Loss of Imprinting of IGF2 Sense and Antisense Transcripts in Wilms’ Tumor

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ABSTRACT

Human insulin-like growth factor II gene (IGF2) is overexpressed, and its imprinting is disrupted in many tumors, including Wilms’ tumor. A transcript that is antisense to IGF2, IGF2-antisense (IGF2-AS), is transcribed from within IGF2 in a reverse orientation. This transcript is also maternally imprinted and overexpressed in Wilms’ tumor. IGF2-AS was detected as a 2.2 kb mRNA in Hep 3B cells by Northern blotting, and it encodes a putative 168 amino acid peptide. An alternative splicing mRNA observed predominantly in adult liver encodes an additional putative 199 amino acid peptide. We have examined the expression of IGF2 and IGF2-AS in normal tissue, breast and ovarian tumors, and 25 informative, well-characterized Wilms’ tumors and determined the relationship between IGF2 and IGF2-AS imprinting. IGF2-AS was expressed at levels comparable with IGF2 sense expression derived from promoters P1 and P2 in normal tissue and in breast, ovarian, and Wilms’ tumor tissues. In Wilms’ tumors that demonstrate maintenance of imprinting of IGF2, IGF2-AS was imprinted. In contrast, in tumors which demonstrate LOI of IGF2, only two of six tumors showed loss of imprinting of IGF2-AS, whereas four of six tumors demonstrated maintenance of imprinting for IGF2-AS. The discrepancy between IGF2 and IGF2-AS loss of imprinting in some tumors demonstrates the control complexity of the imprinting status of the various transcripts derived from the IGF2 gene.

INTRODUCTION

Imprinted genes exhibit parental allele-specific expression (1). The mechanism underlying this form of allelic silencing is not fully understood, but it is clear that imprinted genes have a number of unique properties that distinguish them from nonimprinted genes. Most imprinted genes contain differentially methylated regions in which CpG island methylation is present on only one of the two parental alleles (2). Differences in histone acetylation (3, 4), methylation (5), and chromatin structure have also been demonstrated, and short interspersed transposable elements are found in very low abundance in imprinted regions (6). Imprinted genes are frequently associated with the presence of imprinted antisense transcripts and/or noncoding RNA transcripts that may regulate allele-specific gene expression, e.g., sequences upstream of the noncoding RNA H19 regulate the expression of the adjacent gene, Igf2 (7–10). Air, the noncoding RNA that is antisense to the Igf2r gene, regulates the imprinting of Igf2r as well as two other nearby imprinted genes, Slc22a2 and Slc22a3 (11).

IGF2 is overexpressed in many tumors, including Wilms’ tumor (12–14) and colon cancer (15, 16). Although IGF2 is normally only transcribed from the paternal allele (17), this maternal imprinting is lost in many tumors, leading to biallelic expression of the gene (18).

Loss of IGF2 imprinting may also be seen in normal tissue adjacent to the tumor (14), suggesting that loss of IGF2 imprinting leads to an oncogenic diathesis that enhances the risk for neoplastic transformation. Recently, paternally expressed IGF2-AS transcripts have been described in mouse (19) and humans (20). Although the mouse Igf2-as transcripts appear to be noncoding RNAs, the human IGF2-AS contains an open reading frame and has an appropriate Kozak consensus sequence for translation initiation, suggesting a putative IGF2-AS protein (20). This putative IGF2-AS protein would be unique, because none of the known imprinted antisense genes contain a coding sequence. The role of the human IGF2-AS is unknown, and it is not clear how the transcript is regulated in vivo. Because IGF2 overexpression has been implicated in Wilms’ tumor oncogenesis, it is likely that IGF2-AS could also play a role in tumorigenesis. Okutsu et al. (20) found that IGF2-AS was overexpressed in Wilms’ tumor compared with normal kidney, although the level of expression was similar to that seen in fetal kidney. In the two informative samples that they reported, IGF2-AS maintained its imprinted status in Wilms’ tumor; in one of those tumors, IGF2 was also expressed from only one allele. In this study, we examine a larger number of well-characterized samples from patients with Wilms’ tumor to determine the relationship between IGF2 and IGF2-AS imprinting in this form of neoplasia. We have found coordinated LOI of both IGF2 sense and IGF2-AS in some tumors that demonstrate LOI of IGF2; however, in tumors that demonstrate MOI of IGF2, IGF2-AS is imprinted and transcribed from the same paternal allele as IGF2 sense. We have also resequenced the putative coding region of IGF2-AS and redefined the structure of the putative IGF2-AS proteins.

MATERIALS AND METHODS

Human Tissue, TNA, and RNA Extraction. Fresh frozen Wilms’ tumor tissues were obtained from the Cooperative Human Tissue Network (Columbus, OH). Normal fetal tissues were obtained from the Central Laboratory for Human Embryology Tissue, University of Washington, Seattle. TNA was prepared as described previously (21). RNA and DNA were extracted from fresh frozen tissues using Tri-Reagent (Sigma Chemical Co., St. Louis, MO) or by using an RNA preparation kit from Qiagen (Qiagen, Valencia, CA).

Reverse Transcription. RT was performed with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) using both random hexamers and d(T)17 primers as described previously (21). To eliminate any residual genomic DNA, total RNAs (or TNA’s) were treated with DNase I (Life Technologies, Inc.) for 45 min. (two units/1 μg RNA) and then extracted with phenol-chloroform before RT. Various human cDNAs from poly(A+) RNA were also obtained from Clontech (Palo Alto, CA) and Biochain (San Leandro, CA).

Expression of IGF2-sense and IGF2-AS by Multiplex PCR. Multiplex PCR was performed as described previously (21). To identify the PCR products, amplified products from single primer sets were run side by side. Genomic and cDNAs were amplified for 28–30 cycles (95°C for 15 s and 60°C for 2 min), followed by a 5-min extension at 72°C. To verify that the relative levels (ratios) of the multiple PCR products were constant during amplification, cDNA from a fetal liver was amplified for 10, 12, 14, 16, 18, 20, 22, 24, and 28 cycles. The PCR products were labeled by 32P-dCTP (Amersham, Piscataway, NJ) and analyzed on a 5% urea-polyacrylamide gel. After electrophoresis, the polyacrylamide gel was transferred to a blotting paper and covered with saran wrap. The wet gels were exposed to a PhosphorImager.
Allelic Expression of IGF2 and IGF2-AS in Wilms’ Tumor. IGF2-AS transcripts were amplified by RT-PCR (30 cycles of 95°C for 15 s and 60°C for 2 min, followed by a 5-min extension at 72°C) using cross-intron primers (p#4375 and p#475) and a PCR master mix containing no radioisotope. The amplified product (AS-518) was further labeled by primer extension using 32P end-labeled primer p#4376. The primer-extended product (81 b) was digested with Alu I to reveal an undigested allele (allele-c, 81 b) and a digested allele (allele-d, 59 b) on a 5% polyacrylamide-urea gel. Estimation of the digested allele, d(c + d), was based on PhosphorImager scanning density, as reported previously (21).

PCR Cloning and DNA Sequencing. To definitively identify the specific PCR products on urea-polyacrylamide gel, the specific bands were cut out and eluted in sterile water (200 μl at 98°C for 15 min.). Aliquots of 2 μl were reamplified in 10-μl reaction mixture for 35 cycles (95°C for 15 s and 65°C for 2 min), followed by an extension at 72°C for 10 min (22). The PCR products were verified by agarose gel electrophoresis and cloned by TOPO-TA cloning kit (Invitrogen, Carlsbad, CA). DNA sequencing was performed on an ABI 377 sequencer using Big-Dye terminator chemistry (Perkin-Elmer).

Northern Blot Analysis of IGF2 Antisense. Multiple tissue Northern blots containing poly-A(+) RNAs isolated from human adult tissues were obtained from Clontech. Total RNAs (10 μg) from various cell lines were separated on 1.2% agarose-formaldehyde gel and blotted by a standard technique. The filters were hybridized to IGF2-AS cDNA probe (668 b) labeled by PCR amplification (primers p#534 and p#535) using 32P-dCTP for 2 h at 68°C in a Rapid-hyb buffer (Amersham, Piscataway, NJ). The filters were subsequently washed three times with 0.2× SSC containing 0.2% SDS at 60°C for 10 min each and analyzed by a PhosphorImager (Molecular Dynamics).

Oligonucleotide Primer Sequence. The sequences of the primers are as follows:

- p#470 (5’-GAA GGA CAC ACT CGC TGG CGT CA-3’)
- p#475 (5’-AGG CTG CTC AAT CTG CCC AAA GCC A-3’)
- p#1395 (5’-TCC TGT CTC CAA GGG TGG GCC C-3’)
- p#1605 (5’-CAG CCA TGC AGC ACR AGG CGA AGG C-3’)
- p#4182 (5’-CAG TGA TCC CCC GGT CCT TAT C-3’)
- p#4375 (5’-GGG CCG AAG AGT CAC CAG GC-3’)
- p#4376 (5’-GCT GGC AGC AGT TCA GAG CCC TG-3’).

R = A or G

RESULTS

IGF2-AS Is Expressed at Levels Comparable with IGF2 Sense Expression from Promoters P1 and P2. IGF2 is transcribed from four promoters, designated P1–P4. Although in most tissues, transcripts from P3 and P4 are most common, P2 transcripts are found in many fetal tissues, and P1 transcripts are predominant in adult liver (23, 24). IGF2-AS is composed of three exons, overlapping exons 4 and 3 of IGF2 (Fig. 1A). Okutsu et al. (20) have determined that human IGF2-AS is expressed at levels comparable with that of the P1 transcript (~1000 copies in 10 ng of total RNA) in two normal fetal kidneys and Wilms’ tumor. In these fetal kidneys and Wilms’ tumor tissues, total IGF2 sense transcripts are 100-1000-fold greater than the levels of the P1 transcript. We compared the relative levels of expression of IGF2-AS with both P1 and P2 sense transcripts using a multiplex PCR (Fig. 1). Normal fetal kidney cDNA was amplified by ampiclon primers crossing intron: (a) P1, p#4182 and p#4376; (b) P2, p#1605 and p#476; and (c) IGF2-AS, p#472 and p#470 (Fig. 1A). In a control experiment, expected PCR products were observed in single and multiple amplicons (Fig. 1B). DNA sequencing confirmed the

Fig. 1. Expression of IGF2-sense and IGF2-AS in human fetal and adult tissues. A, map of IGF2-sense and IGF2-AS transcripts. IGF2-sense is transcribed from four alternative promoters (P1–P4). Four alternative exons (Exon 1 and 4–6) are spliced to exon 7. Alternative splicing in exon 4′ results in P2a and P2b transcripts. IGF2-AS has the same exons. Exons are identified on top of each box. PCR primers (arrows) and amplified products are identified by sizes (IGF2-sense: P1–150 b, IIP2a-216 b, and IIP2b-381 b; IGF2-AS: IIAs-161 b). The diagram was drawn to scale. kb, kilobase. B, expression of IGF2-sense and IGF2-AS transcripts by a multiplex PCR. In a control experiment, fetal kidney cDNA was amplified by a single primer set (P1–150 b, IAs-161 b, IIP2a-216 b, and IIP2b-381 b) and multiplex primers (P1 + As + P2). IGF2-AS (arrowhead) was expressed in all fetal and adult tissues (two independent sources, I and II). Lane M, 100-b DNA ladder. C, amplification of fetal kidney cDNA by a multiplex PCR at various cycles of amplification. The relative signal ratios of IGF2-AS and IGF2-sense transcripts are constant during the amplification.

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allelic expression of IGF2-AS transcripts in Wilms’ tumors. Wilms’ tumors that demonstrate LOI (Lanes 1–8), maintaining of imprinting (MOI, Lanes 9–19) of IGF2, other tumors (Lanes 20–25), and four sets of tumors (T) and normal (N) adjacent tissues were analyzed by a multiplex PCR. Bottom, PhosphorImager scanning of IGF2-AS, IGF2 sense promoter P1 (II P1), and IGF2 sense promoter P2 (II P2). The expression of IGF2-AS was represented as a percentage of contribution (black panels) to the total of IGF2-AS + II P1 + II P2 (a + b, gray and white panels). IGF2-AS transcripts are abundant in all Wilms’ tumors.

sequence of the amplified products. An alternative exon 4' of 165 b was identified (GenBank gi#14160138, NT 56230–56394) downstream of IGF2 exon 4 (promoter P2), which creates the P2b-381 b (216 + 165) product (Fig. 1, B and C). In our multiplex PCR, both IGF2 sense (P1 and P2a + P2b) and antisense (AS) were amplified in a log phase, and their relative levels were independent of the number of PCR cycles (Fig. 1C).

IGF2-AS was expressed ubiquitously in all fetal tissues, at levels similar to the IGF2 sense transcripts derived from P2a. In contrast, the expression of P1 was more restricted to fetal brain, kidney, liver, and muscle (Fig. 1B, fetal). Transcripts from the alternative splice site P2b were more abundant than P2a transcripts in fetal liver and muscle and very low in brain, lung, and spleen, suggesting tissue-specific alternative splicing. The transcription start site of the IGF2-AS is located 0.7 kb downstream of the transcription start site of P2 in an opposite strand and 18 kb downstream of P1. It is likely, therefore, that IGF2-AS and IGF2-P2 are regulated by the same chromatin domain.

In adult tissues, IGF2-AS was more ubiquitously expressed than IGF2 P2 and P1 sense transcripts (Fig. 1B, adult). Results are consistent with pooled tissues from two different sources. The expression of P2 was much reduced, compared with IGF2-AS, whereas P1 was more abundant in liver and brain. IGF2-AS had an alternative splicing form in which the antisense intron-2 (740 b) failed to be spliced out. This unspliced IGF2-AS transcript was detected as a 951 b band in Fig. 1B (adult livers) and reproducibly observed by a single primer set for IGF2-AS (IIAs-161, p#722/p#470; data not shown). The 951-b band was cut out, cloned, and sequenced to confirm the identity of the unspliced IGF2-AS transcript. It is interesting to note that the unspliced form was unique, detected as a major transcript only in adult livers.

Expression of IGF2-AS in Wilms’ Tumor. We then examined the abundance of IGF2-AS transcripts in Wilms’ tumors. We divided the Wilms’ tumors into three groups according to the IGF2 imprinting status revealed by the Apa I polymorphic site in the 3'-untranslated region of IGF2 in exon 9: (a) those in which IGF2 imprinting was lost (LOI); (b) those in which IGF2 imprinting was maintained (MOI); and (c) other (noninformative) tumors. Because all transcripts derived from promoters P1–P4 of IGF2 have exon 9 (and Apa I site) in common, the IGF2 imprinting status represents total IGF2 transcripts. Twenty-five informative subjects that were reported previously (21) and four sets of Wilms’ tumor and adjacent normal tissues were analyzed by the multiplex PCR. The gel was analyzed by PhosphorImager scanning, and expression of IGF2-AS was represented as a percentage of contribution (Fig. 2, black panels) to the total of
IGF2-AS + II P1 + II P2 (a + b).

All the expression of IGF2-AS in Wilms’ Tumor. Next, we compared the allelic expression of IGF2-AS with that of IGF2 in Wilms’ tumors. Allelic expression from promoter P1 was examined previously by using the Alu I polymorphic site in IGF2 exon 3 (21).

Because the IGF2-AS also encompassed the same Alu I site in IGF2-AS exon 3, we were able to use the Alu I polymorphism to distinguish the two IGF2-AS alleles (Fig. 3a). PCR products (AS-518, primers #4375 and #475) were labeled and end-labeled primer p#4376 to yield an Alu I-undigested allele-c or digested allele-d.

Within the IGF2 LOI group, there were six informative tumors (tumors 2–4 and 6–8), which had equal expression of both parental alleles of IGF2-AS (Fig. 4a, b/a + b = 0.48–0.58; Ref. 21). Two LOI tumors demonstrated equal expression (LOI) of both IGF2-AS parental alleles (tumors 3 and 7). The other four tumors showed maintenance of IGF2-AS imprinting. All six LOI tumors demonstrated biallelic expression of IGF2 sense transcripts from promoter P1 (Alu I, d/c + d) = 0.25–0.52 (21). In normal tissues, P1 is always expressed biallelically (23).

Within the IGF2 MOI group, six Alu I-informative tumors demonstrated a complete MOI of IGF2-AS (Fig. 3b; MOI tumors 10 and 15–19). Unlike normal tissues, transcripts from P1 from these tumors were expressed from only one parental allele (gain of imprinting; Ref. 25). The allelic expression of IGF2-AS was identical to the allelic expression of the IGF2 P1 promoter-specific transcript. Both IGF2 P1-sense and IGF2-AS were derived from the same parental allele. Within the Apa I informative (but Alu I informative) group, tumors that demonstrated LOI of IGF2-AS also demonstrated biallelic expression of IGF2 P1 promoter-specific transcripts (Fig. 3b; tumors 21–23 and 25).

Expression of IGF2-AS in Malignant Tissues. In four pairs (tumor and adjacent normal tissues) of malignant breast and 12 pairs of benign ovarian tumors, IGF2-AS was expressed in all samples at levels comparable with IGF2-P2 transcript (P2a and P2b; Fig. 4, Lanes 1–32). In a set of 8 cDNAs from poly(A+) RNA from malignant tissues, including breast cancer, colon adenoma (2), lung carcinoma (2), ovarian cancer, pancreatic cancer, and prostate adenoma, IGF2-AS was expressed in all tissues at levels comparable with IGF2 P1 and P2 transcripts (Fig. 4, Lanes 33–40).

Although IGF2-AS was expressed ubiquitously in all normal and malignant tissues, the level of its expression was low in all tissues. We further tested seven cell lines for the expression of IGF2-AS and found that it was most highly expressed in the Hep 3B, compared with the other cell lines (NCI-H596, K-562, Raji, U-937, T-47D, and MCF7; 1903).

Putative IGF2-AS Protein. To determine the coding potential of IGF2-AS, we sequenced DNA derived from a fetal kidney and an adult liver by RT-PCR cloning. Our results (GenBank bankit 487966 and bankit 487986) are slightly different from the sequence published previously (GenBank GI #6855329). In addition to the known polymorphic Alu I site (GI #6855329, NT #1177 G/A), we have found three deletion sites in the previous sequence (GI #6855329, GC deletion at NT #612–613, C deletion at NT #1004–1005, and T deletion at NT #1042–1043). Our results are consistent with the genomic sequence available from the human genome project. Our new sequences would result in a shorter putative IGF2-AS protein of 168 amino acids in contrast to the 237 amino acid protein predicted previously. To resolve this protein coding discrepancy, we further genotyped 128 chromosomes (44 fetal and 40 maternal subjects) by PCR-restriction fragment length polymorphism (primers p#1395 and p#471), using Tsp 45 I restriction enzyme to probe the sequence GTCAc, GC-deleted sequence, versus TGCAGc, our new sequence). None of the tested 128 chromosomes had the TGCAC sequence (data not shown), suggesting that the IGF2-AS sequence (GenBank GI # 6855329) published previously represents either a very rare allele or is simply a sequencing error. Alternative splicing in adult but not in fetal liver results in a tissue-specific IGF2-AS mRNA that codes for another putative 199 amino acid peptide on the same reading frame of the 168 amino acid peptide (GenBank # bankit 487986). We analyzed the 1-kb putative promoter sequence in the IGF2-AS region (GenBank #AC130303, NT #149513–148514) for potential transcription factor motifs. The promoter sequence of IGF2-AS lacks typical TATA and CAAT sequences. A GCF-binding site (GCGCCGCG, GC-binding factor), an Sp1-binding site (CCCGC), and two AP2-binding sites (CCCCCGCCG and CCCCCCTCGC), and an EGR-1 site (CGCCCCGGC, early growth response transcription factor-1) were found −10–150 b upstream of the IGF2-AS transcript. No secretory signal sequences or homologous sequence motifs are found in the GenBank database for either of the putative peptides.

DISCUSSION

It is not known whether biallelic expression of imprinted genes in tumors is an important etiologic process in oncogenesis or whether it is merely an epiphenomenon and not of etiologic significance. Loss of IGF2 imprinting is seen in ~40–60% of Wilms’ tumors, especially those demonstrating a later stage of renal development (26). In our

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study, loss of IGF2-AS imprinting also occurred in two of six tumors that demonstrated IGF2 LOI. Loss of IGF2-AS imprinting also occurred in two tumors that showed biallelic expression of the IGF2 transcript derived from promoter P1. Although loss of IGF2 sense imprinting was not always accompanied by loss of IGF2-AS imprinting, in every instance where IGF2 imprinting was maintained, IGF2-AS imprinting was also maintained.

The fact that IGF2-AS imprinting was maintained in four tumors in which IGF2 sense imprinting was lost indicates dissociation in the regulation of the two imprinting loci. We and others (23, 24) had shown that in normal tissues, IGF2 sense imprinting is promoter specific; only transcripts derived from promoters P2–P4 are imprinted. In the Wilms’ tumor group that demonstrated LOI of IGF2, all three transcripts derived from promoters P2–P4 demonstrated LOI (21). IGF2-AS is transcribed in the vicinity of IGF2 promoter P2 (within 1 kb), on the same parental allele but on the opposite DNA strand. It is intriguing, therefore, that the alteration of imprinting (LOI) during tumorigenesis was not identical in these two opposite and overlapping transcripts. This lack of coordinated allele-specific transcription suggests that the mechanisms underlying LOI may be even more complicated than those regulating imprinting. Imprinted transcripts, both sense and antisense, within a chromosomal domain appear to be governed as a single unit known as an imprinted domain. This is true for the sense transcripts derived from promoters P2–P4 of IGF2 as mentioned before. This is also true for the sense and antisense units in the same imprinted domain. We (27) and others (28, 29) have reported that the antisense transcript associated with PEG1 (PEG1-AS) that is transcribed from a site 1.5 kb upstream of the P2 sense transcript of PEG1 is maternally imprinted as is the P2 transcript. Methylation of CpGs in the 1.5-kb region of the PEG1 gene abolished the expression in both orientations, suggesting a bi-directional function of this imprinted domain (27). Human (and mouse) GNAS-AS is transcribed from the same paternal allele as the sense Gα that is located ~2 kb upstream of the GNAS-AS. In the cases of both PEG1-AS and GNAS-AS, the antisense does not overlap with the nearby sense transcript, and there have been no reports concerning the LOI (and association/dissociation of LOI) in these PEG- and GNAS-imprinted domains. The mouse Igf2 receptor sense and antisense (Air) transcripts overlap but are reciprocally imprinted and originate from two separate, reciprocal differentially methylated regions (23). The discrepancy between IGF2 sense and antisense LOI in some tumors suggests that the overlapping sense and antisense transcripts originate from different promoters in the same imprinted domain are regulated by distinct imprinting elements that can account for the dissociation of LOI of the sense and antisense transcripts.

IGF2-AS is a ubiquitous transcript, found in normal tissue, as well as neoplasms, although it is present with an abundance only of 1–10% that of IGF2. Its potential physiological role is unknown. In theory, IGF2-AS could act through its encoded protein after being translated, or, like many imprinted antisense molecules, it could act as a regulating noncoding RNA (30, 31). Of the six noncoding and imprinted antisense RNAs, the roles of Igf2 antisense (23) and LIT1 (32, 33) in imprinting have been demonstrated by gene deletion experiments. The UBE3A-AS (19, 34) may function as tissue-specific small nuclear RNAs. The potential functions of Gnas-as (also known as Nespas; Refs. 27, 29, and 35), PEG1-AS (also known as MESTIT1; Refs. 36 and 37), and mouse Igf2-as (23) in imprinting have not been demonstrated. Imprinted X-chromosome inactivation is regulated by Tsix (38–41), an antisense to Xist. Unique among other imprinted antisense transcripts, IGF2-AS encodes putative polypeptides, but it is not yet known whether IGF2-AS is in fact translated into protein. It is intriguing to speculate that these putative peptides might regulate the allele-specific expression of IGF2.

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