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Gene Expression Profiling of Favorable Histology Wilms Tumors and Its Correlation with Clinical Features

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ABSTRACT

The aims of this study were to understand the underlying molecular mechanisms of favorable histology Wilms tumors (WTs) and to classify them based on their molecular signatures. We studied a total of 15 favorable histology WTs using microarrays containing 19,968 cDNAs. First, we found commonly altered genes in WT. A total of 267 cDNAs were significantly overexpressed at least 3-fold in all of the tumors compared with noncancerous kidney and contained known WT-related genes such as *IGF II* and *WT1*. The gene with the highest expression change compared with noncancerous kidney was *topoisomerase IIα*. By hierarchical clustering, there was a clear distinction between high-stage and low-stage tumors. A total of 30 cDNAs were found differentially expressed between the high- and low-stage groups. One of them, *Stathmin 1*, which is involved in the microtubule system, was highly expressed in high-stage tumors compared with the low-stage tumors. The present chemotherapy regimens for WT consist mainly of topoisomerase II inhibitors (*i.e.*, actinomycin D, doxorubicin, and etoposide) and antimicrotubule agents (*i.e.*, vincristine and paclitaxel). Our data suggest that high expression of *topoisomerase IIα* and microtubule-related genes such as *tubulin* and *stathmin 1* may be related to the high chemosensitivity of WT. In addition, retinol-related genes such as *CRABP2* and *retinol-binding protein 1* were overexpressed in WT, and *CRABP2* was more highly expressed in the poor outcome patients, which suggests that retinoid acid may be a potential drug. In summary, our findings suggest that the integration of gene expression data and clinical parameters could aid in detecting aggressive tumors among favorable histology WT and lead to the discovery of new drugs for WT.

INTRODUCTION

WT,[2] also known as nephroblastoma, is the most common cancer of the urinary tract affecting 7/1,000,000 children under the age of 15 (1). Representing 6–8% of all pediatric solid neoplasms, it occurs most commonly in children between 1 and 5 years of age and occurs with an equal male:female ratio. The majority of cases are of the sporadic form (99%). A small number of cases are hereditary and may be associated with Beckwith-Wiedeman, Denys-Drash, and WAGR syndromes. Histologically, WT can be divided into two subtypes: one with favorable histology and one with anaplasia. The anaplastic subtype, characterized by tumors containing larger nuclei, increased nuclear chromatin content, and polyploid mitotic figures, is associated with poor prognosis. The favorable type of WT lacks these nuclear features.

Much of our understanding of the genetics of WT comes from studies of the hereditary forms. Three distinct genetic loci are associated with WT: 11p13, 11p15, and 17q (2). The *WT1* gene maps to chromosome 11p13 and is mutated in patients with WAGR syndrome. In addition, specific point mutations within the DNA binding domain of *WT1* in Denys-Drash syndrome result in severe abnormalities in sexual and renal development. It encodes a zinc finger-containing transcription factor, which plays a critical role in the development of kidneys and gonads (3). The second WT-related gene is located on chromosome 11p15 and is mutated in families with Beckwith-Wiedeman syndrome. The disease-causing gene at 11p15 has yet to be identified, but other genes located in this region such as *IGF II* gene, which is imprinted (only expressed from the paternally derived allele), have been implicated in WT. In Beckwith-Wiedeman disease, some patients constitutionally lose the imprinting of *IGF II*. In addition, some sporadic WT also show the loss of imprinting of *IGF II*, and this may result in high expression of *IGF II*. Alteration in two other genes in this region, *p57* and *H19*, are also linked to WT. The hereditary WT gene (*WT1*) in chromosome 17 remains unknown (2).

Prognostic factors for WT include anaplasia in tumor cells (4), primary tumor extension (5), lymph node involvement, and hematogenous metastases. To find additional prognostic factors, a number of cytogenetic studies have been carried out, and genetic imbalances including losses of chromosomes 1p, 11q, 16q, 22 and gain of 1q have been reported to associate with poor outcome. Recently, a large cytogenetic study of WT has shown that only 1q gain and monosomy 22 are significant prognostic factors (6). In addition, the overexpression of several genes (*e.g.*, *Bcl-2*, *WT1*, and *survivin*), and a high level of serum VEGF after surgery are associated with recurrence and poor outcome (7–10).

Despite the cloning of *WT1* gene more than 10 years ago and the subsequent identification of several WT-associated genes, the molecular basis for WT remains obscure. We have previously demonstrated the molecular signature of clear-cell renal cell carcinoma by using gene expression microarray technology (11). In this study, we used a similar approach to characterize the global gene expression profiles of WT and sought to identify genes commonly altered in WT and specific gene sets that correlate with clinical parameters.

MATERIALS AND METHODS

Tumor Samples and RNA Preparation. Fifteen frozen WT tissues and 1 matched noncancerous kidney tissue were obtained from the University of Chicago (Chicago, IL), the Medical University of South Carolina (Charleston, SC), and the Karolinska Institute (Stockholm, Sweden). Internal Review Boards of these institutions and the Van Andel Research Institute approved all of the samples used in this study. As reference for the experiments, noncancerous adult kidney

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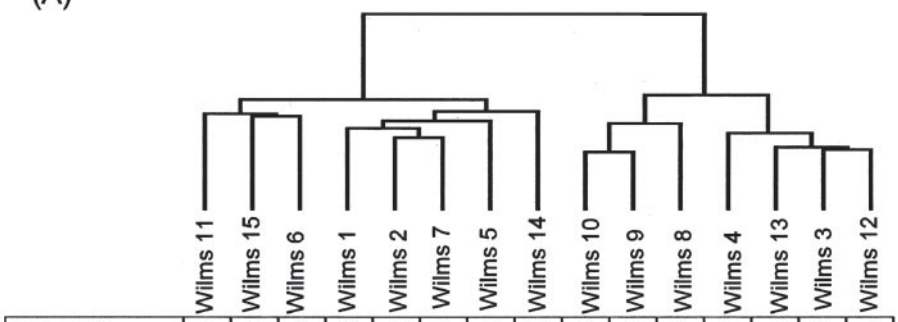
[2] The abbreviations used are: WT, Wilms tumor; Topo, topoisomerase; IGF II, insulin-like growth factor II; CRABP2, cellular retinoic acid-binding protein 2; DBCCR1, deleted in bladder cancer chromosome region candidate 1; FDR, false discovery rate; MMP, matrix metalloproteinase; BNIP3, BCL2/adenovirus E1B M_r 19kD interacting protein; WAGR, WT, aniridia, genitourinary anomalies, and retardation; RT-PCR, reverse transcription-PCR.

(A)



	Wilms 11	Wilms 15	Wilms 6	Wilms 1	Wilms 2	Wilms 7	Wilms 5	Wilms 14	Wilms 10	Wilms 9	Wilms 8	Wilms 4	Wilms 13	Wilms 3	Wilms 12
Age (yrs)	1	3	1	2	6	5	7	11	2	1	8	1	7	3	1
Stage	3	4	3	3	3	3	2	2	2	2	1	1	2	2	1
Outcome	A	D	D	A	A	A	A	D	A	A	A	A	N/A	A	A
Recurrence	R	N/A	R	NE	NE	NE	NE	R	NE	NE	NE	NE	N/A	NE	NE
Followup (yrs)	6	4.0	4	2	1	5	10	3	6	8	7	13	N/A	8	8

(B)

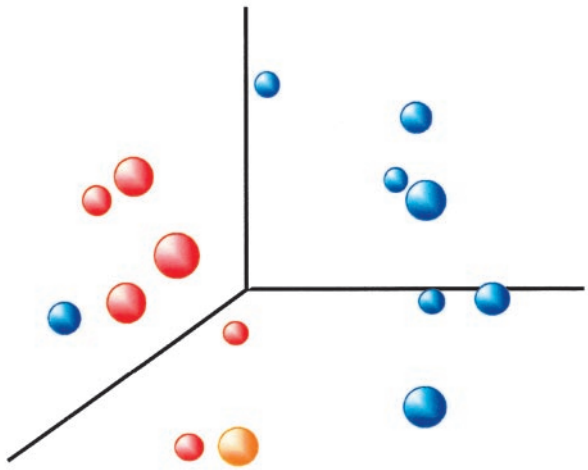


Fig. 1. *A*, clustering of 15 WTs and clinical information; *B*, three-dimensional clustering of these cases. The clustering of patients (using Pearson's correlation) is based on global gene expression profiles consisting of median polished data of 5,594 well-measured spots. The tumors clustered into approximately two main groups with one group consisting of mostly tumors with high stage (stage III, IV) and the other consisting of mostly tumors with low stage (stage I, II). Among high-stage tumors, two patients who died of cancer (Wilms 6, 15) and one patient who had recurrence (Wilms 11) were closely clustered together. One patient (Wilms 14 in *A* and represented by orange circle in *B*), who had stage II disease but developed recurrence and died of cancer, was clustered with high-stage tumors. Abbreviations in *A*: *D*, died from disease; *A*, alive; *R*, recurrence; *N/A*, not available; *NE*, no evidence of recurrence.

tissues from five patients with renal cell carcinoma were used for all of the cases. In addition, in one case (Wilms 2), matched noncancerous kidney RNA was also used for comparison. All of the tissues were accompanied by clinical information and pathological reports. The samples were made anonymous before the study. Part of each tumor sample was frozen in liquid nitrogen immediately after surgery and stored at −80°C. Total RNA was isolated from the frozen tissues using Trizol reagent (Invitrogen, Carlsbad, CA). Then total RNA was purified with 2.5 M final concentration of lithium chloride. The National Wilms Tumor Staging System (12) was used for tumor staging: stage I, tumor limited to kidney and completely excised; stage II, tumor extends beyond the kidney but is completely removed; stage III, residual nonhematogenous tumor confined to the abdomen; stage IV, hematogenous metastasis. All of the patients except two have been followed-up for 9.6 to 159 months (median, 71.2 months). Clinical information is shown in Fig. 1.

cDNA Microarray Fabrication and Procedures. Microarray production was performed as described previously (13, 14) with slight modification. Briefly, 19,968 cDNA clones were PCR amplified directly from bacterial stocks purchased from Research Genetics (Huntsville, AL). After ethanol precipitation and transfer to 384 well plates, clones were printed onto aminosilane-coated glass slides using a home-built robotic microarrayer. Slides were chemically blocked using succinic anhydrate after UV cross-linking. RNA from five noncancerous kidney tissues was mixed and pooled for use as a common reference. RNA from the matched noncancerous kidney tissue of one patient was also prepared. Fifty μg of total RNA from each WT and 50 μg of either pooled total RNA from noncancerous kidney tissues or total RNA from one matched noncancerous kidney were reverse transcribed with oligo(dT) primer and SuperScript II (Invitrogen, Carlsbad, CA) in the presence of Cy5-dCTP and Cy3-dCTP (Amersham Pharmacia Biotech, Peapack, NJ), respectively. The Cy5- and Cy3-labeled cDNA probes were mixed with probe hybridization solution containing formamide and hybridized to prewarmed (50°C) slides for 20 h at 50°C. After hybridization, slides were washed in 1× SSC, 0.1% SDS at 50°C for 5 min followed by 0.2× SSC, 0.1% SDS at room temperature for 5 min, 0.2× SSC at room temperature for 5 min twice, and 0.1× SSC at room temperature for 5 min. Slides were dried by snap-centrifugation and scanned immediately using Scan Array Lite, operating at 532 nm and 635 nm wavelengths (GSI Lumonics, Billerica, MA).

Data Analysis. Images were analyzed using the software Genepix Pro 3.0 (Axon, Union City, CA). The local background was subtracted for all spots. Spots of which the background-subtracted intensities in either Cy5 or Cy3 channel were less than 150 were excluded from the analysis. The ratio of Cy5:Cy3 intensity was calculated for each spot, representing tumor RNA expression relative to the noncancerous kidney tissue pool. Ratios were log transformed (base 2) and normalized so that the median log-transformed ratio equaled zero. Gene expression values that were present in at least 75% of the tumors, had expression ratios that varied at least 2-fold in at least two tumors, and had maximum-ratio-minus-minimum-ratio values greater than 2-fold were selected for the global clustering analysis (5594 genes). The gene expression ratios were median centered across all of the samples. Gene expression values were manipulated and visualized using the CLUSTER and TREEVIEW software.[3] The correlation distances were calculated as $1 - r$, where r indicates the Pearson correlation coefficient (15).

The CIT software was used to find genes that were differentially expressed

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(using Student's *t* test) between two patient groups (16). In this study, the patient groupings were based on clinical parameters. To find significant discriminating genes, 10,000 T-statistics were calculated by randomly placing patients into two groups (17). A 99.5% significance threshold ($\alpha < 0.05$) was used to identify genes that could significantly distinguish between two patient groups *versus* the random patient groupings. The FDR was also calculated to estimate the expected percentage of false positives identified from a multiple testing procedure. Given a set of genes, *n*, identified at a given significance, α, of *m* genes analyzed, the FDR $= n/\alpha \times m$.

Quantitative Real-Time PCR (TaqMan). Two μg of total RNA from tumors and the pooled noncancerous kidney, which was also used for microarray experiments, was reverse transcribed in a 100-μl reaction mixture containing 1× room temperature buffer, 5.5 mM $MgCl_2$, 500 μM each dNTP, 2.5 μM random hexamers, 0.4 units/μl RNase inhibitor, and 3.125 units/μl MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA). The room temperature thermal cycling conditions were as follows: 25°C for 10 min, 48°C for 60 min, and 95°C for 5min. Real-time relative quantitative PCR was performed in triplicate using the ABI PRISM 7700 Sequence Detection system according to the manufacturer's instruction, and data were averaged. Primers and a TaqMan probe were specifically designed for each of six genes: *Topo IIα*, *IGF II*, *stathmin 1*, *integrin α-8*, *CRABP2*, and *DBCCR1* using Primer Express v1.5a (Applied Biosystems).

The following primers and probes were used: (*a*) *Topo IIα*, 5′-CTTTGTCTTTGTA CCTGGTTCTCTTTT-3′ (forward primer), 5′-CCCTCAAGAAGATGGTGTGGAA-3′ (reverse primer), 5′-6FAM-AATCTTTGTTTTAGGCCTTCT-3′ (probe); (*b*) *IGF II*, 5′-GGGAGGGTATGTGAAGGGTGTT-3′ (forward primer), 5′-TGAGGAAGCACAGCAG CATCT-3′ (reverse primer), 5′-6FAM-AATCGATTTTGTACATGTTTG-3′ (probe); (*c*) *stathmin 1*, 5′-GACAGAATTGGGATTGAAAAGTGA-3′ (forward primer), 5′-AAAAG AGTATGTAGTGGCTTCTTTTGAA-3′ (reverse primer), 5′-6FAM-CAGATATTCAGC ATCTAACA-3′ (probe); (*d*) *integrin α-8*, 5′-TGCAGGCAGATACCG TTTGA-3′ (forward primer), 5′-GGTTCTTTGGTTCCATTAACTCTGA-3′ (reverse primer), 5′-6FAM-ACCACCAACAACAGAAA-3′ (probe); (*e*) *CRABP2*, 5′-CCCGGAATGGG TGATCTG-3′ (forward primer), 5′-TTCTCTCCTCCTTTACACCAACAAA-3′ (reverse primer), 5′-6FAM-TTGCAGCCATTCCT-3′ (probe); and (*f*) *DBCCR1*, 5′-GGTTAGC AGAGTTTGGCTGTCA-3′ (forward primer), 5′-GACCTGTAC AACACGGAGATCCT-3′ (reverse primer), 5′-6FAM-CTGGTCCGACTGTTT-3′ (probe). One hundred ng of each cDNA was amplified using PCR Master Mix according to the following the PCR conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Because *18S* rRNA resulted in the least variation throughout the samples among a total of 11 housekeeping genes using Taqman Human Endogenous Control Plate, this gene was used as the endogenous control (Applied Biosystems). Each threshold cycle (C_T), which indicates the cycle at which an increase in reporter fluorescence goes slightly over the optimal value line, was determined. The C_T value of *18S* rRNA was subtracted from each C_T value of tumor or noncancerous kidney tissue for normalization and the ratio of tumor: noncancerous kidney RNA expression was calculated to compare with microarray data.

The Mann-Whitney *U* test was used to examine the significant difference of gene expression ratios between two groups of tumors. Statistical significance was defined as $P \leq 0.05$.

RESULTS

Because only one matched noncancerous kidney tissue (Wilms 2) was available for this study, an experiment was carried out to compare the expression profiles of a sample tumor (*T*) using total RNA from a matched noncancerous kidney (*T*/*Rm*) and pooled total RNA from adult noncancerous kidneys (*T*/*Rp*). The tumor:reference ratios of all present spots were log-transformed and a correlation coefficient between the *T/Rm* and *T/Rp* expression values was calculated. The expression ratios obtained using the difference references were very similar (correlation coefficient = 0.86; data not shown). This data suggested that genes that are found altered in the *T/Rp* samples may be similar to those that would have been found in a study that consists entirely of *T/Rm* samples. Because adult kidney tissues are easier to obtain, they may be more suitable to use as reference. This will allow comparisons between microarray experiments performed by different groups.

Commonly Altered Genes Compared with Noncancerous Kidney Tissue. We first examined gene expression alterations that were common to most WTs. A sign test was used to select genes that were significantly ($\alpha \leq 0.001$) over- or underexpressed at least 2-fold when compared with noncancerous kidney tissue. Genes that had an interquartile mean expression change of at least 3-fold were further analyzed. A total of 267 overexpressed and 591 underexpressed cDNAs met these criteria. A number of interesting genes are present in the overexpressed gene list. Several genes in this list have been previously shown to be closely involved in WT, and other genes are related to present chemotherapy agents for WT (see "Discussion"). Among the underexpressed genes, there are a number of genes related to normal kidney functions such as the solute carrier family, metallothionein, and enzymes including phenylalanine hydroxylase. The list consisting of the top 40 overexpressed cDNA in WT is shown in Table 1.

Molecular Classification of Diverse Favorable Histology WTs (Unsupervised Analysis). We used hierarchical clustering (15) to classify WTs based on their gene expression profiles using the expression ratios of a selected 5594 cDNA set (see "Materials and Methods"). The clustering algorithm groups both genes and tumors by similarity in expression pattern. Clustering based on the total gene expression profile is shown in Fig. 1*A*. The tumors clustered into two main groups, with one group consisting of mostly tumors with high stage (stage III, IV) and the other consisting of mostly tumors with low stage (stage I, II). Among high-stage tumors, two patients who died of cancer (Wilms 6, 15) and one patient who had recurrence (Wilms 11) were closely clustered together. We also used multidimensional scaling (MDS) to visualize the relationship between the tumors' gene expression patterns. Three-dimensional visualization of the MDS data showed mainly two groups based on staging (Fig. 1*B*). One patient with stage II tumor, who died of cancer, was grouped in the high-stage group by both cluster tree and three-dimensional imaging, which suggests that this tumor was aggressive.

Identification of Differentially Expressed Genes on the Basis of Clinical Parameters in Favorable Histology WT (Supervised Analysis). Identifying differentially expressed genes based on clinical parameters may lead to the discovery of genes that are involved with tumor invasion, metastasis. By global-clustering analysis using 5594 cDNA data sets, two main groupings seemed to be related to tumor staging.

Correlation with Staging. We sought to identify the genes that express differentially between low-stage (stage I, II) and high-stage (stage III, IV) tumors by using the CIT software. A total of 30 cDNAs were significantly differentially expressed between the two groups ($P < 0.05$; Fig. 2; see "Materials and Methods"). Among them, several genes that are associated with tumor progression were found such as *stathmin1/oncoprotein 18*, *BNIP3*, and *MMP 16* (see "Discussion").

Correlation with Outcome. Three patients died of cancer within 4 years after surgery, whereas nine patients are alive without cancer for more than 5 years. At the time of this study, two patients had short follow-ups of less than 3 years and were excluded from this analysis. We examined the genes that may be differentially expressed between favorable histology WT with poor outcome and those with good outcome. Eighty cDNAs were differentially expressed between the poor-outcome and good-outcome groups, and 40 of them are listed in Table 2. Some of the interesting genes are discussed in the "Discussion" section.

Table 1 *Significantly overexpressed genes in WTs*

Top 40 cDNAs that are significantly overexpressed in Wilms tumors ($n = 15$). A sign test for a one-sample mean was used to identify genes whose expression changed at least 2-fold in a significant number of samples ($\alpha \leq 0.001$).

Accession ID[a]	Gene name	Average fold[b] (T/N)
AA026682	*Topo* (DNA) II α (M_r 170,000)	80.8
H98218	high-mobility group (nonhistone chromosomal) protein	36.2
AA504348	*Topo* (DNA) II α (M_r 170,000)	32.1
AA419229	*G protein-coupled receptor 39* (*GPR39*)	25.2
AA701455	*centromere protein F* (M_r 350,000/400,000, mitosin) (*CENPF*)	25.1
AA071486	*serine/threonine kinase 12* (*STK12*)	23.5
N67487	*microfibrillar-associated protein 2* (*MFAP2*)	22.1
N74623	*IGF II* (somatomedin A)	21.9
W68220	*ESTs*	18.5
AA460685	*baculoviral IAP repeat-containing 5* (*survivin*)	16.2
H73968	*chromosome 20 open reading frame 1 C20orf1*	15.3
N40940	*transmembrane protein with EGF-like*	15.3
AA598674	*ESTs*	14.5
AA430744	*enhancer of zeste* (*Drosophila*) *homolog 2 (EZH2*)	12.7
AA598945	*metallocarboxypeptidase CPX-1* (*CPX-1*)	12.4
AA598974	not found	12.0
AA873060	*stathmin 1/oncoprotein 18* (*STMN1*)	11.4
AA598508	*CRABP2*	11.2
W58368	*eukaryotic translation initiation factor 2B, subunit 3*	11.2
T67524	*zinc finger protein 205* (*ZNF205*)	11.1
AA629262	*ESTs*	11.0
AA485665	*ephrin-B3* (*EFNB3*), mRNA, *EPHRIN B1,2,3*	11.0
AA449336	*ESTs*	10.9
H80685	*p311*	10.4
AA430032	*ESTs*	10.4
N91887	*thymosin* β identified in neuroblastoma cells	10.1
N94616	*laminin* α *4* (*LAMA4*)	9.8
AA454572	*minichromosome maintenance deficient* (*Saccharomyces cerevisiae*) *2*	9.5
AA700832	*retinol-binding protein 1*, cellular (*RBP1*)	9.5
T87341	*ESTs*	9.5
H92234	*ESTs*	9.3
AA102130	*ectodermal-neural cortex* (with *BTB*-like domain)	9.2
AA598610	*mesoderm-specific transcript* (mouse) *homolog* (*MEST*)	8.9
AA460849	*mitochondrial ribosomal protein L9*	8.8
R62603	*collagen type VI* α3 (*COL6A3*)	8.7
AA101875	*chondroitin sulfate proteoglycan 2* (*versican*) (*CSPG2*)	8.6
R85090	*ectodermal-neural cortex* (with *BTB*-like domain)	8.4
R45941	*protein tyrosine phosphatase receptor type, N*	8.1
R41787	*cadherin 13, H-cadherin* (heart) (*CDH13*)	8.0
AA453170	*hypothetical protein FLJ14299*	7.9

[a] ID, identification.
[b] Average fold shows the interquartile mean of the tumor *versus* non-cancerous tissue (T/N) gene expression values.
[c] EST, expressed sequence tag.

Confirmation Study by Real-Time Relative Quantitative PCR. Four genes were selected to confirm the microarray results with real-time relative quantitative PCR. *Topo IIα* and *IGF II* were selected from the list of commonly overexpressed cDNA (Table 1). *Stathmin 1/oncoprotein 18* and *integrin α-8* were selected from the list of differentially expressed cDNA between low stage and high stage (Fig. 2). *CRABP2* and *DBCCR1* were from the differentially expressed genes between favorable histology WT with poor outcome and those with good outcome.

To compare the gene expression ratios obtained by microarray with the RT-PCR data, the expression ratio of tumor:pooled noncancerous kidney was calculated by real-time PCR (Fig. 3). *Topo IIα* and *IGF II* were overexpressed in all of the tumors confirming microarray data, although some microarray data were missing (Fig. 3, *A* and *B*). Real-time PCR showed higher expression ratio of *Topo IIα* ($P = 0.0771$) in high-stage tumors, and *IGF II* was significantly more highly expressed in high-stage tumors ($P = 0.0251$). *Stathmin 1* and *integrin α-8* were overexpressed in most WTs and were more highly expressed in high-stage tumors ($P = 0.0451, 0.0133$; Fig. 3, *C* and *D*). *CRABP2* and *DBCCR1* were also overexpressed in most WTs. *DBCCR1* was significantly more highly expressed in WTs with poor outcome ($P = 0.0335$; Fig. 3*F*) and *CRABP2* tended to be more highly expressed in WTs with poor outcome ($P = 0.079$; Fig. 3*E*). All of the real-time PCR data were consistent with microarray data.

Some expression ratios calculated by real-time PCR were 10 times higher than those obtained by microarray. This may be caused by spot saturation, competitive hybridization of microarray experiments, or amplification methods of PCR.

DISCUSSION

Microarray studies have uncovered the molecular signatures and molecular subtypes correlated with their clinical parameters of many types of cancers (17–19). Our study also sought to uncover the underlying molecular mechanism of favorable histology WT. To date, two articles on gene expression profiling for WT have been published to identify WT-related genes (20–21). In the first report, Rigolet *et al.* found 153 differentially expressed cDNA common for WT as compared with adult noncancerous kidney by using a 588-cDNA microarray (20). Among them, genes related to cell cycle/growth regulators such as *cell division protein kinase 4*, *proliferating cell nuclear antigen*, and *growth arrest-specific protein 1* have been found overexpressed compared with reference. Also, *versican* and *fibronectin*, genes that are related to cell adhesion and cell invasion, have been shown overexpressed in WT. Those data were consistent with our results (data are not shown). However, the study used a small number of cDNAs and samples without clinical information for microarray and found only limited common molecular features for WT. In the second report, Li *et al.* used a more comprehensive microarray with 12,000 probe sets and found 27 genes that were highly expressed in

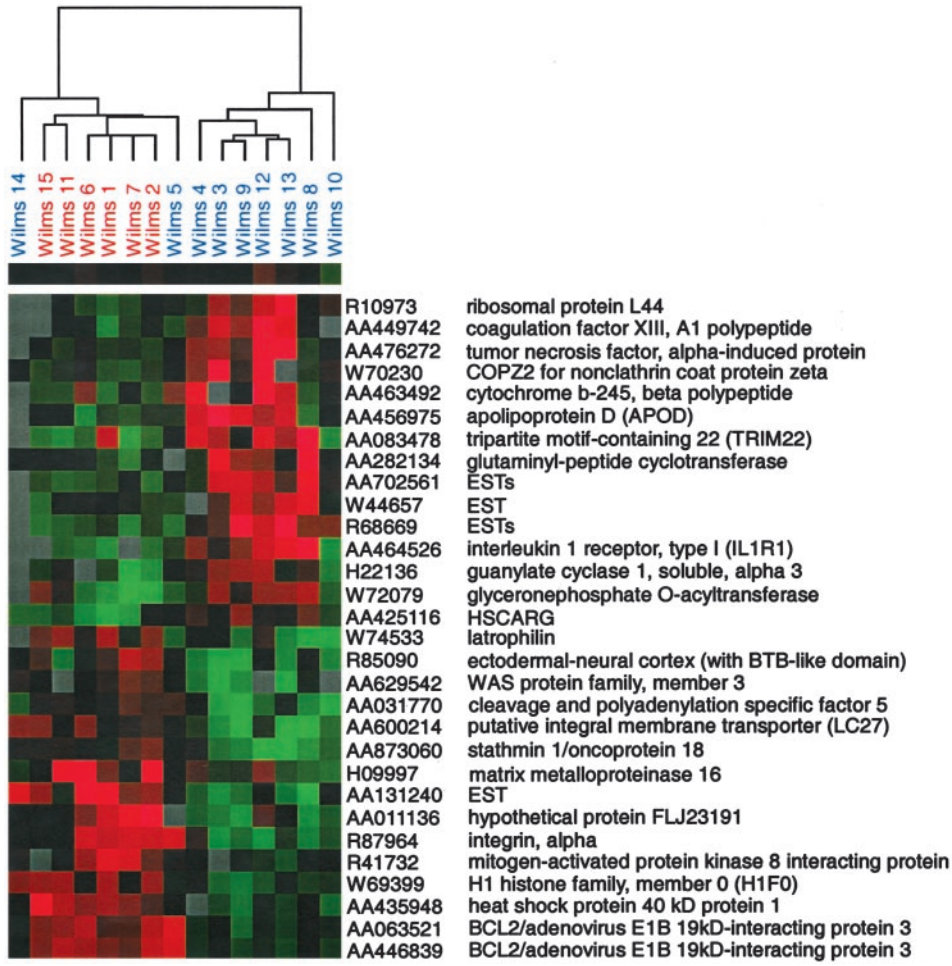

Fig. 2. The 30 cDNAs that are significantly differentially expressed between high stage (stage III, IV) and low stage (stage I, II), with the clustering of the 30 cDNA expression levels. *Rows*, individual cDNA; *columns*, individual tumor samples. *The color of each square*, the median-polished, normalized ratio of gene expression in a tumor relative to noncancerous kidney tissue: *red*, expression levels greater than the median; *green*, expression levels below the median; *black*, expression levels equal to the median; *gray*, inadequate or missing data. The *color saturation* indicates the degree of divergence from the median. The estimated FDR for this set of genes ($\alpha \leq 0.05$) is 9.0%.

WT compared with fetal kidney, heterologous tumor, and normal tissue. High expression of *SIX 1*, *SYA1*, *SALL2*, and *HOXA 11*, which have been shown to be essential for metanephric development, was found. The report may reflect the numerical predominance of neoplastic cells arrested at a specific developmental stage (21). Our findings contained 14 genes of the 27 genes that they listed. Ten genes among them were also overexpressed throughout the WT samples and 2 genes were overexpressed in one-half of the samples compared with adult noncancerous kidney, which suggests the similarity of the results. In this study, in addition to the comprehensive gene-expression profiling of favorable histology WT, we also focused on molecular subtypes related to clinical parameters that have not been discussed.

To validate our microarray data, we first examined the known genes that have been previously reported to be associated with WT. Compared with noncancerous kidney, *IGF II* was highly expressed in 95% of tumors with an average 26-fold increase, which is in agreement with previous studies (22, 23). In addition, the *WT1* gene was found highly expressed in the majority of WTs as reported previously. Interestingly, the four cases that had relatively low expression were low-stage tumors (Fig. 1). Next, we attempted to identify commonly altered genes that may serve as molecular markers for the diseased state or play a causal role in its transformation, therefore, serving as candidate therapeutic targets. Interestingly, the gene with the highest expression in all of the WT samples, which was also confirmed by real time RT-PCR, is *Topo IIα*. *Topo IIα* is one of the nuclear enzymes that controls and alters the topological state of DNA (*e.g.*, relaxation of supercoiled DNA) to facilitate cellular processes such as transcription, replication, and repair recombination. *Topo IIα* amplification has been shown to correlate with increased sensitivity to Topo II inhibitors, a major group of antitumor agents (24), whereas resistance to these drugs may occur through mutation in *Topo IIα*, decreased Topo II production, or through production of P-glycoprotein, conferring multidrug resistance (25). Therefore, *Topo IIα* expression is an indicator of cell proliferation and chemosensitivity to Topo II inhibitors. In the 1960s, Farber (26) reported a dramatic improvement in the survival rate (>80%) by the use of actinomycin D in combination with surgery and radiation. Actinomycin D is a Topo II inhibitor and has been used as the main chemotherapeutic agent for treatment of WT. Doxorubicin is another main chemotherapeutic agent that is also a Topo II inhibitor. The other Topo II inhibitor, etoposide, is generally used for diffuse anaplastic tumors with stage II-IV. Thus, Topo II inhibitors constitute the main chemotherapeutic agents for WT. Our demonstration that *Topo IIα* is overexpressed in all cases of WT may provide a molecular mechanism to explain why the development of Topo IIα inhibitor-based treatment of WT significantly improved the survival of WT patients. This validates the use of gene expression profiling to identify new molecular targets for treatment in WT and other cancers.

We also found that a number of extracellular matrix-related genes were also highly expressed including *laminin*, *collagen type III*, *microfibrillar-associated protein*, and *metallocarboxypeptidase* in the majority of WTs. These proteins may also be potential targets to design new chemotherapeutic agents.

Correlation of Gene Expressions with Clinical Phenotypes. The histological classification, solely based on finding a few anaplastic tumor cells, is prone to sampling errors and dependent on the expe-

Table 2 *Differentially expressed genes between different outcome groups at 5 years*[a]

A total of 80 cDNAs were found to be differentially expressed between the different outcome groups at 5 years.

Accession ID[b]	Gene name	Fold change[c]	*P*	FDR[d] (%)
AA131240	*EST*	6.4	0.0001	0.1
AA598508	*CRABP2*	5.6	0.0130	3.9
AA621201	*solute carrier family 30* (*zinc transporter*) *member 3*	5.2	0.0110	4.1
H10959	deleted in bladder cancer chromosome region candidate	5.1	0.0170	4.7
AA448394	*Homo sapiens mRNA* for *KIAA1783 protein, partial cds*	−4.4	0.0001	0.1
AA436401	*TU3A protein* (*TU3A*)	4.2	0.0180	4.6
R98074	*betaine-homocysteine methyltransferase 2* (*BHMT2*)	−4.2	0.0001	0.1
AA454572	*minichromosome maintenance deficient* (*Saccharomyces cerevisiae*) *2*	3.9	0.0093	3.1
AA252470	*Homo sapiens cDNA FLJ11606 fis*	−3.7	0.0001	0.1
AA600214	*putative integral membrane transporter* (*LC27*)	3.7	0.0290	6.4
H93086	*EST*	−3.5	0.0047	2.1
AA456088	*EPHB3*	3.4	0.0290	6.4
AA446005	*DKFZP434N061 protein*	−3.3	0.0001	0.1
AA449463	*PI-3-kinase-related kinase SMG-1*	−3.3	0.0040	1.9
T94293	*ESTs*, highly similar to *PA2X*	−3.3	0.0040	1.9
H21943	*thymopoietin*	3.2	0.0300	7.1
N91887	*thymosin* β identified in neuroblastoma cells	3.1	0.0360	7.1
R73672	*Homo sapiens, clone IMAGE:3881549*	3.1	0.0140	4.1
T67045	*DKFZP434N061 protein*	−3.1	0.0001	0.1
N52394	*hypothetical protein FLJ20008*	−3	0.0300	7
N53581	*phosphodiesterase 4C, cAMP-specific*	−3	0.0001	0.1
N21550	*EST*	−2.9	0.0001	0.1
T86714	*EST*	−2.9	0.0350	7
AA487590	*novel human gene mapping to chromosome 13*	−2.8	0.0460	8.7
H19105	*ESTs*, weakly similar to *TNP1*	2.8	0.0001	0.1
H84154	*cyclin D2* (*CCND2*)	2.8	0.0450	8.7
W15574	*EST*	−2.8	0.0056	2.1
AA062802	*KIAA0354 KELCH-related*	2.7	0.0001	0.1
AA481758	*heat shock* M_r 40,000 *protein 1* (*HSPF1*)	2.7	0.0001	0.1
AA490144	*EST*	2.7	0.0001	0.1
H10045	*vav3* oncogene (*VAV3*)	−2.7	0.0160	4.3
R31673	*EST*	−2.7	0.0050	2
R97226	*PI-3-kinase-related kinase SMG-1*	−2.7	0.0140	4
T91807	*FLJ10154*	−2.7	0.0001	0.1
T99793	*meningioma-expressed antigen 6*	−2.7	0.0040	1.9
AA429657	*KIAA0080 protein*	2.6	0.0240	5.6
AA478950	*nuclear factor of activated T-cells 5*	−2.6	0.0001	0.1
AA844831	*carboxypeptidase A2* (*pancreatic*) (*CPA2*)	2.6	0.0190	4.8
N69661	*TPA*-regulated locus	−2.6	0.0001	0.1
R55334	ESTs, weakly similar to *B34087* hypothetical protein	−2.6	0.0001	0.1

[a] The 40 most differential expressed cDNAs between the two groups.
[b] ID, identification.
[c] A positive fold change indicates the poor outcome group had relatively higher expression; a negative value indicates the good outcome group had relatively higher expression.
[d] An estimate of the FDR shown for each gene.

rience of a pathologist. Some of the favorable histology WT in this study clearly demonstrated an unfavorable clinical outcome despite being labeled as "favorable" histology. The gene expression profiling of this group of tumors supports the heterogeneity in their behavior.

Using unsupervised clustering, two patient groups with favorable histology WT that correlate with their stage were identified: one group with high-stage tumors and the other with low-stage tumors (Fig. 1). In the high-stage group, three patients died of the disease, and one developed recurrence. Because global gene expression profiles showed two main groups based on staging, and staging is one of the prognostic factors, we sought to find differentially expressed genes between low stage (stage I, II) and high stage (stage III, IV; Fig. 2). Thirty cDNAs had significantly different expression between the two groups. Among them are the genes *stathmin1/oncoprotein 18*, *BNIP3*, *integrin* α*-8*, and *MMP 16*. All of the genes had higher expression in the high-stage group and *stathmin 1/oncoprotein 18* and *integrin* α*-8* were confirmed by real time RT-PCR.

Stathmin1/oncoprotein 18 gene encodes an abundant cytoplasmic tubulin-binding phosphoprotein. It plays an important role in cellular proliferation and functions as a depolymerizer of the microtubules, the integral components of mitotic spindles. *Stathmin 1* was highly expressed in 90% of WTs compared with noncancerous kidney in this study and more highly expressed in high-stage tumors. High protein expression of stathmin has been found correlated with general predictive factors in breast cancers (27), and the stathmin-transfected lung cancer cell line increases the sensitivity to *Vinca* alkaloids (28). Interestingly, antisense inhibition of stathmin expression has been shown to have a synergistic apoptotic effect along with paclitaxel (29).

Tubulin α and β subunits are the main constituents of microtubules, and those genes showed at least a 2-fold overexpression in more than 75% of the tumors. Tubulin is the biochemical target for several clinically used anticancer drugs, including paclitaxel and the *Vinca* alkaloids vincristine and vinblastine. The increasing level of *tubulin* β*3* subtype (30) or gene mutations of *tubulin* β (31) has been shown to have a positive correlation with increasing resistance to paclitaxel. Antisense oligonucleotides have been shown to sensitize drug-resistant cells to paclitaxel (32). Recently, a new synthetic compound, which selectively modifies the β1, β2, and β4 isotypes of tubulin β, has been shown to exhibit cytotoxicity against cancer cell lines chemoresistant for vinblastine, paclitaxel, doxorubicin, and actinomycin D, which suggests their potential clinical use for treating WT (33).

Stathmin 1 and *tubulin* are closely related to microtubule function and support a model suggesting that high expression of these genes contributes to the high chemosensitivity of this tumor type for antimicrotubule agents, such as vincristine, in all stages, and etoposide, in high stages as a agent of combination chemotherapy. Although paclitaxel has not been widely used for treatment of WT, a case with adult WT who showed dramatic response to paclitaxel was recently reported (34). Antimicrotubule therapy such as paclitaxel and anti-

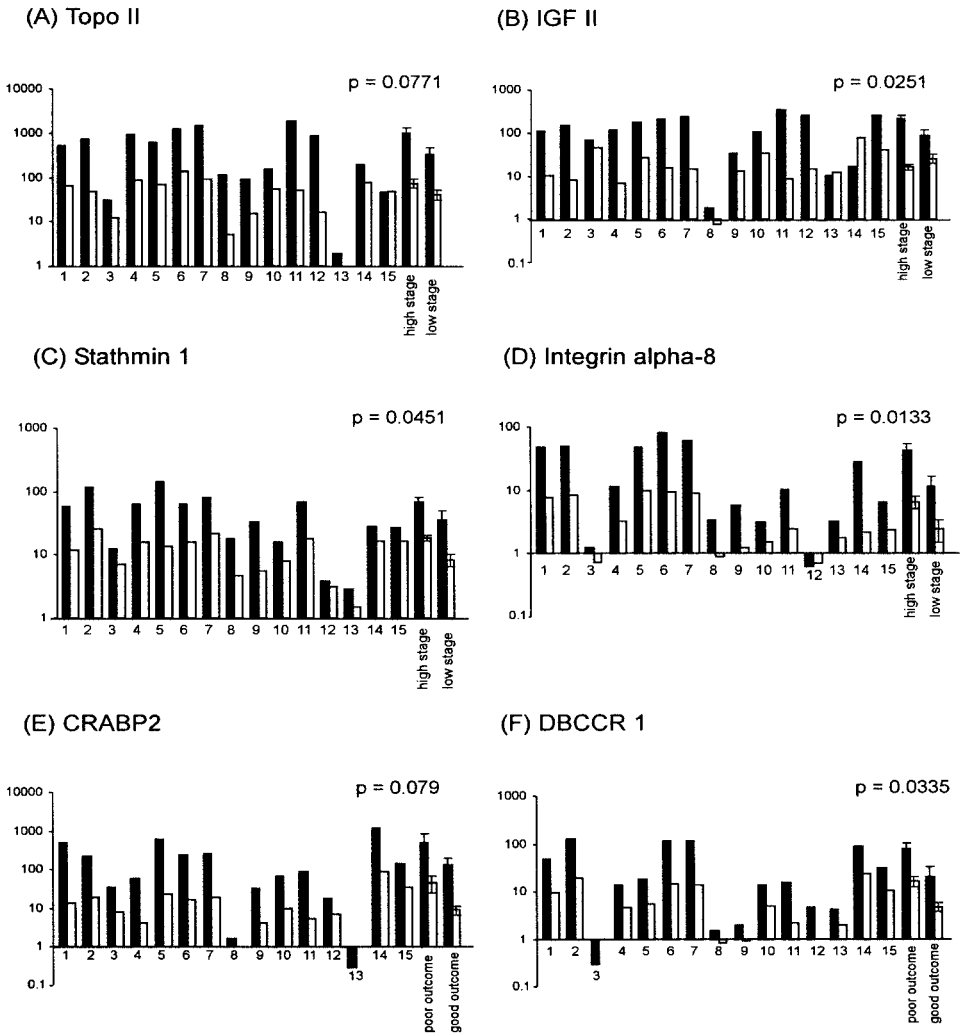

Fig. 3. Real-time relative quantitative PCR analysis of *Topo IIα* (*A*), *IGF II* (IB), *stathmin 1* (*C*), *Integrin α-8* (*D*), *CRABP2* (*E*), *DBCCR1* (*F*). The left side bar in black of each sample, the expression ratio of tumor to noncancerous kidney by real-time PCR; *the right side bar in white*, the expression ratio of tumor to noncancerous kidney by microarray experiments. Data grouped based on clinical parameters are given as the mean and the SE. They are evaluated statistically using the Mann-Whitney *U* test. Real-time PCR was performed in triplicate and each expression data were normalized against the endogenous control, *18S* rRNA. The data were averaged and compared with microarray data. *Topo IIα* and *IGF II* express much higher in WT compared with noncancerous kidney through all of the samples by microarray and real-time PCR. Real-time PCR showed a higher expression ratio of *Topo IIα* ($P = 0.0771$) in high-stage tumors, and *IGF II* was significantly more highly expressed in high-stage tumors ($P = 0.0251$; *A* and *B*). *Stathmin 1* and *integrin α-8* were overexpressed in most WTs and were significantly more highly expressed in high-stage tumors ($P = 0.0451$ and 0.0133, respectively; *C* and *D*). *CRABP2* and *DBCCR1* were also overexpressed in most WTs. *DBCCR1* was significantly more highly expressed in WT with poor outcome compared with reference ($P = 0.0335$; *F*) and *CRABP2* tended to be more highly expressed in WT with poor outcome ($P = 0.079$; *E*). All of the real-time PCR data were consistent with microarray data.

stathmin therapy may be promising agents to improve the cure rate of this tumor.

Integrins are a class of cell surface glycoproteins that mediate cell-cell and cell-extracellular matrix interactions. *Integrin α-8* is expressed particularly in mesenchymal cells bordering on epithelial cell sheets of the kidney. Mutant mice showed defective epithelialization of kidney mesenchymal cells (35). *Integrin α-8* has been found to be the novel target gene of WT1 by the experiment with WT1 expressing fibroblasts (36). In this study, most WT showed high expression of *integrin α-8* compared with reference and are significantly more highly expressed in high-stage tumors, which suggests that *integrin α-8* may be involved in the tumorigenesis and progression of WT.

BNIP3 gene, which had higher expression in the high-stage tumors, is a proapoptotic protein induced by hypoxia and can interact with *Bcl-2*. Interestingly, it has recently been found to be an independent prognostic marker for clinical progression of WTs (8). *MMP 16* is one of the membrane-type MMPs with a transmembrane domain and has been thought to activate *MMP 2* and be related to cancer invasion and metastasis (37). Because these genes have higher expression in the high-stage group, the inhibitors of these genes may serve as good therapeutic targets for advanced disease.

We also examined the specific genes correlated with poor-outcome cases. The list of differentially expressed genes (Table 2) includes some genes that overlap with those on the list for staging. These include *CRABP2* and *heat shock protein* M_r *40,000 protein 1* (*HSPF1*), which may explain their contribution to both tumor progression and metastasis. *CRABP2* is a transcription factor and a regulator of retinoic acid signaling. It was highly expressed in the majority of WTs, which was consistent with previous WT microarray data (21), but had even higher expression in tumors with poor outcome. Another retinol gene, *retinol-binding protein 1* (*RBP-1*) is also highly expressed in the majority of WTs. Recently, the α/β selective retinoids have been shown to dramatically lower the effective dose of paclitaxel to induce cytotoxicity, and this synergy was specific to tubulin-modifying agents (38). That suggests retinoic acid may be a good candidate drug for WT with a combination of conventional chemotherapy.

The *DBCCR1* gene, which was first isolated from chromosome 9q32-q33, is in a frequently deleted region in bladder cancer (39). It is considered a tumor suppressor gene involved in cell cycle control (40). Although the role of *DBCCR1* is unclear, it was significantly more highly expressed in WT with poor outcome and may be one of the prognostic factors. Additional studies are warranted to understand its role in WT.

In conclusion, we have characterized the gene expression profiles of WT, which may reflect the behavior of these tumors. Some of the genes we identified may explain the sensitivity of WT to the present chemotherapy regimen, some may serve as markers for early diagnosis of WT, and some may be potential targets to design new thera-

peutic agents. The sets of differentially expressed genes that correlate with several clinical parameters may explain the underlying molecular mechanisms that contribute to their phenotypes. Obviously, additional molecular, cellular, and even animal studies are warranted to fully make use of this information to understand its pathogenesis and to find a better diagnosis and treatment for this disease.

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Gene Expression Profiling of Favorable Histology Wilms Tumors and Its Correlation with Clinical Features

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