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Short Communication

No Mutations of Lysophosphatidic Acid Receptor Genes in Lung Adenocarcinomas Induced by N-Nitrosobis(2-hydroxypropyl)amine in Rats

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Abstract: Lysophosphatidic acid (LPA) is a bioactive phospholipid that stimulates cell proliferation and migration, and protects cells from apoptosis. It interacts with specific G protein-coupled transmembrane receptors. Recently, frequent mutations of the LPA receptor-1 (LPA1) gene were detected in rat lung adenocarcinomas induced by N-nitrosobis(2-hydroxypropyl)amine (BHP). In this study, to evaluate the involvement of other LPA receptor gene alterations during lung carcinogenesis, we investigated mutations of the LPA2, LPA3, LPA4 and LPA5 genes in lung adenocarcinomas induced by BHP in rats. Fifteen male Wistar rats, 6 weeks of age, were given 2000 ppm BHP in their drinking water for 12 weeks and then maintained without further treatment until sacrifice at 25 weeks, and 15 adenocarcinomas were obtained. Genomic DNAs were extracted from frozen tissues, and the LPA2, LPA3, LPA4 and LPA5 genes were examined for mutations, using polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis. No mutations of LPA2, LPA3, LPA4 and LPA5 were detected in the 15 adenocarcinomas. These results suggest that alterations due to LPA2, LPA3, LPA4 and LPA5 gene mutations might not be involved in the development of lung adenocarcinomas induced by BHP in rats. (J Toxicol Pathol 2010; **23**: 63–66)

Key words: lysophosphatidic acid receptor, mutation, lung adenocarcinoma, N-nitrosobis(2-hydroxypropyl)amine, rat

Introduction

Lung cancer is one of the most common human malignancies, but the rate-limiting molecular events involved in its development remain largely unknown. The experimental model that we have used in several of our studies features the development of non-small cell lung cancers in rats given N-nitrosobis(2-hydroxypropyl)amine (BHP) in their drinking water, with high yields of adenomatous lesions, including adenocarcinomas[1,2]. As the step by step development of lung malignancies is accessible with this model, the molecular mechanisms involved can be readily investigated. Taking advantage of this model, we have been able to accumulate data on genetic and epigenetic alterations during carcinogenesis, including Ki-ras gene mutations[3], alterations in genes associated with transforming growth factor-β signaling pathway[4,5] and aberrant DNA methylation patterns of E-cadherin, p16 and Tslc1 genes, associated with reduced expressions[6,7].

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[8–13]. Since LPA can induce cell proliferation, migration, invasion and production of angiogenic factors in human ovarian cancer cell lines, it has been suggested that LPA may play an important role in the development of tumor cells[9,10,14]. LPA interacts with at least five G protein-coupled transmembrane receptors, lysophosphatidic acid receptor-1 (LPA1), LPA2, LPA3, LPA4 and LPA5[15–18]. LPA1 is ubiquitously expressed in normal tissues, but the expressions of the other LPA receptor subtypes are relatively restricted, suggesting these receptors may have different biological functions in regard to LPA[15–18]. Aberrant expressions of LPA receptors have been reported in human cancers, including ovarian, colorectal and thyroid cancers, indicating that alterations of LPA receptor expression might be important in the malignant transformation of tumor cells as well as LPA *per se*[9,10,13,14,19]. Moreover, we detected that the 5' upstream

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Table 1. Primer Sequences Used in This Study

Genes		Primers		Product size (bp)	Annealing temperature (℃)
LPA2	ex2-1	F: 5'-AAACCCTGCTTTCCGAGTAG-3'	R: 5'-GAAGCGTCGGTTGGAGGCAA-3'	393	60
	ex2-2	F: 5'-ACAATGAGACCATCGGCTTT-3'	R: 5'-CACACTGCGGTGCCGTTCCA-3'	376	58
	ex2-3	F: 5'-AAAGGCTGGTTCCTGCGACA-3'	R: 5'-TGCTGTGCCATGCGTTCAAC-3'	373	62
	ex2-4	F: 5'-CTGTTCAGCCGCTCCTACCT-3'	R: 5'-CTTACTGCTACCTCGGGTCA-3'	266	62
	ex3-1	F: 5'-CACATAGTCAATCCCCCACT-3'	R: 5'-GCATGATCCGGGTGCTGGCA-3'	350	63
LPA3	ex1-1	F: 5'-GACAAGCGGATGGACTTTT-3'	R: 5'-GGAGAAGCCAGCGGTTGACA-3'	297	56
	ex1-2	F: 5'-GCCCGGTGTCGAAAACCTTGA-3	R: 5'-GATGAAGAAGGCCAGGAGGTTT	334	66
	ex1-3	F: 5'-CTGGAATTGCCTCTGCAACATC-	R: 5'-GGGTTGTCTGCCACGTGATCTG	303	66
	ex2-1	F: 5'-CTGAGCAGCGCTTACTCCTT-3'	R: 5'-GATCATCTTCCGCATGGTGT-3'	225	60
	ex2-2	F: 5'-CTCGTACAAGGACGAGGACAT-3	R: 5'-TGAGACAGGCAAGGACTCTTA-3	252	62
LPA4	ex1-1	F: 5'-GCAGGCTCTCTGGGAAAATC-3'	R: 5'-AGTGGCGGTTAAAGTTGTAA-3'	333	56
	ex1-2	F: 5'-CTTTTTGTCTGTACCCTACC-3'	R: 5'-AAGTGGTGGTCGCATTGTTG-3'	306	58
	ex1-3	F: 5'-AGTGGTGGTATTTCAGCGTC-3'	R: 5'-AGTAATGGCTTGGGAACGTA-3'	342	58
	ex1-4	F: 5'-ACCCTCCGCAAGCCTGCAAC-3'	R: 5'-ACAGCGACTCCATCCTTATG-3'	315	60
	ex1-5	F: 5'-AATGTACCCGATTGCCTTGT-3'	R: 5'-ACACTGTAGCTGAACCTGAA-3'	278	56
LPA5	ex2-1	F: 5'-TGGCTTGTCTCCTCACTCCA-3'	R: 5'-GTTGCCAACACCAAGCTGTA-3'	356	60
	ex2-2	F: 5'-CATCGTTTGCATATGGTGG-3'	R: 5'-GCGAACAGCAGGATGAGAGC-3'	397	56
	ex2-3	F: 5'-GTGCATCCGCTGAGACTGC-3'	R: 5'-GTGGAGTTGTAGGGCACGAA-3'	391	63
	ex2-4	F: 5'-CTGGCCAACCTCATCATCTT-3'	R: 5'-TCAGAGGGCTGAATCTTGGG-3'	401	60

region of the LPA1 gene was highly methylated in rat tumor cell lines, which showed undetectable LPA1 expression, suggesting aberrant DNA methylation may be involved in silencing of the LPA1 gene[20].

Recently, we have also reported that LPA1 gene mutations occurred in not only adenocarcinomas but also adenomas during rat lung carcinogenesis induced by BHP[21]. In the present study, to evaluate the involvement of other LPA receptor gene alterations during lung carcinogenesis, we investigated mutations of the LPA2, LPA3, LPA4 and LPA5 genes in lung adenocarcinomas of rats.

A total of 18 male Wistar rats, at 5 weeks of age, were purchased from Japan SLC Inc. (Shizuoka, Japan) and housed 3–5 per cage in an air-conditioned room, with a constant temperature of 25°C and a 12-h light-dark cycle. Food and water were provided *ad libitum* throughout the study. After a one week acclimation period on a basal diet in pellet form (CF-2 Diet; Clea Japan, Tokyo, Japan), fifteen animals received drinking water containing BHP (Nacalai Tesque, Inc., Kyoto, Japan) at a concentration of 2000 ppm for 12 weeks; they received drinking water without BHP thereafter. In order to obtain normal lung tissue, three animals were maintained free from carcinogen exposure throughout the experimental period.

All rats were exsanguinated from the abdominal aorta under light ether anesthesia 25 weeks after the beginning of the experiment[1,2]. The lungs were immediately excised and grossly apparent tumors were dissected from their surrounding tissue. Samples were frozen in liquid nitrogen and stored at –80°C until analysis. Portions of the tumors were fixed in 10% neutrally buffered formalin at 4°C, routinely processed for hematoxylin and eosin staining, and histopathologically evaluated according to diagnostic criteria previously described[1–3]. All experiments and procedures carried out on the animals were approved by the Animal Care Committee of Kinki University. All the samples were the same as those used in a previous study[21].

Genomic DNA was extracted from frozen tissues of 15 adenocarcinomas and 3 normal lung tissues as described previously[6,7]. Polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis was conducted to look for mutations in the LPA2, LPA 3, LPA 4 and LPA 5 genes. The primers used in the present study were designed to amplify the open reading frames of the LPA2, LPA3, LPA4 and LPA5 genes with intron sequences flanking coding exons (NCBI accession numbers NC_005115, NC_005101, NC_005120 and NC_005103, respectively; Table1). Briefly, PCR for SSCP analysis 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., Tokyo, Japan), 2.5 units of AmpliTaq (Applied Biosystems Japan) and 0.5 μl of extracted genomic DNA under the following reaction conditions: primary denaturation for 2 min at 96°C followed by 36 cycles of 15 s of denaturation at 96°C, 15 s of annealing at 56–66°C and 30 s of extension at 72°C, and a final extension period of 2 min at 72°C. PCR products were diluted with 10 μl of loading solution containing 90 % formamide, 20 mM EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue. Aliquots containing 6 μl of diluted products were electrophoresed on polyacrylamide gels using a GeneGel Excel 12.5/24 kit (GE Healthcare UK Ltd., Buckinghamshire, England) at 8, 15, 18 and 20°C for 90 min at 15W, 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.)[21].

There were a total of fifteen lung tumors, and all were histologically well-differentiated adenocarcinomas. Three normal lung tissues obtained from untreated rats were used as controls to eliminate the possibility of contamination with macroscopically undetected cancerous tissue.

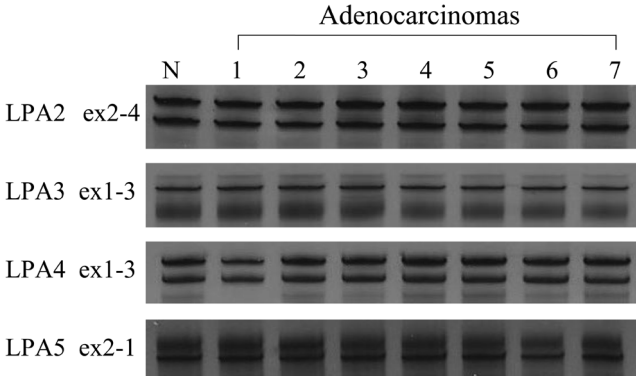

Fig. 1. Representative results of PCR-SSCP analysis of LPA2, LPA3, LPA4 and LPA5 gene mutations in lung adenocarcinomas induced by BHP in rats. N, normal lung tissue.

Using primers to amplify coding regions of LPA2, LPA3, LPA4 and LPA5, the PCR products showed clear single bands in 1% agarose gels (data not shown). Representative results of the PCR-SSCP analysis are shown in Fig. 1. No abnormal band shifts were detected in the 15 adenocarcinomas, indicative of no mutations in the LPA2, LPA3, LPA4 and LPA5 genes. We also confirmed by direct nucleotide sequencing that all amplified PCR products contained the normal LPA2, LPA3, LPA4 and LPA5 sequences (data not shown).

In a recent study, we reported that LPA1 gene mutations were found in 2 out of 12 adenomas (16.7%) and 7 out of 17 adenocarcinomas (41.2%), which suggests that mutations of the LPA1 gene may be involved in the acquisition of growth advantage from adenomas to adenocarcinomas in lung carcinogenesis induced in rats by BHP[21]. Moreover, we also detected frequent mutations of the LPA1 gene in hepatocellular carcinomas induced by N-nitrosodiethylamine in rats[22]. Therefore, this evidence suggests that the LPA1 gene is a target of mutations by nitroso-compounds in the development of lung and liver tumors in rats, but not the LPA2, LPA3, LPA4 and LPA5 genes. Previously, we reported aberrant expressions of the LPA1, LPA2 and LPA3 genes in lung adenocarcinomas induced by BHP in rats[23]. Although the expression levels of the LPA4 and LPA5 genes have not yet been clarified, it seems that aberrant expressions of LPA2, LPA3, LPA4 and LPA5 may be involved in rat lung carcinogenesis rather than mutational alterations. We are currently investigating the expression levels of the LPA4 and LPA5 genes in rat lung adenocarcinomas induced by BHP.

In conclusion, the present study detected no mutations of the LPA2, LPA3, LPA4 and LPA5 genes in rat lung adenocarcinomas induced by BHP. Recently, we reported LPA1 gene mutations in pancreatic duct adenocarcinomas induced by N-nitrosobis(2-oxopropyl)amine in hamsters[24]. To better understand the involvement of LPA receptor genes during carcinogenesis, we are currently investigating LPA2, LPA3, LPA4 and LPA5 gene mutations in hamster pancreatic duct adenocarcinomas.

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