Alterations in the Promoter-specific Imprinting of the Insulin-like Growth Factor-II Gene in Wilms’ Tumor*

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The human IGF2 gene, which encodes a mitogenic peptide required for normal fetal development, is overexpressed in many types of tumors. IGF2 is transcribed from four promoters (P1-P4), and in most tissues, the gene is imprinted. In this study, we have analyzed promoter usage and determined the allelic expression from each promoter in 19 IGF2 tumors. Loss of IGF2 imprinting (LOI) was observed in 8 Apal-informative tumors. In these tumors, each parental allele was expressed in equal abundance, indicating that there was complete relaxation of IGF2 imprinting. In each LOI tumor, expression from promoter P1 as well as from the normally imprinted promoters P2-P4 was biallelic. In the 11 Apal-informative tumors which maintained IGF2 imprinting (maintenance of imprinting), transcription from promoters P2-P4 was always monoallelic, while transcripts from P1 were derived from either one or both alleles. The lack of consistency of IGF2 imprinting of promoter P1 in maintenance of imprinting tumors was also observed in normal fetal tissues of 6-12 weeks gestation, suggesting a similarity in IGF2 regulation between Wilms’ tumors and embryonic tissue development. These data suggest that the increased expression of IGF2 in Wilms’ tumors may be caused either by biallelic gene expression in LOI tumors from promoters P2-P4 and/or by a reversion to an earlier stage of development which is characterized by increased synthesis of this fetal growth factor.

The human insulin-like growth factor-II gene (IGF2)1 is large and complex, spanning ~30 kilobases at chromosome 11p15.5 (1). The gene is flanked by the insulin gene upstream and immediately downstream by H19, a developmentally-regulated gene which codes for an RNA that lacks appropriate protein reading frames. H19 is highly expressed in fetal tissues but is often not transcribed in adult tissues (2). IGF2 has nine exons, four promoters, and two functional polyadenylation addition signals, leading to a variety of mRNA species (3). Promoter usage has been shown to be tissue-specific and developmentally regulated. For example, in fetal liver, IGF-II mRNA is transcribed predominately from promoters 2, 3, and 4. After birth, hepatic IGF-II synthesis continues, but the activity of promoters 2-4 declines, and promoter 1 becomes the predominant hepatic promoter (4). In other tissues, however, promoters 3 and 4 predominate during adult life. Despite the use of multiple promoters and the production of a large number of IGF-II mRNA species, a single prepro-IGF-II peptide is synthesized in most cells. In addition to the most abundant 7.5-kDa 67 amino acid mature IGF-II, 15- and 10-kDa variant human IGF-II peptides have also been found (5).

It has recently been demonstrated that IGF2 is maternally imprinted (6). In most normal human tissues except for liver and central nervous system, IGF-II mRNA is only transcribed from the paternally-derived allele (7). We have shown that IGF2 imprinting is promoter-dependent; in normal human liver and chondrocytes, when promoter P1 is used, IGF2 is expressed biallelically, but when P2, P3, or P4 are employed, only monoallelic expression is seen (8).

While IGF-II is a mitogenic peptide that is required for normal fetal growth (9), its role in adult physiology is unknown. A variety of human malignancies contain increased concentrations of IGF-II mRNA compared to normal tissues, and the subsequent excessive production of the pro-IGF-II peptide has been implicated as the cause of non-islet cell tumor hypoglycemia (10). Furthermore, it has been suggested that IGF2 overexpression is either an initiator of oncogenesis (11) or perhaps a marker for malignant transformation.

Wilms’ tumor is the most common intra-abdominal tumor in childhood. The malignancy is derived from persistent primitive blastemic cells in nephrogenic rests (12). IGF2 is overexpressed in many Wilms’ tumors. Moreover, IGF2 imprinting is relaxed and the IGF2 gene product is transcribed from both paternal and maternal alleles in approximately 70% of Wilms’ tumors, suggesting that loss of IGF2 imprinting may play an important role in the development or maintenance of this tumor (13, 14). To understand the molecular mechanisms underlying this alteration of imprinting in these tumors, we have examined the promoter-specific allelic expression of IGF2 in Wilms’ tumors which exhibit maintenance as well as loss of IGF2 imprinting. Our results indicate that while there was an increased usage of promoter P1 in some Wilms’ tumors, the biallelic expression of IGF2 in tumors with loss of imprinting (LOI) was caused primarily by the biallelic activation of promoters P2-P4. These results are in contrast to the apparent LOI in normal adult liver in which P1 promoter usage and not P3-P4 biallelic expression was observed. In those tumors which demonstrate MOI, promoters P2-P4 were imprinted while transcripts from P1 were derived from either one or both alleles. This variation in promoter P1 imprinting in these tumors was also observed in some early embryonic and fetal tissues.
EXPERIMENTAL PROCEDURES

Human Tissues—Fresh frozen Wilms’ tumor and normal kidney tissues were obtained from the Cooperative Human Tissue Network (Children’s Hospital, Columbus, OH). Normal fetal tissues (6–12 weeks of gestation) were obtained from the Central Laboratory for Human Embryology Tissue, University of Washington, Seattle.

RNA and DNA Preparation—Total RNA was extracted by homogenizing the fresh frozen tissues in an acid-guanidinium thiocyanate buffer according to the protocol of Chomczynski and Sacchi (15). Both RNA and DNA were also prepared using RNA-STAT and DNA-STAT kits (TEL-TEST B., Inc., Friendswood, TX). The RNA samples were treated with RNase-free DNase I (Life Technologies, Inc.) in the presence of RNase inhibitor (Life Technologies, Inc.).

During the course of this study we also developed a simple procedure to prepare a total nucleic acid fraction (termed TNA). Our TNA preparations, which contain both RNA and DNA, served as starting materials for RNA and DNA preparations using the RNA and DNA-STAT kits. The TNAs were used directly for genotyping by PCR. We compared genotyping results from both DNAs and TNAs. The TNA preparations yielded more reliable PCR data. The presence of RNA in the TNA preparations, unexpectedly, did not interfere or compromise the quality of the genotyping analyses, provided that DNA polymerases without reverse transcriptase activity (e.g. Taq or Tfl DNA polymerases) were used in the PCR reactions. It is more convenient to screen a large number of samples for informative heterozygosity using the TNA preparations, since we then need to prepare RNAs only from the informative samples.

Our TNAs also provide high quality, degraded RNAs which can be analyzed directly by eliminating DNAs with RNase-free DNase I in the presence of RNase inhibitor. Reverse transcription of the TNAs provide both cDNA of the RNA and the genomic DNA which can be used as an internal control in PCR analyses.

Total Nucleic Acid Extraction—TNA from fresh frozen tissues by homogenizing tissues in 10–20 volumes of an RNA extraction solution without reverse transcriptase activity (e.g. Taq or Tfl DNA polymerases) were used in the PCR reactions. It is more convenient to screen a large number of samples for informative heterozygosity using the TNA preparations, since we then need to prepare RNAs only from the informative samples.

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Polymerase Chain Reaction Genotyping—We analyzed the IGF2 (Alu and Ava) and H19 (Alu) polymorphisms by PCR using a DNA or TNA stock solutions of 10 ng/ml of the DNA or TNA stock solutions of 1 ng/ml. The reactions were carried out using a 96-well PCR plate (M. Research, Inc., Boston, MA) under liquid wax barrier of “chill-out 14” (MJ Research) with an internal non-polymorphic DNA control template (300 base pairs) was added to the reaction at 60°C. Since only a fraction of a microliter of the diluted cDNA was analyzed by our standard PCR reaction, the reaction was amplified for 30 cycles at 95°C for 30 s and 60°C for 1 min, followed by a 5-min extension at 72°C. The PCR products were digested with restriction enzymes by simply adding 5 µl of 1× containing 1 unit of the enzyme, incubating at 37°C for 1 h (Apa) or 6 h (Alu). Ten µl of formamide sequencing buffer were added to the PCR products, and aliquots of 4 µl were analyzed on a 5% or 6% polyacrylamide-urea gel. After electrophoresis, the polyacrylamide gels were analyzed by PhosphorImager screen and analyzed by PhosphorImager Scanner (Molecular Dynamics, Sunnyvale, CA). The gels were also exposed to x-ray films (Amersham) with an intensifying screen for 12–24 h.

Reverse Transcriptase PCR—We made cDNA stocks by using total RNA, random hexamers, or specific primers, and murine leukemia virus reverse transcriptase (Life Technologies, Inc.). Total RNA (7 µg of 100–200 µg/ml stock solution) protected with the liquid wax (10 µl) was heat denatured at 75°C for 1 min, and then cooled on ice. At room temperature, a cDNA master mixture (3.0 µl) containing first strand buffer, dithiothreitol, all 4 dNTPs, RNase inhibitor, random hexamer (or specific oligo primer), and the reverse transcriptase was added to the RNA, at the concentrations recommended by the supplier (Life Technologies, Inc.). The reaction tubes were incubated at 20°C for 5 min, then 37°C for 25 min, followed by 5 cycles (50°C for 20 s and 37°C for 5 min), and then 99°C for 5 min as described previously (8). After enzyme deactivation, the cDNA samples were diluted 10-fold by adding 45 µl of sterile water.

One microliter of the diluted cDNA was analyzed by our standard 2.5-µl PCR reaction in standard 0.2-ml thin-wall tubes, using 0.50 µl of 32P-end-labeled primer (labeled by [γ-32P]ATP and T4 polynucleotide kinase) and 1.0 µl of the PCR master mixture without [γ-32P]ATP. The PCR amplification and restriction digestion were essentially under the same conditions as described in the PCR genotyping section. Nested PCR was performed as described previously (8), which were specific for human IGF2. To ensure complete digestion of Apal and Avall, an internal DNA control template (300 base pairs) was added to the digestion reaction (8). The Alu digestion was monitored by an internal non-polymorphic Alu site which resulted in a 112-base pair band in Fig. 4. The polyacrylamide gels were analyzed by PhosphorImager Scanner and quantitation was based on the scanning density.

Multiplex PCR Assay for Promoter Usage—To assay for IGF2 promoter usage in Wilms’ tumors and normal tissues, we have developed a multiplex PCR. The cDNAs, which were made by random hexamer priming, were amplified with 4 promoter-specific 5’-sense primers (primers p1a, p2a, p3c, and p4c, see below), and a common 3’-antisense primer (p10 or p11). The 3’-common primer was end-labeled with [γ-32P]ATP and [γ-32P]ATP was added as a mix to the reaction at 60°C. Since only a fraction of a microliter of the diluted cDNA was analyzed by our standard PCR reaction, the reaction was amplified for 28 cycles (95°C for 30 s, 60°C for 1 min, 72°C for 1 min) under similar conditions as described in RT-PCR.

To multiplex the Primers (as recommended for promoter P1 to P4 are p1a, 5’-CAG TGA TTC GGT CTT CCT TTT C-3’; p1b, 5’-CAG TCC TGA GAG CTG CTG TGG C-3’; p2c, 5’-CCC AAT TGG TGG CCG AGA CAC-3’; p3d, 5’-GGG CCG AGG AGT CAC CAC CGA-3’; p4a, 5’-GCT GGC AGC GAG TCA TG TGC CCC-3’; p5a, 5’-AGC CAG CTC TGG TCC CCA GTC TCG CTA GAC-3’; p6a, 5’-GGG GCA CCC CAA TCC GCA ACC-3’; p7a, 5’-GCA CGC ACA TGT CAC GCT CTG-3’; p8a, 5’-GCA-GGG CCG AGA GTG AGG ATG CTG-3’; p9a, 5’-GCA-GGG CCG AGA GTG AGG ATG CTG-3’; p10, 5’-GCA GCA GCC TCC AAG TCC CCA GTC TCG CTA GAC-3’; p11, 5’-GCA GCC TCC AAG TCC CCA GTC TCG CTA GAC-3’; p12, 5’-GCA GCC TCC AAG TCC CCA GTC TCG CTA GAC-3’; p13, 5’-GCA GCC TCC AAG TCC CCA GTC TCG CTA GAC-3’; p14, 5’-GCA GCC TCC AAG TCC CCA GTC TCG CTA GAC-3’. The primers in exon 9 are as follows: p5a, 5’-CTT GGA TGT TGT GAC TTT TTC-3’; p6a, 5’-GCA TGG AGG ATG CTG-3’; p7a, 5’-GCA TGG AGG ATG CTG-3’; p8a, 5’-GCA TGG AGG ATG CTG-3’; p9a, 5’-GCA TGG AGG ATG CTG-3’; p10, 5’-GCA TGG AGG ATG CTG-3’; p11, 5’-GCA TGG AGG ATG CTG-3’; p12, 5’-GCA TGG AGG ATG CTG-3’; p13, 5’-GCA TGG AGG ATG CTG-3’; p14, 5’-GCA TGG AGG ATG CTG-3’.

In a control experiment, single primer sets comprising a single 5’-primer and a common p11 primer were used to amplify each promoter-specific transcript, and to compare with the amplification efficiency in the multiplex PCR. We also performed a standard dilution experiment, in which each of four P1-P4 templates were diluted in a series of 1/1, 1/3, and 1/10 before mixing in a multiplex PCR. PCR amplification (5 µl of reaction) was performed for 28 cycles (95°C for 30 s, 60°C for 1 min, 72°C for 1 min) under similar conditions as described in RT-PCR.

RESULTS

IGF2 Imprinting in Wilms’ Tumor—We screened 70 Wilms’ tumors and 6 normal adjacent kidney tissues for the presence of the Apal polymorphism (16) in IGF2 exon 9 (Fig. 1A). The Alu polymorphism (17) in IGF2 exon 3, the H19 Alu polymorphism (18), and the WT1 Hinfl polymorphism (19). The human
IGF2, H19, and WT1 genes are located on chromosome 11p and loss of heterozygosity at this locus appears frequently in Wilms' tumor. Three of the six pairs of Wilms' tumors and the adjacent normal kidney showed loss of heterozygosity for WT1, H19, or both IGF2 and H19 (data not shown). Twenty tumors were heterozygous for the Apal polymorphism and 19 were examined for IGF2 allelic expression (Table I). Eight of the Apal heterozygous tumors showed biallelic expression of IGF2 (LOI) while 11 of these tumors showed MOI. Fig. 1B shows representative data from subjects 5 and 6 (LOI) and 9 and 11 (MOI). Sixteen of the 19 tumors were also IGF2 Alu heterozygotes, a polymorphism which facilitates the analysis of allelic transcripts from IGF2 promoter P1. Six other Wilms' tumors were IGF2 Alu heterozygotes but Apal homozygotes (Table I).

Since genomic imprinting has been defined as a parent of origin effect on the differential expression of the two parental alleles, it is appropriate to analyze the imprinting quantitatively. To determine the relative contribution of the two parental alleles in genomic DNA and in cDNAs by PCR, we used primers flanking the polymorphic Apal site in IGF2 exon 9 (Fig. 1A). The IGF2 Apal heterozygosity has a unique feature: the alleles differ by a single base (GGGCC and GGACCC) can be digested by two different restriction enzymes, Apal and Aval. This specific feature allowed a more accurate quantitation of the two alleles. Aliquots of the PCR products which were labeled at one end by a $^{32}$P end-labeled primer were digested with Apal and Aval enzymes. The extent of the enzyme digestion was monitored by an internal control template in each digestion tube. Since the a allele, which is not digested by Apal is cut by Aval (and the b allele is digested by Apal and not by Aval), the average values from the two separate enzymatic digestions of the same PCR products eliminates any artifactual interference of undigested heteroduplexes which have been noted in previous studies (13).

In all 8 LOI tumors, the two parental alleles of IGF2 demonstrated quantitatively equal expression, indicating a complete disruption of imprinting in these tumors. The ratios of the two allelic mRNA transcripts were close to 50% and were similar to the ratios of the two alleles from the genomic DNA (Table I). In all 11 MOI tumors, on the other hand, IGF2 was transcribed from only one parental allele (Table I). Leaky expression of the suppressed allele (average 2.2%, Table I) may be attributable in part to the biallelic expression of IGF2 that occurred from promoter P1.

IGF2 Promoter Usage in Wilms’ Tumor—Since IGF2 imprinting has been shown to be promoter specific in a variety of tissues, we were interested to learn if the reversal of the imprinting process involved all four promoters. We hypothesized that LOI was a result of either: 1) promoter switching, whereby transcription from the imprinted promoters P2-P4 declined and
transcription from the non-imprinted promoter P1 greatly increased; or, 2) biallelic expression from all four promoters. Therefore, we determined IGF2 promoter usage in all informative tumors.

In order to estimate the relative abundance of IGF2 transcripts derived from each promoter, we developed a multiplex PCR assay. A panel of oligonucleotide primers was synthesized, all of which have a similar Tm and which were specific for each of the promoters. These were used in conjunction with a downstream primer, p11, in exon 7 (Fig. 2A). Similar results were obtained using downstream primer p10 (not shown). The transcripts corresponding to promoters P1-P4 were amplified separately and together in a multiplex PCR with similar efficiency (Fig. 2B, lanes 1-5). The quantitative nature of this assay was demonstrated by performing standard dilutions of each of the specific templates in a multiplex mixture (Fig. 2B, P1-P4). In each case, proportionally decreasing amounts of the specific PCR product was observed. For the purpose of quantitation of promoter usage, a slight difference in PCR amplification efficiency was adjusted by conversion factors which varied from 0.66 to 2.68 (Table I). Similar results were also obtained without this adjustment.

We determined the IGF2 promoter usage in each tissue by PhosphorImager scanning of the specific PCR products (Table I). As expected, in human adult liver, IGF2 was transcribed predominantly from promoter P1 (90.3%), whereas P3 and P4 accounted for 9.7% of the total transcripts. In contrast, in newborn liver and in some fetal tissues (54 days of gestation), P3 and P4 accounted for more than 90% of the total IGF2 transcripts, with P1 accounting for ≤1%.

In the Wilms' tumors (Fig. 2C), just as in normal tissues, IGF2 was transcribed predominantly from promoter P3 (≥80%). Interestingly, in comparison to fetal kidney, most of the Wilms' tumors showed a dramatic increase in the use of promoters P1 and P3 (Table I). However, except for tumor 2, in most cases, promoters P1 and P3 each accounted for <15% of the total transcripts. There was no difference in promoter usage in those tumors which demonstrated LOI for IGF2 (tumors 1-6) compared with those in which IGF2 remained unimprinted (tumors 9-11). Thus, despite varying promoter usage, relaxation of IGF2 imprinting in Wilms' tumor was not simply accomplished by promoter switching from the mononucleically expressed promoters P2, P3, and P4 to the biallelically expressed promoter P1.

Promoter-specific Alteration of Imprinting—To examine the allelic expression of IGF2 from individual promoters P1-P4 in Wilms' tumors, we amplified full-length PCR transcripts from each promoter using a nested PCR as described previously (8). Full-length transcripts from P1 were of low abundance and were observed in tumors from subjects 1 (not shown), 2 and 3 (Fig. 3). The nature of the double nested PCR technique does not allow an accurate quantitation of the expression of the two alleles. In the tumors which demonstrate LOI, IGF2 was biallelically expressed from all four promoters (subjects 2 and 3 in Fig. 3; and 5 and 6, not shown). Since promoter P1 is not normally imprinted in liver and chondrocytes (8), in fetal pancreas, and in some fetal kidneys (see below), LOI in Wilms' tumor therefore represents the relaxation of imprinting from

| Tissue        | Subject | DNA Genotype | b/(a+b) | Expression | RNA Genotype | b/(a+b) | d/(c+d) |
|---------------|---------|--------------|---------|------------|--------------|---------|---------|
| LOI tumors    | 1       | a/b          | 49.8    | a/b        | 50.0         | d/d     | NI      |
|               | 2       | a/b          | 46.9    | a/b        | 47.7         | c/d     | 25.7    |
|               | 3       | a/b          | 48.1    | a/b        | 47.9         | c/d     | 30.2    |
|               | 4       | a/b          | 50.4    | a/b        | 49.0         | c/d     | 32.8    |
|               | 5       | a/b          | 40.1    | a/b        | 52.5         | c/d     | 22.5    |
|               | 6       | a/b          | 47.7    | a/b        | 47.6         | c/d     | 32.8    |
|               | 7       | a/b          | 47.8    | a/b        | 49.2         | c/d     | 48.3    |
|               | 8       | a/b          | 48.4    | a/b        | 49.9         | c/d     | 51.5    |
| MOI tumors    | 9       | a/b          | 48.5    | b          | 99.0         | c/d     | 0.0     |
|               | 10      | a/b          | 49.7    | b          | 98.0         | c/d     | 8.5     |
|               | 11      | a/b          | 47.7    | a          | 2.0          | d/d     | NI      |
|               | 12      | a/b          | 48.0    | b          | 98.0         | c/d     | 1.0     |
|               | 13      | a/b          | 48.2    | b          | 99.0         | c/d     | 12.4    |
|               | 14      | a/b          | 49.3    | b          | 98.0         | c/d     | 27.9    |
|               | 15      | a/b          | 49.0    | b          | 100          | d/d     | 96.3    |
|               | 16      | a/b          | 51.2    | a          | 3.0          | c/d     | 19.1    |
|               | 17      | a/b          | 49.9    | b          | 96.0         | c/d     | 98.5    |
|               | 18      | a/b          | 50.2    | a          | 6.0          | d/c     | 0.0     |
|               | 19      | a/b          | ND      | a          | 2.0          | d/c     | 100     |
| Other tumors  | 20      | bb           | NI      | NI         | NI           | d/d     | 16.4    |
|               | 21      | bb           | NI      | NI         | NI           | d/d     | 31.6    |
|               | 22      | bb           | NI      | NI         | NI           | d/d     | 46.2    |
|               | 23      | bb           | NI      | NI         | NI           | d/d     | 52.5    |
|               | 24      | bb           | NI      | NI         | NI           | d/d     | 82.0    |
|               | 25      | bb           | NI      | NI         | NI           | d/d     | 6.3     |

*a* NI, not informative.

*b* ND, not determined.

*c* Selected Alu heterozygous Wilms' tumor.
promoters P2-P4 with the maintenance of biallelic expression from P1. In each of the tumors in which IGF2 remained imprinted, IGF2 was transcribed exclusively from one allele from promoters P2, P3, and P4 (Fig. 3, subjects 9 and 10).

Fluctuation of Allelic IGF2 Gene Expression from Promoter P1—To further investigate allelic expression from promoter P1, we took advantage of a recently discovered AluI polymorphism in exon 3 of the IGF2 gene (17). The propinquity of this polymorphic site to the region of promoter P1 allows a direct analysis of a relatively short P1 transcript. We screened DNA from the Wilms' tumors for the AluI polymorphism using primers spanning 81 bases of exon 3 (Fig. 4A). Sixteen of the 19 Apal heterozygous tumors were also AluI heterozygotes (Table I). The AluI and Apal polymorphic sites may exist under disequilibrium as in the case of many polymorphic sites in the insulin gene which is located 2 kilobases 5' of the IGF2 gene (17, 20).
IGF2 promoter usage is represented as a percent of total promoter-specific transcripts (P1–P4) and was determined by a multiplex PCR using P1–P4 specific primers and a α-32P end-labeled primer pI1. Since each P1–P4 primer, although similar in Tm, is slightly different in PCR amplification efficiency, amplification conversion factors for P1–P4 promoters were calculated. Values were normalized for the conversion factors. All fetal tissues were from one fetus at 54 days of gestation.

| Normal tissues | Promoter usage, % | Promoter-specific transcript |
|----------------|------------------|----------------------------|
| Adult liver    | 90.3             | P1 0.7  P2 19.8  P3 5.3  P4 0.6 |
| Newborn liver  | 7.6              | P1 0.3  P2 19.5  P3 5.3  P4 0.6 |
| Fetal heart    | 6.3              | P1 0.5  P2 19.5  P3 5.3  P4 0.6 |
| Fetal renal    | 9.4              | P1 0.4  P2 19.5  P3 5.3  P4 0.6 |
| Fetal pancreas | 9.0              | P1 0.2  P2 19.5  P3 5.3  P4 0.6 |

| LOI tumors     | Promoter usage, % | Promoter-specific transcript |
|----------------|------------------|----------------------------|
| 1              | 8.2              | P1 3.6  P2 19.8  P3 5.3  P4 0.6 |
| 2              | 19.8             | P1 2.8  P2 19.5  P3 5.3  P4 0.6 |
| 3              | 5.3              | P1 5.3  P2 19.5  P3 5.3  P4 0.6 |
| 6              | 0.6              | P1 11.9 P2 19.5  P3 5.3  P4 0.6 |

| MOI tumors     | Promoter usage, % | Promoter-specific transcript |
|----------------|------------------|----------------------------|
| 9              | 10.5             | P1 7.3  P2 19.8  P3 5.3  P4 0.6 |
| 10             | 6.7              | P1 12.5 P2 19.5  P3 5.3  P4 0.6 |
| 11             | 0.0              | P1 3.8  P2 19.5  P3 5.3  P4 0.6 |

| Multiplex PCR efficiency | Promoter usage, % | Promoter-specific transcript |
|--------------------------|------------------|----------------------------|
| Template before PCR, %   | 18.66            | P1 5.44  P2 30.21  P3 31.69  P4 20.38 |
| Multiple PCR product %   | 8.06             | P1 2.03  P2 19.5  P3 5.3  P4 0.6 |
| Conversion factor, a/b   | 2.32             | P1 2.08  P2 19.5  P3 5.3  P4 0.6 |

Transcripts from P1 were analyzed by RT-PCR using primers spanning exons 2 and 3. An internal Alu site in exon 2 served as an internal restriction control (Fig. 4A). In LOI tumors, transcripts from P1 were derived from both alleles (Table I, subjects 2–8; Fig. 4B, lanes 3–5). In contrast, in 5 of 9 Alu-informative MOI tumors, more than 95% of P1 transcripts were from one allele only (Table I and Fig. 4B, lanes 6–9).

Since the genomic imprinting of IGF2 is specific for promoters P2–P4, we speculated that there may exist variations in IGF2 imprinting during early development in some specific tissues. Using the Alu polymorphism, we have observed fluctuation of imprinting of promoter P1 in various normal tissues. As early as 6 to 8 weeks of gestation, biallelic IGF2 expression of P1 was observed in brain and liver (not shown), as well as in kidney and adrenal from different subjects (Fig. 4B, lanes 10 and 11). In a subject at 12 weeks of embryonic development, the fluctuation was tissue specific: both parental alleles showed equal expression from P1 in kidney (lane 12), but unequal expression in other tissues (lanes 13–15). Such fluctuation of imprinting was specific for the promoter P1 region. There was no indication of variation in imprinting in transcripts from promoters P2–P4. Analysis of total IGF2 transcripts which derived predominantly from promoters P3 and P4 in various normal fetal tissues (liver, lung, kidney, pancreas, heart, limb, adrenal, spleen, and gut) showed only monoallelic expression (data not shown).

IGF2 Imprinting in Other Tumors—To compare the disruption of imprinting in Wilms’ tumor with the imprinting alteration in other malignancies, we also examined the promoter allele expression of IGF2 in lung tumors, hepatoblastomas, and in several normal tissues. In addition to normal livers and chondrocytes, we have observed IGF2 promoter-specific imprinting in an adrenal gland from a 19-week-old fetus (data not shown) and in a pancreas from an 8-week-old fetus (Fig. 5A). In a hepatoblastoma which demonstrated persistent imprinting, we found monoallelic expression of IGF2 from promoters P2–P4 (98–100%) and biallelic expression from P1. This hepatoblastoma (Fig. 5B) maintained the promoter-specific imprinting which has been reported in liver. In an informative LOI lung tumor, IGF2 transcripts were derived from P1–P4 promoters of both parental alleles (Fig. 5C). The imprinting alteration in this lung cancer, therefore, is similar to the LOI seen in Wilms’ tumor.

DISCUSSION

Genomic imprinting refers to a phenomenon whereby only one of the two parental alleles is expressed, while the other allele is transcriptionally silenced. In some circumstances, it appears that the imprinting process is completely efficient, leaving one allele totally suppressed. Some investigators, however, have suggested that imprinting may be incomplete, or “leaky”; it has been estimated that in the mouse, the amount of IGF2 expressed from the imprinted maternal allele was about 2-4% of that expressed from the non-imprinted paternal allele (21). Although it may be difficult to quantitate accurately this small amount of expression from the generally imprinted allele, the presence of this escape from total imprinting suggests that imprinting should not be considered an all-or-none event. Instead, imprinting may be more usefully defined as the differential expression of two parental alleles.

Using the multiplex PCR, we have shown that in most normal tissues (except for adult liver) as well as in Wilms’ tumor, P3 is the major promoter utilized in the transcription of IGF2. In general, more than 80% of the transcripts derive from P3, with <10% derived from P1 and P2. Therefore, when IGF2 imprinting is studied without regard to promoter usage, i.e. when all of the IGF2 transcripts are examined together, there is an overwhelming predominance of P3-derived mRNAs, and the imprinting status of the sample will reflect that of P3; the contribution to imprinting or LOI by P1, for example, may not be apparent because of the low abundance of P1-derived transcripts.

Our studies confirm previous reports that there is loss of IGF2 imprinting in some but not all Wilms’ tumors (13, 14). Having previously shown that IGF2 imprinting was promoter-specific in some tissues (8), we hypothesized that the loss of imprinting in Wilms’ tumors might be due to an alteration in promoter usage, switching from the imprinted promoters P2–P4 to the biallelically transcribed promoter P1. Our data, however, do not confirm that theory. Instead, we have shown that in LOI neoplastic tissue, IGF2 is transcribed primarily from the same promoters as in normal kidney (albeit with an increased use of promoters P1 and P2), but that these promoters are now biallelically expressed; promoter P1 continues to be expressed from both parental alleles in these tumors.

Interestingly, in LOI tumors, transcripts from both parental alleles are equally abundant. Since all three promoters, P2–P4, show equal allelic expression (Fig. 3), in LOI tumors the normally imprinted promoters demonstrate complete relaxation of imprinting. This suggests that the IGF2 imprinting process, which is governed by some unidentified imprinting factors, must have been reversed.

The significance of monoallelic expression from P1 in MOI tumors is unclear. Except in adult liver and chondrocytes, P1 appears to play a small role in the overall transcription of IGF2, and it seems unlikely that the total abundance of IGF2 transcripts would be altered significantly if P1 were expressed biallelically or monoallelically. As we have shown, P1 may be expressed from a single allele in some organs during early fetal development, and it is likely that there are tissue- and development-specific factors that can modulate the imprinting signal or imprinting box responsible for the differential allelic
expression from the IGF2 gene. In this light, it will be of interest to study the tissue-specific ontogeny of methylation of the IGF2 gene.

Since tumors may demonstrate relaxation of IGF2 imprinting, it has been suggested that the appearance of biallelic expression of IGF2 may be an early event in tumorigenesis. According to this hypothesis, one function of genomic imprinting is to repress the expression of one of two copies of a putative tumor-enhancing growth factor. With the loss of imprinting, the growth factor is overexpressed, contributing to the growth and/or development of the neoplasm (22). IGF-II is necessary for the initiation of cell proliferation in transgenic mice which express the SV-40 large T antigen oncogene and develop pancreatic tumors. As the pancreatic cells begin to proliferate, IGF2 imprinting is relaxed, and the gene is expressed biallelically, suggesting that LOI may be an early and perhaps etiologic event in malignant transformation (23, 24). Constitutional relaxation of IGF2 imprinting has been reported in skin fibroblasts (25), normal kidney tissue, and in peripheral blood leukocytes (26) of patients with Beckwith-Wiedemann syndrome and Wilms' tumor, a finding that suggests that the generalized loss of IGF2 imprinting may predispose to the development of neoplasia. However, since biallelic expression of IGF2 normally occurs in central nervous system, it is clear that this abrogation of the normal imprinting status is not a sufficient signal for oncogenesis.

IGF2 gene expression is variably increased in Wilms' tumors which demonstrate biallelic expression of IGF2. As might be expected, tumors with LOI may contain as much as twice the amount of IGF2 mRNA as do tumors in which the gene is imprinted, but there is a very wide range of expression in these tumors, and the reported increase among all of the tumors was not statistically significant (27). In some but not all cases, increased amounts of IGF-II peptide can be extracted from these tumors.

It is also possible, of course, that the LOI of IGF2 is an epigenetic event and is not itself oncogenic. The mechanism underlying the imprinting of IGF2 is unknown, although CpG methylation has been suggested as a final pathway or "mark" for the imprint. If methylation of some CpG sites in the IGF2 gene has a mechanistic implication in the suppression of the imprinted allele, the reversion to complete activation in LOI tumors indicates a complete demethylation at CpG sites. This is in accordance with the observation of extensive hypomethylation of both IGF2 alleles in LOI tumors in contrast to a specific maternal methylation pattern in MOI tumors (28). A number of studies have indicated that there is reciprocal regulation of IGF2 and H19, a paternally-imprinted gene immediately downstream from IGF2. Studies have suggested that a common imprinting mechanism regulates these two genes in a coordinate but opposite fashion (29). H19 is a tumor suppressor gene (30), and its expression is substantially reduced in tumors in which IGF2 is biallelically expressed. In the mouse, it appears that H19 is itself responsible for imprinting IGF2, since H19 knockout mice do not display IGF2 genomic imprinting (31). The mouse, however, does not have a homolog of the nonimprinted human promoter P1, and it is not known how H19 itself could differentially alter IGF2 promoter-dependent imprinting. The promoter-specific imprinting of human IGF2 is not related to H19 expression and is not likely to be controlled by H19 (32). Finally, the tumor suppressor gene, WT1 has been shown to suppress IGF2 gene expression (33). WT1 may also be
imprinted (34), and it is possible that alterations in IGF2 imprinting, WT1 expression, and silencing of H19 may all contribute to Wilms' tumor progression.

We have recently reported differential transcription of the two parental IGF2 alleles in the mouse central nervous system (35). In some parts of the central nervous system, the maternal allele is predominantly expressed, while in other parts, the paternal allele is predominant. We have also observed biallelic expression of IGF2, which is transcribed primarily from promoters P3 and P4, in the human central nervous system. Thus LOI of promoters P2-P4 is not restricted to neoplastic tissues.

With the exception of the central nervous system, we have not observed any alteration of P2-P4 imprinting in normal tissues, although Ekstrom et al. (32) have reported potential allelic switching involving these promoters in two adult liver specimens. Promoter-specific LOI of P2-P4 is, therefore, most likely a consequence of an alteration of regional imprinting factors which may be modulated by tissue-specific factors or by local, potentially epigenetic changes in the IGF2 gene.

In the human fetus, biallelic expression of P1 is seen as early as 6 weeks, but in some tissues, IGF2 P1 expression varied greatly, with predominant expression of one of the parental alleles occasionally observed. Fluctuation of imprinting of promoter P1 was observed in some tissues in early development at

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3 T. H. Vu and A. R. Hoffman, unpublished results.
6–12 weeks of gestation. However, we have not observed a specific development stage for the changing of imprinting of promoter P1; rather mono- and biallelic expression from promoter P1 were observed in different tissues from the same fetus. It appears that tissue-specific factors may play an important role in this epigenetic phenomenon.

Taniguchi et al. (36) recently reported biallelic expression of IGF2 from all four promoters in LOI Wilms’ tumors and monoallelic expression from all four promoters in MOI tumors. Furthermore, they found monoallelic expression from promoter P1 in tissues from fetuses of 12 weeks gestation. While our results are consistent with their findings in LOI tumors, our informative MOI tumors showed either monoallelic or biallelic expression from promoter P1. Moreover, we found biallelic expression of IGF2 in developing kidneys of 8 and 12 weeks gestational age, and both mono- and biallelic expression of P1 in various other embryonic tissues. Therefore, imprinting of promoter P1 is mosaic, developmentally dependent, and tissue-specific.

It has always been assumed that both the paternal and maternal alleles are expressed in an equivalent fashion, with the exception of X-chromosome genes which are silenced during lyonization. However, it is possible that other genes, which may or may not be imprinted, are also subject to differential allelic expression, allowing “dominance” of one set of parental genes. The tissue- and developmental-specific fluctuations of imprinting may provide a window for understanding the physiology of local factors which modulate the imprinting process and for intervening in an attempt to reverse the oncogenic state by initiating a reversion to normal genomic imprinting.

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