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K-*ras* Gene Mutation in Early Ductal Lesions Induced in a Rapid Production Model for Pancreatic Carcinomas in Syrian Hamsters

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The presence of K-*ras* gene mutation was examined in experimentally induced preneoplastic pancreatic ductal lesions. Syrian hamsters received 70 mg/kg of *N*-nitrosobis(2-oxopropyl)amine (BOP) followed by repeated exposure to an augmentation pressure regimen consisting of choline-deficient diet combined with DL-ethionine and L-methionine and administration of 20 mg/kg BOP. After two augmentation pressure cycles, pancreatic ductal cell hyperplasias appeared and after three cycles, atypical hyperplasias of pancreatic ductal cells and intraductal carcinomas developed. K-*ras* mutations were detected by single-strand conformation polymorphism analysis of polymerase chain reaction products and nucleotide sequencing. The results showed that K-*ras* mutation had occurred in one of 9 simple hyperplasias of pancreatic ductal epithelium, in 5 of 9 atypical hyperplasias, and in 4 of 8 intraductal carcinomas. The findings thus suggested that K-*ras* is activated in association with very early stage malignant transformation of pancreatic ductal cells in hamsters.

Key words: K-*ras* — Hamster — Pancreatic carcinoma — Preneoplastic lesion

Oncogene activation and tumor suppressor gene loss during carcinogenesis are key events in the mechanisms underlying cancer development. In 1988 Almoguera *et al.*[1] first reported that most human carcinomas of the exocrine pancreas contain a mutant K-*ras* gene, and subsequently high incidences of K-*ras* mutation in pancreatic duct carcinomas have been confirmed by several clinical investigations.[2–5] The hamster pancreatic cancer model is well-known as a useful system for studies of the mechanisms of ductal carcinoma development, and in line with human findings, recent studies have shown frequent point mutations of the K-*ras* gene in experimentally induced carcinomas.[6,7] Therefore, this is a typical example of correlative molecular events occurring during carcinogenesis in both humans and hamsters.

Previously, we established a rapid production model for pancreatic duct carcinomas in hamsters[8–10] and reported a sequence of well-characterized changes in ductal morphology.[11] In the present experiment, we studied the possible role of K-*ras* mutation in preneoplastic and early neoplastic ductal lesions induced using this rapid production model.

Female Syrian golden hamsters (Nihon SLC, Shizuoka) weighing approximately 90 g at the commencement were used. The animals were housed, five per plastic cage, in an air-conditioned room at 24°C and 60% relative humidity with alternating 12 h periods of light/darkness, and given food and water *ad libitum*. The experimental protocol is shown in Fig. 1, being based on the published rapid production model for hamster pancreatic carcinogenesis.[8,11] The basal diet, Oriental MF, was purchased from Oriental Yeast Co. Ltd., Tokyo. DL-Ethionine and L-methionine were purchased from Nakarai Chemical Co. Ltd., Kyoto. All groups received 70 mg/kg body weight of BOP,[1] as the initiation dose, and were then exposed to two or three cycles of augmentation pressure. Eleven days after BOP initiation, hamsters received four daily i.p. injections of 500 mg/kg of ethionine while being maintained on the CD diet (Dyets Inc., Bethlehem, PA). The animals were then returned to the basal diet and were given a single i.p. injection of 800 mg/kg of methionine followed by a single i.p. injection of 20 mg/kg of BOP at day 5 after the beginning of the augmentation pressure cycle. Group 1, consisting of 25 hamsters, received 70 mg/kg of BOP following by two cycles of augmentation pressure and the animals were killed on day 32 after the beginning of the experiment. Groups 2 and 3 consisting of 15 hamsters each received 70 mg/kg of BOP followed in both cases by three augmentation pressure cycles. The 10 hamsters of group 4 received 0.9% NaCl solution (saline) followed by three cycles of augmentation pressure. Group 2 ham-

[1] Abbreviations: BOP, N-nitrosobis(2-oxopropyl)amine; CD diet, choline-deficient diet; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.

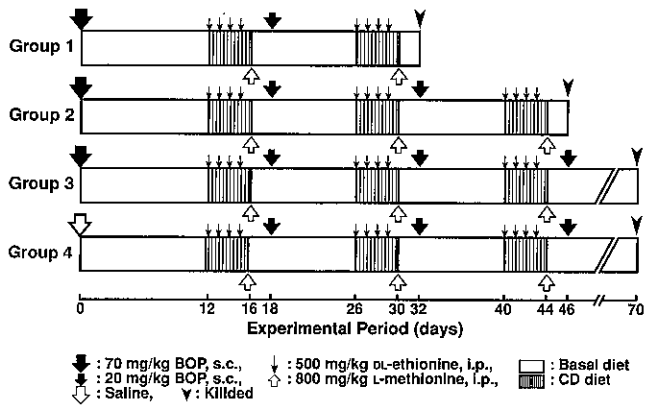

Fig. 1. Experimental protocol of the rapid production model for pancreatic duct carcinogenesis induced by BOP in hamsters.

sters were killed on day 46 and group 3 and 4 animals were killed on day 70 after the beginning of the experiment. All hamsters were killed under ether anesthesia, and the pancreas tissues were immediately removed, fixed in 100% ethanol at 4°C, and routinely processed for paraffin embedding. Two serial thin sections were made, one cut at 5 μm thickness and stained with hematoxylin and eosin (HE) for histopathological examination, and the other cut at 20 μm thickness for DNA extraction. The method of DNA extraction was as follows. Each lesion was dissected out from the paraffin-embedded thick section under the microscope, and processed according to the protocol described by Wright *et al.*[12] The methods used for PCR-SSCP analysis were described previously.[13–15] As primers, appropriate oligonucleotide sequences, 5-AGGCCTGCTGAAAATGACTG-3 and 5-GCAGCGTTACCTCTATCGTA-3, were used for specific amplification of hamster K-*ras* gene exon 1. Polyacrylamide gel (5%) containing 45 m*M* Tris-borate (pH 8.3), 4 m*M* EDTA, and 10% glycerol was prepared, and gel electrophoresis was performed at 30 W for about 4 h at 30°C (Macrophore DNA electrophoresis system, Pharmacia Co. Ltd.) The gel was dried and exposed to X-ray film at −80°C for about 2 days. To confirm results of SSCP analysis, direct nucleotide sequencing was done as described previously.[16]

In group 1, only simple hyperplasias of pancreatic ductal epithelium were observed (Fig. 2a) and no adenocarcinomas developed. In group 2, atypical hyperplasias of the ductal epithelium (Fig. 2b) and intraductal carcinomas were observed. In group 3, intraductal carcinomas (Fig. 2c) and invasive ductal carcinomas were evident. In the control group 4, not given the BOP initiation step, no ductal lesions developed.

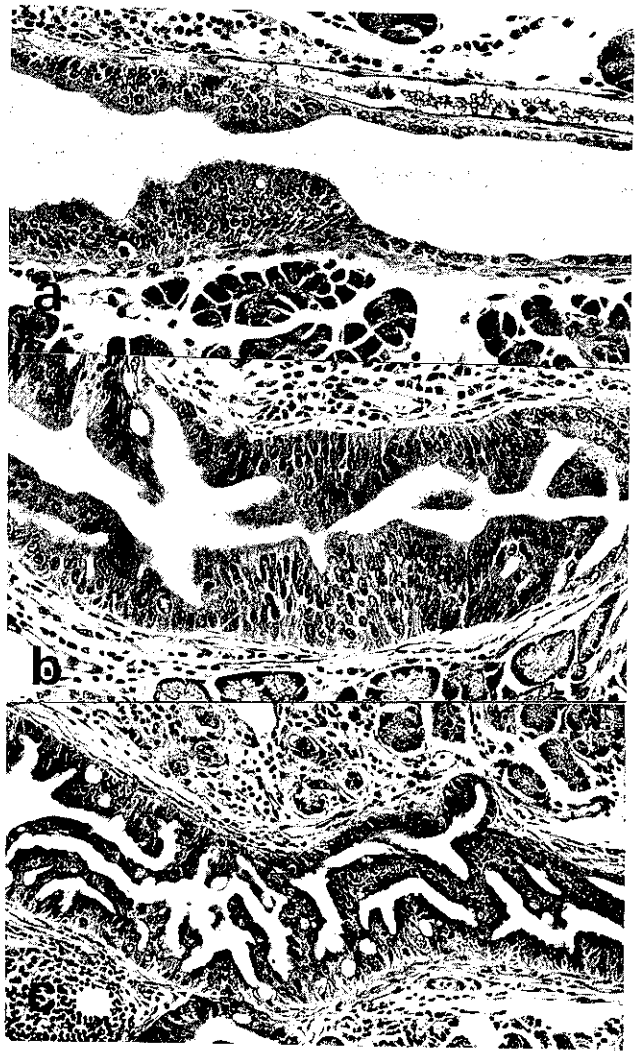

Fig. 2. Photomicrographs of pancreatic ductal lesions in hamsters induced by BOP. (a) Simple hyperplasia of pancreatic duct epithelium (HE staining ×250). (b) Atypical hyperplasia of pancreatic duct epithelium (HE staining ×250). (c) Intraductal carcinoma (HE staining ×150).

The results of PCR-SSCP analysis of the K-*ras* exon 1 containing codon 12 are summarized in Table I. In pancreatic ductal cell hyperplasias, which were observed in hamsters killed at 32 days after the beginning of the experiment, as shown in Fig. 3a, paired complementary strands were obtained from all lesions. In one case of simple hyperplasia, a faint band with abnormal mobility shift was observed (Fig. 3a) and this abnormal fragment was indicative of the presence of gene mutation in K-*ras* gene exon 1. In 5 out of 9 atypical hyperplasias, which were observed in hamsters killed at 46 days after the beginning of the experiment, fragments with abnormal

Table I. Incidence of K-*ras* Mutation in Early Ductal Lesions of a Rapid Production Model for Pancreatic Carcinomas in Hamsters

Group	Hyperplasia		Atypical hyperplasia		Intraductal carcinoma	
	Number	Incidence (%)	Number	Incidence (%)	Number	Incidence (%)
1	9	1 (11)	—	—	—	—
2	NE	NE	9	5 (56)	1	0 (0)
3	NE	NE	NE	NE	7	4 (57)
4	—	—	—	—	—	—

NE: Not examined. —: Ductal lesions did not develop.

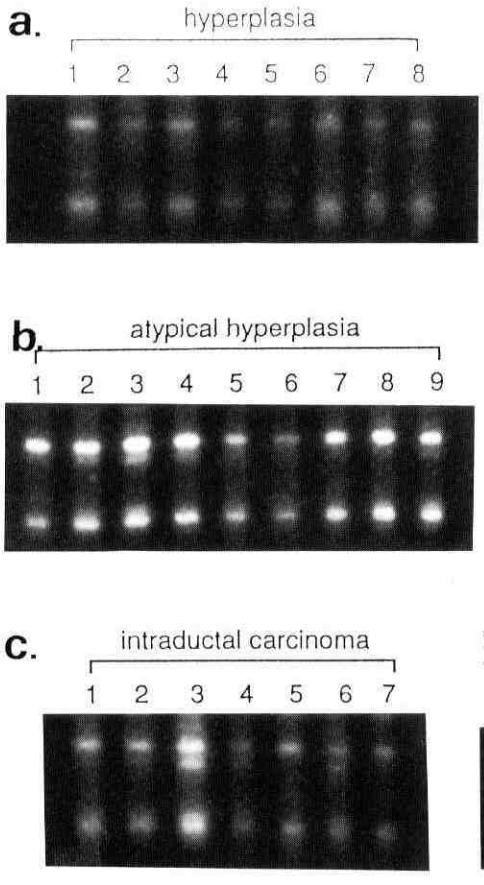

Fig. 3. PCR-SSCP analysis of K-*ras* gene (exon 1) in pancreatic ductal lesions in hamster. (a) Areas of hyperplasia of pancreatic duct epithelium. Lanes 1–7: Fragments with abnormal mobility shifts were not detected. Lane 8: A faint fragment with abnormal mobility shift is observed. (b) Atypical hyperplasias of pancreatic duct epithelium. Fragments with abnormal mobility shifts are observed in lanes 2, 3, 4, 5 and 9. (c) Intraductal carcinomas. One intraductal carcinoma developed in a group 2 animal (lane 1), and the other carcinomas developed in group 3 animals (lane 2–7). Fragments with abnormal mobility shift were observed in lanes 2, 3, 4 and 6. As the positive control, invasive carcinomas which developed in group 3 animals were used. Fragments with abnormal mobility shifts are observed.

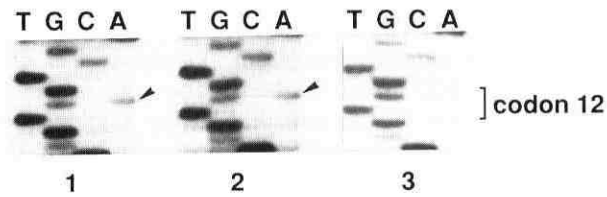

Fig. 4. Nucleotide sequence around codon 12 of K-*ras* gene. In preneoplastic lesions showing an abnormal band in PCR-SSCP analysis, G-to-A transition was detected in the second position of codon 12, indicated by the arrow. Lane 1, simple hyperplasia; lane 2, atypical hyperplasia; lane 3, normal pancreatic duct in the hamster without BOP-initiation.

mobilities were observed (Fig. 3b). In 4 out of the 7 intraductal carcinomas and 2 of 2 invasive carcinomas observed in hamsters killed at 70 days after the beginning of the experiment, similar fragments showing abnormal mobilities were also observed (Fig. 3c). All results of SSCP analysis were confirmed by nucleotide sequencing. The results of nucleotide sequencing showed G-to-A transition of the second position of codon 12 in K-*ras* gene; other types of point mutation were not observed (Fig. 4).

The finding of frequent mutation of the K-*ras* gene in both human and nitrosamine-induced hamster pancreatic duct adenocarcinomas has excited interest in the role of the *ras* gene in pancreatic carcinogenesis. The hamster model provides a very useful system, since it facilitates examination of pancreatic ductal lesions which progress from preneoplastic lesions to invasive carcinomas. In the present study, K-*ras* gene mutations could already be detected in simple hyperplasias and the occurrence of a *ras* gene mutation might correspond to an increase of structural atypia of ductal lesions, such as bridge formation of duct epithelial cells. These results suggest that K-*ras* gene mutation is an early event in the process of neoplastic transformation, but since simple hyperplasias were positive with low incidence, it might not be initiation-linked, and further studies using more sensitive

methods for detection of K-*ras* gene mutation are needed.

The present results confirmed and extended the findings reported by Cerny *et al.*[17] They also detected K-*ras* mutation in hyperplasias, using the oligonucleotide hybridization method. In this work, we applied PCR-SSCP analysis for detection of K-*ras* gene mutation, because this approach offers a rapid and sensitive method for detection of alterations of various genes, including simple point mutation in the *ras* gene.[13, 14]

The present findings are also in line with the reports of K-*ras* gene mutation in human pancreatic tumors, including intraductal lesions. Tada *et al.*,[18] for example, described the presence of K-*ras* mutations in 3 of 5 cases of intraductal papillary neoplasm. Yanagisawa *et al.*[19] found K-*ras* activation in ductectatic-type mucinous cystic neoplasms of the pancreas. They suggested an adenoma-carcinoma sequence in the evolution of this type of neoplasm and speculated that K-*ras* activation might be an important event in adenoma development. Moreover, they found that K-*ras* activation had occurred in nonatypical mucous cell hyperplasia of pancreas suffering from chronic inflammation.[20] These results are in concert with the present result in hamsters and suggest that the clonal proliferation of cells comprising the ductal cell hyperplasia is neoplastic or preneoplastic in nature. Since K-*ras* activation occurs so early in pancreatic carcinogenesis, other oncogene changes might also be necessary for development of pancreatic cancer in both human and hamster. On the other hand, Lemoine *et al.*[21] reported no K-*ras* mutation in 5 cases of intraductal neoplasia, whereas they did find K-*ras* activation in pre-invasive pancreatic cancers. They therefore concluded that K-*ras* activation might be important in the tumorigenesis of ductal adenocarcinoma of the pancreas but is not required in the pathogenesis of ductal papillary hyperplasia or intraductal papillary neoplasms. This question clearly requires further attention. As one possibility to explain discrepancies in the literature, it has been suggested that diagnosis of intraductal lesions is different between Japan and Europe. As another possibility, other factors involved in pancreatic carcinogenesis, including racial and cultural differences, could be crucial. However, the available data lead to the conclusion that K-*ras* activation is an early event in pancreatic carcinogenesis in man as well as in hamsters. The intraductal papillary neoplasms observed in the human pancreas are unfortunately not a feature of the hamster model, whereas the intraductal lesions in the hamster are very similar to very early common-type pancreatic carcinomas in man.[22, 23] In conclusion, our data suggest that K-*ras* activation may be an important event in the malignant transformation of pancreatic ductal cells induced by the DNA alkylating agent, BOP. This carcinogen primarily produces methyl adducts at the N^7 and O^6 positions of guanine.[24, 25] How such adducts might generate K-*ras* point mutations during the early phase of pancreatic carcinogenesis in hamsters is a subject for future investigation.

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