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Promoter-Dependent Tissue-Specific Expressive Nature of Imprinting Gene, Insulin-like Growth Factor II, in Human Tissues

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The insulin-like growth factor II (IGF2) is a polypeptide with structure homology to insulin which possesses mitogen activity, and imprinted with paternal allele. In order to elucidate the distribution of imprinting pattern and relationship between allele- and tissue-specific expression of *IGF2* in growth and maturation of human tissues, we investigated allele-specific expression of *IGF2* in a wide spectrum of normal maturated human tissues by a PCR-based assay and found monoallelic expression in all eight-type tissues tested except human adult liver. Moreover, when a RT-PCR based sensitive allele-specific primer extension for an *Apa* I polymorphism within exon 9 of *IGF2* was used, the analysis revealed the gene was normally imprinted in placenta; in contrast to the finding with placenta, *IGF2* transcripts were biallelically expressed in human adult liver. Our results have clearly demonstrated preferential paternal expression and tissue-specific imprinting pattern of *IGF2* in all human tissues tested in this study. Collectively, since *IGF2* expression in developing fetal and adult liver is specified by distinct promoters, these extensive observations definitively indicate that transcriptional imprinting of *IGF2* is more likely a promoter dependent manner.

IGF2 is a polypeptide growth factor which appears to be a major embryonic mitogen, at least in rodent (1). In the rat, *IGF2* is highly expressed in fetal life, and levels fall rapidly after birth (2). In humans, the majority of fetal tissues express *IGF2*, but significant circulating levels of *IGF2* (300∼500 μg/ml) are also detectable in the serum of adults (3). The regulation of *IGF2* expression is complex, involving both developmentally regulated promoters and alternative splicing of different 5′-untranslated leader sequences (4). The human *IGF2* gene is transcribed at least four promoters a developmental and tissue specific fashion (5). Accordingly, the promoter P1 which may be unique to humans, is transcriptionally active in postnatal liver (6) and in the fetal choroid plexus/leptomenings (7). The transcript from this promoter includes non-translated exons 1, 2, and 3, which are not found in the rodent (8). Promoters P2∼P4 are transcriptionally active in many embryonic/fetal and adult tissues, including liver (9) but an overall down regulation is evident after birth suggesting a role for *IGF2* in prenatal development. In transgenic mouse model, SV49 Tag induced tumor in the islet of Langerhans showed focal activation of the *IGF2* gene which correlated to proliferation of the tumor and inducing apotosis *in vivo* (10). Thus, the *IGF2* peptide has been suggested to be an important factor in both embryonic/fetal growth as well as in certain types of tumors in both human and mouse.

Genomic imprinting in mammals refers to an epigenetic modification of a gene or chromosome that can affect its expression in a stable, heritable fashion. These modifications appear to contribute to the regulation of a variety of processes during normal development and differentiation (11). The first molecular evidence was derived from studies of transgenes in mice (12), some of which are imprinted and associated with methylation of nonexpressed allele (13). Recently, both *IGF2* and *H19* have been sown to be imprinted in humans, with reversal expression of the paternal *IGF2* (14, 15) and maternal *H19* alleles (14, 16). In addition, *IGF2* and *H19* undergo loss of imprinting (LOI) in Beckwith-Wiedemann syndrome (17) and most Wilms'

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Abbreviations used: IGF2, insulin-like growth factor II gene; LOI, loss of imprinting; gDNA-PCR, genomic DNA-polymerase chain reaction (PCR); cDNA-PCR, complementary DNA-PCR; IGF2-P1, -P2, -P3, and -P4, IGF2 promoter(s)-1, -2, -3, and -4.

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tumors (14, 15). Considering that 'one-copy' dosage of imprinted gene is normal, altered gene dosage or disruption of the molecular system involved in the correct identification and maintenance of the imprint could have deleterious effects on cellular function. Because *IGF2* is an autocrine growth factor, it has been proposed that LOI of *IGF2* leads to overexpression of *IGF2* and Wilms' tumorigenesis.

The mechanisms by which the gene is imprinted remain unknown. Two proposed mechanisms involving alteration of imprinting processing system have been suggested to play a role during development and tumorigenesis (18). In order to elucidate the distribution of imprinting pattern and relationship between allele- and tissue-specific expression of *IGF2* in growth and maturation of human tissues, we studied allele- and tissue-specific expression of *IGF2* in eight-type of human matured tissues with a PCR-based assay and employed a RT-PCR based sensitive allele-specific primer extension adjacent to a common *Apa* I polymorphism within *IGF2* cDNA-PCR products to directly assay expression profiles, revealing that both parental alleles of *IGF2* are normally transcribed in the human adult liver.

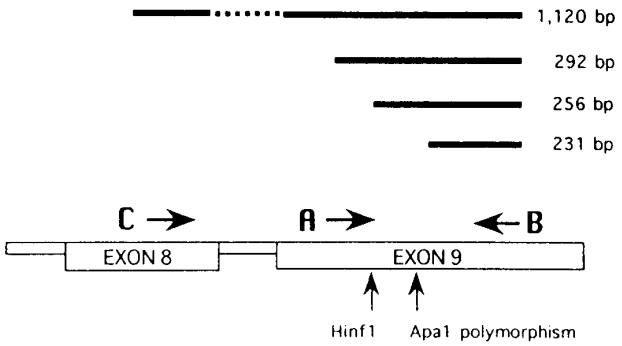

FIG. 1. Strategy for examination of allele expression of *IGF2*. Allele expression was identified using an *Apa* I polymorphism in exon 9 of the *IGF2* gene, essentially as described by Davies (20). Primers A and B amplify a 292-bp product from gDNA, which yield a 256-bp band after digestion with *Hinf* I. Primers B and C were used to amplify a 1.12-kb band from cDNA, which yield a 256-bp band after digestion with *Hinf* I. Different primers were used to amplify cDNA as primer B and C span an intron, allowing gDNA and cDNA derived products to be distinguished by agarose gel electrophoresis and to exclude any gDNA contamination (1.4-kb) of cDNA preparation. Digestion of the 256-bp band with *Apa* I will yield a 231-bp if the *Apa* I polymorphism is present. Expressed allele can be identified in heterozygous individuals by comparison of the cDNA *Apa* I digestion pattern with the gDNA pattern.

MATERIALS AND METHODS

Human tissue samples. Three liver biopsies included in this study were from normal adult donors for transplantation. Other fresh human adult tissues (three of each kind), including normal brain, heart, kidney, peripheral blood leukocyte, placenta, and tongue were obtained either from clinical biopsies or from routine deliveries in the Departments of Pathology and Medical Genetics, the Hospital for Sick Children following informed consent according to the guidelines of University of Toronto. Fibroblast cell lines were established from the skin biopsies that were diagnosed without defined disease. Mononuclear cells from peripheral blood leukocytes were separated by Ficoll-hypaque centrifugation.

Isolation of gDNA and gDNA-PCR. High molecular weight genomic DNA (gDNA) from various human tissues was isolated by proteinase K-sodium dodecyl sulfate treatment followed by phenol-chloroform extraction and ethanol precipitation (19). gDNA-PCR reaction were performed on 0.3 μg of quality gDNA essentially as described elsewhere (15, 20) using primers A (5′-TCC TGG AGA CGT ACT GTG CTA-3′) and B (5′-CTT GGA CTT TGA AGT CAA ATT GG-3′) (denoted P2 and P3, in ref. 14, 15, 20) which flank a transcribed *Apa* I polymorphism in exon 9 of *IGF2* gene (Fig. 1). The cycling condition were as previously cited (20). The resultant 292-bp DNA products were digested with *Apa* I-*Hinf* I, electrophresed through 3% agarose gels and stained with 0.5 μg/ml of ethidium bromide. Tissue samples yielding polymorphic bands of 256- and 231-bp in size (allele *a* and *b* respectively), indicated heterozygosity for the *Apa* I polymorphism.

cDNA-PCR and determination of allele-specific expression of IGF2. Total RNA of the human tissues was extracted from various human adult tissues using the single step acid guanidinium thiocyanate-phenol-chloroform procedure and treated with RNase-free DNase. The complementary DNA (cDNA) of the human tissues was directly synthesized as previously described (19). Briefly, 3 μg of RNA was reversibly transcribed with primer B to cDNA using an reverse transcription (RT) system (Gibco-BRL, Burlington, ON, Canada) (Fig. 1). RT-PCR was performed on 1 μl of the first strand cDNA with primers B and C (5′-GGT CGT GCC AAT TAC ATT TCA-3′) and the same PCR cycling conditions as for DNA-PCR to yield a 1.12-kb *IGF2* RT-PCR product (20), which was confirmed by sequencing (data not shown). As these primers span an intron, gDNA-specific PCR product (1.4-kb) could be detected separately from cDNA-specific product (1.12-kb) by agarose gel electrophoresis, and so made it possible to ensure that the nucleic acid source for the cDNA was RNA and not contaminating gDNA. None of our RT-PCR preparations yielded a 1.4-kb gDNA band, indicating that there was no gDNA contamination and that DNase treatment was successful. Restriction digest analysis using *Apa* I-*Hinf* I revealed either monoallelic or biallelic usage of parental alleles and allele-specific expression of *IGF2* was designated as *a* and *b* (256- and 231-bp bands respectively). This experiment was repeated for each tissue sample.

Primer extension analysis of IGF2. cDNA-PCR product generated as described above that is specific for the 292-bp fragment in exon 9 of human *IGF2* gene (15, 20) was subcloned into the pCR™ II transcription vector (Invtrogen, USA). Following the manufacturers instructions the MEGAscript *in vitro* transcription kit (Ambion Inc., USA) was used to generate sense and antisense RNA for control templates (Fig. 2). The protected target RNA fragments were identified by polyacrylamide gel electrophoresis. Primer extension experiment was performed on 1 μg of mRNA purified from 2 mg of total human liver and placenta RNA with the following modifications (21). Instead of all four nucleotides being included in the extension mixture only dGTP, dCTP, and dTTP were used in order to let extension reaction proceed to the first adenine nucleotide. In the case of allele *a* the product was extended by four nucleotides, for allele *b* seven nucleotides. The end-labelled antisense oligonucleotide used is shown below and is based on nt 8761~8798 of human *IGF2* gene. Autoradiography was performed at −80°C for 24 hr using Kodak XAR film unless otherwise noted.

RESULTS

Identification of informative samples for IGF2. *IGF2* contains a transition polymorphism which gener-

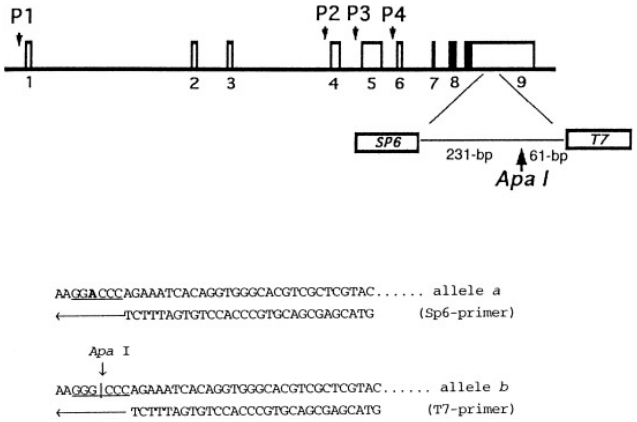

FIG. 2. Schematic diagram of *IGF2* gene based on the published maps (15, 20) indicates the position of the four *IGF2* promoters, the exon structure (boxes) and coding region (solid boxes). The non-coding part of exon 9 contains the *Apa* I-*Hinf* I fragment in these studies. RT-PCR product was subcloned into the transcription vector described in the methods, sense and antisense *IGF2* RNA(s) were generated by SP6 and T7 primers respectively.

ates a fragment digested with *Apa* I-*Hinf* I by DNA-specific PCR assay (20). The frequency of heterozygosity of this polymorphism is 57% and it has been used to identify the parental origin of each *IGF2* allele in informative heterozygotes (15, 20). In total, 24 randomly selected samples containing eight-type of human tissues were investigated for biallelic expression of *IGF2*. Following gDNA-PCR amplification of a 292-bp fragment encompassing an *Apa* I polymorphism site in exon 9 of *IGF2, Apa* I-*Hinf* I restriction digestion identified informative samples. This was evidence from the presence of two polymorphism bands of 256- and 231-bp in size (allele *a* and *b* respectively), indicating retention of both copies of *IGF2*. Using this PCR-based allele-specific approach (20), heterozygosity was demonstrated in 58.3% (14/24) of eight-type human adult tissues. Representative gDNA-PCR electrophoretic analysis of *Apa* I-*Hinf* I genotypes of *IGF2* are shown in Fig. 3. Both total RNA and mRNA were extracted from each of the heterozygotes and studies further.

Determination of allele-specific expression of IGF2 in informative human tissues. To eliminate possible contaminating gDNA in the RNA preparations, the samples were treated with DNase prior to reverse transcription and subsequent RT-PCR amplification. As anticipated, the use of intron-spaning primers (B and C) generated a 1.12-kb RT-PCR product, confirming the cDNA source as RNA. To determine allele-specific expression of *IGF2* gene, the *Apa* I transcribed polymorphic site was regenerated from the 1.12-kb RT-PCR product using primers B and C (20), to yield a RNA-specific PCR product of 292-bp digested with *Hinf* I only in size which, on *Apa* I digestion and electrophoresis in 3% agarose gel, allowed biallelic and monoallelic expression to be assessed. In cDNA-PCR analysis, all seven-type of human tissues examined in our study were presented as monoallelic expression of *IGF2* (homozygosity), including brain, fibroblast, heart, kidney, peripheral blood leukocyte, placenta and tongue. Conversely, biallelic expression of the gene (heterozygosity) was only observed in adult liver tissues. This implicated that normal adult liver keeps the biallelic expression of *IGF2* gene in both gDNA and RNA (Fig. 3).

Primer extension assay for allele-specific expression of IGF2 in liver and placenta. RNA-specific PCR product (292-bp) amplified using *IGF2* primers A and B without *Apa* I-*Hinf* I digestion (15, 20) was subcloned into a transcription vector to generate sense and antisense RNA for control templates as described in materials and methods. The position of these constructs in relation to the published gene structure of *IGF2* is shown in the schematic of Fig. 2. An antisense oligonucleotide primer containing 4-bp in 3′-side of the *Apa* I polymorphism of *IGF2* cDNA was synthesized and used in the primer extension assay of RNA(s) and cDNA-PCR products. Primer extension analysis of control template RNA generated from constructs (Fig. 4. lanes 3, 4, 12 and 13) and total RNA obtained from snap frozen heterozygous human tissues was performed. For both placenta samples, paternal allele-specific expression of *IGF2* was detected (Fig. 4. lanes 6 and 7) showing monoallelic expression of *IGF2* in human placenta determined by cDNA-PCR assays. In contrast, mRNA from three informative adult livers exhibited biallelic expression of *IGF2* by sensitive primer extension analysis indicating significant expression from both paternal and maternal alleles and an absence of *IGF2* imprinting in matured human liver (Fig. 4. lanes 8, 9 and 10).

DISCUSSION

The human *IGF2* gene is located on chromosome 11p15, just downstream of the insulin gene, and spans 30-kb. It consists of nine exons, of which, exons 7, 8, and of exon 9 code for the *IGF2* precursor protein. Exons 1, 4, 5, and 6 are each preceded by a distinct promoter (P1∼P4) (22, 23). Multiple transcripts are synthesized as a result of alternate promoter usage and the splicing of a unique 5′-untranslated region to common coding exons. Expression of the *IGF2* gene is regulated in a development-dependent and tissue-specific manner. Promoter 1 is active only in adult liver, while P2, P3, and P4 are active in most fetal tissues. There are a few adult tissues that express low amounts of *IGF2* transcripts from promoters P2, P3, and P4 (24, 25). Our results of allele-specific expression of *IGF2* in a wide spectrum of human adult tissues, taken together with other studies in humans (14, 15) and mice (12, 13), strongly suggest that transcriptional inactivity of the

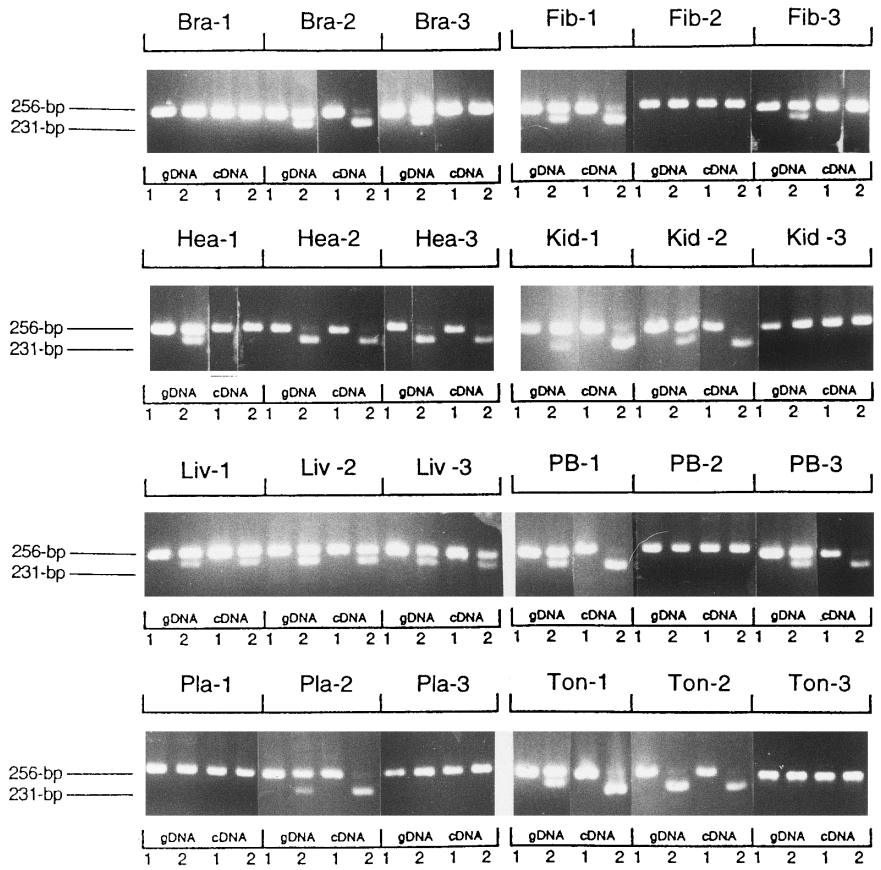

FIG. 3. Analysis of genotyping (gDNA-specific PCR) and allele-specific expression (cDNA-specific PCR) of human *IGF2* gene in eight-type of human adult tissues by PCR-based assay. Three gene-specific primers, *IGF2*-A, -B and -C were used to amplify gDNA and cDNA-PCR fragments of *IGF2* from twenty-four of normal human specimens. Heterozygous genotype of *IGF2* is identified with a noticeable 3:1 ratio of staining intensity for the fragment of 256-bp in comparison to 231-bp fragment. This is thought to occur because heteroduplex DNA is uncut by *Apa* I (20, 21). Lane 1 indicates gDNA or cDNA products of *IGF2* digested with both *Hinf* I only. Lane 2 indicates gDNA or cDNA products of *IGF2* digested with *Hinf* I and *Apa* I. From top to bottom: normal human brains (Bra), fibroblast cells (Fib), hearts (Hea), kidneys (Kid), livers (Liv), peripheral blood leukocytes (PB), placentas (Pla), tongues (Tog). Relative positions of 256- and 231-bp bands are indicated on the left.

maternal *IGF2* allele is the normal pattern of expression for most tissues. In contrast, normal adult human liver expresses both paternal and maternal transcripts in approximately equal amounts, demonstrating that unlike most matured tissues, *IGF2* is not imprinted in adult liver. This finding contrasts with what has been observed in human and rodent fetal liver where monoallelic *IGF2* expression is seen (12-15). Since *IGF2* is normally transcribed from distinct fetal and adult promoters in human liver (6, 7, 24, 25), not only our results indicate that *IGF2* imprinting is stage specific, but also further suggest that *IGF2* imprinting may be promoter restricted.

The progressive changes in imprinting gene methylation and establishment of monoallelic expression pattern indicate that gene imprinting is determined, in large part, by events that translate the original marks that denote parental chromosome original into differences in allele expression. An additional level of complexity has become apparent with regard to these modifications, namely the tissue-specific nature of imprinting. For example, in adult choroid plexus, both alleles of the *IGF2* gene are heavily methylated at upstream sites, while only the paternal allele is methylated at the same sites in other tissues (26). Since the level of methylation at these sites, particularly in the promoters, is correlated with gene expression (26), these results indicate that biallelic expression of the *IGF2* gene in the choroid plexus is the result of tissue-specific mechanisms that direct methylation of the maternal as well as paternal allele. Both rat and human *IGF2* gene is biallelically expressed in the liver beginning at several months after birth (2, 27), and this apparently involves the usage of a distinct non-imprinting promoter (28). A recent study reported that all four promoters of human *IGF2* gene are imprinted initially but that during development imprinting of P1 promoter become relaxed (29). It will be interesting to

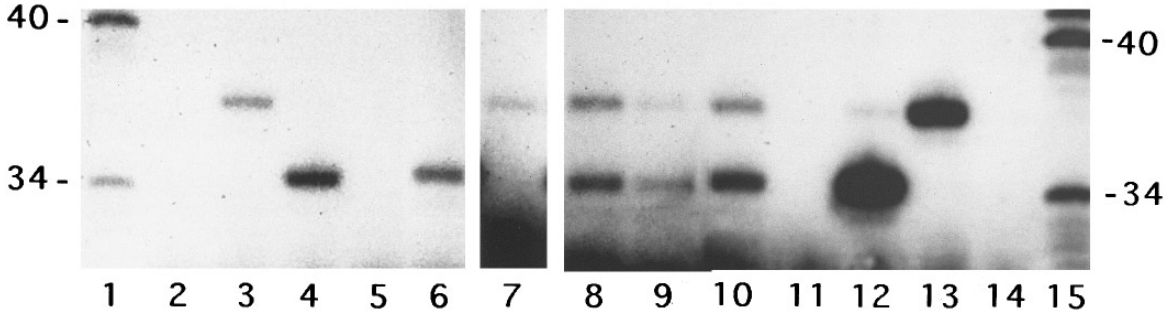

FIG. 4. Primer extension analysis of mRNA from liver and placental tissues, as well as exon 9 antisense controls. Sense and antisense mRNA(s) was generated by SP6 and T7 primers respectively for use as control template. The antisense oligonucleotide primer 4-bp 3′- end of the *Apa* I polymorphism was used in the extension analyses of RNA. For detecting an allele-specific mRNA, a shorter extension product of 34-bp is obtained from allele *b*. RNA from allele *a* mRNA extends further, resulting in a 37-base pair product. Lanes 1 and 15 are sequencing ladders used as molecular weight standards. Primer extension analysis of control template mRNA generated from allele *a* transcription constructs (lanes 3 and 13) and allele *b* constructs (lanes 4 and 12). Paternal allele-specific expression of *IGF2* was detected in placenta mRNA from two individuals (lanes 6 and 7, the latter was exposed for 72 hr at −80°C). In all three normal liver mRNA samples (lanes 8, 9 and 10) both extension products indicating equal expression from both paternal and maternal alleles (Lanes 8–15 were developed after 48 hr exposure at −80°C). Lanes 2, 5, 11, and 14 contain total RNA controls.

know how the P1 promoter can circumvent imprinting of the *IFG2* gene. As *IGF2* transcripts in human adult liver are almost exclusively derived from the adult specific P1 promoter (7, 9, 29, 30), biallelic P1 transcription is the simplest and most probable explanation for our observations. Moreover, we also used a PCR-based sensitive allele-specific primer extension assay to detect endogenous liver mRNA, and then to determine the expression levels of each parental *IGF2* allele (Fig. 4). The observed constant ratio of the *IGF2* alleles seen in three independent liver samples is consistent with expected uniform biallelic P1 transcription. The maintenance of constant allelic proportions makes it less likely that biallelic *IGF2* transcription is a result of relaxed imprinting of one or more of the fetal P2∼P4 promoters which have very low activity in adult liver. Hence, biallelic *IGF2* expression in human matured liver that we have solely illustrated here is most likely corespondent to transcription from the P1 promoter (24, 25, 29, 30). Most importantly, our findings clarify precise delineation of promoter specific imprinting of *IGF2* as a mechanism for its controlling developmental expression.

In the present experiments, we have solely demonstrated that *IGF2* is monoallelically expressed in a variety of human tissues tested. Thus, in addition to stage specificity, human *IGF2* imprinting may also be highly tissue restricted. Presumably, such variation in imprinting regimens reflect distinct *IGF2* gene functions in different tissues and at different stages of development. Although the potent mitogen properties of *IGF2* is well documented (1) the precise biological role of *IGF2* remains unknown. The persistent expression of *IGF2* in certain matured organs, such as the human adult liver, suggests *IGF2* may also has differential specific tissue-functions. Expression studies of liver show that while *IGF2* transcripts are seen in all cell types in the relatively immature liver (6, 7), *IGF2* expression is primarily seen in the more specialized perisinusoidal cells of the developed liver (26, 27). It is possible that the developmental variation in imprinting may serve to regulate the primarily proliferative functions of *IGF2* during fetal development.

Enhanced levels of P3 and P4 promoters driven *IGF2* mRNA has also been detected in many tumors of different origins, which suggested a role for *IGF2* in autocrine or paracrine growth stimulation. Demonstrated LOI of *IGF2* in various human tissues from tumor and overgrowth syndromes attest to the critical role of imprinting for regulating *IGF2* gene dosage during fetal/embryogenesis (14-17). The fact that biallelic *IGF2* transcription was observed in human adult liver in this study is providing a supportive notion that stage dependent relaxation of gene imprints may be a normal developmental event that facilitates proper organ maturation and function. It thus suggests that LOI is only consequential when they occur within a restricted developmental window. Furthermore, as *IGF2* in hepatic tissue normally undergoes a promoter switch concomitant with the onset of tissue maturation, these findings clearly indicate that tissue-specific imprinting of the human *IGF2* gene may be in a promoter-dependent manner.

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