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H19 and IGF-2 allele-specific expression in hepatoblastoma

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Summary Patterns of allele-specific expression of H19 and insulin-like growth factor-2 (IGF-2) were examined in tissue obtained from 30 children diagnosed with hepatoblastoma. All informative tumours demonstrated monoallelic expression of H19. In contrast, variable patterns of allele-specific expression of IGF-2 were seen in tumours from children of different ages. © 2000 Cancer Research Campaign

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Genomic imprinting is the differential expression of a gene depending on the parent of origin. H19 and insulin-like growth factor-2 (IGF-2) are imprinted genes that map to a 90-kilobase (kb) region on chromosome 11p15 (Zemel et al, 1992). In the majority of human tissues, only the maternal allele of the H19 gene is expressed and only the paternal allele of the IGF-2 gene is expressed. In adult human liver and fetal choroid plexus and leptomeninges, however, IGF-2 is biallelically expressed (Ohlsson et al, 1993; Ekstrom et al, 1995).

In human liver, IGF-2 is monoallelically expressed at birth, with a switch to biallelic expression some time during the first year of postnatal life (Ohlsson et al, 1993; Davies, 1994; Li et al, 1996). IGF-2 expression is regulated by four promoters, P1–P4, which are expressed, and hence imprinted, differently. P1 is biallelically expressed and is active in adult human liver. In fetal and early postnatal liver, however, the pattern of P1 expression is complex. Although it has been demonstrated that the P1 promoter directs expression from both parental alleles in prenatal as well as postnatal liver specimens (Ekstrom et al, 1995), some studies suggest that the P1 promoter is not used in early fetal life (Li et al, 1996, 1998), while others have detected P1-derived transcripts in fetal and newborn liver tissue (Taniguchi et al, 1995; Vu and Hoffman, 1996). In contrast, promoters P2–P4 are expressed monoallelically and are used in fetal and infant tissues, but in older infants (> 18 months) and adults imprinting of P2–P4 exhibits plasticity. Both monoallelic and biallelic expression have been described, as have instances of monoallelic expression from opposite parental alleles. Switching from monoallelic to biallelic IGF-2 expression in normal human liver in the first year of life involves increased use of P1 and the development of plasticity in P2–P4. In contrast, H19 is imprinted in normal human liver throughout life (Ekstrom et al, 1995). The precise mechanism by which genes and their regulatory elements are imprinted is currently unknown, although available data suggest that methylation of DNA may determine the imprint (Hu, 1996).

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Biallelic expression of IGF-2 and H19 has been demonstrated in several paediatric and adult malignancies, including Wilms' tumour, Ewing sarcoma, embryonal rhabdomyosarcoma, germ cell tumours, lung cancer, oesophageal cancer, glioma and renal cell carcinoma (Glassman et al, 1996; Uyeno et al, 1996; Nonomura et al, 1997; Ross et al, 1999). In contrast, for hepatoblastoma, an embryonal liver tumour that usually occurs in the first 5 years of life, monoallelic expression of IGF-2 has generally been reported, with biallelic expression observed in only a minority of cases (Davies, 1993; Li et al, 1995; Rainier et al, 1995). In this study, we examined expression of IGF-2 and H19 in informative tumour specimens and normal matched tissue (if available) from children of different ages diagnosed with hepatoblastoma.

MATERIALS AND METHODS

Tumour samples

Hepatoblastoma tissue (and normal matched tissue, if available), frozen at –70°C, was obtained from the Cooperative Human Tissue Network (CHTN; Columbus, OH, USA). Tissue underwent institutional and central pathological review to ensure uniformity of diagnosis. Normal liver tissue from a 14-year-old traffic accident victim was obtained from the Liver Tissue Procurement and Distribution System (NIH Grant N01-DK-6-2274).

Allele-specific gene expression

Genomic DNA (gDNA) was extracted using standard techniques, and samples were screened for heterozygosity for a known *Apa*I polymorphism within exon 9 of IGF-2 as previously described (Ogawa et al, 1993). If heterozygous (and hence informative), reverse transcription polymerase chain reaction (RT-PCR) was performed as described by Nonomura et al (1997). PCR products were digested with *Apa*I (yielding either a 236-bp fragment or a 173- and 63-bp fragment), electrophoresed through a 1.5% agarose gel, and visualized with ethidium bromide. To monitor for gDNA contamination of the cDNA preparation, RT-PCR was performed both with and without reverse transcriptase; no amplification was observed from samples without reverse transcriptase (Figure 1A).

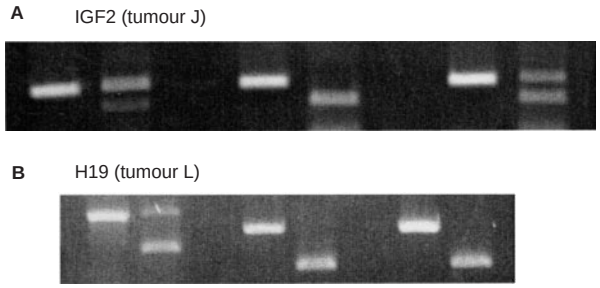

Figure 1 (**A**) IGF-2, tumour J. Each panel contains nine lanes: Lane 1 contains primer-amplified gDNA, Lane 2 contains primer amplified gDNA digested with *Apa*I to demonstrate heterozygosity, Lane 3 is a control to monitor for gDNA contamination of the tumour cDNA product (cDNA without reverse transcriptase), Lane 4 contains primer-amplified cDNA product, Lane 5 contains primer-amplified cDNA tumour product digested with *Apa*I, Lane 6 is a control to monitor for gDNA contamination of cDNA from normal liver adjacent to the tumour, Lane 7 contains primer-amplified cDNA from normal tissue, and Lane 8 contains primer-amplified cDNA from normal tissue digested with *Apa*I (**B**) H19, tumour L. Each panel contains nine lanes: Lane 1 contains primer-amplified gDNA, Lane 2 contains primer amplified gDNA digested with *Rsa*I to demonstrate heterozygosity, Lane 3 is a control to monitor for gDNA contamination of the tumour cDNA product (cDNA without reverse transcriptase), Lane 4 contains primer-amplified cDNA product, Lane 5 contains primer-amplified cDNA tumour product digested with the *Rsa*I, Lane 6 is a control to monitor for gDNA contamination of cDNA from normal liver tissue adjacent to the tumour, Lane 7 contains primer-amplified cDNA from normal tissue, and Lane 8 contains primer-amplified cDNA from normal tissue digested with *Rsa*I

IGF-2 promoter usage was evaluated using the method of Hu et al (1996) (Figure 2). In brief, a multiplex PCR was performed in which cDNAs were amplified using four promoter-specific 5′-primers and a common 3′-primer end-labelled with [γ-^{32}P]ATP. Oligonucleotide primers used were: CAGTCCTGAGGTGAGCTGCTGTGGC (hP1), ACCGGGCATTGCCCCCAGTCTCC (hP2), CGTCGCACATTCGGCCCCCGCGACT (hP3), TCCTCCTCCTCCTGCCCCAGCG (hP4), CAGCAATGCAGCACGAGGCGAAGCC (3′ primer).

Amplification of H19 was performed as described by Uyeno et al (1996). PCR products were digested with *Rsa*I (yielding either a 655-bp product or a 485- and 170-bp fragment). If heterozygous, cDNA was reverse transcribed and amplified in the same manner as gDNA producing 575- or 405-bp products after digestion with *Rsa*I. As the primers span the last intron of H19, gDNA contamination is excluded by the absence of a 655 bp product (Figure 1B).

For all experiments, PCR amplification and digests were repeated in duplicate to exclude the possibility of incomplete digestions yielding misleading results. In addition, all samples were digested in the presence of a positive control homozygous for the smaller fragment to ensure complete digestions. To monitor for potential cross-sample DNA contamination of PCR reactions, control amplifications were performed without DNA template and were shown to be negative for each experiment.

RESULTS

Genomic DNA from 30 hepatoblastomas was amplified by PCR and digested with *Apa*I and *Rsa*I to identify the presence of previously described polymorphisms in IGF-2 and H19 respectively (Ogawa et al, 1993; Rainier et al, 1993). Thirteen (43%) tumours were informative (heterozygous) for H19, and 13 were informative for IGF-2. Six (20%) hepatoblastomas were heterozygous for both IGF-2 and H19. Four tumours had normal adjacent liver tissue available (Table 1). Demographic data on individuals with these informative tumours are provided in Table 1. The majority (80%) of patients were male and the median age at diagnosis was 1.8 years (range 1 month to 9 years).

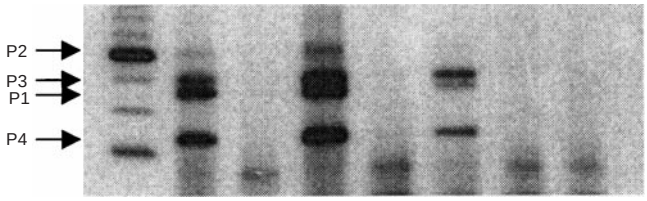

Figure 2 IGF-2 promoter usage. Lane 1 contains a size marker, Lane 2 contains promoter-specific primer amplified cDNA product (P2 = 277 bp, P3 = 211 bp, P1 = 186 bp, P4 = 119 bp) of liver tissue obtained from a 14-year-old traffic accident victim, Lanes 3, 5 and 7 are controls to monitor for gDNA contamination of the cDNA product, Lane 4 contains promoter-specific primer amplified cDNA product from normal liver tissue adjacent to tumour M, Lane 6 contains promoter-specific primer amplified cDNA product from normal liver tissue adjacent to tumour G, Lane 8 is a PCR-negative control. (Although P2 appears faint in Lane 6, it is clearly apparent with longer exposure)

For all tissue examined (including both malignant and normal adjacent liver tissue), H19 was monoallelically expressed. In contrast, variable patterns of allele-specific expression at IGF-2 were observed. The majority (10/13) of informative tumours demonstrated monoallelic expression of IGF-2. Tumours demonstrating biallelic expression of IGF-2 (E, I and T) were diagnosed in children aged 10 months, 18 months and 9 years respectively. In two cases (J and L; diagnosed at ages 18 months and 2 years) normal adjacent liver tissue showed biallelic expression of IGF-2, while expression was monoallelic in the tumour tissue. However, two others (G and M; diagnosed at ages 13 months and 2 years) showed monoallelic IGF-2 expression in tumour tissue and in adjacent normal liver tissue.

Examination of IGF-2 promoter usage in the adjacent normal liver tissue of G and M showed usage of all promoters, including P1 (Figure 2).

DISCUSSION

This study represents one of the largest investigations of H19 and IGF-2 allele expression in hepatoblastoma. Allele-specific expression in hepatoblastoma is of particular interest since monoallelic expression of IGF2 is typically observed in normal fetal liver, with a change to biallelic expression occurring during the first year of life. This pattern is in direct contrast to that observed in most other normal tissues where monoallelic expression of IGF-2 continues throughout life. We examined allele-specific expression of IGF-2 and H19 in a wide age range of hepatoblastomas including children diagnosed in the first few months of life, as well as tumours diagnosed at 2 years of age or greater, where biallelic IGF-2 expression is expected in normal liver.

We found monoallelic expression of H19 was maintained in all tissue (malignant and normal) studied. Few previous studies have specifically examined H19 allele expression in hepatoblastoma. Rainier et al (1995) examined five hepatoblastomas and found that H19 was biallelically expressed in one tumour that demonstrated biallelic expression of IGF-2. In contrast, in a study of three tumours, Li et al (1995) reported that monoallelic expression was

Table 1 Allelic usage of IGF2 and H19 in 20 informative hepatoblastoma tumours

ID	Gender	Normal tissue	Age at Dx	IGF-2 informative	H19 informative	IGF-2 (malignant tissue)	H19 (malignant tissue)	IGF-2 (normal adjacent tissue)	H19 (normal adjacent tissue)
A	M		1 months	Yes	No	Monoallelic			
B	M		7 months	Yes	Yes	Monoallelic	Monoallelic		
C	M		8 months	No	Yes		Monoallelic		
D	M		10 months	No	Yes		Monoallelic		
E	F		10 months	Yes	Yes	Biallelic	Monoallelic		
F	M		12 months	Yes	Yes	Monoallelic	Monoallelic		
G	M	Yes	13 months	Yes	No	Monoallelic		Monoallelic	
H	F		15 months	No	Yes		Monoallelic		
I	M		18 months	Yes	No	Biallelic			
J	M	Yes	18 months	Yes	Yes	Monoallelic	Monoallelic	Biallelic	Monoallelic
K	F		2 years	Yes	No	Monoallelic			
L	F	Yes	2 years	Yes	Yes	Monoallelic	Monoallelic	Biallelic	Monoallelic
M	M	Yes	2 years	Yes	Yes	Monoallelic	Monoallelic	Monoallelic	Monoallelic
N	F		2 years	Yes	No	Monoallelic			
O	F		2 years	No	Yes		Monoallelic		
P	M		2.5 years	Yes	No	Monoallelic			
Q	M		2.5 years	No	Yes		Monoallelic		
R	M		3 years	No	Yes		Monoallelic		
S	M		4 years	No	Yes		Monoallelic		
T	M		9 years	Yes	No	Biallelic			

maintained for H19 in a tumour that demonstrated biallelic expression of IGF-2. Given that all of our informative hepatoblastomas demonstrated monoallelic expression at H19, it is unlikely that alterations in allele-specific expression of H19 plays a major role in the development of hepatoblastoma.

In contrast to H19, variable patterns of allele-specific expression of IGF-2 were observed. The majority of tumours in our study demonstrated monoallelic expression of IGF-2, supporting observations reported in smaller studies (Davies, 1993; Li et al, 1995; Rainier et al, 1995). Biallelic expression of IGF-2 in malignant tissue at most sites is abnormal and is considered part of the malignant phenotype, potentially contributing to the progression of disease. In contrast, biallelic expression of IGF-2 is considered normal in post-natal human liver and fetal choroid plexus/leptomeninges (Ekstrom et al, 1995). Switching from the fetal pattern of monoallelic IGF-2 expression to biallelic expression in normal human liver development entails increased use of the biallelically expressed promoter P1, together with plasticity of imprinting of P2–P4 (Li et al, 1998). The precise age at which this typically occurs is controversial and has only been studied in a small number of cases. Some studies suggest that biallelic IGF-2 expression begins between 6 months and 1 year post-natal age (Ohlsson et al, 1993; Davies, 1994), whereas others observed biallelic IGF-2 expression in the liver tissue of a 2-month-old (Li et al, 1996). In the latter study, however, the tissue evaluated was adjacent normal liver from a child with hepatoblastoma, which may not be appropriate for determination of developmental patterns in healthy individuals.

In our study, the observation of monoallelic IGF-2 expression in hepatoblastoma tissue at ages when normal liver expresses IGF-2 biallelically (tumours J, K, L, M, N, P; all 18 months of age or older) may indicate a failure to follow the normal sequence of change in promoter usage (e.g. increased usage of P1). In such cases, it is possible that carcinogenesis is initiated at an earlier developmental time point than in cases in which normal progression to biallelic usage is seen. Moreover, the observation of monoallelic IGF-2 expression in normal liver adjacent to hepatoblastoma may indicate premalignant change in a larger area of the liver than that morphologically involved with tumour. Nevertheless, for the two cases (G and M; ages 13 months and 2 years) that showed monoallelic expression of IGF-2, all four promoters were utilized in the normal adjacent liver tissue. If the age at which promoter switching occurs is more variable than that reported to date, it is possible that the normal liver in these cases has simply not undergone the switch from monoallelic to biallelic IGF-2 expression and that the allele usage observed in both tumour and normal liver is appropriate, and not related to the malignant phenotype. Clearly, larger studies of normal liver tissue obtained in the first few years of life are needed to accurately evaluate the variability of timing of promoter silencing and switching of IGF-2.

In malignancies other than hepatoblastoma, allele-specific expression patterns for H19 and IGF-2 appear to vary, with coupling of expression frequently observed. For example, in testicular germ cell tumours (both paediatric and adult), as well as in Wilms' tumour, biallelic expression of both H19 and IGF-2 has been frequently observed (van Gurp et al, 1994; Nonomura et al, 1997, Ross et al, 1999). In childhood embryonal tumours in particular, biallelic expression of IGF-2 is thought to be related to elevated levels of IGF-2 production, and potentially to contribute to growth and progression of the tumour (Leisenring et al, 1994). An alternative interpretation of these data is that biallelic expression of IGF-2 represents non-specific gene dysregulation, perhaps due to genome-wide hypomethylation as part of the malignant phenotype as was shown in hepatocellular carcinoma (Takeda et al, 1996; Li et al, 1997). While our data regarding hepatoblastomas could represent non-specific dysregulation of gene expression, the continuation of monoallelic expression would argue against hypomethylation as a mechanism. Li et al (1995) suggest that for hepatoblastoma (unlike Wilms' tumour) the level of H19 expression is not a prerequisite for maintaining a monoallelic IGF-2 expression. Moreover, as in our study, they report monoallelic expression of H19 in a case of hepatoblastoma that showed biallelic expression of IGF2.

In summary, we conclude that monoallelic expression of H19 is maintained in the vast majority of hepatoblastomas, suggesting that alterations of allele-specific expression of H19 are unlikely to play a major role in carcinogenesis at this site. Moreover, we report variable allele-specific expression patterns for IGF-2 with respect to age at diagnosis. To further understand the importance of IGF-2 allele-specific expression in the malignant phenotype, larger studies evaluating the age at which the switch from monoallelic to biallelic IGF-2 expression occurs in normal liver are warranted.

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