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Expression and Imprinting Status of Human *PEG8/IGF2AS*, a Paternally Expressed Antisense Transcript from the *IGF2* Locus, in Wilms' Tumors[1]

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A large imprinted gene cluster in human chromosome 11p15.5 has been implicated in Beckwith-Wiedemann syndrome and Wilms' tumor. We have identified a paternally expressed imprinted gene, *PEG8/IGF2AS*, in this locus. It is transcribed in the opposite direction to the *IGF2* transcripts and some genomic regions are shared with the *IGF2* gene, as in the case of the mouse imprinted *Igf2as* gene reported previously by T. Moore *et al.* As to the relationship between these genomic regions, the human and mouse genes are very similar but there is no homology in their middle parts. Interestingly, *PEG8/IGF2AS* and *IGF2* were found to be overexpressed in Wilms' tumor samples, at levels over ten and a hundred times higher than that in normal kidney tissues neighboring the tumors, respectively. These findings indicate that *PEG8/IGF2AS* is a good marker of Wilms' tumor and also suggest the possibility of *PEG8/IGF2AS* being one of the candidate Wilms' tumor genes.

Key words: genomic imprinting, *IGF2*, *IGF2AS*, imprinted genes, Wilms' tumor.

Genomic imprinting and imprinted genes play an important role in mammalian development, growth, and behavior, as well as in some human genetic diseases (*1–5*). Many studies have been carried out on imprinted genes on human chromosome 11p15.5 because it has been reported to be responsible for Beckwith-Wiedemann syndrome (BWS) (*6–9*) and Wilms' tumor (*8–12*). Eight human imprinted genes, comprising paternally expressed *IGF2* (*13–16*) and *LIT1* (*17*) and maternally expressed *H19* (*18*, *19*), *HASH2* (*20*, *21*), *p57^{KIP2}* (*22–24*), *KVLQT1* (*25*), *IPL* (*26*), and *IMPT1* (*27*), have been identified so far in this region, making a large imprinted gene cluster.

Balanced translocations of human 11p15.5 have been found in familial cases of BWS (*28*, *29*). These translocations cause the disease when maternally inherited but have no apparent effect when paternally inherited. For sporadic cases, there have been some reports of trisomies of 11p15.5 (with an excess of the paternal chromosome) (*8*, *30*) and paternal disomies of chromosome 11, although they are rare (*9*, *31*). Therefore, it has been thought that overexpression of some paternally expressed gene(s) (the BWS gene) and/or loss of a maternally expressed gene(s) whose product functions as a *cis*-acting suppressor may be the cause of the disease (*32*, *33*). The *IGF2* gene is the best candidate for the gene responsible for BWS, because it is paternally expressed and its product functions as a growth factor. It has been demonstrated that *Igf2* transgenic mice show the characteristic symptoms of BWS, such as gigantism and organ overgrowth (*34*). Another candidate gene is *p57^{KIP2}*, because knockout of the *p57^{Kip2}* gene results in neonatal

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Abbreviations: LOI, loss of imprinting; LOH, loss of heterozygosity; PEG, paternally expressed gene.

lethality with omphalocele, and altered cell differentiation and proliferation, which are other features of BWS (*35*, *36*).

Wilms' tumor and several childhood tumors have been frequently observed (10%) in BWS patients (*7*, *37*). Loss of maternal chromosome 11 (LOH) and subsequent duplication of paternal chromosome 11 in many Wilms' tumor patients have been reported (*8–12*). Therefore, overexpression of paternally expressed gene(s) and loss of maternally expressed repressor genes (tumor suppressor genes) have been postulated as the mechanism responsible for Wilms' tumor as well as BWS. The most plausible candidate for such a paternally expressed gene is also the *IGF2* gene. In 60% of Wilms' tumor cases, heterozygosity of the 11p region is maintained. However, loss of imprinting of the *IGF2* gene was observed in 70% of such cases (*38–40*). In the present report, we have shown that human *PEG8/IGF2AS* is a paternally expressed imprinted gene, and that there is a high correlation between increased expression of *PEG8/IGF2AS* and Wilms' tumor as well as between *IGF2* and Wilms' tumor.

MATERIALS AND METHODS

Human Samples—Human embryos, chorionic villi, and fetal kidney samples were obtained through therapeutic terminations. Wilms' tumor tissues and normal kidney tissues neighboring the tumors were obtained at surgery.

RNA Extraction—ISOGEN (Nippon Gene) was used to extract total RNA from the tissues. One hundred milligram tissue was homogenized with 1 ml of ISOGEN, 200 µl of chloroform was added, and then the sample was shaken vigorously, followed by incubation for 5 min on ice. The suspension was centrifuged at 12,000 ×*g* at 4°C for 15 min. The RNA in the aqueous phase was transferred to a clean tube, precipitated with an equal volume of 2-propanol, stored for 15 min at 4°C, and then pelleted by centrifugation at 12,000 ×*g* at 4°C for 10 min. The RNA pellet was washed with 75% ethanol, air dried, and then resuspended in 100 µl of DEPC-treated water. Dynabeads oligo (dT) 25 (DYNAL) was used to isolate poly A^+ RNA from total RNA.

Northern Blotting—Wilms' tumor and normal kidney poly A^+ RNAs, 1–3 µg of each, were analyzed by Northern blotting using a probe specific for *PEG8/IGF2AS*. The blots were washed at 65°C in 0.2 × SSC/0.1% SDS, exposed overnight to an imaging plate (FUJI), and then analyzed with a BAS2000A system (FUJI).

Genomic PCR and RT-PCR—Genomic PCR and RT-PCR were performed using TaKaRa *Ex Taq*. Fifty nanograms of genomic DNA was used for genomic PCR. One microgram of total RNA was used to synthesize cDNA using an oligo (dT) 12-18 and SUPERSCRIPT preamplification system (GIBCO BRL), and one one-hundredth or one one-thousandth of the resulting material was used for RT-PCR. Amplification consisted of 30–34 cycles of 96°C for 15 s, 65°C for 30 s, and 72°C for 60 s. The primers used were: F1, CTCTCCTCCTCCACGTCAAC; F2, CAAGGTGACCTGAAGGAACC; F3, GCACAGCAGCATCTTCAAAC; F4, CCTGGGAATGCTCATTCATG; R1, AGCAGCTCACCTCAGGACTG; R2, TTGGTCTTACTGGGTCCCTC; R3, TGGACACACAGCTCTGCTTG. The PCR primer sets used for the amplification of genomic DNAs and cDNAs were F2-R1, F4-R3 (for *PEG8/IGF2AS*), F1-R1 (for the region of *IGF2* transcribed from the P1 promoter), and F3-R2 (for total *IGF2*).

Selective Amplification of Antisense or Sense Transcripts by RT-PCR—Reverse transcription was performed using the SUPERSCRIPT preamplification system (GIBCO BRL) with specific reverse (R1) or forward (F2) primers, followed by amplification by PCR using F2-R1 primers. To avoid non-specific annealing of the primers, the extension reaction was performed at 55°C. The PCR conditions were the same as above except that the number of cycles was increased to 35.

Verification of Imprinting of PEG8/IGF2AS and IGF2—Analysis of *PEG8/IGF2AS* and *IGF2* was carried out by amplifying the DNA fragments from genomic DNA by PCR or by production of cDNA. The PCR primers used for the amplification of genomic DNAs and cDNAs were F2-R1 (for *PEG8/IGF2AS*), F1-R1 (for the region of *IGF2* transcribed from the P1 promoter), and F3-R2 (for total *IGF2*). The amplified products were digested with *Alu*I (*41*) (in the case of *PEG8/IGF2AS* or the region of *IGF2* transcribed from the P1 promoter) or *Apa*I (*42*) (in the case of total *IGF2*).

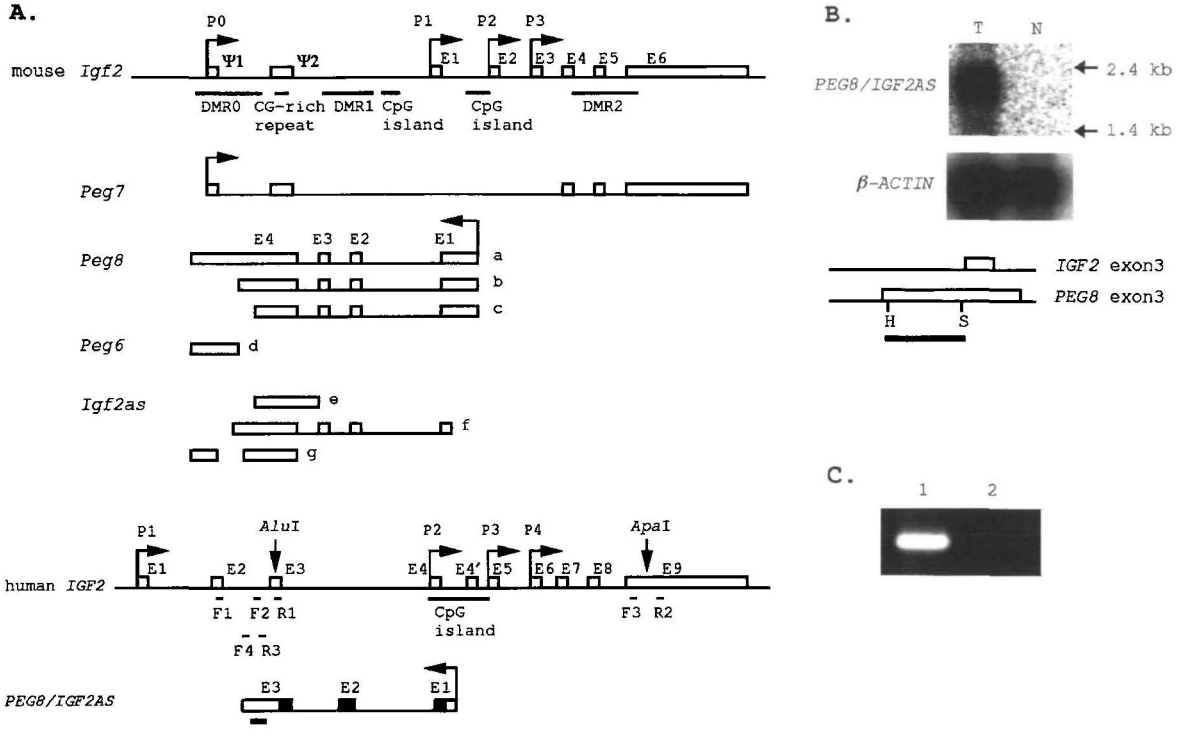
Real Time Quantitative PCR—Expression of *PEG8/IGF2AS* and *IGF2* in Wilms' tumor tissue and in normal kidney tissue neighboring the tumor was analyzed with an ABI PRISM 7700 using SYBR Green PCR Core Reagents (PE Applied Biosystems), designed to detect cDNA (*43–45*). *PEG8/IGF2AS*, *IGF2* (P1), and *IGF2* were cloned in plasmids, and then the concentration and copy number were calculated. These plasmids were used to prepare standard curves. In the case of each sample, the level of expression was measured as the increase in the strength of fluorescence upon binding of SYBR Green to double-stranded

Fig. 1. **Organization of the *PEG8/IGF2AS* and *IGF2* region in human and mouse genomic DNA.** A: Genomic structure of mouse and human *Igf2/IGF2*, and multiple sense and antisense (a–g) transcripts. The genomic structure of the *PEG8/IGF2AS-IGF2* region was established based on the sequence of 2.1 kb *PEG8/IGF2AS* transcripts and genomic DNA sequences (Genbank: X03423, M22372, Y13633, U80851, X53038, X03562, and X07868). *Peg6* (d) and *Peg8* (a–c) are paternally expressed genes isolated in our screening that are identical to the *Igf2as* (e–g) reported by Moore *et al.* (*50*). *Peg7* is identical to the placenta-specific *Igf2* transcript (*50*) and is paternally expressed. Open boxes on the genomic DNA indicate the exons of each transcript. Black boxes indicate ORF of *PEG8/IGF2AS*. The transcription start sites and directions are indicated by arrows. An *Alu*I polymorphic site in human *IGF2* exon 3 is indicated by an arrow, by means of which *PEG8/IGF2AS* and *IGF2* (P1) imprinting was examined (Figs. 3 and 5). An *Apa*I polymorphic site in human *IGF2* exon 9 is also indicated by another arrow, by means of which *IGF2* (total) imprinting was examined (Fig. 5). The positions of the forward and reverse primers designed for the detection of *PEG8/IGF2AS*, F2-R1 and F4-R3, and the primers for the detection of *IGF2* (P1), F1-R1, and the primers for the detection of *IGF2* (total), F3-R2, are shown. DMR0 was reported to be maternally hypermethylated in the placenta (*50*). DMR1 and 2 are regions that show paternally hypermethylation in the fetus (*53*, *54*). B: Northern hybridization of *PEG8/IGF2AS* in Wilms' tumor tissue (T) and corresponding normal kidney tissue neighboring the tumor (N) using a *Hin*dIII (H)–*Sma*I (S) 0.7 kb fragment as a probe (thick bar). C: Detection of the human *PEG8/IGF2AS* transcript and determination of the direction of transcription were carried out by selective amplification of antisense or sense transcripts by RT-PCR. The human *PEG8/IGF2AS* transcript was only detected in the case of F2 priming of the reverse transcription reaction, a band corresponding to a 272 bp fragment being obtained (lane 1), whereas R1 priming resulted in no band (lane 2), indicating that *PEG8/IGF2AS* is transcribed in the opposite direction to the *IGF2* transcript. D: Total cDNA sequence and ORF of *PEG8/IGF2AS*

DNA in the reaction mixture. The copy number was calculated from C_T (threshold cycle), which is the point at which the amplification plot crosses the threshold on the standard curve. Each sample was analyzed three times and the mean C_T value was calculated. Amplification consisted of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 65°C for 30 s, and 72°C for 60 s. The primers used were F4-R3 (for *PEG8/IGF2AS*, described above), F3-R2 (for *IGF2*, described above), F1-R1 (for the region of *IGF2* transcribed from the P1 promoter), and *β-ACTIN* (for normalization of the cDNA concentration in each sample).

RESULTS

Isolation and Mapping of Human PEG8/IGF2AS—Systematic screening of imprinted genes in the mouse has been carried out using our subtraction-hybridization method (*46*), day 8.5 fertilized and parthenogenetic embryos be-



D.

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                                                  M  S  K  R  K  W  R  G  F  R  G  A  Q  Q  E  R  A
CAGCCGCCAGCCGCAAGTCCGCAGCCGTGTCCCGCGCCCCACGCCGGCCTCCCCGGCGGCAGCCGGAGACGAGCGCCAGCACCGGCGGGA
Q  P  P  A  A  S  P  Q  P  C  P  A  P  H  A  G  L  P  G  G  S  R  R  R  A  P  A  P  A  G
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Q  Q  Q  M  R  A  E  S  R  S  G  A  Q  R  R  R  G  S  A  R  R  G  A  H  R  E  A  G  G  C
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V  R  G  R  T  R  S  S  G  S  E  R  S  N  A  L  W  Q  A  V  D  A  A  E  A  L  A  L  S  S
CCGCTCCGCCGTCCGTGGGACCAAGCCCAGCATTTTACAAACCCAGCTCCTGTCTCCAAGGGTCCTCAGAGCGCCCCTCCGTCACCCCCT
P  L  R  R  P  W  D  Q  A  Q  H  F  T  N  P  A  P  V  S  K  G  P  Q  S  A  P  P  S  P  P
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A  G  R  R  R  R  G  A  D  L  A  L  A  P  L  A  G  E  G  H  T  R  W  R  H  R  P  A  W  E
GTAGGACTAAGGACCCGAACTGCAACCCTCCACACCAGACAGCACAGACCACCCCAAGGCTGCTCAATCTGCCCAAAGCCAAAAGAGCTC
V  G  L  R  T  R  T  A  T  L  H  T  R  Q  H  R  P  P  Q  G  C  S  I  C  P  K  P  K  E  L
CCCTGCCAGGACCTCGGTGCCGTGGAGACTGCGGGGAACCTGCTCTGCCCACACCTGCTCTGGCCCCTAGACGGAAGCACCTGGAGCCAA
P  C  Q  D  L  G  A  V  E  T  A  G  N  L  L  C  P  H  L  L  W  P  L  D  G  S  T  W  S  Q
CCACGGAACTCCAGATCCCAGCTGTGTGACTGAATCCACGCCAGCCTCTCTGAGCCAAAGCTGCTTGCAGAAGGGGGAGATCCCAGTTCG
P  R  N  S  R  S  Q  L  C  D  *
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ing examined, and five paternally expressed genes, *Peg1/Mest* (*46*), *Igf2*, *Peg3* (*47*), *Snrpn* (*48*), and *Peg5/Nnat* (*49*), including three novel imprinted ones have been identified. In the course of further screening, we found three novel transcripts (*Peg6-8*) in the region upstream of the *Igf2* gene (Fig. 1A). They were identical to the transcripts previously reported by Moore *et al.* (*50*). *Peg7* was identical to the placenta-specific *Igf2* transcript, and *Peg6* and *Peg8* were identical to the *Igf2* antisense transcripts (Fig. 1A, *Igf2as*). We have also confirmed the paternal expression of these transcripts. They showed that there were several antisense transcripts [Fig. 1A, *Igf2as* (e–g)] in this region (*50*), and we also isolated clones of several lengths, such as *Peg8* (a–c) and *Peg6* (d). Initially, we separated the clones into two groups (*Peg6* and *Peg8*). However, these transcripts may be parts of the same continuous transcript because the longest transcript of *Peg8* recently isolated [Fig. 1A, *Peg8* (a)] contains the full length *Peg6* sequence. They commented that there was no human *IGF2AS* in the corresponding region. However, we identified a human homologue of *PEG8/IGF-2AS* in the region corresponding to mouse *Igf2as* (*Peg8*).

We designed PCR primers (Fig. 1A, F2-R1 primers) corresponding to the human *PEG8/IGF2AS* region by comparing the mouse (Genbank: U71085) and human (Genbank: X03423 and M22372) genomic DNA sequences. RT-PCR was carried out using chorionic villi cDNA and a 272 bp DNA fragment was cloned. After removing the region coinciding with *IGF2* exon 3, the resulting 120 bp fragment was obtained. Using this fragment as a probe, we screened a human 20–26 weak fetal kidney cDNA library and isolated a 2.1-kb *PEG8/IGF2AS* cDNA. Northern blot analysis of Wilms' tumor samples (Fig. 1B) and 5′-RACE experiments (data not shown) suggested that this was a full-length cDNA clone. The genomic structure of human *PEG8/IGF2AS* is compared with that of mouse in Figs. 1A and 2.

Human *PEG8/IGF2AS* appeared to be transcribed from the region between human *IGF2* exon 4 (corresponding to mouse exon 1) and exon 4′ (no corresponding exon in the mouse) in the opposite direction to the *IGF2* transcript, judging from the position of the poly A sequences. This was confirmed by the following RT-PCR experiment. As *PEG8/IGF2AS* completely overlapped the *IGF2* transcript from the P1 promoter (sense transcript) within the region of human *IGF2* exon 3, we performed RT-PCR with samples using direction-specific priming (F2 or R1 priming) in the reverse transcription reaction (Fig. 1C). It should be noted that the F2 primer itself was designed based on an intron region of the *IGF2* gene, as shown in Fig. 1A. Human *PEG8/IGF2AS* was detected by PCR using the F2-R1 primer set in the case of the F2 priming samples only (Fig. 1C, lane 1). No bands were detected in the case of the R1 priming samples (Fig. 1C, lane 2). Thus, *PEG8/IGF2AS* is apparently an antisense transcript of *IGF2* from the P1 promoter. Human *PEG8/IGF2AS* has an open reading frame encoding a putative protein consisting of 273 a.a. (Fig. 1D), and positions –3 (G) and –2 (C) from the ATG codon were consistent with the Kozak consensus sequence (*51*), whereas the corresponding *Igf2as* (*Peg8*) in mouse does not have any such ORF (*50*).

Human *PEG8/IGF2AS* exhibits about 50% homology with mouse *Igf2as* (*Peg8*) but lacks an apparent tandem repeat in the middle part (Fig. 2A). The transcription start site of human *PEG8/IGF2AS* is different from that of mouse *Igf2as* (*Peg8*), but the poly A addition site of *PEG8/IGF2AS* is almost the same as one of the poly A addition sites of our mouse *Peg8* clones (Fig. 1A, b). Human *PEG8/IGF2AS* exon 1 overlaps *IGF2* exon 4 by 91bp, and mouse *Igf2as* (*Peg8*) exon 1 overlaps *Igf2* exon 1 by 97bp (Fig. 2B). The transcription start sites of both mouse *Igf2as* (*Peg8*) and human *PEG8/IGF2AS* are located near CpG islands.

Imprinting of Human PEG8/IGF2AS—Using the *Alu*I DNA polymorphism site (*41*) in human *IGF2* exon 3 (arrow in Fig. 1A), we could examine the imprinting status of *PEG8/IGF2AS* using the F2-R1 primer set. We analyzed the DNA polymorphism of *PEG8/IGF2AS* in several human genomic DNAs from embryos (or chorionic villi) and found two informative familial cases. As shown in Fig. 3, pattern A (two bands; 202 and 70 bp) and pattern B (three bands; 144, 70 and 58 bp) were distinguished on *Alu*I digestion of RT-PCR products. In family one, the genomic DNA pattern of the mother, father and fetus was A/A, A/B, and A/B, respectively, while only the B pattern was detected in the case of cDNA from 7 week villi samples, indicating paternal expression of *PEG8/IGF2AS*. The same paternal expression of the B pattern as that in the case of the villi samples was also observed on analysis in family 2. In this case, the genomic pattern of the mother, father and 9 week fetus was A/A, B/B, and A/B, respectively. Thus, it was demonstrated that human *PEG8/IGF2AS* was imprinted and paternally expressed as in the case of *Igf2as* (*Peg8*) in the mouse.

Human PEG8/IGF2AS Expression in Wilms' Tumor Samples—The high frequency of loss of heterozygosity (LOH) or loss of imprinting (LOI) of *IGF2* resulting in overexpression of *IGF2* in Wilms' tumor cells has been reported (*10*, *38*, *39*). Because a paternally expressed imprinted gene(s) is a candidate for the Wilms' tumor gene, we examined the expression of *PEG8/IGF2AS* and *IGF2* transcripts from the P1 promoter, *IGF2* (P1), in Wilms' tumor samples compared with the total level of expression of *IGF2*, including four different transcripts from the P1-P4 promoters, *IGF2* (total), using F3-R2 primers specific for the exon 9 region of *IGF2* (Fig. 1A).

We examined the levels of expression by means of a quantitative PCR method using a GeneAmp 7700 system (PE Applied Biosystems). Using four Wilms' tumor samples, we compared the expression levels in the tumor tissues and those in normal neighboring kidney tissues (Fig. 4, 1T-4T, 1N-4N). Normal fetal kidney samples (Fig. 4, FK1-2) and chorionic villi samples (Fig. 4, V1-4) were also examined to assess the expression levels during fetal stages. A high level of expression of *PEG8/IGF2AS* was detected in all the tumor tissues (1T-4T), whereas very low (2N-4N) or almost no expression (1N) was observed in the normal kidney tissues neighboring the tumors. High levels of expression of *IGF2* (P1 and total) were also detected in the tumor tissues (1T-3T), whereas very low (2N and 3N) or almost no expression (1N) was observed in the control tissues. However, the expression levels of these transcripts in 4T were almost the same as those in 4N. It should be noted that the expression copy number of *IGF2* (total) even in the neighboring control kidney tissues was much higher than that of *PEG8/IGF2AS* or *IGF2* (P1) in the tumors (Fig. 4). In the remaining five Wilms' tumor samples (Fig. 4, 5T-9T), the same high level of expression of these transcripts was observed. We confirmed that the expression levels of these

three transcripts were similar to or at most within several fold of that detected in placental samples in the early stages of gestation (4–10 weeks) and fetal kidney samples (14W and 19W6D).

Imprinting Status of PEG8/IGF2AS and IGF2 in Wilms' Tumor—We also checked the imprinting of *PEG8/IGF2AS*, *IGF2* (P1), and *IGF2* (total) in samples that had heterozygous DNA polymorphism patterns (Fig. 5). On the basis of the *Alu*I polymorphism [for *PEG8/IGF2AS*, *IGF2* (P1)] and *Apa*I polymorphism (*42*) [for *IGF2* (total)] described in Fig.

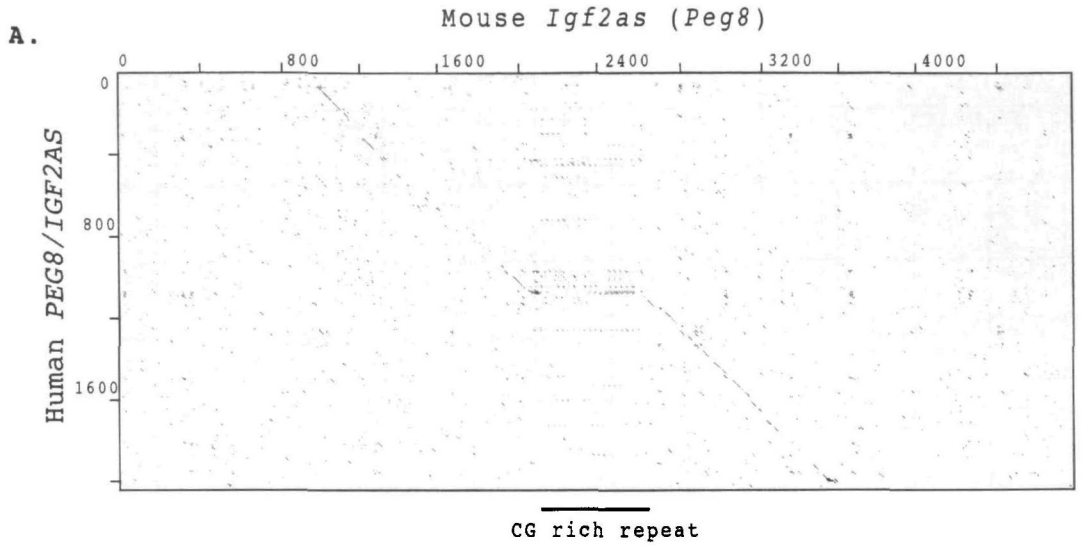

Fig. 2. **Comparison between human *PEG8/IGF2AS* and mouse *Igf2as* (*Peg8*).** A: Comparison of the cDNA sequences, the *Peg8*-a clone (Fig. 1A, a) and human *PEG8/IGF2AS* were analyzed. B: Genomic DNA sequence of the 5' upstream regions.

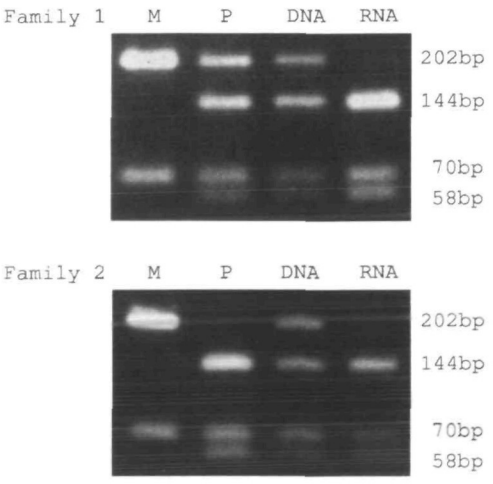

Fig. 3. **Verification of imprinting of *PEG8/IGF2AS*.** The *Alu*I polymorphic site, shown in Fig. 1A, was examined in two families to demonstrate that *PEG8/IGF2AS* was imprinted and paternally expressed. The primer set used for PCR was F2-R1 in Fig. 1A. Pattern A consisted of two bands (202 and 70 bp) and pattern B of three bands (144, 70, and 58 bp). M and P indicate genomic DNA from the mothers and fathers, respectively. DNA and RNA indicate the genomic PCR and RT-PCR of villi samples, respectively.

1A, we found two informative cases (Fig. 5, 5T and 6T) for *PEG8/IGF2AS* and *IGF2* (P1), and three (Fig. 5, 1N-1T, 2N-2T, and 6T) for *IGF2* (total). As shown in Fig. 5, both *PEG8/IGF2AS* and *IGF2* (P1) showed monoallelic expression in both 5T and 6T. Because we confirmed that the genomic DNA of each sample retained both heterozygous alleles, it is apparent that there are no LOH or LOI in these two samples (Figs. 4 and 5).

Among the three informative cases, *IGF2* (total) showed monoallelic expression in one sample (6T) as well as *PEG8/IGF2AS* and *IGF2* (P1). Biallelic expression (LOI) was observed in two samples (1N-1T and 2N-2T), however, apparently, the level of expression of one of the two alleles was higher than that of the other allele in the case of 1N-1T. On the other hand, completely biallelic expression was observed in the case of 2T-2N. Interestingly, the degree of

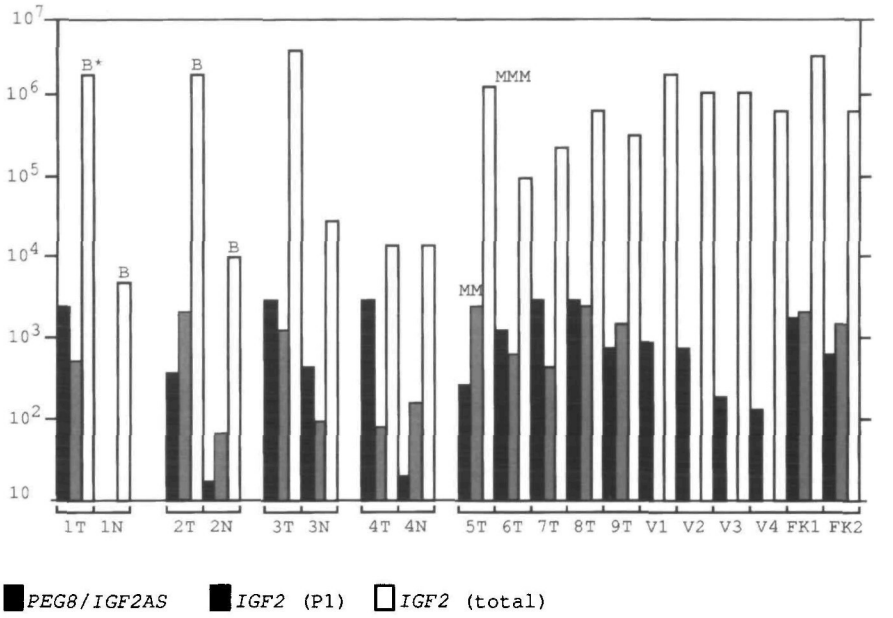

Fig. 4. **Expression of human *PEG8/IGF2AS* and *IGF2* in Wilms' tumor samples.** Expression of *PEG8/IGF2AS*, *IGF2* (P1), and *IGF2* (total) in Wilms' tumor and control samples was assayed by means of a quantitative PCR method and the expression copy number per 10 ng of total RNA is shown on the log scale. Expression of *PEG8/IGF2AS*, *IGF2* (P1), and *IGF2* (total) was examined in four Wilms' tumor samples, comparing the levels in the tumors and those in normal kidney tissues neighboring the tumors (tumors: 1T–4T; control kidney tissues: 1N–4N). Five other Wilms' tumor samples (5T–9T) were also examined. Almost no expression of *IGF2* transcripts (P1 and total) was observed in 1N. In 2T and 3T, a 10- to 60-fold higher level of expression of *PEG8/IGF2AS* or *IGF2* (P1) was detected compared with in the corresponding normal kidney tissues neighboring the tumors. *IGF2* (total) was also highly expressed (at least 100-fold more) in the tumor samples (1T–3T) compared with in the neighboring normal kidney tissues. However, almost the same levels of expression of *IGF2* transcripts (P1 and total) were observed in 4T, whereas a over 200-fold higher level of expression of *PEG8/IGF2AS* was detected. The primer sets used were F4-R3 (*PEG8/IGF2AS*), F1–R1 [*IGF2* (P1)], and F3-R2 [*IGF2* (total)] in Fig. 1A. M or B indicates that monoallelic or biallelic expression was confirmed. * indicates biallelic but biased expression of *IGF2* (see Fig. 5).

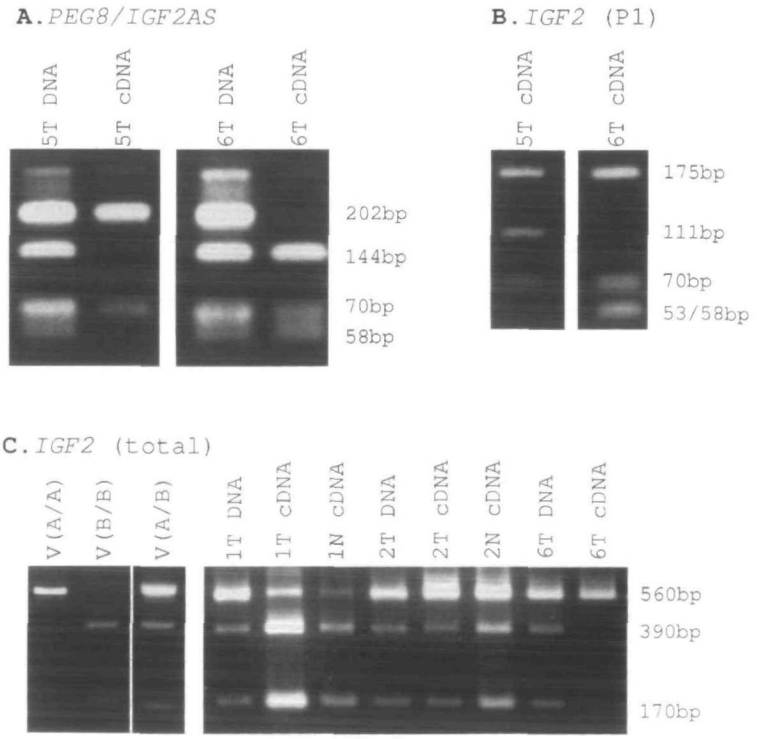

Fig. 5. **Imprinting status of *PEG8/IGF2AS* and *IGF2* in Wilms' tumor samples.** A: *PEG8/IGF2AS* imprinting in 5T and 6T samples. The primer set used for PCR was F2–R1 in Fig. 1A. Genomic PCR or RT-PCR products were digested with *Alu*I. The digestion patterns were the same as those in Fig. 3. Left lane, genomic DNA. Right lane, cDNA. B: *IGF2* (P1) imprinting in 5T and 6T samples. The primer set used was F1–R1 in Fig. 1A. RT-PCR products were digested with *Alu*I. Pattern A consisted of three bands (175, 111, 70 bp) and pattern B of four bands (175, 70, 58, and 53 bp). The genomic DNA of 5T and 6T was heterozygous, as seen upon examination of *PEG8/IGF2AS* imprinting. Only the A pattern was detected in 5T and only the B pattern in 6T. C: *IGF2* (total) imprinting in 1T, 1N, 2T, 2N, and 6T. The primer set used for PCR was F3–R2 in Fig. 1A. PCR products were digested with *Apa*I. 560 bp band (pattern A), and 390 and 170 bp bands (pattern B) were observed. V(A/A), V(B/B), and V(A/B) indicate the genotypes of genomic DNA of control chorionic villi. In 1T and 1N, the intensity of the B pattern was much greater than that of the A pattern, suggesting biallelic but biased expression of *IGF2*.

biallelic expression (LOI) in these two tumors was the same as that in normal tissues, suggesting that LOI occurred before tumorigenesis.

DISCUSSION

In all nine Wilms' tumor samples, increased expression of *PEG8/IGF2AS* was observed. Overexpression of *IGF2* (P1) and *IGF2* (total) was also confirmed in eight of nine samples. There was one sample (4T) that showed increased expression of *PEG8/IGF2AS* but not *IGF2* transcripts (P1 and total). In this study, we used a quantitative PCR method to assess the levels of gene expression in tumor samples, neighboring normal kidney tissues, normal fetal kidney samples and normal chorionic villi samples. We confirmed that the levels of expression of *PEG8/IGF2AS, IGF2* (P1), and *IGF2* (total) were 10- to 200-fold higher, 10- to 60-fold higher, and more than 100-fold higher, respectively, in the tumor tissues compared with in the neighboring normal kidney tissues. Therefore, the levels of expression observed in the tumors were extremely high. However, it is important to note that the levels of expression were almost the same as those in the normal fetal kidney samples at 14 and 19 weeks gestation and the villi samples at 4–10 weeks gestation. This indicates that the levels observed in the tumors are not abnormal *per se*, because such levels actually occur in some tissues including kidney tissues during normal development. It is possible that the control of expression had changed back to that in immature tissues when the tumors arose, and the resulting overexpression of *IGF2* and/or *PEG8/IGF2AS* caused Wilms' tumors. On the other hand, it is also possible that the mechanism of gene regulation in immature tissues remains in Wilms' tumor (*52*), and that high levels of expression of these transcript simply reflect the character of the fetal stage of development. In the 4T sample, no overexpression of *IGF2* (P1) and *IGF2* (total) was detected, whereas a high level of *PEG8/IGF2AS* was observed. Apparently, *IGF2* was not the cause of the tumor in this case. Therefore, it is possible that *PEG8/IGF2AS* rather than *IGF2* was associated with the tumor progression in this case. It is still unclear why the levels of expression of these transcripts among the tumor samples varied so much and there seemed no correlation of the expression levels among these three transcripts. From results of analysis of normal villi and normal fetal kidney samples, it is suggested that the levels of their expression seemed to differ to some extent during development. Therefore, more detailed analysis will be required on the relationship between *IGF2-PEG8/IGF2AS* expression and the tumorigenesis of Wilms' tumors.

Interestingly, human *PEG8/IGF2AS* has an open reading frame encoding a putative polypeptide consisting of 273 a.a., including many basic amino acids (47 a.a.), whereas the corresponding *Igf2as* (*Peg8*) in mouse does not have any such ORF. Although there is no homology with known proteins, it is possible that *PEG8/IGF2AS* plays an important role in tumorigenesis through this putative protein product. It will be also necessary to identify the protein product to show that *PEG8/IGF2AS* has a real coding frame.

We have observed a similar level of increased expression of *PEG8/IGF2AS* in some other childhood tumors, such as clear cell sarcomas of the kidney, congenital mesoblastic nephromas (CMN), and renal cell carcinomas (Okutsu *et al.*, unpublished data), as well as *IGF2*. Thus, it is indicated that *PEG8/IGF2AS* is a good marker of such fetal tumors and it is suggested that there is a common mechanism of resulting increased expression of *PEG8/IGF2AS-IGF2* in fetal tumors.

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