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High frequency of β-catenin mutations in hepatoblastoma

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Abstract Hepatoblastoma (HB) is an embryonic neoplasm representing the most frequent malignant liver tumour in childhood. Its tumourigenesis at the molecular level is still poorly understood, and candidate genes are yet to be identified. According to recent reports describing β-catenin mutations (BCM) at hot-spot regions involving exon 3 in several types of malignancies including HB, we investigated BCM in 16 HBs classified into different histological types. One tumour had been previously confirmed to harbour adenomatous polyposis coli (APC) mutations that exhibit a similar oncogenic effect to that of BCM. Mutations in both exon 3 and its flanking region of the β-catenin gene were investigated and determined. Twelve tumours (75%) revealed pathogenic BCM, including 5 with missense mutations at codons 32, 34, or 37 and 7 with interstitial deletions that partially or totally affected exon 3. All 7 deletions were in-frame deletions without frameshift. A single nucleotide change at codon 31 regarded as non-pathogenic polymorphism was detected in the tumour possessing APC mutations. Therefore, a total of 13 tumours (81%) were compromised by an enhanced β-catenin-mediated transcription pathway. Mutations were observed in every histological type of HB. The very high frequency without correlation to histological type indicates that BCM are crucial events in the tumourigenesis of HB.

Keywords 8-Catenin • Hepatoblastoma • Adenomatous polyposis coli • Mutation • Deletion

Introduction

Hepatoblastomas (HBs) are embryonic tumours affecting young children and represent the most frequent malignant liver tumours in childhood. Although several instances of molecular evidence for other embryonic tumours, such as RB mutations in retinoblastoma and alterations of the WT1 gene in Wilms’ tumour, have been provided, tumourigenesis of HB at the molecular level has not been well understood. Because some HBs are associated with the Beckwith-Wiedemann syndrome [21] and the genetic locus of the syndrome has been localised at 11p15.5 [14], this region has been investigated, and loss of heterozygosity has been described in HBs [1,6]. However, candidate genes have not been clarified, and other genetic factors associated with tumourigenesis are yet to be identified.

Beta-catenin, a protein associated with E-cadherin involved in cell–cell adhesion, has been found to have an additional role as a member of the Wnt signal transduction pathway [3,16,23]. Through interaction with transcription factors of the T-cell factor family, β-catenin serves as an activator of transcription [3,16,23]. Increased levels of β-catenin lead to enhanced activation of downstream transcriptional targets such as the c-myc oncogene [7] and possess oncogenic properties. The intracellular p-catenin level is tightly downregulated by its degradation, which requires phosphorylation within N-terminal residues including codons 32-45 of β-catenin through the action of glycogen synthase kinase-3β (GSK-3β) and wild-type adenomatous polyposis coli (APC) activity [3,16,23]. Therefore, both inactivating mutations of APC and β-catenin mutations (BCM) involving the GSK-3β phosphorylation sites prevent β-catenin degradation and result in an increase in intracellular β-catenin levels relating to tumourigenesis [3,16,23].

Biallelic inactivation of APC was previously demonstrated in a HB case [12], and BCM have recently been reported in several types of malignancies [8,15,22].
in 16 HBs, including 1 case with APC mutations, in order to determine the role of the β-catenin gene in HB.

**Materials and methods**

Sixteen cases of HB were analysed (Table 1), 8 in males and 8 in females. The ages of the patients ranged from 4 to 120 months, averaging 22.6 months. The disease status of 16 tumours classified by the staging system adopted by the Japan Society of Pediatric Surgeons\[17\] was stage I in 3, stage II in 8, stage III in 3, and stage IV in 2 tumours. Both stage IV cases had multiple metastatic tumours in both lungs. Histologically\[5,10\], 10 tumours were epithelial and the other 6 were mixed-type (epithelial and mesenchymal components). Ten epithelial tumours were subclassified into a fetal pattern in 5 cases, including 2 pure fetal tumours, an embryonal pattern in 4, and a macrotrabecular pattern in 1. Mixed-type tumours included 1 with teratoid features. In 1 tumour (HB6), biallelic inactivating mutations of the APC gene had been previously confirmed\[12\].

Tumour specimens were collected at tumour resection and/or biopsy performed at Osaka University Hospital or its affiliated hospitals. A portion of each specimen was used for histopathological examination and another part was immediately frozen in liquid nitrogen and stored at -80°C until DNA/RNA extraction was performed.

Genomic DNA from frozen specimens was prepared by standard proteinase K digestion and the phenol/chloroform extraction method. Total cellular RNA from later-indicated samples was prepared using a commercially supplied kit (RNAeasy TOTAL PURE, Bioline). RNA was applied to cDNA synthesis according to the recommended protocol (1-strand cDNA Synthesis Kit, Clontech), which used the oligo (dT) primer. All possible precautions were taken to avoid RNase contamination during the procedure.

To detect small gene alterations such as point mutations, the polymerase chain reaction–single-strand conformational polymorphism (PCR-SSCP) method was used. From genomic DNA of each sample, a 228 bp PCR fragment of exon 3 of the 8-catenin gene was amplified using the following primer pair (BCAT-1/BCAT-2): 5'-GATTTGATGGAGTTGGACATGG-3' and 5'-TGTTCTTGAGTGAAGGACTGAG-3'. This primer pair produces a PCR fragment of 228 bp encompassing the sequence of the most activating change of this case does not cause a replacement of amino acid, and is regarded as non-pathogenic polymorphism; inactivating mutations of the APC gene has been previously confirmed in this case\[12\].

To detect larger interstitial deletions, genomic DNA was also amplified using another primer pair (BCAT-3/BCAT-4): 5'-AAAATCCAGCGTGCAATGG-3' and 5'-TGTGCGAGTTCTGCCATCATC-3'. This primer pair produces a PCR fragment of 1153 bp encompassing exon 3, the adjacent introns, and parts of exons 2 and 4 of the β-catenin gene\[11,18\]. If an interstitial deletion occurred within this region, a smaller-sized PCR product than normal would be observed. The PCR conditions in this case were similar to those described for BCAT-1/BCAT-2 except for the cycle numbers (37 PCR cycles) and some buffer components (1.0 mM MgCl2 and 1.0% DMSO). PCR products were visualised on 1.5% agarose gel with ethidium bromide to detect samples with size-altered products.

In tumour samples suspected to have interstitial deletions of the p-catenin gene, consequent production of corresponding mRNA of altered size is expected. To confirm this, PCR amplification using the same primer pair and CDNA as a template, which normally yielded 467 bp fragments, was further performed for these tumours, and product sizes were examined on agarose gel.

For samples that showed abnormal band patterns on SSCP analysis and those that yielded PCR products of altered size, using PCR products as a template cycle sequencing (ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems) was performed. Each sequencing product was run on an ABI sequencer Genetic Analyzer 310 (Applied Biosystems), and the nature of the alterations was determined.

**Table 1** Clinical data and mutational status of the β-catenin gene in 16 hepatoblastomas (mixed mixed epithelial and mesenchymal type. Epith epithelial type)

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex/age (months)</th>
<th>Stage</th>
<th>Histology</th>
<th>Nucleotide change</th>
<th>Affected codon</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB1</td>
<td>M/24</td>
<td>II</td>
<td>Mixed</td>
<td>A 330 bp</td>
<td>5-80</td>
<td>Alive</td>
</tr>
<tr>
<td>HB2</td>
<td>F/19</td>
<td>I</td>
<td>Epith (pure fetal)</td>
<td>No</td>
<td>Alive</td>
<td></td>
</tr>
<tr>
<td>HB3</td>
<td>F/4</td>
<td>III</td>
<td>Mixed (teratoid)</td>
<td>GGA→AGA</td>
<td>Gly34Arg</td>
<td>Alive</td>
</tr>
<tr>
<td>HB4</td>
<td>F/20</td>
<td>II</td>
<td>Epith (embryonal)</td>
<td>A 690 bp</td>
<td>5-80</td>
<td>Alive</td>
</tr>
<tr>
<td>HB5</td>
<td>M/19</td>
<td>II</td>
<td>Epith (fetal)</td>
<td>A 24 bp</td>
<td>25-32</td>
<td>Alive</td>
</tr>
<tr>
<td>HB6</td>
<td>F/26</td>
<td>IV</td>
<td>Epith (embryonal)</td>
<td>CTG→TTG</td>
<td>Leu31Leu</td>
<td>Dead of disease</td>
</tr>
<tr>
<td>HB7</td>
<td>M/8</td>
<td>I</td>
<td>Epith (fetal)</td>
<td>A 138 bp</td>
<td>5-40</td>
<td>Dead of disease</td>
</tr>
<tr>
<td>HB8</td>
<td>M/18</td>
<td>II</td>
<td>Mixed</td>
<td>No</td>
<td>Alive</td>
<td></td>
</tr>
<tr>
<td>HB9</td>
<td>M/8</td>
<td>II</td>
<td>Mixed</td>
<td>TCT→TTT</td>
<td>Ser37Phe</td>
<td>Dead of disease</td>
</tr>
<tr>
<td>HB11</td>
<td>M/34</td>
<td>IV</td>
<td>Epith</td>
<td>TCT→TTT</td>
<td>Ser37Phe</td>
<td>Dead of disease</td>
</tr>
</tbody>
</table>

\* A, deletion
\* A, nucleotide change of this case does not cause a replacement of amino acid, and is regarded as non-pathogenic polymorphism; inactivating mutations of the APC gene has been previously confirmed in this case\[12\].
Fig. 1 SSCP analysis of exon 3 of the β-catenin gene in 16 hepatoblastomas. Six samples showed abnormal patterns with band shifts.

Fig. 2 Sequence results confirming mutations. Results of four samples. In HB12 and HB19, the same point mutation at codon 34 (GGA→GAA) was confirmed, and in HB11 a point mutation at codon 37 (TCT→TTT) was detected. In HB3, which initially showed no abnormal findings in both SSCP and deletion analyses, a point mutation at codon 34 (GGA→AGA) was confirmed by subsequently performed sequence analysis.

Fig. 3 Deletion analysis using PCR fragments amplified from genomic DNA. In 6 samples (HB1, HB4, HB8, HB14, HB20 and HB21), PCR fragments with reduced size were identified besides normal product of 1115 bp.

Results

On SSCP analysis, 6 of the 16 HB samples showed abnormal band patterns (Fig. 1). Sequence analyses of these samples confirmed point mutations at codons 31, 32, 34, or 37 in 5 cases and an interstitial deletion of 24 bp in 1 (Fig. 2). Subsequent sequencing of the 4 samples in which no abnormal findings had been observed on both SSCP and deletion analyses detected another point mutation at codon 34 in 1 case (HB3) (Fig. 2). Details of the mutations confirmed in 7 cases (Table I) were as follows: Asp32Ala (GAC→GCC) in 1 case (HB18), Gly34Arg (GGA→AGA) in 1 (HB3), Gly34Glu (GGA→GAA) in 2 (HB12, 19), Ser37Phe (TCT→TTT) in 1 (HB11), a single nucleotide change at codon 31 (Leu: CTG→TTG) without replacement of an amino acid in 1 (HB11), and an in-frame interstitial deletion of 24 bp at codons 25-32 in 1 (HB3).
Using the primer pair BCAT3/BCAT4 and genomic DNA, PCR products with reduced size were identified in 6 other specimens (Fig. 3). PCR products derived from corresponding cDNAs also revealed a reduction in size (Fig. 4), and these results provided additional evidence of interstitial deletions. The length of interstitial deletions confirmed by sequencing varied from 138 to 1040 bp, and exon 3 was partially or totally deleted in these 6 cases (HB1, HB4, HB6, HB14, HB20, HB21). All of these deletions were in-frame deletions without frameshift.

Clinical data and all β-catenin gene alterations detected are summarised in Table 1.

Discussion

In this study, 16 HBs were investigated for BCM in both exon 3 and its flanking region by a combination of several methods. Firstly, using PCR-SSCP which is an effective method for detecting small mutations, we found 5 point mutations and a small deletion of 24 bp, all of which were confined to exon 3. To detect larger interstitial deletions involving exon 3, size reductions of a 1115 bp PCR fragment amplified from genomic DNA were investigated. Six additional gene alterations were identified. In these 6 cases, PCR fragments derived from corresponding cDNA also revealed size reductions which provided further evidence of interstitial deletions. Finally, performing sequence analysis, another point mutation was confirmed in 1 of the 4 samples in which an abnormality was not previously observed on both SSCP and deletion analyses.

Therefore, a total of 13 β-catenin gene alterations were confirmed in our 16 HBs. Of these, 12 gene alterations (12/16, 75%) that cause amino-acid replacements at codons 32, 34, or 37 in 5 cases and partial or total deletions of exon 3 are considered to be associated with the development of HB. All these alterations involve essential residues for phosphorylation by GSK-3β and are considered to result in stabilisation of mutated p-catenin protein [3, 16, 23]. Importantly, because these are either point mutations or in-frame deletions without frameshift, the structure and function of the proteins, except at mutated sites, are preserved in these cases. Therefore, the accumulated p-catenin in tumour cells in these 12 cases is considered to be transcriptionally active and to present oncogenic activity.

In the remaining gene alteration of a single nucleotide change at codon 31 (CTG→TTG), no amino-acid replacement was detected. This nucleotide change should be regarded as a non-pathogenic polymorphism. In the tumour with this change (HB6), however, inactivating mutations of the APC gene had previously been demonstrated [12]. Because inactivation of APC also leads to stabilisation of p-catenin and exhibits a similar oncogenic effect [3, 16, 23], a total of 13 HBs in our series (13/16, 81%) were compromised by enhancement of β-catenin-related transcriptional activation.

To date, several other cytogenetic and genetic events associated with the pathogenesis of HB have been reported: trisomy of chromosomes 2 [19], 20 [19] and 18 [20] and loss of heterozygosity at the 11p15.5 locus [1, 6]. The latter locus is suggested to include genes responsible for Beckwith-Wiedemann syndrome [14], and embryonic malignancies including HB are often encountered in patients with this syndrome [21]. These cytogenetic and genetic events account for only a subset of HBs, however, and specific gene mutations, except APC and BCM, have not yet been demonstrated. Even in the p53 gene, whose mutations have been most widely detected in a variety of malignancies, gene abnormalities have not been well confirmed [10, 13].

Compared to these previous findings, BCM were observed very frequently (75%) in our series. Similarly, Jeng et al. [9] found BCM in 8 of 9 HB cases (89%) in their study, and Wei et al. [24] also reported mutations in 12 or 18 tumors (67%). Taken together, this accumulating evidence suggests that alterations of the β-catenin gene are common genetic events in HB.

Our HB series consisted of several different histological types, i.e., a fetal pattern in 5 including 2 pure fetal types, an embryonal pattern in 4, a macrotrabecular pattern in 1, and 6 mixed-type tumours including 1 teratoid tumour. Mutations were found in every histological type, and we could not identify any correlation between the occurrence of mutations and specific histological types. These findings may indicate that BCM can occur in pluripotent hepatic blastematal Cells, which subsequently differentiate into a wide spectrum of tumourous cell types.

In summary, the detection of BCM at a very high frequency in HB without correlation to histological type strongly indicates that enhancement of β-catenin-mediated transcriptional activation may be an important event in the development of HB, and these mutational events may occur at an early stage of tumourigenesis in this malignancy.
References