Increased Expression of the Insulin-like Growth Factor-II Gene in Wilms’ Tumor Is Not Dependent on Loss of Genomic Imprinting or Loss of Heterozygosity*

Wan-Heng Wang, Jian-Xiong Duan, Thanh H. Vu, and Andrew R. Hoffman†

From the Medical Service and GRECC, Veterans Affairs Palo Alto Health Care System and Department of Medicine, Stanford University School of Medicine, Palo Alto, California 94304

Loss of imprinting of insulin-like growth factor-II gene (IGF2) and/or loss of heterozygosity at the 11p15 loci have been postulated to be responsible for IGF2 overexpression in Wilms’ tumor. In order to delineate the mechanism of IGF2 overexpression in Wilms’ tumors, we have genotyped the 11p15-11p13 chromosomal region and determined allelic expression of IGF2 and H19 in both tumor tissue and in normal adjacent kidney tissue from 40 patients with Wilms’ tumor. In five of the eight subjects informative for the Apa I IGF2 polymorphism, loss of imprinting of IGF2 was observed in both normal and tumor tissues. A significant increase (>5-fold) in IGF2 expression in tumor tissues compared to the normal adjacent kidney tissue was observed regardless of the IGF2 imprinting or the chromosome 11p15 heterozygosity status. In each case, the overexpression of IGF2 in the tumors was accompanied by activation of all four IGF2 promoters. Our data indicate that alterations of IGF2 imprinting occurred in normal adjacent kidney tissue before tumorigenesis and that the IGF2 overexpression in Wilms’ tumor tissue occurs through a loss of heterozygosity- or loss of imprinting-independent process.

Wilms’ tumor, one of the most common childhood tumors (1:10,000, 8% of childhood tumors), is associated with abnormalities in the short arm of human chromosome 11 (1). Somatic chromosomal events leading to the loss of constitutional heterozygosity (LOH)1 of this chromosomal region occur in 30–57% of Wilms’ tumors (1–3). Among the cluster of genes residing at this chromosomal region that have been implicated in tumorigenesis are IGF2 and H19. These genes are located within 200 kilobases of each other on chromosome 11p15.5, and both genes are imprinted. IGF2, spanning ~30 kilobases at this region, codes for a 67-amino acid mitogenic peptide that plays an important role in fetal growth and development (4). In most normal human tissues except for liver and CNS, IGF2 is transcribed exclusively from the paternally derived allele (5, 6). A number of malignant tumors, including Wilms’ tumor, have been shown to contain abnormally high levels of IGF2 mRNA, and loss of IGF2 imprinting, where both paternal and maternal alleles are transcribed, has been observed in many of these tumors (7, 8). The regulation of IGF2 expression in Wilms’ tumor has been studied extensively as a model system for examining mechanisms of genomic imprinting and loss of imprinting.

In an earlier study, we quantitated the allelic and promoter-specific expression of IGF2 in Wilms’ tumor, comparing tumors in which imprinting was lost (LOI) with those in which imprinting was maintained (MOI, maintenance of imprinting). We demonstrated that while the normal imprinting of IGF2 is promoter-specific and may fluctuate during early development, all four IGF2 promoters are expressed biallelically when there is LOI in Wilms’ tumors (9). Coordinate LOI of all four IGF2 promoters in tumor has also been reported by others (10, 11). IGF2 may act as an autocrine or paracrine growth factor when it is produced by tumors, potentially maintaining and enhancing tumor growth (12, 13). It has been suggested that the increased expression of IGF2 observed in some of these tumors is associated with, or caused by, the relaxation of genomic imprinting and the subsequent biallelic expression of the gene. In theory, biallelic IGF2 expression should lead to increased mRNA and protein synthesis.

H19 is a gene which is highly expressed during development in a number of fetal tissues but not in most adult tissues (14–16). It is thought that H19 exerts its effects as an RNA molecule and not as a protein, since there is no significant open reading frame in the gene (17). In contrast to IGF2, only the maternal allele of the H19 gene is transcribed (18). Alterations in H19 imprinting have also been reported in Wilms’ tumor (7, 8). Interestingly, loss of imprinting of IGF2 has been linked to reduced expression and abnormal methylation of H19 in Wilms’ tumors (19, 20). A similar linkage of overexpression and LOI of IGF2 has been found in children with IGF2-induced overgrowth disorder (21).

In this report we have compared the allelic expression of IGF2 and H19 in 40 sets of Wilms’ tumor specimens. In order to ascertain the role of LOI and LOH in the overproduction of IGF2 in Wilms’ tumor, we quantitated IGF2 mRNA levels in the two groups of Wilms’ tumors: LOH and non-LOH (LOI and MOI groups). IGF2 expression was measured in tumor and in normal adjacent kidney tissue (NAT) from the same patient. We demonstrate that LOI can be observed in some NAT samples and that IGF2 expression is increased in all categories of Wilms’ tumor tissues compared to NAT, suggesting that mechanisms other than LOI and LOH with consequent biallelic expression of IGF2 can result in increased IGF2 mRNA abundance in Wilms’ tumors.
human tissue and nucleic acid preparation—Forty pairs of fresh frozen Wilms' tumors and their normal adjacent kidney tissues were obtained from the Cross Cancer Institute, Northern Alberta Cancer Foundation. Total nucleic acid (TNA, containing RNA and DNA) was extracted from fresh frozen tissues as described previously (9). From the TNA preparations, RNA and DNA were prepared using RNA-DNA STAT (Tel-Test, Friendswood, TX). RNA was also prepared by simply removing DNA from TNA using DNase I treatment (9). cDNA was synthesized by RNA reverse transcription and diluted 10-fold before PCR amplification as described previously (9).

PCR Polymorphism Analysis—Eight polymorphisms involving five genes on chromosome 11 were examined. They were TH-CATT repeat (22), INS-HpaI (23), INS-PstI (24), IGF2-AuI (25), IGF2-ApaI (26), H19-AuI (27), H19-RsaI (17), and WT1-Hinfl (28) (see Fig. 1). PCR reaction, restriction enzyme digestion, electrophoresis, and quantitation analysis were performed using established methodology as described previously (9, 29, 30). Briefly, PCR amplification for polymorphism determination was performed in 96-well microtiter plates (MJ Research Inc., Boston, MA), each 3-μl reaction containing 10–20 ng of DNA for genotyping or cDNA for examining allelic expression, 50 ml volume at 37°C for 4 h. Each digested product was detected or quantitated by PhosphorImager 445SI (Molecular Dynamics, Sunnyvale, CA) after electrophoresis in a 5% polyacrylamide urea gel. To quantitate deletion-duplication of LOH, TH-CATT repeat or IGF2-ApaI, the DNA target, and 18 S fragment were amplified simultaneously using their 5’ primers end-labeled with [γ-32P]ATP in the PCR reaction.

Measurement of Relative mRNA Levels by PCR Analysis—To quantify the relative mRNA levels of IGF2 and H19 in tumors and normal tissues, we simultaneously amplified cDNAs derived from IGF2, H19, and 18 S rRNA in a multiplex PCR reaction. Variation among PCR reactions and sampling was therefore minimized. We used a mixture of two sets of primers of IGF2 (p23-p24) and H19 (p11-p12) at a 0.08 μM final concentration, and an internal control 18 S RNA (18SF and 18SR) at a 0.04 μM concentration. The IGF2 and H19 primers were designed to amplify DNA crossing-intron borders to differentiate genomic DNA and cDNA products. Amplification and analysis of PCR products were performed as described above, omitting the restriction digestion.

Analysis of Promoter Usage—To compare IGF2 promoter usage in tumor tissues versus NAT, we utilized a multiplex PCR (9). Tumor and NAT cDNAs were amplified with four promoter-specific 5’ primers (p17, p18, p19, and p20) and a common 3′ primer (p22) end-labeled with [γ-32P]ATP. In the PCR reaction, the four promoter-specific 5’ primers compete with each other for the one end-labeled 3′ primer, thereby reflecting the relative abundance of the promoter-specific derived transcripts (31). The PCR conditions were the same as those used for quantitation analysis.

Oligonucleotide Primers—The oligonucleotide primers were: p1 (5134), 5’-GCT ATC TGG GCT CGT GGA GTG TAT TTC-3’, p2 (5135), 5’-GCT CAC AGG GAA CAC ACA CTG CAT G-3’, p3 (5155), 5’-GCC ATC AAG CAG GTG TCC TGC AAG-3’, p4 (5255), 5’-CTT GGG TGT GTA GAA GAA GCC TCG-3’, p5 (3836), 5’-CCT GCA GAA GGG TGG CAT TGT G-3’, p6 (3741), 5’-GCT GGT TCA GGG TTA TTC CAT C-3’, p7 (4375), 5’-GGG CCG AAG ATG CAT CAC CGA GC-3’, p8 (4376), 5’-GCT GCC ACG CAT TCA GGG CGC TG-3’, p9 (3039), 5’-CTG CTA TGA GCA GGC TCC CCA CCA C-3’, p10 (2839), 5’-CTT TAC AAC CAC TCT GCT ACT ACC TGA-3’, p11 (2838), 5’-GAT GGC TTT GAT GTT GGG CTC-3’, p12 (2388), 5’-GAT GGC TCT CCT GAT TGG GCG C-3’, p13 (2350), 5’-GGA GTG TAT GAC ACC ACG TGT AGT-3’, p14 (2490), 5’-CCA TCA GCC CCC CCA ACA AGT AG-3’, p15 (3232), 5’-TCT GAC AAG CCA GCC ATG AGT-3’, p16 (3233), 5’-ATA CAG AGG TAC TGG TTA GGT CT-3’, p17 (3240), 5’-CAG TCC TGG GAT CAG CGT CCG TCG-3’, p18 (1873), 5’-ACC GCC CAT TCC CAG CGT TCT CC-3’, p19 (1872), 5’-CAG GAG CAT TCC TGG GAC CT-3’, p20 (1870), 5’-TCC TCC TCT CGC TCC (CTG CAC GAC-3’, p21 (1668), 5’-CGA CCT CCC CA/TCC TGG GAC CAG CAG C-3’, p22 (1655), 5’-CAG GAA TGC AGC ACCAGA) AGA CGG AGA C-3’, p23 (3038), 5’-TGG CCC TTC TCG TGA CAG CAT/ACT GTG C-3’, p24 (2384), 5’-TTG GAA GAA CTT CCT CAC GGC GTA TC-3’, 18SF (3967), 5’-ATC CTG CCA GCA TGA TAT GCT GTT CT-3’, 18SR (3968), 5’-TTA TCC AAC AGG AAG AGG AGC GAG C-3’. Mixed bases in parentheses were designed for both human and mouse.

RESULTS

Genotyping of TH, INS, IGF2, H19, and WT1 Genes—LOH in Wilms’ tumor has been located within the 11p15 and 11p13 regions (1, 3). To identify LOH in our 40 subjects with Wilms’ tumor, we determined the allelic genotypes of tyrosine hydroxylase (TH), insulin (INS), IGF2, and H19 at 11p15.5, and WT1 at 11p13 in the 40 Wilms’ tumor samples and in their paired normal adjacent kidney tissues (Fig. 1). For each DNA polymorphic site, undigested alleles were designated as A and digested allele as B. The TH gene has five major alleles, designated as A (121 bp), B (117 bp), C (113 bp), D (109 bp), and E (105 bp) (Fig. 2).

Table I shows the DNA polymorphisms found within these five genes in the 40 pairs of Wilms’ tumor and normal adjacent tissues. Heterozygous specimens were informative for further analysis of loss of heterozygosity and imprinting status. Among the eight DNA markers, the most informative site was the TH-CATT repeat site at which 65% (26/40) of cases were shown to be heterozygous, and the least informative site was INS-PstI, where only 20% (8/40) were informative (Table II). We observed eight paired specimens to be heterozygous for the ApaI polymorphism of IGF2, and eight pairs for the H19-AuI polymorphism, which were thus informative for further examination of genomic imprinting (Table I).

Loss of Heterozygosity Involving Deletion-Duplication in Tumorigenesis—To determine the presence or absence of LOH at chromosome 11p, we compared genotypes in the tumors with those in their paired normal tissues. When LOH was observed in one polymorphic site, all other sites, if informative, also showed LOH, suggesting that the LOH in the Wilms’ tumor DNA extended from the TH through the WT1 locus except for patient 10, in which LOH did not involve 11p13 (WT1); these findings are consistent with previous studies (2, 32, 33). Considering all of the cases which were informative for these eight DNA markers, 14/47 (37.8%) of Wilms’ tumors demonstrated LOH for the short arm of chromosome 11. Because of the strong association of LOH of these genes across the region from 11p15.5 to 11p13, we classified tissues showing LOH in any of the eight DNA markers into the LOH group. There were 14 cases of LOH and 23 cases of maintenance of heterozygosity.
Gene expression in tumor demonstrating loss of heterozygosity was quantified by densitometric analysis. The average ratio of the density of the remaining allele bands in eight tumors with LOH for \( H19-\text{Alu} \)I and five tumors with LOH for \( WT1-\text{Hin} \)fI to that of the two allele bands in their corresponding normal tissues was 0.96 ± 0.07 and 1.24 ± 0.13, respectively, suggesting that the loss of one allele was accompanied by the duplication of the other allele (isodisomy). To confirm the presence of LOH with deletion-duplication in tumors as previously reported (34), we used end-labeled primers and an internal control 18 S ribosomal RNA to assess gene duplication in the LOH samples at \( TH-CATT \) and \( IGF2-\text{Apa} \)I sites (Fig. 3). The normalized ratio revealed that the intensity of the band of the one remaining allele in the tumor was equal to the total density of the two alleles seen in normal adjacent tissue DNA (\( p = 0.84 \)), confirming the presence of a deletion-duplication in LOH (35).

Loss of Imprinting of IGF2 Predisposes to Tumorigenesis—Allelic expression of IGF2 and \( H19 \) was investigated using primers flanking the polymorphic \( IGF2-\text{Apa} \)I (p9, p10) and \( H19-\text{Alu} \)I (p11, p12) sites, respectively (Fig. 4). Among eight informative cases for \( IGF2 \), monoallelic expression of \( IGF2 \) was found in two paired tissues (patients 15 and 20), while the other six tumors showed biallelic expression of \( IGF2 \) (LOI). Interestingly, LOI was also observed in five of the six normal tissues adjacent to the tumors that showed LOI for \( IGF2 \) (Table I).

**Table I  Genotypes of 40 pairs of Wilms' tumor and their normal adjacent tissues**

| Patient no. and tissue | DNA polymorphism | Genotypes | Genotypes | Genotypes | Genotypes | Genotypes | Genotypes |
|-----------------------|------------------|-----------|-----------|-----------|-----------|-----------|-----------|
|                       | TH-\text{CATT}   | INS-\text{Hph}  | INS-\text{Pst} | IGF2-\text{Alu} | IGF2-\text{Apa} | H19-\text{Alu} | H19-\text{Rsa} | WT1-\text{Hin}fI |
| Loss of heterozygosity: |                  |           |           |           |           |           |           |           |
| 1 N                   | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 2 N                   | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 3 N                   | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 4 N                   | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 5 N                   | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 6 N                   | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 7 N                   | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 8 N                   | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 9 N                   | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 10 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 11 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 12 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 13 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 14 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 15 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 16 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 17 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 18 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 19 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 20 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 21 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 22 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 23 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 24 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 25 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 26 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 27 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 28 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 29 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 30 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 31 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 32 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 33 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 34 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 35 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 36 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 37 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 38 N                  | CD               | AB        | AA        | AA        | AA        | AA        | AA        | AA        |
| Others:               |                  |           |           |           |           |           |           |           |
| 2 N                   | DD               | BB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 3 N                   | DD               | BB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 4 N                   | DD               | BB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 5 N                   | DD               | BB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 6 N                   | DD               | BB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 7 N                   | DD               | BB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 8 N                   | DD               | BB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 9 N                   | DD               | BB        | AA        | AA        | AA        | AA        | AA        | AA        |
| 10 N                  | DD               | BB        | AA        | AA        | AA        | AA        | AA        | AA        |

\( ^{a} \) N, adjacent normal tissue; T, tumor tissue.

\( ^{b} \) Boxes indicate the sites showing LOH, and circles, MOH in LOH group. Specimens informative for \( IGF2-\text{Apa} \)I and \( H19-\text{Alu} \)I are underlined.
Increased Expression of IGF2 and LOI/LOH in Wilms’ Tumor

III. To exclude possible contamination of the cDNA samples with genomic DNA, cDNAs from these samples were subjected to PCR using primers spanning intron 8 of IGF2 (p23 and p24, see Fig. 4, IGF2). Reverse transcription PCR with these primers produced only the 115-bp fragments characteristic of the cDNA without any detectable band derived from genomic DNA (408 bases, data not shown). Tumor tissue contamination of the NAT was not considered likely because the genotyping data of LOH patients clearly showed that the normal tissues differed from tumor tissues (for polymorphism heterozygosity analysis see Table I). In addition, the analysis of IGF2 abundance argues against the possibility of tissue cross-contamination in our specimens (see below). Thus, these data indicate that LOI frequently occurs in tissue adjacent to Wilms’ tumors, suggesting the possibility that LOI leads to a proclivity for tumorigenesis.

As shown in Table III, of the eight tissue pairs informative for the H19-AluI polymorphism, only one tumor (patient 15) displayed biallelic expression of H19; there was maintenance of IGF2 imprinting in this case. H19 was monoallelically expressed in all remaining tumors and in all normal adjacent tissues including subjects 30, 31, and 32, where there was a loss of IGF2 imprinting, suggesting a lack of association or concordance between IGF2 and H19 genomic imprinting.

Overexpression of IGF2 in Wilms’ Tumors—We next examined the relative abundance of IGF2 and H19 transcripts in the 40 pairs of Wilms’ tumors and adjacent normal tissues using quantitative reverse transcription PCR. To exclude interference from genomic DNA contamination during PCR transcription quantitation, we used specific primers which cross exon/ intron borders for each gene. The PCR products derived from cDNA and DNA are therefore of different sizes (Fig. 4). We have optimized our PCR conditions to amplify simultaneously the transcripts of IGF2, H19, and 18 S ribosomal RNA (as an internal control) in a quantitative manner in the same tube and subsequently evaluate them in the same gel lane (Fig. 5). As shown in Table III, a significantly increased abundance of IGF2 mRNA was observed in 40 tumors regardless of their imprinting or heterozygosity status (average 5.26 ± 0.76-fold increase; p < 0.0001) compared to their NAT. No consistent changes in H19 gene expression could be discerned. Twenty-eight of the 40 tumors showed decreased (≤1.0-fold) expression of H19 and 12 displayed an increase (>1.0 fold) in H19 abundance when compared to their normal adjacent tissue. The average H19 abundance in Wilms’ tumors was 90% relative to the normal tissues, but there was no significant difference between them (p > 0.05).

To determine the effects of LOH and LOI on the expression of IGF2 and H19 in Wilms’ tumor, we analyzed the quantitation data by subgroups according to allele expression analysis (Table III) and genotype data (Table I). Tumors which demonstrated LOH had a significant increase of IGF2 expression (6.74 ± 1.66-fold compared to normal tissues) than did tumors which maintained heterozygosity (4.04 ± 0.51-fold compared to normal tissues), indicating that LOH and isodisomic reduplication contributed to the overexpression of IGF2 in tumors. There was a significant decrease in H19 abundance in Wilms’ tumors with LOH (29%, p < 0.05) and in those tumors which demonstrated loss of IGF2 imprinting (26%, p < 0.05) when compared with their normal adjacent tissues. No consistent changes in H19 gene expression could be discerned in the other MOH tumor group, confirming previous work which showed that the decrease of H19 in Wilms’ tumors was related to LOH and LOI of IGF2 (19, 20, 36). However, we did not observe any relationship between the increase in IGF2 or the decrease in H19 in Wilms’ tumors with their LOI/LOH imprinting status. In summary, these data suggest that the overexpression of IGF2 in Wilms’ tumor cannot be directly or solely attributable to LOI or LOH mechanisms.

Activation of IGF2 Promoters in Tumorigenesis—The IGF2 gene has four promoters (37). Previously we observed that transcripts from promoters P1–P4 are derived from both alleles in Wilms’ tumors with LOI. In contrast, in tumors with MOI, transcripts from P2–P4 originate from only one allele, while P1 transcripts were from one or both alleles (9). To see which promoter contributes to the increase of IGF2 expression in Wilms’ tumors, we examined the promoter usage of IGF2 using the competitive PCR with end-labeled primers (Fig. 4, IGF2). Using this methodology, transcript products from only promoters P3 and P4 were observed in tumors or in normal tissues. Transcripts derived from promoters P1 and P2 were not detectable in either normal or malignant of tissues (Fig. 6A), confirming that P3 and P4 were dominant promoters in both normal and malignant kidney tissues (9, 37). The expression P3–derived IGF2 transcripts was 6.42 ± 1.0-fold higher in tumors than in the normal tissues, while expression from P4 was 3.48 ± 0.83-fold higher in tumors. A similar trend was observed in tumors with either LOI (patients 11, 30, and 31) or MOI

Table II

| Polymorphism sites | Tissue | Genotypes | Allele frequency | LOH/informative in tumor (%) |
|-------------------|--------|-----------|-----------------|-----------------------------|
| TH-CATT<sup>a</sup> | N      | AA        | 0.41            | 9/26 (34.6)                |
|                   | T      | BB        | 0.59            |                            |
|                   |        | AB        | 0.50            |                            |
| INS-HphI          | N      | 11        | 0.41            | 5/11 (45.5)                |
|                   | T      | 12        | 0.59            |                            |
| INS-PstI          | N      | 30        | 0.51            |                            |
|                   | T      | 30        | 0.54            |                            |
| IGF2-AluI         | N      | 14        | 0.47            |                            |
|                   | T      | 10        | 0.76            |                            |
| IGF2-ApaI         | N      | 13        | 0.55            |                            |
|                   | T      | 22        | 0.43            |                            |
| H19-AluI          | N      | 14        | 0.51            |                            |
|                   | T      | 15        | 0.59            |                            |
| H19-RsaI          | N      | 16        | 0.59            |                            |
|                   | T      | 16        | 0.51            |                            |
| WT1-HinII         | N      | 19        | 0.51            |                            |
|                   | T      | 21        | 0.76            |                            |

Total 14/57 (29.4)

<sup>a</sup> Frequency of five alleles, a, b, c, d, and e, in normal tissues for TH were 37.5%, 25.5%, 7.5%, 52.5% and 40%, respectively.

**Fig. 3.** Quantitation of LOH in Wilms’ tumor by PCR analysis using γ-<sup>32</sup>P-end-labeled primers of TH-CATT repeat and IGF2-ApaI with the 18 S internal control. T, Wilms’ tumor tissue; N, normal adjacent kidney tissue.
To focus on whether promoter P1 and P2 were activated in Wilms’ tumors, we examined each promoter by a more sensitive PCR analysis using single primer sets. As we have previously shown, IGF2 transcripts derived from P1 and P2 were observed in Wilms’ tumors, but these transcripts could not be detected in normal adjacent tissues (Fig. 6B). The contribution of promoters P1 and P2 to the overexpression of IGF2 in Wilms’ tumors was much less than the contribution from promoters P3 and P4.

**DISCUSSION**

IGF-II is a mitogenic peptide which is overexpressed in many types of tumors, and it has been speculated that the peptide can act in an autocrine or paracrine manner to initiate and/or maintain oncogenesis and tumor growth (12, 13). The recent observation that IGF2 imprinting is lost in a majority of Wilms’ tumors appears to have provided one potential mechanism for the increased abundance of IGF2 in such tumors since biallelic gene expression would presumably lead to twice the amount of mRNA as would be transcribed from an imprinted gene (7, 8, 35, 38). In this study, we have characterized 40 sets of Wilms’ tumors and their normal adjacent tissues. We confirm earlier findings that LOH is accompanied by reduplication of the remaining (presumably paternal) allele and by increased expression of the normally maternally imprinted IGF2 (1, 39). Moreover, we have shown that the abundance of IGF2 was >5 times greater in the tumor tissue than in the normal kidney tissue. This increased IGF2 expression occurred in tumor tissue irrespective of imprinting or heterozygosity status. Even NATs which demonstrated loss of IGF2 imprinting contained less IGF2 than did the tumor tissue which also manifested LOI, suggesting that LOH of IGF2 imprinting may not directly alter IGF2 gene expression (or mRNA abundance) in Wilms’ tumor. Instead, it appears that other mechanisms not associated with imprinting must contribute to the overexpression of IGF2.

In Wilms’ tumors, LOH was restricted to the short arm of...
TABLE III
Expression of IGF2 and $H_19$ in Wilms' tumors

| Patient | IGF2 | | H19 | | RNA-ApiI$^b$ | | T/N$^a$ | | RNA-ApiI | | T/N | | Normal Tumor | | Normal Tumor |
|---------|------|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I. MOH: | | | | | | | | | | | | | | |
| a). MOI of IGF2: | | | | | | | | | | | | | | |
| 15 | 2.36 | A– | A– | 1.08 | A– | AB | | | | | | | | |
| 20 | 4.55 | A– | A– | 1.13 | B– | B– | | | | | | | | |
| Average | 3.46 ± 1.10 | | 1.11 ± 0.03 | | | | | | | | | | | |
| b). LOI Of IGF2: | | | | | | | | | | | | | | |
| 5 | 1.70 | A– | AB | 0.88 | | | | | | | | | | |
| 11 | 2.36 | AB | AB | 0.59 | | | | | | | | | | |
| 30 | 2.55 | AB | AB | 0.66 | B– | B– | | | | | | | | |
| 31 | 3.95 | AB | AB | 0.73 | B– | B– | | | | | | | | |
| 32 | 4.66 | AB | AB | 0.65 | B– | B– | | | | | | | | |
| 37 | 2.17 | AB | AB | 0.92 | | | | | | | | | | |
| Average | 2.90 ± 0.47 | | 0.74 ± 0.05 | | | | | | | | | | | |
| c). Others: | | | | | | | | | | | | | | |
| 4 | 4.68 | | | 0.69 | | | | | | | | | | |
| 6 | 4.55 | | | 1.09 | | | | | | | | | | |
| 8 | 10.8 | | | 0.71 | | | | | | | | | | |
| 12 | 2.43 | | | 0.73 | | | | | | | | | | |
| 17 | 5.72 | | | 1.47 | | | | | | | | | | |
| 21 | 1.08 | | | 0.93 | | | | | | | | | | |
| 22 | 3.93 | | | 1.21 | | | | | | | | | | |
| 25 | 6.94 | | | 1.40 | | | | | | | | | | |
| 26 | 8.55 | | | 1.12 | | | | | | | | | | |
| 27 | 1.19 | | | 1.60 | | | | | | | | | | |
| 29 | 3.43 | | | 0.91 | | | | | | | | | | |
| 33 | 3.45 | | | 0.72 | | | | | | | | | | |
| 34 | 7.27 | | | 1.13 | | | | | | | | | | |
| 36 | 1.65 | | | 1.56 | | | | | | | | | | |
| 38 | 2.62 | | | 1.51 | | | | | | | | | | |
| Average | 4.57 ± 0.73 | | 1.12 ± 0.09 | | | | | | | | | | | |
| II. LOH: | | | | | | | | | | | | | | |
| 1 | 4.44 | | | 0.97 | | | | | | | | | | |
| 3 | 3.69 | | | 1.01 | | | | | | | | | | |
| 7 | 3.53 | | | 0.85 | | | | | | | | | | |
| 9 | 15.7 | | | 0.65 | | | | | | | | | | |
| 10 | 5.90 | | | 0.59 | | | | | | | | | | |
| 14 | 3.81 | | | 0.71 | | | | | | | | | | |
| 16 | 2.24 | | | 0.88 | | | | | | | | | | |
| 18 | 6.64 | | | 0.53 | | | | | | | | | | |
| 19 | 3.96 | | | 0.27 | | | | | | | | | | |
| 23 | 1.64 | | | 0.37 | | | | | | | | | | |
| 28 | 7.15 | | | 0.68 | | | | | | | | | | |
| 35 | 24.0 | | | 0.68 | | | | | | | | | | |
| 39 | 1.96 | | | 0.91 | | | | | | | | | | |
| 40 | 10.47 | | | 0.96 | | | | | | | | | | |
| Average | 6.74 ± 1.66 | | 0.71 ± 0.06 | | | | | | | | | | | |
| III. Others: | | | | | | | | | | | | | | |
| 2 | 18.5 | | | 1.00 | | | | | | | | | | |
| 13 | 1.09 | | | 0.90 | | | | | | | | | | |
| 24 | 3.12 | | | 0.83 | | | | | | | | | | |
| Average | 7.57 ± 5.52 | | 0.91 ± 0.05 | | | | | | | | | | | |

Total average 5.26 ± 0.76 0.90 ± 0.05

$^a$ Relative transcription abundance which were normalized to 18 S RNA under identical condition of PCR amplification. Radioactivity intensity was measured using a PhosphorImager.

$^b$ Only informative cases were examined for their allelic expression.

$^c$ Average ± standard error of the mean.

Increased expression of IGF2 and LOI/LOH in Wilms’ tumor

Chromosome 11 (2). LOH can be detected at 11p15 to 13 in 30–57% of informative samples of Wilms’ tumor (1–3). In this study, we identified LOH in 37.8% (14 of 37) informative tumors, and we found linkage of LOH at 11p13 to 11p15. Due to the limited number of samples which were informative at each polymorphic site, the frequency of LOH that we determined at each polymorphic site might not reflect the exact incidence of LOH at these loci in Wilms’ tumors. For example, the apparent low LOH rate at $H_P h1$ site (1/8, 12.5%) was probably due to the relatively few patients who were informative (i.e., heterozygous) at this site because another polymorphic site ($H_p h1$) of the INS gene displayed a higher incidence of LOH (8/11,
samples, we were able to observe a highly significant increase of IGF2. Furthermore, our data also revealed a significant increase in the expression of IGF2 in tumor groups which maintained heterozygosity as well as imprinting. In each case, the overexpression of IGF2 in the malignant tissue was due to activation of all four promoters of IGF2, regardless of their LOI/MOI status. In our previous study (9), we demonstrated that, in every tumor that demonstrates LOI of IGF2, expression from promoter P1 as well as from the normally imprinted promoters P2–P4 was biallelic, but we did not examine promoter-specific gene expression in the NAT samples.

LOH at chromosome 11p15 in Wilms’ tumor is a result of a loss of the maternal chromosome (39, 44). Since H19 is paternally imprinted, LOH should result in elimination of expression of H19. Previous studies have shown little or very low expression of H19 in tumors with LOH, although these studies did not report the measurement of H19 in paired normal tissues (20, 36). We observed reduced (71% of the NAT) expression of H19 in 14 tumor specimens with LOH (Table III), suggesting that while LOH may have been responsible for the reduction in the abundance of H19 RNA in the tumor, some expression of H19 from the paternal chromosome (loss of imprinting) may also have occurred. Similar results have previously been reported (19). The association of LOI of IGF2 with a downregulation of H19 expression has been reported by others (19, 20). However, our data showed that the abundance of IGF2 transcripts was not related to H19 expression in Wilms’ tumors. While numerous studies have shown that H19 may control IGF2 imprinting (20, 36, 45), we could not confirm that H19 regulates the abundance of IGF2 mRNA in Wilms’ tumors.

It is unclear what mechanism(s) is involved in enhancing the expression of IGF2 in Wilms’ tumors since it appears that this activation is independent of LOH or LOI. Differential DNA methylation has been proposed as a candidate for epigenetic activation is independent of LOH or LOI. Differential DNA methylation in Wilm’s tumor (data not shown). Increased Expression of IGF2 and LOI/LOH in Wilms’ Tumor

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