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# Point Mutation at Codon 12 of the c-Ha-ras gene in Human Gastric Cancers[1]

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***The molecular mechanisms of the carcinogeneic process of gastric cancer have not been fully understood yet. In order to know whether c-Ha-ras gene is being involved in the process of gastric carcinogenesis, 8 gastric cancer cell lines, 8 cases of gastric cancer and same number of adjacent dysplasia were analyzed for the presence of mutation at codon 12, 13 and 61 of the c-Ha-ras gene by using polymerase chain reaction (PCR) and mutant-specific oligonucleotide hybridization. Point mutations at codon 12 of the c-Ha-ras gene were found in 2 out of 8 gastric cancer cell lines. The c-Ha-ras mutation at codon 12 was detected in both cancer and dysplasia samples in one case, but we found no mutation at codon 13 or 61 of the c-Ha-ras gene. These results suggest that the frequency of mutation of the c-Ha-ras gene detected by sensitive PCR technique is low indeed, however it would be notable that such a genetic change has been detected in the dysplastic lesion of the gastric cancer patient.***

**Key Words:** *c-Ha-ras gene, point mutation, codon, polymerase chain reaction*

## INTRODUCTION

Gastric adenocarcinoma is the leading cause of cancer death in Korea. The causative factors and molecular mechanisms of gastric carcinogenesis have not yet been fully determined. Though multiple, heterogeneous genes have been found to be correlated with these carcinogeneic processes, no dominant gene having significant role in carcinogenesis has been documented in gastric cancer (Ranzani et al., 1990). Several research efforts were focused on ras gene for last few years, for instance, dietary nitrosamine and mycotoxins that have been implicated as gastrointestinal carcinogens (Zarbl et al., 1985; McMahon et al., 1986; Vousden et al., 1986) can induce mutation of the ras gene.

Mutated ras p21 protein or abnormally high-expressed normal p21 have been reported to induce transformation of NIH 3T3 cells in vitro (Chang et al., 1982; Feramisco et al., 1984; Theodorescu et al., 1990). In addition enhanced expression of c-Ha-ras p21 has been commonly demonstrated in gastric cancers and the level of ras p21 was known to be correlated with tumor progression (Tahara et al., 1986; Ohuchi et al., 1987). Employing the RNA-RNA in situ hybridization technique, Ohuchi et al. (1987) have demonstrated that the expressed ras p21 protein in gastric cancers and adjacent dysplasia might be a product of the c-Ha-ras gene. In this context, we aimed to study the role of the c-Ha-ras gene in gastric carcinogenesis.

A few cases concerning the mutation of the K-ras (Bos et al., 1986), N-ras (O'hara et al., 1986), and c-Ha-ras genes (Deng et al., 1987) in gastric cancer have been reported, primarily by transfection assay. Since transfection assay has certain limitations in terms of sensitivity and speed, a more proper methodology has been pursued. Recently, with the development of polymerase chain reaction (PCR) technology, gene mutation could be detected even in paraffin-embedded tissue or in extremely tiny tissue sample of premalig-

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nant lesion as well as in tumor tissue (Impraim et al., 1987; Meltzer et al., 1990; Shibata et al., 1990). By using of PCR and mutant-specific oligonucleotide dot-blot analysis that allows detection of single-base mutation, we made an attempt to study the mutation of the c-Ha-ras gene in 16 cases of gastric cancer and 8 cases of dysplasia for the purpose of better understanding of the carcinogenesis of gastric cancer.

## MATERIALS AND METHOD

### Cell Lines

Eight human gastric cancer cell lines were established by us in culture after isolation from patients' ascites with gastric adenocarcinoma. They have now demonstrated stable growth for more than 1 year. These cells were cultured in minimal essential medium (MEM, Hazalton Biologics, Denver, PA) supplemented with 10% fetal calf serum (CSL, Melbourne, Australia). Antibiotics: 100u/ml of penicillin and 10ug/ml of streptomycin, were added to the growth medium. Four cell lines were intestinal type, poorly to well differentiated adenocarcinomas, and the other four were diffuse type, signet ring cell carcinomas. T-24, bladder cancer cell line (Capon et al., 1983) containing mutated c-Ha-ras gene at codon 12 (gly→val) was available as a positive control for mutant-specific hybridization procedure.

### Microdissection

Tumor tissues from endoscopic biopsies or surgical resections were obtained from 8 gastric cancer patients at Yonsei University Medical Center, Seoul, Korea. Microdissection was performed on paraffin-embedded specimens and each block was sectioned and stained with hematoxylin and eosin. All stained slides were reviewed by a pathologist. Gastric dysplasia was classified according to the criteria of the World Health Organization (WHO) as mild, moderate or severe. Biopsied specimens containing moderate and severe dysplasia were selected, and cancerous regions were microdissected from surgical sections under the microscope. Tissue sections on the slides were scraped off with a scalpel and collected into eppendorf tubes.

### Extraction of DNA

Extraction of DNA from gastric cancer cell lines was done as described elsewhere (Sambrook et al., 1989). Tissues from paraffin-embedded sections were incubated overnight in lysis buffer (60ug/ml proteinase K, 0.05% Tween 20, 100mM Tris-Hcl (pH 8.5)) at 60°C, made to an adequate dilution and used as a template (Imprain et al., 1987).

### Oligonucleotide primer

Oligonucleotide primers near codon 12 and 13 of the c-Ha-ras gene were synthesized on an Applied Biosystem, Model 380B DNA synthesizer and those near codon 61 were purchased from Clontech (Palo Alto, CA). The sequences of primers were the following: c-Ha-ras codon 12 and 13: 5′-GCAGGCCCCT-GAGGAGCGAT, 3′-AATGGTTCTGGATCAGCTGG, codon 61:5′-AGGTGGTCATTGATGGGGAG, 3′-AGGAAGCCCTCCCCGGTGCG.

### Polymerase Chain Reaction

One hundred microliters of the reaction mixture containing 1$\mu$g of genomic DNA in 10mM Tris (pH 8.4), 50mM KCl, 2.5mM MgCl2, 0.02% gelatin, 25pmol of each primer, 200mM deoxyribonucleoside triphosphate (dATP, dCTP, dTTP, dGTP) were amplified with Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) as described by SaiKi et al ( 8). To prevent DNA contamination, all procedures were done under the hood and disposible pipet tips, centrifuge tubes were used. All buffers were autoclaved before use. All reagents were prepared and stored in small aliquots, and discarded aliquots after use. The heating cycle consisted of 1-min denaturing at 95°C, 2-min annealing at 65°C, and 3-min polymerase reaction at 72°C. After 30 cycles of amplification, 20ml of the amplified DNA was resolved by electrophoresis in 3% agarose gel and stained with ethidium bromide to check the presence of amplified DNA. After PCR amplification around 12 and 13 of the c-Ha-ras gene, control samples were digested with Hpa II to confirm whether the correct sequence was amplified. Hpa II recognition site is located in the center of the amplified 100bp fragment, at 12th codon. Thus, the 100bp fragment from the wild type of the c-Ha-ras gene could be cleaved into two 50bp fragments by Hpa II and those from the mutated c-Ha-ras gene could not be cleaved.

### Dot-Blot Hybridization

Twenty microliters of the final PCR mixture, containing 100ng of the orignal DNA was adjusted to 0.4M NaOH/25mM EDTA in a 200ul volume, heated at 95°C for 5 minutes, and applied under vacuum to a nylon membrane in a dot-blot apparatus. Each filter was prehybridized in 10ml solution consisting of 5 x SSPE, 5 x Denharts, 0.5% SDS, 100mM sodium pyrophosphate (pH 7.5) at Tm-10°C for 1-2 hours. $10^7$ cpm of labeled probe was then added and hybridized at Tm-10°C for 2 hours. The filter was washed twice in

**Table 1.** Sequences of mutant-specific oligonucleotide probes of each codon of the c-Ha-ras gene

| codon | Sequences of probe | Amino acid |
|---|---|---|
| 12 | GGC GCC GGC GGT GTG GGC AA | gly (wild) |
| | AGC | ser |
| | TGC | cys |
| | CGC | arg |
| | GAC | asp |
| | GTC | val |
| | GCC | ala |
| 13 | GGC GCC GGC GGT GTG GGC AA | gly (wild) |
| | AGT | ser |
| | TGT | cys |
| | CGT | arg |
| | GAT | asp |
| | GCT | ala |
| | GTT | val |
| 61 | ACC GCC GGC CAG GAG GAG TA | gln (wild) |
| | GAG | glu |
| | AAG | lys |
| | CGG | arg |
| | CTG | leu |
| | CCG | pro |
| | CAT | his |
| | CAC | his |

2×SSC at room temperature and washed again with 3M tetraethylammonium chloride (TEAC) at Tm −10°C for 10 minutes. Finally the filters were exposed to X-Ray film at −70°C.

### Mutant-specific Oligonucleotides

Oligonucleotides to screen for the presence of specific point mutations of c-Ha-ras gene were purchased from Clontech (USA). Amplified DNAs were analyzed with a set of oligonucleotides each designed to be complementary to a different point mutation within sequences around codon 12, 13, and 61 respectively (Table 1). The oligonucleotide probes were 5′-end labeled with [r-$P^{32}$] ATP and T4 polynucleotide kinase (New England Biolabs, Beverly, MA).

## RESULTS

### Confirmation of PCR-amplified DNA

The PCR product was analyzed on a 3% agarose gel to make sure the amplification was successful (Fig. 1). The plasmid pHLTR (normal c-Ha-ras, codon 12) and pEJ and genomic DNA extracted form T-24cell line (mutant c-Ha-ras codon 12, GGC (gly)→GTC (val) were used as negative and positive controls respectively. After PCR amplification around codon 12 and 13 of the c-Ha-ras gene, the control samples were digested with Hpa II and electrophoresed in an 8% polyacrylamide gel. The wild type (pHLTR) was cleaved into two 50bp fragments, but the mutated types (pEJ

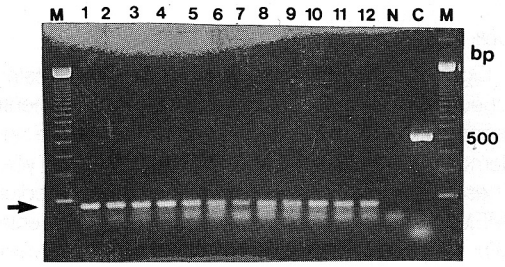


**Fig. 1.** Amplified products of polymerase chain reaction. DNAs from the gastric cancers were amplified using primers around codon 12 and 13 of c-Ha-ras and electrophoresed in a 3% agarose gel. Lane M: marker, 123bp DNA ladder.

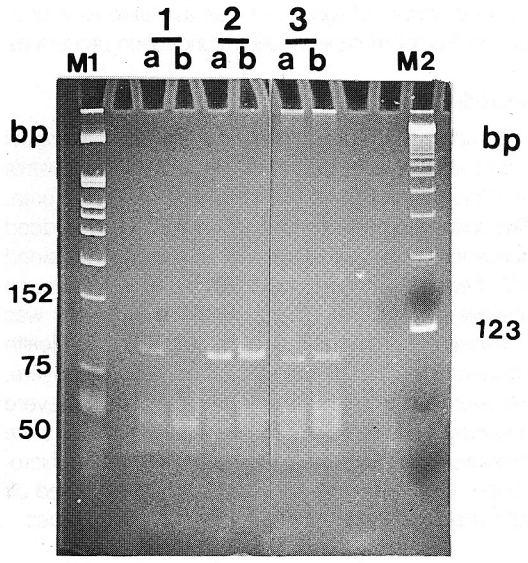


**Fig. 2.** Analysis of amplified products by HpaII, whose restriction site is located at codon 12. After digestion with HpaII, the amplified products were electrophoresed in an 8% polyacrylamide gel. The 100bp fragment from the wild type of c-Ha-ras (pHLTR) was digested into two 50bp fragments (lane 1-a,b), but those from the mutated c-Ha-ras at codon 12 (pEJ and T-24) were not cleaved (lane 2-a,b and 3-a,b). Marker, M1: pBR 322 DNA-Hinf I digest, M2: 123bp ladder.

and T-24) were not (Fig. 2).

**Analysis for mutation of the c-Ha-ras gene in DNA samples using mutant-specific oligonucleotide hybridization**

Following PCR amplification around codon 12, 13, and 61 of the Ha-ras gene, Twenty microliters of the amplified DNA mixtures containing 100ng of the original DNA were blotted onto a nylon membrane and hybridized with the various mutant-specific oligonucleotides to analyze the frequency and type of mutation. All 24 samples hybridized strongly to the normal 12 (gly) 13 (gly) and 61 (gln) specific probes, although no hybridization was obtained with the plasmid pEJ containing mutation at codon 12. Dot-blot hybridization employing the same amplified DNA was repeated using the mutant-specific probes of each codon. The positive control plasmid pEJ was hybridized strongly to the mutant probe (val) at codon 12. These results showed that the mutant specific probes hyridized exclusively to those DNA sequences that fully matched the corresponding c-Ha-ras gene. A glycine to valine amino acid substitution at codon 12 of the c-Ha-ras gene was detected in 2 out of 8 gastric cancer cell lines derived from malignant ascites, and mutation at codon 12 of the c-Ha-ras gene was also demonstrated (Fig. 3). One c-Ha-ras mutation at codon 12 occured both in high-grade dysplasia and cancer lesion of one patient's specimen (Fig. 4). No mutations at codon 13 or 61 of the c-Ha-ras gene was detected in any samples.

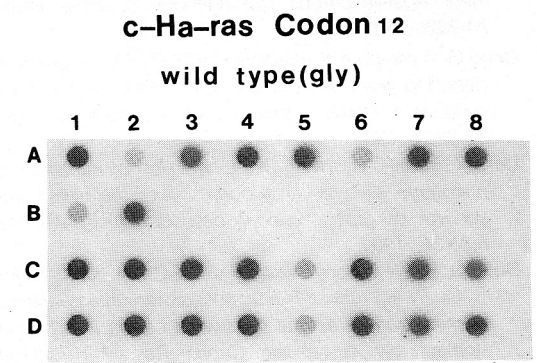


**Fig. 3.** Dot-blot hybridization of a normal (gly) oligonucleotide probe to PCR-amplified DNA from cell lines (A1-B2) and carcinoma (C1-C8) and dysplastic tissues (D1-D8). A1: pHLTR (normal c-Ha-ras 12); A2: pEJ mutant c-Ha-ras 12 (val).

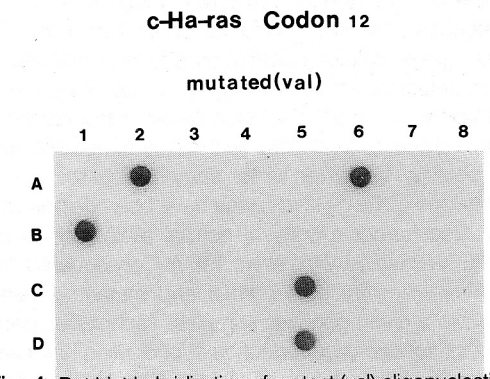


**Fig. 4.** Dot-blot hybridization of mutant (val) oligonucleotide probe to PCR-amplified DNA from cell lines (A1-B2) and carcinoma (C1-C8) and dysplastic tissues (D1-D8). Detection of the mutation at codon 12 of the c-Ha-ras (gly→val) in two gastric cell lines (A6 & B1), gastric cancer and adjacent dysplasia (C5 & D5) of one patient. A2: pEJ (mutant c-Ha-ras 12, Val).

## DISCUSSION

The role of p21 protein in neoplastic development has been explained in terms of its activation caused by single point mutation of the ras gene (Tabin et al., 1982). Mutated ras p21 has been recognized as having a transforming activity (Stacy and Kung, 1984), metastatic potential (Price et al., 1989), and increased mitosis (Hagag et al., 1990) which could be responsible for further genetic errors (Preston-Martin et al., 1990). By using an evolutional model of colonic carcinogenesis, Vogelstein et al., (1987) explained as follow; Mutations of the c-K-ras gene might be involved in the initial event of carcinogenesis, and additional genetic changes would be involved in further progression to invasive carcinoma.

Mutated ras gene has only been reported in isolated cases and it seemed to play a minor role in gastric carcinogenesis. The commonly used biological assay based on DNA transfection of cells that either form morphologically transformed foci or induce tumors in nude mice, is too laborious for large-scale screening and has problem in its sensitivity. Moreover we are in need of information about the incidence of ras gene both in gastric cancer and precancerous lesion in order to understand the role of the mutated ras gene in gastric cariciogenesis. In many cases, preparation of DNA was done from paraffin-embedded microsections of tumor, in which the proportion and type of cancer and non- cancerous cells could be assessed accurately.

Therefore the amount of DNA obtained from the precancerous lesion might be under 1$\mu$g. The PCR technology makes it possible amplify target sequence and detect in very tiny samples. In addition, tumor sample may consist of a heterogeneous population of cancer and normal cells such as lymphocytes and fibroblasts. Moreover tumor samples in which point mutations of the c-Ha-ras gene have been demonstrated, also harbor a copy of normal allele (Bos et al., 1986; Verlaan-de Vries et al., 1989). Consequently, the PCR will amplify both normal and mutant c-Ha-ras allele in a given genomic samples. Techniques using mutant-specific oligonucleotide hybridization has been reported to detect mutant allele at 10% level. In contrast, direct sequencing could detect the mutant allele if present in 25% or greater of the cells within a mixed population (Cogswell et al., 1989; Collins et al., 1989). In addition, this approach might have an advantages of screening large number of sample easily and its high sensitivity that allows detection of all possible single-base mutations at codon 12, 13 and 61 (Bos et al., 1985; Verlaan-de Vries et al., 1989). In this study, we also found that oligonucleotide dot-blot hybridization could be useful to detect mutation of ras gane, especially in very tiny precancerons tissue samples under proper hybridization condition.

In spite of using more sensitive PCR technique to detect ras mutation, the incidence of point mutation of the ras gene in gastric cancer has been reported to be low (Nanus et al., 1990; Victor et al., 1990), although some papers reported that significant number of mutations of the ras gene could be detected in gastric cancer (Deng, 1988; Deng et al.,). Recently Soman et al. (1991) found significant number of ras mutations in gastric precancerous lesion by using PCR and direct sequencing.

We have used a sensitive technique based on PCR amplification of target DNA sequences followed by mutant-specific oligonucleotide dot-blot analysis to study the c-Ha-ras gene mutation in human gastric cancer and adjacent dysplastic lesion. Mutation at codon 12 of the c-Ha-ras gene resulting in glycine to valine amino acid substitution, was found in 2 out of 8 gastric cancer cell lines derived from malignant ascites. Since such a mutation was detected in established cell lines, it is unclear whether the mutation could occur in vivo or continue to appear during the in vitro passages of the cell lines.

Numerous reports suggest that oncogenes are involved in human cancers both in the initial conversion of normal to malignant phenotype, and in the progression of less aggressive cell types to more agressive ones (Garett, 1986). Likewise we have found that point mutation of the c-Ha-ras gene occuring even in dysplastic lesion as well as in gastric cancer and cell lines, although the incidence was quite low. This finding suggests that mutation of the c-Ha-ras gene could occur in the premalignant stage, thus might be regarded as an initial event of gastric carcinogenesis in some cases. Therefore, further study to investigate the role of the c-Ha-ras mutation in precancerous lesion would be warranted.

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