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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*).[3] 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 gene[16] or the mannose 6-phosphate/insulin-like growth factor 2 receptor gene[17] 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 acetate[21] 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-

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ered formalin and embedded in paraffin for histological analysis and DNA extraction. A total of 35 liver tumors were microdissected from 5 μm-thick paraffin sections. Liver tumors were histologically classified into hepatocellular adenomas and hepatocellular carcinomas using criteria defined previously.[23, 24] Twenty-eight tumors were adenomas and the remaining 7 tumors were carcinomas histologically. For negative controls, adjacent non-neoplastic liver tissues were also dissected in the same manner. Their DNAs were extracted by means of the Pinpoint Slide DNA Isolation System (Zymo Research, Orange, CA) according to the manufacturer's protocol.

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[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′-GCTGACGTCGTACTCAG-GCA-3′) and R3 (reverse; 5′-TCCACATCCTCTTCCTCAGG-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 m*M* Tris-HCl, pH 9.0; 50 m*M* KCl; 1.5 m*M* $MgCl_2$; 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 extention (72°C 2 min) using a thermal cycler (Perkin Elmer Cetus). Five microliters of the PCR products was mixed with the same volume of SSCP buffer (0.1% SDS and 10 m*M* EDTA), and this in turn was mixed with 2.5 μl of formamide dye (10 ml formamide, 10 mg xylene cyanol, 10 mg bromophenol blue, 10 m*M* 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. For positive controls, DNAs that contain G-to-A transition at the first position of codon 32 and G-to-A transition at the second position of codon 34 were obtained from AOM-induced colon tumors.

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 *Eco*RI when the mutated products represent G-to-A transition at codon 32 (GAT to AAT).

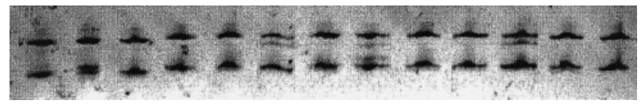

Fig. 1. PCR-SSCP analysis of *Ctnnb1* in DEN-induced liver tumors of the F344 rat. Band shifts were observed in lanes 1, 5, 7, 10, and 11. N, adjacent non-neoplastic liver tissue. Bands with altered mobilities were excised from the gel for sequencing analysis.

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 of 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 *Eco*RI (data not shown). The remaining two tumors exhibited T-to-C transition at codon 35 (ATC to ACC) and C-to-T transition affecting codon 41 (ACC to ATC) respectively (Fig. 2). These point mutations were expected to result in amino acid substitutions, that is $\text{Ser}^{37}\rightarrow\text{Pro}$, $\text{Asp}^{32}\rightarrow\text{Asn}$, and $\text{Thr}^{41}\rightarrow\text{Ile}$, $\text{Ile}^{35}\rightarrow\text{Thr}$ 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.[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.[10–12, 19, 25–27] Five mutations affecting codon 37 and one mutation at codon 41 were expected to result in $\text{Ser}^{37}\rightarrow\text{Pro}$ and $\text{Thr}^{41}\rightarrow\text{Ile}$ 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.[28, 29] Thus, loss of

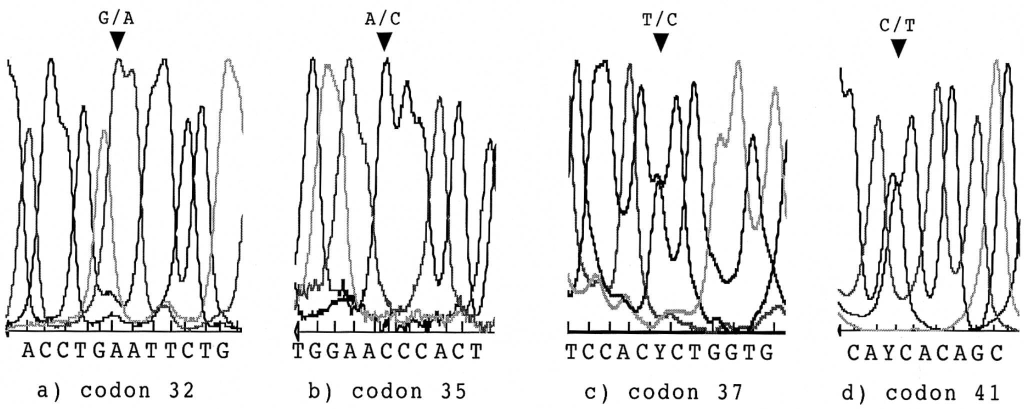

Fig. 2. Sequencing analysis of PCR fragments from *Ctnnb1* exon 3 showing the types of mutation in DEN-induced liver tumors. Arrow, the position of the altered nucleotide.

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		GAT	ATC	TCT	ACC
Protein (wild-type)		Asp	Ile	**Ser**	**Thr**
Mutations	Adenomas	AAT	ACC	CCT	
	(8/28)	AAT		CCT	
		AAT		CCT	
		AAT			
	Carcinomas			CCT	ATC
	(3/7)			CCT	
Substituted residues		Asn	Thr	Pro	Ile

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.[28]

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 NH_2-terminal six-amino acid region of β-catenin, which is almost identical to a motif in protein IκBα, targeting β-catenin for ubiquitination.[8, 30] Furthermore, frequent mutation affecting codon 32 has been reported in human and mouse HCCs,[19, 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.

In colon carcinogenesis, mutation in the APC gene or the β-catenin gene, which may activate the β-catenin-Tcf pathway, is also considered to be involved in the relatively early stage.[36] 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 germline 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 the colon, which is thought to be downstream of APC. Analysis of other pathway(s) involving p53 is, therefore, necessary for understanding the characteristics of DEN-induced hepatocarcinogenesis.

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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