Abstract. To evaluate an involvement of LKB1 gene alteration during pancreatic carcinogenesis, mutations in the LKB1 gene in hamster pancreatic duct adenocarcinomas (PDAs) induced by N-nitrosobis(2-oxopropyl)amine (BOP) were investigated. Female Syrian golden hamsters received 30 mg/kg of BOP followed by repeated exposure to an augmentation pressure regimen consisting of a choline-deficient diet combined with DL-ethionine then L-methionine and a further administration of 20 mg/kg BOP. A total of 10 PDAs obtained 10 weeks after beginning the experiment were examined for mutations using reverse transcription (RT)–polymerase chain reaction (PCR)–single-strand conformation polymorphism (SSCP) analysis. Mutations were detected in 3 out of the 10 PDAs (30.0%). Sequence analysis revealed the identity of these mutations to be a CCC to CCT (Pro to Pro) transition at codon 221, a CCG to CAG (Pro to Gln) transversion at codon 324 and a GAC to GGC (Asp to Gly) transition at codon 343. The LKB1 gene may be involved in the development of PDAs induced by BOP in hamsters.

The LKB1 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) (1). LKB1 has several biological functions, including the regulation of cell polarity, p53-mediated apoptosis and cell proliferation (1, 2). Germline mutations of the LKB1 gene cause the autosomal dominant Peutz-Jeghers syndrome (PJS) characterized by gastrointestinal hemartomatous polyposis and melanin pigmentation (3-5). PJS has also been associated with an increased risk of developing neoplasms, including gastrointestinal, lung, liver, breast and pancreatic carcinomas, suggesting that LKB1 may act as a tumor suppressor in PJS (5-7). Additionally, LKB1 mutations have been detected in a variety of sporadic tumors, but their frequency is low (8). Therefore, the role of LKB1 gene inactivation in the development of tumors has not been clarified.

Pancreatic duct adenocarcinomas (PDAs) have one of the lowest cure rates among human malignancies (9). It is important to understand the molecular mechanisms underlying pancreatic carcinogenesis. However, at present, information on rate-limiting molecular events is exceedingly limited. Experimental models suitable for the investigation of human PDA development have been established in hamsters using the carcinogen N-nitrosobis(2-oxopropyl)amine (BOP) (10) and to facilitate studies of the underlying mechanisms, we previously developed a rapid production approach (11). Indeed, we have reported several genetic changes in this model. For example, a high frequency of Ki-ras mutations was found at early stages, along with p53 mutations, during pancreatic ductal carcinogenesis (12, 13). In addition, we have shown aberrant DNA methylation in tumor suppressor genes, such as p16, E-cadherin, Tslc1 and Rassf1a (14-17).

In the present study, to assess an involvement of the Lkb1 gene in pancreatic carcinogenesis, mutations of the Lkb1 gene in hamster PDAs induced by BOP were investigated.
Materials and Methods

Animals and treatment. A total of 12 female Syrian golden hamsters, weighing approximately 100 g each, were used (Japan SLC Inc., Shizuoka, Japan). PDAs were induced in 10 of the animals according to the rapid production model (11). Briefly, BOP (30 mg/kg body weight) (Nacalai Tesque, Inc., Kyoto, Japan) was administered subcutaneously as the initiation, followed by two cycles of augmentation pressure which consisted of choline-deficient diet administration and ethanoline then methionine and a further BOP (20 mg/kg) injection. To obtain normal control tissues including the liver and pancreas, the remaining 2 animals were untreated and maintained free from carcinogen exposure throughout the experimental period. All the hamsters were sacrificed under light ether anesthesia at 10 weeks after the beginning of the experiment and the pancreas was immediately excised. Macroscopically apparent tumors were dissected from the surrounding tissue and frozen in liquid nitrogen. Portions of the tumors were also fixed in 10% neutrally buffered formalin at 4˚C, routinely processed for embedding in paraffin, sectioned and stained with hematoxylin and eosin for histological examination.

RNA extraction and detection of hamster Lkb1 gene cDNA. Total RNA was prepared from the frozen normal liver tissue using an ISOGEN kit (Nippon Gene, Inc., Toyama, Japan) and first-strand cDNA was synthesized from 0.2 μg aliquots with Ready-To-Go Your-Prime First-Strand Beads (GE Healthcare UK Ltd.). To eliminate possible false positives caused by residual genomic DNA, all the samples were treated with DNase.

Reverse transcription (RT)–PCR–single-strand conformation polymorphism (SSCP) analysis. The total RNA was prepared from the 10 frozen PDA samples and 2 normal pancreases using an ISOGEN kit (Nippon Gene, Inc.), then first-strand cDNA was synthesized from 0.2 μg aliquots with Ready-To-Go Your-Prime First-Strand Beads (GE Healthcare UK Ltd.). To confirm the results, the PCR amplification was repeated using the same DNA nucleotide sequencing.

RT-PCR-SSCP analysis was performed with the primers listed in Table I. All were designated from the hamster Lkb1 cDNA sequence obtained in the above analysis. Briefly, the PCR for the SSCP was performed in 10 μl of reaction mixture containing 1 μM of each primer, 200 μM of each dNTP, 1xPCR buffer (Applied Biosystems Japan Ltd.), 2.5 units of Ampli Taq (Applied Biosystems Japan Ltd.) and 0.5 μl of synthesized cDNA mixture under the following reaction conditions; primary denaturation for 2 min at 95˚C, 36 cycles of denaturation at 95˚C, 15 s annealing at 60-64˚C and 1 min extension at 72˚C, and a final extension for 5 min at 72˚C. The PCR products were diluted with 10 μl of loading solution containing 90% formamide, 20 mM EDTA and 0.05% xylene cyanol and bromophenol blue. Aliquots containing 6 μl of the diluted products were electrophoresed on polyacrylamide gel using a GeneGel Excel 12.5/24 kit (GE Healthcare UK Ltd.) at 5, 10, 15 and 20˚C for 90 min at 15W with a GenePhor Electrophoresis Unit (GE Healthcare UK Ltd.). After electrophoresis, the gels were stained with a DNA Silver Staining kit (GE Healthcare UK Ltd.).

DNA nucleotide sequencing. Following the RT-PCR-SSCP analysis, DNA fragment from the abnormal shift band in the gel was extracted and reamplified. The obtained PCR product was directly sequenced using a BigDye terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems Japan Ltd.) and an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan Ltd.). To confirm the results, the PCR amplification was repeated using the same samples and each PCR product was sequenced with the forward and reverse primers at least twice. The products obtained from PCR amplification with each primer set were also directly sequenced.

Results and Discussion

PDAs developed in all 10 hamsters treated with BOP, and a total of 10 (one from each hamster) were subjected to RT-PCR-SSCP analysis. All the PDAs used in this study were histologically well-differentiated. The ORF of the hamster Lkb1 gene cDNA sequence (GenBank: accession number AB459534) was identified and thus it was possible to designate...
primers for the RT-PCR-SSCP analysis (Table I). The amplified PCR products indicated a clear single band in 1% agarose gel. Homozygous deletion was not found. No changes of Lkb1 gene expression were found in any of the PDAs, compared with the normal pancreatic tissues (data not shown).

Representative results of the RT-PCR-SSCP and sequencing analysis are shown in Figure 1 (A) and (B), respectively. Three out of the 10 PDAs (30% incidence) indicated an abnormal band shift using the primer sets of 3F-3R and 4F-4R. Sequence analysis revealed the mutation to be a CCC to CCT (Pro to Pro) transition at codon 221, a CCG to CAG (Pro to Gln) transversion at codon 324 and a GAC to GGC (Asp to Gly) transition at codon 343. These three mutations indicated two missense mutations and one silent mutation. The two missense mutations were in exon 8. This exon is likely to constitute a regulatory domain, which contains a conserved phosphorylation consensus sequences for cAMP-dependent protein kinase close to its carboxyl-terminal end (5). Normal sized PCR products amplified from 1F-1R and 2F-2R indicated no mutations (data not shown). These results are summarized in Table II.

In several human carcinomas, somatic mutations of the LKB1 gene have been reported (8). Although frequent mutations of LKB1 were detected in primary non-small cell lung carcinomas (39%) and their cell lines (30%), it has been demonstrated that LKB mutations are a rare event in most sporadic tumor types (18). In pancreatic tumors, LKB1 mutations were present in 4 out of 100 primary tumors from xenografts (4%) (19); one exhibited homozygous deletion of LKB1, one non-sense and two frameshift mutations. Loss of heterozygosity existed in LKB1 gene locus in these four cases, while no mutations were found in 11 pancreatic cancer cell lines (19). Elsewhere in the literature, one out of 12 pancreatic tumors (8.3%) showed a C1257T change without amino acid substitution (20). Therefore, it seems that the low frequency of LKB1 mutations in pancreatic tumors may indicate that other components of the LKB1 pathway are preferentially altered in these tumors (8).

It has been considered that G/C to A/T transition is a common mutation induced by nitrilosomocompounds (21). Previously, we reported that Ki-ras mutations were all G/C to A/T transitions at codon 12 in hamster pancreatic and rat lung tumors induced by nitroso compounds (12, 22). In the present study, one out of the three Lkb1 mutations was a G/C to A/T transition, suggesting that this mutation might have been caused by BOP per se. The remaining two mutations were A/T to G/C transition and C/G to A/T transversion. It

Figure 1. Mutation of the Lkb1 gene in hamster PDAs induced by BOP. A, Representative results of RT-PCR-SSCP analysis. The arrowhead indicates an abnormal band shift. N: Normal pancreas; PDAs: pancreatic ductal adenocarcinomas. B, The mutation patterns of the Lkb1 gene detected by the sequencing analysis. wild: Normal pancreatic tissue; PDAs: pancreatic ductal adenocarcinomas.
seems that these mutations might have been attributable to some other factors, such as DNA damage caused by chronic oxidative stress, acting during the pancreatic carcinogenesis induced by BOP.

The frequency of Lkb1 mutations in the hamster PDAs was somewhat higher than that in human cases. It is possible that this discrepancy may be due to a species difference. To address this issue, we are currently investigating Lkb1 mutations in rat lung and liver tumors induced by nitroso compounds. In conclusion, the Lkb1 mutations may play a role in hamster pancreatic carcinogenesis induced by BOP.

Acknowledgements

This study was supported in part by the Foundation for Promotion of Cancer Research in Japan, and a Grant-in-Aid (20591765) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by grants (RK-027) from the Faculty of Science and Engineering, Kinki University.

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