

Loss of Imprinting of Insulin-Like Growth Factor-II (IGF2) Gene in Distinguishing Specific Biologic Subtypes of Wilms Tumor

Jason D. Ravenel, Karl W. Broman, Elizabeth J. Perlman, Emily L. Niemitz, Tilanthi M. Jayawardena, Daphne W. Bell, Daniel A. Haber, Hiroshi Uejima, Andrew P. Feinberg

Background: Loss of imprinting (LOI) of the insulin-like growth factor-II (IGF2) gene, an epigenetic alteration associated with expression of the normally silent maternal allele, was observed first in Wilms tumor. Although LOI has subsequently been detected in most adult tumors, the biologic role of LOI in cancer remains obscure. We analyzed the imprinting status of Wilms tumors with respect to pathologic subtype, stage, and patient's age at diagnosis and examined the expression of genes potentially affected by LOI. **Methods:** Of 60 Wilms tumors examined, 25 were informative for an *ApaI* polymorphism in the IGF2 gene, allowing analysis of allele-specific gene expression, and could be classified by pathologic subtype. Gene expression was measured quantitatively by real-time polymerase chain reaction, and pathologic analysis was blinded for genetic status. All statistical tests were two-sided. **Results:** We observed LOI of IGF2 in nine (90%) of 10 Wilms tumors classified as having a pathologic subtype associated with a later stage of renal development and in only one (6.7%) of 15 Wilms tumors with a pathologic subtype associated with an earlier stage of renal development ($P < .001$). LOI was associated with a 2.2-fold increase (95% confidence interval [CI] = 1.6-fold to 3.1-fold) in IGF2 expression ($P < .001$). Children whose Wilms tumors displayed LOI of IGF2 were statistically significantly older at diagnosis (median = 65 months; interquartile range [IQR] = 47–83 months) than children whose tumors displayed normal imprinting (median = 24 months; IQR = 13–35 months; $P < .001$). **Conclusions:** These data demonstrate a clear relationship between LOI and altered expression of IGF2 in Wilms tumors and provide a molecular basis for understanding the divergent pathogenesis of this cancer. Analysis of LOI could provide a valuable molecular tool for the classification of Wilms tumor. [J Natl Cancer Inst 2001;93:1698–703]

Genomic imprinting is an epigenetic alteration in DNA; i.e., it is stably transmitted during cell division, but it does not involve DNA sequence *per se*. Imprinting of a specific parental allele of a gene in the gamete or zygote leads to the silencing of that allele in somatic cells of the offspring. Thus, imprinting can lead to the complete or partial silencing of one parental allele; consequently, imprinted genes do not follow traditional Mendelian rules of inheritance. Loss of imprinting (LOI) is a common alteration in cancer that typically involves the activation of the normally silent maternal allele of the insulin-like growth factor-II (IGF2) gene, which encodes an important autocrine growth

factor in cancer. However, LOI can also involve silencing of the normally active copy of growth-inhibitory genes, such as p57^{KIP2}, a cyclin-dependent kinase inhibitor. Tumors with LOI of IGF2 display biallelic, rather than monoallelic, expression of the IGF2 gene. Although LOI of IGF2 presumably promotes tumorigenesis by increasing IGF2 expression, that has not been proven previously.

LOI of IGF2 was first described by us and other investigators to occur in Wilms tumor (1–3), the most common solid tumor of childhood. Subsequently, we and others (4–24) have found that LOI of IGF2 is one of the most common molecular alterations in cancer, including other embryonal tumors of childhood, such as hepatoblastoma, rhabdomyosarcoma, and Ewing's sarcoma, and major malignancies in adults, such as uterine, cervical, esophageal, prostate, lung, and germ cell tumors. However, despite the prevalence of this alteration, the biologic role of LOI in cancer remains obscure.

Wilms tumor provides a unique opportunity to address the biologic role of LOI in cancer for several reasons. First, it is the first tumor in which LOI was observed (1,2). Second, Wilms tumors are characterized by two distinct subgroups that are distinguishable both by pathology and by the precursor lesions, termed "nephrogenic rests," from which the tumors arise (25). Third, Wilms tumor was the first type of tumor examined by Knudson to formulate his two-hit hypothesis of cancer to explain the bimodal age distribution of children at diagnosis (26). We, therefore, hoped to relate LOI to the known epidemiology of Wilms tumor.

Intralobar nephrogenic rest (ILNR)-like Wilms tumors contain heterologous elements, such as smooth muscle, cartilage, and adipocytes, and are often associated with ILNRs that arise within the interior of the renal lobe early in fetal development. In contrast, perilobar nephrogenic rest (PLNR)-like Wilms tumors lack these heterologous elements and are often associated with

Affiliations of authors: J. D. Ravenel, E. L. Niemitz, T. M. Jayawardena, H. Uejima, A. P. Feinberg (Institute of Genetic Medicine), E. J. Perlman (Department of Pathology), The Johns Hopkins University School of Medicine, Baltimore, MD; K. W. Broman, Department of Biostatistics, Bloomberg School of Public Health, The Johns Hopkins University; D. W. Bell, D. A. Haber, Massachusetts General Hospital Cancer Center and Harvard Medical School, Charlestown.

Correspondence to: Andrew P. Feinberg, M.D., M.P.H., Institute of Genetic Medicine, The Johns Hopkins University School of Medicine, Ross 1064, 720 Rutland Ave., Baltimore, MD 21205 (e-mail: afeinberg@jhu.edu).

See "Notes" following "References."

© Oxford University Press

PLNRs that arise at the periphery of the renal lobe later in fetal development (25).

We hypothesized that, if LOI of IGF2 is important in Wilms tumorigenesis, LOI might distinguish the two subtypes of Wilms tumors, perhaps providing molecular insight into the pathogenesis of this malignancy. We, therefore, examined the relationship between LOI and the pathologic subtype and stage of Wilms tumor and the patient's age at diagnosis. We also addressed the relationship between LOI in cancer and the abnormal expression of IGF2 and other imprinted genes in the same chromosomal region, including p57^{KIP2}, that might also be affected by LOI, by quantifying and comparing their expression in tumors with LOI with that in tumors with normal imprinting.

MATERIALS AND METHODS

Tissue, DNA, RNA, and Complementary DNA

We obtained snap-frozen surgical specimens of 99 Wilms tumors and matched normal kidneys from the same patients from the National Wilms Tumor Study tissue bank (Alberta, Canada) and the Cooperative Human Tissue Network (Columbus, OH). Pathology reports and information on Wilms tumor stage and the patient's age at diagnosis, but no patient identifiers, were provided with the tissue specimens. The Study pathologist (E. J. Perlman) examined all of the tissue samples to verify their identity and homogeneity. PLNR lesions were isolated by microdissecting matched normal kidney tissue.

Homogenized tissue samples were digested overnight at 55 °C with 10 mg/mL proteinase K, and genomic DNA was isolated from them with the use of the Puregene DNA Isolation Kit (Gentra Systems, Research Triangle Park, NC) according to the manufacturer's instructions and resuspended in 10 mM Tris-HCl and 1 mM EDTA (pH 8.0). Total RNA was isolated from homogenized tissue samples with the use of the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and resuspended in 50 µL ribonuclease (RNase)-free water. Twenty-four microliters of RNA was digested with 3 U of Amplification Grade Deoxyribonuclease I (Life Technologies, Inc. [GIBCO BRL], Rockville, MD) in 30 µL according to the manufacturer's instructions. We synthesized complementary DNA (cDNA) in a 40-µL reaction volume that included 5 µL of deoxyribonuclease (DNase) I-digested RNA and 200 ng of random hexamers and either contained or lacked 200 U Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) for 2 hours at 37 °C. cDNA was diluted to a final volume of 200 µL with DNase-free and RNase-free water.

Quantitation of Allele-Specific IGF2 RNA Expression to Determine Imprinting Status

We determined the imprinting status of IGF2 by measuring the relative abundance of the two alleles of IGF2 with the use of cDNA derived from Wilms tumor and normal kidney specimens and the Hot-Stop polymerase chain reaction (PCR) method followed by *ApaI* digestion (27). We amplified 1 µL of each cDNA in a 10-µL PCR reaction that contained 1 pmol each of the unlabeled oligonucleotide primers P3 (5'-CTTGACTTTGAGTCAAATTGG-3') and P4 (5'-GGTCGTGCCAATTACATTTCA-3'), which are complementary to exon 9 of IGF2, 0.4 µL of the deoxynucleoside triphosphates (2.5 mM each of deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate), 0.6 µL of MgCl₂, 1 µL of 10× PCR Buffer (Applied Biosystems, Foster City, CA), and 0.5 U AmpliTaq Polymerase (Applied Biosystems). PCR cycle conditions were as follows: denaturation for 2 minutes at 94 °C and 35 amplification cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds, followed by a final elongation step of 10 minutes at 72 °C. An additional primer extension cycle was performed with the use of 1 pmol of ³²P end-labeled oligonucleotide primer P4. We end-labeled 20 pmol of oligonucleotide primer P4 in a 20-µL reaction mixture that contained 4 µL of 5× Forward Reaction Buffer (Invitrogen Corp., Carlsbad, CA), 10 U of T4 polynucleotide kinase (Life Technologies, Inc.), and 25 µCi [³²P]adenosine triphosphate (Amersham Pharmacia, Piscataway, NJ) for 10 minutes at 37 °C and then heat-inactivated the kinase by incubating the reaction mixture for 10 minutes at 70 °C. The PCR products were then digested at 37 °C overnight in a 10-µL volume that contained 1 µL of PCR product, 0.5 µL of 10× *ApaI* diges-

tion buffer, and 10 U of *ApaI* (Stratagene, La Jolla, CA). We combined 5 µL of *ApaI*-digested PCR product with an equal amount of Gel Loading Buffer II (Ambion, Austin, TX), incubated the mixture for 5 minutes at 95 °C, and resolved the DNAs on a 6% acrylamide-8M urea gel for 1.5 hours at 75 W. Labeled PCR products were visualized by autoradiography and quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Loss of Heterozygosity Analysis of Wilms Tumors

Loss of heterozygosity (LOH) for 11p was determined by a comparison of tumor and normal DNA at the same *ApaI* polymorphism used for IGF2 analysis. Samples that were not informative for this marker were examined with the use of the microsatellite marker D11S922 (AFM217yb10, UniSTS). Genomic DNA (100 ng) was PCR amplified including a fluorescently labeled primer. PCR products were separated on an ABI Prism 377 Sequencer and analyzed with Genotyper Software (Applied Biosystems).

Mutational Analysis of the WT1 Gene

The coding region of WT1 that encompasses exons 1 through 10 was amplified in two overlapping fragments by reverse transcription (RT)-PCR with the use of 50 ng of cDNA as a template and two sets of oligonucleotide primer pairs. Primers 2F (5'-CCAGTTCAGTGGCACAGCCGGAGCCTGTGCG-3') and 2R (5'-GTACCCTGTGCTGTGGTTGCTCTGCCCTTC-3') were used to produce WT1 fragment 2, which contains the transactivation domain; primers 3F (5'-GCCACCTTAAAGGGAGTTGCTGCTGGGAGC-3') and 3R (5'-CTTGGAAGTTGGATGAAGAAGATCAACTG-3') were used to produce WT1 fragment 3, which contains the zinc finger domain. The following conditions were used for PCR amplification: 95 °C for 2 minutes, followed by 35 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 1 minute. Because of its high GC content, exon 1 of WT1 was amplified from genomic DNA in a PCR reaction mixture that included 10% dimethyl sulfoxide and oligonucleotide primers 1F (5'-GTGCCTACAGCAGCCAGAGCAGCAGGGA-GTC-3') and 1R (5'-GATTGCGAATAGCGGGCTGGCTCTCGAGG-3'). Amplification conditions were 95 °C for 2 minutes, followed by 35 cycles of 95 °C for 30 seconds, 57 °C for 30 seconds, and 72 °C for 45 seconds.

PCR products were resolved by gel electrophoresis, eluted from the gel, and sequenced with the use of BigDye Terminator Cycle sequencing chemistry (Applied Biosystems) on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Fragment 1 was sequenced with the use of primers 1F and 1R, fragment 2 was sequenced with the use of primers 2F and 2R, and fragment 3 was sequenced with the use of primers 3F, 3R, and 3aR (5'-GTATGAGTCCTGTGTGGTCTTCAGGTGG-3').

Real-Time Quantitative PCR Analysis to Measure Gene Expression

Real-time quantitative PCR was performed in triplicate in 96-well plates; each reaction consisted of 12.5 µL of Taqman Master Mix (Applied Biosystems), 2.5 µL each of 900 nM forward and reverse primers, 2.5 µL of 200 nM Taqman probe, and 5 µL of cDNA. Sequences of the primers and probes that were designed to span and detect intron-exon boundaries of IGF2 were as follows: 5'-CCCCTCGACCGTGCT-3' (forward primer), 5'-TCATATTGGAAGAACTTGCCCA-3' (reverse primer), and VIC-5'-CCGGACAACCTCCCCGAGATACCC-3'-TAMRA (probe). The primers and probe for β-actin were purchased from Applied Biosystems. The sequences of the primers and probes used to amplify and detect expression of p57^{KIP2}, WT1, TSSC3, TSSC4, TSSC5, TSSC6, and TAPA1 are provided in supplementary Table 1 at the Journal's Web site (<http://jnci.oup-journals.org>). Each 96-well real-time quantitative PCR plate included serial dilutions of fetal kidney cDNA, which were used to generate a standard curve for gene expression. The level of IGF2 gene expression was normalized to that of β-actin, after it was demonstrated that there was no statistically significant difference in the expression of IGF2 compared with 10 other housekeeping genes assayed in the multiendogenous control plate (Applied Biosystems) in any of the tissues examined. Expression analysis was performed on an Applied Biosystems Prism 7700 Sequence Detection System. We confirmed reproducibility by comparing the experimental results obtained by two investigators several weeks apart ($r = .97$).

Statistical Analysis

Statistical significances of differences were assessed by two-sided Fisher's exact tests (for proportions) or permutation tests with the *t* statistic (for continu-

ous variables). In the permutation tests [see (28)], we shuffled (randomized) group status relative to the quantitative variable and calculated a two-sample *t* statistic. This procedure was repeated for a total of 10 000 replicates. The cited *P* value was the proportion of the replicates for which the absolute value of the *t* statistic based on randomized data exceeded the absolute value of the observed *t* statistic. We used permutation tests to avoid assumptions of normality. However, we calculated confidence intervals (CIs) by using the normal assumptions; this inconsistency is not a concern, because the *P* values obtained by the permutation tests were virtually indistinguishable from those calculated via the *t* distribution. All statistical tests were two-sided and were performed with the statistical software R (52), version 1.3.1 (see: <http://www.R-project.org>).

RESULTS

Relationship Between LOI and Wilms Tumor Pathology

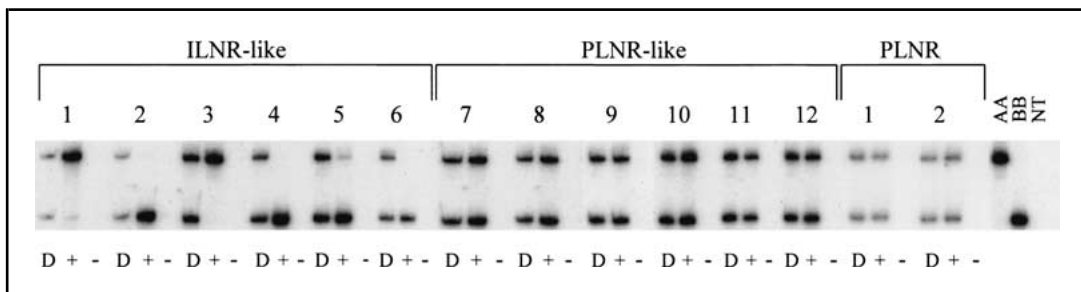
To analyze imprinting of IGF2, we first identified 60 Wilms tumors that could be classified as ILNR-like or PLNR-like. Of these, 36 tumors were heterozygous for a transcribed *ApaI* polymorphism (1) in IGF2, allowing us to distinguish between the two alleles of the gene and subsequently to determine their imprinting status. Using a quantitative PCR assay (27), we analyzed imprinting of these 36 tumors. Tumors with normal imprinting (*n* = 21) showed essentially monoallelic expression of IGF2 (i.e., a >5:1 ratio of either of the two alleles), whereas tumors with LOI (*n* = 15) showed equal biallelic expression of the two alleles of IGF2. The tumors also underwent pathologic evaluation by the chief pathologist for the National Wilms Tumor Study (E. J. Perlman). The tumors were scored as objectively as possible, with the use of a double-blinded analysis and conservative criteria (i.e., presence or absence of heterologous elements and presence or absence of nephrogenic rests) to classify them as ILNR-like or PLNR-like. Currently, only approximately 60% of Wilms tumors can be classified into these two groups with the use of conventional histologic approaches; thus, a molecular tool that could improve this rate of classification would be of great value clinically. We found that 25 of the 36 tumors informative for IGF2 imprinting could be classified as being either ILNR-like or PLNR-like. It was remarkable that there was virtually complete segregation of ILNR-like and PLNR-like tumors, depending on the imprinting status, with nine (90%) of 10 PLNR-like tumors showing LOI but only one (6.7%) of 15 ILNR-like tumors showing LOI (Fig. 1). Examples of ILNR-like tumors with normal imprinting and PLNR-like tumors with LOI are shown in Fig. 1. This difference in the IGF2-imprinting status between these two pathologic subtypes of Wilms tumors was statistically significant (*P* < .001; Fisher's

exact test), indicating a fundamental biologic difference between Wilms tumors with LOI of IGF2 and those with normal imprinting. LOI was not associated with the stage of Wilms tumor and, thus, was not related to tumor progression.

Wilms tumor was used originally as a model of Knudson two-hit kinetics (26,29) for tumor suppressor genes because mutations (e.g., in WT1) and consistent LOH, particularly of chromosome 11p, were observed in these tumors (30,31). Because we found that a subtype of Wilms tumor displayed epigenetic alterations (i.e., LOI), we naturally asked which of the 36 tumors showed classic genetic changes (i.e., LOH and WT1 mutations). The frequency of LOH of 11p is approximately 30% in Wilms tumor, and 11p LOH represents the majority of LOH in Wilms tumor (31,32). Although the presence of LOH suggests loss of a tumor suppressor gene following Knudson's model (29,31), the absence of LOH does not rule that out. Nevertheless, analysis of LOH should give an indication of the type of tumors that show conventional genetic alterations (LOH). Among the 35 Wilms tumors that could be scored as either ILNR-like or PLNR-like and that were informative for polymorphisms allowing analysis of LOH, nine (26%) showed LOH of 11p and 26 (74%) did not (data not shown). Remarkably, all nine (100%) of the tumors that showed LOH of 11p were ILNR-like, while only 15 (58%) of the 26 tumors that showed no LOH of 11p were ILNR-like (*P* = .03; Fisher's exact test). Furthermore, none of 12 PLNR-like tumors showed LOH. This is consistent with an earlier observation that tumors with ILNRs are associated with LOH (33). Therefore, just as PLNR-like tumors are predominantly associated with LOI, ILNR-like tumors are predominantly associated with LOH.

To determine whether there was overlap between those tumors with abnormal imprinting and those with WT1 mutations, we also examined Wilms tumors directly for the presence of specific inactivating mutations in WT1. We found that only one (6%) of 16 tumors had such a mutation (data not shown). This frequency of mutated WT1 in patients with Wilms tumors is consistent with that reported in a previous study (34). The WT1 mutation that we identified was a tandem duplication of 46 nucleotides inserted within the stop codon, and it resulted in a codon frameshift that would be expected to generate a WT1-like protein containing 83 additional amino acids at the C-terminus. This mutation occurred in an ILNR-like tumor that displayed LOH of 11p. Thus, there was virtually complete segregation between, on the one hand, PLNR-like tumors and LOI and, on

Fig. 1. Loss of imprinting is associated with a specific pathologic subtype of Wilms tumor. Intralobar nephrogenic rest (ILNR)-like tumors showed normal imprinting of the insulin-like growth factor-II (IGF2) gene (lanes 1–6), and periobar nephrogenic rest (PLNR)-like tumors showed loss of imprinting (LOI) (lanes 7–12). Hot-Stop polymerase chain reaction (PCR) of genomic DNA (D) and complementary DNA (cDNA) generated from RNA by reverse transcription-PCR with (+) and without (–) reverse transcriptase (control) are shown. Controls include genomic DNA homozygous for the undigested allele (AA), homozygous for the digested allele (BB), and no template (NT). All of the samples were digested with *ApaI*. Two bands of equal intensity were detected in the genomic DNA of all tumor samples, indicating the presence of both alleles of IGF2. Only one allele was observed in the cDNA derived from tumors with normal imprinting of IGF2, and both alleles were observed at equal intensities in the cDNA derived from tumors with LOI.



the other hand, ILNR-like tumors with normal imprinting, LOH, and WT1 mutations.

Relationship Between LOI and Gene Expression in Wilms Tumors

Despite extensive evidence that LOI occurs in a variety of cancers, it has been difficult to directly link altered expression of specific genes to LOI because of problems related to the quantification of relative levels of specific messenger RNAs (mRNAs). We, therefore, designed real-time quantitative PCR assays to quantify the mRNA expression levels of eight genes that have been directly or indirectly linked to Wilms tumor in samples of Wilms tumor. We assayed the expression of the following genes: IGF2, WT1, p57^{KIP2}, TSSC3, TSSC4, TSSC5, TSSC6, and TAPA1. p57^{KIP2}, which encodes a cyclin-dependent kinase inhibitor, is rarely mutated in Beckwith–Wiedemann syndrome (35,36), a genetic disorder that predisposes individuals to Wilms tumor. TSSC3, TSSC4, TSSC5, TSSC6, and TAPA1 are genes that lie within a region of chromosome 11, 11p15, that has been shown by microcell-mediated gene transfer to harbor a tumor suppressor gene (37). Previous studies (6,8,38,39) have not demonstrated a statistically significant difference in the expression of any of these genes in Wilms tumor or other cancers in a comparison of tumors with and without LOI of IGF2. However, those studies used techniques, such as conventional semiquantitative RT-PCR, northern blots, and RNase protection assays, that lack the sensitivity of real-time quantitative PCR (40) and also failed to link changes in IGF2 expression with imprinting status in Wilms tumors (38,39).

We examined 28 Wilms tumor samples that were informative for the IGF2 *ApaI* polymorphism, i.e., in which we could determine the imprinting status of IGF2, and found a 2.2-fold (95% confidence interval [CI] = 1.6-fold to 3.1-fold) increase in IGF2 expression in tumors with LOI compared with those with normal imprinting of IGF2 (Table 1). Whereas the magnitude of the difference in IGF2 expression was small, it was both statistically significant ($P < .001$) and consistent with the activation of the normally silent allele in tumors with LOI. None of the other genes showed a statistically significant difference in expression in tumors with LOI compared with those with normal imprinting of IGF2 (Table 1), suggesting that the alteration in IGF2 expres-

sion is specific for Wilms tumors with LOI and indicating a second fundamental difference in the biology of Wilms tumors with and without LOI.

Relationship Between LOI and Patient's Age at Diagnosis of Wilms Tumor

Patients with Wilms tumor show a bimodal distribution in the age of onset of their tumors, but this has not been fully explained previously. We found a striking difference between the median age at diagnosis of patients who had Wilms tumors with LOI of IGF2 and the median age of patients who had Wilms tumors with normal imprinting. Patients whose tumors showed LOI had a median age at diagnosis (interquartile range [IQR]) of 65 months (47–83 months), which was greater than twice that of patients whose tumors showed normal imprinting, who had a median age at diagnosis (IQR) of 24 months (13–35 months). This difference in median age of occurrence was statistically significant ($P < .001$), and it indicates a third fundamental difference in the biology of Wilms tumors in these two groups of patients with and without LOI in their tumors. The relationship of LOI to age was not related to tumor progression, since it was independent of the stage of Wilms tumor (data not shown).

Relationship Between LOI and Nephrogenic Rests

Because LOI appears to distinguish Wilms tumors with PLNR-like pathology from those with ILNR-like pathology, we hypothesized that LOI might be present in the precursor lesions (i.e., the PLNRs themselves). To test this hypothesis, we microdissected normal tissue from a single kidney from a patient with Wilms tumor and identified two PLNRs, both of which were informative for the *ApaI* polymorphism in IGF2. Both of these rests showed LOI of IGF2 (Fig. 1), confirming that LOI is an early event in Wilms tumorigenesis, in that it occurs in the premalignant lesions themselves.

DISCUSSION

In summary, we found that Wilms tumors with LOI of IGF2 differ in three biologically significant ways from Wilms tumors with normal imprinting: pathologic subtype, expression of IGF2, and age of the patient at diagnosis. Furthermore, we found that tumors with LOI of IGF2 were predominantly PLNR-like, whereas tumors with LOH of chromosome 11p were predominantly ILNR-like. We and other investigators (41,42) have previously linked mutations and reduced expression of WT1 to tumors with ILNR-like pathology and/or heterotypic elements, such as muscle. Here, we show that LOI and increased expression of IGF2 are common features of Wilms tumors with PLNR-like pathology. Of course, these results do not exclude other mechanisms, such as a potential tumor suppressor on chromosome 16q (43) or an as-yet unidentified familial Wilms tumor gene (44), that have also been implicated in the etiology of Wilms tumorigenesis. Moreover, the observation that some Wilms tumors show features of mixed pathology is consistent with the coexistence of independent mechanisms of tumorigenesis within a single tumor. Nevertheless, to our knowledge, this is the first demonstration that a specific pathologic subtype of any cancer is associated with LOI of a specific gene.

Fig. 2 shows a molecular model for the divergent pathogenesis of Wilms tumor that incorporates the present discovery and results from earlier studies of the molecular biology and pathology of this cancer (41,42). According to this model, WT1 mutations, decreased expression of WT1, or other classic tumor suppressor

Table 1. Relationship between loss of imprinting of IGF2 and gene expression in Wilms tumor*

Gene	Fold change in gene expression in Wilms tumors with LOI versus Wilms tumors with normal imprinting†	95% CI	P‡
IGF2	2.2	1.6 to 3.1	<.001
TAPA1	1.0	0.8 to 1.2	.68
TSSC3	1.0	0.4 to 2.2	.97
TSSC4	1.2	0.7 to 2.0	.42
TSSC5	1.1	0.6 to 2.0	.54
TSSC6	0.8	0.4 to 1.8	.61
WT1	1.3	0.8 to 2.2	.33
p57 ^{KIP2}	0.8	0.4 to 1.5	.51

*IGF2 = insulin-like growth factor-II gene; LOI = loss of imprinting; CI = confidence interval.

†Fold change in gene expression, with the expression in tumors with LOI divided by expression in tumors with normal imprinting. Expression of each was determined by quantitative real-time polymerase chain reaction and normalized to β -actin gene expression.

‡Statistical significance was determined with the use of a permutation test.

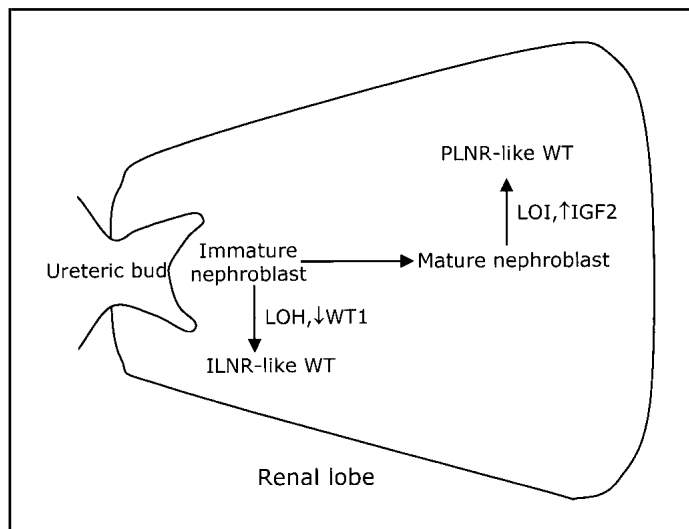


Fig. 2. Molecular model for the divergent pathogenesis of Wilms tumor (WT). Immature nephroblasts develop in proximity to the ureteric bud and migrate to the periphery of the developing renal lobe as they mature. WT1 mutations, decreased WT1 expression, and 11p loss of heterozygosity (LOH) interfere with maturation and lead to intralobar nephrogenic rest (ILNR)-like Wilms tumor derived from pluripotent nephroblasts, accounting for the presence of heterotypic elements, such as muscle and cartilage. In contrast, loss of imprinting of the insulin-like growth factor-II (IGF2) gene in mature nephroblasts leads to increased IGF2 expression, a failure of proliferation arrest, and the development of perilobar nephrogenic rest (PLNR)-like tumors at the periphery of the renal lobe.

gene alterations associated with LOH interfere with the maturation of immature nephroblasts at the interior of the developing renal lobe, leading to ILNR-like Wilms tumors. In contrast, LOI in mature nephroblasts leads to the increased expression of IGF2, a failure of proliferation arrest, and the development of PLNR-like tumors at the periphery of the renal lobe. Here, we show for the first time a clear relationship between LOI and IGF2 overexpression. IGF2 is a mitogen that is normally expressed during the proliferative stage of kidney development, in the periphery of the developing renal lobe, where PLNR-like tumors arise. Furthermore, the regions within the kidney that have increased IGF2 expression are those that show increased proliferation in patients with Beckwith–Wiedemann syndrome and LOI of IGF2 (45).

This division of Wilms tumor into two biologic subtypes according to pathology is consistent with what we know of two rare hereditary disorders that predispose individuals to Wilms tumors. Denys–Drash syndrome, which involves germline mutations in WT1 (30), leads to a failure of normal renal development and renal dysplasia consistent with a developmental block in nephrogenesis and ILNR-like tumors. In contrast, Beckwith–Wiedemann syndrome, which can involve either LOI of IGF2 or mutations in p57^{KIP2} (4), leads to prenatal and postnatal overgrowth of relatively mature kidney, consistent with the continued proliferation of mature nephroblasts seen in PLNR-like tumors. Also, consistent with this division, ILNRs, with or without Wilms tumor, are relatively common in Denys–Drash syndrome, and PLNRs are often found in Beckwith–Wiedemann syndrome (46).

These results also support a somewhat provocative view of Breslow and colleagues (46–48), who analyzed the two-hit kinetics first proposed by Knudson and Strong (26) in 1972 to explain the bimodal age distribution of patients who develop Wilms tumor.

According to Knudson's model, bilateral Wilms tumors arise in younger individuals than do unilateral tumors because individuals with bilateral tumors have a germline mutation of a tumor suppressor gene and those with unilateral tumors have no such germline mutation (26,29). While that is true, the model does not account for the fact that bilateral Wilms tumors are uncommon [7% incidence overall (46) and only one bilateral case among our study subjects] and it does not explain the observed bimodal age distribution of an unselected group of Wilms tumor patients in the general population, i.e., those with primarily unilateral tumors. Our results support the somewhat provocative study of Breslow and colleagues (46,48) that analyzed Knudson's model. Breslow et al. (47) observed that patients with PLNRs develop tumors at a later age than those with ILNRs. Given that rest-associated tumors constitute a minority of those for which we had imprinting data and that such tumors did not alone account for the bimodal age distribution, we suggest that distinguishing between tumors with or without LOI validates observations by Breslow et al. and provides a biologic foundation for them.

In conclusion, this study demonstrates a striking relationship between LOI and Wilms tumor biology that may distinguish the tumors by their pathology, the premalignant lesions associated with them, and the epidemiology and the genetics of Wilms tumors. Finally, because only approximately 60% of Wilms tumors can presently be classified into ILNR-like or PLNR-like subtypes by histologic examination, this study should provide a valuable molecular tool for Wilms tumor evaluation, similar to that provided by the molecular studies of B-cell lymphoma (49).

REFERENCES

- (1) Rainier S, Johnson LA, Dobry CJ, Ping AJ, Grundy PE, Feinberg AP. Relaxation of imprinted genes in human cancer. *Nature* 1993;362:747–9.
- (2) Ogawa O, Eccles MR, Szeto J, McNoe LA, Yun K, Maw MA, et al. Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumour. *Nature* 1993;362:749–51.
- (3) Feinberg AP, Rainier S, DeBaun MR. Genomic imprinting, DNA methylation, and cancer. *J Natl Cancer Inst Monogr* 1995;17:21–6.
- (4) Feinberg AP. Genomic imprinting and cancer. In: Scriver C, editor. *The metabolic and molecular bases of inherited disease*. New York (NY): McGraw-Hill; 2001. p. 525–37.
- (5) Rainier S, Dobry CJ, Feinberg AP. Loss of imprinting in hepatoblastoma. *Cancer Res* 1995;55:1836–8.
- (6) Li X, Adam G, Cui H, Sandstedt B, Ohlsson R, Ekstrom TJ. Expression, promoter usage and parental imprinting status of insulin-like growth factor II (IGF2) in human hepatoblastoma: uncoupling of IGF2 and H19 imprinting. *Oncogene* 1995;11:221–9.
- (7) Zhan SL, Shapiro DN, Helman LJ. Activation of an imprinted allele of the insulin-like growth factor II gene implicated in rhabdomyosarcoma. *J Clin Invest* 1994;94:445–8.
- (8) Zhan S, Shapiro DN, Helman LJ. Loss of imprinting of IGF2 in Ewing's sarcoma. *Oncogene* 1995;11:2503–7.
- (9) Vu TH, Yballe C, Boonyanit S, Hoffman AR. Insulin-like growth factor II in uterine smooth-muscle tumors: maintenance of genomic imprinting in leiomyomata and loss of imprinting in leiomyosarcomata. *J Clin Endocrinol Metab* 1995;80:1670–6.
- (10) Douc-Rasy S, Barrois M, Fogel S, Ahomadegbe JC, Stehelin D, Coll J, et al. High incidence of loss of heterozygosity and abnormal imprinting of H19 and IGF2 genes in invasive cervical carcinomas. Uncoupling of H19 and IGF2 expression and biallelic hypomethylation of H19. *Oncogene* 1996;12:423–30.
- (11) Hibi K, Nakamura H, Hirai A, Fujikake Y, Kasai Y, Akiyama S, et al. Loss of H19 imprinting in esophageal cancer. *Cancer Res* 1996;56:480–2.
- (12) Takeda S, Kondo M, Kumada T, Koshikawa T, Ueda R, Nishio M, et al. Allelic-expression imbalance of the insulin-like growth factor 2 gene in

- hepatocellular carcinoma and underlying disease. *Oncogene* 1996;12:1589–92.
- (13) Hashimoto K, Azuma C, Koyama M, Ohashi K, Kamiura S, Nobunaga T, et al. Loss of imprinting in choriocarcinoma. *Nat Genet* 1995;9:109–10.
- (14) van Gurp RJ, Oosterhuis JW, Kalscheuer V, Mariman EC, Looijenga LH. Biallelic expression of the H19 and IGF2 genes in human testicular germ cell tumors. *J Natl Cancer Inst* 1994;86:1070–5.
- (15) Jarrard DF, Bussemakers MJ, Bova GS, Isaacs WB. Regional loss of imprinting of the insulin-like growth factor II gene occurs in human prostate tissues. *Clin Cancer Res* 1995;1:1471–8.
- (16) Brunner N, Yee D, Kern FG, Spang-Thomsen M, Lippman ME, Cullen KJ. Effect of endocrine therapy on growth of T61 human breast cancer xenografts is directly correlated to a specific down-regulation of insulin-like growth factor II (IGF-II). *Eur J Cancer* 1993;29A:562–9.
- (17) Yee D, Cullen KJ, Paik S, Perdue JF, Hampton B, Schwartz A, et al. Insulin-like growth factor II mRNA expression in human breast cancer. *Cancer Res* 1988;48:6691–6.
- (18) El-Badry OM, Minniti C, Kohn EC, Houghton PJ, Daughaday WH, Helman LJ. Insulin-like growth factor II acts as an autocrine growth and motility factor in human rhabdomyosarcoma tumors. *Cell Growth Differ* 1990;1:325–31.
- (19) El-Badry OM, Helman LJ, Chatten J, Steinberg SM, Evans AE, Israel MA. Insulin-like growth factor II-mediated proliferation of human neuroblastoma. *J Clin Invest* 1991;87:648–57.
- (20) Gelato MC, Vassalotti J. Insulin-like growth factor-II: possible local growth factor in pheochromocytoma. *J Clin Endocrinol Metab* 1990;71:1168–74.
- (21) Lahm H, Amstad P, Wyniger J, Yilmaz A, Fischer JR, Schreyer M, et al. Blockade of the insulin-like growth-factor-I receptor inhibits growth of human colorectal cancer cells: evidence of a functional IGF-II-mediated autocrine loop. *Int J Cancer* 1994;58:452–9.
- (22) Lamonerie T, Lavialle C, Haddada H, Brison O. IGF-2 autocrine stimulation in tumorigenic clones of a human colon-carcinoma cell line. *Int J Cancer* 1995;61:587–92.
- (23) Leventhal PS, Randolph AE, Vesbit TE, Schenone A, Windebank A, Feldman EL. Insulin-like growth factor-II as a paracrine growth factor in human neuroblastoma cells. *Exp Cell Res* 1995;221:179–86.
- (24) Osborne CK, Coronado EB, Kitten LJ, Arteaga CI, Fuqua SA, Ramasharma K, et al. Insulin-like growth factor-II (IGF-II): a potential autocrine/paracrine growth factor for human breast cancer acting via the IGF-I receptor. *Mol Endocrinol* 1989;3:1701–9.
- (25) Beckwith JB, Kiviat NB, Bonadio JF. Nephrogenic rests, nephroblastomatosis, and the pathogenesis of Wilms' tumor. *Pediatr Pathol* 1990;10:1–36.
- (26) Knudson AG, Strong LC. Mutation and cancer: a model for Wilms' tumor of the kidney. *J Natl Cancer Inst* 1972;48:313–24.
- (27) Uejima H, Lee MP, Cui H, Feinberg AP. Hot-stop PCR: a simple and general assay for linear quantitation of allele ratios. *Nat Genet* 2000;25:375–6.
- (28) Becker RA, Chambers JM, Wilks AR. *The new S language*. New York (NY): Chapman & Hall; 1988.
- (29) Knudson AG Jr. Hereditary cancer, oncogenes, and antioncogenes. *Cancer Res* 1985;45:1437–43.
- (30) Pelletier J, Bruening W, Li FP, Haber DA, Glaser T, Housman DE. WT1 mutations contribute to abnormal genital system development and hereditary Wilms' tumour. *Nature* 1991;353:431–4.
- (31) Reeve AE, Sih SA, Raizis AM, Feinberg AP. Loss of allelic heterozygosity at a second locus on chromosome 11 in sporadic Wilms' tumor cells. *Mol Cell Biol* 1989;9:1799–803.
- (32) Mannens M, Devilee P, Bliker J, Mandjes I, de Kraker J, Heyting C, et al. Loss of heterozygosity in Wilms' tumors, studied for six putative tumor suppressor regions, is limited to chromosome 11. *Cancer Res* 1990;50:3279–83.
- (33) Grundy P, Telzerow P, Moksness J, Breslow NE. Clinicopathologic correlates of loss of heterozygosity in Wilms' tumor: a preliminary analysis. *Med Pediatr Oncol* 1996;27:429–33.
- (34) Bickmore WA, Oghene K, Little MH, Seawright A, van Heyningen V, Hastie ND. Modulation of DNA binding specificity by alternative splicing of the Wilms tumor wt1 gene transcript. *Science* 1992;257:235–7.
- (35) Lee MP, DeBaun M, Randhawa GS, Reichard BA, Elledge SJ, Feinberg AP. Low frequency of p57^{KIP2} mutation in Beckwith–Wiedemann syndrome. *Am J Hum Genet* 1997;61:304–9.
- (36) Hatada I, Ohashi H, Fukushima Y, Kaneko Y, Inoue M, Komoto Y, et al. An imprinted gene p57KIP2 is mutated in Beckwith–Wiedemann syndrome. *Nat Genet* 1996;14:171–3.
- (37) Koi M, Johnson LA, Kalikin LM, Little PF, Nakamura Y, Feinberg AP. Tumor cell growth arrest caused by subchromosomal transferable DNA fragments from human chromosome 11. *Science* 1993;260:361–4.
- (38) Steenman MJ, Rainier S, Dobry CJ, Grundy P, Horon IL, Feinberg AP. Loss of imprinting of IGF2 is linked to reduced expression and abnormal methylation of H19 in Wilms' tumour. *Nat Genet* 1994;7:433–9.
- (39) Nonomura N, Nishimura N, Miki T, Kanno N, Kojima Y, Yokoyama M, et al. Loss of imprinting of the insulin-like growth factor II gene in renal cell cancer. *Cancer Res* 1997;57:2575–7.
- (40) Wang T, Brown MJ. mRNA quantification by real time TaqMan polymerase chain reaction: validation and comparison with RNase protection. *Anal Biochem* 1999;269:198–201.
- (41) Huang A, Campbell CE, Bonetta L, McAndrews-Hill MS, Chilton-MacNeill S, Coppes MJ, et al. Tissue, developmental, and tumor-specific expression of divergent transcripts in Wilms tumor. *Science* 1990;250:991–4.
- (42) Miyagawa K, Kent J, Moore A, Charlier JP, Little MH, Williamson KA, et al. Loss of WT1 function leads to ectopic myogenesis in Wilms' tumour. *Nat Genet* 1998;18:15–7.
- (43) Maw MA, Grundy PE, Millow LJ, Eccles MR, Dunn RS, Smith PJ, et al. A third Wilms' tumor locus on chromosome 16q. *Cancer Res* 1992;52:3094–8.
- (44) Grundy P, Koufos A, Morgan K, Li FP, Meadows AT, Cavenee W. Familial predisposition to Wilms' tumour does not map to the short arm of chromosome 11. *Nature* 1988;336:374–6.
- (45) Hedborg F, Holmgren L, Sandstedt B, Ohlsson R. The cell type-specific IGF2 expression during early human development correlates to the pattern of overgrowth and neoplasia in the Beckwith–Wiedemann syndrome. *Am J Pathol* 1994;145:802–17.
- (46) Breslow NE, Beckwith JB. Epidemiological features of Wilms' tumor: results of the National Wilms' Tumor Study. *J Natl Cancer Inst* 1982;68:429–36.
- (47) Breslow N, Beckwith JB, Ciol M, Sharples K. Age distribution of Wilms' tumor: report from the National Wilms' tumor study. *Cancer Res* 1988;48:1653–7.
- (48) Pritchard-Jones K, Breslow N. Wilms tumour and other childhood renal tumours. 2001. In: *Eeles RA, Easton DF, Eng C, Ponder B, editors. Genetic predisposition to cancer*. London (U.K.): Arnold; 2001.
- (49) Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000;403:503–11.

NOTES

Supported by Public Health Service grant CA65145 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services. H. Uejima was supported by a fellowship from the Japan Society for the Promotion of Science.

We thank Hengmi Cui (The Johns Hopkins University, Baltimore, MD) for helpful discussion and Tracy Litz (The Johns Hopkins University) for technical assistance.

Manuscript received September 7, 2001; revised October 12, 2001; accepted October 15, 2001.