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Short Communication

Infrequent Mutation of Lysophosphatidic Acid Receptor-1 Gene in Hamster Pancreatic Duct Adenocarcinomas and Established Cell Lines

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Abstract: To evaluate the involvement of lysophosphatidic acid receptor-1 (LPA1) gene alteration in pancreatic carcinogenesis, we investigated mutations in the LPA1 gene in hamster pancreatic duct adenocarcinomas (PDAs) and established cell lines. Female Syrian golden hamsters received 30 mg/kg of N-nitrosobis(2-oxopropyl)amine (BOP) followed by repeated exposure to an augmentation pressure regimen consisting of a choline-deficient diet combined with DL-ethionine and then L-methionine and a further administration of 20 mg/kg BOP. A total of 10 PDAs obtained 10 weeks after beginning the experiment and three cell lines established from subcutaneously transplantable PDAs in syngeneic hamsters were examined for mutations using reverse transcription-polymerase chain reaction-single strand conformation polymorphism (RT-PCR-SSCP) analysis. A mutation was detected in only one PDA (1/10, 10%) in the form of a GGA to GTA (Gly to Val) transversion at codon 355, and no mutations were detected in the three cell lines. These results suggest that the LPA1 gene mutation may play roles in a limited fraction of BOP-induced pancreatic duct carcinogenesis in hamsters. (J Toxicol Pathol 2009; **22**: 89–92)

Key words: pancreatic duct adenocaricinoma, LPA1, mutation, hamster, nitrosamine

Pancreatic duct adenocarcinomas (PDAs) have one of the lowest cure rates among human malignancies[1]. It is important to understand the molecular mechanisms underlying pancreatic carcinogenesis. However, at present, very little information about rate-limiting molecular events is available. Experimental models suitable for investigation of human PDA development have been established in hamsters using the carcinogen N-nitrosobis(2-oxopropyl)amine (BOP)[2], and to facilitate studies on the underlying mechanisms, a rapid production approach has previously been developed[3,4]. Indeed, we have reported several genetic and epigenetic changes in this model. For example, Ki-ras mutations are frequently found in the early stages of pancreatic ductal carcinogenesis, but infrequent mutation of Smad4 gene is detected in PDAs[5,6]. In addition, we have provided aberrant DNA methylation in tumor suppressor genes, such as p16, E-cadherin, Tslc1 and Rassf1a[7–10].

Lysophosphatidic acid (LPA) is a bioactive mediator that induces diverse cellular effects, including regulation of cell proliferation, differentiation, transcellular migration, morphogenesis and protection from apoptosis[11–16]. LPA can induce cell proliferation, migration, invasion and production of angiogenic factors in human ovarian cancer cell lines, suggesting that LPA may play an important role in the development of tumor cells[12,13,15–19]. LPA also interacts with at least five G protein-coupled transmembrane receptors, lysophosphatidic acid receptor-1 (LPA1), LPA2, LPA3, LPA4 and LPA5[18–21]. LPA1 is ubiquitously expressed in normal tissues, but the expressions of other LPA receptor subtypes are relatively restricted, suggesting that these receptors may have different biological functions regarding LPA[7,18,19]. Recently, aberrant expressions of LPA1 have been reported in human and rat tumors, demonstrating that

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alteration of LPA1 expression might be important in malignant transformation of tumor cells as well as LPA *per se*[12,13,16,17,22,23]. Moreover, we have reported that loss of LPA1 expression is due to its aberrant DNA methylation in rat tumor cell lines[24].

Recently, we reported frequent mutations of the LPA1 gene in rat hepatocellular carcinomas (HCCs) induced by N-nitrosodiethylamine and a choline-deficient L-amino acid defined diet[25]. In the present study, to assess the involvement of the LPA1 gene in pancreatic carcinogenesis, we investigated mutations of the LPA1 gene in hamster PDAs induced by BOP and three established cell lines.

A total of 12 female Syrian golden hamsters, weighing approximately 100 g each, were used in the present study (Japan SLC Inc., Shizuoka, Japan). PDAs were induced in 10 animals according to the rapid production model[3,4]. Briefly, BOP (30 mg/kg body weight) (Nacalai Tesque, Inc., Kyoto, Japan) was given subcutaneously as the initiation, followed by two cycles of augmentation pressure consisting of choline-deficient diet administration and ethionine-methionine-BOP injection. To obtain normal control tissues, including the pancreas, the remaining 2 animals were untreated and maintained free from carcinogen exposure throughout the experimental period. All hamsters were sacrificed under light ether anesthesia 10 wk after the beginning of the experiment, and their pancreases were immediately excised. Macroscopically apparent nodules were dissected from the surrounding tissue and frozen in liquid nitrogen. Portions of the nodules 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.

The details of establishment of the three cell lines, HPD-1NR, HPD-2NR and HPD-3NR, have been reported previously[26]. Frozen cell lines were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (Flow Laboratories, McLean, VA, USA), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin sulfate.

Total RNA was prepared from frozen normal liver tissue using an ISOGEN kit (Nippon Gene, Inc. Toyama, Japan), and first-strand cDNA was synthesized from 0.5 μg aliquots with Ready-To-Go Your-Prime First-Strand Beads (GE Healthcare UK Ltd., Buckinghamshire, England). To determine the sequences of the open reading frame (ORF) and 5' upstream and 3' downstream regions, PCR amplifications were performed with primer sets based on the rat LPA1 cDNA sequence (NCBI accession number NM_053936) as described previously[8–10]. The amplified products were separated on 1% NuSieve agarose gels (BMA, Rockland, ME, USA) containing 0.05 μg/ml ethidium bromide, extracted and directly sequenced with a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems Japan Ltd., Tokyo, Japan) and an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan Ltd.).

Total RNA was prepared from the 10 frozen PDA samples, 3 cell lines and 2 normal pancreases using an ISOGEN kit (Nippon Gene, Inc.), and then the 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 samples were treated with DNase.

Table 1. Primers Used for RT-PCR-SSCP Analysis

cDNA location	Primers	Annealing temperature (°C)
nt -20 - 315	1F: 5'-TTTCAGACTACAGCACCGTC-3'	60
	1R: 5'-CAGGTAGAAGTAGGCCAACC-3'	
nt 260 - 573	2F: 5'-TGATGGCCAACCTGGCCGCG-3'	62
	2R: 5'-ATCACAGATGCAGTTCCAGC-3'	
nt 533 - 852	3F: 5'-TGGGTGCCATACCCAGCGTG-3'	64
	3R: 5'-GCAGCACACATCCTGCAGTA-3'	
nt 780-1092	4F: 5'-TGCTTGGTGCCTTCATTGTC-3'	58
	4R: 5'-CTAAACCACAGAGTGGTCATT-3'	

RT-PCR-SSCP analysis was performed with the primers listed in Table 1. All were designed from the hamster LPA1 cDNA sequence obtained in the above analysis. Briefly, PCR for SSCP was performed in 10 μl of reaction mixture containing 1 μM of each primer, 200 μM of each dNTP, 1×PCR 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 15 s denaturation at 95°C, 15 s annealing at 58–64°C and 1 min extension at 72°C; and a final extension for 5 min at 72°C. PCR products were diluted with 10 μl of loading solution containing 90% formide, 20 mM EDTA and 0.05% xylene cyanol and bromophenol blue. Aliquots containing 6 μl of 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 15 W 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.).

Following RT-PCR-SSCP analysis, the 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, PCR amplification was repeated using the same samples, and each PCR product was sequenced with the forward and reverse primers at least twice.

Nodules developed in all 10 hamsters treated with BOP. These lesions were evaluated according to the diagnostic criteria described previously[3,4]. All nodules obtained were histologically well-differentiated PDAs. Since there is no genetic information available about the hamster LPA1 gene, we first identified the ORF of the hamster LPA1 gene cDNA sequence (GenBank accession number AB257088). Based

on this sequence, primers for the RT-PCR-SSCP analysis were designed (Table 1). The amplified PCR products with these primer sets indicated a clear single band in 1% agarose gel. Homozygous deletion was not found. No changes of LPA1 gene expression were found any of the PDAs compared with normal pancreatic tissues (data not shown).

Representative results of the RT-PCR-SSCP and sequencing analyses are shown in Fig.1 (A) and (B), respectively. One out of 10 PDAs (10% incidence) produced an abnormal band shift using the primer set of 4F-4R. Sequence analysis revealed the mutation to be a GGA to GTA (Gly to Val) transversion at codon 355. Although this codon is located in the intracellular domain of the carboxyl-terminal end, which is important in activation of several biological signaling pathways[11,27], it is unclear whether this mutation can affect LPA1 function. Normal sized PCR products amplified from 1F-1R, 2F-2R and 3F-3R produced no mutations (data not shown).

In human tumors, aberrant expression levels have been reported for the LPA1 gene[12,16,17,22], whereas there have been no reports of LPA1 mutations. Although the reported expression level of LPA1 has varied, no consistent change between normal and transformed epithelial ovarian cancer cells has been found in ovarian cancer cells[12,16,17]. By contrast, reduced expression of LPA1 gene has been detected in human colorectal cancers, suggesting that reduction of LPA1 expression may occur during malignant transformation[22]. Previously, we reported that the promoter region of the LPA1 gene is highly methylated in rat tumor cell lines, correlating with loss of LPA1 expression[24]. In our recent study, we detected frequent mutations of LPA1 gene in HCCs induced by N-nitrosodiethylamine and a choline deficient L-amino acid defined diet (46.7% and 41.7% incidences, respectively)[25]. Therefore, if LPA1 gene acts as a tumor suppressor gene, mutation of the LPA1 gene may play important roles in inactivating LPA1 during tumorigenesis.

G/C to A/T transition is considered a common mutation induced by ethylating N-nitroso compounds[28]. However, in the present study, one mutation of LPA1 was a G/C to T/A transversion. By contrast, the Ki-ras mutations were all G/C to A/T transitions at codon 12[5]. Therefore, it seems that the Ki-ras mutations were caused by BOP per se and that the LPA1 mutation may have been due to some other factors, such as DNA damage caused by chronic oxidative stress, acting during pancreatic carcinogenesis as a result of BOP.

In conclusion, we found infrequent mutation of the LPA1 gene in hamster PDAs induced by BOP, suggesting that LPA1 gene mutation may play roles in a limited fraction of BOP-induced pancreatic duct carcinogenesis in hamsters. The observed mutation frequency was markedly lower than that of rat liver tumor cases. It seems this discrepancy may be due to organ or species differences. To better understand the involvement of LPA receptors in pancreatic duct carcinogenesis, alterations of other receptors, such as LPA2 and LPA3, should be further studied.

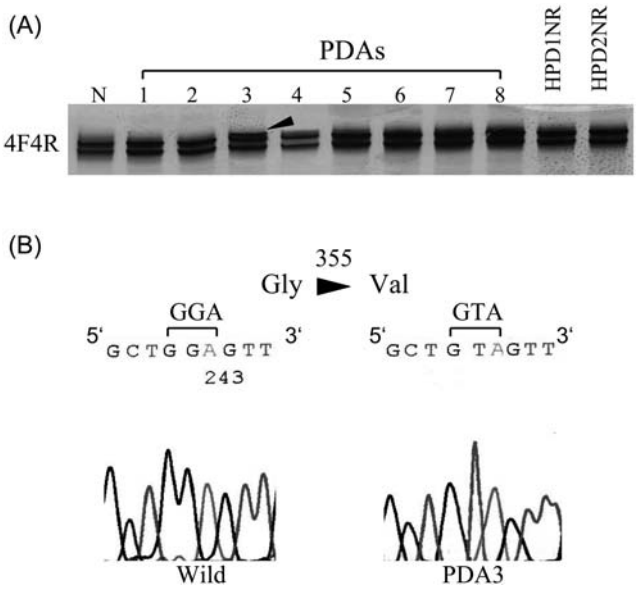

Fig. 1. Mutation of the LPA1 gene in hamster PDAs and established cell lines. (A) Representative results of RT-PCR-SSCP analysis. The arrowhead indicates an abnormal band shift. N: normal pancreatic tissue. PDAs: pancreatic ductal adenocarcinomas. HPD: hamster PDA cell line. (B) The mutation pattern of the LPA1 gene detected by the sequencing analysis. Wild: normal pancreatic tissue. PDA: pancreatic ductal adenocarcinoma.

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