β-Catenin (Ctnnb1) Gene Mutations in Diethylnitrosamine (DEN)-induced Liver Tumors in Male F344 Rats

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Alterations in multiple phosphorylation sites on exon 3 of the β-catenin gene have recently been implicated in hepatocarcinogenesis in humans as well as mice. To identify genetic alterations which could be involved in the chemical-induced hepatocarcinogenesis of rats, we analyzed the status of the sites in the β-catenin gene (Ctnnb1) of liver neoplasms induced by diethylnitrosamine (DEN) in male F344 rats, using the polymerase chain reaction-single strand conformation polymorphism method. In the present investigation, we examined 35 hepatocellular neoplasms (28 adenomas and 7 carcinomas) for the expression of mutations in the region of the β-catenin gene. Point mutation at codon 32, 35, 37 or 41, which has been reported in human and mouse liver cell carcinomas and/or other cancers, was recognized in eleven (31%) out of 35 lesions (8 adenomas and 3 carcinomas).

Our results indicate that Ctnnb1 mutations may contribute to hepatocarcinogenesis in rats. Our finding that Ctnnb1 mutation was present in adenomas as well as carcinomas also suggests that the mutation is a relatively early event in DEN-induced hepatocarcinogenesis in rats.

Key words: β-Catenin — Mutation — Hepatocarcinogenesis — Rat — Diethylnitrosamine

β-Catenin, which was originally discovered as a cadherin-binding protein, has recently been proved to function as a transcriptional activator when complexed with members of the T cell factor (Tcf) family of DNA binding proteins.1, 2 It is known that hTCF is expressed in normal and neoplastic colorectal epithelium, and β-catenin-Tcf complexes affect gene expression.3 These complexes may play important roles in cell proliferation and/or apoptosis.1, 4–6 Activation of the β-catenin-Tcf pathway is considered to depend mainly on free β-catenin levels. It is also known that β-catenin levels are regulated by degradation of this protein through the ubiquitin-proteasome pathway,7, 8 and intact adenomatous polyposis coli (APC) cooperates with glycogen synthase kinase-3β (GSK-3β) to regulate this degradation via multiple phosphorylation sites on exon 3 of the β-catenin gene (CTNNB1).9 Meanwhile, APC mutations are known to repress β-catenin degradation and to induce activation of the β-catenin-Tcf pathway.9 Mutations in the β-catenin gene that alter functionally significant phosphorylation sites on exon 3, have been shown to activate the β-catenin-Tcf pathway and to contribute to the development of human colon cancers.9 Furthermore, mutations in these sites have been demonstrated in various types of neoplasms, including human medulloblastomas,10 endometrioid ovarian carcinomas,11 and prostate cancers,12 suggesting that CTNNB1 may act as an oncogene for the development of malignant tumors.

A carcinogenesis model with the use of diethylnitrosamine (DEN) is well established for hepatocarcinogenesis.13–15 However, little is known regarding the genetic alterations that occur in tumorigenesis by DEN, although mutations in the connexin 32 gene16 or the mannose 6-phosphate/insulin-like growth factor 2 receptor gene17 have been reported.

Very recently, alterations of multiple phosphorylation sites on exon 3 of the β-catenin gene have been demonstrated in human and mouse hepatocellular carcinomas (HCCs).18, 19 These findings suggest important roles of the gene alterations in hepatocarcinogenesis. Furthermore, such mutation was also confirmed in rat colon tumors induced by azoxymethane (AOM),20 methylazoxymethanol acetate21 and heterocyclic amines.22 In order to determine the possible involvement of such mutations in rat hepatocarcinogenesis, we performed mutational analyses of multiple phosphorylation sites on exon 3 of Ctnnb1 in DEN-induced liver tumors of male F344 rats, using the polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) method.

MATERIALS AND METHODS

Materials The examined tumor materials were obtained from a total of 28 male F344 rats (Shizuoka SLC, Co., Shizuoka), which had received i.p. injections of DEN (100 mg/kg body weight) (Nacalai Tesque Inc., Kyoto) once a week for 3 weeks, at the age of 6 weeks, and had been killed 21 weeks later. The tumors were fixed in 10% buff-
PCR-SSCP analysis  Extracted DNAs were amplified with primers designed to produce a 211-bp product of rat Ctnnb1 corresponding to functionally important phosphorylation sites in CTNNB1. The primers used here were the same as those in previous studies\(^\text{21, 22}\) and were designed to PCR-amplify the regions corresponding to exons 2 and 3 (codons 1–57) of Ctnnb1 including intron 2. IF4 (forward; 5′-GCTGACGTCTCAGGA-3′ and R3 (reverse; 5′-TCCACATCCTTCTTCAGG-3′) were included in the following PCR reaction mixture, contained in a total volume of 50 μl: 20 μM of each primer, 200 μM of each deoxynucleotide triphosphate, 1 unit of Taq polymerase in 1× PCR buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl; 1.5 mM MgCl\(_2\); Pharmacia Biotech, Tokyo), and 50 ng of template DNA. The mixture was heated at 94°C for 5 min and subjected to 30 cycles of denaturation (94°C 45 s), annealing (57°C 45 s) and extension (72°C 2 min) using a thermal cycler (Perkin Elmer Cetus). Five microliters of the PCR products was mixed with the same volume of formamide dye (10 ml blue, 10 ml green, 10 ml xylene cyanol, 10 ml bromophenol blue, 10 μM EDTA). After denaturation at 90°C for 3 min, samples were applied to a 10% polyacrylamide gel with 1% or 10% glycerol. DNAs extracted from adjacent non-neoplastic liver tissues were used as negative controls. Their DNAs were extracted by means of the Pinpoint Slide DNA Isolation System (Zymo Research, Orange, CA) according to the manufacturer’s protocol.

Sequencing analysis  When the pattern of migration was abnormal, the corresponding PCR products were purified, amplified again by PCR using IF4 and R3 primers, and sequenced. Sequencing was performed using an ALF Express DNA sequencer (Pharmacia Biotech) and the sequencing was repeated more than twice, including the use of forward and reverse primers. When the mutated products were underrepresented, bands were purified and cloned into pCR2.1 TA-vector (Invitrogen, San Diego, CA) before sequencing. Mutations were checked by restriction enzyme analyses using EcoRI when the mutated products represent G-to-A transition at codon 32 (GAT to AAT).

RESULTS

Aberrant PCR-fragments of Ctnnb1 in SSCP analysis were detected in eleven (31%) out of 35 liver tumors (Fig. 1). The aberrant fragments were not found in the adjacent non-neoplastic liver tissues. Sequencing analysis revealed the presence of point mutations in the β-catenin gene; five regions had T-to-C transition affecting codon 37 (TCT to CCT). Four mutations were transitions from G to A at codon 32 (GAT to AAT). These mutations were checked by restriction enzyme analyses using EcoRI (data not shown). The remaining two tumors exhibited T-to-C transition at codon 35 (ATC to ACT) affecting codon 41 (ACC to ATC) respectively (Fig. 2). These point mutations were expected to result in amino acid substitutions, that is Ser\(^{37}\)→Pro, Asp\(^{32}\)→Asn, and Thr\(^{41}\)→Ile, Ile\(^{35}\)→Thr respectively. Such mutations were found in 8 of 28 adenomas (29%) and 3 of 7 carcinomas (43%) (Table I). It has been shown that interstitial deletion of the multiple GSK-3β phosphorylation sites is related to human hepatocarcinogenesis.\(^\text{25}\) However, deletion of the sites was not detected by the primers used in the present study.

DISCUSSION

In this study, we found that eleven (31%) out of 35 lesions (8 adenomas and 3 carcinomas) of the liver induced by DEN in male F344 rats had point mutations in the β-catenin gene. These point mutations were detected at codon 32, 35, 37 or 41, sites which are mutated in human and mouse liver cell carcinomas and/or other cancers.\(^\text{10–12, 19, 25–27}\) Five mutations affecting codon 37 and one mutation at codon 41 were expected to result in Ser\(^{37}\)→Pro and Thr\(^{41}\)→Ile substitutions, respectively. Ser\(^{37}\) and Thr\(^{41}\) were within a series of serines or threonines found near the NH\(_2\)-terminus of β-catenin. These residues have been implicated as substrates for GSK-3β and are thought to play a central role in the down-regulation of β-catenin.\(^\text{28, 29}\) Thus, loss of
these phosphorylation sites might inhibit the down-regulation by GSK-3β and lead to activation of the β-catenin-Tcf pathway. In contrast, the remaining five mutations did not occur at serine or threonine residues; four of these mutations result in Asp→Asn substitutions at amino acid 32 and one mutation results in Ile→Thr substitution at amino acid 35. However, in this study, they occurred within the NH₁-terminal six-amino acid region of β-catenin, which is almost identical to a motif in protein IkBα, targeting β-catenin for ubiquitination.⁸, 30) Furthermore, frequent mutation affecting codon 32 has been reported in human and mouse HCCs,¹⁰, 2⁵) and codon 35 mutation has been reported in rat colon tumors induced by chemical carcinogens.²¹, ²²) Thus, mutations affecting codon 32 or codon 35 seem to yield a protein that is refractory to proteosomal degradation, especially in chemical carcinogenesis including hepatocarcinogenesis.

Recently, c-myc has been identified as a target gene of the β-catenin-Tcf pathway.³¹) Among the proto-oncogenes examined by northern blot analysis, c-myc has been reported to be activated in rat HCCs induced by DEN.³², ³³) These findings suggest that activation of the β-catenin-Tcf pathway due to alterations of multiple phosphorylation sites of the β-catenin gene leads to c-myc overexpression and contributes to the hepatocarcinogenesis by DEN, although amplification of c-myc is present in DEN-induced liver tumors.³⁴) In this study, we have shown the presence of Ctnnb1 mutation in adenomas, suggesting that these alterations are a relatively early event in liver carcinogenesis. Furthermore, the levels of c-myc mRNA were increased not only in adenomas, but also in altered hepatic foci, which are regarded as a pre-neoplastic cell population,³⁵) implying that the involvement of the pathway is related to an earlier stage of hepatocarcinogenesis.

Table I. Summary of Ctnnb1 Mutations in DEN-induced Liver Tumors and the Corresponding Amino Acid Substitutions in the β-Catenin Protein

| Codon | 32 | 35 | 37 | 41 |
|-------|----|----|----|----|
| Wild-type Protein (wild-type) | GAT | ATC | TCT | ACC |
| Mutations Adenomas (8/28) | AAT | ACC | CCT | Ser |
| Mutations Carcinomas (3/7) | AAT | CCT | CCT | Thr |

The eleven point mutations found in DEN-induced liver tumors and the corresponding amino acid substitutions in the β-catenin protein. The residues in bold type have been demonstrated to affect down-regulation of β-catenin through GSK-3β phosphorylation in Xenopus embryos.⁵⁹)}

Fig. 2. Sequencing analysis of PCR fragments from Ctnnb1 exon 3 showing the types of mutation in DEN-induced liver tumors. Arrow, the position of the altered nucleotide.
In colon carcinogenesis, mutation in the APC gene or the β-catenin gene, which may activate the β-catenin-Tcf pathwy, is also considered to be involved in the relatively early stage.\(^{30}\) However, the frequency of Ctnnb1 mutations demonstrated in this study (31%) was much lower than that (more than 80%)\(^{20-22}\) in chemically induced colon tumors in rats. In addition, no predisposition to HCCs has been recognized in patients with familial adenomatous polyposis or Min mouse,\(^{36-38}\) carrying a germ-line mutation of the APC genes. These findings indicate that Ctnnb1 mutations in DEN-induced hepatocarcinogenesis may play a different role from the gatekeeper role in colon tumors in rats. In addition, no predisposition to APC colon tumors in rats. In addition, no predisposition to adenomas and carcinomas also implies that such genetic alteration is a relatively early event in the carcinogenesis.

In summary, we examined Ctnnb1 mutation in 35 rat liver tumors induced by DEN and found mutants in 31% of the tumors. These data suggest that Ctnnb1 mutations may be related to hepatocarcinogenesis in rodents. The fact that Ctnnb1 mutations were confirmed in both adenomas and carcinomas also implies that such genetic alteration is a relatively early event in the carcinogenesis.

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