Mutations and Aberrant Transcriptions of *Stk11* (*Lkb1*) Gene in Rat Liver Tumors

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Abstract. *To clarify the involvement of the Stk11/Lkb1 gene in the development of hepatocellular carcinomas (HCCs), its alteration in rat HCCs induced by exogenous and endogenous liver carcinogenesis models was investigated. Materials and Methods: Fifteen HCCs induced by N-nitrosodiethylamine (DEN) and 12 HCCs induced by a choline-deficient L-amino acid-defined (CDAA) diet were obtained. To assess mutations and aberrant transcriptions of the Stk11 gene, polymerase chain reaction (PCR)–single strand conformation polymorphism (SSCP) and reverse transcription (RT)–PCR analyses were performed, respectively. Results: A mutation was detected in only 1 out of 15 HCCs by DEN, but no mutations in 12 HCCs by the CDAA diet. Aberrant transcripts were found in 4 out of 15 HCCs by DEN and in 3 out of 12 HCCs by the CDAA diet. Conclusion: These results suggest that alterations of the Stk11 gene may play a limited role in both exogenous and endogenous rat liver carcinogenesis.*

It is known that germline mutations of serine/threonine kinase 11 (*STK11/LKB1*) gene cause the autosomal dominant Peutz-Jeghers syndrome (PJS) characterized by gastrointestinal hamartomatous polyposis and melanin pigmentation (1-3). The STK11 protein has serine-threonine kinase activity and possesses a nuclear localization signal in the N-terminal noncatalytic region (residues 38-43) and a kinase domain (residues 49-308) (4). STK11 has a role in several biological functions, such as cell proliferation, regulation of cell polarity and p53-mediated apoptosis (4, 5).

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PJS is also associated with an increased risk of developing tumors, including gastrointestinal, lung, liver, breast and pancreatic cancers, suggesting that STK11 may act as a tumor suppressor in PJS (3, 6, 7). By contrast, although *STK11* mutations have been also detected in several sporadic tumors, their frequency is low (8). Therefore, it is unclear whether the *STK11* gene may play an important role in the development of sporadic tumors. In rodents, our recent studies indicated that alterations of the *STK11* gene occurred in pancreatic and lung tumors induced by chemical carcinogens (9, 10).

In the present study, to elucidate an involvement of the *Stk11* gene in liver carcinogenesis, we sought mutations and investigated expression patterns of the *Stk11* gene in hepatocellular carcinomas (HCCs) induced by *N*-nitrosodiethylamine (DEN) and a choline-deficient L-amino acid-defined (CDAA) in rats (11, 12). *N*-Nitrosodiethylamine (DEN) is one of the best-known liver carcinogens in rats. On the other hand, unequivocal HCCs associated with cirrhosis can be induced by prolonged feeding of rats with a choline-deficient L-amino acid-defined (CDAA) diet that does not contain any known carcinogens.

Materials and Methods

Animals and treatment. The method for the production of HCCs using exogenous carcinogens was as described previously (11). A total of 15 male F344 rats, at 6 weeks of age (Japan SLC Inc. Shizuoka, Japan), received DEN (Wako Pure Chemical Co., Ltd., Kyoto, Japan) *i.p.* at a dose of 10 mg/kg body weight, and underwent a partial hepatectomy 4 h later (13). Colchicine (Sigma Chemical Co., St. Louis, MO, USA) was injected *i.p.* at 1 and 3 days after DEN treatment at a dose of 0.5 mg/kg body weight, followed by the procedure described by Cayama *et al.* (14); rats were killed 42 weeks after the beginning of the experiment.

In order to produce HCCs by means of endogenous carcinogens, 15 male F344 rats, at 6 weeks of age (Japan SLC Inc.), were continuously given a CDAA diet (12). Subgroups of 3 and 12 rats were killed at 50 and 75 weeks after the beginning of experiments, respectively.

To obtain normal liver tissues, 6 rats were also maintained free from carcinogen exposure throughout the experimental period.

Tissue preparation. All animals were sacrificed under light ether anesthesia at the end of the experiment. Livers were immediately excised and grossly apparent tumors were dissected from their surrounding tissues. Samples were frozen in liquid nitrogen and stored at –80˚C until analysis. All experiments and procedures carried out on the animals were approved by the Animal Care Committee of Kinki University.

Polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis. Genomic DNAs were extracted from frozen tissue using a DNeasy Tissue Kit (QIAGEN). PCR-SSCP analysis was conducted to look for mutations in the *Stk11* gene as described previously (10). The primers used in this study were designed to amplify exons 1 through 9 of the *Stk11* gene with intron sequences flanking coding exons (10). The PCR products were electrophoresed on polyacrylamide gels using a GeneGel Excel 12.5/24 kit (GE Healthcare UK Ltd., Buckinghamshire, UK) at 8, 15, 18 and 20˚C for 90 min at 15 W, using a GenePhor Electrophoresis Unit (GE Healthcare UK Ltd.). After electrophoresis, the gels were stained with a DNA Silver Staining kit (GE Healthcare UK Ltd.).

Reverse transcription (RT)-PCR amplification of aberrant transcripts of the Stk11 gene. Total RNA was extracted from frozen tissue using ISOGEN (Nippon Gene, Inc. Toyama, Japan) and first-strand cDNA was synthesized from 0.5 μg samples with Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Co. Ltd., Mannheim, Germany).

The cDNAs were assessed for aberrant splicing of the *Stk11* gene by nested RT-PCR analysis using the primers 1F(5'-CGGGTGGGGGAAATTTGAAC-3'), 1R(5'-CAGACAGGCCCGGAATCAGT-3'), 2F(5'-AAGAATTGGCGCTCCCGAAG-3') and 2R(5'-GGACACCTGCCCAAAGATCC-3') designed according to the rat *Stk11* cDNA sequence (10). The PCR products were then separated on 2% NuSieve agarose gels (BMA, Rockland, ME, USA) containing 0.05 μg/ml ethidium bromide.

DNA nucleotide sequencing. Following the PCR-SSCP and the RT-PCR analyses, DNA fragments from abnormally shifted bands and aberrant transcription bands in the gel were extracted and reamplified. The obtained PCR products were directly sequenced using a BigDye terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems Japan, Tokyo, Japan) and an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan).

Results and Discussion

In this study, to clarify any involvement of *Stk11* gene alterations in the development of HCCs, we employed two different liver cancer models of rats. DEN induced HCCs with relatively short latency period, and the CDAA diet induced HCCs with cirrhotic background (11, 12). The fifteen HCCs induced by DEN in 15 rats and the 12 HCCs induced by the CDAA diet in 12 rats were used. All tumors were histologically well-differentiated. Six normal liver tissues, obtained from untreated rats, were used as controls, to eliminate the possibility of contamination with microscopically undetected cancerous lesions. To obtain cirrhotic tissues, three rats fed the CDAA diet for 50 weeks were also used.

Table I. *Results for aberrant transcripts from the Stk11 gene in rat HCC*.

Sample no.	cDNA alteration
DEN3	1014 bp deletion (nt 289-1302)
DEN4	1244 bp deletion (nt 216-1459)
DEN6	1014 bp deletion (nt 289-1302)
DEN8	1014 bp deletion (nt 289-1302)
CDAA2	1014 bp deletion (nt 289-1302)
CDAA3	922 bp deletion (nt 502-1423)
CDAA5	1014 bp deletion (nt 289-1302)

Representative results of the PCR-SSCP and sequencing analyses of *Stk11* gene mutations are shwn in Figure 1 A and B, respectively. A mutation was detected in only 1 out of 15 HCCs induced by DEN (6.7%) in the form of a GGG to GAG (Gly to Glu) transition at codon 270. It is considered that G/C to A/T transition is a common mutation induced by nitrosocompounds (15). Our previous reports indicated that *Ki-ras* mutations were all G/C to A/T transitions at codon 12 in rat lung and hamster pancreatic tumors induced by nitrosocompounds (16, 17). Therefore, it suggests that this mutation was also caused by DEN *per se*. By contrast, no mutations were detected in 12 HCCs induced by the CDAA diet. In several types of cancer, somatic mutations of *STK11* gene have been reported (8). It has been demonstrated that *STK11* mutations are rare event in most sporadic tumor types, while high frequency mutations of *STK11* were detected in lung cancer cell lines (30%), especially in non-small cell cancer (39%) (18). In HCCs, one missense mutation in 80 cases (1.3%) and allelic loss in 6 out of 27 cases (22%) were found (19). In a HCC cell line and an immortalized liver cell, homozygous deletions of the *STK11* gene were detected (20). These results suggest that genetic alterations of the *STK11* gene may play an important role in tumor development or progression of a subset of HCCs. In the present study, we detected only one mutation of the *Stk11* gene in HCCs induced by DEN in rats, but no mutations in HCCs by the CDAA diet. In rodents, we have reported that mutations of the *Stk11* gene were not or only infrequently detected in hamster pancreatic and rat lung adenocarcinomas induced by nitroso-compounds (9, 10). Therefore, it seems that the inactivation of the STK11 gene due to mutation may be a rare event in exogenous and endogenous liver carcinogenesis in rats, as well as in chemical-induced pancreatic and lung tumors. By contrast, it has been reported that *Stk11* (+/–) knockout mice had a high frequency of HCCs (21).

The functional domains of 10 exons of STK11 protein include a central catalytic domain, nuclear localization signal, putative cytoplasmic retention signal, and a prenylation motif (4). A catalytic kinase domain is located at nt 111 to 944 and a regulatory domain at nt 945 to 1301, which contains a

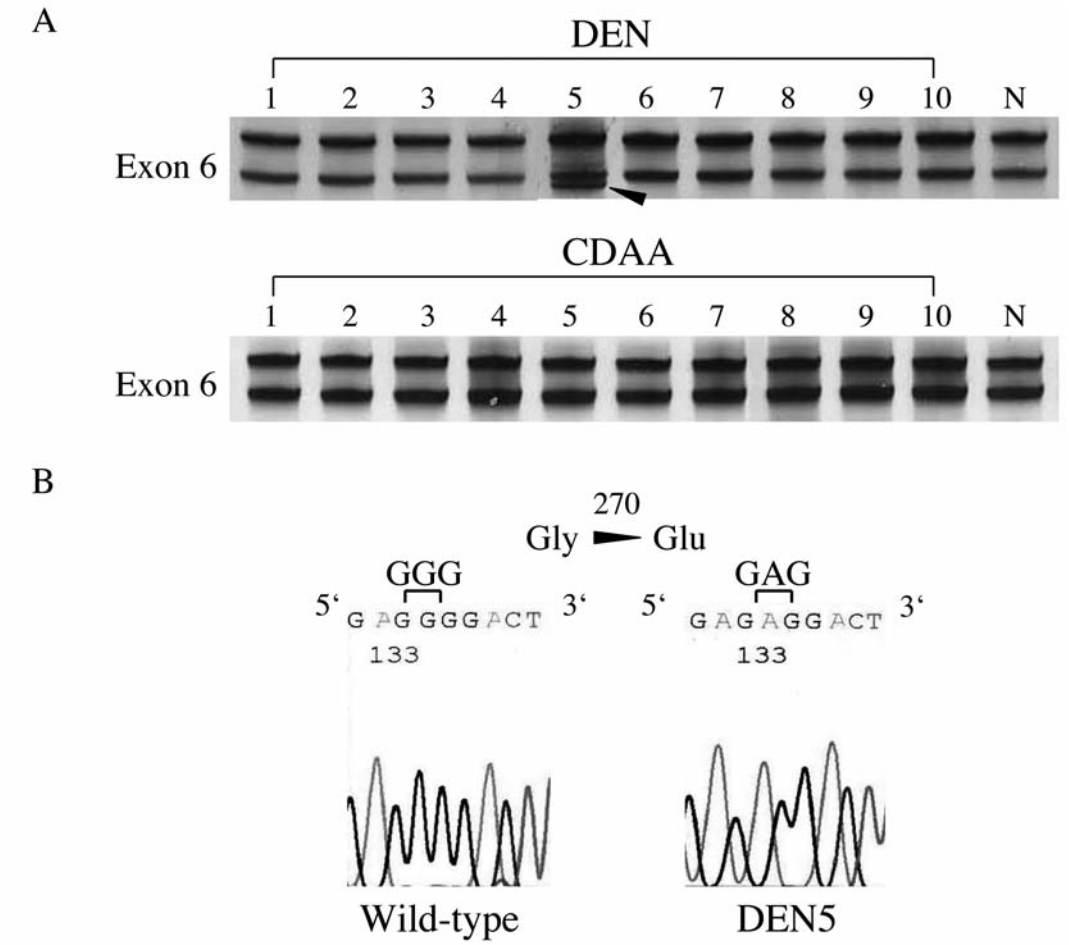

Figure 1. *A: Representative results of PCR-SSCP analysis for the Stk11 mutation in rat HCCs. The arrowhead indicates an abnormal band shift. N: Normal liver tissue. B: The mutation patterns of the Stk11 gene detected by the sequencing analysis. Wild-type: Normal liver tissue.*

conserved phosphorylation consensus sequences for cAMP-dependent protein kinase close to its carboxyl-terminal end (3, 8). In human lung cancer, a large number of *STK11* alterations, including missense mutations, were located in the kinase domain (8). Missense mutations in this region abolish the ability of the *STK11* to autophosphorylate (22, 23). In this study, a mutation detected in DEN-induced HCC is located at codon 270 in this kinase domain.

Altered expressions of the *STK11* gene have been also reported in lung cancer cell lines (18). In seventy lung cancer cell lines, shorter-sized RT-PCR products were found in eight cell lines (11%), and nine cell lines (13%) showed absence of the products, which implied no or decreased expression of *STK11* mRNA (18). In rat lung tumors, we detected aberrant transcripts of the *Stk11* gene in 5 of 15 adenocarcinomas (33.3%) (10). Therefore, we performed RT-PCR analysis to assess altered expression of the *Stk11* gene. Representative results of nested RT-PCR and DNA sequencing analyses are shown in Figure 2. The RT-PCR analysis revealed that whereas only the normal-sized product band was amplified at 1597 bp in all 6 normal liver tissues and 3 cirrhotic tissues in 3 rats fed the CDAA diet for 50 weeks, an additional abnormal-sized product was evident in 4 out of 15 HCCs induced by DEN (26.7% incidence) and 3 out of 12 HCCs induced by the CDAA diet (25.0% incidence). Such abnormal-sized transcripts were seen at three different positions of 341, 581 and 673 bp. Sequencing analysis revealed that these aberrant fragments were due to deletions in the regions of nt 216 to 1459, 289 to 1302 and 502 to 1423, respectively (Table I). Lack of or reduced expression of *Stk11* gene was not detected (data not shown). In human lung cancer cell lines, a lack of or abnormal-sized mRNA expression was due to homozygous deletions in the region of exon 1 and/or a 0.5 kb upstream, or intragenic homozygous deletions (18, 24). In contrast with cell lines, no large deletions of the *STK11* gene were detected in tumor specimens of lung cancer, suggesting

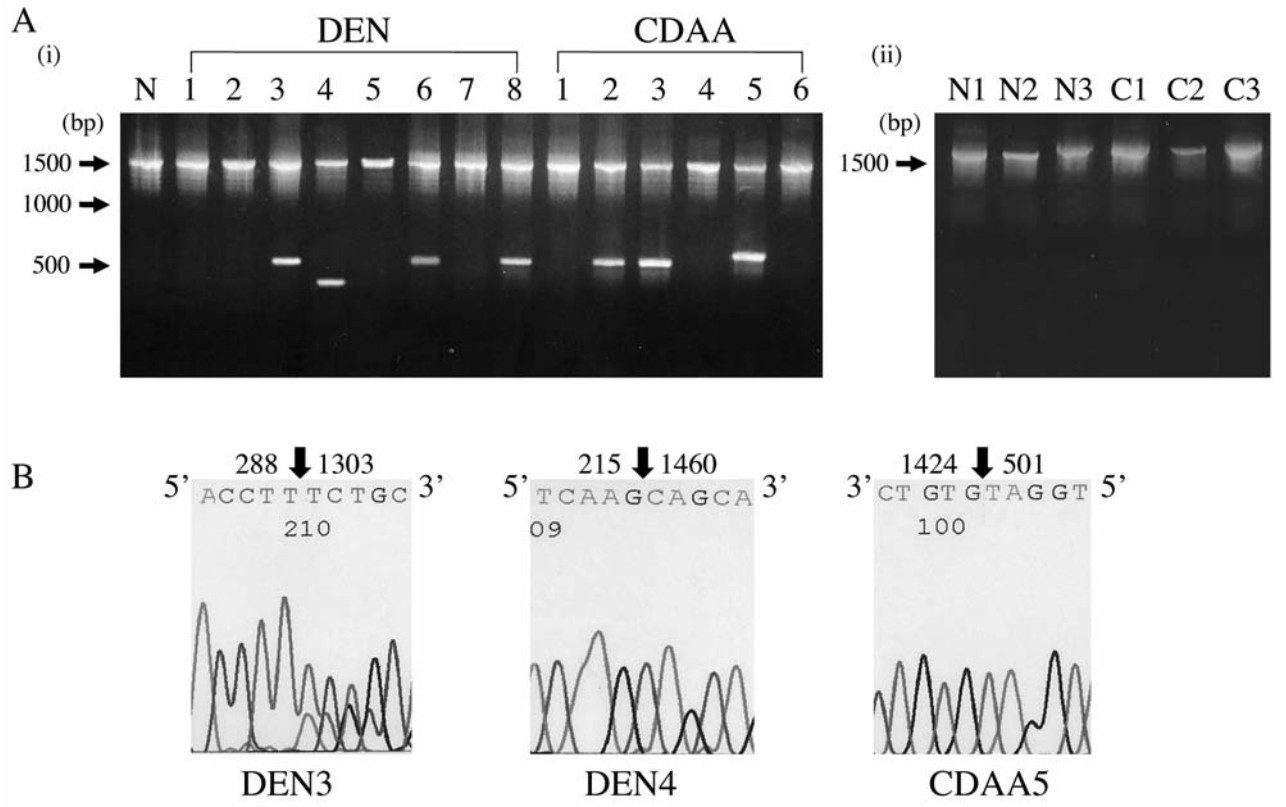

Figure 2. *Aberrant transcription of the Stk11 gene. A: (i) Representative results of nested RT-PCR analysis. N: Normal liver tissue obtained from rat aged 81 wk. (ii) N1: Normal liver tissue obtained from rat aged 81 weeks. N2 and N3: Normal liver tissue obtained from rat aged 48 weeks. C1, C2, C3: Cirrhotic tissues obtained from 3 rats fed the CDAA diet for 50 weeks. B: Patterns of aberrant transcripts detected by DNA sequencing analysis. Arrows indicate the junction between nt 288 and 1303 (DEN3), 215 and 1460 (DEN4), and 501 and 1424 (CDAA5) in the aberrant transcripts.*

that the contamination of noncancerous cells in the tumor specimens masked large deletions (18). In the present study, homozygous deletions were not found in PCR-SSCP analysis, and all abnormal-sized bands displayed wild-type *Stk11* in RT-PCR analysis. Therefore, these results may be due to the contamination of HCC samples with normal cells.

In conclusion, the present study indicated mutations of *Stk11* gene and aberrant transcripts in rat HCCs induced by DEN and the CDAA diet. However, we were unable to find any significant differences in *Stk11* gene alterations between exogenously and endogenously induced liver carcinomas. Therefore, these results suggest that *Stk11* gene alterations may play a limited role in rat liver carcinogenesis.

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