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Rapid Detection of H-*RAS* Point Mutation Using Two-Step Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

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Abstract: Mutations in codon 12, 13 and 61 of one of the three *ras* genes, H-*ras*, K-*ras* and N-*ras*, convert these genes into active oncogenes. The presence of H-*ras* gene mutations have important prognostic implications in various cancers. In this study, the H-*ras* gene mutations were investigated by two-step PCR-RFLP in patients with bladder and stomach cancer. For the control experiments, T24 and SK2 cell lines were used. In a total of 36 bladder cancer patient cases, five (13.9%) mutations were found by this method. Of these, point 12 mutations were two (5.6%) cases and point 61 mutations were three (8.3%) cases. On the other hand, H-*ras* mutation was not found in 29 cases of stomach cancer. The results of the mutated H-*ras* gene confirmed by direct sequencing analysis were correlated well with PCR analysis. From the sensitivity test, the H-*ras* mutation was found to have about 0.2% of mutated DNA mingled in normal DNA. In conclusion, the H-*ras* mutation has a higher clinical significance in bladder cancer than stomach cancer. Moreover the two-step PCR-RFLP method is sensitive, rapid and relatively simple for clinical work in detecting H-*ras* point mutations.

Key words: H-*ras* point mutation, two-step PCR-RFLP.

The most frequently identified transformation-associated genes found in human solid tumors are members of the *ras* family, H-*ras*, K-*ras* and N-*ras* (Bos, 1989). It has been well established that specific alterations in members of the *ras* gene family can convert them into active oncogenes (Shimizu *et al.*, 1983; Uchida *et al.*, 1995). The fact that *ras* sequences are expressed suggests an important physiological function for members of this proto-oncogene. Point mutations at codon 12, 13, and 61 of the *ras* gene result in an equilibrium shift of *ras* proteins (p21) toward the activated state, which constitutively activates the mitogenic signal transduction pathway (McGrath *et al.*, 1984). Such mutations contribute to progression of tumors rather than initiation of malignancy. The *ras* mutations were found to be approximately 10~18% in human solid tumors (Saito, 1992; Burchill *et al.*, 1994). Mutated *ras* proteins have been reported to induce transformation of NIH/3T3 cells *in vitro* (Shih and Weinberg, 1982). Since these studies utilize a DNA transfection assay in which some of the *ras* oncogenes may escape detection, the incidence of point mutation must be investigated by a more accurate method. Recently, with the development of polymerase chain reaction (PCR) technology (Radnall *et al.*, 1989), several methods, single strand conformation polymorphism (SSCP) (Sugano *et al.*, 1993), dot hybridization (Koh *et al.*, 1992), and direct sequencing (McMahon *et al.*, 1987) have been introduced. In this study, a restriction fragment length polymorphism (RFLP) technique was used for the detection of the H-*ras* gene mutation. We have extended and modified that method for what is called two-step PCR-RFLP (Saito, 1992; Cerutti *et al.*, 1994) and the usefulness of this technique was evaluated in patients with bladder and stomach cancers.

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Materials and Methods

Cell lines and samples

The bladder cell line T24 (Evans *et al.*, 1977) which contains GGC (Gly) to GTC (Val) substitution at the codon 12 and the melanoma cell line SK2 which contains CAG (Gln) to CTG (Leu) substitution at codon 61 of H-*ras* (Sekiya *et al.*, 1984) were used as positive controls (Reddy *et al.*, 1982). Thirty six cases of bladder cancer and 29 cases of stomach cancer were used for the evaluation of clinical usefulness.

Table 1. Oligonucleotide primers for the detection of H-*ras* gene mutation

	Primer	Sequence (5'→3')
Point 12	outer, sense	AGGAGCGATGACGGAATATAAGC
	antisense	GGCTCACCTCTATAGTGGGGTCGTATT
	inner, sense	AATATAAGCTGGTGGTGGTGGGCGC
	antisense	GGGGTCGTATTCGTCCACAAAATG
Point 61	outer, sense	GTGGTCATTGATGGGGAGACGTGC
	antisense	CTCACGGGGTTCACCTGTACTGGT
	inner, sence	TGCCTGTTGGACATCCGGGATACCGCC
	antisense	CTGGTGGATGTCCTCAAAAGACTTG

Synthesis of primers

The sequences of the oligomers are listed in Table 1. Amplification primers were synthesized using a 392 DNA/RNA synthesizer and purified by an oligonucleotide purification cartridge (Applied Biosystem, Foster City, USA).

Extraction and amplification of DNA

Genomic DNA was prepared from tumor cells and peripheral blood mononuclear cells by proteinase K digestion and phenol/chloroform extractions (Laborca and Paigen, 1980). The PCR reactions were carried out in 20 ul volumes, containing 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% Triton X-100, 1.5 mM $MgCl_2$), 0.2 mM of each dNTPs, 0.5 U of *Taq* DNA polymerase, 10 pmol of primers and a 0.2 μg of target DNA. The reaction mix was heated to 95℃ for 5 min to denature the DNA. PCR was performed for 12 cycles at 95℃ for 1 min (denature), 57℃ for 1.5 min (annealing), and 72℃ for 2 min (extension) using a GeneAmp PCR system 9600 (Perkin-Elmer Cetus Ins, Norwalk, USA). After first digestion of the PCR product, the second PCR was carried out for 30 cycles with the same conditions as the first PCR and digested with the same restriction enzymes used for the first PCR. Each typing was run with a negative control containing distilled water and a normal control containing normal type DNA. Fig. 1 shows the principle of two-step PCR-RFLP used in this study.

Restriction enzyme analysis and visualization

The nucleotide sequences of the H-*ras* gene were analyzed using the PC/GENE computer program (Intelligenetics Inc., Moutain View, USA) to search for restriction enzymes which have cleavage sites in point 12 and 61. Two different restriction enzymes, *Nae*I and *Msp*I, were selected for H-*ras* point 12. In order to differentiate the H-*ras* point 61, restriction enzymes *Bst*NI and *Eae*I were used. Five microliter aliquots of the PCR product were digested by 5 units of restriction enzymes in the appropriate buffer for 4 h at each optimum incubation temperature in a final volume of 20 μl. The amplified DNAs cleaved by the restriction enzymes were subjected to 13% polyacrylamide gel at 100 volts for 3 h in a Mini-Protean II cell apparatus (Bio Rad Co. Hercules, USA) for precise analysis of RFLP bands. The DNA fragments were visualized by staining with ethidium bromide and compared with a positive and normal control (Elder and Southern, 1983).

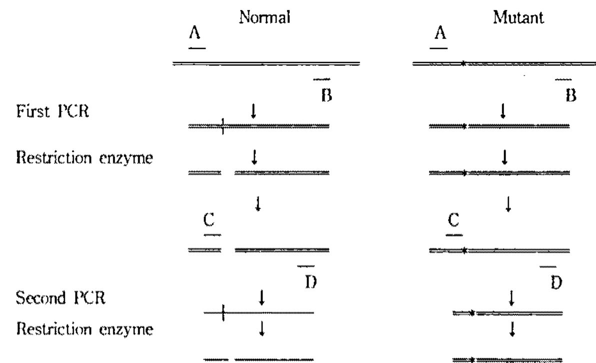

Fig. 1. H-*ras* mutation analysis using two-step PCR-RFLP. The DNA, which may contain a mutation (*), is amplified using outer primers A and B. Incubation with the restriction enzyme cleaves the amplified wild type sequence and leaves mutant sequence intact. The inner primers C and D are used in a second PCR, and now the uncleaved mutant sequence is amplified. The products of the second PCR are incubated with the restriction enzyme. The restriction enzyme-resistant DNA fragment is diagnostic for the presence of a mutant.

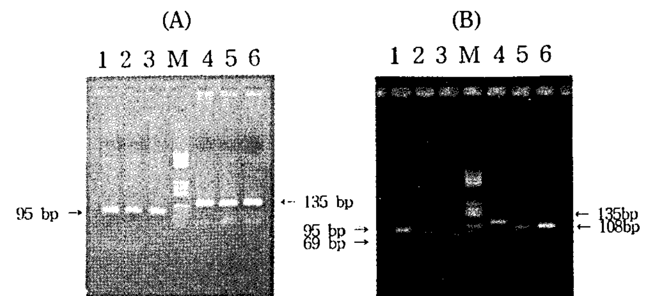

Fig. 2. Analysis of amplified DNA including codon 12 or codon 61. The DNA bands were analysed by 2% agarose gel electrophoresis and stained with ethidium bromide. Size marker (M) was used with pBR322 digested with *Hae*III. (A) Lane 1: codon 12 of T24; lanes 2, 3: codon 12 of normal cell; lane 4: codon 61 of SK2; lanes 5 and 6: codon 61 of normal cell. (B) Lane 1: T24 sample restricted with *Nae*I; lane 2: normal sample restricted with *Nae*I; lane 3: normal sample restricted with *Msp*I; lane 4: SK2 sample restricted with *Eae*I; lane 5: normal sample restricted with *Eae*I; lane 6: normal sample restricted with *Bst*NI.

The sensitivity of two step PCR-RFLP

The sensitivity of PCR for the detection of H-*ras*

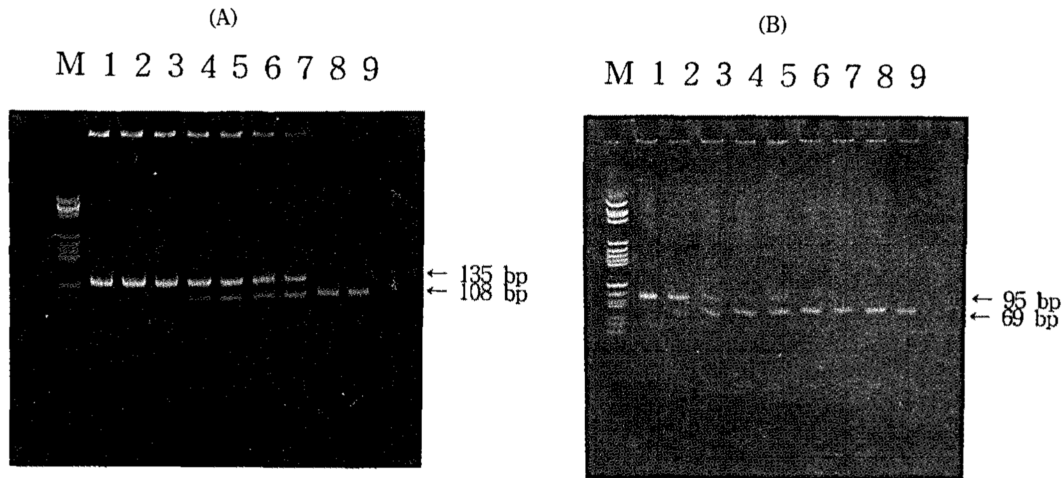

Fig. 3. The sensitivity of PCR for the detection of H-*ras* gene mutated cells contaminated with normal cells. The PCR bands were detected by 13% polyacrylamide gel electrophoresis stained with ethidium bromide. pBR322 digested with *Hae*III was used as size marker. (A) DNA from SK2 and normal cell were mixed at each consistency as below table. Amplified PCR products were digested with *Eae*I at codon 61. (B) DNA from T24 and normal cell were mixed at each consistency, and amplified PCR products were digested with *Nae*I at codon 12.

	1	2	3	4	5	6	7	8	9
Normal	0	250	450	490	495	499	499.5	499.9	500 ng
Control	500	250	50	10	5	1	0.5	0.1	0 ng

gene mutation was analysed using standard cell lines. DNAs isolated from positive control cells, T24 and SK2 cells, and normal cells were mixed as shown in Fig. 3.

Direct sequencing analysis

Direct sequencing of the PCR product was analyzed using a commercial kit (USB, Cleveland, USA). The PCR products by inner primer were purified by a DNA purification system (Wizard PCR preps, Promega, Medison, USA). Ten microliter aliquots of purified PCR product was treated with 1 μl of exonuclease I (1.0 U/mL) for 15 min at 37℃ and at 80℃ for 15 min. The same procedure was repeated with 1 μl of alkaline phosphatase (2.0 U/μl). A sample of 5 μl of PCR product was mixed with 4 μl of distilled water and 1 μl of primer (5 pmol/μl). After boiling for 2~3 min, the mixture was cooled as quickly as possible by placing the vial in ice for 5 min. Then 10 μl of DNA mixture was mixed with 2 μl of sequencing reaction buffer (80 mM of Tris-HCl [pH 8.3], 140 mM of KCl and 10 mM of $MgCl_2$) to which was added 1 μl of 10 mM dithiothreitol (DTT), 2 μl of diluted dGTP labelling mixture and 0.5 μl of ^{35}S-dATP (250 μCi/μl). The amplified PCR products were incubated to anneal with the primer at room temperature for 5 min, and 2 μl each of four dideoxy nucleotide mixtures was added. Ten units of DNA polymerase were added to each dideoxy reaction mixtures and, incubated at 37℃ for 20 min. The sequence information was obtained after electrophoresis of 2 μl of the final reaction mixtures onto a 7% polyacrylamide-urea sequencing gel followed by autoradiography (Feinberg and Vogelstein, 1984).

Results

T24 and SK2 control cell lines have mutations in point 12 and point 61, respectively. Fig. 2A and B shows amplified DNA fragments by second PCR and the DNA fragments of control cell digested by restriction enzymes-*Nae*I and *Msp*I for point 12 and *Bst*NI, *Eae*I for point 61. The DNA fragments of PCR products restricted by specific enzymes are listed in Table 2. First and second PCR products of point 12 were 125 bp and 95 bp, respectively. On the other hand, the PCR products of point 61 were 175 bp in the first PCR and 135 bp in the second PCR. The restricted size of a second PCR product by specific enzymes was 69 bp on point 12 and 108 bp on point 61 (Table 2). In the sensitivity test for two-step PCR-RFLP, serial dilutions were run with control cells as described in the Method section. Mutations are readily detectable even when diluted with 1 ng of control cell DNA in 499 ng with normal cell DNA (Fig. 3A and 3B).

The frequencies of H-*ras* gene mutation in bladder and stomach cancer were analyzed. Five (13.9%) muta-

Table 2. DNA fragments by two step PCR-RFLP for the detection of H-*ras* mutations

Mutation position of H-*ras*	PCR	DNA fragment of PCR product	Restricted size with enzyme
Point 12	1st PCR	125	85, 40
	2nd PCR	95	69, 26
Point 61	1st PCR	175	127, 48
	2nd PCR	135	108, 27

Table 3. Frequency of H-*ras* point mutations using two step PCR-RFLP

Amino acid	Bladder cancer (n=36)	Stomach cancer (n=29)
Codon 12	2 (5.6%)	0
Codon 61	3 (8.3%)	0
Total	5 (13.9%)	0

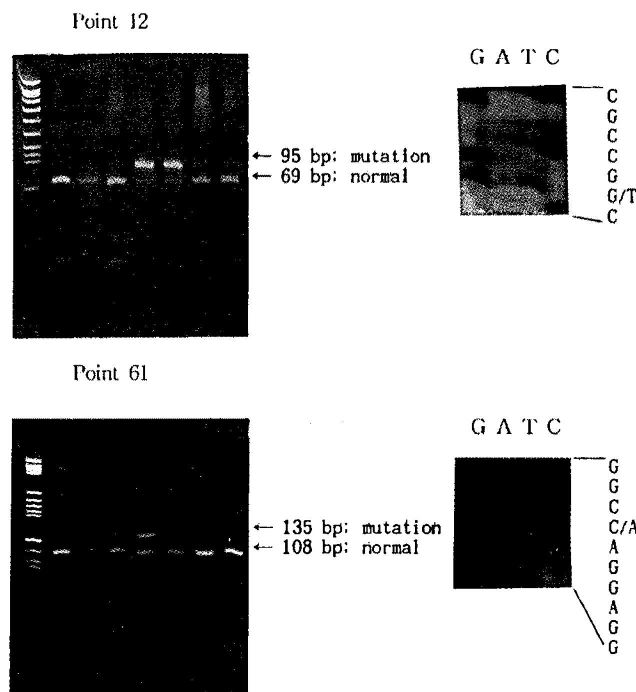

Fig. 4. Two-step PCR-RFLP and direct sequencing analysis of H-*ras* point mutation from patient samples contaminated with normal tissues. The amplified DNAs were cleaved by *NaeI* (point 12) or *BstNI* (point 61), and analysed by 13% polyacrylamide gel electrophoresis stained with ethidium bromide. Two bands were generated from mutated H-*ras* tumor samples contaminated with normal tissues. Only one bands were amplified in tumor samples with normal H-*ras* gene and normal tissues. From direct sequencing, mutated bases were found at point 12, GGC (Gly) to GTC (Val), and point 61, CAG (Gln) to AAG (Lys).

tions were detected among 36 bladder cancers. Of these, two (5.6%) were found in point 12 and three (8.3%) were in point 61 (Table 3). On the other hand, H-*ras* mutation was not found in 29 cases of stomach cancer (Table 3). Fig. 4 shows the point mutation in bladder cancer samples and the DNA sequences of PCR products confirmed by direct sequencing analysis. The glycine (GGC) was converted with valine (GTC) at codon 12, and glutamine (CAG) was altered into lysine (AAG) at codon 61.

Discussion

The *ras* oncogenes originally were isolated from Harvey and Kirsten murine sarcoma viruses (Bos, 1989). The *ras* sequences in these viruses were derived from rat DNA that had been transduced by murine retroviruses (Sekiya *et al.*, 1984). Subsequently, sequences corresponding to both Harvey (H-) and Kirsten (K-) *ras* genes were detected in human, avian, murine, and nonvertebrate genomes. A third member of the *ras* family, N-*ras*, was detected by its expression in human neuroblastoma and sarcoma cell lines (Mannem *et al.*, 1985). The *ras* genes are of three varieties, each with a similar exon-intron structure and each encoding a protein of 21 kilodaltons (p21). An H-*ras* gene from a bladder carcinoma cell line was isolated and characterized by its ability to transform the murine cell line NIH/3T3 (Shih and Weinberg, 1982). The H-*ras* genes are more preferentially activated in urinary tract tumor as compared to N-*ras* or K-*ras* genes (Visvanathan *et al.*, 1988). And codon 12 and 61 of the H-*ras* are the "hot spots" for the incidence of point mutation (Fujita *et al.*, 1984). In this study, detection of point mutations at codon 12 and 61 of the H-*ras* gene was attempted by a simple method involving PCR amplification. Adequate conditions for PCR, restriction enzyme digestion and direct sequencing analysis were established. The incidence of mutation of the H-*ras* gene in bladder cancer is estimated to be 5 to 17% (Visvanathan *et al.*, 1988). Nagata *et al.* (1990) reported that the incidence was approximately 12% using an NIH/3T 3 transfection assay. This rate is rather low compared with other cancers such as pancreatic cancer (90%) (Yamada *et al.*, 1986) and colon adenoma (40%) (Yuasa *et al.*, 1986). In the present study, five (13.9%) mutations in 36 bladder cancer samples were detected using two-step PCR-RFLP (Table 3). Of these, point 12 mutations constituted two cases and point 61 mutations 3 cases. Activated H-*ras* genes have been found occasionally in fresh gastric tumor tissue (O'Hara *et al.*, 1986). Several studies, however, have indicated the frequency of mutated *ras* genes in stomach cancer to be zero or extremely low (Nanus *et al.*, 1990). The NIH/3T3 assay failed to detect any activated *ras* genes

in a study comprising 26 primary stomach carcinomas (Sakato *et al.*, 1986). Two (12.5%) of 16 stomach cancer samples were determined by Koh *et al.* (1992). On the other hand, no *ras* mutations were reported in serveral studies (Sakato *et al.*, 1986; Jiang *et al.*, 1989; Nanus *et al.*, 1990). In this experiment, an H-*ras* mutation was also not found (Table 3). It is desirable to repeat this study in a larger group of stomach cancer samples. Consequently, mutational activation of *ras* genes presumably does not play a major role in the pathogenesis of stomach cancer.

The original assay to identify altered *ras* genes was based on the ability of the genes to transform the established mouse cell line NIH/3T3 (Shih and Weinberg, 1982). This assay was not suitable for the analysis of large numbers of tumors, mainly due to the laboriousness of the assay. The development of techniques for the detection of molecular lesions made it possible to diagnose many germlines and somatic diseases. PCR is one of such techniques that provide a simple, accurate and rapid assay. Presently, point mutations are usually detected by selective hybridization with synthetic oligodeoxynucleotide probes (Bos *et al.*, 1984), RNase mismatch cleavage (Winter *et al.*, 1985), dot blot analysis (Koh *et al.*, 1992), direct sequencing analysis (McMahon *et al.*, 1987; Meltzer *et al.*, 1990; Manam and Nicols, 1991), PCR-SSCP (Korn *et al.*, 1993; Sugano *et al.*, 1993) and PCR-RFLP (Saito, 1992). It is desirable to establish a simple method for reinspection of the incidence of point mutations in the *ras* gene. A simple assay to detect and characterize *ras* gene mutation should also be useful for a further understanding of their roles in carcinogenesis (Saito, 1992). In this study, new and rapid assay systems for the identification of a mutated H-*ras* gene using two-step PCR-RFLP was developed (Fig. 1). The PCR-RFLP, which depends mainly on the cleavage sites of the amplified segment identified by informative restriction enzymes, is relatively simple, fast and does not require the use of radioisotopes. We employed four pairs of primers to selectively amplify point 12 and 61 the of H-*ras* gene. A small amount of the mutated *ras* gene can be found by this two-step PCR-RFLP method. Moreover, this method is so simple and rapid that it can be used to detect mutations in blood, tissue and even paraffin block specimen. The results of control cells, T24 and SK2 detected by PCR-RFLP, were well correlated with previous reports (Reddy *et al.*, 1982). In the sensitivity test, less than 0.2% of mutated DNA mingled in normal DNA is readily detectable (Fig. 3A and 3B). Direct sequencing analysis to confirm the amplified PCR product showed the single base changes at points 12 and 61 of the H-*ras* gene in control cells (Fig. 4). In conclusion, this two-step PCR-RFLP method has made it possible to analyze large numbers of tumor samples for detection of the presence of H-*ras* genes. Moreover it is more simple, accurate and convenient for mutation study and can be used for routine clinical work.

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