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Comparison of K-*ras* Oncogene Activation in Pancreatic Duct Carcinomas and Cholangiocarcinomas Induced in Hamsters by N-Nitrosobis(2-hydroxypropyl)amine

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The presence of K-*ras* point mutations in pancreatic duct carcinomas and cholangiocarcinomas induced by N-nitrosobis(2-hydroxypropyl)amine (BHP) in Syrian hamsters was investigated by single-strand conformation polymorphism analysis of polymerase chain reaction products from frozen fresh materials in order to clarify the K-*ras* mutation rates in those two carcinomas induced simultaneously by one carcinogen, BHP. In the examined pancreatic duct carcinomas, 10 out of 16 were positive for a mutation in codon 12 while 3 out of 12 cholangiocarcinomas demonstrated mutation of K-*ras* gene. G-to-A transition was detected in the second position of codon 12 in both pancreatic carcinomas and cholangiocarcinomas. These results suggest that the role of genetic alteration in carcinogenesis may differ with the target organ, even when initiation is with the same carcinogen.

Key words: *ras* gene — Pancreatic cancer — Cholangiocarcinoma — Hamster — PCR-SSCP

Point mutations in members of the *ras* gene family commonly occur in spontaneous and chemically induced carcinomas in a variety of tissues. Ki-*ras* activation is particularly frequent in human pancreatic duct carcinomas, for which multiple gene changes have been reported.[1)] Previously, we described Ki-*ras* activation at codons 12 and 13 in pancreatic duct carcinomas of Syrian hamsters induced by BHP[4] at high incidence. All mutations involved a G-to-A transition at the second position of codon 12, resulting in a change at the amino acid level from glycine to aspartic acid.[2)]

BHP and related compounds are known to be primarily metabolized in the liver, initiating neoplasia not only in pancreatic duct cells but also in bile-duct epithelial cells. The aim of this experiment was to study the incidence of *ras* gene mutation in pancreatic carcinomas and cholangiocarcinomas induced by a single carcinogen, BHP, since it has been suggested that the different rates of K-*ras* mutation between pancreatic and bile duct lesions may depend on different etiological factors.

MATERIALS AND METHODS

Animals Six-week-old female Syrian hamsters (SLC, Shizuoka) were injected subcutaneously with 500 mg/kg body weight of BHP (Nakarai Chemical Co. Ltd., Kyoto) once a week for 15 weeks. Animals were killed at 24 weeks after the beginning of the experiment and parts of macroscopic pancreatic and liver tumors were removed and frozen in liquid nitrogen for oncogene analysis. Residual tumor and non-tumor tissues were fixed in ethanol and stained with hematoxylin and eosin for histological examination.

PCR-SSCP analysis of *ras* oncogenes DNA was extracted by incubation in PCR buffer with the non-ionic detergents, Tween 20 and NP-40. A portion of the resultant extraction buffer (1 μl) was subjected directly to the PCR to amplify the regions of *ras* oncogenes. Mixtures (5 μl) were prepared using a Gene Amp Kit (Cetus), and [α-^{32}P]dCTP was added for radio-labeling of PCR products. As primers, the pair of appropriate oligonucleotides shown in Table I was used. The primer sequences used in this study were derived from the genomic DNA sequence and contained both intron and exon sequences. PCR products were diluted with 100 volumes of loading solution containing 90% formamide, 20 m*M* EDTA, and 0.05% xylene cyanol and bromophenol blue, denatured at 80°C and applied to 5% polyacrylamide gel containing 0.5×Tris-borate EDTA buffer with or without 10% glycerol. Electrophoesis was performed at 30 W for about 3 h at 30°C or 4°C (LKB macroelectrophoresis system, Pharmacia). The gel was dried on filter paper and exposed to X-ray film at −80°C as descrived previously.[3)]

Direct DNA sequencing The DNA fragment was extracted from the mobility-shifted band in the polyacrylamide gel. PCR was performed ad the amplification

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[4] Abbreviations: BHP, N-nitrosobis(2-hydroxypropyl)amine; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.

reaction mixture was deionized in a Microcon 100 (Amicon). For determination of nucleotide sequences, a dsDNA cycle sequencing system (GIBCO BRL) was used and products were analyzed in 6% polyacrylamide gel containing 7 *M* urea.

RESULTS

The results of PCR-SSCP analysis of the K-*ras* exon 1 containing codon 12 in pancreatic samples are shown in Fig 1. Two complementary strands were obtained from all tumors and normal pancreas tissue. Fragments showing abnormal mobilities, indicative of gene mutation in K-*ras* exon 1, were observed in 10 out of the 16 pancreatic duct carcinoma cases. In clear contrast, 3 out of the 12 cholangiocarcinomas showed fragments with abnormal mobilities (Fig. 2). In 4 cases of cholangiocarcinoma (cases 17, 25, 32 and 40), pancreatic duct carcinomas occurred in the same animals, and in these animals, K-*ras* gene mutation was detected only in pancreatic carcinomas. With all observed fragments showing abnormal mobility, two normal complementary strands and only one abnormal strand were observed. Theoretically, another fragment complementary to that with an abnormal mobility should be evident but it is possible that the faster-moving strand carrying a structural change did not show a mobility shift. Such false-negative results are sometimes obtained with PCR-SSCP analysis. Nevertheless, all known nucleotide substitutions, including point mutations and DNA polymorphism, so far analyzed could be detected by a mobility shift of one of the complementary strands.[3] No other type of mobility shift was observed in the pancreas adenocarcinomas examined in this study.

Table I. Primers of Rodent *ras* Genes for PCR-SSCP Analysis

H-*ras* exon 1	5′ TGATT CTCAT TGGCA GGTGG 3′
	5′ AGGTA GTCAG AGCTC ACCTC 3′
H-*ras* exon 2	5′ AGGAC TCCTA CCGGA AACAG 3′
	5′ ACCTG TACTG ATGGA TGTCT 3′
K-*ras* exon 1	5′ AGGCC TGCTG AAAAT GACTG 3′
	5′ GCAGC GTTAC CTCTA TCGTA 3′
K-*ras* exon 2	5′ CCTAC AGGAA ACAAG TAGTA 3′
	5′ TAAAC CCACC TATAA TGGTG 3′
N-*ras* exon 1	5′ ACAAA CTGGT GGTGG TTGGA 3′
	5′ CTCTA TGGTG GGATC ATATT 3′
N-*ras* exon 2	5′ GATTC TTACC GAAAG CAAGT 3′
	5′ CCAGT ACCTG TAGAG GTTAA 3′

The results of PCR-SSCP analysis were confirmed by direct DNA sequencing. The results of nucleotide sequencing in representative cases are shown in Fig. 3. G-to-A transition at the second position of codon 12 was detected in both pancreatic carcinoma cases and cholangiocarcinoma cases in which an abnormal band was detected by PCR-SSCP analysis.

The results of PCR-SSCP analysis of K-*ras* exon 2 containing codon 61, H-*ras* exons 1 and 2, and N-*ras* exons 1 and 2 did not reveal any abnormal mobility shifts, indicating that no gene mutation occurred in these regions, in either pancreatic adenocarcinomas or cholangiocarcinomas.

DISCUSSION

In human pancreatic cancers, an activating point mutation in the K-*ras* gene has been observed at high frequency.[4-9] The altered p21 protein encoded by this mutated K-*ras* gene is therefore considered to play an impor-

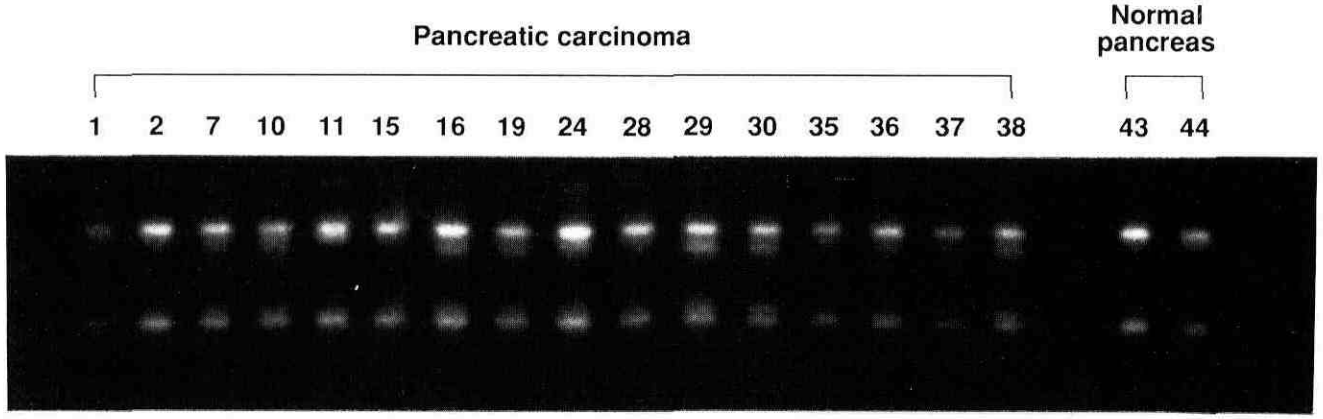

Fig. 1. PCR-SSCP analysis of K-*ras* gene (exon 1) in hamster pancreatic adenocarcinoma induced by BHP. In case numbers 7, 10, 16, 19, 24, 29, 30, 36, 37 and 38, fragments showing abnormal mobility shift were observed.

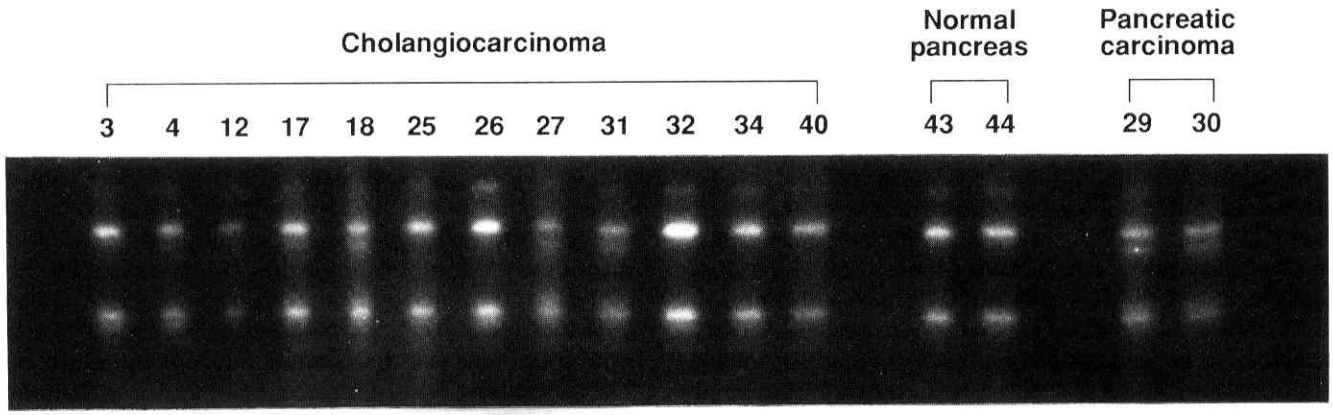

Fig. 2. PCR-SSCP analysis of K-*ras* gene (exon 2) in hamster cholangiocarcinoma induced by BHP. In 3 cases of cholangiocarcinoma (numbers 18, 27, 31), and 2 pancreatic carcinomas, fragments showing abnormal mobility shift were observed.

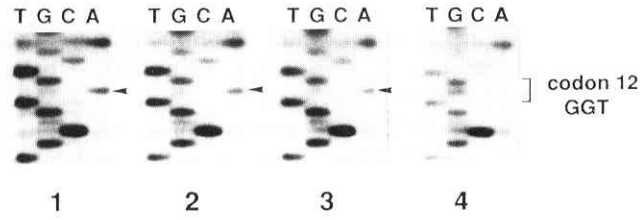

Fig. 3. Nucleotide sequence around codon 12 of K-*ras*. In carcinomas showing an abnormal band on PCR-SSCP analysis, G-to-A transition was detected at the second position of codon 12 (indicated by the arrow). Lane numbers: 1 and 2, pancreatic carcinoma; 3, cholangiocarcinoma; 4, normal pancreas.

tant role in human pancreatic carcinogenesis. However, in human cholangiocarcinomas developing in the intrahepatic and extrahepatic bile ducts, as well as in gall bladder cancers, the frequency of *ras* gene mutation is relatively low.[10, 11] Possible reasons for this difference include variation in the etiology or the causal agent. In this study, to eliminate this factor we used a hamster model for pancreatic ductal and bile-ductal carcinogenesis. In spite of the fact that both tumors were induced by the same carcinogen, the fraquency of mutation of the K-*ras* gene in pancreatic adenocarcinomas and cholangiocarcinomas was obviously different. This result suggests that the role of activation of K-*ras* gene differs with the cell type from which the tumor originates, even when they may be morphologically very similar. It was suggested that the role of *ras* gene activation during cancer development was different in different tissues or species. Dimethylbenz[a]anthracene induced both H-*ras* and K-*ras* gene mutations but only K-*ras* gene mutation contributed to Balb/c 3T3 cell transformation.[12] In rat hepatocellular neoplasms induced by *N*-methyl-*N*-nitrosourea, mutation of H-*ras* and K-*ras* gene was not detected.[13, 14] These results suggested that the initiated cell with the most advantageous genetic changes was selected and recruited in a tissue-specific manner.[15]

N-Nitrosamines with two propyl chains, such as *N*-nitrosobis(2-oxopropyl)amine (BOP), *N*-nitrosobis(2-hydroxypropyl)(2-oxopropyl)amine (HPOP) and BHP, are metabolized in the liver.[16, 17] Analysis of the adducts formed after treatment of Syrian hamsters with BOP and HPOP suggests that the major proximate carcinogens of these two compounds methylate DNA to from 7-methylguanine and O^6-methylguanine.[18] A low capacity of pancreatic duct cells for repairing O^6-methylguanine might be responsible for their susceptibility to induction of carcinomas by BOP,[19] non-repaired DNA adducts giving rise to gene mutations including that leading to an activated K-*ras* gene, which might endow the cells with a selective growth advantage. There is some evidence to suggest that activation of the *ras* gene is a relatively early event during multi-stage carcinogenesis in the mammary gland,[20] colorectal tissues,[20-22] and skin.[23] Further study is required to clarify at which stage of pancreatic carcinogenesis *ras* genes become activated. It is still not clear whether BHP-induced DNA adducts in bile duct cells are similar to those in the pancreatic duct cells. However, conceivably, the same K-*ras* activation could occur but, because of the difference in micro-environment, does not confer a growth advantage. Other mutations might be neccessary for the bile duct cell to express a phenotype leading to cancer. An alternative possibility is that repair is more efficient.

In terms of the type of *ras* oncogene activation, a specific G-to-A transition at the second position of codon 12 of K-*ras* has been reported for hamster pancreatic adenocarcinomas induced by both BOP[13, 24-26] and

BHP.[2] On the other hand, a G-to-A transition at the second position of codon 13 was found in a tumor line from a pancreatic carcinoma that developed spontaneously in a Syrian hamster.[24] In the present study, only one type of fragment showing abnormal mobility was detected in the PCR-SSCP analysis. This result suggests that all pancreatic tumors containing a mutated K-*ras* gene are characterized by a specific G-to-A transition at the second position of codon 12, and no other transition occurs. In the 4 adenocarcinoma cases where no mutated K-*ras* gene was detected, the accompanying pancreatitis was severe, resulting in the infiltration of large numbers of inflammatory cells and abundant fibroblasts. Therefore, there is a possibility that the presence of all these other type could have hindered the demonstration of any K-*ras* mutation. On the other hand, normal K-*ras* gene alleles were detected in all the pancreatic adenocarcinomas. At least three possibilities should be considered to explain this: 1) included normal cells, for example fibroblasts, inflammatory cells, blood vessels, and so on, account for the normal alleles, 2) cancer cells having a mutated gene co-exist with cancer cells without a mutated gene in the same tumor, 3) normal K-*ras* gene alleles remain in the cancer cells. This requires further study. However, the important point is that the mutation observed in hamster pancreatic adenocarcinomas is the same as that occurring in human pancreatic adenocarcinoma, Therefore, the present findings reinforce other findings in indicating that pancreatic adenocarcinomas in both man hamsters are essentially similar lesions with shared susceptibility to exogenous agents, and common underlying carcinogenic processes including genetic changes.

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