediate resistance to DNA damaging agents because telomerase can act as a chromosome healing enzyme.[33-35] In support of this premise, studies of in vitro drug sensitivity have shown that cell lines become more sensitive to various classes of chemotherapy agents upon the inhibition of telomerase.[36-39] Finally, recent studies using mouse models have shown that the telomerase complex may promote tumorigenicity and metastatic potential in a manner independent of telomere lengthening.[40;41]

The optimal assay for telomerase expression has not been established and may vary according to tumor type. We assessed three measures of telomerase expression, each with distinct advantages and disadvantages. Telomerase enzyme activity, assessed by the TRAP assay, is the most widely used measure for telomerase expression because it provides a functional readout of the protein and was the first telomerase assay to be developed. In contrast to the findings in other cancers, TRAP level was not predictive of relapse in our study of Wilms tumor. A potential explanation for this lack of correlation is that TRAP activity is subject to inactivation with heat and time [13], which could be

particularly problematic in a multi-institutional study such as ours in which the processing of tumor tissue was not uniform. Additionally, Wilms tumor may differ from other tumors with regard to the extent of variation of telomerase activity level between tumors. Nearly all Wilms tumors in our study had TRAP activity, whereas other cancers had a more dichotomous pattern of activity (either absent or present).[26;28;30;42-45] When quantitative, rather than qualitative, measurement of activity is important, the TRAP assay may have limitations.

The second telomerase assay we undertook was quantitative RT-PCR to measure expression level of *TERT* mRNA, which encodes the catalytic component of the telomerase enzyme complex. *TERT* mRNA expression level was more predictive of relapse than TRAP activity in a univariate analysis, but was not predictive after adjustment for patient age and tumor stage, corroborating the findings of our pilot study. [9] A limitation of the *TERT* mRNA expression assay is that *TERT* mRNA undergoes alternative splicing, generating splice forms that encode inactive protein. We attempted to correct for this by exclusively amplifying the α+β+ splice form, which is predicted to encode active protein. However, in contrast to results for neuroblastoma [28], our analysis of the α+β+ splice form did not reveal a strong correlation with patient outcome. An important caveat to the α+β+ splice form analysis is that it accounted for only two of the known *TERT* mRNA splice variants. It is possible that a subset of the "active" α+β+ *TERT* splice forms contained other insertions or deletions that would render them inactive. Unfortunately, the distance between *TERT* mRNA alternative splice sites precludes the development of PCR primer sets that amplify mRNA species that exclusively encode active protein.

The third telomerase assay used in our study was quantitative PCR of TERC, the RNA template component of the telomerase enzyme complex. Interestingly, TERC expression was the best prognosticator of the three telomerase measures, which differs from the conclusions of our pilot study. [9] This difference likely relates to the larger sample size and improved assays (ie, real-time quantitative PCR) in the current study. However, the confidence intervals indicate that the differences observed between the two studies are within the bounds of expected statistical variation. Although TERC is constitutively expressed in both normal and malignant cells, TERC is known to be upregulated during

the process of tumorigenesis. [46-50] A high level of TERC expression has been linked with adverse outcome in neuroblastoma and breast cancer.[27;51-53] Recent studies of individuals with the autosomal dominant form of dyskeratosis congenita have revealed that TERC haploinsufficiency results in impaired telomere length maintenance and clinical phenotype, indicating that the *level* of TERC expression is physiologically important.[54;55] Likewise, in a mouse model system, haploinsufficiency of TERC was limiting for telomere maintenance.[56] Although correlative, our findings suggest that level of TERC expression may be of biological significance in human cancer and that measurement of TERC level deserves further consideration as a prognostic indicator.

In summary, our findings indicate that high TERC expression level in primary favorable histology Wilms tumors is predictive of relapse, even after adjustment for patient age and tumor stage. These results suggest that measurement of TERC expression may provide a clinically useful adjunct to the current risk stratification schema for favorable histology Wilms tumor. Because this is the first study demonstrating a correlation between TERC level and relapse in Wilms tumor, further validation of this molecular marker will be required before the results of this test can be utilized for treatment stratification.

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CHAPTER 3

Improved Survival for Patients with Recurrent Wilms Tumor: The Experience at St. Jude Children's Research Hospital

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ABSTRACT

Purpose

Reported estimates of survival for patients with recurrent Wilms tumor (WT) are 24% to 43%. Because published survival data are more than a decade old and do not reflect advances in therapy, we reviewed our experience in treating recurrent WT to determine whether the probability of survival has increased.

Patients and Methods

We reviewed the cases of 54 patients with recurrent WT who were treated on one of six consecutive clinical trials at St. Jude Children's Research Hospital between 1969 and 2000.

Results

Five-year overall survival estimates after relapse were 63.6% ± 15.7% for patients treated during or after 1984 (n=20) and 20.6% ± 6.5% for patients treated before 1984 (n=34) (p=0.002). When the analysis was restricted to patients with high-risk clinical features, 5-year overall survival estimates were 47.6% ± 15.7% for those treated in the modern era (n=16) and 11.1% ± 5.2% for those treated in the earlier era (n=25) (p=0.005). Only three patients received high-dose chemotherapy with autologous stem cell rescue; one survived. No patients with recurrent anaplastic histology disease survived.

Conclusions

Significant progress has been achieved in the treatment of recurrent favorable histology WT using multi-modality salvage regimens with conventional doses of chemotherapy. Novel therapeutic strategies will be necessary to cure patients with recurrent anaplastic Wilms tumor.

INTRODUCTION

Because of advances in therapy, approximately 90% of patients with Wilms tumor can now be cured. Despite this remarkable success, reported estimates of durable survival after relapse are only 24% to 43% (1-4). Patients who have favorable histology disease, long duration of remission, isolated pulmonary recurrence, abdominal recurrence in the absence of prior irradiation, low-stage disease at presentation, or have had up-front treatment with only vincristine and actinomycin D have a relatively good prognosis after relapse (1-4).

Before the mid-1980s, recurrent Wilms tumor was treated with combinations of vincristine, actinomycin D, doxorubicin, radiation therapy, or surgery. In many cases, the identical chemotherapy agents were used for the treatment of both primary and recurrent disease. In recent years, cyclophosphamide, ifosfamide, cisplatin, carboplatin, and etoposide have been used to treat recurrent Wilms tumor, but their impact on long-term survival remains poorly defined (5). Trials of high-dose chemotherapy with autologous stem cell rescue have yielded markedly improved survival estimates compared to historical controls (6, 7), but it is uncertain whether this approach is superior to conventional chemotherapy with the newer agents.

In 1985, we reported a survival rate of 25% among 32 patients with recurrent Wilms tumor treated at St. Jude Children's Research Hospital (2). Because published survival data are more than a decade old and do not reflect advances in therapy, we reexamined our experience in treating recurrent Wilms tumor to determine whether the probability of survival has increased.

PATIENTS AND METHODS

Patients

The records of 388 children with renal tumors treated at St. Jude Children's Research Hospital on one of six consecutive clinical trials between 1969 and 2000 were reviewed. As of March 2000, 65 of these patients (16.8%) had experienced recurrence. Seven of the 65 patients were excluded from this study because either their histologic diagnoses were not

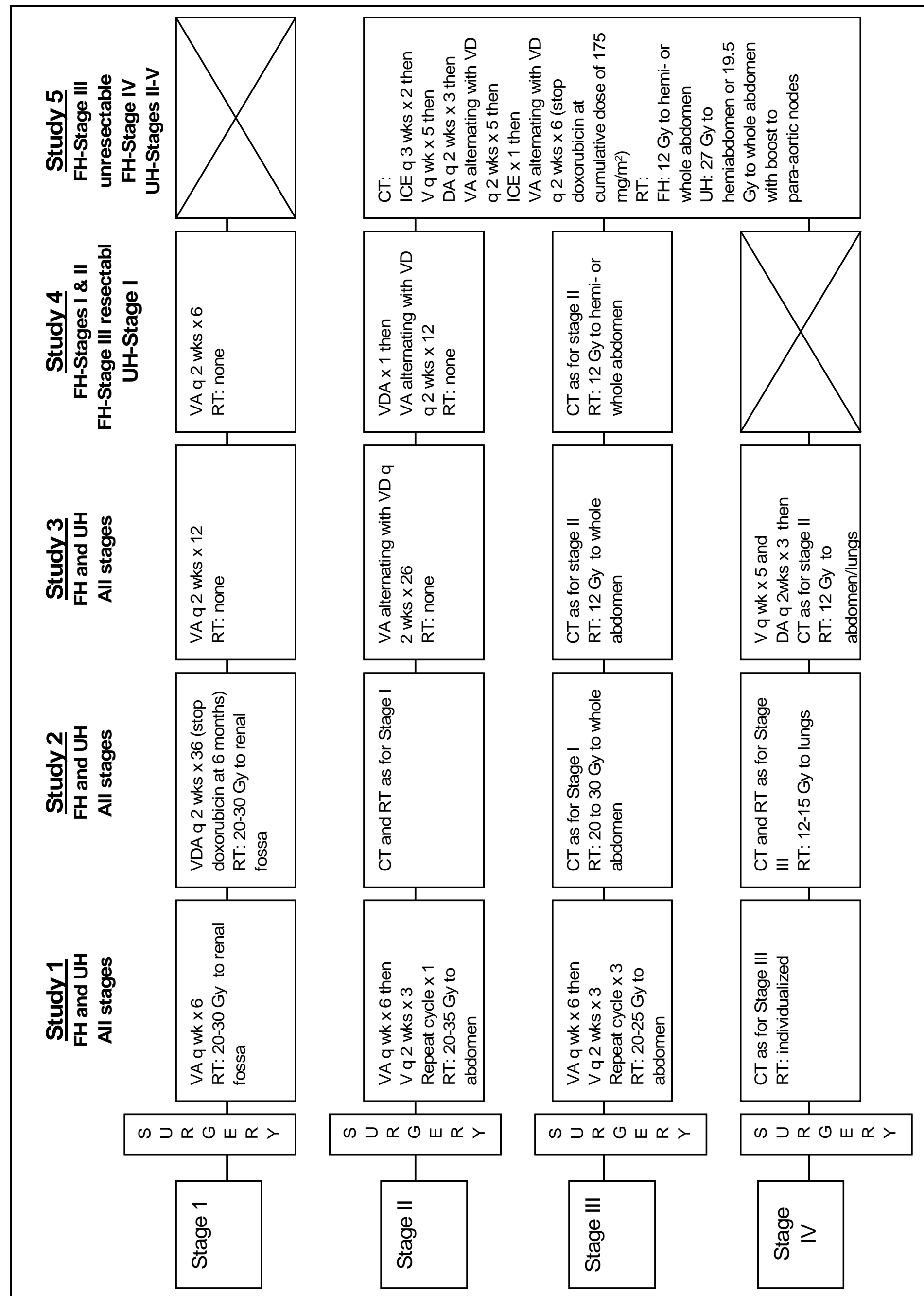
consistent with Wilms tumor (n=4) or because they did not have a complete response to primary treatment (n=3). An additional four patients with bilateral Wilms tumor were excluded because their recurrences involved the kidney and an unequivocal distinction between new primary tumors and recurrent disease could not be made. Thus, 54 patients were included in this analysis.

Wilms Tumor Treatment Protocols

Nephrectomy was generally performed before chemotherapy and radiation therapy (RT) were given. Tumor stage and histologic type were assigned according to the guidelines of the National Wilms Tumor Study Group (NWTSG). Patients were treated on the St. Jude institutional Wilms-1 (1969-1972), Wilms-2 (1973-1978), Wilms-3 (1979-1988), Wilms-4 (1989-1995), and Wilms-5 (1994-1998) protocols, which are outlined in Figure 1. Some patients with measurable disease on the Wilms-3 study, including one patient in this analysis, were treated with an up-front window of etoposide. The Wilms-4 and Wilms-5 studies were complementary: patients with stages I-III favorable histology disease and patients with stage I anaplastic histology disease were enrolled on the Wilms-4 study; patients with unresectable (including stage IV) favorable histology disease and patients with stages II-V anaplastic histology disease were enrolled on the Wilms-5 study. After the Wilms-4 and Wilms-5 studies closed, eligible patients were enrolled on the National Wilms Tumor Study (NWTS)-5 (8).

Treatment for recurrent Wilms tumor was individualized. In the earlier era, treatment generally included chemotherapy with vincristine and actinomycin D, with or without doxorubicin. In more recent years, patients have often been treated with combinations such as ifosfamide, carboplatin, and etoposide; cisplatin and etoposide; or cyclophosphamide, carboplatin, and etoposide. There was no uniform approach to surgery or radiation therapy at the time of relapse. The use of these modalities was based on the resectability of the tumor and on whether the recurrence occurred in a previously irradiated site.

Figure 1. Outline of the St. Jude Children's Research Hospital Wilms tumor protocols 1 through 5 (1969-1998). FH= favorable histology, UH= unfavorable histology, RT= radiation therapy, CT= chemotherapy



Abbreviations used in Figure 1

V= vincristine 1.5 mg/m²; A= actinomycin D, 0.4 mg/m² (0.6 mg/m² in Wilms-5 study and first three doses of Wilms 4 study for Stage IV patients); D= doxorubicin, 25 mg/m², I= ifosfamide 2g/m² /day x 3 days ; C= carboplatin targeted to an area under the curve (AUC) of 6 mg/ml x min x 1 day ; E= etoposide 100 mg/m² /day x 3 days.

Analysis of Factors Predictive of Survival after Recurrence

To determine the effect of treatment era on survival, we classified the 54 patient records according to tumor recurrence during the modern treatment era (on or after January 1, 1984; n=20) or during the previous era (1983 or before; n=34). This date was selected because combinations of new and effective chemotherapy agents first came into routine use at St. Jude in 1984, which was also the year in which our previous analysis of recurrent Wilms tumor ended.

We examined the patient records to investigate whether factors previously shown to affect patient outcome were predictive in our study group. These factors were initial tumor stage, tumor histology, chemotherapy and radiation therapy used to treat the primary disease, duration of complete response, site of recurrence, and number of pulmonary metastases. We also assessed whether the extent of surgical resection of recurrent tumor and the use of RT to treat recurrence were predictive of survival. Finally, we created a new variable: we analyzed the number of active chemotherapy agents that were administered at the time of recurrence, but not during primary therapy. By measuring the effect of using drugs to which the tumor cells were naive, this variable assessed the impact of the expanded repertoire of drugs available in the modern era. For this analysis, we considered vincristine, actinomycin D, doxorubicin, cyclophosphamide, ifosfamide, carboplatin, cisplatin, and etoposide to be active against Wilms tumor (5, 9-14).

Statistical Methods

The duration of overall survival (OS) after relapse was defined as the interval between the date of first relapse and either the date of death from any cause or the date of the patient's most recent follow-up contact. The duration of event-free survival (EFS) after relapse was defined as the interval between the date of first relapse and either the date of subsequent disease progression or relapse, as documented by radiologic studies, or the date of the most recent follow-up contact, or the date of death. The probabilities of OS and EFS (± one standard error) were estimated by the method of Kaplan and Meier (15). The predictive value of prognostic factors for OS was analyzed by using the exact log-rank test or the Mantel-Haenszel test for factors with more than two levels. Pairwise comparisons were made only if the overall p-value was less than 0.05. No adjustments

for multiple comparisons were made. Risk ratios estimated from univariate Cox models (16) are presented with 95% confidence intervals. Associations among variables were examined by Fisher's exact test, the exact chi-square test, the exact Wilcoxon rank sum test, or the Kruskal-Wallis test. SAS (version 6.12, SAS Institute, Cary, NC) and StatXact (version 4, CYTEL Software Corporation, Cambridge, MA) software were used for statistical analysis.

RESULTS

Patients

Among 388 children with renal tumors treated on one of six consecutive clinical trials between 1969 and 2000, 5-year event-free survival estimates were 73.8% ± 3.4% for patients treated before 1984 and 81.4% ± 3.1% for patients treated during or after 1984 (p=0.054). The 5-year overall survival estimates were 79.3% ± 3.2% for patients treated in the earlier era and 90.6% ± 2.3% for patients treated in the modern era (p=0.008).

The clinical characteristics of the 54 patients with recurrent Wilms tumor who were included in our analysis are described in Table 1. There were no detectable differences in tumor histology, age at diagnosis, gender, or race. However, a comparison of patients treated in the modern and previous eras revealed a trend toward lower-stage disease in the patients who were treated in the modern era (p=0.060). The median time between primary diagnosis and recurrence was 9.9 months (range, 3-83.5 months). Interestingly, the median time to recurrence was greater among patients whose relapses occurred after 1983 (16.9 months; range, 4.1- 60.0 months) than among patients in the earlier group (9.4 months; range, 3.0-83.5 months) (p=0.060). Thirty-seven patients had isolated pulmonary recurrence; these included 2 patients with hilar disease only. Twelve patients had local recurrence; these included one patient with both abdominal and pulmonary recurrence. Five patients had recurrence at other sites (bone, brain, or liver).

Table 1

Characteristics of the Study Group Overall and by Treatment Era

Characteristic	Entire Study Population (N=54)	Study Population According to Treatment Era		
		Before 1984 (N=34)	1984 and after (N=20)	
	N	N	N	P-value*
Primary disease stage				
I	7	2	5	0.060
II	12	8	4	
III	23	14	9	
IV	12	10	2	
Histology				
Favorable	45	29	16	0.71
Anaplastic	9	5	4	
Gender				
Female	29	19	10	0.78
Male	25	15	10	
Race				
Caucasian	33	23	10	0.25
African-American	21	11	10	
Age at diagnosis (years)				
Median	4.5	4.0	5.5	0.47
Range	0.7 – 13.9	0.9 – 13.9	0.7 – 13.3	
Time from diagnosis to recurrence (months)				
Median	9.9	9.4	16.9	0.060
Range	3-83.5	3.0-83.5	4.1-60.0	
Site of relapse				
Lung/hilum only	37	24	13	0.9
Abdomen	12	7	5	
Other	5	3	2	
Survival status				
Alive	20	7	13	0.002
Expired	34	27	7	

* P values were obtained by using the exact Kruskal-Wallis test, Fisher's exact test, exact Wilcoxon rank sum test, or the exact log rank test.

There was no detectable difference between treatment-era groups in the sites of recurrence (p=0.9). Twenty of the 54 patients remained alive with a median follow-up of 7.2 years (range, 1.3-27.4 years) after relapse. The median follow-up of survivors treated for recurrence before 1984 was 22.0 years (range, 17.7-27.4 years) whereas that for patients treated in the modern era was 4.0 years (range 1.3-13.4 years). Fourteen of the 20 survivors had been followed for more than 4 years after relapse. Seventeen of the 20 survivors had had follow-up contact within the past 12 months; the other three survivors were last contacted in 1995, 1998, and 1999.

Treatment of Recurrence

Patients were treated for relapsed disease on individualized treatment plans. Thirty-eight patients (70.3%) received chemotherapy. Not surprisingly, patients whose disease recurred in the later era were more likely to have received two or more active agents that were not included in their primary treatment regimens (p<0.001). Forty-two patients (77.8%) received radiation therapy at relapse. Nineteen of 20 (95%) patients received RT for relapsed disease in the modern era, as compared to 23 of 34 (67.6%) in the previous era (p=0.022). All patients except one received RT with curative intent. Twenty patients (37.0%) had a complete (gross total) surgical resection of recurrent tumor, eight (14.8%) had a partial resection, eight (14.8%) had a biopsy, and 18 (33.3%) had no surgery. We found no statistically significant difference between the two treatment-era groups in the frequency of complete surgical resection (p=0.56). Three patients in the modern era underwent high-dose chemotherapy with autologous stem cell rescue as part of their salvage therapy.

Patient Outcomes

The patient outcomes are summarized in Figure 2. Thirty-eight of the patients had a second complete response (CR). There was no significant difference in the percentage of patients who had a second complete response according to treatment era (Table 2). Of the 16 patients who did not have a second complete response, 15 died of Wilms tumor. The single surviving patient in this group showed evidence of recurrent disease at the most recent follow-up examination.

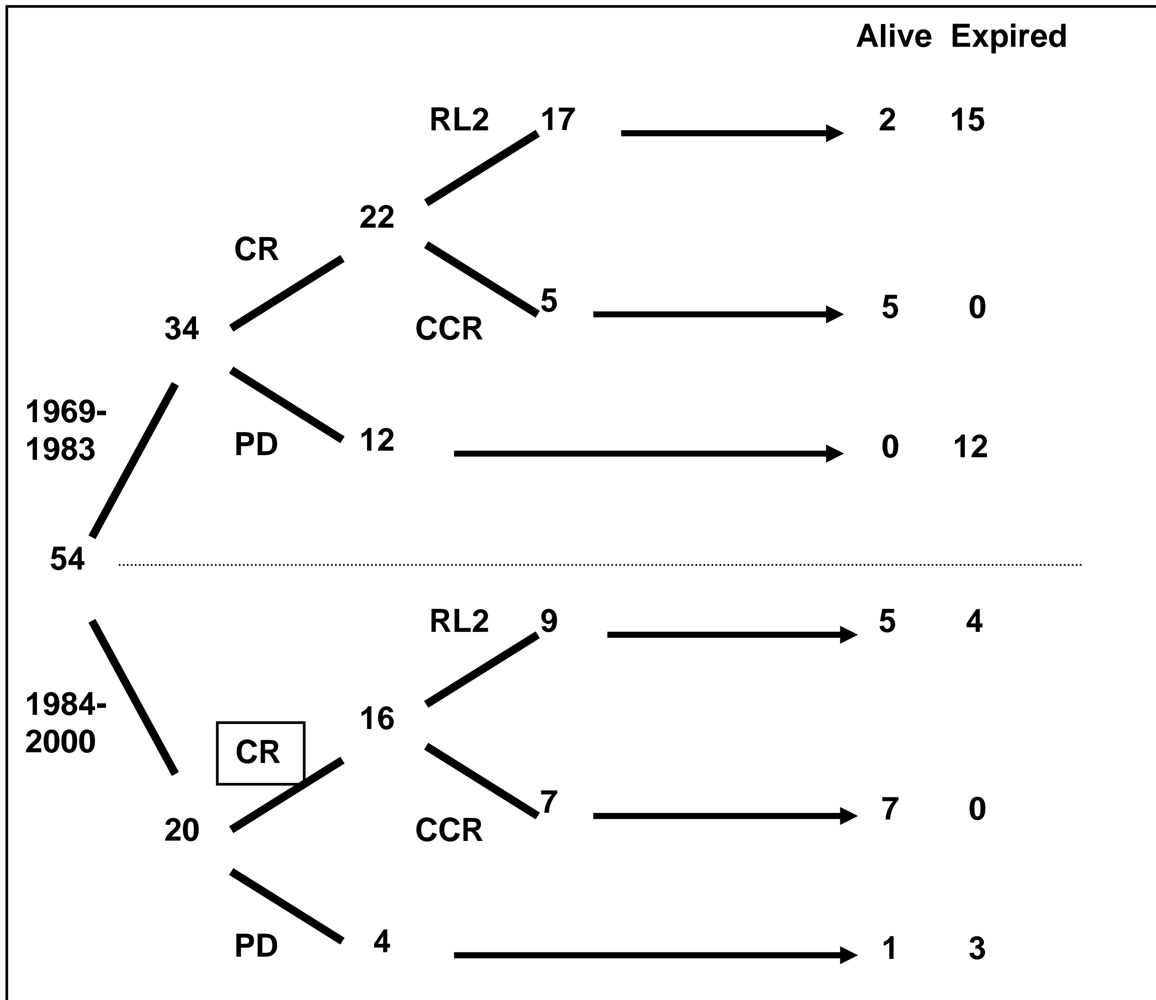


Figure 2. Outcomes of patients with recurrent Wilms tumor. CR= complete response, PD= progressive disease, CCR= continuous complete response, RL2= second relapse.

Twenty-six of the 38 patients who achieved a second complete response had subsequent disease recurrence. The 12 patients who did not have subsequent disease recurrence were alive without disease at their last follow-up examinations. Of the 26 patients who had a second recurrence, 19 died of Wilms tumor at a median time of 0.8 years (range 0.01-2.2 years) from their second recurrence and seven were alive with no evidence of disease at 0.9, 1.3, 2.1, 4.8, 5.8, 20.5, and 26.4 years after their second recurrence.

Table 2

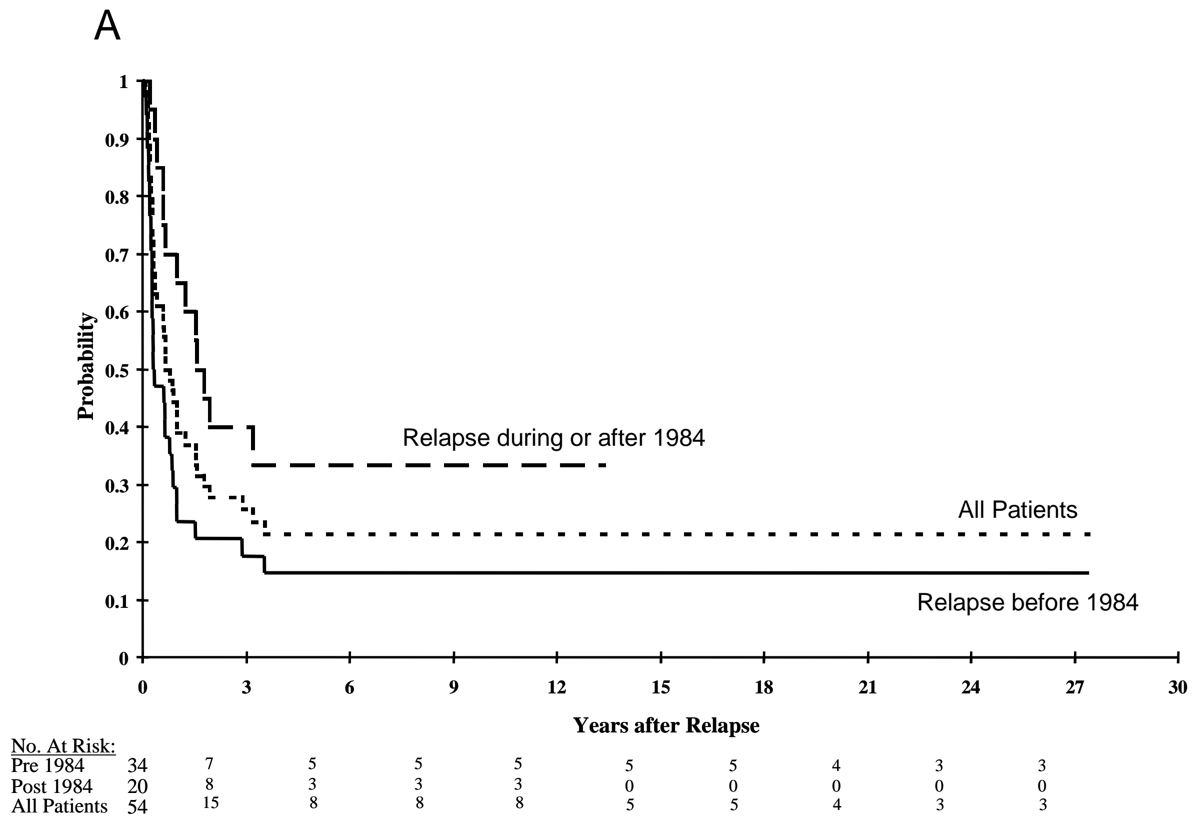
Comparison of Patient Outcomes By Treatment Era

	Pre-1984	1984-2000	p-value
2nd Complete Response	22/34 (64.7%)	16/20 (80.0%)	0.356*
2nd Relapse	17/22 (77.2%)	9/16 (56.2%)	0.064**
Survival after 2nd Relapse	2/17 (11.7%)	5/9 (55.6%)	0.069**

*Fisher's exact test

** Exact log rank test

The second recurrences of the seven survivors were treated with surgery only (1), RT only (1), surgery and RT (2), surgery and chemotherapy (1), or surgery, RT, and chemotherapy (2). None of the survivors of second recurrences received consolidation with high-dose therapy and stem cell rescue. Patients treated during the modern era were less likely to have a second recurrence, but more likely to survive after a second recurrence, than were patients treated during the previous era; these differences were marginally significant (Table 2).



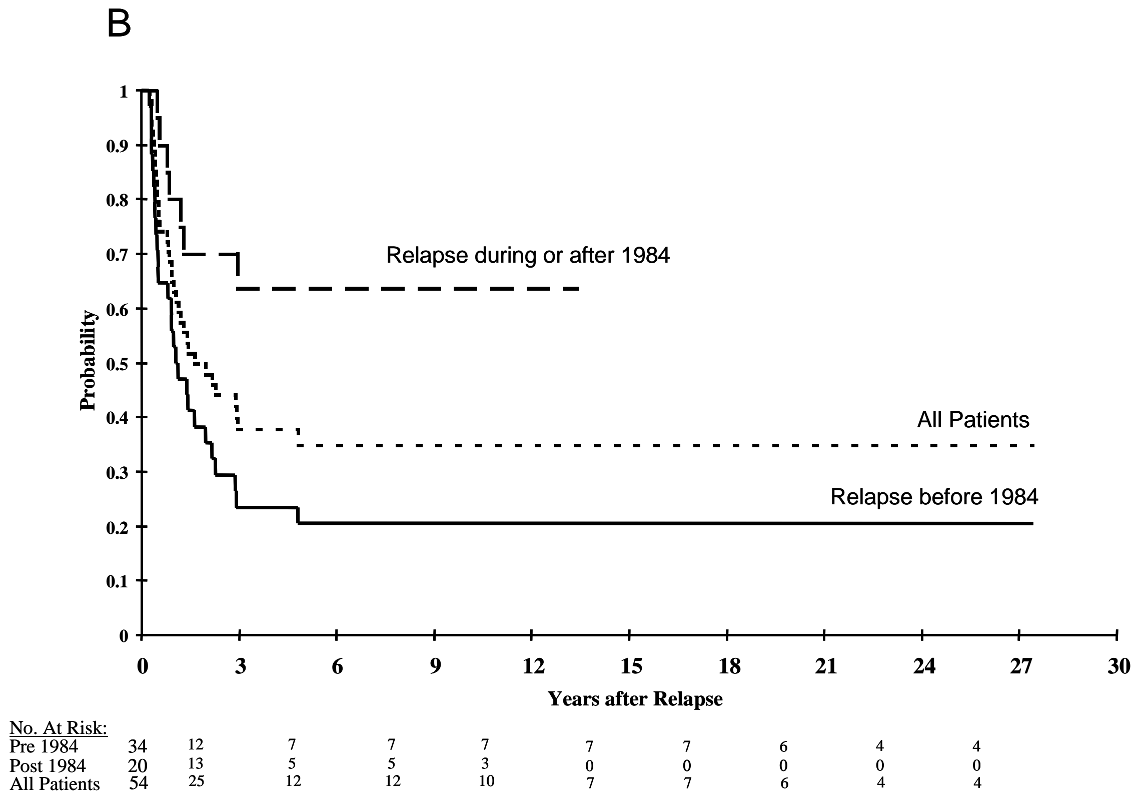


Figure 3. Estimated event-free survival (A) and overall survival (B) after recurrence of Wilms tumor. Both event-free survival (p=0.012) and overall survival (p=0.002) increased significantly in the more recent treatment era.

As shown in Figure 3, the estimated 5-year EFS was 21.5% ± 6.3% for the entire study group, 33.3% ± 13.6% for patients whose relapses occurred in the modern era, and 14.7% ± 5.5% for patients whose relapses occurred before 1984 (p=0.012). The estimated 5-year OS was 34.9% ± 7.8% for the entire study group, 63.6% ± 15.7% for the modern-era cohort, and 20.6% ± 6.5% for the pre-1984 cohort (p=0.002). It is possible that the shorter duration of follow-up for the patients in the modern era may have overestimated the survival of these patients, particularly those with more than one recurrence. It is notable that of the 13 survivors from the modern era treatment group, only three had been followed for less than three years after their relapses. In the early group, 26/27 deaths occurred within three years of relapse.

The International Society of Paediatric Oncology (SIOP) identified adverse prognostic factors for recurrent Wilms tumor including initial stage IV disease, unfavorable histology, time to recurrence 6 months or less after diagnosis, and recurrence in multiple

organs, liver, bone, brain, lymph nodes, or a previously irradiated field (17). In our study group, 13 patients (24.1%) had none of these adverse factors and 41 patients (75.9%) had at least one factor. Patients with none of the adverse factors had a 5-year survival estimate of 71.4% ± 14.4%, compared to 23.4% ± 7.7% for patients with at least one adverse factor ($p<0.001$). Among the latter group, 5-year survival estimates were 47.6% ± 17.2% for patients treated in the modern era (n=16) and 11.1% ± 5.2% for those treated in the earlier era (n=25) ($p=0.005$).

Factors Predictive of Survival after Relapse

Table 3 summarizes the univariate analysis of prognostic factors. Factors that were significantly predictive of survival after relapse were time from diagnosis to recurrence, tumor histology, treatment era, initial stage, extent of resection of recurrent disease, and the number of active drugs given for the first time at relapse. There was a trend toward improved survival in patients who did not receive RT for their primary disease. Moreover, of 15 patients who experienced recurrence within a previously irradiated field, only one survived.

Table 3

Univariate Analysis of Prognostic Factors for Patients with Relapsed Wilms Tumor

Factor	N	5-year OS Estimate (SE) (%)	Relative Risk (95% CI)	P-value[==]
Stage			2.1 (1.0 – 4.6)	0.039
I, II[=]	19	47.8 (14.1)		
III, IV	35	26.8 (8.1)		
Histology			4.2 (1.9 – 9.6)	0.004
Favorable[=]	45	41.9 (8.9)		
Unfavorable	9	0 (0)		
Treatment era			3.3 (1.4 – 7.5)	0.002
Relapse after 1984[=]	20	63.6 (15.7)		
Relapse before 1984	34	20.6 (6.5)		
Time from Dx to recurrence				0.004[#]
≤ 6 months	10	##	4.1 (1.6 – 10.3)	
>6 to ≤12 months	20	24.0 (9.4)	2.7 (1.2 – 6.0)	
> 12 months[=]	24	54.6 (12.3)	1.0	
Chemotherapy for the primary tumor			0.7 (0.3 – 1.4)	0.28
No drugs	1	###		
One or two drugs	18	42.8 (11.4)		
Three or more drugs[=]	35	30.1 (10.3)		
Irradiation during initial therapy			0.5 (0.2 – 1.1)	0.070
No	14	57.1 (18.7)		
Yes[=]	40	27.9 (7.5)		
Surgical response at relapse			3.3 (1.5 – 7.3)	0.001
Partial resection, biopsy, no surgery	34	19.6 (7.9)		
Complete resection[=]	20	60.0 (12.6)		
Irradiation at relapse			1.5 (0.7 – 3.1)	0.35
No	12	25.0 (10.8)		
Yes[=]	42	37.7 (9.4)		
Radiation field at relapse			2.6 (1.3 – 5.1)	0.009
Previously irradiated field or no RT	21	16.7 (7.6)		
Previously unirradiated field[=]	33	46.0 (10.7)		
Active drugs first given at relapse			3.2 (1.3 – 7.6)	0.005
None or one	36	22.2 (6.5)		
Two or more[=]	18	65.0 (17.2)		
First relapse site				0.169[#]
Lung/Hilum only[=]	37	36.5 (8.8)	1.0	
Abdomen	12	####	1.9 (0.9 – 4.1)	
Other	5	60.0 (21.9)	0.6 (0.1 – 2.5)	
Number of pulmonary lesions[*]			3.2 (1.3 – 7.6)	0.009
Single[=]	18	53.5 (12.9)		
Multiple	17	17.6 (9.2)		

[=] Reference group
[==] *P* values were obtained by exact log-rank tests except where indicated by superscript [#]. Pairwise comparisons for analyses with multiple variables are indicated by brackets.
[#] *P* values were obtained by Mantel-Haenszel tests.
[##] No five-year estimate was available; 9 of 10 patients died within 3 years and one patient is alive 38.1 months after recurrence.
[###] No five-year estimate was available; only one patient did not receive chemotherapy at initial presentation; this patient is alive 38.1 months post-recurrence. Comparisons for this variable exclude this one patient
[####] No five-year estimate was available; 9 of 12 patients died within 3 years and three patients are alive 15.8 (with disease), 29.3, and 38.1 months post-recurrence.
* Subset analysis for patients with lung recurrence only.

Although the use of RT in general to treat recurrent disease was not prognostically significant, the use of RT in a previously unirradiated field was associated with a higher probability of survival than was RT in a previously irradiated field or no RT. The relatively small number of patients in this study precluded a multiple regression analysis of all of the prognostic variables.

DISCUSSION

Our findings indicate that substantial progress has been made in the treatment of relapsed Wilms tumor at St. Jude Children's Research Hospital. Patients treated for recurrence during or after 1984 had significantly improved event-free and overall survival estimates compared to patients treated in the previous era. The improvement in survival after recurrence occurred concurrently with a decrease in the primary relapse rate. The combination of the lower relapse rate and the higher salvage rate translated into significantly improved overall survival for the Wilms tumor patient population as a whole. The improvement in survival after Wilms tumor recurrence may be attributed to the decreased second recurrence rate and the increased salvage rate after second recurrences (Table 2). Several factors are likely to have contributed to these improvements. First, patients whose tumors recurred in the modern era were more likely to receive at least two agents that were not included in their primary treatment regimens. Most of these patients received combinations of oxazaphosphorines (ifosfamide or cyclophosphamide), platinum drugs (carboplatin or cisplatin), and etoposide, all of which have demonstrated significant activity against Wilms tumor (5, 9-14). Second, patients

whose tumors recurred in the modern era were more likely to receive RT as part of their salvage therapy. In particular, the administration of RT to a previously unirradiated field was associated with a higher probability of survival. The more conservative use of RT for primary therapy in the modern era may have allowed for increased use at relapse, contributing to the improved patient outcomes. Finally, advances in less quantifiable factors such as diagnostic imaging, surgical technique, and supportive care are likely to have improved the outcome of patients with recurrent Wilms tumor.

It is possible that differences in the clinical characteristics between the two treatment-era groups contributed to the observed improvement in survival in the modern era. Although we did not detect differences in tumor histology, site of recurrence, or age at diagnosis, we observed trends toward lower-stage disease and longer interval to recurrence in patients treated in the modern era. When our analysis was restricted to patients with the high-risk clinical features identified by SIOP (17), the improved survival in the modern era persisted. This indicates that improvement in therapy, and not simply clinical differences between the patient groups, accounted for the superior patient outcomes in the modern era.

High-dose chemotherapy with autologous stem cell rescue has been evaluated in several clinical trials for recurrent Wilms tumor, with overall survival estimates ranging from 36-60% (6, 7, 18). Only three patients in our analysis were treated with high-dose therapy; one survived. Although direct comparisons are limited by differences in patient selection, it is interesting that survival in our modern era patients with high-risk clinical features is similar to the survival attained in the autologous transplant studies. A prospective randomized trial is warranted to clarify whether high-dose chemotherapy with stem cell rescue is superior to conventional chemotherapy for the treatment of recurrent Wilms tumor.

Although the outcome for patients with recurrent favorable histology Wilms tumor has improved dramatically over time, it is notable that there were no survivors of recurrent anaplastic histology Wilms tumor in our cohort. Two of the nine patients with recurrent anaplastic histology disease were treated with high-dose therapy with autologous stem

cell rescue. It is apparent that novel therapies will be necessary to improve outcomes for this unfortunate group of patients.

Prognostic factors may help to identify patients with recurrent Wilms tumor who may be candidates for relatively non-intensive treatment. In our study population, favorable histology, a long interval (>12 months) between diagnosis and relapse, and low stage (I or II) of primary disease were associated with improved post-relapse survival. Although site of recurrence was not predictive of outcome, patients with solitary pulmonary nodules had a significantly higher probability of survival than did those with multiple nodules. Our analysis also indicated that patients who underwent a complete surgical resection of recurrent tumor had a higher probability of survival than did patients who had a partial resection or no surgery. It is tempting to speculate that surgery plays an important role in treating recurrent disease, but we cannot exclude the possibility that patients who underwent a complete surgical resection had less aggressive disease than did other patients. A caveat to our analysis of prognostic factors is the relatively small sample size of our study population. Factors such as the site of recurrence and the number of chemotherapy drugs given for primary treatment may have shown prognostic significance had our sample size been larger.

In conclusion, significant progress has been achieved during the past two decades in the treatment of recurrent favorable histology Wilms tumor. The improvement is likely due to a combination of factors including the availability of new chemotherapy agents, the increased use of radiation therapy, and advances in supportive care. Novel treatment approaches will be necessary to improve the outcome for patients with recurrent anaplastic Wilms tumor.

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Chapter 4

Treatment of Anaplastic Histology Wilms Tumor: Results from the Fifth National Wilms Tumor Study

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ABSTRACT

Background

An objective of the fifth National Wilms Tumor Study (NWTS-5) was to evaluate the efficacy of treatment regimens for anaplastic histology Wilms tumor (AH).

Methods

Prospective single-arm studies were conducted. Patients with stage I AH were treated with vincristine/dactinomycin x 18 weeks. Patients with stages II-IV diffuse AH were treated with vincristine/doxorubicin/cyclophosphamide/etoposide x 24 weeks and flank/abdominal radiation.

Results

2596 patients with Wilms tumor were enrolled on NWTS-5, of whom 281 (10.8%) had AH. Four-year event-free (EFS) and overall survival (OS) estimates for evaluable patients with stage I AH (n=29) were 69.5% (95% CI 46.9, 84.0) and 82.6% (95% CI 63.1, 92.4). By comparison, 4-year EFS and OS estimates for patients with stage I favorable histology (FH) (n=473) were 92.4% (95% CI, 89.5 to 94.5) and 98.3% (95% CI, 96.4 to 99.2). Four-year EFS estimates for patients who underwent immediate nephrectomy with stages II (n=23), III (n=43), and IV (n=15) diffuse AH were 82.6% (95% CI 60.1, 93.1), 64.7% (95% CI 48.3, 77.7), and 33.3% (95% CI 12.2, 56.4). OS was similar to EFS for these groups. There were no local recurrences among patients with stage II AH. Four-year EFS and OS estimates for patients with bilateral AH (n=29) were 43.8% (95% CI 24.2, 61.8) and 55.2% (95% CI 34.8, 71.7).

Conclusions

The prognosis for patients with stage I AH is worse than that for patients with stage I FH. Novel treatment strategies are needed to improve outcomes for patients with AH, especially those with stages III-V disease.

Introduction

In 1978, Beckwith and Palmer published a detailed histopathological review of Wilms tumors that were collected on the first National Wilms Tumor Study (NWTS-1). [1] Approximately 6% of the tumors had cells with nuclear enlargement, nuclear atypia, and irregular mitotic figures and were considered to have anaplastic histology (AH). The presence of anaplasia was prognostically significant; 44% of patients with AH died of disease, whereas only 7.1% of patients without anaplasia, the so-called "favorable histology (FH)" subtype, died of disease. [1] Subsequent studies from the NWTSG and other groups have confirmed the adverse prognostic significance of anaplasia. [2-5]

The first NWTS to stratify patients with AH into a distinct treatment group was NWTS-3 (1979-1986). On this study, and on NWTS-4 (1986-1993), patients received 15 months of vincristine (VCR), dactinomycin (AMD), and doxorubicin (DOX), and were randomized to receive or not receive cyclophosphamide (CYCLO). [6] Patients with stages II-IV diffuse AH had an estimated 4-year overall survival of 27.1% when treated without CYCLO, compared with 52.2% when treated with CYCLO (p=0.04). [7] On NWTS-4, patients with stage I AH had good outcomes when treated with only VCR and AMD, with 2-year OS estimates of 85.5 to 93.3%, depending on the AMD administration regimen. [8]

Although the addition of CYCLO provided a clear benefit for patients with stages II-IV diffuse AH, about half of these patients experienced tumor recurrence and disease-related death. A primary objective of NWTS-5 was to improve the outcomes for these patients using a new treatment regimen containing the combination of CYCLO and etoposide, agents shown to be active against recurrent Wilms tumor in phase II studies.[9;10] In this report, we present the outcomes of patients with AH who were treated on NWTS-5.

Patients and Methods

Patients

NWTS-5 was open to accrual between August, 1995 and June, 2002. Each participating institution obtained local Institutional Review Board approval to conduct the study. Eligibility criteria included no prior chemotherapy or radiation therapy before study

enrollment; histologic diagnosis of Wilms tumor (favorable or anaplastic), clear cell sarcoma of the kidney (CCSK), or rhabdoid tumor of the kidney (RTK); nephrectomy or biopsy performed, and provision of informed consent to participate by a parent/legal guardian.

Tumor Stage and Histologic Classification

Patients underwent nephrectomy before chemotherapy using previously described surgical guidelines [11] unless the tumor was considered to be unresectable by the treating surgeon, in which case a biopsy was obtained. A tumor stage was assigned using the NWTSG surgical-pathological staging system.[11]

Pathology slides, institutional pathology reports, and NWTSG pathology forms were reviewed by the study pathologists. The designation of anaplasia was applied to tumors with cells having major diameters at least three times those of adjacent cells, increased chromatin content (hyperchromaticity), and the presence of atypical polyploid mitotic figures. The criteria distinguishing focal from diffuse anaplasia were based on the distribution of anaplasia within a tumor sample. [12] Tumors with focal anaplasia had anaplastic changes confined to sharply restricted foci within the primary tumor sample. Anaplasia occurring outside the primary tumor, in an extra-renal site such as vessels of the renal sinus, or in a random biopsy specimen was considered to be diffuse anaplasia.

Treatment Regimens

The treatment regimens are outlined in Figure 1. Patients who received pre-nephrectomy chemotherapy received Regimen DD-4A. All regimens called for 50% reductions of AMD and DOX doses during the six weeks following irradiation if the radiation field included the whole lung or whole abdomen. All treatment regimens recommended 50% chemotherapy dose reductions in infants <12 months old. Patients with stage I focal or diffuse AH did not receive radiation therapy. Patients with stages II-IV focal or diffuse AH received 1080 cGy to the abdomen or flank, depending upon the extent of disease, with a boost of 1080 cGy to areas of bulky residual tumor. Patients with lung metastases received 1200 cGy to the whole lung.

Regimen EE-4A: Stage I focal or diffuse anaplastic histology

Week	0	1	2	3	4	5	6	7	8	9	10	11	12	15	18
	A			A			A			A			A	A	A
		V	V	V	V	V	V	V	V	V	V		V*	V*	V*

Regimen DD-4A: Stages II-IV focal anaplastic histology

Week	0	1	2	3	4	5	6	7	8	9	10	11	12	15	18	21	24
	A			D*			A			D*			A	D	A	D	A
		V	V	V	V	V	V	V	V	V	V		V*	V*	V*	V*	V*
	XRT																

Regimen I: Stages II-IV diffuse anaplastic histology

Week	0	1	2	3	4	5	6	7	8	9	10	11	12	13	15	18	21	24
	D*						D*						D*			D*		D*
		V	V		V	V	V	V	V		V	V	V*	V*		V*		V*
				C*			C			C*			C		C*	C	C*	C
				E						E					E		E	
	XRT																	

Figure 1. Schema for Regimen I. D-Doxorubicin (1.5 mg/kg; 45mg/m^2 for patients>30kg); V-Vincristine (0.05 mg/kg; 1.5mg/m^2 for patients>30kg, max dose 2mg); V*-Vincristine (0.067 mg/kg; 2 mg/m^2 for patients>30kg, max dose 2mg)
C -Cyclophosphamide (14.7 mg/kg/day x 5; 440mg/m^2/day for pts>30kg);
C*-Cyclophosphamide (14.7 mg/kg/day x 3; 440mg/m^2/day for pts>30kg);
E-Etoposide (3.3 mg/kg/day x 5; 100mg/m^2/day for patients>30kg)
XRT- 1080 cGy to flank/abdomen.

Statistical Design and Analysis

The study was a prospective single-arm study to evaluate the efficacy of Regimen I, a novel treatment regimen for patients with stages II-IV diffuse AH. The study also included descriptive analyses of patients with stage I AH, stage V (bilateral) AH, and stages II-IV focal AH. Of 281 patients with AH enrolled, eight with focal AH and 73 with diffuse AH were not considered evaluable for the outcomes analyses because they had major protocol violations such as a late change in treatment protocol after central pathology review (n=60), administration of the incorrect treatment regimen (n=9), or other violations such as incomplete data submission (n=12). Sixty-five patients received pre-operative chemotherapy because their primary tumors were considered to be unresectable. As recommended by the protocol, most of these patients started treatment

with Regimen DD-4A. Patients were considered evaluable as long as they changed to the correct treatment regimen upon nephrectomy (n=47). One patient over 16 years of age at diagnosis was excluded as an evaluable patient because previous NWTSG studies excluded such patients.

Event free survival (EFS) and overall survival (OS) percentages at 4 years past diagnosis were estimated by actuarial methods of Kaplan and Meier. Comparisons of EFS and OS between patient subgroups were made with the log-rank test. Comparisons of mean age at diagnosis by histology were made using the t-test. Comparisons of gender and stage distribution by histology were made using the Fisher exact and related tests.

Results

Patient characteristics

2596 patients with Wilms tumor were enrolled on NWTS-5, of which 59 had focal AH and 222 had diffuse AH by central pathology review. Anaplasia was not originally recognized by institutional pathologists in 74 of 190 (38.9%) patients who underwent immediate nephrectomy and were considered to have anaplastic histology by central pathology reviewers. An additional nine patients were considered to have focal AH by institutional pathologists, but diffuse AH by central reviewers. The analyses in this report are based on the central pathology histology designation. Among 158 patients with unilateral anaplastic Wilms tumor for whom a local tumor stage was assigned (regardless of distant metastases), discordance between institutional stage and central pathology stage was noted in 30 patients (19%). The analyses in this report use the overall stage assigned by the treating institution, which was based on local pathology stage and the presence of distant metastases.

More patients with unilateral Wilms tumor had AH on NWTS-5 (10.1%) compared to NWTS-4 (7.5%). More patients with unilateral Wilms tumor received pre-nephrectomy chemotherapy on NWTS-5 (14.0%) compared to NWTS-4 (9.0%). Anaplasia was more frequently detected in unilateral tumors after pre-operative chemotherapy (18.6%) than in tumors resected immediately (8.7%).

The clinical characteristics of the patients with AH are described in Table 1. For demographic comparison, patients with FH Wilms tumor are included in this table. The female to male ratio among patients with AH was 2 to1; in comparison, the female to male ratio among patients with FH was 1.2 to 1. Of patients with unilateral AH, 65.4% presented with high-stage (III+IV) disease, whereas 45.6% of patients with unilateral FH presented with high-stage disease (OR 2.26, p<0.001). Stage V (bilateral) disease was present in 12.5% of patients with AH and 5.6 % of patients with FH. The mean age at presentation for patients with AH was 56.5 months compared to 43.3 months for patients with FH (p<0.001).

Table 1

Demographics of patients with Wilms tumor enrolled on NWTS-5*

	Favorable Histology (n=2315)	Focal Anaplasia (n=59)	Diffuse Anaplasia (n=222)
Gender			
Male	1036 (44.8%)	15 (25.4%)	78 (35.1%)
Female	1279 (55.2%)	44 (74.6%)	144 (64.9%)
Median Age at Diagnosis (months)	38.0	45.0	52.5
Age at Diagnosis (years)			
0 – 1	693 (29.9%)	3 (5.1%)	20 (9.0%)
2 – 3	743 (32.1%)	31 (52.5%)	70 (31.5%)
> 4	879 (38.0%)	25 (42.4%)	132 (59.5%)
Average Age at Diagnosis (months)	43.30	50.49	58.04
Stage			
I	511 (22.1%)	12 (20.3%)	32 (14.4%)
II	678 (29.3%)	5 (8.5%)	36 (16.2%)
III	693 (29.9%)	16 (27.1%)	88 (39.6%)
IV	304 (13.1%)	15 (25.4%)	42 (18.9%)
V	129 (5.6%)	11 (18.6%)	24 (10.8%)

*Excludes patients 16 and older at diagnosis

Patient outcomes

Of the 200 patients considered evaluable for outcome analyses, 118 were followed alive beyond two years from diagnosis and 63 beyond 4 years. Three of 77 events were due to death in the absence of Wilms tumor; one patient with stage IV diffuse AH died of secondary acute myelogenous leukemia (AML) one patient with stage II diffuse AH died of rhabdomyosarcoma, and one patient with stage V diffuse AH died of infectious complications while on dialysis after bilateral nephrectomy. The outcomes according to stage and histologic subtype are summarized in Table 2.

Patients with stage I focal or diffuse AH were treated with VCR/AMD without irradiation, based on satisfactory results with this approach in previous NWTSG studies. The 4-year EFS and OS estimates for 29 patients in this group were 69.5% (95% CI, 46.9 to 84.0) and 82.6% (95% CI, 63.1 to 92.4), respectively (Figure 2).

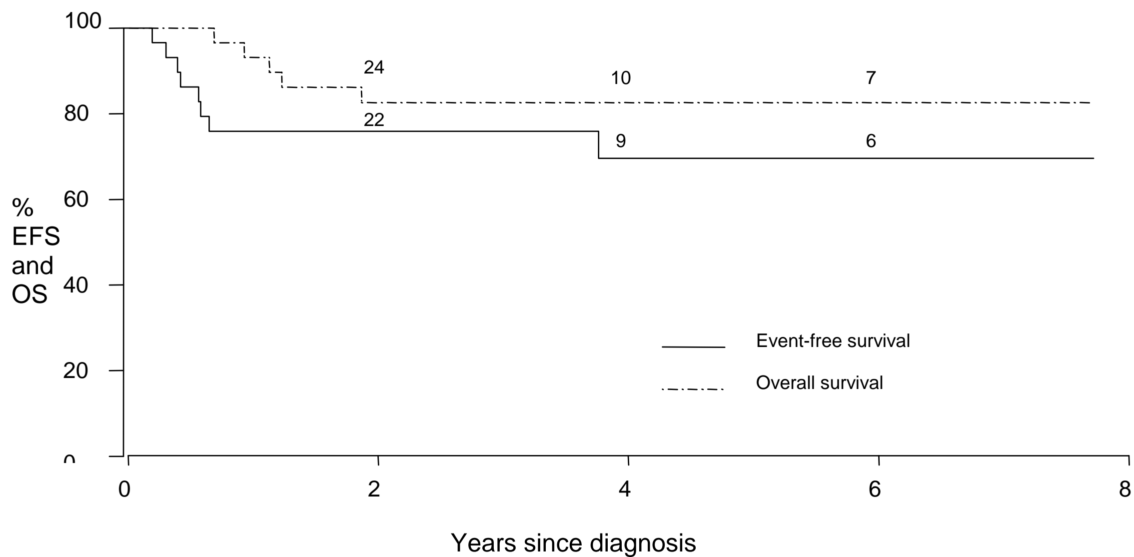

Figure 2. Event-free and overall survival for patients with stage I focal or diffuse anaplastic Wilms tumor (n=29).

By contrast, 4-year EFS and OS estimates for 473 evaluable patients with stage I FH Wilms tumor were 92.4% (95% CI, 89.5 to 94.5) and 98.3% (95% CI, 96.4 to 99.2), respectively.

Table 2

EFS and OS by stage and subtype of anaplastic Wilms tumor

	# patients			EFS at 4 years		OS at 4 years	
	Total	Events	Deaths	%	95% CI	%	95% CI
Overall	200	77	67	59.9	(56.8,70.2)	65.9	(58.6,72.2)
Focal AH	51	12	8	74.9	(59.9,85.0)	82.4	(67.6,90.8)
Diffuse AH	149	65	59	54.9	(46.2,62.7)	60.4	(51.8,67.9)
Stage I	29	8	5	69.5	(46.9,84.0)	82.6	(63.1,92.4)
Focal AH	10	2	1	67.5*	(16.2,91.9)	88.9*	(43.3,98.4)
Diffuse AH	19	6	4	68.4	(42.8,84.4)	78.9	(53.2,91.5)
Stage II	28	5	5	82.1	(62.3,92.1)	81.2	(60.3,91.7)
Focal AH	5	1	1	80.0*	(20.4,96.9)	80.0*	(20.4,96.9)
Diffuse AH	23	4	4	82.6	(60.1,93.1)	81.5	(57.7,92.6)
Stage III- IN	51	16	14	68.3	(53.6,79.3)	72.0	(57.3,82.4)
Focal AH	8	1	0	87.5	(38.7,98.1)	100.0	-
Diffuse AH	43	15	14	64.7	(48.3,77.7)	66.7	(50.2,78.8)
Stage III- POCT	23	10	9	54.2	(31.6,72.2)	58.0	(34.6,75.6)
Focal AH	7	2	2	71.4	(25.8,92.0)	71.4	(25.8,92.0)
Diffuse AH	16	8	7	45.7*	(20.1,68.3)	53.3*	(26.3,74.4)
Stage IV-IN	16	10	10	37.5*	(15.4,59.8)	37.5	(15.4,59.8)
Focal AH	1	0	0	-	-	-	-
Diffuse AH	15	10	10	33.3*	(12.2,56.4)	33.3*	(12.2,56.4)
Stage IV-POCT	24	13	11	44.6	(24.3,63.2)	55.9	(33.1,73.6)
Focal AH	11	4	3	61.4	(26.6,83.5)	71.6	(35.0,89.9)
Diffuse AH	13	9	8	30.8*	(9.5,55.4)	44.0*	(16.8,68.4)
Stage V	29	15	13	43.8	(24.2,61.8)	55.2	(34.8,71.7)
Focal AH	9	2	1	76.2*	(33.2,93.5)	87.5*	(38.7,98.1)
Diffuse AH	20	13	12	25.1*	(5.88,51.0)	41.6*	(19.7,62.2)

*Fewer than 5 patients have survived 4 years: result must be interpreted with caution

AH: anaplastic histology; EFS: event-free survival; OS: overall survival; CI: confidence interval

Comparison of EFS and OS curves between patients with Stage I FH and Stage I AH demonstrated a highly significant difference ($p<0.001$).

Patients with stages II-IV diffuse AH were treated with the novel regimen I. The EFS and OS estimates for these patients are shown in Table 2 and Figure 3. Forty seven evaluable patients with unilateral AH underwent tumor biopsy and preoperative chemotherapy before tumor resection was performed. Among 39 evaluable patients for whom both biopsy and nephrectomy histology results were available, only four (10.3%) had anaplasia detected in the biopsy sample. Patients who received preoperative chemotherapy were analyzed separately because most switched treatment regimens as a result of the change in histologic diagnosis. No difference in outcome was observed between patients who received preoperative chemotherapy and those who had immediate nephrectomy; the estimated hazard ratios for preoperative chemotherapy versus immediate nephrectomy (stratified by stage) were 0.991 for EFS (p=0.972) and 0.952 for OS (p=0.862).

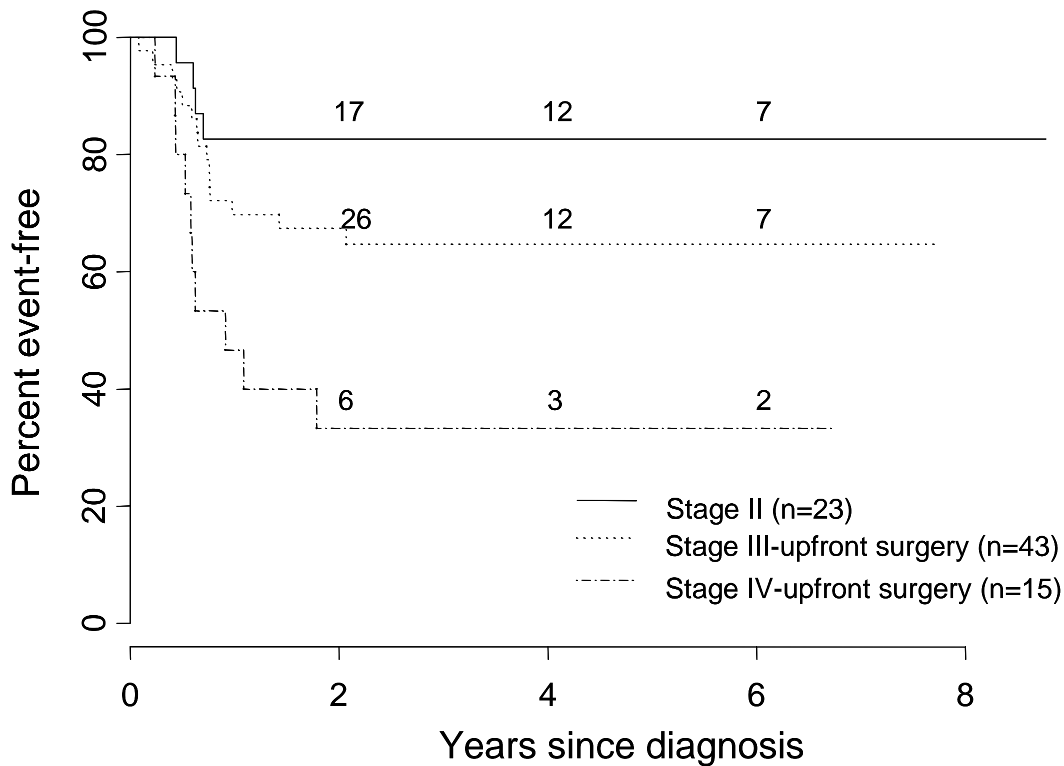

Figure 3. Event-free survival for patients with stages II-IV diffuse anaplastic Wilms tumor.

Patients with bilateral (stage V) AH were treated heterogeneously. Among 25 patients with bilateral AH for whom biopsy and nephrectomy histology were available for review, only two had anaplasia detected in the initial biopsy sample (8%). Upon definitive surgery (nephrectomy or partial nephrectomy), anaplasia was present on both sides in 3 patients, on one side in 8 patients, and the status of one of the sides was unknown in 14 patients. Among the 26 patients with bilateral AH who were evaluable for response, 4/6 patients who started treatment with Regimen EE-4A, 2/17 patients who started treatment with Regimen DD-4A, and 0/3 patients who started treatment with Regimen I had progressive disease. The survival estimates for patients with bilateral AH are shown in Table 2 and Figure 4.

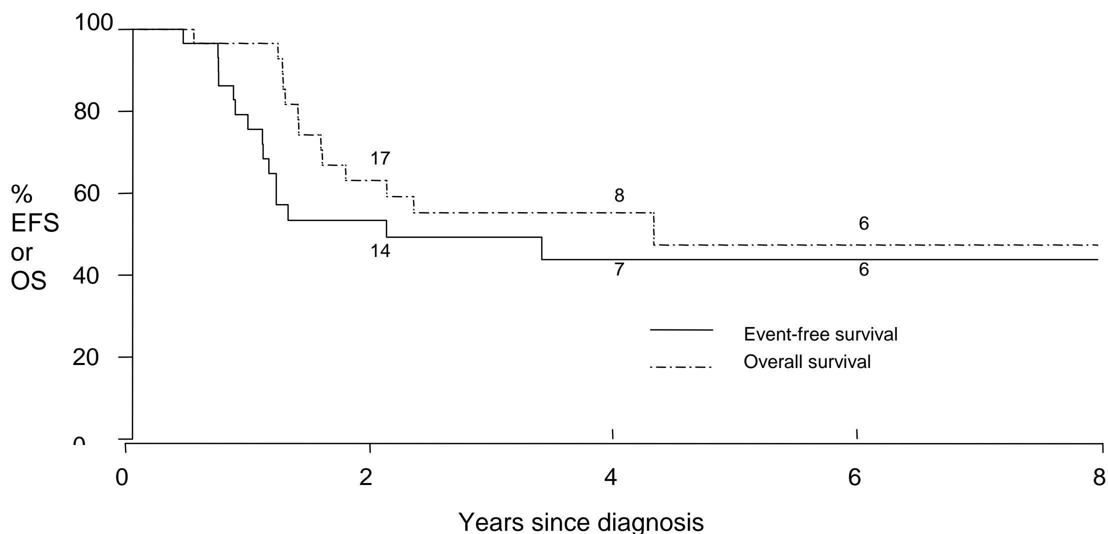

Figure 4. Event-free and overall survival for patients with stage V (bilateral) anaplastic Wilms tumor.

Because a substantial proportion of patients were considered "non-evaluable," we assessed the difference in outcomes between the non-evaluable and evaluable patients. Only 8 patients with focal AH were non-evaluable; of these, 4 had events and died. The 4-year EFS and OS estimates for the 73 non-evaluable patients with diffuse AH were 57.6% (95% CI, 44.5 to 68.7) and 67.5% (95% CI, 54.5 to 77.5), respectively. These estimates are very similar to those for the evaluable patients with diffuse AH.

Patterns of Recurrence

On NWTS-3 and -4, the prescribed dose of flank/abdominal radiation for patients with anaplastic Wilms tumor increased with patient age. The frequency of operative bed relapse was not greater among patients treated with lower, compared to higher, radiation doses. [7] Based on this observation, NWTS-5 prescribed a uniform dose of 10.8 Gy for all patients with stages II-IV AH. To estimate the effectiveness of local control, we analyzed the rates of recurrence in the operative bed or the abdomen/pelvis outside the operative bed, which also may have been included in the radiation field (Table 3). None of the patients with stage II disease had first recurrences in the operative bed or abdomen, indicating that local control for these patients was excellent with 10.8 Gy. The rates of operative bed or abdominal recurrences for patients with stages III, IV, and V disease were 12/74 (16.2%), 6/40 (15%), and 11/29 (37.9%), respectively.

Table 3

Sites of initial recurrence by stage in evaluable patients with anaplastic Wilms tumor*

Stage	# of patients	# of patients with recurrence or progression					
		All Sites	Lung	Op bed	Abd/pelvis outside op bed	Liver	Other sites
I	29	8	1	1	2	2	2
II	28	4	3	0	0	1	0
III-immediate nephrectomy	51	16	9	2	3	2	0
III-preoperative chemotherapy	23	10	1	5	2	1	1
IV-immediate nephrectomy	16	9	2	1	1	0	1
IV-preoperative chemotherapy	24	13	0	3	1	2	0
V	29	14	1	6	5	2	0
Total	200	74	17	18	14	10	4

* Two patients with lung recurrence had contemporaneous recurrence in the lung and other sites. 11 patients with stage IV tumors had persistent Wilms tumor or progressive disease. Other sites include bone and the contralateral kidney.

Toxicity of Regimen I

Among 91 patients who received Regimen I as their initial treatment regimen, common grade 3 or 4 toxicities were absolute neutrophil count (n=65), total white blood cell count (n=49), hemoglobin level (n=55), platelet count (n=27), and infection (n=32). Other grade 3 or 4 toxicities occurred in fewer than 5% of patients. Two patients had second malignant neoplasms. One of these patients with stage IV diffuse AH developed AML 14 months from the diagnosis of Wilms tumor. The other patient had stage II diffuse AH with a focus of rhabdomyosarcoma within the primary tumor (not therapy-related). It is possible that this focus represented Wilms tumor with muscle differentiation, but the histologic appearance was more consistent with rhabdomyosarcoma. This patient subsequently developed disease consistent with rhabdomyosarcoma in the orbit, which is an unusual site for Wilms tumor.

Discussion

Despite remarkable success in the treatment of FH Wilms tumor, the treatment of AH Wilms tumor remains a clinical challenge. A primary objective of NWTS-5 was to improve the outcomes of patients with stages II-IV diffuse AH using Regimen I, a novel treatment regimen containing the CYCLO/etoposide combination. The outcomes of patients treated with Regimen I compared favorably to historical controls. On NWTS-3 and -4, patients with diffuse AH treated with nephrectomy followed by VCR, AMD, DOX, and CYCLO had 4-year OS estimates of 70.1% for stage II (n=11), 56.3% for stage III (n=13), and 16.7% for stage IV (n=6) disease. [7] By comparison, the 4-year OS estimates for patients treated on NWTS-5 with immediate nephrectomy and Regimen I were 81.5% for stage II (n=23), 66.7% for stage III (n=43), and 33.3% for stage IV (n=15) disease. The local control rate among patients with stage II disease was 100% with a radiation dose of 10.8 Gy.

Although Regimen I itself likely contributed to the observed improvement in outcomes between NWTS-4 and NWTS-5, the effect of shifts in the patient population cannot be discounted. A higher percentage of patients with unilateral tumors received pre-nephrectomy chemotherapy on NWTS-5 (14%) compared to NWTS-4 (9%). The reason for this alteration in clinical practice is unclear, but the result was a migration from stage II to stage III because pre-operative chemotherapy was a defining criterion for stage III disease. Additionally, there was a higher prevalence of AH among patients enrolled on NWTS-5 (10.1%) compared to NWTS-4 (7.5%), which is partially explained by the increased detection of anaplasia in tumors treated with pre-operative chemotherapy.

What was the cost, in terms of adverse effects, of the switch from Regimen J to Regimen I? The key differences between the two regimens are that Regimen I incorporated etoposide and used a higher cumulative dose of CYCLO. While augmenting these components of therapy, Regimen I eliminated AMD, lowered the cumulative dose of DOX, lowered the flank radiation doses for most patients, and shortened the duration of therapy (Table 4). Short-term toxicities of Regimen I were manageable, though one patient developed secondary AML, an uncommon complication that occurs in Wilms tumor patients even without etoposide therapy. [13] The effects on fertility of the higher cumulative CYCLO dose remain to be determined.

Table 4

Comparison of Regimens J and I

	Cumulative Doses (mg/kg)	
Drug	Regimen J	Regimen I
Cyclophosphamide	300	467
Doxorubicin	10	7.5
Vincristine	1.4	0.7
Etoposide	0	67
Dactinomycin	0.46	0
Flank Radiation	variable, mostly> 32 Gy	10.8 Gy
Duration of Therapy (weeks)	67	25

Patients with stage I AH had significantly worse outcomes compared to patients with stage I FH. This finding was unexpected because previous NWTSG studies showed that patients with stage I AH had good outcomes.[6;8;14] It is unclear why the EFS estimate for patients with stage I AH on NWTS-5 (n=29, 4-year EFS 69.9%) was inferior to the EFS estimates reported for patients with stage I AH treated on NWTS-4 (n=21, 2-year EFS 87.5% or 93.8%, depending on the AMD schedule).[8] The apparent discrepancy may relate to the small number of patients studied and the wide confidence intervals surrounding the survival estimates. Among 23 patients with stage I AH treated on the International Society of Paediatric Oncology (SIOP) -6 and -9 trials, six had recurrence and four died of disease. [4] These results are similar to the results of NWTS-5. A recent report from the SIOP 93-01 study suggested that patients with stage I AH had good outcomes, but the number of patients with anaplastic histology and their outcomes were not specified.[15]

Previous NWTSG studies have suggested that anaplasia *per se* is not a marker of aggressiveness. [12;14] The somewhat higher than expected recurrence and death rates for patients with stage I AH on NWTS-5 seem to question that suggestion. Ongoing studies of molecular prognostic markers of tumor invasiveness and metastatic potential will help clarify whether anaplastic Wilms tumors are inherently aggressive.

The molecular biology of anaplastic Wilms tumor is only beginning to be defined. Approximately 65% of anaplastic Wilms tumors studied to date had detectable mutations of the *TP53* tumor suppressor gene, whereas such mutations were rare in FH Wilms tumors. [16-19] The restriction of *TP53* mutations to areas of anaplasia within a Wilms tumor indicates that anaplasia arises in a clonal fashion on a background of favorable histology. [20] Because p53 protein plays a central role in the cellular response to DNA-damaging agents [21], it is likely that *TP53* mutations contribute to the relative unresponsiveness of anaplastic Wilms tumors to treatment. However, 35% of anaplastic Wilms tumors lack detectable *TP53* mutations. While these tumors may harbor alterations of other molecules in the p53 pathway, it is possible that *TP53* mutations are neither necessary nor sufficient to generate anaplasia. The 2:1 female to male ratio observed in patients with anaplastic Wilms tumor raises the possibility that gender is a determinant of susceptibility to anaplasia. Gene expression studies have uncovered

several candidate genes associated with anaplasia, but their role in the pathogenesis of anaplasia remains to be confirmed. [22]

Anaplasia was not detected by institutional pathologists in about 40% of immediate nephrectomy specimens deemed to have anaplasia by the central reviewers. As a result, a substantial proportion of patients switched treatment regimens in the middle of the study and were considered non-evaluable for the primary outcomes analysis. Interestingly, comparison of outcomes in the evaluable and non-evaluable patients showed that survival rates were essentially identical between the two groups. Similarly, patients who received pre-operative chemotherapy for what eventually proved to be AH did not have compromised outcomes, even though most of these patients initially received treatment regimens for FH. The upcoming Children's Oncology Group (COG) studies will require central pathology review to be completed before treatment is initiated.

The results of NWTS-5 provide the framework for future COG studies of anaplastic Wilms tumor. Based on the lower than expected survival rate for patients with stage I AH, the upcoming study will augment therapy for this group of patients. Although NWTS-5 outcomes for patients with stage II-IV diffuse AH were improved compared to historical regimens, a considerable percentage of patients experienced disease recurrence. A new treatment regimen including carboplatin, which has shown activity against Wilms tumor [23-25], will be used for this patient group. Patients with stage IV disease are particularly challenging to treat. A priority of future pre-clinical and clinical studies will be to identify novel agents.

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Chapter 5

Concordance of Genomic Copy Number and Gene Expression Profiles Between Wilms Tumor Xenografts and Matched Primary Tumors

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ABSTRACT

Purpose

Despite overall success in Wilms tumor (WT) treatment, some patients have poor clinical outcomes. To assist prioritization of novel drugs for Phase II studies, WT xenograft models were generated for preclinical testing. This study was conducted to evaluate the extent of genetic change that occurs during the establishment of WT xenografts from primary tumors.

Experimental Design

Nine WT xenografts were generated from primary tumor specimens. Genomic copy number was compared in six xenograft/primary tumor pairs using Affymetrix GeneChip Human Mapping 100K single nucleotide polymorphism arrays. Gene expression analysis was performed using Affymetrix HG-U133A GeneChip microarrays. Antitumor activity of several classes of chemotherapy agents was determined.

Results

Unsupervised hierarchical clustering analysis according to gene copy number showed that xenografts invariably clustered adjacent to their primary tumors. Gene expression analysis showed no evidence of differential expression between primary WT/xenograft pairs beyond that expected by chance; 291 out of 22,215 probe sets (<2%) were differentially expressed at a p-value <0.01 (estimated false discovery rate 71.6%). Unsupervised hierarchical clustering analysis according to gene expression level showed that WT xenografts clustered with other WT, but not always adjacent to their corresponding parent tumors. Expression levels of genes encoding proteins targeted by agents undergoing phase I and II clinical testing were not significantly different between xenografts and primary tumors. The xenograft models demonstrated sensitivity to vincristine, dactinomycin, cyclophosphamide, and topotecan, consistent with the known clinical efficacy of these agents.

Conclusions

Gene copy number and expression levels were concordant between WT xenografts and parent primary tumors. The results suggest that the WT xenografts provide a biologically relevant model for preclinical testing.

Introduction

Wilms tumor accounts for 7% of pediatric cancers in children under age 15 (1). In spite of the overall success of Wilms tumor treatment, approximately 15% of patients have tumors that are resistant to current treatment regimens (2). The identification of novel active agents for this group of patients is warranted.

The number of new anti-cancer drugs has grown tremendously in the past decade. While the overall progress in the area of cancer therapeutics has been remarkable, drug development for pediatric malignancies is hampered by the relative paucity of patients available for clinical trials (3). The conduct of phase II clinical studies for pediatric cancer often requires a several year commitment, underscoring the need to rationally prioritize agents for clinical testing. Toward this end, preclinical models of pediatric cancers including cell lines, genetically engineered mice, and mice bearing human tumors (xenografts) have been developed to screen agents for potential antitumor activity (3).

The preclinical testing of novel agents for Wilms tumor has been limited by a lack of Wilms tumor cell lines. Only a few Wilms tumor cell lines have been reported (4-8), and some of these were subsequently shown to be derived from other tumor types, such as malignant rhabdoid tumor (9). It was recently demonstrated that SK-NEP1, a widely used anaplastic Wilms tumor line, has a gene expression profile similar to Ewing sarcoma and contains the characteristic t(11;22) translocation (10). Mice heterozygous for the Wilms tumor suppressor gene (*WT1*), do not develop Wilms tumor (11;12) nor do mice bearing *WT1* mutations of Denys Drash Syndrome, which confers a strong Wilms tumor predisposition in humans (13). Likewise, mouse models of Beckwith-Wiedemann Syndrome do not develop Wilms tumor (14).

Given the dearth of preclinical models for Wilms tumor, we established Wilms tumor xenografts from the primary tumors of nine different patients. Four of these models have been included in the xenograft panel of the National Cancer Institute-sponsored Pediatric Preclinical Testing Program (PPTP) (15). Previous gene expression profiling analyses have indicated that Wilms tumor xenografts cluster with other Wilms tumor xenografts (16) and with unmatched primary Wilms tumors (17). To gain further insight into the extent of

genetic evolution that occurs during the process of xenograft establishment, we compared gene copy number and expression profiles between Wilms tumor xenografts and their matched parent tumors. We also report the pre-clinical testing results of a panel of cytotoxic drugs with known activity against Wilms tumor to assess the accuracy of the xenograft models in predicting true clinical response in patients.

Patients and Methods

Establishment of Wilms Tumor Xenografts

To screen novel anti-cancer agents for activity against Wilms tumor, xenograft models were generated from the primary tumors of nine patients with Wilms tumor (8 favorable histology, 1 anaplastic histology) who were treated at St. Jude Children's Research Hospital. The legal guardians of these patients provided informed consent to participate in the St. Jude Bank 97 study, a tumor banking protocol that was approved by the Institutional Review Board. Eight xenografts were derived from patients whose nephrectomies were performed before chemotherapy was given and one was derived from a patient who had undergone treatment with ifosfamide, carboplatin, etoposide, doxorubicin, vincristine, and cyclophosphamide before nephrectomy. The procedures for xenograft generation and determination of chemosensitivity have been reported previously (18;19). Briefly, CB17/Icr female SCID mice (Charles River, Wilmington, MA) were implanted with single tumor fragments subcutaneously in the flank. The mice were observed until tumors were 0.20-1 cm in diameter before being treated with chemotherapy drugs. All mice were maintained under barrier conditions. All experiments were conducted using protocols and conditions approved by the Institutional Animal Care and Use Committee.

Single Nucelotide Polymorphism (SNP) Arrays

SNP analysis was performed in the St. Jude Hartwell Center core laboratory using the Affymetrix GeneChip Human Mapping 100K assay. Purity and integrity of DNA samples was confirmed by UV spectrophotometry and by agarose gel electrophoresis. Processing of samples was performed according to the Affymetrix 100K SNP protocol (20). In brief, 250 ng genomic DNA was digested to completion with either *XbaI* or *HindIII*. After ligation of

adapters, digested DNA was amplified using thermocycling conditions that enrich fragments 250 to 2000 bp in size. Forty micrograms of amplified DNA were fragmented by digestion with DNaseI and end-labeled with biotinylated nucleotide using terminal deoxynucleotidyl transferase. Labeled DNA was added to a hybridization cocktail containing probe array controls and blocking agents, then incubated overnight at 48 C on a GeneChip array. Arrays were washed, stained with streptavidin-phycoerythrin, washed again and then scanned using the Affymetrix GeneChip Scanner 3000. Genotype calls were determined using the Affymetrix Gene DNA Analysis Software (GDAS) version 3.0. DNA copy number estimates were determined using the Copy Number Analysis Tool version 2.0 (21;22) in conjunction with the diploid reference dataset supplied by Affymetrix.

Gene Expression Analysis

Gene expression analysis was performed using the Affymetrix HG-U133A GeneChip microarray. This array contains 22,215 probesets that interrogate 18,400 transcripts and variants (full details available at www.affymetrix.com). Total RNA was prepared from tumor samples using the TriReagent method (Molecular Research Center). RNA quality was confirmed by UV spectrophotometry and by analysis on the Agilent 2100 Bioanalyzer. Processing of RNA samples was performed according to the Affymetrix gene expression protocol (23). In brief, 5ug of total RNA were annealed to a T7-oligodT(24) primer and double-stranded cDNA generated using the SuperScript II cDNA synthesis kit according to the manufacturer's conditions (Invitrogen). Following phenol/chloroform extraction, the cDNA was used as template to synthesize biotin-labeled anti-sense cRNA by using the T7 RNA polymerase Bioarray high-yield kit (ENZO Diagnostics, Inc). Ten micrograms of the biotin-labeled cRNA was fragmented by heating and metal induced hydrolysis, added to a hybridization cocktail containing probe array controls and blocking agents and then incubated overnight at 45 C on a GeneChip array. Following hybridization arrays were washed, stained with streptavidin-phycoerythrin, washed again and then scanned using an Affymetrix GeneChip Scanner 3000. Expression signals were calculated using the MAS5 statistical algorithm in the Affymetrix GCOS software (version 1.1). Signal values were scaled using the global normalization method using a 2% trimmed mean target value of 500. Detection calls for each gene (absent, marginal, or present) were determined using the default parameters of the software.

Hierarchical Clustering Analysis

Unsupervised hierarchical clustering was performed using GeneMaths XT software version 1.6 (Applied Maths, Belgium). Similarity between samples or genes was calculated using the Pearson's correlation method while linkage between groups was calculated using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) method. For expression analyses, log2-transformed Affymetrix signals were scaled across arrays to a mean of zero and a standard deviation of one. Cluster analysis was performed using the expression profiles of 15592 probesets with at least one Present Call across the dataset. For copy number analysis, SNP signal intensity relative to the reference dataset as reported by the Affymetrix copy number tool (Log_2Ratio) was used for cluster analysis as described above. Clustering was performed using 761 SNPs with high variation across the samples (variance of Log_2Ratio >1).

The microarrays for the primary Wilms tumor and xenograft samples were conducted at St. Jude. The microarrays for the fetal kidneys, clear cell sarcomas of the kidney, and hyperplastic perilobar nephrogenic rests were conducted at Children's Memorial Hospital in Chicago as previously described (24).

Statistical Analysis

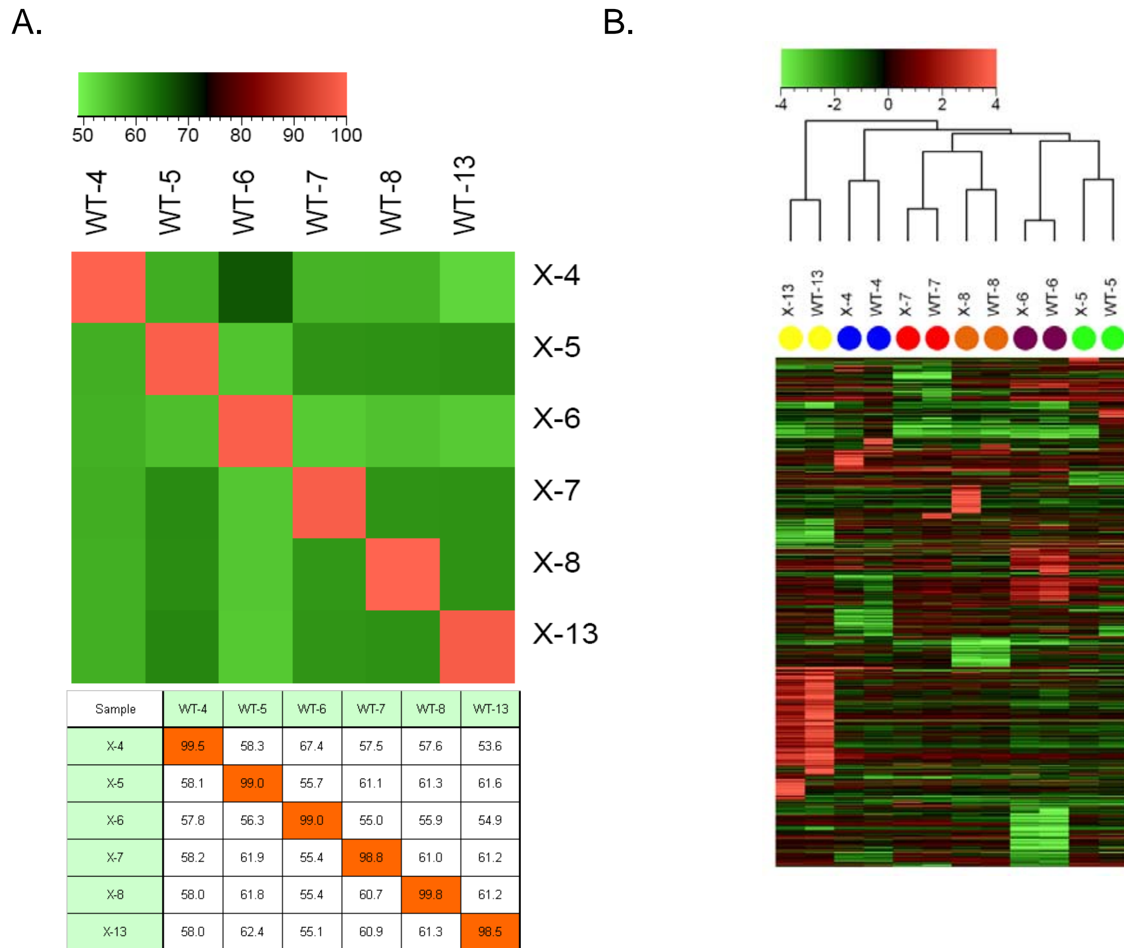
Similarities in global gene expressions between Wilms tumor xenografts and their parent primary tumors and between Wilms tumor xenografts and other primary kidney tumors were assessed by Pearson's correlation coefficient. Differential gene expression between Wilms tumor xenografts and primary tumors was analyzed by paired t tests, and differential gene expression between Wilms tumor xenografts and other tumors was analyzed by analysis of variance (ANOVA). False discovery rates (FDR) were estimated by the method of Cheng (25).

Results

Comparison of genomic stability between Wilms tumor xenografts and parent tumors

Among the nine xenograft models established, six had RNA from both primary tumor and matching xenograft that passed quality control tests for gene expression microarray studies. To confirm that these six Wilms tumor xenografts originated from their parent primary tumors, assessment of single nucleotide polymorphisms (SNP) was undertaken

using Affymetrix 100K SNP chips. A strong correlation of genotypes between xenografts and matched primary tumors was observed, as indicated by concordance rates of SNP calls >98% in each of the six xenograft/primary tumor pairs (Figure 1A). By contrast, concordance rates between xenografts and mismatched primary Wilms tumors ranged from 54.9–67.4%. This result provided confidence that the six xenograft/primary tumor pairs were indeed matched.



Sample	WT-4	WT-5	WT-6	WT-7	WT-8	WT-13
X-4	99.5	58.3	67.4	57.5	57.6	53.6
X-5	58.1	99.0	55.7	61.1	61.3	61.6
X-6	57.8	56.3	99.0	55.0	55.9	54.9
X-7	58.2	61.9	55.4	98.8	61.0	61.2
X-8	58.0	61.8	55.4	60.7	99.8	61.2
X-13	58.0	62.4	55.1	60.9	61.3	98.5

Figure 1. 100K single nucleotide polymorphism (SNP) chip analysis of primary Wilms tumor/xenograft pairs. **A**. Heat map showing fraction of concordance in SNP calls between primary tumors and xenografts. **B**. Unsupervised hierarchical cluster analysis of 760 SNPs with log2 ratio of copy number variance >1 (compared to normal). Scale bar represents relative signal intensity of each SNP; units are log2 (sample/diploid reference).

The SNP chip analysis also afforded the opportunity to assess genomic stability between primary tumor/xenograft pairs. An unsupervised hierarchical cluster analysis of gene

copy number changes demonstrated that xenografts invariably clustered adjacent to their primary tumors (Figure 1B). Areas of genomic amplification and deletion within the primary tumors tended to be preserved in the xenografts. Together, these results indicate that the genomes of Wilms tumor xenografts are stable in comparison to their matched primary tumors.

Comparison of gene expression between Wilms tumor xenografts and parent tumors

Correlation analysis was used to evaluate the variation in global gene expression that occurs when primary Wilms tumors are implanted into immunodeficient mice. When the log-transformed signal intensities for probe sets with present calls were considered, the correlation coefficients (r^2) between xenografts and parent tumor pairs were 0.85, 0.76, 0.88, 0.76, 0.80, and 0.78 for pairs 1 though 6, respectively (Figure 2A). This strong correlation of global gene expression may reflect the fact that these tissues all originated from pediatric kidney. To place these results in the context of an unrelated type of pediatric kidney tumor, correlation of gene expression levels between Wilms tumor xenografts and clear cell sarcoma of the kidney (CCSK) was assessed. When probe sets with present calls were considered, the r^2 values between random pairings of the six Wilms tumor xenografts and six primary CCSK tumors were 0.65, 0.65, 0.70, 0.66, 0.62, and 0.70 (Figure 2B). These correlations were weaker than those observed between the xenograft/primary tumor pairs.

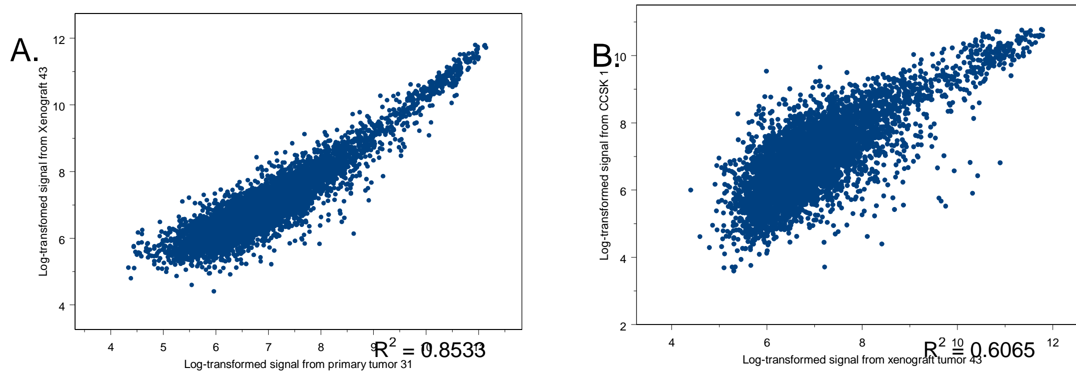


Figure 2. Representative correlation analyses of gene expression between Wilms tumor xenografts and primary tumors. Correlation between Wilms tumor xenograft WT1 and its matching primary tumor using all probe sets (A) or probe sets with present calls (B). Correlation between xenograft WT1 and a primary CCSK sample using all probe sets (C) or only probe sets with present calls (D).

The similarity in gene expression between xenografts and their parent primary tumors was further assessed with paired t test and ANOVA analyses for each individual probe set. Figure 3 shows a histogram of the percentage of probe sets with each p-value. In the comparison between xenografts and primary tumors, the distribution of p-values was nearly uniform; only 291 out of 22215 (less than 2%) of probe sets were differentially expressed at a p-value <0.01 (estimated false discovery rate 70.9%). In this case, the percentage of differentially expressed probe sets did not exceed that which would be expected to occur by chance. By contrast, comparison of expression between Wilms xenografts and primary CCSK samples revealed that 3942 of 22,215 (17.7%) probe sets were differentially expressed with a p-value<0.01 (estimated false discovery rate 2.66%).

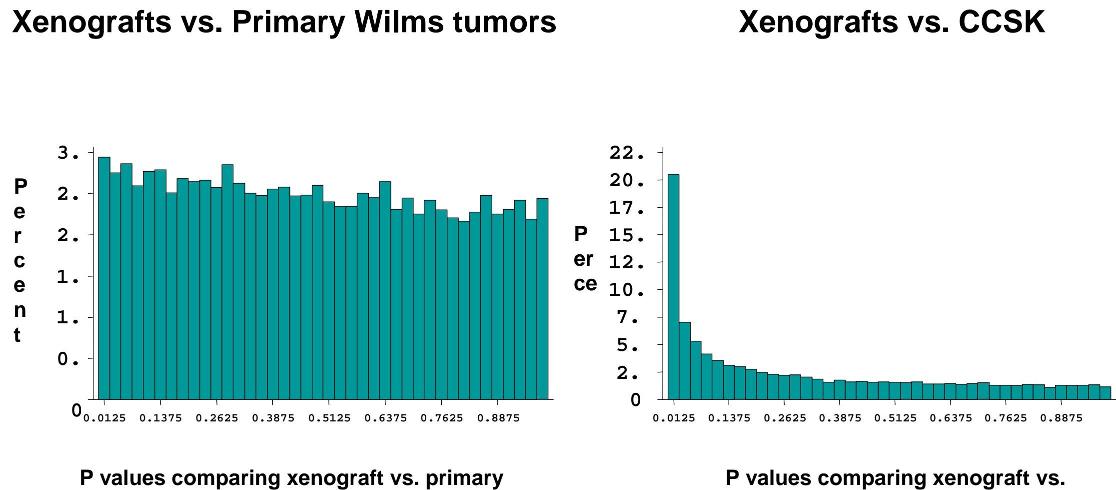


Figure 3. Histogram of p-values to evaluate differential expression of all 22,215 probe sets between 6 Wilms tumor xenografts and matching primary tumors (A) or between 6 Wilms tumor xenografts and 6 CCSK samples (B).

An unsupervised hierarchical cluster analysis, including two fetal kidney samples, three hyperplastic perilobar nephrogenic rests (precursors to Wilms tumor), and the six CCSK samples was performed to evaluate the relationship between Wilms tumor xenografts and their primary tumors. This analysis revealed that the samples clustered into four categories: the CCSKs comprised one, the fetal kidneys, nephrogenic rests, and one Wilms tumor comprised the second, and other Wilms tumors and xenografts comprised the third

and fourth (Figure 4). Four of six xenografts clustered in the same groups as their primary tumors.

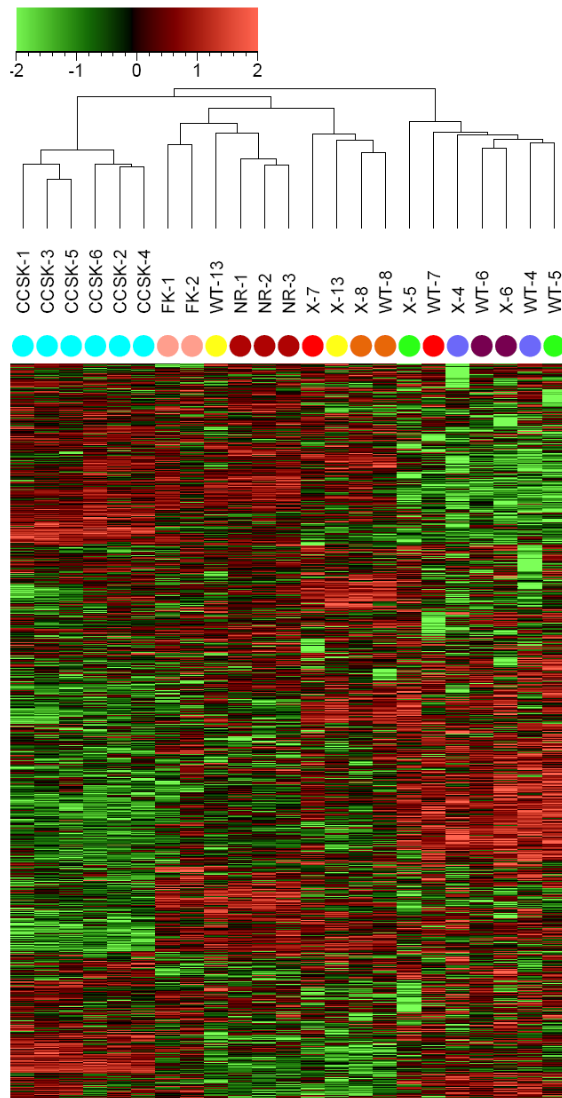


Figure 4. Unsupervised hierarchical cluster analysis including 6 Wilms tumor xenograft/primary tumor pairs, 2 fetal kidney samples, 3 hyperplastic perilobar nephrogenic rests, and 6 clear cell sarcomas of the kidney. Scale bar represents relative expression of transcripts; units are standard deviations from the mean.

Changes in gene expression between primary tumor/xenograft pairs for pathways of clinical interest in Wilms tumor

The previous analyses suggested that on a global scale, gene expression was not substantially altered between Wilms tumor xenografts and parent primary tumors.

Table 1

Gene expression levels for therapeutic targets or modifiers

Drug	Relevant Targets or Modulators	Probe	P values (Primary vs. Xenograft)	Primary median	Xenograft median
Erlotinib, gefitinib, lapatinib	EGFR	201983_s_at	0.89	645.6	665.9
	EGFR	201984_s_at	0.82	413	284.3
	EGFR	210984_x_at	0.60	57.2	59.3
	EGFR	211550_at	0.44	36.4	21.4
	EGFR	211551_at	0.52	382.4	348.1
	EGFR	211607_x_at	0.52	40.1	40.3
Imatinib	KIT	205051_s_at	0.71	438.5	368.5
	ABL1	202123_s_at	0.22	1154.9	1704.8
	PDGFRA	203131_at	0.17	1378.7	844.9
	PDGFRA	211533_at	0.52	33.4	47.7
	PDGFRA	215305_at	0.52	30.2	31.8
Lapatinib	ERBB4	206794_at	0.40	139.8	83.8
	ERBB4	214053_at	0.02	321.7	163.7
Trastuzumab, lapatinib	ERBB2	210930_s_at	0.09	37.6	117.1
	ERBB2	216836_s_at	0.86	839.7	879.3
Bevacizumab	KDR	203934_at	0.08	287.2	183.4
	FLT1	204406_at	0.19	63.4	38.4
	FLT1	210287_s_at	0.21	29.7	22.4
	FLT1	222033_s_at	0.05	386.5	172.3
	VEGF	210512_s_at	0.09	1040.5	421.2
	VEGF	210513_s_at	0.15	528.8	401.6
	VEGF	211527_x_at	0.34	232.1	168.9
	VEGF	212171_x_at	0.12	988	689.5
SAHA, depsipeptide	HDAC1	201209_at	0.70	1020.3	1011.8
	HDAC2	201833_at	0.91	1651.7	1558.7
	HDAC3	216326_s_at	0.12	606.2	965.6
IMCA12, Everolimus, Temsirolimus	FRAP1	202288_at	0.40	186.7	195.4
	FRAP1	215381_at	0.90	29.3	28.6
	PTEN	204053_x_at	0.07	1140.5	678.4
	PTEN	204054_at	0.29	181	123
	PTEN	211711_s_at	0.19	583.1	295.9
	PTEN	222176_at	0.18	49.3	22.5
	FKBP1A	200709_at	0.47	2482.5	2838.7
	FKBP1A	210186_s_at	0.46	734.8	858.8
	FKBP1A	210187_at	0.06	353.2	230.1
	FKBP1A	214119_s_at	0.76	1532.3	1364
	AKT1	207163_s_at	0.38	516.9	730.8

Because the xenografts were developed as pre-clinical models to test new agents, we assessed whether pathways targeted by novel classes of drugs are differentially expressed between xenografts and primary tumors. Table 1 provides a sampling of genes that encode proteins that are targeted by agents undergoing phase I/II pediatric testing. Only the *ERBB4* gene, which had lower expression levels in xenografts compared to primary tumors, showed differential expression at a p-value <0.05. This differential expression was observed in only one of two *ERBB4* probe sets. Interestingly, the VEGF receptor genes *FLT1* and *KDR* showed trends towards lower expression in xenografts compared to primary tumors. The gene for VEGF itself also showed a trend towards lower expression in the xenografts. One of the probe sets for the PTEN tumor suppressor gene showed lower expression in xenografts compared to primary tumors (p=0.07).

Anti-tumor activity in xenografts of agents with known clinical responsiveness

The gene expression data indicated that the Wilms tumor xenograft models provide a reasonable representation of the parent primary tumors. To gain insight into how faithfully the xenograft models correlate with clinical responsiveness, we subjected nine Wilms tumor models to a battery of conventional cytotoxic drugs, many of which have known clinical response rates from phase II trials. The xenografts were divided into two categories based on the clinical responsiveness of the primary tumors in actual patients. Six "clinically responsive" favorable histology tumors were derived from patients who did not experience tumor recurrence. Three "clinically resistant" tumors included two favorable histology tumors and one anaplastic histology tumor taken from patients who ultimately had tumor recurrence. Figure 5 shows representative growth curves for one of the xenografts, WT-7.

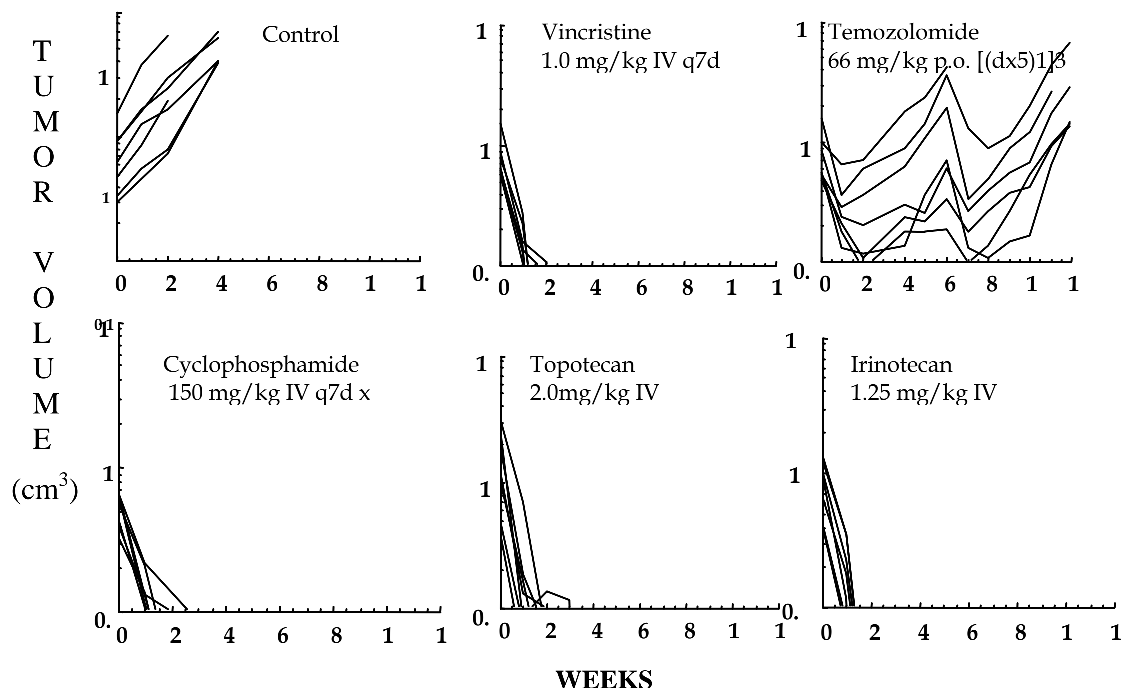


Figure 5. Representative tumor response assays for xenograft model WT7. A minimum of 5 xenografts were tested for each agent.

A summary of xenograft responsiveness to a panel of cytotoxic chemotherapy agents is shown in Figure 6. Overall, the "clinically responsive" xenografts were more sensitive to cytoxic drugs than the "clinically resistant" xenografts, although there were exceptions.

	Clinically Sensitive						Clinically Resistant		
Tumor Line	**WT1**	**WT5**	**WT7**	**WT8**	**WT9**	**WT10**	**WT6**	**WT11**	**WT13**
Histology	**FH**	**FH**	**FH**	**FH**	**FH**	**FH**	**FH***	**FH***	**AH**
Vincristine	MCR	PD	MCR	MCR	MCR	CR	PR	PR	PD
Doxorubicin	PR	PD	PD	MCR	No Data	PD	PD	PD	No Data
Dactinomycin	No Data	PD	No Data	CR	PD	PD	MCR	MCR	No Data
Cyclophosphamid	PD	MCR	MCR	MCR	MCR	MCR	PD	PD	MCR
Etoposide	PD	PD	PD	No Data	No Data	PR	SD	SD	PD
Paclitaxel	No Data	PD	PD	PD	No Data	PD	PD	PD	No Data
Cisplatin	PD	PD	PD	MCR	No Data	MCR	PD	PD	No Data
BCNU	PD	PD	PD	No Data	No Data	PD	PD	PD	PD
Topotecan	MCR	MCR	MCR	MCR	MCR	MCR	PD	PD	MCR
Irinotecan	MCR	MCR	MCR	MCR	No Data	MCR	SD	SD	PR
Temozolomide	SD	SD	SD	MCR	PD	PD	PD	PD	PR
Ixabepilone	No Data	PD	MCR	MCR	MCR	PD	PR	PR	No Data

KEY: MCR | CR | PR | SD | PD | No Data

Figure 6. Heat map of sensitivity of Wilms tumor xenografts to cytotoxic agents.

FH: favorable histology; AH anaplastic histology; MCR: maintained complete response; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease. The doses and schedules of drugs used were as follows:

Drug	**Dose (mg/kg)**	**Schedule**[a]	**Route of administration**
Vincristine	1	Q7days x 6[b]	I.V.
Doxorubicin	4	Q7days x 2	I.V.
Dactinomycin	0.375	Q21 days x 2	I.P.
Cyclophosphamide	150	Q7 days x 6	I.V.
Etoposide	10	Daily x 5 Q 21d	I.V.
Paclitaxel	15	Q4 days x 3 Q21 days	I.V.
Cisplatin	7	Q 21 days	I.V.
BCNU	35	Single administration	I.V.
Topotecan	0.6	Daily x 5 x 2 Q 21 days	I.V.
Irinotecan	1.25	Daily x 5 x 2 Q 21 days	I.V.
Temozolomide	200	Daily x 5 Q 21 days	P.O
Ixabepilone	10	Q4 days x 3 q21days	I.V.

Table 2 compares the phase II response rates to various drugs in Wilms tumor patients to the response rates in the xenograft models.

Table 2

Single-agent response rates in Wilms tumor xenografts and clinical trials

Drug	**Xenograft Response Rate***	**Clinical Response Rate***	**Reference**
BCNU	0/7 (0%)	0/1 (0%)	Green, 1985
Doxorubicin	2/8 (25%)	31/52 (60%)	Green, 1985
Dactinomycin	3/7 (43%)	17/44 (39%)	Green, 1985
Cisplatin	2/8 (25%)	3/19 (15.8%)	Green, 1985
Cyclophosphamide	7/10 (70%)	10/37 (27%)	Green, 1985
Etoposide	1/8 (12.5%)	15/38 (39.5%)	Pein, 1993
Ixabepilone	5/7 (71.4%)	0/10 (0%)	Widemann, 2008
Paclitaxel	0/7 (0%)	2/15 (13.3%)	Harris, 1999
Vincristine	7/10 (70%)	17/27 (63%)	Green, 1985

* Partial and complete responses are considered to be responses

Discussion

We developed Wilms tumor xenograft models with which to screen new drugs for anti-tumor activity. A potential limitation of xenografts is that they may undergo extensive genetic change in the process of establishment from primary tumors. Whiteford et al studied gene expression profiles of a large panel of pediatric xenografts with disparate histologic types and found that like histiotypes nearly always clustered together by hierarchical cluster analysis (16). Six of eight Wilms tumor xenografts clustered with other Wilms tumor xenografts, whereas two clustered with rhabdomyosarcoma xenografts. Neale et al subsequently conducted molecular characterization of the xenograft and cell line panel that is utilized in the Pediatric Preclinical Testing Program (PPTP), an NCI-sponsored program that screens new agents. The four Wilms tumor xenografts included in the PPTP panel, which were also included in the present report, were shown to cluster together when analyzed among 87 samples of various histiotypes (17). The Wilms tumor xenografts also clustered with unrelated primary Wilms tumors, though within the Wilms tumor group, the xenografts were segregated from the primary tumors. A similar observation was made with other histiotypes; immune-related genes and angiogenesis-related genes were found to be expressed at higher levels in primary tumors as compared to xenografts.

Our current data expand on the previous results by comparing genomic and gene expression profiles in xenografts matched with their parent tumors. Genomic copy number was strikingly similar in xenograft/ primary tumor pairs, with concordance rates of SNP calls >98% (Figure 1). There was less correlation of gene expression, though the number of differentially expressed genes between xenografts and primary tumors was not greater than that expected by chance. Hierarchical clustering analysis showed that Wilms xenografts clustered with other Wilms tumors as opposed to clear cell sarcomas of the kidney, though the xenografts did not uniformly cluster adjacent to their matched primary tumors. This discordance could be due to selective growth of certain Wilms tumor cell types (blastemal, epithelial, or stromal) in xenografts, a change in gene expression when tumors are grown in an ectopic milieu, the lack of an immune response in the SCID mouse models, or the inefficiency of the microarray to detect infiltrating stromal cells derived from the mouse host.

Because the xenografts were developed with the intent of screening novel therapeutic agents, we interrogated whether molecular pathways of biological and clinical interest are differentially expressed between xenografts and primary tumors. Gene expression levels of members of the IGF, EGFR, and VEGF pathways, pathways known to be altered in Wilms tumor, were not significantly different between primary tumors and xenografts.

Although the global similarity in gene copy number and expression between xenografts and parent tumors was encouraging, several caveats should be considered. First, relatively few xenograft/primary tumor pairs were available for the current study. The number of genetically characterized xenografts was too small to meaningfully correlate gene expression profile with responsiveness to specific agents. A future goal is to expand the number of Wilms tumor models to enable this type of analysis. Second, the xenografts tested were obtained at early passages after implantation into mice (passage 2 or 3). It is possible that as these xenografts are passaged further, their gene expression profiles will diverge from those of their parent tumors. Third, the gene copy number and expression studies did not account for potential changes in protein expression or post-translational modifications.

The correlation between xenograft and clinical chemo-responsiveness varies according to the tumor type and the agent tested. The National Cancer Institute's screening initiative of adult tumors showed that with the exception of non-small cell lung cancer (NSCLC), the xenografts did not correlate with phase II clinical data of the same tumor type (26). A retrospective literature-based study comparing published results of xenograft models with phase II studies showed that xenograft models were predictive for NSCLC and ovarian cancer, but not breast and colon cancer (27). Studies of pediatric xenografts have been predictive of clinical responses, notably for camptothecin analogues in neuroblastoma and rhabdomyosarcoma (19;28-30). The panel of nine Wilms tumor xenografts presented here identified vincristine, dactinomycin, and cyclophosphamide as active agents, in agreement with clinical efficacy data. Overall, tumors that were sensitive to therapy in patients (defined as lack of recurrence) showed greater responsiveness in the xenograft models. Among the agents tested in Wilms tumor xenograft panel were the topoisomerase I poisons topotecan and irinotecan. When administered on the protracted schedule of

administration (daily x 5 for two consecutive weeks) at dose levels that give clinically relevant systemic exposures to their active metabolites, both agents showed significant activity. In subsequent phase II testing, topotecan has demonstrated good activity against Wilms tumor at relapse, pointing to the value of the preclinical models (31).

Not all agents, however, showed concordant results between mice and humans. Doxorubicin and etoposide, agents with known activity in Wilms tumor, showed weak activity in the xenograft models. This may relate to the very different pharmacology of these agents in mouse and man. SCID mice are extremely sensitive to doxorubicin, where the maximum tolerated dose is ~40% that tolerated in athymic mice. Etoposide is rapidly cleared in mouse ($T_{1/2}$ ~12 min) compared to humans, where clearance is far slower ($T_{1/2}$ ~ 6 hr). Thus, in SCID mice the systemic exposure to these agents may be significantly lower than can be achieved in children. Conversely, another problem in accurately translating preclinical activity to clinical activity is that often the systemic exposure to drugs in mice cannot be achieved in patients due to host toxicity (32;33). Evaluating the activity of drugs in human tumor models should attempt to use dose levels that approximate clinically achievable systemic exposures. There also was discordance between the high level of ixabepilone activity observed in the xenograft models and the low level of activity seen in the recently completely phase II study. It is possible that different administration schedules (daily x 5 in the clinical study, every 4 days in the xenograft study) are responsible for this discordance.

In summary, gene copy number and expression levels were concordant between WT xenografts and parent primary tumors. Agents with known clinical efficacy in patients with WT were generally found to be active in the xenograft models, though the models were not predictive for all agents tested. Together, the results suggest that the WT xenografts provide a biologically and clinically relevant model for future preclinical testing.

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CHAPTER 6

Topotecan is active against Wilms Tumor: Results of a Multi-Institutional Phase II Study

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Purpose

A phase II study was conducted to evaluate the activity and safety of topotecan in pediatric patients with recurrent Wilms tumor.

Patients and Methods

Patients with favorable histology Wilms tumor (FHWT) and recurrence after at least one salvage chemotherapy regimen or anaplastic histology Wilms tumor (AHWT) in first or subsequent recurrence were eligible. Patients were stratified according to histology, with statistical considerations based on the FHWT stratum. Topotecan was administered intravenously over 30 minutes for 5 days on two consecutive weeks [(daily x 5) x 2]. Treatment dosages were adjusted to achieve a target area under the curve (AUC) of 80 ± 10 ng-hr/ml. Tumor responses were measured after 2 cycles of treatment.

Results

Thirty-seven patients (26 FHWT) were enrolled and received a total of 94 cycles of topotecan (range, 1 - 6 cycles). The median topotecan dosage required to achieve the target AUC was 1.8 mg/m^2 (range, 0.7 to 3.2 mg/m^2). Of 25 assessable patients with FHWT, 12 had partial response (PR), 6 had stable disease (SD), and 7 had progressive disease (PD), for an overall response rate of 48% (95% confidence interval, 27.8-68.7%). Of 11 assessable patients with AHWT, 2 had PR, 1 had SD, and 8 had PD. The main toxicities were grade 3 and 4 neutropenia (median duration 13 days) and thrombocytopenia (median duration 7.5 days).

Conclusion

Topotecan given on a protracted schedule is active against recurrent FHWT. Inclusion of topotecan in front-line clinical trials for patients with recurrent Wilms tumor should be considered.

INTRODUCTION

The treatment of Wilms tumor is one of the great success stories in oncology, but certain subgroups of patients do not fare well, including those with anaplastic histology, bilateral disease, and recurrent disease.[1-3] For patients with recurrent Wilms tumor, relapse-free survival (RFS) has improved significantly since the 1980s with the use of intensive chemotherapy or high-dose therapy with autologous stem cell rescue.[1,4-9] Despite the use of modern treatment regimens, 4-year RFS for patients treated initially with vincristine/dactinomycin is about 70% and 4-year RFS for patients treated initially with vincristine/dactinomycin/doxorubicin is about 40%.[7,10] Patients with recurrent anaplastic Wilms tumor have particularly poor salvage rates; fewer than 15% of such patients achieve durable survival.[2] Novel agents and treatment strategies are needed for patients with high-risk or recurrent Wilms tumor.

Topotecan is a camptothecin analogue that interacts with topoisomerase I and causes DNA double-strand breaks in an S-phase-dependent manner.[11] Topotecan has previously shown activity against various pediatric solid tumors including neuroblastoma, rhabdomyosarcoma, Ewing sarcoma, and medulloblastoma.[12-16] Xenograft studies have suggested that the activity of topotecan is schedule-dependent, producing a higher frequency of responses when given on a protracted schedule of administration rather than an intermittent high-dose regimen.[17] In Wilms tumor xenograft models, six of eight favorable histology models and one anaplastic histology model responded to topotecan at systemic exposures that are achievable in patients. [18]

Based on the pre-clinical data and promising results of phase I studies,[19] we conducted a phase II study to estimate the response rate of topotecan in patients with recurrent Wilms tumor.

MATERIAL AND METHODS

Patient selection

The study of topotecan in children with recurrent Wilms tumor (WILTOP) was a multi-institutional phase II trial including St. Jude Children's Research Hospital, Dana Farber

Cancer Institute, Alberta Children's Hospital, Texas Children's Hospital, Children's Hospital of Atlanta, and the Hospital for Sick Children in Toronto. Patients were eligible if they had recurrent or progressive favorable histology Wilms tumor (FHWT) after primary treatment and at least one standard salvage treatment regimen or if they had recurrent or progressive anaplastic histology Wilms tumor (AHWT) after primary treatment. Other eligibility requirements included age $\leq$ 21 years, absolute neutrophil count (ANC) $\geq$ 1,000 /mm^3 and platelet count $\geq$ 100,000 /mm^3 unsupported by transfusion, a serum bilirubin < 1.5 times the upper limit of normal for age and an ECOG performance status[20] of 0 to 2.

The protocol was approved by the Institutional Review Boards of all participating institutions and all patients, parents or guardians, as appropriate, were required to provide written informed consent in accordance with institutional and federal guidance.

Treatment Regimen

Topotecan was administered intravenously over 30 minutes daily for 5 days for each of 2 consecutive weeks [(daily x 5) x 2]. The initial dosage (2.4 mg/m^2/day, later modified to 1.8 mg/m^2/day) was adjusted to attain a target topotecan lactone systemic exposure (AUC) of 70 to 90 ng-hr/ml. Although a phase I study recommended a topotecan lactone AUC of 100 ng-hr/ml as the systemic exposure to target in phase II studies,[19] the current study used a target AUC of 70 to 90 ng-hr/ml based on early clinical experience showing significant toxicity in patients with recurrent Wilms tumor at the higher systemic exposure (Dome, unpublished data). Subsequent cycles of topotecan were given approximately 28 days after the beginning of the previous cycle once patients had achieved an ANC > 1,000 /mm^3 and platelet count > 50,000 /mm^3. Patients received filgrastim at 5 mcg/kg/day subcutaneously 24 hours after the last dose of topotecan until the ANC exceeded 5,000 /mm^3 after the expected nadir. Trimethoprim-sulfamethoxazole for *Pneumocystis carinii* prophylaxis was withheld during the 2 weeks of topotecan administration.[21] Aerosolized pentamidine was used as an alternative prophylactic regimen.

Pharmacokinetically Guided Topotecan Dosing

Pharmacokinetically guided topotecan dosing was performed as previously described.[15,19] During the first and second cycle, plasma samples (2.5ml) were obtained prior to infusion,

at 5 minutes, 2 hours and 3 hours after the end of topotecan infusion and processed immediately.[15,19] If the single day topotecan lactone AUC was within target range after the first dose, then no dose adjustment and no further pharmacokinetic sampling was necessary for that cycle. If not, then the topotecan dosage was adjusted linearly based on the patient's topotecan lactone clearance to attain the target AUC and repeat pharmacokinetic studies were performed until the patient's topotecan systemic exposure was within the target range. Up to three dose adjustments were permitted per cycle. Patients who required dose adjustments on cycle 2 also had pharmacokinetic studies performed in cycle 3. No pharmacokinetic studies were performed beyond the third cycle.

A two-compartment model was fit to the topotecan lactone plasma concentration using a maximum a posteriori Bayesian algorithm as implemented in ADAPT II[22] with published values (mean and variance) used as the Bayesian priors.[19] Model parameters estimated for each patient included the volume of the central compartment, elimination rate constant, and the intercompartment rate constants. These parameters were used to simulate the plasma concentration-time profile for each patient, from which the AUC from time zero to infinity ($AUC_{0\to\infty}$) was calculated. As in our previous studies, we used the following equation to adjust topotecan dosage: adjusted dosage (mg/m^2) = current topotecan dosage (mg/m^2)/current AUC X target AUC.[15,19]

Evaluations during Study

Baseline evaluations included a complete medical history and physical examination; computed tomography (CT) of the chest, abdomen, and pelvis; complete blood count (CBC) with differential; complete metabolic panel including electrolytes, liver and kidney function studies; urinalysis; and glomerular filtration rate (GFR) determined either by a Tc99m-DTPA renal/plasma clearance study or by a 24-hour urine collection for creatinine measurement. At the completion of 2 cycles of topotecan therapy, patients underwent diagnostic imaging of the primary and metastatic sites. Toxicity was assessed according to the National Cancer Institute Common Toxicity Criteria, version 2.0.

Response Criteria

Response to treatment was defined according to the Response Evaluation Criteria in Solid

Tumors (RECIST).[23] Diagnostic, end of the first cycle (when available), second cycle and off therapy images were centrally reviewed by the study radiologist (FAH) at St Jude. A measurable lesion was defined as a lesion whose longest diameter was greater than or equal to twice the CT scan slice diameter. The longest diameter in the axial plane was recorded. All measurable lesions up to a maximum of five lesions per organ and 10 lesions in total were defined as target lesions, measured, and recorded at baseline. At baseline, a sum of the longest diameter for all target lesions was calculated and reported. All other lesions were identified as non-target lesions and were recorded at baseline without measurement. In the evaluation of target lesions, complete response (CR) was defined as the complete regression of all apparent tumor, a more than 30% decrease in the sum of the longest diameter of target lesions constituted a partial response (PR), a greater than 20% increase in the sum of the longest diameter represented progressive disease (PD), and stable disease (SD) was anything that did not qualify for either a PR or PD. In the evaluation of non-target lesions, the disappearance of all non-target lesions represented a CR; incomplete response or SD was considered, when one or more non-target lesions persisted; and the appearance of any new lesion and/or unequivocal progression of existing non-target lesions represented PD.

Statistical Considerations

This trial was designed to estimate the response rate after 2 cycles of topotecan in patients with FHWT. Based on a four-stage group sequential design[24] with a type I error rate of 10% and 90% power, 25 patients were needed to test whether the true response rate was less than 10%; a response rate of 30% was considered promising. The estimated response rate was presented with an exact binomial 95% confidence interval (CI). The rarity of AHWT precluded a formal statistical design for this group of patients.

Survival was defined as the time interval from date of study enrollment to date of death from any cause or to the last follow-up date. Event-free survival (EFS) was defined as the time interval from date of study enrollment to date of first event (relapsed or progressive disease, or death from any cause) or to the last follow-up date. Survival and EFS were estimated using the method of Kaplan and Meier. Fisher's exact test, the exact Wilcoxon rank sum test, and the exact Kruskal-Wallis test were used to compare characteristics between responders and non-responders. Responders were defined as those patients who

achieved at least a PR after 2 cycles of topotecan; non-responders were those who had either SD or PD after one or 2 cycles of topotecan.

RESULTS

Patient Characteristics

Between March of 2003 and March of 2006, 37 eligible patients were enrolled; 30 of the patients were enrolled at St. Jude and the other centers enrolled one or two patients each. Twenty-six (70%) patients had FHWT and 11 (30%) had diffuse AHWT. Patient and treatment characteristics for all patients and for patients by histology are shown in Table 1. Sixty percent of patients (n=22) were female and most were white (n=30; 81%). The median age at diagnosis of Wilms tumor was 4.8 years and the median age at enrollment on WILTOP was 6.1 years.

Table 1

Patient and Treatment Characteristics

Characteristic	**All Patients** (n=37) (%)	**Favorable Histology** (n=26) (%)	**Anaplastic Histology** (n=11) (%)
Gender			
Male	15 (40.5)	10 (38.5)	5 (45.5)
Female	22 (59.5)	16 (61.5)	6 (54.5)
Race			
White	30 (81.1)	20 (76.9)	10 (90.9)
Black	3 (8.1)	3 (11.5)	0 (0.0)
Other	4 (10.8)	3 (11.5)	1 (9.1)
Age at initial diagnosis (yrs)			
Median	4.8	4.3	4.9
Range	0.4 – 14.7	0.4 – 14.7	3.9 – 7.2
Age at study enrollment (yrs)			
Median	6.1	6.6	5.8
Range	1.3 – 19.0	1.3 – 19.0	4.7 – 7.9

Sites of involvement at study enrollment[a]			
Local	6 (16.2)	3 (11.5)	3 (27.3)
Distant	19 (51.4)	14 (53.9)	5 (45.4)
Local + Distant	12 (32.4)	9 (34.6)	3 (27.3)
Stage at initial diagnosis			
I	4 (10.8)	2 (7.7)	2 (18.2)
II	7 (18.9)	4 (15.4)	3 (27.3)
III	6 (16.2)	3 (11.5)	3 (27.3)
IV	12 (32.4)	12 (46.2)	0 (0.0)
V	8 (21.6)	5 (19.2)	3 (27.3)
Prior exposure to topotecan			
Yes	1 (2.7)	1 (3.9)	0 (0)
No	36 (97.3)	25 (96.1)	11 (100)
Previous A-SCT			
Yes	4 (10.8)	4 (15.4)	0 (0)
No	33 (89.2)	22 (84.6)	11 (0)
No. of prior recurrences			
PD	11 (29.7)	7 (26.9)	4 (36.4)
1	18 (48.7)	11 (42.3)	7 (63.6)
2	8 (21.6)	8 (30.8)	0 (0)

Local = original tumor bed site; Distant = outside the original tumor site.
A-SCT = autologous stem cell transplant; PD=progressive disease

Study Withdrawals, Eligibility, and Assessability

Seven patients discontinued treatment before the end of the second topotecan cycle due to progressive disease (three before completing the first cycle, three at the end of the first cycle, and one during the second cycle). One patient was removed from the study during the second cycle after suffering a stroke from a hemorrhage within a frontal lobe metastasis. This patient was not assessable for response because the CNS lesion could not be accurately measured after the hemorrhage and she did not complete two full topotecan cycles. In total, 22 patients with FHWT and 7 patients with AHWT completed at least 2 cycles of topotecan (Table 2).

Table 2

Patient Disposition

Patient Disposition	No. of patients	
	Favorable Histology	**Anaplastic Histology**
Patients enrolled	**26**	**11**
Patients withdrawing before the end of first cycle for PD	2	1
Patients treated with ≥ 1 cycle	**24**	**10**
Patients withdrawing after first cycle for PD	1	2
Patient withdrawing at the end of the first cycle for PD	0	1
Drug-related adverse event before end of second cycle	1	0
Patients treated with ≥ 2 cycles	**22**	**7**
Patients treated with ≥ 4 cycles	9	1

PD=progressive disease

Topotecan Pharmacokinetics

The inter- and intrapatient variability in topotecan lactone clearance was assessed using the mixed-effect model, which allowed us to account for possible correlations between topotecan clearance and cycle with repeated measurements within each subject. The population average topotecan lactone systemic clearance was 20.7 L/hr/m^2 with a range of 7.8 to 43.9 L/hr/m^2. The estimated intersubject and intrasubject variances were 30.3% and 15.7%, respectively. This finding is consistent with several of our other studies in which intersubject variability in topotecan clearance exceeded intrasubject variability.[13,25] In the 37 children enrolled on this study, we performed a total of 127 pharmacokinetic studies. The first pharmacokinetic study in each patient (n=37) was performed after a fixed topotecan dosage (n=9 at 2.4 mg/m^2 or n=28 at 1.8 mg/m^2). All patients studied at the 2.4 mg/m^2 initial dosage were above the topotecan target (range 97 to 250 ng-hr/ml), whereas when the initial dosage was reduced to 1.8 mg/m^2, fifteen patients (54%) were within the target range on first dose. In subsequent studies using pharmacokinetically-guided dosing, the overall pharmacokinetic targeting success rate was 70.2% (AUCs in 59 of 84 evaluable studies were in the target range), though the target AUC was ultimately achieved in all cycles. The median topotecan dosage in the cycles in which the target AUC range was achieved was 1.8 mg/m^2 (range, 0.7 to 3.2 mg/m^2).

Since this patient population was likely to have altered renal function and potentially decreased topotecan clearance (and elevated topotecan AUC values), one concern was that these patients would be "over-dosed." However, only 30 (24%) pharmacokinetic studies showed AUCs that were above the target range (i.e., > 90 ng-hr/ml) and only 19 (15%) showed AUCs that were more than 10% above the upper end of the target range. All of the AUCs in these patients were brought within the target with further pharmacokinetic studies. Conversely, only eight (6%) pharmacokinetic studies were more than 10% below the lower end of the target range (i.e., < 60 ng-hr/ml). Of these eight studies, three were with the initial fixed topotecan dosage, and the remaining five occurred after course 1 dose 2 (n=1), course 2 dose 1 (n=2), course 2 dose 3 (n=1), and course 3 dose 1 (n=1). In all eight cases, the topotecan target value was attained on subsequent pharmacokinetic studies.

Topotecan Response

Thirty-six of 37 patients were assessable for response (Table 3).

Table 3

Tumor responses in favorable and anaplastic histology Wilms tumor

Response	**Favorable Histology** (n=26) Number observed	**Anaplastic Histology** (n=11) Number observed
Complete Response	0	0
Partial Response	12	2
Stable Disease	6	1
Progressive Disease	7	8
Not Assessable[a]	1	0
Total Response[b]	**12/25 (48%)**	**2/11 (18%)**

[a] Patient removed from study during the second cycle and before response assessment after suffering a stroke from a hemorrhage within a frontal lobe metastasis.
[b] Total Response = complete and partial response

The observed response rate for patients with favorable histology tumors (25 patients) was 48.0% (95% confidence interval, 27.8-68.7%); 12 patients had partial response (PR), 6 patients had stable disease (SD), and 7 patients had progressive disease (PD). Among patients with AHWT, 2 patients had PR, one patient had SD, and 8 patients had PD. The

median duration of response was 158 days (range, 18 days to 899 days). It was not feasible to measure the duration of response specifically to topotecan because most responders received additional treatment after discontinuing protocol therapy, including surgery, radiation therapy, or high-dose chemotherapy with autologous stem cell rescue. Twelve of 37 patients (32%) were alive with a median follow-up of 11.7 months (range, 1.9 to 37.7 months). Six of the survivors had no evidence of disease at last follow up and six were alive with disease. All survivors had been seen or contacted within 10 months of the analysis. Estimates of survival and EFS for all patients at one year were 29.5% ± 8.3% and 16.4% ± 6.1% respectively.

Table 4 shows patient characteristics among responders and non-responders for the 36 assessable patients. The only significant difference between responders and non-responders was a longer time from initial diagnosis to topotecan study therapy (median 30.5 months versus 11.9 months) and a longer time from last treatment to study therapy (median 3.2 months versus 1.3 months) for responders. We were not able to detect a relationship between topotecan systemic exposure and antitumor response (data not shown), given that we maintained a very narrow range of systemic exposure values (AUC).

Topotecan Toxicity

Table 5 summarizes the most common grade 3 and 4 toxicities encountered in a total of 94 cycles of topotecan administered. The main toxicity was hematologic; all 37 patients had grade 3 or 4 toxicities. The median duration of grade 3 or 4 neutropenia was 13 days per episode (range, 2 – 31 days) and the median duration of grade 3 or 4 thrombocytopenia was 7.5 days (range, 1 – 40 days). There were 12 episodes of grade 3 bleeding/hemorrhage associated with thrombocytopenia, mostly skin bruises, nosebleeds, and mucosal bleeds. As described above, one patient had hemorrhage into a brain metastasis. There were 61 admissions for febrile neutropenia reported in 27 patients. Thirteen patients (35%) had a total of 18 episodes of documented infection (6 catheter-related, two infections without neutropenia, and 10 episodes related to neutropenia).

Table 4

Characteristics of Patients Assessable for Response to Topotecan

	Responders[a] (n= 14)		Non-responders (n= 22)		*P*
	No.	%	No.	%	
Gender					1.0*
Male	6	43	9	41	
Female	8	57	13	59	
Race					0.39*
White	10	71	19	86	
Non-white	4	29	3	14	
Age at initial diagnosis (years)		--		--	0.74^
Median	4.8		4.4		
Range	0.4 – 13.0		0.9 – 14.7		
Histology					0.142*
Favorable	12	86	13	59	
Anaplastic	2	14	9	41	
Stage at initial diagnosis					1.0[b#]
I/II	5	36	6	27	0.44[c*]
III/IV	5	36	12	54	
V	4	29	4	18	
Months from initial diagnosis to study treatment					**0.001**^
Median	30.5	--	11.9	--	
Range	7.4 – 193.1		5.1 – 34.0		
Months from last treatment to study treatment					**0.030^**
Median	3.2	--	1.3	--	
Range	0.8 – 19.4		0.4 – 13.7		
Ever CR prior to WILTOP					0.142*
Yes	12	86	13	59	
No	2	14	9	41	
Sites of disease at study entry					1.0[d*]
Local	2	14	4	18	0.096[e#]
Distant	5	36	14	64	
Local + Distant	7	50	4	18	
Survival					
No. alive	7	50	5	23	-

[a] Responders=partial response; non-responders= stable or progressive disease
[b] Comparison of stages I/II *vs*. stages III/IV *vs*. stage V
[c] Comparison of stages I/II *vs*. stages III/IV
[d] Comparison of local only *vs*. distant/local + distant
[e] Comparison of local *vs*. distant *vs*. local + distant as an ordered categorical variable
* P-value derived from Fisher's exact test; ^ P-value derived from the exact Wilcoxon rank sum test; # P-value derived from the exact Kruskal-Wallis test

Renal toxicity consisted mainly of electrolyte imbalance partly attributable to the patients' underlying renal disease and previous therapy. One patient had a creatinine of

3.5 mg/dL at study entry and had progressive disease that compromised the function of her sole remaining kidney, leading to grade 3 creatinine elevation. There were no toxic deaths.

Table 5

Grade 3 and 4 toxicities observed during a total of 94 administered cycles

	No. of patients (%)	**No. of episodes**
Hematologic	37 (100%)	318
Anemia	36	98
Thrombocytopenia	37	101
Neutropenia	36	100
Renal	9 (24%)	21
Electrolytes	9	19
Creatinine	1	2
GI	15 (41%)	36
Diarrhea	4	4
Nausea/Vomiting	12	14
Abdominal pain	5	7
Other*	6	11
Anorexia	9 (24%)	9
Infection	13 (35%)	18

*Other toxicities included colitis (3 episodes), typhlitis (2 episodes), ileus (1 episode), mucositis/stomatitis (3 episodes), elevated gamma glutamic transferase (GGT) (1 episode), and not otherwise specified (1 episode).

DISCUSSION

This study demonstrates that topotecan has significant activity in children with FHWT when administered on a protracted schedule. The 48% response rate is especially promising given that the responses were observed in a population of heavily pre-treated patients whose disease progressed after at least one salvage chemotherapy regimen. The response rate is comparable to response rates seen with other single agents that are commonly used for the treatment of Wilms tumor including ifosfamide (20 to 50%),[26-28] etoposide (42%),[29] carboplatin (52%),[30] and doxorubicin (54%).[31] Among patients with AHWT, two responses were seen among 11 patients. Although the study was not statistically powered to assess response rate in patients with AHWT, the results suggest that topotecan has modest activity in this high-risk subgroup.

The results of the present trial differ from previous topotecan trials, which showed no responses in five patients with recurrent Wilms tumor.[14,16,32] In contrast to the protracted schedule [(daily x 5) x 2] that we describe, topotecan was administered on a daily x 5 schedule (2 mg/m^2/day) or as a 72-hour continuous infusion (1.3-1.9 mg/m^2/day) in the earlier trials. It is possible that the higher cumulative topotecan dosage in the current trial improved the response rate. It is also possible that the protracted topotecan schedule was more active than the shorter schedules used in the previous studies. The selective cytotoxic action of the topoisomerase I poisons during S-phase suggests that prolonged exposure to these drugs would maximize the number of cells susceptible to drug-induced death. [11,33]

Our study featured pharmacokinetically-guided dosing of topotecan. The Wilms tumor patient population was ideal for individualized topotecan therapy because the patients had only one kidney, and topotecan primarily undergoes renal elimination. The intersubject variance in topotecan lactone clearance was 30.2% and a range of dosages (0.7-3.2 mg/m^2, median 1.8mg/m2) was required to achieve the desired AUC. Despite this variability, only 15% of pharmacokinetic studies showed topotecan AUC values more than 10% above the upper end of the target range and only 6% of studies showed AUC values more than 10% below the lower end of the target range. It would be helpful to have a reliable predictor of topotecan clearance (e.g., serum creatinine or GFR), but no predictive relationship could be established (data not shown).

To guide future use of topotecan in patients with recurrent Wilms tumor, we assessed predictors of topotecan response. The only significant differences between responders and non-responders were the time from initial diagnosis to study therapy and the time from most recent treatment to study therapy. There are several potential mechanisms of resistance to topotecan, which can be inherent to the tumor or the host. Mutations in topoisomerase I,[34] decreased levels of cellular topoisomerase,[35-37] and decreased cellular camptothecin accumulation [38] have all been described, however studies of in vivo mechanisms of resistance were not performed and warrant further investigation in prospective trials.

In conclusion, topotecan is active against recurrent FHWT. Introduction of topotecan using this protracted schedule to front-line trials of high risk recurrent Wilms tumor should be considered.

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CHAPTER 7

Summary, Conclusions, Perspectives, and Future Directions

The oft-quoted 90% survival rate for Wilms tumor imparts a false sense of comfort to pediatric oncologists and affected patients. The 90% figure is largely influenced by the extraordinary outcomes for patients with stage I and II favorable histology (FH) Wilms tumor, who enjoy predicted survival rates around 98%. However, it is underappreciated that nearly one-third of Wilms tumor patients have event-free survival estimates <70% and overall survival estimates <80%. This high-risk group includes patients with anaplastic histology (AH), bilateral, and recurrent FH Wilms tumor.

This thesis defense focuses on the patients with high-risk Wilms tumor. Chapters 1 and 2 describe a potential prognostic factor (telomerase) to define a group of patients with high-risk FH Wilms tumor. Chapters 3 and 4 report on outcomes of two high-risk Wilms tumor populations: those with relapsed disease and those with AH. Chapters 5 and 6 describe efforts to identify novel agents for high-risk Wilms tumor.

Chapters 1 and 2: Telomerase as a prognostic marker for Wilms tumor

The two most powerful and widely used prognostic factors for Wilms tumor are stage and histology. Tumor stage has long been known to predict outcome and has been used to assign risk-based therapy since the earliest cooperative group trials. Histology was recognized as an important factor in the 1970s after a retrospective analysis of NWTS-1 revealed that anaplasia was strongly predictive of relapse and patient death (1). More recently, blastemal predominance after pre-operative chemotherapy has been identified as a powerful prognostic factor (2). Despite the strength of stage and histology as prognostic factors, many patients experience recurrence without readily apparent unfavorable features. This has led investigators to seek molecular and genetic predictors of recurrence. Loss of heterozygosity (LOH) at 16q, and to a lesser extent 1p, was noted to predict recurrence in several studies in the 1990s (3;4). Based on these results, the fifth

National Wilms Tumor Study (NWTS-5) sought to prospectively analyze LOH at 1p and 16q as a prognostic marker (5). Analysis of more than 1700 study participants with FH Wilms tumor confirmed the prognostic significance of LOH, with the strongest effect seen in tumors with combined LOH at both loci. A shortcoming of LOH is that only 4-5% of tumors have LOH at both loci and LOH predicts only 8% of Wilms tumor recurrences. Moreover, the mechanism for how LOH at 1p/16q confers adverse outcome is unknown. It is possible that LOH is merely a bystander effect for a more important change, such as gain of chromosome 1q, another promising adverse prognostic indicator for Wilms tumor (6;7). The United Kingdom Children's Cancer and Leukemia Group (CCLG) recently found that LOH of 16q, but not 1p, is predictive of survival (8). It is possible that the patient numbers were insufficient to detect an effect or the treatment context was different.

Although the identification of LOH represents an advance in the field, it would be desirable to identify additional prognostic factors for Wilms tumor. Toward this end, we evaluated telomerase expression level as a prognostic marker. Telomerase is a specialized reverse transcriptase that adds nucleotide repeats to telomeres, counteracting the progressive loss of DNA that occurs during replication. The enzyme, which plays a key role in cellular immortalization, is minimally composed of a catalytic subunit (TERT), and an RNA subunit (TERC/hTR), which provides the template for nucleotide repeat generation. Because telomerase is expressed in approximately 85-95% of cancer specimens, but is absent in most normal tissue (9;10), it has been proposed as a tumor marker and therapeutic target. The presence of telomerase expression has emerged as a predictor of adverse outcome in a variety of adult and pediatric malignancies, including neuroblastoma (11).

Chapter 1 describes a pilot study of 78 favorable histology Wilms tumor samples for which telomerase activity level, TERC level, and *TERT* mRNA level were evaluated (12). The study found that nearly all Wilms tumors expressed telomerase activity, which differs from the situation in neuroblastoma in which a dichotomy of telomerase-positive and telomerase-negative tumors was seen. The Wilms tumor samples exhibited a wide range of telomerase expression levels. Univariate analysis of *TERT* mRNA level as a continuous variable suggested that each unit increase in *TERT* mRNA level increased the risk of

relapse by a factor of 1.66 (95% confidence interval (CI) 1.2-2.3, p<0.005). This study did not detect an association between patient outcome and levels of telomerase enzyme activity or TERC, but was limited by its relatively small sample size. Moreover, the study pre-dated the era of real-time quantitative polymerase chain reaction (PCR), so the results were only semi-quantitative.

The promising results of Chapter 1 led us to design the follow-up study described in Chapter 2. This study used a case-cohort design involving nearly 300 samples to compare telomerase expression in primary tumors with and without eventual recurrence (13). As with the original study, three measures of telomerase expression were assessed: telomerase enzyme activity, TERC expression level, and TERT mRNA expression level. In this study, quantitative real-time PCR using Taqman methodology was utilized. The study demonstrated a positive correlation between risk of recurrence and tumor expression level of telomerase RNA (TERC) and, to a lesser extent, *TERT* mRNA. The third of patients with the highest TERC expression level had twice the risk of relapse compared to those with the lowest TERC expression level. TERC expression level remained a significant predictor of relapse after adjustment for the known prognostic factors of patient age and tumor stage. As seen in the first study, telomerase enzyme activity level was not predictive of recurrence.

These results suggested that measurement of TERC expression may be a useful prognostic factor, though they raise several questions. The original pilot study did not find TERC expression level to be predictive of outcome. This difference likely related to the larger sample size and improved assay techniques in the second study. It was also somewhat surprising that TERC emerged as the superior prognosticator compared to TERT because TERC is constitutively expressed in both normal and malignant cells, whereas TERT is more restricted to cancer cells. However, several lines of evidence indicate that level of TERC expression is biologically relevant, most notably in patients with the autosomal dominant form of dyskeratosis congenital in whom TERC haploinsufficiency results in impaired telomere length maintenance and clinical phenotype (14;15).

In 2008, Wittmann and colleagues conducted quantitative PCR analysis on 102 tumor samples to evaluate 40 prognostic markers that were previously identified for Wilms tumor (16). Levels of only two markers, TERT and HEY2, were predictive of relapse and death

after correction of p-values for multiple testing error. TERT expression level remained predictive of outcome in a multivariate analysis, though was not prognostically significant in a smaller confirmatory study of 74 independent samples. The authors concluded that further efforts should be undertaken to evaluate TERT as a potential stratifying marker.

The relevant clinical question is whether the predictive value of telomerase expression is sufficiently strong to provoke a change in clinical practice. The third of patients with the highest level of TERC expression had a relatively risk of recurrence of 2.06, which translates to a 4-year relapse-free survival of 79% in the high-TERC group compared to 90% in the low-TERC group. By comparison, among patients with stage I/II FH Wilms tumor, patients with LOH at both 1p and 16q had a 4-year RFS of 75% compared to 91% in patients without LOH. Among patients with stage III/IV FH Wilms tumor, patients with LOH at both 1p and 16q had a 4-year RFS of 66% compared to 83% in patients without LOH. Hence, the magnitude of the LOH effect is greater than that of the telomerase effect. LOH was also predictive of overall survival, whereas telomerase expression was not, though there were very few deaths in the telomerase case-cohort study. Interestingly, an unpublished multivariate analysis indicated that both TERC expression level and LOH were predictive of relapse.

In conclusion, telomerase expression seems to be a real prognostic marker for Wilms tumor that lies at the threshold of clinical utility. Given the absence of other clear prognostic factors, telomerase expression deserves further study, especially for AH Wilms tumor because this group has not been studied.

Chapter 3: Improved survival for relapsed Wilms tumor

Approximately 10% of patients with favorable histology Wilms tumor and 40% of patients with AH Wilms tumor develop recurrent disease. Historical estimates indicated that only 24% to 43% of patients survive after relapse (17-20). Before the mid-1980s, recurrent Wilms tumor was treated with combinations of vincristine, dactinomycin, doxorubicin, radiation therapy, or surgery. In many cases, the identical chemotherapy agents were used for the treatment of both primary and recurrent disease. In recent years, cyclophosphamide, ifosfamide, cisplatin, carboplatin, and etoposide have been used to

treat recurrent Wilms tumor, but their impact on long-term survival remained poorly defined (21).

To better understand the effect that contemporary agents have on the outcomes of patients with relapsed Wilms tumor, we conducted a retrospective study of 54 patients with relapsed Wilms tumor treated over years at St. Jude Children's Research Hospital (22). Patients treated for recurrence after 1984 had significantly higher survival estimates than patients treated in the previous era (63.6% versus 20.6%). The improvement was associated with the use of additional chemotherapy agents. The outcomes of patients with high-risk features treated in the modern era in the St. Jude study (47.6% overall survival) was similar to outcomes reported in the recently published series from the NWTSG (48% overall survival) (23).

These figures are slightly inferior to results reported for high-dose therapy with stem cell rescue (48%-63% overall survival) (24-28). However, the stem cell transplant series have an inherent selection bias such that only patients with adequate disease control were able to undergo transplant. The only way to decipher whether stem cell transplant offers a survival benefit will be to perform a randomized study comparing conventional chemotherapy to stem cell transplant, which does not appear to be feasible within a reasonable time frame. We will probably never fully comprehend the benefit of stem cell transplant for recurrent Wilms tumor.

Although the St. Jude study indicated that we have made substantial progress in the treatment of recurrent Wilms tumor, an overall survival estimate of 64% is still unsatisfactory. It is possible that additional cytotoxic drugs such as topotecan (see Chapter 6) will improve survival to some degree, but it is likely that new cytotoxic agents will not be the cure-all. A combination of chemotherapy and molecularly targeted therapies will likely be necessary to push the post-relapse survival rate beyond 90%. The challenge in the next decade will be to identify the best targets and therapies.

Chapter 4: Treatment of anaplastic Wilms tumor

Anaplasia has been recognized as an adverse prognostic factor for Wilms tumor since 1978, but we continue to learn about the biology and treatment of this entity. Beckwith

proposed that anaplasia is a marker of resistance to therapy, but not tumor aggressiveness (29). This model was based largely on the observation that patients with stage I AH Wilms tumor had good outcomes. The analysis of AH Wilms tumor described in Chapter 4, the largest cohort of AH Wilms tumor studied to date, provides several important insights into the treatment and biology of this Wilms tumor subtype. Key lessons learned from the study are as follows (30).

Insight 1. Anaplasia is likely a marker of both resistance to therapy AND tumor aggressiveness.

One of the major observations from NWTS-5 is that patients with stage I anaplastic Wilms tumor did not fare as well as previously believed. The 4-year EFS and OS estimates for 29 patients with stage I focal or diffuse anaplasia were 69.5% and 82.6%, respectively. By contrast, 4-year EFS and OS estimates for 473 evaluable patients with stage I FH Wilms tumor treated with the same regimen (EE-4A, vincristine/dactinomycin) were 92.4% (95% CI, 89.5 to 94.5) and 98.3% (95% CI, 96.4 to 99.2), respectively. Comparison of EFS and OS curves between patients with Stage I FH and Stage I AH demonstrated a highly significant difference ($p<0.001$). The poor outcomes for stage I anaplastic Wilms tumor suggest that these patients had micrometastic disease that was unsatisfactorily controlled by vincristine and dactinomycin. It is possible that the stage I FH and AH tumors had equal prevalence of micrometastatic disease, but that the FH micrometastatic disease was sensitive to chemotherapy whereas the AH micrometastatic disease was not, consistent with Beckwith's theory. However, examination of the stage distribution of the favorable histology tumors as compared to the AH tumors revealed that 65.4% of patients with AH presented with high-stage (III+IV) disease, whereas only 45.6% of patients with FH presented with high-stage disease (OR 2.26, $p<0.001$). If the histologic types were equally aggressive, one would expect a similar stage distribution.

Insight 2. Anaplasia is a marker of relative, but not complete, resistance to chemotherapy.

The table below summarizes the outcomes of patients with stage II-IV AH Wilms tumor on successive treatment regimens from NWTS 3-5. The randomized study demonstrating the benefit of cyclophosphamide in NWTS- 3 and -4 is the most cogent demonstration that

additional chemotherapy can be of benefit for this disease. The more modest improvement between Regimen J and Regimen I suggests a benefit of adding etoposide, but this was not a randomized comparison. Other evidence that chemotherapy benefits patients with AH Wilms tumor is that patients with stage II disease (treated with vincristine/doxorubicin/cyclophosphamide/etoposide/radiation) fared better than patients with stage I disease (treated with vincristine/dactinomycin). The fact that AH Wilms tumor is only relatively chemo-resistant is an important consideration for planning future studies. We may not have optimized the use of conventional cytotoxic agents for AH Wilms tumor, though we seem to be reaching a point of diminishing returns with cytoxic therapy and will likely need to turn to molecularly targeted therapy in the next generation of clinical trials.

Table 1.

4-year RFS % for Patients with Diffuse Anaplastic Wilms Tumor on NWTS 3-5

Stage	Regimen DD-RT (# of pts.)	Regimen J (# of pts.)	Regimen I (# of pts.)	RegimenEE-4A (# of pts.)
	VDA/XRT	**VDAC/XRT**	**VDCE/XRT**	**VA**
I	80% (10)	100% (5)	---	68.4% (19)
II	40% (12)	72% (11)	82.6% (23)	---
III	33% (9)	59% (13)	64.7% (43)	---
IV	0% (8)	17% (6)	33.3% (15)	---

V-vincristine, D-doxorubicin, A-dactinomycin, C-cyclophosphamide, E-etoposide, XRT-radiation therapy

Insight 3. Central pathology review is important for the identification of anaplasia.

In an effort to save costs and improve efficiency, the leaders of the COG have questioned the need for central pathology review for Wilms tumor. The data from NWTS-5 indicate that central pathology review remains important; anaplasia was not originally recognized by institutional pathologists in 74 of 190 (38.9%) patients who underwent immediate nephrectomy. An additional nine patients were considered to have focal AH by institutional pathologists, but diffuse AH by central reviewers. Among 158 patients with unilateral AH Wilms tumor for whom a local tumor stage was assigned (regardless of

distant metastases), discordance between institutional stage and central pathology stage was noted in 30 patients (19%). Overall, approximately 50% of patients with AH Wilms tumor had an adjustment in therapy based on central pathology review. Preliminary review of patients enrolled on the current COG AREN03B2 Biology and Classification study indicates a similar discordance. Although clear descriptions of the criteria for anaplasia have been included in the protocol documents, institutional pathologists are likely to see just one case every few years, thereby necessitating the need for central review.

Chapters 5 and 6: Developmental therapeutics for Wilms tumor

As described in Chapters 3 and 4, substantial progress has been made in the treatment of recurrent Wilms tumor and AH Wilms tumor. However, survival rates for these groups remain less than 70%. New treatment approaches are needed.

Table 2.

Results of COG phase II studies for relapsed disease published since 2000

Agent	Tumor Types	Response Rate	Reference
Gemcitabine/vinorelbine	**Hodgkin**	**19/25 (76%)**	**Cole, 2009**
TNF/dactinomycin	Wilms	3/19 (15.8%)	Meany, 2008
Irinotecan	Solid tumors	8/161 (5%)	Bomgaars, 2007
Temozolomide	Gliomas	6/104 (5.8%)	Nicholson, 2007
Docetaxel	Leukemia	0/12 (0%)	Franklin, 2008
Rebeccamycin	Solid tumors	4/126 (3.2%)	Langevin, 2008
Imatinib	Solid tumors	1/59 (1.7%)	Bond, 2008
ATRA/IFN-alfa	Wilms/neuroblastoma	0/30 (0%)	Adamson, 2007
Oxaliplain	Brain tumors	2/43 (4.7%)	Fouladi, 2006
Cytarabine/cisplat/etoposide	**Hodgkin**	**31 (68%)**	**Wimmer, 2006**
Docetaxel	Solid tumors	8/173 (4.6%)	Zwerdline, 2006
Topotecan (21 day)	Sarcomas	2/42 (4.8%)	Hawkins, 2006
Lobradimil/carboplatin	Brain tumors	0/38 (0%)	Warren, 2006
Gemcitabine	Leukemia	1/30 (3.3%)	Angiolillo, 2006
Ifos/carboplat/etoposide	**Sarcomas**	**91 (51%)**	**VanWinkle, 2005**
Nelarabine	**T-Leukemia**	**106 (>50%)**	**Berg, 2005**
Idarubicin	Brain tumors	91 (<10%)	Dreyer, 2003
Homoharringtonine	AML	5/28 (18%)	Bell, 2001
Cyclophos/topotecan	**Solid tumors**	**25/83 (30.1%)**	**Saylors, 2001**
Pyrazolozcridine	Solid tumors	0/47 (0%)	Berg, 2000
Oral methotrexate	Brain tumors	4/78 (5.1%)	Mulne, 2000

TNF-tumor necrosis factor, ATRA-all trans retinoic acid, IFN-interferon

Combinations with response rates >20% in bold

A challenge we face in developing novel agents for Wilms tumor is the paucity of patients available to participate in phase I and II trials. WILTOP, a phase II study of topotecan for recurrent Wilms tumor (Chapter 6) required 4 years and significant financial investment to complete. The result was positive, but the majority of recent phase II studies for relapsed disease conducted by the COG have yielded negative results (Table 2). With a success rate less than 25 % in phase II studies for relapsed disease, the COG clearly needs a more efficient way to develop new agents.

To maximize the chance for success before embarking on new clinical studies, it is essential that new agents be chosen wisely and that the proper dosage and administration schedule be utilized. There is no perfect way to accomplish this, but the development of pre-clinical models may help inform the prioritization of new agents for Wilms tumor. To this end, we wrote a protocol to develop Wilms tumor xenografts from primary tumors resected at St. Jude Children's Research Hospital and collaborated with Dr. Peter Houghton to use these models to screen new agents.

A potential shortcoming of the xenograft models is that they may undergo extensive genetic change in the process of establishment from primary tumors. The research described in Chapter 5 indicates that this is not the case (manuscript in preparation for submission). The single nucleotide polymorphisms (SNP) chips indicated that there is a very high degree of genomic stability during the establishment of xenografts, with concordance of SNP calls >98%. Gene expression levels were less concordant, but the number of differentially expressed genes was not greater than what would be expected by chance. Importantly, genes in pathways that are targeted therapies, like the insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), mammalian target of rapamycin (mTOR), and epidermal growth factor receptor (EGFR) pathways were not differentially expressed. A caveat to these studies is that they did not evaluate protein expression of post-translational modifications.

A screen of agents with known clinical activity in Wilms tumor revealed that for the most part, activity level in xenograft models correlated with activity level seen in phase II studies. Caution must be taken in ensuring that the pharmacokinetics of agents in mice

is similar to that in humans before translating information from xenograft models to human trials.

The agent found to be most active in the xenograft models was topotecan. Further characterization of topotecan in the mice indicated that the schedule of administration was important; topotecan administered for 10 days produced superior responses to topotecan administered for 5 days, even at the same cumulative dose. This pre-clinical data led to the development of the WILTOP trial (Chapter 6), a phase II study of protracted-schedule topotecan for recurrent Wilms tumor (31). The topotecan dosing was pharmacokinetically-guided and designed to achieve an area under the curve (AUC) that mirrored active levels in the pre-clinical models. Patients were stratified into two arms, one for FH and one for AH. The statistical design was based solely on the FH stratum, which was wise because the FH tumors had a higher response rate than the AH tumors. The study found that 12/25 patients with FH achieved a partial response, which was very impressive considering that the patients enrolled in the study were heavily pre-treated, including several patients who previously underwent high-dose therapy with autologous stem cell rescue. Two of 11 patients with AH responded, suggesting that topotecan has some activity in this subset of patients.

A disadvantage of the topotecan regimen is that it requires 10 days of IV administration, meaning frequent visits to the pediatric oncology clinic. Another key shortcoming is that this dose and schedule is associated with significant hematologic toxicity, mainly neutropenia and thrombocytopenia. As expected, many patients were hospitalized for neutropenic fever and infection, but there were no toxic deaths.

The promising activity of topotecan has led the COG and SIOP to propose a trial to test the feasibility of ifosfamide/carboplatin/etoposide alternating with topotecan (ICE-T) for patients with recurrent Wilms tumor. If ICE-T is feasible to administer (ie, not associated with excessive toxicity) and produces satisfactory disease control, we anticipate that the regimen will serve as a backbone upon which to incorporate new biologic agents such as tyrosine kinase inhibitors, antibodies directed against IGF1R, Wnt pathway inhibitors, or mTOR pathway inhibitors.

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The initial telomerase results were very intriguing, so I contacted Dan Green and Paul Grundy to inquire whether I may obtain more samples from the National Wilms Tumor Study Group Biology Bank. Both Dan and Paul were incredibly supportive and invited me to join the NWTSG committee and attend a meeting in Seattle. I was awestruck sitting around the table with Drs. D'Angio, Green, Breslow, Beckwith, Grundy, Shamberger, and others. It was fascinating to match the faces and personalities to the names on the many papers, protocols, and textbook chapters that I had read. To this day, I am exceptionally grateful to the leaders of the NWTSG for taking me under their wings, listening to my ideas, and giving me the opportunity to take a leadership role.

One of the Wilms tumor investigators I met for the first time at the NWTSG table was a physician-scientist from Calgary. I recall thinking that Max Coppes was very outspoken, but I enjoyed what he had to say. Over the years, we got to know each other well and we had the opportunity to work together on several projects. Then several years ago, he tried

to recruit me to Calgary but the weather (too cold) and distance to family in the northeastern part of the United States, made me decide to stay in Memphis. Being persistent in nature, Max tried to recruit me again after he had moved to Children's National Medical Center in Washington, DC. This time the opportunity was too exciting to pass. Max, thank you for your trust in me over the years and for your continued guidance. I greatly value our friendship and your mentorship.

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BIOGRAPHY

Jeffrey Stuart Dome was born and raised in New York City. He graduated from the Bronx High School of Science in 1983 and received the school's awards for Excellence in Biology and Excellence in Statistics. During his high school years, he worked in the laboratory of Dr. Ronald Birke at the City College of New York and completed a project entitled "Raman Spectroscopic Studies of Cadmium Sulfide Films on a Cadmium Electrode," for which he was a semi-finalist in the Westinghouse Science Talent Search competition.

Dr. Dome obtained a BA degree in Biochemistry (with Honors) at the University of Pennsylvania (1987) and completed an independent study project in the laboratory of Drs. Joseph and Jean Sanger on the assembly of cytoskeletal proteins into cleavage furrows during mitosis. He received an MD degree from the University of Pennsylvania School of Medicine in 1991. Dr. Dome did residency training in Pediatrics at Yale-New Haven Hospital (1991-1994) and a fellowship in Pediatric Hematology/Oncology at Johns Hopkins Hospital (1994-1997). During fellowship, he worked in the laboratory of Dr. Sara Sukumar on two projects: "RNA of the *WT1* gene in Wilms tumor" and "Telomerase Expression as a Prognostic Factor for Wilms tumor." He was awarded an American Society of Clinical Oncology Young Investigator Award in 1997. After completing fellowship, Dr. Dome served on the faculty at Johns Hopkins until 1998, at which time he joined the faculty at St. Jude Children's Research Hospital in Memphis, Tennessee. Working in the Divisions of Molecular Therapeutics and Solid Tumors, he established his own laboratory to study telomerase expression in Wilms tumor, telomere signaling pathways in cellular senescence, and telomere maintenance mechanisms in osteosarcoma.

Dr. Dome became a member of the National Wilms Tumor Study Group (NWTSG) in 1997. When the NWTSG merged into the Renal Tumor Committee of the Children's Oncology Group (COG), he served as Committee Vice-Chair (2001-2006) and Chair of the Renal Tumor Biology Committee. In 2006, he assumed the role of Committee Chair. Dr. Dome is a member of the American Society of Clinical Oncology (ASCO), the American Association for Cancer Research (AACR), the American Society of Pediatric

Hematology/Oncology (ASPHO), and the International Society of Paediatric Oncology (SIOP).

In December 2006, Dr. Dome became Chief of the Division of Oncology at Children's National Medical Center in Washington, DC. He is Associate Professor of Pediatrics at George Washington University School of Medicine.