Concordant Loss of Imprinting of the Human Insulin-like Growth Factor II Gene Promoters in Cancer*

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The human insulin-like growth factor II (IGFII) gene has been shown to be imprinted for the promoters P2, P3, and P4 but not for the promoter P1 in liver and chondrocytes. Loss of imprinting of the IGFII gene has been found in a number of human tumors including rhabdomyosarcoma and lung cancer. In this report, we determined whether loss of imprinting in tumors displays a promoter-specific pattern. We examined allelic expression of all four IGFII promoters in rhabdomyosarcoma, lung cancer, and normal skeletal muscle. We demonstrated that the imprinting of all IGFII promoters is relaxed in rhabdomyosarcoma and lung cancer. These data suggest that loss of imprinting of IGFII gene promoters may be regulated coordinately by a common mechanism in these tumors. Unexpectedly, we also found that P1, in addition to P2, P3, and P4 is monoallelically expressed in three informative adult skeletal muscle tissues. This indicates that imprinting of the IGFII promoter P1 occurs in a tissue-specific manner.

Human insulin-like growth factor II (IGFII),1 a 67-amino acid mitogenic peptide, appears to be involved in normal fetal growth and development (1, 2). In addition, it has been shown that abnormally high levels of IGFII mRNA are expressed in a number of human malignant tumors, and it has been suggested that IGFII may act as an autocrine or paracrine growth factor, maintaining and enhancing tumor growth (3–5). An important role for IGFII in oncogenesis was further supported by the finding that IGFII acts as a second important signal in SV40 large T antigen-induced tumorigenesis (6).

Genomic imprinting, or the differential expression of parental alleles of a gene in somatic cells, is now thought to play a role in human disease and cancer (7). Prader-Willi syndrome and Angelman syndrome are associated with genomic imprinting on chromosome 15q11-q13 (8, 9). Imprinting has also been proposed to be a potential mechanism for Beckwith-Wiedemann syndrome with paternal chromosome 11 iso-disomy (10). It has been shown that the IGFII gene is maternally imprinted in both mice and humans (11–13). Altered imprinting of the IGFII gene has been found in a number of tumors where IGFII is believed to play a role in pathogenesis, such as Wilms’ tumors, rhabdomyosarcoma (RMS), lung cancer, and leiomyosarcoma (12–16). Furthermore, it has recently been shown that in Wilms’ tumors, loss of imprinting (LOI) of the IGFII gene is associated with reduced expression of H19 mRNA (17, 18), which has been suggested to act as a tumor suppressor gene (19). More recently, an important role for LOI of IGFII in cancer was supported by the evidence that transgenic mice expressing SV40 large T-antigen develop insulinomas and that biallelic expression of IGFII appears to be required for progression from adenoma to carcinoma (20). These studies suggest that deregulation of IGFII genomic imprinting may play an important role in the development of some tumors.

Tissue-specific imprinting of all three IGFII promoters has been reported in both rats and mice (21, 22). All three rodent IGFII promoters were found to be expressed from the paternal allele exclusively in all tissues except in the choroid plexus and leptomeninges of the central nervous system. While the human IGFII promoters P2, P3, and P4 have their counterparts in the rodent, the promoter P1 appears to be unique to human (1). It has also been documented that the IGFII promoter P1 directs expression from both parental alleles while the promoters P2, P3, and P4 transcribe mRNA from only one parental allele in liver and chondrocytes (23, 24). Relaxation of imprinting of IGFII occurs at high frequency in some human tumors overexpressing IGFII (12–16). To explore whether or not this relaxation involved all promoters, we examined the allelic expression status of all four IGFII promoters in RMS, lung cancer, and normal skeletal muscle.

**EXPERIMENTAL PROCEDURES**

Materials—All tumor tissues and adult muscle tissues were obtained from a tumor tissue bank of samples from patients treated at St. Jude Children’s Research Hospital (Memphis, TN) or at NIH, or from the Cooperative Human Tissue Network, and all tumor samples had been confirmed to contain viable tumor.

Extraction of DNA and RNA—Chromosomal DNA was extracted from tumor tissues or adult muscles using the “QuickClean DNA Extraction System” (Oncogene Science, Inc., New York) according to the manufacturer’s recommendations. Total cellular RNA was isolated as described previously (25).

PCR Analyses—To screen normal muscle tissues and tumor tissues for informative heterozygosity, amplification of DNA was performed using oligonucleotides p5 and end-labeled p7 and the same conditions as described previously (14). Labeled PCR products were digested with Hinfl or HinfI + Apal (New England Biolabs, Beverly, MA) and electrophoresed on 6% polyacrylamide-urea gel. For analysis of allelic usage, the four IGFII promoters, 1 μg of total RNA was reverse-transcribed using random primers at 42°C for 15 min, and aliquots of 4-fold diluted cDNA were amplified using p8 and each of four specific primers (p1a, p2a, p3a, and p4a) for 30 cycles at 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min followed by a final extension at 72°C for 7 min. Nested PCR (20 cycles) was performed using 32P end-labeled p9 and each of the following following primers: p1b, p2b, p3b, and p4b. Labeled PCR products were purified on 1% agarose gel, digested with Hinfl or HinfI + Apal at 37°C for 30 min, and analyzed on a 6% polyacrylamide-urea gel. Primers used were: p1a, 5′-CGA ATT CTG GGC ACC AGT GAC TCC CCG-3′; p2a, 5′-TTC CAG GCA CAA TGA GAA TCT-3′; p3a, 5′-GAG GCT TGC TGT GCA TCC AAA-3′; p4a, 5′-CGG CGA CTC TCC GAT GCA G-3′.
RESULTS

PCR Amplification of Promoter-specific IGFII Transcripts—We used a modified technique described previously to determine allelic expression of the four IGFII promoters (23). The exon organization of the human IGFII gene and the primers used for amplification of the promoter-specific transcripts generated by the four different promoters are shown in Fig. 1A. IGFII cDNA was generated from total RNA by reverse transcription. Promoter-specific PCR products were then generated by amplification using p8 and each of four promoter-specific primers (Fig. 1B), followed by a nested PCR using 32P end-labeled p9 and each of additional promoter-specific primers (p1b, p2b, p3b, and p4b). C, 50-fold diluted first round PCR products were amplified by nested PCR using 32P end-labeled p9 (asterisks) and one of four promoter-specific primers (p1b, p2b, p3b, and p4b). HinfI digestion resulted in a 141-bp end-labeled fragment, which was digested further with Apal. Transcripts that do not demonstrate genomic imprinting show two alleles: an Apal-undigested allele A (141 bp) and an Apal-digested allele B (108 bp). D, to rule out partial digestion by Apal, control template B/B alleles were amplified using p5 and 32P end-labeled p7 (asterisks). This product was added to all enzymatic reactions and generated a 268-bp end-labeled HinfI fragment and a 235-bp end-labeled Apal fragment. E, amplification of IGFII from total RNA of adult muscle. The nested PCR products were sized on a 1% agarose gel with a DNA molecular weight ladder (lane M). Amplified transcripts from promoters P1, P2, P3, and P4 show expected 1350, 1306, 1291, and 1266 bp, respectively.

p1b, 5'-CAG TCC TGT GAG GTG CTC-3'; p2a, 5'-GTT CCC GGA ACC TGA GGA CGT CGT-3'; p2b, 5'-AGA GCG TTC TAT GTC CGC TCG CCT GCT G-3'; p3a, 5'-CGG ACA ATA GCC TGG CCG CTC-3'; p4a, 5'-GGT TGG CGA CAC GCA GCA GGA AGT G-3'; p4b, 5'-GTT CCC GGA GCG TTC TGG GCT GAT G-3'; p5, 5'-CTT CGG CTA GTG ATG AGA AAG GGA GGA GTG AGC C-3'; p6, 5'-GAG GAG CCA GTG TGG GTT GCT A-3'.

Restriction Context Template

\[ \text{Restriction Context Template} \]

Fig. 1. Structure of the human IGFII gene. A, the numbered boxes indicate the nine exons of the IGFII gene. The locations of four promoters (P1–P4) are indicated. The coding regions which encode prepro-IGFII are shown as shaded boxes. Parental alleles were distinguished by digestion using a common HinfI site and the polymorphic Apal site. B, total RNA was reverse-transcribed into cDNA. Each promoter-specific cDNA transcript was amplified using a common primer p8 and one of four promoter-specific primers (p1a, p2a, p3a, and p4a). C, 50-fold diluted first round PCR products were amplified by nested PCR using 32P end-labeled p9 (asterisks) and one of four promoter-specific primers (p1b, p2b, p3b, and p4b). HinfI digestion resulted in a 141-bp end-labeled fragment, which was digested further with Apal. Transcripts that do not demonstrate genomic imprinting show two alleles: an Apal-undigested allele A (141 bp) and an Apal-digested allele B (108 bp). D, to rule out partial digestion by Apal, control template B/B alleles were amplified using p5 and 32P end-labeled p7 (asterisks). This product was added to all enzymatic reactions and generated a 268-bp end-labeled HinfI fragment and a 235-bp end-labeled Apal fragment. E, amplification of IGFII from total RNA of adult muscle. The nested PCR products were sized on a 1% agarose gel with a DNA molecular weight ladder (lane M). Amplified transcripts from promoters P1, P2, P3, and P4 show expected 1350, 1306, 1291, and 1266 bp, respectively.

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Fig. 2. Allele usage in the four IGFII promoters in adult skeletal muscles. A, genomic DNA extracted from adult skeletal muscles of subjects 1 and 2 was PCR-amplified with primer p5 and 32P end-labeled primer p7. Labeled PCR products were purified and digested with HinfI or HinfI + Apal and analyzed on a 6% polyacrylamide-urea gel and demonstrate heterozygosity for the Apal site. B, total RNA from adult muscle of subject 1 was reverse-transcribed into cDNA, and nested PCR was performed as described in Fig. 1. Nested PCR products from four promoters (P1–P4) were digested with HinfI (H, lanes 1, 3, 5, and 7) or HinfI + Apal (HA, lanes 2, 4, 6, and 8) in the presence of internal control template and analyzed on 6% polyacrylamide-urea gel. Lane M shows a 268-bp end-labeled ladder. Note that the internal control template generates a 268-bp HinfI fragment and a 235-bp HinfI + Apal fragment to indicate complete digestion.

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Global IGFII Promoter Imprinting in Normal Human Skeletal Muscle—Three normal muscles were informative for heterozygosity at the Apal restriction site. Fig. 2A shows two such muscle tissues that contain the Apal polymorphism. Total RNA from informative muscle tissues was used to determine allele-specific expression in all four IGFII promoters. In adult muscle of subject 1, full-length PCR transcripts from P2, P3, and P4 were digested to a 141-bp end-labeled fragment with HinfI (Fig. 2B, lanes 3, 5, and 7) and showed that only allele B was present after further digestion with Apal (Fig. 2B, lanes 4, 6, and 8). These data are consistent with the previously described results in normal liver (23). In contrast, however, the PCR transcript of the IGFII P1 promoter showed that only allele B was present after digestion with both HinfI and Apal (Fig. 2B, lane 2). This result was confirmed by analysis of the other two informative adult muscles (Table I). Since the IGFII P1 promoter is expressed from both parental alleles in liver and chondrocytes (23, 24), we conclude that imprinting of the IGFII promoter P1 appears to be tissue-specific.

Global Loss of IGFII Promoter Imprinting in RMS—We have
shown that all four promoters are imprinted in normal skeletal muscle. In addition, we have previously demonstrated LOI in RMS, which is thought to arise from skeletal muscle precursors. To determine whether LOI in RMS includes all four promoters or displays a promoter-specific pattern, we examined allele usage of the IGFII promoters in RMS tumors with LOI. Transcripts from all four IGFII promoters showed that both A and B alleles are expressed (Fig. 3A, lanes 2, 4, 6, and 8). This observation was confirmed for the other three RMS tumors (Table I). Since it has been shown that all four IGFII promoters are imprinted in normal adult skeletal muscle, these data indicate the deficiency of IGFII genomic imprinting in RMS involves all four promoters.

It has been demonstrated that biallelic expression of IGFII in Wilms’ tumor was associated with hypermethylation of the H19 promoter region (17, 18). To determine if Wilms’ tumor and RMS share common characteristics, we have studied the methylation status of H19 in RMS tumors as well as in normal skeletal muscle. In contrast with Wilms’ tumors, no differences in the methylation pattern between the normal skeletal muscle and the RMS tumors with LOI of IGFII were observed either in the promoter region or in the 3'-half region of the H19 gene (Table I). Therefore, we conclude that biallelic IGFII expression is not linked to H19 hypermethylation in RMS.

**Biallelic Expression of All Four IGFII Promoters in Lung Carcinoma**—It has been demonstrated that relaxation of IGFII imprinting also occurs in lung cancer, a common adult malignancy (15). We therefore sought to determine whether all four IGFII promoters are biallelically expressed in these tumors. Fig. 3B demonstrates biallelic expression of IGFII transcripts from all four promoters, P1–P4. This result was confirmed in two other lung carcinomas with LOI (Table I). These data demonstrate that biallelic expression of all four IGFII promoters also occurs in lung cancer and suggests that disruption of IGFII imprinting in these two tumor types may be regulated by similar mechanisms.

**DISCUSSION**

Our data demonstrate that silent alleles of all four IGFII promoters are coordinately activated in RMS, indicating that LOI of IGFII promoters may be regulated coordinately in this tumor. Our data also show biallelic expression of all four promoters in lung cancer. Since IGFII promoters P1–P4 span a ~20-kb DNA region, our data support the hypothesis that imprinting may be regulated in a regional manner (27). A recent study has shown that deletion of the H19 gene region in mice disrupted the imprinting of two other genes, IGFII and insulin, located over 100 kb upstream, implying an imprinting control center around the H19 gene (27). A group of investigators has also identified an imprinting center within a locus on chromosome 15 responsible for Prader-Willi syndrome and Angelman syndrome (28). Furthermore, it has recently been shown that LOI of IGFII in Wilms’ tumor is linked to increased methylation of the maternal H19 allele (17), which is located ~200 kb downstream of the IGFII gene (29). Since epigenetic alterations at the H19 locus can regulate neighboring imprinted genes located more than 100 kb away, it is not surprising to find that LOI involves all IGFII promoters.

The results reported here document the existence of concordant imprinting of all four IGFII promoters in human adult skeletal muscle. While the IGFII promoter P1 can direct expression from both parental alleles in liver and chondrocytes (23), all informative adult muscles examined expressed P1-specific IGFII transcripts monallelically. These data demonstrate that imprinting of the IGFII promoter P1 is tissue-
specific. Tissue-specific imprinting has also been found in all three rodent IGFII promoters and the mouse insulin-2 gene (30). All these genes or their promoters express mRNA biallylically in some specific tissues. On the other hand, a number of tumors also transcribed IGFII from both parental alleles. Although the mechanisms responsible for biallelic expression in normal tissues and in cancer are unknown, the processes involved may be similar. A recent study indicated that allele-specific inactivation occurs early postimplantation (31). After the establishment of imprinting in the early embryo, a repressed allele of an imprinted gene is reactivated in the precursor cells of specific tissues, and this activation is maintained throughout adult life. To date, no association between LOI and stages of tumor development have been found. This implies that activation of a silenced allele may also occur in the precursor of tumor cells.

In Wilms’ tumor, biallelic expression of IGFII was shown to be linked to reduced expression of H19 and increased methylation of H19 promoter region (17, 18). In the RMS tumors with LOI, we did not observe any H19 methylation differences between normal skeletal muscle tissues and tumor tissues. In addition, overexpression of H19 mRNA was observed by Northern blot analysis in the two RMS tumors with biallelic expression of IGFII, using normal muscle tissue as a control (data not shown). These findings indicate that the mechanisms for LOI of IGFII in RMS may be distinct from those in Wilms’ tumor in which the model of enhancer competition between H19 and IGFII is supported. One possible explanation is that LOI of IGFII in RMS may involve IGFII methylation but not H19 methylation. We are currently investigating the methylation pattern of four IGFII promoter regions.

Thus the mechanisms for biallelic expression in normal tissues and cancer as well as the precise molecular mechanisms involved in the process of genomic imprinting remain to be determined, and identification of these mechanisms may have important implications for understanding the biology of these tumors.

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