Loss of imprinting of IGF2 correlates with hypermethylation of the H19 differentially methylated region in hepatoblastoma

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IGF2, a maternally imprinted foetal growth factor gene, is implicated in many childhood tumours including hepatoblastoma (HB); however, the genetic and epigenetic alterations have not comprehensively been studied. We analysed the methylation status of the H19 differentially methylated region (DMR), loss of heterozygosity (LOH) and allelic expression of IGF2 in 54 HB tumours, and found that 12 tumours (22%) with LOH, 9 (17%) with loss of imprinting (LOI) and 33 (61%) with retention of imprinting (ROI). Biallelic and monoallelic IGF2 expressions correlated with hypermethylation and normal methylation of H19 DMR, respectively, in two tumours with LOI and seven tumours with ROI. Quantitative RT–PCR analysis showed minimal expression of H19 mRNA and substantial expression of IGF2 mRNA in tumours with LOH or LOI, and substantial expression of both H19 and IGF2 mRNAs in tumours with ROI. Increased IGF2 expression with predominant embryonic P3 transcript was found in the majority of HBs with ROI and foetal livers. In contrast to the earlier reports, our findings suggest that the disruption of the enhancer competition model reported in Wilms' tumour may also occur in HB. Both frequencies of LOH and LOI seem to be lower in HB than in Wilms' tumour, reflecting the different tissue origins.

Keywords: hepatoblastoma; IGF2; H19; loss of heterozygosity; loss of imprinting

Hepatoblastoma (HB) is a rare malignant neoplasm of the liver, with an incidence of 0.5–1.5 per million children (Perilongo and Shafford, 1999). Remarkable progress in clinical outcome has been achieved in the past 20 years because of advances in chemotherapy and surgical procedures; however, the mortality rate remains 20–30% and treatment results in patients in advanced stages who are refractory to standard preoperative chemotherapy regimens are unsatisfactory (Perilongo et al, 2000; Fuchs et al, 2002). To improve the mortality of these patients, innovative treatment based on a specific molecular target is needed. The molecular mechanism involved in the development and progression of HB includes overexpression of insulin-like growth factor-II (IGF2) (Li et al, 1998b; Gray et al, 2000; Hartmann et al, 2000), downregulation of RASSF1A by promoter hypermethylation (Sugawara et al, 2007; Hayashi et al, 2008) and alterations of genes in the Wnt signalling pathway; most notably, the high incidence of CTNNB1 (catenin, β1) mutation (Koch et al, 1999; Taniguchi et al, 2002).

IGF2 is a maternally imprinted gene and encodes a foetal peptide hormone that regulates cellular proliferation and differentiation (Foulstone et al, 2005). IGF2 has four promoter regions and P3 is the most active promoter in the foetal liver, followed by P2 and P4 promoters (Li et al, 1998a). PLAG1 encodes a developmentally regulated transcription factor, which positively regulates IGF2 through binding the P3 promoter region. Although IGF2 is downregulated in normal tissues after birth, except for liver tissues, it is overexpressed in a wide variety of childhood and adult cancers and serves as a tumour enhancer through autocrine and paracrine mechanisms (Toretsky and Helman, 1996). IGF2 has been studied extensively over the past decade as a key molecule involving HB and Wilms’ tumour (WT) pathogenesis.

The allelic expression of IGF2 is regulated by the methylation status of the sixth CTCF (CCCTC-binding factor) site in the H19 differentially methylated region (DMR) that represents the parental origin of the IGF2 allele; whereas the paternal CTCF6 allele is methylated, the maternal allele is unmethylated in normal tissues (Bell and Felsenfeld, 2000; Hark et al, 2000; Takai et al, 2001). Using the enhancer competition model, IGF2 and H19 promoters compete on the same chromosome for a shared enhancer, and access of the maternal IGF2 allele to this enhancer is blocked by H19 DMR when unmethylated because of the insulator activity of CTCF binding to unmethylated H19 DMR (Bell and Felsenfeld, 2000; Hark et al, 2000). It has been proved in many WTs that aberrant methylation of the maternal CTCF6 prevents the insulator binding and leads to loss of imprinting (LOI), resulting in the overexpression of IGF2 (Steenman et al, 1994; Ravenel et al, 2001). Although LOI of IGF2 was reported in HB, the mechanism of LOI, the concurrent overexpression of IGF2 mRNA and loss of
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H19 mRNA expression are uncertain because of the limited number of HB tumours examined and the low frequency of the heterozygous IGF2 polymorphic site in general populations (Davies, 1993; Montagna et al., 1994; Rainier et al., 1995; Li et al., 1995, 1998b; Fukuzawa et al., 1999; Gray et al., 2000; Hartmann et al., 2000; Ross et al., 2000; Albrecht et al., 2004; Suzuki et al., 2008), and some investigators stated earlier that the mechanisms of IGF2 upregulation by LOI found in WT do not apply to HB (Li et al., 1995; Hartmann et al., 2000).

Loss of imprinting was reported in 32–38% of WTs (Ravenel et al., 2001; Fukuzawa et al., 2004; Yuan et al., 2005), and loss of heterozygosity (LOH), leading to uniparental disomy (UPD) of the paternal IGF2, was reported in 36–50% of WTs (Grundy et al., 1996; Fukuzawa et al., 2004; Yuan et al., 2005). In HB, although LOH of IGF2 was reported in 20–30%, the incidence of LOI of IGF2 was uncertain because each series included only a small number of HB tumours. In addition, it is also uncertain whether the same mechanism of LOI is involved in both WT and HB tumorigenesis because the methylation status of H19 DMR in HB has rarely been examined (Li et al., 1995, 1998b; Fukuzawa et al., 1999).

To determine whether the alterations of IGF2 and H19 loci identified in WT are also found in HB, we examined the LOI and LOH status of IGF2 using combined bisulphite restriction assay (COBRA) of the CTCF6 region that can determine the methylation status of H19 DMR more efficiently than the method using methylation-specific restriction enzymes and Southern blot in 54 HB tumours. In addition, we evaluated promoter-specific IGF2 transcripts, the methylation status of IGF2 promoters and PLAG1 mRNA expression. Our results showed that the genetic and epigenetic alterations in the IGF2-H19 region with elevated expression of IGF2 mRNA identified in WTs were also found in the great majority of HB tumours, although the incidences of LOH and LOI may be lower in HBs than in WTs.

MATERIALS AND METHODS

Patients and samples

Tumour tissues were obtained from 54 Japanese children with HB, and adjacent normal liver tissues were available from 5 patients. Eighteen tumour and five matched normal liver specimens were supplied by institutions affiliated with Saitama Cancer Center. DNA and RNA were extracted from tumour and normal liver tissues adjacent to HB; the age of the patients was 16, 24 or 26 months. Of the 20 tumours, 3 and 16 were obtained before and after chemotherapy, respectively, and the chemotherapy status was unknown in the other 3 tumours. Pathologists in each institution and/or the JPLT pathology panel made the diagnosis of HB and verified that each sample contained 70% or more tumour cells. Informed consent was obtained from the parents, and the study design was approved by the ethics committee of Saitama Cancer Center.

COBRA of the CTCF6 site at H19 DMR

We performed COBRA to determine the methylation status of the CTCF6 binding site at H19 DMR, as described earlier (Watanabe et al., 2007). COBRA of CTCF6 showed that the mean methylation percentage ± 2 s.d. of five normal livers was 52.8 ± 15.0%, and we defined more than the mean percentage ± 2 s.d. as the hypermethylated state.

LOH analysis of IGF2

High-resolution single nucleotide polymorphism (SNP) array, Affymetrix Mapping 50K-Xba array (Affymetrix, Santa Clara, CA, USA), was used to analyse chromosomal aberrations of 11p15.5 where IGF2 resides. Genomic DNA in 43 of 54 tumours and 2 cell lines was assayed according to the manufacturer’s protocol, and the genomic status of IGF2 was determined as described earlier (Haruta et al., 2008).

Allelic expression analysis of IGF2 and quantitative real-time reverse transcription-PCR analysis of IGF2 and H19 mRNA

The ApaI/AvaI polymorphic site in exon 9 of IGF2 was used to evaluate the allelic expression of IGF2 mRNA in 21 tumours whose DNA was available for this study, as described earlier (Watanabe et al., 2006). Quantitative real-time reverse transcription-PCR was performed to evaluate the total IGF2 and H19 mRNA levels in 20 tumour tissues, 2 HB cell lines (HuH6 and HepG2), foetal liver total RNA pooled from 34 foetuses (Clontech, Ohtsu, Japan) and 3 normal liver tissues adjacent to HB; the age of the patients was 16, 24 or 26 months. Of the 20 tumours, 3 and 16 were obtained before and after chemotherapy, respectively, and the chemotherapy status was unknown in 1. The primers and TaqMan probes used for IGF2 and H19 mRNA were described earlier (Watanabe et al., 2007; Haruta et al., 2008). The expression of IGF2 and H19 mRNAs was normalised with GAPDH.

Methylation-specific PCR and bisulphite sequencing analysis of IGF2 promoter regions

Genomic DNA from tumour and normal liver samples was treated with sodium bisulphite (Herman et al., 1996), and the methylation status of the P2–P4 promoter regions of IGF2 was analysed by methylation-specific PCR (MSP), as described earlier (Beeghly et al., 2007). Polymerase chain reaction products were run on 2% agarose gels and visualised after staining with ethidium bromide. We confirmed the results of MSP analysis of P3 promoter by bisulphite sequencing of eight or more subcloned plasmids.

Semiquantitative RT–PCR analysis of promoter-specific transcripts of IGF2 and PLAG1

P1 and P3 promoter specific expressions of IGF2 mRNA were analysed using the primer sets described elsewhere (Lu et al., 2006). The primer sequences for P2-specific transcript were derived from exons 4 and 5: forward, 5′-CCCTCGAGACGTTGACAG-3′; reverse, 5′-CTTGGGTTGGACCTGCAATG-3′ and the primer sequences for P4-specific transcript were derived from exons 7, 8 and 9: forward, 5′-CGAGCCCTTCTGCTGAGCTAC-3′; reverse, 5′-CGGAAAGCAGCACTCTCAAC-3′. PLAG1 mRNA expression was analysed using the following primer sets: forward, 5′-AACGTAAGCGTGGTGAAACC-3′; reverse, 5′-TGACATTCCTCCGAGACTTA-3′ (Zatkova et al., 2004). Polymerase chain reaction products were run on polyacrylamide gels and visualised after ethidium bromide staining. The intensity of each band was examined using a fluorescence image analyser, FLA-3000G (Fujiﬁlm, Tokyo, Japan). Dividing the intensity of the target transcript by that of GAPDH calculated the level of each transcript.

Mutation analysis of the CTNNB1 gene

To detect point mutations and deletions of the CTNNB1 gene, genomic DNA from each tumour sample was ampliﬁed using two sets of primers, F1, 5′-TGGCTATCATTCTGCTTTTCTTG-3′ and R1, 5′-CTCTTTTTCCTCACCAGACATTTT-3′, and BCAT-3, 5′-AA
AATCCAGGCTGGACAATGG-3' and BCAT-4, 5'-TGTTGCAAGTT CTGGCATCATC-3', respectively (Koch et al., 1999; Satoh et al., 2003). The PCR products were either directly sequenced or inserted into a vector (pGEM- (R)- T Easy Vector System (Promega, Madison, WI, USA)), and six or more clones were sequenced.

Statistical analysis

Student’s t-test or Welch’s t-test compared mRNA levels of IGF2 and H19 between tumours with or without IGF2 alterations or other characteristics and the levels of IGF2 promoter-specific transcripts between tumours with or without PLAG1 mRNA expression. We also assessed the association between total IGF2 mRNA levels and P2-, P3- or P4-specific IGF2 mRNA levels by determining the Spearman rank correlation coefficient and associated P-value. Differences in the incidence of tumours with unmethylated P3 promoter were examined between tumours with hypermethylated H19 DMR and tumours with normally methylated H19 DMR by the χ² test. Differences in the incidences of tumours with CTNNB1 mutation were examined between any two of three groups of tumours classified on the basis of the IGF2 status by the χ² test.

RESULTS

Methylation status of the CTCF6 binding site at H19 DMR, LOH analysis using SNP array and allelic expression analysis of IGF2

Combined bisulphite restriction assay showed that 21 and 33 tumours had hypermethylation and normal methylation at CTCF6, indicating LOH or LOI and retention of IGF2 imprinting (ROI), respectively (Table 1 and Figure 1). Single nucleotide polymorphism array analysis was performed in 43 of 54 tumours; all 21 tumours with hypermethylated CTCF6 and 22 of 33 tumours with normally methylated CTCF6. Combined results of both analyses indicated that 12 tumours had LOH (10 hypermethylated CTCF6 and UPD 2 hypermethylated CTCF6 and hemizygous 11p15 deletion), 9 had LOI (hypermethylated CTCF6 and retention of heterozygosity (ROH)) and 22 had ROI (normally methylated CTCF6 and ROH). Of 21 tumours whose RNA was available, 9 and 12 tumours had heterozygous and homozygous Apal/AvalI sites in exon 9 of IGF2, respectively. Of the nine heterozygous tumours, seven showed monoallelic expression of IGF2, indicating ROI, and two showed biallelic expression of IGF2, indicating LOI, and the results were consistent with those examined by COBRA and SNP array analyses (Table 1). From these findings, 11 tumours with normally methylated CTCF6, in which SNP array analysis was not performed, were classified as those with ROI. Thus, combined results of COBRA, SNP array and allelic expression analyses showed 12 tumours with LOH, 9 tumours with LOI and 33 tumours with ROI. In addition, one cell line (HuH6) had LOI, and the other (HepG2) had LOH (UPD) of IGF2.

The mean age was compared between any two of three groups of patients (i.e., LOH, LOI or ROI) by Student’s t-test. There was no difference in the mean age between any two of the three groups of patients.

Correlation between IGF2 and H19 mRNA levels and the IGF2 status (LOH, LOI or ROI)

Quantitative real-time reverse transcription-PCR analysis showed that although 15 of 20 tumours had a higher level of IGF2 mRNA than normal liver tissues, 15 of 20 tumours had a lower level of H19 mRNA than normal liver tissues (Table 1 and Figure 2). All 3 tumours with UPD, 1 of 1 with 11p15 loss, 1 of 3 with LOI and 10 of 13 with ROI, expressed higher levels of IGF2 mRNA than normal liver tissues. There was no significant difference in IGF2 mRNA levels between 3 tumours with UPD or 7 tumours with IGF2 alterations; that is UPD, 11p15 loss or LOI, and 13 tumours with ROI. In contrast, 7 tumours with IGF2 alterations expressed very low levels of H19 mRNA, whereas 11 of 13 tumours with ROI expressed a substantial amount of H19 mRNA; 2 tumours (nos. 25 and 27) with ROI expressed very low levels of H19 mRNA. H19 mRNA levels were higher in 13 HB tumours with ROI than in 7 HB tumours with IGF2 alterations (P < 0.01 by Welch’s t-test). Although HepG2 with UPD had a higher level of IGF2 mRNA than normal liver tissues, HuH6 with LOI had a very low level of IGF2 mRNA. H19 mRNA levels were very low in both cell lines.

Semiquantitative RT-PCR analysis of promoter-specific IGF2 transcripts

Because the IGF2 gene has four kinds of promoters, promoter-specific IGF2 transcripts were analysed to determine the usage of each promoter. Representative results of the P3 transcript are shown in Figure 3A. All 20 tumours showed undetectable or lower levels of P1 transcripts than 3 normal liver tissues. The levels of P2, P3 and P4 transcripts were higher in 13, 15 and 10 of the 20 tumours, respectively, than those of normal liver tissues. Polymerase chain reaction cycle numbers to obtain visible levels of PCR products were 40 for P2 transcripts, 30 for P3 transcripts and 35 for P4 transcripts, indicating that the amounts of P3 transcripts were high, those of P2 transcripts were low and those of P4 transcripts were intermediate. The Spearman correlation coefficient analysis showed that the expression levels of the P2, P3 and P4 transcripts correlated with the levels of total IGF2 mRNA (P2, rS = 0.730; P3, rS = 0.773 and P4, rS = 0.664) (Figure 3B and C; data for the P2 and P4 transcripts are not shown).

The methylation status of IGF2 promoters and its correlation with the levels of promoter-specific transcripts

In the MSP analysis of each promoter, the P2 promoter region was partially methylated in 19 tumours and normal liver tissues and the P4 promoter region was unmethylated in all 20 tumours and normal liver tissues. Therefore, the methylation status of P2 or P4 promoter region was not correlated with the expression level of P2- or P4-specific transcripts. The P3 promoter region was partially methylated in 11 tumours, HuH6 and normal liver tissues and unmethylated in 9 tumours and HepG2 (Table 1, Figure 4A and B). The results of MSP analysis in one tumour (no. 1) and HuH6 were confirmed by bisulphite sequencing (Figure 4C). Nine tumours with the unmethylated P3 promoter had higher levels of P3 transcripts than 11 tumours with the partially methylated P3 promoter (P = 0.005) (Figure 4D). The P3 promoter was unmethylated in 5 of 7 tumours with IGF2 alterations; UPD, 11p15 loss or LOI, but in 4 of 13 tumours with ROI. Thus, the incidence of tumours with unmethylated P3 promoter tended to be higher in tumours with hypermethylated H19 DMR than in tumours with normally methylated H19 DMR (P = 0.1).

Semiquantitative RT-PCR analysis of PLAG1 mRNA

PLAG1 positively regulates IGF2, and its expression was detected in 12 tumours, foetal liver RNA and 2 cell lines, but not in 8 tumour and 3 normal liver tissues (Table 1 and Figure 5). The 12 tumours with PLAG1 mRNA expression showed higher levels of P4-specific IGF2 transcripts (P = 0.01) and tended to show higher levels of P3-specific IGF2 transcripts (P = 0.051) than the 8 tumours without PLAG1 expression. There was no significant difference in P2- or P1-specific transcript levels between tumours with and without PLAG1 mRNA expression.
Table 1 Genetic and epigenetic status of the IGF2-H19 region in 54 hepatoblastoma tumours

| Patients | Age in months | Chemo | %methyl | 11p15 SNP | Apol site | IGF2 RT-PCR | IGF2 status | IGF2 mRNA | P1E | P2M | P2E | P3M | P3E | P4E | H19 mRNA | PLAG1 mRNA | CTNNB1 status |
|----------|---------------|-------|---------|--------|----------|-------------|-------------|-----------|-----|-----|-----|-----|-----|-----|---------|-----------|----------------|
| 1        | 48/F          | +     | 82.8    | UPD    | Homo     | ND          | UPD         | 11.3      | 0.6 | MU | 17.7 | U   | 7   | 3.1 | 0       | +         | M              |
| 2        | 5/M           |       | 93.4    | UPD    | Homo     | ND          | UPD         | 3.9       | 0   | MU | 0   | 2.1 | 0.9 | 0.1 | 0       |          |               |
| 3        | 24/M          |       | 76.7    | UPD    | Homo     | ND          | UPD         | 3.2       | 0   | MU | 2.4 | MU  | 2.3 | 1.2 | 0       | +         | M              |
| 4–10     | 5–96/M6, F1   | +6, -1| 72–90   | UPD    | ND       | ND          | UPD         | ND        | ND  | ND | ND  | ND  | ND  | ND  | ND      | ND        | M, N3           |
| 11       | 27/M          |       | 87.8    | Loss chr II | Homo | ND          | Loss        | 6.1       | 0   | MU | 2.3 | U   | 4.4 | 1.3 | 0       | +         | M              |
| 12       | 24/M          |       | 81.9    | Loss chr II | ND   | ND          | Loss        | ND        | ND  | ND | ND  | ND  | ND  | ND  | ND      | ND        | M              |
| 13       | 12/F          |       | 91.4    | ROH    | Homo     | ND          | LOI (m)     | 9.7       | 0   | U  | 1.1 | U   | 2.3 | 0.6 | 0       | −         | ND             |
| 14       | 16/M          |       | 86.1    | ROH    | Homo     | ND          | LOI (m)     | 1.0       | 0   | MU | 0.3 | U   | 1.3 | 0.9 | 0       | −         | ND             |
| 15       | 26/F          |       | 83.1    | ROH    | Hetero   | LOI (m)     | 0.8        | 0.5       | 0   | MU | 0.6 | U   | 0   | 0   | 0       | −         | M              |
| 16       | 24/M          |       | 70.9    | ROH    | Hetero   | LOI (m)     | 0.8        | 0.5       | 0   | MU | 0.6 | U   | 0   | 0   | 0       | −         | M              |
| 17–21    | 12–84/M4, F1  | +1, −3, UK1 | 71–91 | ROH    | ND       | ND          | LOI (m)     | 9.7       | 0   | U  | 1.1 | U   | 2.3 | 0.6 | 0       | −         | ND             |
| 22       | 12/F          |       | 52.5    | ND     | Homo     | ND          | ROI (m)     | 9.2       | 0.8 | MU | 8.4 | MU  | 2.2 | 3.5 | +       | N         |                 |
| 23       | 109/F         |       | 49.1    | ND     | Homo     | ND          | ROI (m)     | 8.4       | 0   | MU | 4.3 | U   | 4.6 | 2.8 | 0.5     | +         | M              |
| 24       | 12/M          |       | 56.3    | ND     | Hetero   | ROI         | ROI (m, p)  | 7.4       | 0   | MU | 13.2 | U   | 4.6 | 2.4 | 1.9     | +         | M              |
| 25       | 15/M          |       | 55.9    | ROH    | Hetero   | ROI         | ROI (m, p)  | 5.7       | 0   | MU | 6.5 | MU  | 5.1 | 2.2 | 0       | +         | ND             |
| 26       | 6/M           | UK    | 51.1    | ND     | Hetero   | ROI         | ROI (m, p)  | 5.6       | 0.1 | MU | 4.7 | U   | 1.2 | 0.5 | 2.7     | +         | ND             |
| 27       | 10/M          |       | 62.1    | ROH    | Homo     | ND          | ROI (m)     | 5.0       | 0   | MU | 0.9 | U   | 5   | 1.1 | 0       | −         | ND             |
| 28       | 29/F          |       | 61.5    | ND     | Homo     | ND          | ROI (m)     | 3.7       | 0   | MU | 1.5 | U   | 2.5 | 1.4 | 2.5     | +         | M              |
| 29       | 26/M          |       | 55.4    | ND     | Homo     | ND          | ROI (m)     | 3.1       | 0.2 | MU | 9.3 | MU  | 0.4 | 1.2 | 0.7     | −         | M              |
| 30       | 18/M          |       | 48.7    | ND     | Hetero   | ROI         | ROI (m, p)  | 2.4       | 0.1 | MU | 1.2 | MU  | 1.1 | 0.8 | 0       | −         | ND             |
| 31       | 13/M          |       | 55.3    | ND     | Hetero   | ROI         | ROI (m, p)  | 2.2       | 0   | MU | 1.1 | MU  | 1.5 | 1   | 0.6     | +         | N              |
| 32       | 60/F          |       | 55.7    | ND     | Hetero   | ROI         | ROI (m, p)  | 0.7       | 0.2 | MU | 0.2 | MU  | 0.3 | 0.9 | 0.4     | +         | N              |
| 33       | 29/M          |       | 56.4    | ND     | Homo     | ND          | ROI (m)     | 0.5       | 1   | MU | 0.1 | MU  | 0   | 0.5 | 1.2     | −         | M              |
| 34       | 9/M           |       | 56.7    | ND     | Hetero   | ROI         | ROI (m, p)  | 0.5       | 0   | MU | 0   | MU  | 0.1 | 0.2 | 0.2     | +         | M              |
| 35–54    | 4–156/M11, F9 | +12, −7, UK1 | 41–65 | ROH    | ND       | ND          | ND          | ND        | ND  | ND | ND  | ND  | ND  | ND  | ND      | ND        | M11, N9         |
| Normal livers |       |       | 52.8    | ND     | ND       | ND          | ND          | ND        | ND  | ND | ND  | ND  | ND  | ND  | ND      | ND        | M              |
| Fetal livers |       |       | 52.8    | ND     | ND       | ND          | 59.2       | 0.2     | 6.4 | ND  | 4.8 | 1.9 | 2.4 | ND      |           |                 |
| HuH6     | 87.3         | ROH   | Homo    | ND     | ND       | ND          | ND          | ND        | ND  | ND | ND  | ND  | ND  | ND  | ND      | ND        | M              |
| HepG2    | 89.5         | UPD   | ND      | UPD    | ND       | ND          | ND          | 2.4      | 0   | U  | 1.5 | U   | 5   | 2.8 | 0       | +         | M              |

F = female; M = male; M = methylated; ROH = retention of heterozygosity; ROI = retention of imprinting; U = unmethylated; ND = not done; UK = unknown. All 20 tumours showed unmethylated promoter 4; UPD, uniparental disomy; loss chr 11, loss of chromosome 11 or 11p15; LOI, loss of imprinting. *Age in months. **Chemo, chemotherapy before surgery; +6, −1 indicates that six and one tumours were treated and untreated, respectively, with chemotherapy before surgery. %methyl CTCF indicates % methylated CTCF6 allele. Results of SNP array. **Homo, homozygosity at Apal/Aval1 site; hetero, heterozygosity. Results of SNP array analysis, methylation analysis of CTCF6 (m) and Apal/Aval2 polymorphism site analysis (p). **P1E, promoter 1-specific transcript. **P2M, the methylation status of promoter 2. CTNNB1 status: M, mutated; N, normal.
Incidences of tumours with CTNNB1 mutation between any two groups of tumours classified on the basis of the IGF2 status

DNA was available for CTNNB1 mutation analysis in 48 of 54 HB tumours. The results are described in Table 1. There were no differences in the incidences of CTNNB1 mutation between 7 tumours with IGF2-LOI and 29 tumours with IGF2-ROI or 12 tumours with IGF2-LOH, and between 29 tumours with IGF2-ROI and 12 tumours with IGF2-LOH.

DISCUSSION

In this study, biallelic and monoallelic IGF2 expressions correlated with hypermethylation and normal methylation of CTCF6,
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Figure 3  (A) Representative data of RT–PCR analysis of P3 transcripts. (B) Expression levels of P3 transcripts (upper lane) and total IGF2 mRNA (lower lane) are plotted in 20 tumours, in 2 cell lines, in foetal liver tissues and normal liver tissues (a mean value of 3 samples). Tumours are arranged in order by total levels of IGF2 mRNA. Numbers below X axis indicate the tumour number.  (C) Correlation between levels of P3 transcript (X axis) and total IGF2 mRNA (Y axis).

respectively, in two tumours with LOI and seven tumours with ROI (Table 1, Figure 1). In addition, the paternal origin of the duplicated IGF2 loci was confirmed by the hypermethylated CTCF6 in 10 tumours with UPD. Furthermore, very low expression levels of H19 mRNAs and substantial expression levels of IGF2 mRNAs in HB tumours with UPD or LOI, and substantial expression levels of both IGF2 and H19 mRNA in HB tumours with ROI were found (Table 1 and Figure 2). Two (nos. 14 and 15) of three HB tumours with LOI expressed IGF2 mRNA levels comparable to but not higher than those of IGF2 mRNA in normal liver tissues. In addition, one cell line, HuH6, with LOI expressed minimal expression of IGF2 mRNA, although Hartmann et al (2000) found the moderate expression in the same cell line. These findings may be explained by the speculation that such tumours expressed increased levels of IGF2 mRNA at the critical time of tumorigenesis, but not at the time of surgical resection or after many passages of cell culture. From these findings, the hypothesis established for WT that the hypermethylation of maternal H19 DMR causes LOI, and that LOI or duplication of paternal IGF2 (UPD) results in overexpression of IGF2, may be also applied to HB.

Although the expression levels of IGF2 mRNA were reported to be higher in WTs with UPD than in WTs with ROI in two series of WTs (Wang et al, 1996; Haruta et al, 2008), conflicting results were reported in IGF2 mRNA levels between WTs with LOI and WTs with ROI (Wang et al, 1996; Ravenel et al, 2001). The present and earlier studies showed that all HB tumours with UPD and the majority of HB tumours with LOI or ROI expressed the higher levels of IGF2 mRNA than normal liver tissues (Li et al, 1998b; Gray et al, 2000; Hartmann et al, 2000). This study also showed that P3 transcripts predominated in total IGF2 mRNAs in HB tumours irrespective of the IGF2 status (i.e., UPD, 11p15 loss, LOI or ROI); these findings were similar to those reported in foetal liver tissues showing elevated expression of IGF2 mRNA with predominance of the P3 transcript (Li et al, 1998a). Thus, the high IGF2 mRNA expression of many HB tumours with ROI may mimic the upregulation of IGF2 expression in embryonic liver tissues, from which HB may arise.

In this study of 54 HB tumours, we found LOH in 12 (22.2%), LOI in 9 (16.7%) and ROI in 33 (61.1%). Hepatoblastoma tumours can be classified into those with LOH and those with ROH, and tumours with ROH can be further classified into those with LOI and those with ROI. For data comparison, the frequencies of LOH and LOI in the earlier and present series of HB tumours are shown in Tables 2 and 3, respectively (Davies, 1993; Montagna et al, 1994; Li et al, 1995; Rainier et al, 1995; Fukuzawa et al, 1999; Gray et al, 2000; Hartmann et al, 2000; Ross et al, 2000; Albrecht et al, 2004; Suzuki et al, 2008). Both frequencies of LOH and LOI were similar between the earlier and present series of HB tumours. When we compared the frequencies of LOH and LOI in HB and WT, the frequencies of LOH and LOI are lower in HB tumours than in WT tumours (Table 4). The present and earlier studies showed that levels of IGF2 mRNA are higher in normal liver tissues than in normal kidney tissues, and in foetal liver tissues than in foetal kidney tissues, but showed similarly high levels in both WTs and HBs (part of the data not shown) (Hedborg et al, 1994; Haruta
et al., 2008), indicating that embryonal kidney tissues might be more susceptible to IGF2 stimulation than embryonal liver tissues. These findings might be related to higher incidences of UPD or LOI in WT than in HB.

The IGF2 gene has four promoter regions and each promoter can initiate transcription producing a distinct IGF2 transcript with different 5’-untranslated regions with a common translated region in the 3’-side (Li et al., 1998a). The IGF2 gene is transcriptionally regulated in a development-dependent and tissue-specific manner. In the foetal liver, promoters P2, P3, and P4 are active and expressed monoallelically; P3 is the most active promoter and P1 is inactive. However, in the adult liver, P1 becomes dominant and is biallelically expressed, and P2, P3 and P4 activities are decreased or lost (Li et al., 1998a). In foetal liver tissues, P3 promoter methylation is inversely correlated with the P3 transcript expression. The inverse correlation between P3 promoter methylation and P3 transcript expression was reported earlier in seven HB tumours (Li et al., 1998b). This study confirmed the upregulation of P2, P3 and P4 transcripts and downregulation of P1 transcript, and the inverse correlation between P3 promoter methylation and P3 transcript expression in the majority of 20 HB tumours. Although P2, P3 and P4 transcripts were all correlated to the total amount of IGF2 mRNAs, the earlier and present studies showed that the P3 transcript was most abundant and seemed to play a major role in the tumorigenesis of HB (Li et al., 1998b). Increased IGF2 expression with the predominant P3 transcript was reported earlier in WTs with LOI or ROI (Vu and Hoffman, 1999). This study also showed that HB tumours with hypermethylated H19 DMR tended to have an unmethylated P3 promoter, indicating that

![Diagram of the IGF2 P3 promoter region.](image)

**Figure 4** (A) Diagram of the IGF2 P3 promoter region. Individual CpG dinucleotides located upstream of exon 6 (from −1068 to −702 bp) are represented by circles. Horizontal arrows indicate locations of PCR primers used for MSP and bisulphite sequencing. (B) Examples of the promoter methylation status using methylation-specific PCR. Polymerase chain reaction products of methylated or unmethylated P3 promoters from HB tumours are shown. Numbers above horizontal bars indicate the tumour number: M, methylated promoter; U, unmethylated promoter. (C) Bisulphite sequencing analysis of the methylation status of P3 promoter in HuH6 and one tumour (no. 1), which displayed complete methylation and complete unmethylation, respectively. Open and closed circles indicate unmethylated and methylated CpG dinucleotides, respectively. (D) Levels of P3 transcripts in tumours with partially methylated P3 promoter and tumours with unmethylated P3 promoter.

![Representative data of RT–PCR analysis of PLAG1 mRNA.](image)

**Figure 5** Representative data of RT–PCR analysis of PLAG1 mRNA. Numbers below lanes indicate the tumour number.

| References          | Total number | LOH of IGF2a | No-LOH of IGF2 | %   |
|---------------------|--------------|--------------|----------------|-----|
| Hepatoblastoma      |              |              |                |     |
| Montagna et al. (1994) | 13           | 3            | 10             | 23.1|
| Fukuzawa et al. (1999) | 7            | 2            | 5              | 28.6|
| Gray et al. (2000)  | 10           | 2            | 8              | 20.0|
| Hartmann et al. (2000) | 24           | 6            | 18             | 25.0|
| Albrecht et al. (2004) | 56           | 13           | 43             | 23.2|
| Suzuki et al. (2008) | 17           | 4            | 13             | 23.5|
| Total number        | 127          | 30           | 97             | 23.6|
| Present study       | 54           | 12           | 42             | 22.2|
| Wilms’ tumour       |              |              |                |     |
| Grundy et al. (1996) | 260          | 93           | 167            | 35.8|
| Yuan et al. (2005)  | 62           | 26           | 36             | 41.9|

*Tumours with LOH of 11p15, but no informative IGF2 locus are included.

Table 2 Incidences of LOH of IGF2 in previous and present series of hepatoblastoma and Wilms’ tumours

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the paternal P3 promoter or the maternal P3 promoter upstream of the aberrantly methylated H19 DMR is likely to be unmethylated, probably because of stimulation of the enhancer signal. In contrast, the significance of unmethylation in the P3 promoter found in 4 (nos. 23, 24, 26 and 27) of 13 HB tumours with normally methylated H19 DMR (ROI) remains unresolved.

PLAG1 located in 8q11 encodes a developmentally regulated transcription factor, and positively regulates IGF2. The P3 promoter region of IGF2 contains PLAG1 consensus-binding sites, and PLAG1 transactivates the transcription from embryonic IGF2 promoter P3 in HB cell lines, HuH6 and HepG2 (Zatkova et al., 2004). PLAG1 mRNA was highly expressed in most HB tumours compared with normal liver tissues. In this study, HB tumours with PLAG1 mRNA expression showed and tended to show higher levels of P4 and P3 transcripts, respectively. Thus, the correlation of PLAG1 mRNA expression with increased levels of P3 transcripts reported by Zatkova et al. (2004) may be confirmed; furthermore, the correlation of PLAG1 mRNA expression with increased levels of P4 transcripts was also suggested.

WTs can be classified at least into two groups; one has intralobar nephrogenic rest that is associated with WT1 abnormality and the other has perilobar nephrogenic rest associated with IGF2-LOI (Ravenel et al., 2001). CTNNB1 mutation is frequently found in WTs with WT1 abnormality, but rare in WT tumours without WT1 abnormality (Maiti et al., 2000). These findings suggest that WTs with no WT1 abnormality may include a substantial number of WTs with IGF2-LOI, and that CTNNB1 mutation and IGF2-LOI may be mutually exclusive in WT and also in HB. However, there were no differences in the incidences of CTNNB1 mutation between HBs with IGF2-LOI and those with IGF2-ROI, or those with IGF2-LOH. We have recently reported a paper describing the occurrence of duplication of paternal IGF2 or IGF2-LOI in half of WTs with WT1 abnormalities (Haruta et al., 2008). Of two WTs with IGF2-LOI and WT1 abnormality reported in that paper, one had CTNNB1 mutation and the other had not. These findings suggest that CTNNB1 mutation and IGF2-LOI may not be mutually exclusive in either WT or HB.

The IGF signalling pathway is activated in various cancers, and monoclonal antibodies targeting IGF1R have been recently developed; IGF1R is a transmembrane tyrosine kinase receptor, and both IGF1 and IGF2 are ligands for IGF1R (Foulstone et al., 2005). Early clinical trials using anti-IGF1R monoclonal antibodies showed promising results in refractory Ewing’s sarcomas and rhabdomyosarcomas (Ryan and Goss, 2008). Because 20–30% of HB tumours do not respond to the current chemotherapy consisting of cisplatin and adriamycin (Perilongo et al., 2000; Fuchs et al., 2002), and the great majority of HB tumours overexpresses IGF2, as shown in the present and earlier studies, HB may be the next target tumour for antibody therapy.

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Table 3 Incidences of LOI of IGF2 in previous and present series of hepatoblastomas and Wilms’ tumours

| References | Total number | LOI of IGF2 | ROI of IGF2 |
|------------|--------------|-------------|-------------|
| Hepatoblastoma | | | |
| Davies (1993) | 3 | 0 | 3 |
| Montagna et al. (1994) | 5 | 1 | 4 |
| Ranier et al. (1995) | 5 | 1 | 4 |
| Li et al. (1995) | 3 | 1 | 2 |
| Fukuzawa et al. (1999) | 4 | 1 | 3 |
| Ross et al. (2000) | 13 | 3 | 10 |
| Hartmann et al. (2000) | 5 | 3 | 2 |
| Total number | 38 | 10 | 28 |
| Present study | 42 | 9 | 33 |

| References | Total number | LOI of IGF2 | ROI of IGF2 |
|------------|--------------|-------------|-------------|
| Wilms’ tumour | | | |
| Ravenel et al. (2001) | 36 | 15 | 21 |
| Yuan et al. (2005) | 29 | 22 | 7 |

Table 4 Incidences of LOH, LOI and ROI of IGF2 in hepatoblastoma and Wilms’ tumours

| References | Total number | LOH of IGF2 | LOI of IGF2 | ROI of IGF2 |
|------------|--------------|-------------|-------------|-------------|
| Hepatoblastoma | | | |
| Present study | 54 | 12 (22.2%) | 9 (16.7%) | 33 (61.1%) |

| References | Total number | LOH of IGF2 | LOI of IGF2 | ROI of IGF2 |
|------------|--------------|-------------|-------------|-------------|
| Wilms’ tumour | | | |
| Fukuzawa et al. (2004) | 51 | 17 (41.5%) | 13 (31.7%) | 11 (26.8%) |
| Yuan et al. (2005) | 58 | 29 (50.0%) | 22 (37.9%) | 7 (12.1%) |
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