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Ki-*ras* Activation in Pancreatic Carcinomas of Syrian Hamsters Induced by *N*-Nitrosobis(2-hydroxypropyl)amine

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Five pancreatic carcinomas induced by *N*-nitrosobis(2-hydroxypropyl)amine in Syrian golden hamsters were analyzed for activation of Ki-*ras* at codons 12 and 13, using the polymerase chain reaction and direct sequencing. The Ki-*ras* gene was shown to be activated in four of the five carcinomas, and the results were further confirmed by subcloning and sequencing. All the mutations involved a G-to-A transition at the second position of codon 12, which resulted in a change at the amino acid level from glycine to aspartic acid. This mutation is identical with that reported for pancreatic tumors of Syrian hamsters induced by *N*-nitrosobis(2-oxopropyl)amine.

Key words: Ki-*ras* — Hamster — Pancreatic carcinoma — *N*-Nitrosobis(2-hydroxypropyl)amine

Most human pancreatic malignancies originate from ductal cells and Ki-*ras* activation has frequently been found in these exocrine carcinomas.[1-6] However, there is also one report of direct sequencing analysis indicating an unexpectedly low rate of activation of Ki-*ras*.[7] Two possible reasons for this discrepancy may be considered. One concerns differences in etiology, or causal agent, and the other, the degree of progression of the populations of neoplastic cells having mutated Ki-*ras*; namely the stage of pancreatic tumor development. Animal systems are of great help in solving these problems since we can experimentally assess both chronological aspects of pancreatic carcinogenesis and the influence of inducing carcinogen on Ki-*ras* activation.

N-Nitrosamines with two propyl chains, such as *N*-nitrosobis(2-oxopropyl)amine (BOP),[6] *N*-nitroso(2-hydroxypropyl)(2-oxopropyl)amine (HPOP), and *N*-nitrosobis(2-hydroxypropyl)amine (BHP), are the basis of reliable models for human pancreatic carcinomas utilizing the Syrian hamster.[8-10] The tumors induced by these chemicals are mostly exocrine ductal cell carcinomas, the same pathological type as the majority of human pancreatic carcinomas,[11] and showing similar patterns of staining for mucoid substances.[12] BOP is reduced to HPOP, and then to BHP *in vivo* and also *in vitro*.[13,14] Analysis of the adducts formed after treatment of Syrian hamsters with BOP and HPOP suggested that the major proximate carcinogen of these two compounds would be diazomethane, which would be produced via a mono(2-oxopropyl)nitrosamine intermediate and could methylate DNA to form 7-methylguanine and O^6-methylguanine.[15] The selective induction of pancreatic ductal cell carcinomas by BOP might be due to the low capacity of ductal cells for repairing O^6-methylguanine.[16] However, it is still not clear what is the key DNA adduct in the pancreas after administration of BHP and the proximate carcinogenic form of BHP similarly remains to be elucidated.

As for oncogene activation, G-to-A transition at the second position of codon 12 of Ki-*ras* was demonstrated in a tumor line derived from a Syrian hamster pancreatic ductal carcinoma induced by BOP.[17] The same mutation was also detected in all of 10 primary tumors arising in the Syrian golden hamster pancreas after BOP treatment when DNA extracted from paraffin-embedded samples was amplified.[18] On the other hand, a G-to-A substitution at the second position of codon 13 was found in a tumor line from a pancreatic carcinoma occurring spontaneously in a Syrian golden hamster.[17] This might be a reflection of the difference in causal agent. In the case of BHP, it is not known what kind of mutation is caused.

In this investigation, we demonstrated Ki-*ras* activation in BHP-induced primary pancreatic carcinomas of

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[6] Abbreviations: BOP, *N*-nitrosobis(2-oxopropyl)amine; BHP, *N*-nitrosobis(2-hydroxypropyl)amine; HPOP, *N*-nitroso(2-hydroxypropyl)(2-oxopropyl)amine; PCR, polymerase chain reaction.

Syrian golden hamsters, by applying the polymerase chain reaction (PCR) to the DNA of these carcinomas.

Five primary pancreatic ductal carcinomas were obtained by injecting 500 mg/kg BHP subcutaneously into Syrian golden hamsters, fifteen times from 6 weeks through 20 weeks of age. The animals were killed at 34 weeks, then pancreatic tumors were excised and the surrounding non-tumorous tissues were trimmed off as far as possible. The obtained tumors were cut into two pieces; one underwent microscopic examination and the other was frozen and stored until required for the following analysis. Although pancreatic tumors were observed in all of the 20 treated animals, five ductal cell carcinomas of large size, which were approximately 2–3 mm in diameter and whose pathology was confirmed by microscopic analysis, were selected for the analysis.

DNA was extracted by incubating tissues in PCR buffer with non-ionic detergents, Tween 20 (Bio-Rad) and NP-40 (Sigma).[19] About 100 ng of DNA was amplified by PCR with 2.5 units of *Taq* polymerase (Takara) according to the manufacturer's instructions, using 50 and 5 pmol of 5′(5′-GCCTGCTGAAAATGACTGAG-3′) and 3′(5′-CGTAGGATCATATTCATCCA-3′) primers, respectively. The primer sequences were based on rat c-Ki-*ras* gene,[20] the former corresponding to nucleotides −11 to 9 and the latter to codons 29 to 35. The reaction mixture was subjected to 50 cycles of reaction, consisting of a 30-s denaturing step at 94°C, a 1-min annealing step at 55°C and a 2-min polymerization step at 72°C, using a thermal cycler (Perkin Elmer/Cetus). After purification of the product by phenol/chloroform extraction and ethanol precipitation, unincorporated nucleotides and primers were removed by filtration through a polysulfone filter with an MW cut-off of 10,000 (Ultrafree C3GC, Millipore Japan). The primer for sequencing (5′-AGTGATTCTGAATTAGCTGT-3′) was end-labeled, and the sequencing reaction was performed with Sequenase Ver. 2.0 (USB) according to the manufacturer's instructions.

For subcloning of PCR product, 35 cycles of PCR were performed with equimolar (50 pmol) phosphorylated 5′ and 3′ primers. The product was blunt-ended with T4 polymerase (Takara), and ligated into Bluescript SKM13(+)(Stratagene). Plasmids with inserts were sequenced with Sequenase Ver. 2.0, using α[^{35}S]dCTP (Amersham).

Direct sequencing detected G-to-A transition at the second position of codon 12, resulting in the substitution of glycine by aspartic acid, in four out of five carcinomas analyzed (carcinomas #1, #2, #3 and #5; Fig. 1). A faint band in the same position was also observed in carcinoma #4. The bands in the A lanes at the second position of codon 12 in these carcinoma samples were not seen in negative controls, and were reproducible in three independent PCR and direct sequencing procedures using the same source of DNA. Further, we detected mutated clones in all four carcinomas which were suspected to have mutations on direct sequencing, by subcloning and sequencing of the PCR products (Table I).

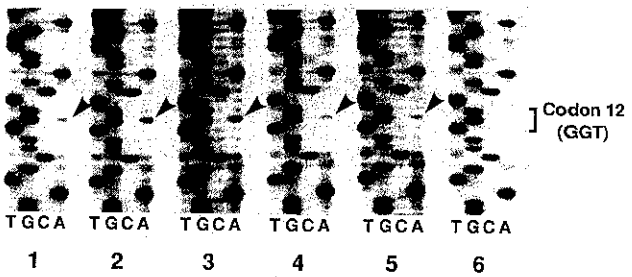

Fig. 1. Nucleotide sequence around codon 12 of Ki-*ras*. Samples 1, 2, 3, 4, 5, and 6 correspond to carcinomas #1, #2, #3, #4, #5, and normal pancreas of an untreated syngeneic hamster, respectively. DNA was extracted with PCR buffer containing non-ionic detergents, and then asymmetrically amplified by PCR. Direct sequencing was performed with a 3′ sequencing primer. Lanes from left to right are T, G, C, and A of the sense strand. The second position of codon 12 of carcinomas #1, #2, #3, and #5 showed a clear additional band in the A lane (indicated by the arrows) whereas that of carcinoma #4 showed an ambiguous band (also indicated by the arrow).

Table I. Positive Rates of Ki-*ras* Mutation in Pancreatic Carcinomas of Syrian Golden Hamsters Induced by BHP

Sample	Number of clones sequenced	Number of mutated clones	Positive rate (%)
Carcinoma #1	7	1	14
Carcinoma #2	8	5	62
Carcinoma #3	8	2	25
Carcinoma #4	16	0	0
Carcinoma #5	8	1	13
Normal pancreas	7	0	0

PCR products of pancreatic carcinomas were subcloned into plasmids and then sequenced to determine the presence or absence of a mutated sequence.

During PCR amplification or small-scale bacterial culture in subcloning of the PCR-amplified product, at least one control sample from normal pancreas of a non-treated animal was included in the same set of reactions or in the same multi-well culture dish with carcinoma samples. However, no mutation was observed in any sample derived from normal pancreas of a non-treated animal. Thus, the existence of mutation in carcinomas #1, #2, #3 and #5 was definitely confirmed.

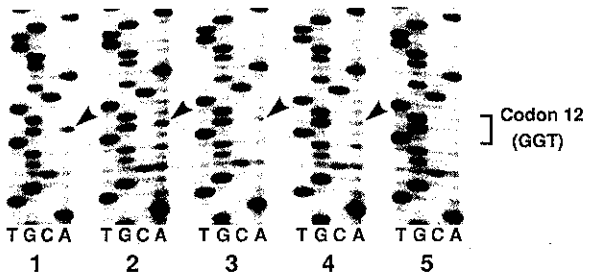

Fig. 2. Detection limit of the direct sequencing method. Plasmid with mutated Ki-*ras* was mixed with various amounts of that with normal Ki-*ras*, and then sequenced by the direct sequencing method after PCR. Samples 1, 2, 3, 4 and 5 correspond to plasmid mixtures containing mutated DNA at levels of 50, 20, 10, 5 and 0%, respectively. Lanes from left to right are T, G, C, and A of the sense strand. Mutated bands are marked with arrows. Mutated allele can be discerned at the level of 10% or more.

Other ambiguous bands observed on direct sequencing, such as the band in the A lane at the third position of codon 13 of carcinoma samples in Fig. 1, were not reproducible and not observed in any of the subclones isolated. Thus extra bands other than the bands mentioned above were suggested to be artefacts arising during direct sequencing.

Any tumor sample usually also contains normal cells and, even within carcinoma tissues, cells might be heterogeneous for Ki-*ras* mutation. This could explain the variation in mutation rate of carcinoma samples found on subcloning and sequencing, and could cause negative results even when some cells within a carcinoma have a mutation. Thus, it is important to know the detection limit of direct sequencing for minor populations. To determine sensitivity, two kinds of plasmid, one with the normal sequence and the other with a mutated sequence, were mixed in various ratios, and subjected to analysis. The mutated sequence was detected when it existed in more than about 10% of the total DNA (Fig. 2).

Based on this result, mutated DNA in carcinoma #4 was concluded to exist, if at all, at a level of less than 10%. Subcloning and sequencing (Table I) also indicated no mutated DNA at 6.3% (1/16). Re-examination of the histology showed that the pathological features of this carcinoma #4 were the same as for the other carcinomas, diagnosis being of a ductal adenocarcinoma, but that the accompanying pancreatitis was particularly severe in this carcinoma, resulting in the infiltration of large amounts of inflammatory cells. There is therefore a possibility that these inflammatory cells could have hindered the detection of any Ki-*ras* mutation. In view of the uniformity of BOP effects,[18] this speculation deserves consideration, although the possibility still remains that carcinoma #4 might be an exception, not containing any Ki-*ras* mutation.

The detected mutation in Ki-*ras* was identical with that described in tumors induced by BOP.[17,18] This specific G-to-A transition at the second position of codon 12 presumably reflects the mechanism of action of BHP, suggesting that this carcinogen also causes mutation of Ki-*ras* via the same proximate form as BOP, diazomethane, or through a compound which has similar properties to diazomethane.

The mutation demonstrated for experimental pancreatic tumors induced in Syrian hamsters by BHP or BOP is the same as that occurring in human pancreatic tumors.[2-6] The rat pancreatic carcinomas of acinar origin induced by azaserine, in contrast, are reported not to have this mutation of Ki-*ras*.[21] In addition to the similarity between the pathological characters of human and hamster pancreatic tumors,[11,12] the present findings reinforce the idea that these tumors of the two species may share other common features, such as susceptibility to various agents, underlying carcinogenic processes and further genetic changes in addition to that involving Ki-*ras*. The hamster model system is therefore useful for understanding human pancreatic carcinogenesis. Since the mutation rate is very high, it should be possible to clarify at which step of pancreatic carcinogenesis the Ki-*ras* alteration takes place using this model.

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