Low Incidence of Point Mutation of c-Ki-ras and N-ras Oncogenes in Human Hepatocellular Carcinoma

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We examined the incidence of point mutation in codons 12, 13 and 61 of c-Ki-ras and N-ras genes in human hepatocellular carcinoma (HCC) using the polymerase chain reaction and oligonucleotide hybridization techniques. Among 34 tissue specimens surgically resected from 30 patients and 5 cell lines of human HCC, only two had *ras* point mutations; in one case, codon 12 of c-Ki-ras was altered from GGT, coding glycine, to GTT, coding valine; in the other case, codon 61 of N-ras was altered from CAA, coding glutamine, to AAA, coding lysine. Thus, point-mutational activation of *ras* oncogenes is an uncommon event in human HCC.

Key words: *ras* oncogene — Mutational activation — Human hepatocellular carcinoma

In human pancreatic carcinomas, the c-Ki-ras gene has been found to show point mutation at codon 12 in almost all cases.¹⁾ Since both the pancreas and the liver originate from the distal end of the foregut during the process of embryonal development, there may be a common mechanism of carcinogenesis between hepatocellular carcinoma (HCC) and pancreatic carcinoma. We previously examined *ras* activation by immunoblotting analysis using a monoclonal antibody reactive with *ras* p21 protein, and two out of 21 cases of HCC were suspected to have activated *ras* genes as revealed by a change in electrophoretic mobility of *ras* p21.²⁾ In the present study, using the polymerase chain reaction and oligonucleotide hybridization techniques, we examined the incidence of point mutation in the *ras* gene family in cases of human HCC in order to clarify the role of *ras* gene activation in human hepatocarcinogenesis.

High-molecular-weight DNA was extracted by the standard method³⁾ from 5 human HCC cell lines including the PLC/PRF/5 cell line⁴⁾ and 4 others established in our laboratory, i.e., C-Li-7, C-Li-21, C-Li-22 and C-Li-24, and from 34 resected tissue specimens of HCC from 30 patients who had been undergone partial hepatic resection at the National Cancer Center Hospital, Tokyo, from 1985 through 1988. One microgram of DNA was amplified *in vitro* using the polymerase chain reaction method described by Kogan *et al.*⁵⁾ The sequences of specific oligonucleotide primers and probes corresponding to all of the possible amino acid sequences at codons 12, 13 and 61 for each of c-Ki-ras and N-ras were the same as those described by Verlaan-de Vries *et al.*⁶⁾ In

total, 4 kinds of pairs of primers and 44 kinds of probes were synthesized on an Applied Biosystems model 380A DNA synthesizer (Applied Biosystems Japan Inc., Tokyo) by the β -cyanoethyl phosphoramidite method. The amplified DNA samples were subsequently spotted onto nylon filters (Gene Screen Plus, New England Nuclear, Boston) using a Bio-Dot apparatus (Nippon Bio-Rad Laboratories, Tokyo). The hybridization technique employed, using an oligonucleotide probe labeled with [γ -³²P]ATP, has also been described by Verlaan-de Vries *et al.*⁶⁾ and Wood *et al.*⁷⁾ As a positive control for this experiment, DNA was isolated from 4 other cell lines, C-Lu-65 (human giant cell lung carcinoma with c-Ki-ras mutation at codon 12, GGT to TGT), HL60 (human promyelocytic leukemia with N-ras mutation at codon 61, CAA to CTA), NTF-7 (NIH3T3 transformant carrying mutated N-ras at codon 61, CAA to AAA, of the human neuroblastoma cell line SK-N-SH), and 8C-7-2 (NIH3T3 transformant carrying mutated N-ras at codon 13, GGT to CGT, derived from human rectal carcinoma).⁸⁻¹¹⁾

In each sample of positive control DNA, we were able to detect the mutant codon in c-Ki-ras or N-ras in an identical way to that previously described. Among the 34 HCC specimens from 30 patients and 5 human HCC cell lines, point mutation of c-Ki-ras or N-ras was detected in only two cases (Table I). In one case (sample 7), encoded glycine (GGT) was substituted by valine (GTT) at codon 12 of c-Ki-ras (Fig. 1A). In the other case (sample 29), encoded glutamine (CAA) was substituted by lysine (AAA) at codon 61 of N-ras (Fig. 1B). In both cases (samples number 7 and 29), wild-type c-Ki-ras and N-ras were also detected. It is uncertain whether the presence of wild-type c-Ki-ras and N-ras was due to

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Table I. Clinical and Pathological Features of Two Cases of Hepatocellular Carcinoma Carrying Point-mutated *ras* Gene

Case/Age/Sex	Codon	Histologic grade ¹⁶⁾	Size (cm)	Non-cancerous liver	Preoperative therapy
14/64/F	c-Ki- <i>ras</i> (GGT ₁₂ -GTT)	M/D	3.5	cirrhosis	TAE
23/68/M	N- <i>ras</i> (CAA ₆₁ -AAA)	M/D	7.5	cirrhosis	TAE

TAE, transcatheter arterial embolization.

M/D, moderately differentiated.

Fig. 1. Dot-blot analysis of enzymatically amplified DNA samples from HCC specimens. DNAs were isolated from 34 resected samples from 30 patients who underwent surgery for the tumor, 5 samples of human HCC cell lines, and 4 control cell lines carrying activated *ras* genes. The DNA was enzymatically amplified *in vitro* by the polymerase chain reaction (PCR) method⁵⁾ and screened for mutations in codons 12, 13 and 61 of c-Ki-*ras* (A) and N-*ras* (B). Figure 1A shows hybridization of amplified DNA samples to oligonucleotide probes corresponding to the wild-type sequence GGT of c-Ki-*ras*, codon 12 (upper), to the mutant sequence GTT of c-Ki-*ras*, codon 12 (middle) and to the mutant sequence TGT of c-Ki-*ras*, codon 12 (lower). One sample (number 7) shows a mutation at codon 12, where glycine is substituted by valine. The positive control C-Lu-65 cell line (number 40) also shows point mutation which is of the same type as reported.⁸⁾ Figure 1B shows hybridization of HCC DNA samples to oligonucleotide probes corresponding to the wild-type sequence CAA of N-*ras*, codon 61 (upper), to the mutant sequence AAA of N-*ras*, codon 61 (middle) and to the mutant sequence CTA of N-*ras*, codon 61 (lower). One sample (number 29) shows a mutation at codon 61, where glutamine is substituted by lysine. The positive controls NTF-7 (number 41) and HL-60 (number 42) also show the same point mutations as reported.^{9,10)} Numbers and corresponding amplified DNAs are as follows: 1 to 5, cell lines of human HCC (C-Li-21, C-Li-22, C-Li-24, C-Li-7 and PLC/PRF/5); 6 to 39, surgically resected HCC tissues from 30 different patients. Each of the pairs 18 and 19, 20 and 21, 23 and 24, and 31 and 32 are two samples obtained from different tumors in 4 patients with multiple HCC, 40; C-Lu-65 cell line, 41; NTF-7 cell line, 42; HL-60 cell line. The small signals at the positions of number 16 in the lower part of Fig. 1A and number 19 and 32 in the middle of Fig. 1B are nonspecific radioisotopic contamination. The faint signals at the positions of number 1 to 3 in the lower of Fig. 1A are due to overloading of PCR products.

contamination by non-cancerous cells or to the presence of the normal allele in HCC. There was no point mutation at codon 13 or 61 of c-Ki-ras, or at codons 12 or 13 of N-ras in any of the cases examined. Some of the negative results are also shown in Fig. 1A and 1B.

The clinical and pathological findings in the two cases carrying point-mutated *ras* genes are summarized in Table I. Since there were very few cases with point mutations of *ras* genes, it was not possible at this stage to discuss any correlation between the *ras* mutation and clinical or histological findings.

Point mutation of c-Ki-ras gene is reported to be high in carcinomas of colon and pancreas.^{1,12)} Compared with these results, the incidences of c-Ki-ras and N-ras mutations appear to be very low in human HCC. NIH3T3 transfection assay has failed to show the presence of an activated *ras* gene in human HCC,^{13,14)} except for one report by Gu *et al.*,¹⁵⁾ in which an activated N-ras gene was identified in transfectants from DNAs of 3 HCC cases and one HCC cell line, although they did not mention the alteration of DNA sequence of the transfected N-ras gene. This result was inconsistent with the results from our laboratory and the others. The reasons for the discrepancy remain unknown. The lack of any report of c-Ha-ras activation, which is considered to be very easily detectable by transfection assay, together with the present results, suggests that point-mutational activation of the *ras* gene family is not involved in the carcinogenesis or progression of HCC in the majority of cases. However, the possibility of *ras* gene activation in human HCC by point mutation in different positions, for example, codons 63, 116 to 120 and 145 to 147 still remains to be clarified. Gene amplification was also very

rare in HCC, at least for c-myc, c-erbB-2 and *hst-1*, which we examined (data not shown). Other genetic alterations, such as integration of viral DNA, chromosomal deletion or translocation, seem to be much more important. *lca* gene was isolated from human HCC DNA as a transforming gene, but its importance in human hepatocarcinogenesis is still unclear.¹³⁾ It is of considerable interest that a discrepancy exists in the incidence of mutational activation of *ras* genes between pancreatic carcinoma and HCC. Apart from these two carcinomas, there are several kinds of carcinoma occurring in the hepatobiliary and pancreatic system, i.e., cholangiocellular carcinoma, and carcinoma of the gallbladder, duodenum or Vater's papilla. It will therefore be important to examine *ras* activation in these kinds of carcinoma, in order to clarify the role of *ras* activation in carcinogenesis in the hepatobiliary system.

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