Jpn. J. Cancer Res. **81**, 22–27, January 1990

Point Mutations of c-*ras* Genes in Human Bladder Cancer and Kidney Cancer

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Point mutations of c-*ras* genes at codons 12, 13 and 61 were analyzed in 26 cases of bladder cancer and 16 cases of kidney cancer. DNA prepared from either frozen tissues or 10% formalin-fixed, paraffin-embedded tissues were amplified by means of polymerase chain reaction methods, and mutations were analyzed by dot blot hybridization assays with oligonucleotide probes. In three cases of bladder cancer c-*ras* mutations were found, at codons 13 and 61 of c-Ha-*ras* and at codon 61 of c-Ki-*ras*, while no mutation was found in kidney cancer. No mutation was found in normal bladder epithelial tissues from the same patients. Our findings, taken together, may indicate relative scarcity of c-*ras* mutations in these types of human cancer. The results of dot blot hybridization assays and DNA sequencing showed a G-to-C transition of the first nucleotide at codon 13 of c-Ha-*ras*. This is the first time that such a point mutation has been detected in human cancer tissues.

Key words: c-*ras* — Point mutation — Polymerase chain reaction — Bladder cancer — Kidney cancer

Analysis of cellular DNA of human cancer cells in transformation assays identified activated c-*ras* genes with point mutations.[1] These point mutations have so far been observed only at codons 12, 13 and 61 of the c-*ras* gene family although mutations in other codons were shown to lead to activation of genes in *in vitro* experimental systems.[2–4] The more recently devised polymerase chain reaction (PCR) and hybridization assays with oligonucleotide probes have allowed precise analyses of the profiles of point mutations by examining a wide variety of human cancer tissues as well as cell lines.[5]

Analyses of a large number of human cancer tissues such as colorectal cancer,[6,7] pancreatic cancer[8,9] and lung cancer[10] resulted in the identification of frequent mutations of c-Ki-*ras* at codon 12, confirming the results of the initial analyses of a limited number of samples utilizing transformation assays. On the other hand, in human cancers of ovary,[11] liver,[12] esophagus,[13] mammary glands,[14] and lymphoid tissues[15,16] c-*ras* mutations at the examined codons seem to be much less frequent.

There has been no report of examination of human cancers of the urinary tract in this regard by utilizing PCR and hybridization assays with oligonucleotide probes. We therefore analyzed 26 cases of bladder cancer and 16 cases of kidney cancer in terms of point mutations of codons 12, 13 and 61 of three c-*ras* genes.

MATERIALS AND METHODS

Tumor specimens Frozen specimens and 10% formalin-fixed and paraffin-embedded specimens of bladder cancer and kidney cancer were obtained from the Laboratory of Chemotherapy, Aichi Cancer Center Research Institute, Nagoya (cases 1–17 were bladder cancers and 9 cases were kidney cancers) and also from the Department of Urology, Center for Adult Diseases, Osaka (cases 18–26 were bladder cancers and 7 cases were kidney cancers). Histological classification and grading of malignancies were done according to the General Rules for Clinical and Pathological Studies on Bladder Cancer by the Japanese Urological Association and The Japanese Pathological Society. All examined samples contained more than 50% of cancer cells on a morphological basis.

Preparation of cellular DNA Cellular DNA was prepared from frozen tissues by the method of Blin and Stafford[17] and also from the paraffin-embedded sections according to the method of Shibata *et al.*[18] for PCR-mediated amplification.

Synthetic oligonucleotides The oligonucleotides were synthesized by a DNA synthesizer (model 380 B, Applied Biosystems, Foster City, CA). Sequences of oligonucleotide primers for PCR and oligonucleotide probes for detection of c-*ras* mutations were as described by Verlaan-de Vries *et al.*[19] with slight modifications.[15]

PCR DNA amplification *in vitro* was performed as described previously.[20,21] Briefly, the reaction mixture

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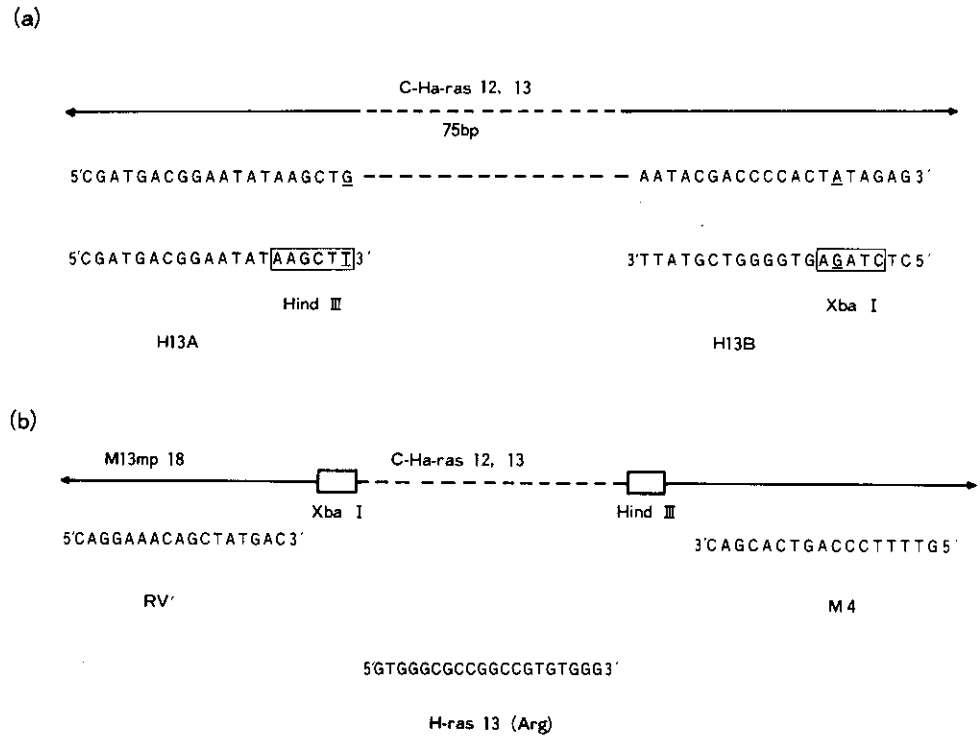

Fig. 1. (a) To determine the sequence of c-Ha-*ras* with the mutation at codon 13, two primers (H13A, H13B) were used for amplification in the PCR. The H13A primer contained a single base change to generate a *Hind* III site (boxed), and the H13B primer also contained a single base change to generate a *Xba* I site (boxed). (b) Two primers (RV', M4) of M13mp18 vector were used in the PCR for the screening of the c-Ha-*ras* gene with the mutated codon 13. An internal oligomer, H-ras 13 (Arg), was used to detect amplified fragments.

contained 1 μg of chromosomal DNA or tissue pellets from paraffin sections, 0.6 μg of each primer, 800 μM of the dNTPs, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 200 μg/ml BSA, and 2 units of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) in a total volume of 100 μl. The mixture was incubated at 95°C for 60 s to denature the DNA and the primers were allowed to anneal to the DNA at 48–55°C for 90 s. Primer extension was performed at 72°C for 90 s. Thirty-five to forty cycles of denaturation, hybridization and extension of DNA were repeated by an automated heat-block (Program Temp Control System PC.500, Astec Inc., Fukuoka).

Dot blot hybridization The method described by Verlaan-de Vries *et al.*[19] was used with slight modifications.[15] The amplified DNA (10 μl) was spotted onto nylon filters (Gene Screen Plus; New England Nuclear, Boston, MA). The filters were dried and prehybridized for 3–16 h at 56°C in 3.0 M tetramethylammonium chloride, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 100 μg/ml of sonicated and denatured salmon sperm DNA, 0.1% SDS and 5×Denhardt's solution, and subsequently hybridized for 1 h at 56°C in the same mixture containing ^{32}P-labeled oligomer probes. The probes were labeled at the 5′ end using [γ-^{32}P]ATP and T4-polynucleotide kinase. The filters were washed twice in 2×SSC, 0.1% SDS for 10 min at room temperature then stringently washed twice in 5×SSC, 0.1% SDS for 10 min at temperatures ranging from 59°C to 71°C. Subsequently, the filters were washed twice in the hybridization buffer without Denhardt's solution and salmon sperm DNA and were incubated for 1 h in the same solution at 59–60°C. The filters were then exposed to Kodak XAR-5 films at −70°C using intensifying screens.

DNA sequencing of the mutated c-Ha-*ras* Two 20-base oligonucleotide primers (H13A, H13B) amplify a 115-bp segment containing a codon 13 of the c-Ha-*ras* gene. Primers were modified with single base changes to produce convenient restriction sites (*Hind* III, *Xba* I) for cloning into M13mp18 sequencing vector (Fig. 1(a)). After amplification, the PCR products were cleaved with the appropriate restriction enzymes. These fragments

were ligated into the M13mp18 vector, transfected into the JM109 host, and plated out. Phage DNA templates from the resulting colorless colonies were amplified for a 214-bp segment using two 17-base M13 primers (RV′, M4) (Fig. 1(b)). The amplified DNAs were spotted onto nylon filters, and were screened by hybridization with a labeled oligonucleotide H-ras 13 (Arg). The detected clone was sequenced by the chain-termination method with dideoxy nucleotides.[22]

RESULTS

Analysis of codons 12, 13 and 61 of c-*ras* genes by oligonucleotides DNAs either obtained from frozen tissues or paraffin-embedded tissue specimens of bladder cancer and kidney cancer were amplified by means of PCR using the primers.[15] The presence of point mutations was examined by dot blot hybridization assays utilizing the synthesized oligonucleotide probes.[15] In all samples, all possible mutations at codons 12, 13 and 61 of c-Ki-*ras*, c-N-*ras* and c-Ha-*ras* genes were examined. By analyzing 26 cases of bladder cancer and 16 cases of kidney cancer, point mutations of c-*ras* genes were found in three cases of bladder cancer, at codons 13 and 61 of c-Ha-*ras* and at codon 61 of c-Ki-*ras*, as shown in Fig. 2 (a) and Table I. No mutations of c-*ras* genes at the 3 codons examined were found in 16 cases of kidney cancer. Normal epithelial bladder tissues of three bladder cancer patients with point mutations showed no mutation of c-*ras* genes at codons 12, 13 or 61 (Fig. 2(b)).

Sequencing of codon 13 of c-Ha-*ras* in a case of bladder cancer While analysis of dot blot hybridization assays with oligonucleotide probes suggested that a point mutation of codon 13 of c-Ha-*ras* found in a case of bladder cancer is a G-to-C transition of the first nucleotide, we confirmed this by sequencing cellular DNA since such a point mutation has never previously been observed in human cancer tissues.

As shown in Fig. 3, a single base change within the 13th codon in the c-Ha-*ras* gene was seen. Instead of the normal GGT at codon 13, a G-to-C transition was detected at the first nucleotide. This should result in an alteration of glycine to arginine at this position in the p21 protein of c-Ha-*ras*.

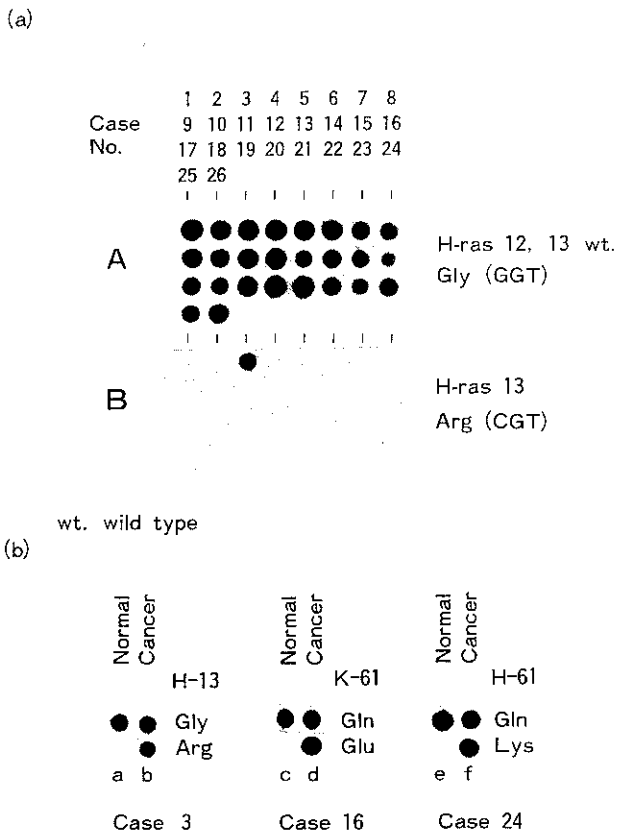

Fig. 2. (a) Characterization of point mutations at codon 13 of the c-Ha-*ras* gene. Panel A shows hybridization of the dot blot to an oligonucleotide probe with the normal (wild-type; wt.) sequence GGT of codon 13. Panel B shows hybridization of the same dot blot to a probe with the mutated sequence CGT of codon 13, revealing the presence of this mutation in one DNA specimen (from case 3). The negative hybridizations are not shown. (b) Oligomer hybridizations to DNA from bladder tumors (dots b, d, f) and normal epithelial tissues of bladder from corresponding individuals (dots a, c, e). Autoradiographs of dot blots of amplified DNA preparations from cases 3, 16 and 24 hybridized to oligomers specific for wild-type H-13, K-61 and H-61 (upper) and also those for mutated types (lower) are shown.

DISCUSSION

In three out of 26 cases of bladder cancer, point mutations of c-*ras* genes were found, while none of the 16 cases of kidney cancer showed the mutation. These results may suggest that point mutations of c-*ras* genes at codons 12, 13 and 61 are rather infrequent in these types of cancer, particularly in kidney cancer. It should be mentioned that in our standardized assays, the presence of c-*ras* point mutations can be detected with confidence when more than 10% of cells in examined samples contain mutations. Though all samples we examined contained more than 50% of cancer cells on a morphological basis, small populations with mutated genes might have been missed. Our findings are, however, essentially compatible with previous reports on human bladder cancer and kidney cancer, in which point mutations of c-*ras* genes were infrequently found by *in vitro* transfection assays.[23, 24]

Table I. A Summary of Mutations of c-*ras* in Human Bladder Cancer

Case	Age	Sex	Histology	Grade	Mutated ras (amino acid)	Others
1	72	M	TCC	G3		
2	61	M	TCC	G3		
3	53	F	TCC	G2	H13 (Arg)	normal(−)
4	60	M	TCC	G2		
5	64	M	TCC	G2		
6	74	M	TCC	G2		
7	57	M	TCC	G3		
8	60	M	TCC	G2		
9	67	F	TCC	G2		
10	77	M	TCC	G2		
11	88	M	TCC	G2		
12	81	M	TCC	G2		
13	68	M	TCC	G3		
14	54	M	TCC	G2		
15	75	M	TCC	G1		
16	64	M	UC	G3	K61 (Glu)	normal(−)
17	52	M	TCC	G2		
18	48	F	TCC	G2		
19	67	F	TCC	G2		
20	65	M	TCC	G1		
21	77	F	TCC	G2		
22	77	F	TCC	G3		
23	75	M	TCC	G1		
24	61	M	inverted papilloma	G1-0	H61 (Lys)	normal(−)
25	82	M	TCC	G3		
26	60	F	TCC	G2		

normal(−): No mutation of c-*ras* in normal bladder epithelial tissues. TCC: transitional cell carcinoma. UC: undifferentiated carcinoma.

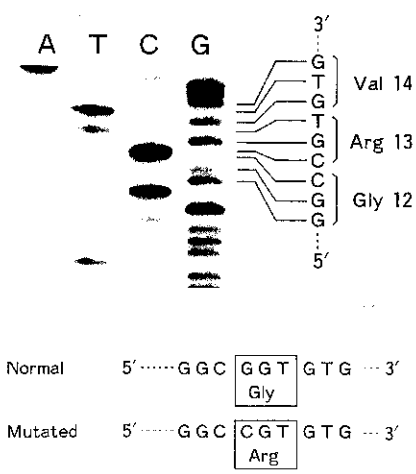

Fig. 3. The nucleotide sequence of the mutated c-Ha-*ras* gene in case 3 of bladder cancer is shown. The sequence was determined by the M13, dideoxy method using vector M13mp18.

Point mutations found in this study were confined to cancer tissues; no mutation was detectable in normal bladder epithelial tissues of the same three patients. The sites and modes of the mutations were variable among the three cases as shown in Table I. Reported analyses of various human cancer tissues and cell lines have indicated certain tendencies of point mutation patterns in terms of cancer types,[5] although no relationship has been strictly proven. For example, mutations at codon 12 of c-Ki-*ras* were most commonly observed in several epithelial malignancies such as colon cancer,[6,7] pancreatic cancer[8,9] and lung cancer.[10] On the other hand, point mutations of c-N-*ras* were relatively common in malignancies of myelogenous cells.[25] Bladder cancer, however, seems to exhibit some contrast to the above cancers. Data collected by us and others, show that mutations of c-*ras* genes in bladder cancer seem to take place in any member of the c-*ras* gene family, although mutations of c-Ha-*ras* gene are the most frequent.[23] Although the machinery to

transform wild-type c-*ras* genes into mutated genes is totally unknown, the multiplicity of mutation sites in bladder cancer might be associated with variations in oncogenic processing in this particular type of cancer.

Experiments with artificial mutagenesis of c-Ha-*ras* gene showed that point mutations at codons 13, 59, 63, 116 and 119, in addition to codons 12 and 61, could activate *ras* genes.[2-4] In these experiments, codon 13 GGC (Gly) was mutated into GAC (Asp), in which case the c-Ha-*ras* gene acquired transforming activities in culture.[2] In two cases of furfural-induced murine liver tumors, activated c-Ha-*ras* genes with a Gly-to-Val mutation or a Gly-to-Arg mutation at codon 13 were observed.[26] Recently, Tadokoro *et al.*[27] reported codon 13 mutation of c-Ha-*ras* for the first time in a cell line of oral squamous cell carcinoma. In their study a Gly-to-Arg mutation was found at codon 13, caused by a G-to-C transition in the first nucleotide of the codon, which was identical with our finding in a bladder cancer tissue. This may indicate that other types of c-Ha-*ras* mutations at codon 13 might also be observed by more intensive analysis of human cancers.

A series of initial experiments with *in vitro* and *in vivo* transformation assays mostly identified mutations at codons 12 and 61 of c-*ras* genes. While these mutations might be dominant in a variety of human cancers, there is no reason to exclude other potential mutations. In fact, in our recent study of gastro-intestinal cancers,[28] point mutations at codon 13 of c-Ki-*ras* were as dominant as those at codon 12 of c-Ki-*ras*, which were previously reported to be dominant in colon cancers.

This is particularly important because it is still unknown whether particular types of amino acid changes caused by changes at these codons result in greater potential transforming activities than other types of amino acid changes. Alternatively, the frequencies and types of mutations might be solely determined at the DNA level, in which case an amino acid change itself is more crucial than the nature of the altered amino acid. In either case, until we obtain solid biological evidence in this regard, various potential changes in c-*ras* have to be looked for in human cancers.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science and Culture, and by grants from the Japanese Ministry of Health and Welfare.

(Received October 6, 1989/Accepted December 1, 1989)

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