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Molecular Characteristics of Tumorigenesis in Human Gastric Carcinomas*

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=국문초록=

인체 위암에서 종양화의 분자생물학적 특성

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위암 발생과 암유전자의 관련 여부 및 기전을 알아 보고자 조직병리학상 위암으로 진단된 34명의 환자 위조직을 대상으로 하여 c-fos, c-erbB, c-myc, c-Ha-ras 및 p53 유전자와 위암과의 관련여부를 조사하였다. Southern 및 slot blot analysis를 한 결과 암 및 항암유전자의 유전자 재배열은 없었으며 실험대상 34명중 한명에서만 c-myc 암유전자의 증폭이 있었다. Northern blot analysis를 실시한 결과 Southern blot analysis상 c-myc 암유전자의 증폭이 관찰된 경우를 제외하고는 어떠한 유전자의 RNA 전사의 이상은 없었다. c-Ha-ras 암유전자의 12번 및 13번 codon의 돌연변이 여부를 조사하고자 PCR을 이용하여 12번 및 13번 codon 주변의 유전자를 증폭한 후 12번 codon을 절단하는 제한효소(HpaII) 처리 및 PCR-single strand conformational polymorphism analysis를 실시한 결과 돌연변이가 발견되지 않았다. 이 실험에서 조사하지 않은 12번 13번 codon의 다른 codon의 점돌연변이로 p21의 발현이상을 알아보고자 Westhern blot analysis를 실시한 결과 위암조직 및 대조군 간에 p21의 발현의 차이는 관찰할 수 없었다. 이상의 결과를 미루어 볼때 한국인에 있어서 위암의 발생은 이 실험에서 조사한 c-fos, c-erbB, c-Ha-ras 및 p53과는 크게 관련이 없다고 생각되며 34명 중 한명에서 c-myc 암유전자의 증폭 및 RNA 전사의 증가로 인한 이상은 어떤 위암의 발생과는 관계가 있다고 생각되나 빈도가 낮아서 단언하기는 어렵다고 생각된다.

Key Words: Stomach cancer, c-myc amplification

INTRODUCTION

Although gastric cancers have been declining in recent decades, it remains one of the most serious health burdens throughout the world and it is still a leading malignant disease in many countries, including Korea, Japan, Northern Europe, and South Africa[1,2]. Especially in Korea, gastric cancer is the leading cause of cancer death, followed by cervical cancer, hepatoma, and lung cancer in decreasing order[3]. Epi-

demiological studies have shown that gastric cancer is strongly associated with various dietary and cultural habits such as excessive salt intake, low intake of ascorbic acid and carotenoid[1], infection with *Helicobacter pylori*[4,5], and frequent exposures of carcinogenic nitrosamines[6].

Cancerous growth is stably inherited at cellular level, so genetic changes are responsible for transformation from normal to malignant phenotypes and there is evidence that genomic changes are important in the development of human cancers[7,8]. The past decade has witnessed great changes in our understanding of the molecular origin of cancer. Much of this progress stems from the discovery of specific genes, the oncogenes[9,10]. Oncogenes identified as viral oncogenes are activated versions of cellular oncogenes(protooncogenes), and their products with abnormal functions or in abnormal amounts are believed to contribute to tumorigenesis[7,11]. Protooncogenes can be activated to oncogenes by a variety of molecular mechanisms in which gene amplification, point mutation, gene rearrangement, and so on are included[7,8,12]. In addition to activated oncogenes, p53 gene, an antioncogene or tumor suppressor gene, which is located at chromosome 17p13[13], has been reported to be a frequent target for genetic abnormalities in many tumors[14~17]. Since the p53 gene can act as a antioncogene, it has been suggested that its inactivation is involved in tumorigenesis[18].

Moreover, recent evidence has demonstrated that gastric tumorigenesis is considered to be not a single but a multistep process of alteration of genes which are involved in growth control and differentiation of cells[1,7,9]. Therefore gastric carcinomas also might arise through the total accumulation of genetic alterations in oncogenes and tumor-suppressor genes, but molecular mechanisms of the gastric carcinogenesis have not been fully understood.

In this study, to understand gastric carcinogenesis and to evaluate the relevance of protooncogene alterations, the presence of the amplifications, rearrangements and overexpressions of 4 representative protooncogenes and p53 in 34 samples of DNAs isolated from primary gastric carcinomas were surveyed. In addition, activation of c-Ha-ras gene by point mutations within coding sequences, an event often linked to initiation or early stage of malignancy[19~21], were analyzed by the HpaII restriction analysis and the single-strand conformational polymorphism(SSCP) using target sequences amplified by polymerase chain reaction(PCR), and the level of expression of ras gene product(p21), using the ras p21 specific monoclonal antibody Y13-259, was determined.

MATERIALS AND METHODS

1) Tumor samples

Fresh tumor and normal tissues from the stomach of the same patients were obtained from 34 patients undergoing surgery for gastric cancer and were frozen in liquid nitrogen tank until analysis. These tissues were provided by Department of Surgery, Keimyung University Medical Center, Korea. The tumors were histologically confirmed to have carcinomatous tissues and the normal mucosa was confirmed to have no evidence of tumor invasion or significant inflammatory involvement.

2) Southern blot analysis

High molecular weight DNAs were prepared by using the phenol-chloroform method after treatment with sodium dodecyl sulfate(SDS) and proteinase K[22]. Genomic DNAs(10 μg) were

digested to completion with appropriate restriction enzymes as specified by the commercial supplier(BRL Co.). Digested DNAs were electrophoresed on 0.9% agarose gel in 40 mM Tris acetate, 20 mM Na-acetate, and 2 mM ethylene diamine-tetraacetic acid(EDTA) buffer(pH8.0). After electrophoresis, DNAs were denatured, neutralized and transferred to nitrocellulose (NC) filters(Hoefer Scientific Co.) according to the method of Southern[23]. The filters were hybridized overnight at 42℃ with DNA probes that were labeled with ^{32}P-dCTP to high specific activity($1-3\times10^8$ cpm/μg) by the nick-translation method. After hybridization, fiters were washed using high strength standard saline citrate(SSC) containing 0.3 M sodium chloride/0.03 M sodium citrate(pH 7.4) at room temperature for 60 minutes with 3 changes and 0.1× SSC/0.25% SDS at 65℃ for 120 minutes with 3 changes and exposed to Kodak XAR-5 films using intensifying screens. The same filters were hybridized repeatedly with probes for several oncogenes to exclude the possibility that the intensities of the bands in different lanes were due to difference in the amounts of DNAs loaded on agarose gel.

3) DNA slot blot analysis

Ten μg of DNA was dissolved in 0.3 M sodium hydroxide, and TE(10 mM Tris and 1 mM EDTA, pH 8.0) was added to make 100 μl of the solution. The samples were incubated at 95℃ for 10 minutes, treated with equal volumn of 2 M ammonium acetate and applied under vacuum to NC filters clamped in a Minifold II apparatus(Schleicher and Schuell). Filters were baked at 80℃ for 2 hours and the procedure of hybridization was the same as that of Southern blot analysis as described above[24]. The levels of amplification were determined by densitometer (Hoefer GS300 scanning densitometer, Hoefer Scientific Co.) and the sum of the densitometric signals of all the band was taken.

4) Northern blot analysis

Total RNAs were isolated by homogenizing in guanidine-HCl buffer(4 M guanidine-HCl, 25 mM sodum citrate, 10 mM EDTA, 0.33% antifoam A, 0.1 M 2-mercaptoethanol and 0.5% sarcosyl, pH7.0) according to the method of Chomczynski and Sacch[25]. RNA was denatured by heating in formaldehyde and formamide, and then electrophoresed on 0.9% denaturing formaldehyde agarose gel. Following transfer of RNA onto NC filter in 20×SSC, and incubation of the dried blot in an 80℃ oven for 2 hours, blots were hybridized and washed essentially as described above. A β-actin gene probe served as a control in assessing gene copy number for protooncogenes.

5) Western blot analysis

Tumors were pulverized and homogenized in a teflon glass homogenizer with 5 volumes of ice cold buffer TEN(0.1 M NaCl, 5 mM $MgCl_2$, 1 % Nonidet P-40, 0.5% sodium deoxycholate, 2 kallikrein inhibitor unit/ml bovine aprotinin, and 20 mM Tris-HCl, pH 7.4). The homogenates were centrifuged at 15,000 rpm for 5 minutes at 4℃, and the resulting supernatants were used as the lysates[26]. Protein concentration of lysates were determined by the method of Lowry et al[27]. Western blot analysis was used to determine the amount of p21 present in the tumors and normal gastric tissues. The lysates were separated by 12% SDS-polyacylamide gel electrophoresis and transferred to NC filter in transfer buffer(25 mM Tris, 192 mM glycine, 20 % methanol, pH7.4). NC filters were washed and first incubated with 5% non fat dried milk in TNT(100 mM Tirs, 0.9%, NaCl, 0.05% Tween 20, pH 7.5) for 1 hour at room temperature and

then sequentially incubated with medium containing p21 monoclonal antibody Y13-2594[28] directly against the Ha-MuSV-encoded p21 for 1 hour at room temperature. Then the avidine-biotin complex immunmoperoxidase assay was poerformed with Vector stain ABC kit for rat IgG(Vector Laboratories, Inc., Burlingame, CA). For visualization of p21 bands 0.5 mg/ml of 3, 3'-diaminobenzidine tetrahydrochloride solution containing 0.002% H_2O_2 was used.

6) PCR-restriction fragments length polymorphism(PCR-RFLP) and PCR-SSCP analysis

To detect the Ha-ras gene point mutation at codon 12 and 13, HpaII RFLP of PCR products were analysed and SSCP analysis was accomplished. Restriction site of HpaII is located at codon 12[29]. Oligonucleotide primers used for PCR amplification of exon 1 of the Ha-ras gene were purchased from supplier(Clontec Inc.). Primers for this PCR were 5'ATGACGGAA-TATAAGCTGGT3'(sense) and 5'CGCCAGGC-TCACCTCTATA3'(anti-sense). Two-tenth of the PCR product was then digested with HpaII for 2 hours at 37℃, and electrophoresed on 3% Nusieve agarose gel(FMC Bioproduct). SSCP analysis was accomplished according to a previously reported method with minor modifications[30]. PCRs were performed with 100 ng of genomic DNA, 10 pmole of each primer, 2.5 μM dNTPs, 1 μCi of [α-^{32}P] dCTP(Amersham; specific acivity, 3,000 Ci/mmole; 1 Ci=37 GBq), 10 mM Tris-HCl(pH 8.8), 50 mM KCl, 1 mM $MgCl_2$, 0.5 unit of Taq polymerase(Cetus Co.), in a final volume of 10 μl. Thirty cycles of denaturation (94℃), annealing(56℃) and extension(72℃) were done using an automated heat block(DNA thermal cycler: Elicomp). The PCR products were diluted 9-fold with dye solution(95% formamide; 20 mM EDTA; 0.05% xylene cyanol; 0.05% bromphenol blue). This was followed by denaturation at 95℃ for 2 mintes, chilled on ice and then the samples were applied to 6% non-denaturing polyacrylamide gel with or without 10% glycerol, and electrophoresed at 40 W for 3 hours with cooling system(4℃). Gels were sujected to drying after electrophoresis and autoradiographed using Kodak XAR-5 film at room temperature with intensifying screens.

7) Probes

The c-myc is an 1.4 kilobase(Kb) ClaI/EcoRI fragment corresponding to the third myc exon from human DNA. The c-Ha-ras probe is a 0.73 Kb SstII/PstI fragment from Harvey rat sarcoma virus. The c-fos probe is an 1.0 Kb PstI/PvuII fragment from FBJ osteosarcoma virus proviral DNA. The c-erbB is an 1.7 Kb SstI/PvuII fragment from avian erythroblastosis virus. The p53 probe was prepared from the plasmid php53B by purifying a 2.2 Kb human p53 cDNA insert. The β-actin probe consisted of an 1.7 Kb BamHI/PstI fragment cloned in the pBR322 plasmid.

RESULTS

1) Southern and slot blot analysis

To determine amplifications and rearrangements of oncogenes in gastric carcinomas, Southern and slot blot analysis were done. High molecular weight DNAs were prepared using phenol-chloroform method after treatment with SDS and proteinase K. Southern(Fig. 1 and 2) and slot blot(Fig. 3) analysis from genomic DNAs of 34 gastric cancers showed that there were found no amplification of oncogenes analyzed except c-myc gene. Out of 34 gastric cancers, only one showed amplification of c-myc gene but no coamplified oncogenes were found.

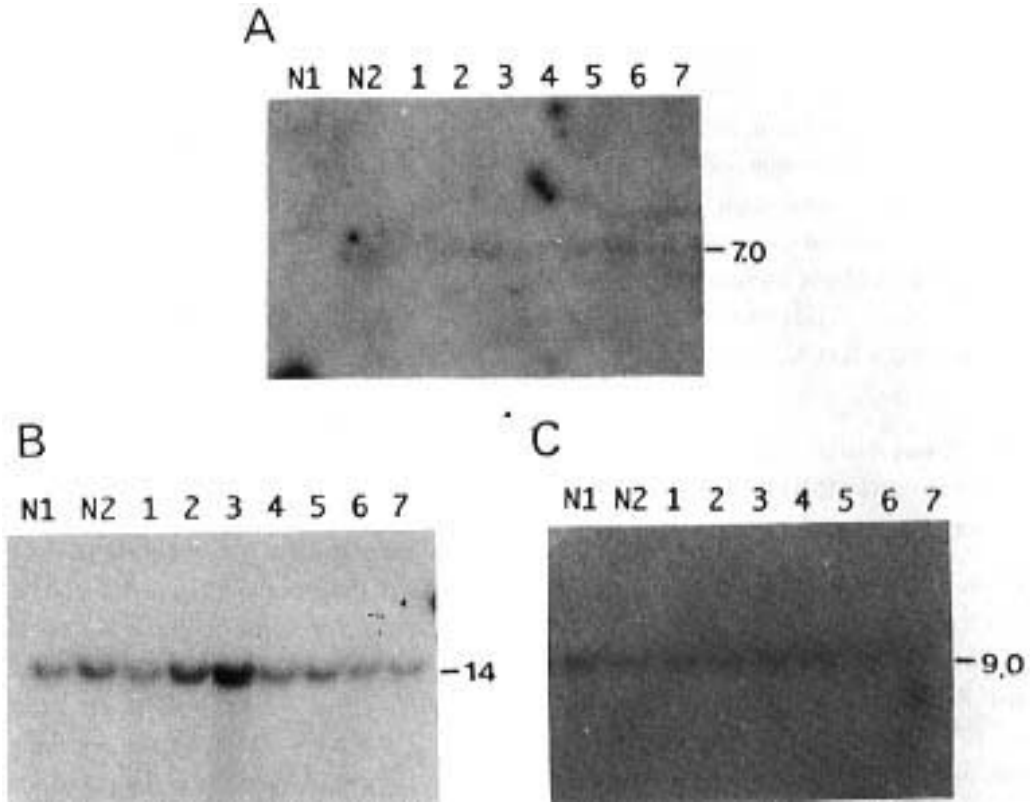

Fig. 1. Southern blot analysis of c-Ha-ras(A), c-myc(B), and c-fos(C) gene in genomic DNA. DNAs were isolated from gastric cancers(lane 1~7) and normal gastric tissues(N1-N2). 20 μg of DNAs digested with BamHI(A) and EcoRI(B and C) was loaded per lane. Electrophoresis, blotting and probing was performed as described in materials and methods. N stands for normal tissues. The numbers on the right show kilobase pair.

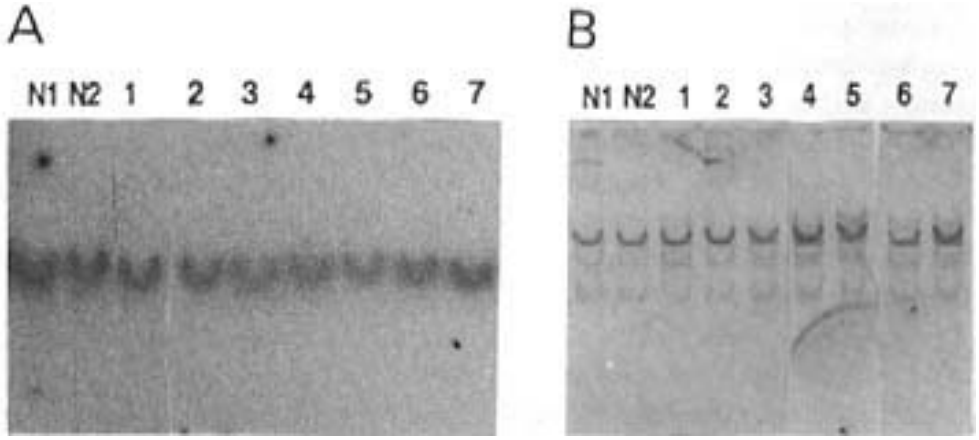

Fig. 2. Southern blot analysis of p53 antioncogene. DNAs were isolated from gastric cancers(lane1-7) and normal gastric tissues(N1-N2). 20 μg of DNAs digested with HindIII(A) and XbaII(B) was loaded per lane. Electrophoresis, blotting, and probing was performed as described in materials and methods.

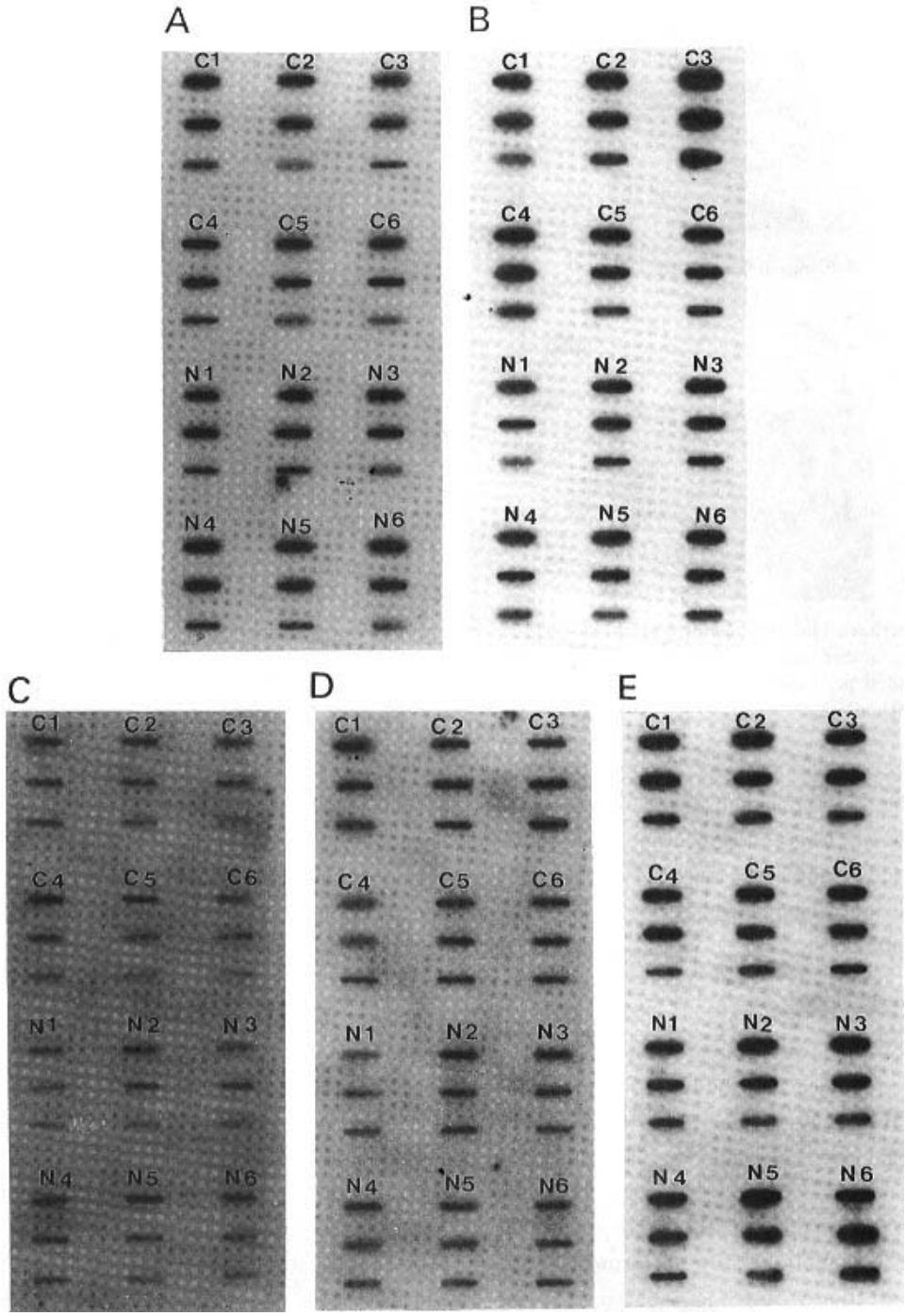

Fig. 3. DNA slot blot analysis results obtained after hybridization of DNAs isolated from gastric cancer tissues(C1-C6) and normal gastric tissues(N1-N6) with ^{32}P-dCTP-labeled c-erbB(A), c-myc(B), c-Ha-ras(C), c-fos(D), and p53(E) cDNA probes. Different amount of DNA, 20, 10, and 5 μg, were dotted on nitrocellulose filters in serial dilution.

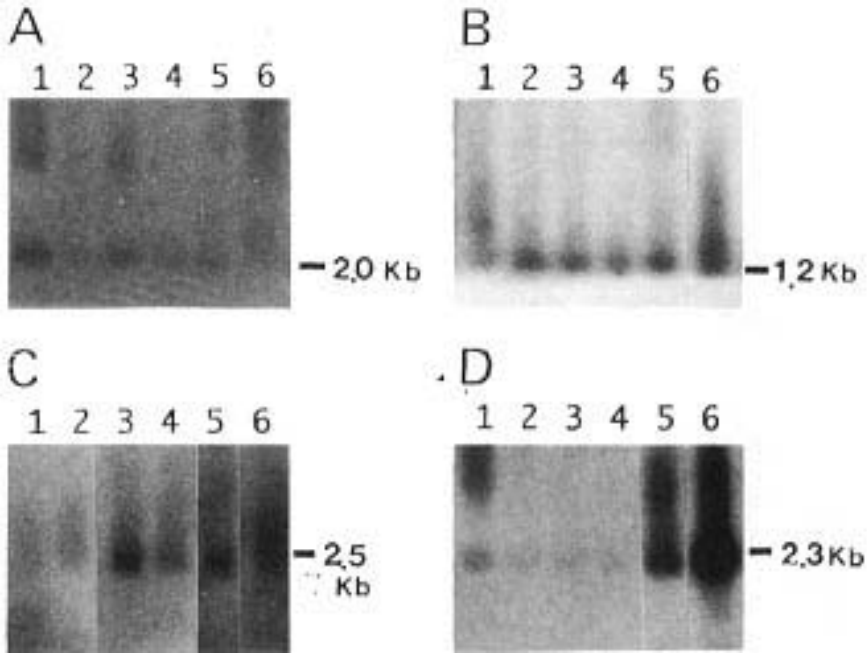

Fig. 4. Northern blot analysis of β-actin(A), c-Ha-ras(B), and p53, and c-myc(D) mRNA transcripts from gastric cancer tissues(lane 2, 4, and 6) and normal gastric tissues(lane 1, 3, and 5). 15 μg of total RNAs was loaded per lane. Electrophoresis, blotting and probing was performed as described in materials and methods

No possible rearrangement of oncogenes tested was detected. And p53, an antioncogene, also was not found any abnormal finding including structural rearrangement or partial or total deletion of alleles.

2) Northern blot analysis

The expression of protooncogenes was measured by Northern blot analysis of electrophoretically fractionated total RNAs from 34 gastric carcinomas. Especially to determine the correlation between amplification of the c-myc gene and abundance of the gene transcript, Northern blot analysis were performed. Northern hybridization with RNAs isolated from gastric cancers demonstrated(Fig. 4) that one out of 34 samples exhibited significantly increased expression of c-myc gene when compared with normal controls($p<0.01$). Judging from the intensity of β-actin, c-myc gene was overexpressed about 10 fold. Hybridization of same blot with β-actin probe indicated that changes in the levels of c-myc transcripts were not due to sample size variation or RNA degradation. But, there were no differences between cancers and normal controls in the expression of genes for c-Ha-ras and p53.

3) PCR-RFLP and PCR-SSCP analysis

To screen the presence of specific point mutations at codon 12 and 13 of c-Ha-ras gene, PCR amplification around codon 12 and 13 of c-Ha-ras gene was performed. Twenty mocroliters of the amplified DNA containing 100 ng of the original DNA were treated with HpaII at 37℃ and electrophoresed on a 3%

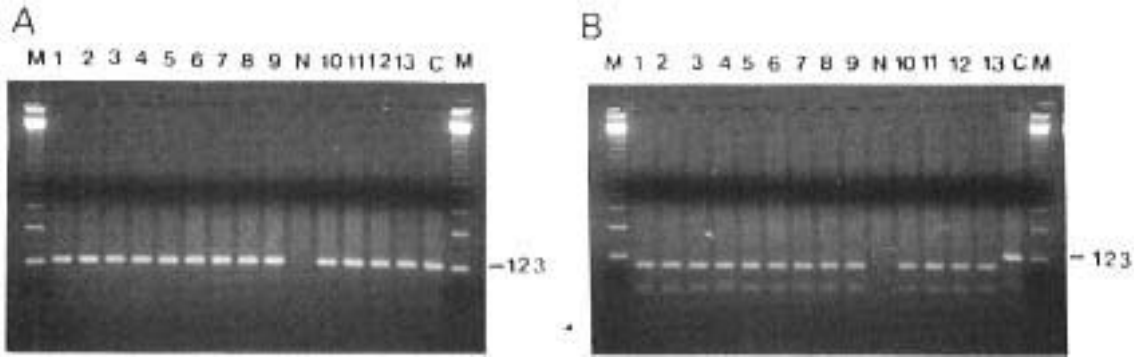

Fig. 5. Analysis of amplified products of PCR(A) and PCR-RFLP analysis of PCR products by HpaII restriction enzyme(B). DNAs from gastric cancers(lane 1-9) and normal gastric tissues(lane 10-13) were amplified using primers around codon 12 and 13 of c-Ha-ras and electrophoresed in a 3% Nusieve agarose gel. C stands for positive control, N for negative control, and M for size marker(123 base pair ladder). The numbers on the right show base pair.

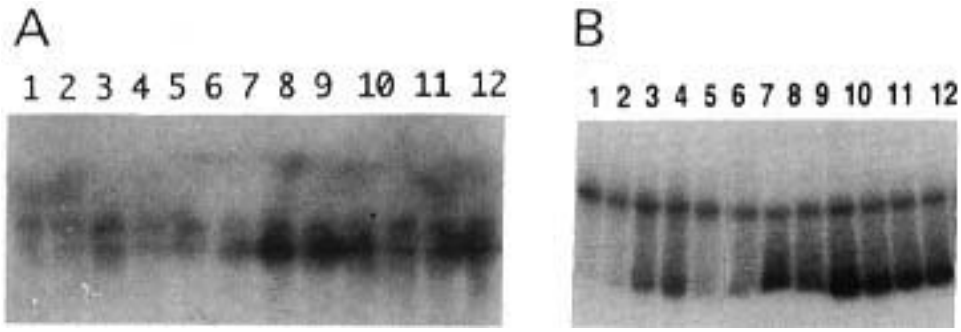

Fig. 6. PCR-SSCP analysis of exon 1 of the c-Ha-ras mutation in gastric cancers. DNAs from gastric cancer tissues(lane 1-8) and normal gastric tissues(lane 9-12)were amplified using primers around codon 12 and 13 of c-Ha-ras in presence of α-^{32}P-dCTP. After PCR amplified products were denaturated by heat and alkaline treatment, and acrylamide gel(6%) electrophoresis was done in the abscence(A) and presence(B) of 10% glycerol.

Nusieve agarose gel containing 0.5 μg/ml ethidium bromide. PCR-RFLP analysis of exon 1 of the c-Ha-ras gene(Fig. 5) from genomic DNAs of 34 gastric cancers showed that amplified PCR products were 123 bp and HpaII treated PCR products showed that two fragments, 90 and 33 bp were separated. If codon 12 of c-Ha-ras gene was mutated, 123 bp of amplified PCR products were not separated into two fragments (90 and 33 bp). So no mutation of codon 12 was found. PCR-SSCP analysis of exon 1 of the c-Ha-ras gene showed that no altered migration bands relative to those amplified from normal controls were found. So there were no mutations in codon 12 and 13 of c-Ha-ras gene.

4) Western blot analysis

Because, in c-Ha-ras gene, there was no DNA amplification found in Southern and DNA slot blot analysis and also no increased expression

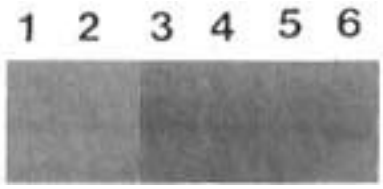

Fig. 7. Expression of p21 in primary gastric carcinomas(1-4) and normal controls(5-6).

of c-Ha-ras mRNA transcipt in Northern blot analysis, and no mutation of codon 12 and 13 in PCR-RFLP and PCR-SSCP analysis, so to determine level of ras protein, Western blot analysis was done. When conmpared with ras protein of normal controls, there was no differences of p21 expression between cancers and normal contros.

DISCUSSION

Over two hundred different kinds of human cancer are recognized and histologically classified according to their origin[7]. Among these neoplasms, gastric cancer is the most frequent and the first leading cause of cancer death in Korea[3]. Epidemiological studies have shown that etiological factors of gastric cancer were different in geographycally distinct areas[31]. Among these factors, excessively salty food and low intake of ascorbic acid and carotenoid are considered as three major etiological factors[32]. In addition, a new major factor, infection with *Helicobacter pylori* has been added[4,5]. In Korea, intake of fungal contaminated foodstuffs and frequent exposure of nitrosamine may also be important.

Cancerous growth is stably inherited at cellular level, so genetic cnagnes are responsible for neoplastic transformation. During past decade, many reports have identified critical genes that play a causal role in tumor development, and then, the application of molecular biology to the study of cancer has focused on finding such genes, oncogenes, and understanding their mechanisms of action in regulating cell proliferation and differentiation[7]. The possibility of alterations of oncogenes such as c-myc, c-Ha-ras, c-fos, c-erbB, and p53 was mainly observed in this study.

Using Southern blot technique, 34 DNAs from primary gastric carcinomas were analysed with 5 different probes(c-myc, c-Ha-ras, c-fos, c-erbB, and p53). The hybridization patterns showed that the gross rearrangements such as deletion and insertion were not occurred in the oncogenes tested. With slot blot analysis, the one unexpected pattern found with c-myc probe can be explained by a genomic alteration of the c-myc locus. Only one of 34 DNAs showed gene amplification of c-myc gene which was about 3-4 folds when compared with normal control. Gene amplification is one of the most common alterations of protooncogenes in cancerous cells. And in some tumors structurally and functionally altered protooncogenes were found to be amplified[33~35]. Examples of consistent tumor specific oncogene amplification include the N-myc gene in neuroblastoma[36], family of myc genes in small cell lung cancer[33,37], and c-erbB in glioblastoma[38]. Amplification of the growth factor receptor gene(c-erbB), c-myc, c-yes and c-ki-ras genes was detected in about 10% of gastric carcinoma tissues[39,40]. In this study, amplification frequency of c-myc(2.9%) was lower than that of Yoshida et al[39] and Tahara[40], but was comparable to those obtained by other authors[41,42], and other oncogenes tested such as c-erbB, c-fos, c-Ha-ras, and p53 were not detected any alterations. Therefore, oncogene amplification in primary gastric cancer might be considered as a relatively rare event. Because gene amplification does not appear to be a normal event in mammalian development[3], although amplifica-

tion frequencies of c-myc in this study were lower than examples of consistent tumor specific oncogene amplification described above. Amplification of c-myc in this case might play a role in development of some gastric cancers. Ramzani et al[41] and Tsuchiya et al[35] reported a gene amplified simutaneously with other genes tested in gastric cancers, so cooperative expression of amplified genes might be involved in the genesis and/or progression of gastric cancer. But in our results, there was no coamplification of other genes with c-myc. Otherwise, other oncogenes which were not analyzed in this study such as hst, c-Ki-ras, and int might be amplified alone or coamplified with c-myc, so further studies for these oncogenes will be needed.

In contrast to the dominant oncogenes, a distinct class of genes have been found that function as negative regulators of neoplastic disease. That is, a class of genes, now called anti-oncogenes, tumor suppresor genes, or recessive oncogenes, whose loss of function lead to tumor development[18,43]. Of these oncogenes, the presence of alteration of p53 was analysed using Southern and slot blot analysis. In p53 any alterations such as amplifications, deletions, or rearrangements were not found in these analyses. Mutation or deletion in p53 has been shown to be one of the most common features of human cancers[13,44,45], so any mutations of p53 should be analysed in order to evaluate a role of recessive oncogene in gastric carcinogenesis.

In this study, no alterations of oncogenes tested were seen except c-myc. So the analysis of the expression of oncogenes using Northern blot technique to evaluate differences of RNA transcripts between normal and cancer tissues and to determine the correlation between amplification of the c-myc gene and abundance of the gene transcript has been performed. Total RNAs were isolated by modified method of Chomczynski and Sacchi[25]. RNAs were denatured, electrophoresed, and transferred to NC paper and then, hybridized with five different probes homologous to c-myc, c-erbB, c-Ha-ras, c-fos, and p53. Elevated levels of the c-myc gene transcript were found in only one primary gastric cancer whcih showed DNA amplification in Southern blot analysis. Gene amplification may be biologically equivalent to overexpression[46]. This analysis exhibited significantly increased expression of c-myc gene when compared with normal controls. Judging from the intensity of β-actin, c-myc gene was overexpressed about 10-fold. Gene rearrangement also can lead to transcriptional deregulation, but there was no rearrangement in Southern blot analysis, therefore, overexpression of c-myc gene in this study was not due to a gene rearrangement but a gene amplification. According to amplification and overexpression of c-myc oncogene in one of the gastric cancers tested, molecular carcinogenesis of this cancer might be associated with alteration of c-myc oncogene. From these results, neither amplification nor rearrangement of p53, c-fos, and c-erbB oncogenes may be the mechanisms by which these oncogenes became activated in this malignancy.

The ras genes have been identified as normal cellular components in a various organisms, including human. These genes encode a group of closely related 21,000 dalton proteins(p21) which bind guanosine diphosphate and guanosine triphosphate(GTP) with high affinity and display low GTPase activity[3,11]. Two possible mechanisms have been proposed to explain how ras genes transform normal cells to malignant cells. The first possible mechanism involves overexpression of the normal protooncogene product, and the second mechanism involves

point mutations causing alterations at amino acids 12, 13, or 61 of the ras product[47, 48]. A single base substitution for their transforming activity, and the activated forms of these oncogenes have been detected in various human cancer by means of the NIH 3T3 transfection assay[48~51]. As DNA transfection assay was a biological assay with some technical difficulties and the extensive time was needed for this assay[52], this led to the development of new methods such as PCR. The advent of PCR and PCR-SSCP greatly enhanced the sensitivity of detection of point mutations of genes. One of the major sites of the genetic lesions responsible for activation of ras oncogenes in human tumors has been localized to position 12 in their coding sequences[50]. In this study, to screen the presence of specific point mutations in codon 12 and 13 of c-Ha-ras gene, HpaII RFLP of PCR products which were amplified around codon 12 and 13 of c-Ha-ras gene was analysed. Twenty microliters of the amplified DNA containing 100ng of the original DNA were digested with HpaII restriction enzyme that cuts -CCGG- and electrophoresed in 3% Nusieve agarose. If codon 12 was mutated, amplified PCR products(123 bp) were not separated into two fragments[29]. All of amplified PCR products in this study were digested by HpaII, so no mutations were found in codon 12 of c-Ha-ras. To confirm this PCR-RFLP analysis and to screen mutations of condon 12, 13 and other codons, PCR-SSCP analysis was performed. The SSCP technique on radiolabeled PCR fragments which allows separation of the mutated molecule prior to sequencing, seems a good approach to screen for mutated sequence in special genes[53,54]. In PCR-SSCP analysis, there were no altered migration bands compared to those of normal controls. From these results, ponit mutation of codon 12 and 13 of c-Ha-ras gene might not be involved in gastric carcinogenesis, and the results were consistent with those of Fujita et al[46], Jiang et al[52] and Victor et al[31], but not with those of Bos et al[19], Deng et al[55], Koh et al[56] and O'Hara et al[57]. But other codons in which point mutations were frequently found such as codon 61 were not screened, so further study would be warrented.

No gross structural rearrangement, gene amplification and overexpression of c-Ha-ras gene could be detected by Southern, slot, and Northern blot analysis. And mutational events in codon 12 and 13 were not found, therefore, the level of p21 products was analysed by Western blot. The expression of proteins encoded by ras oncogenes was examined in 34 fresh human gastric cancers using p21 monoclonal antibody Y13-2594. When compared with normal controls, there were no differences in expression of p21 between cancers and normal controls. Fujita et al[46] reported that transformation of the gastric mucosa from normal to the malignant phenotype is rarely associated with activation of ras genes by point mutation, but is frequently associated with enhancedd expression of p21, and Karayiannis et al[58] and Ohuchi et al[59] suggested that elevated expresssion of ras genes may be important in developement of gastric tumors. But the present study did not show elevated expression of p21 and point mutations at codon 12 and 13 of c-Ha-ras gene in gastric carcinomas tested. So, it appears that gastric carcinogenesis may not generally involve alteration of c-Ha-ras oncogne in these cancers tested.

In this study there were no apparent association between the presence of genetic alterations and tumor developements except c-myc oncogene. Among 34 samples tested, c-myc amplification and overexpression were found in only one case of gastric cancers tested, but alteratins of other oncogenes tested were not

detected. Amplification may be preceded by overexpression of c-myc gene in gastric cancer and overexpression may confer a selective growth advantage to an initiated preneoplastic cell. However, the role of c-myc gene in pathogenesis of gastric cancer remains unclear. Therefore, further study to evaluate the role of c-myc gene in development of gastric cancer and other oncogenes which were not tested in this study would be warranted.

REFERENCES

1) Correa P: *Human gastric carcinogenesis: A multistep and multifactorial process-First american cancer society award lecture on cancer epidemiology and prevention. Cancer Res* **52:** *6735, 1992*

2) Park JG, Frucht H, LaRocca RV, Bilss DP, Kurita Y, Chen T-R, Henslee JG, Trepel JB, Jensen RT, Johnson BE, Bang Y-J, Kim J-P Gazdar AF: *Characteristics of cell lines established from human gastric carcinoma. Cancer Res* **50:** *2773, 1990*

3) Ministry of Health and Social Affairs: *Five years reports for cancer register programme in the Republic of Korea. J Korean Cancer Asso* **21:** *151, 1988*

4) Forman D, Newell DG, Fullerton F, Yarnell JWG, Stacey AR, Wald N, Sitas F: *Association between infection with Helicobacter pylori and risk of gastric cancer: evidence from a prospective investigation. Br Med J* **302:** *1302, 1991*

5) Parsonnett J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK: *Helicobacter pylori infection and the risk of gastric carcinoma. N Eng J Med* **325:** *1127, 1991*

6) Bartsch H, Montesano R: *Relevance of nitrosamines to human cancer. Carcinogenesis* **5:** *1381, 1984*

7) Cooper GM: *Oncogenes. Boston: Jones and Bartlett Publishers, 1990*

8) Tahara E: *Growth factors and oncogenes in human gastrointestinal carcinomas. J Cancer Res Clin Oncol* **116:** *121, 1990*

9) Bishop JM: *The molecular genetics of cancer. Science* **235:** *305, 1987*

10) Friend SH, Dryja TP, Weinberg RA: *Oncogenes and tumor-suppressing genes. New Eng J Med* **318:** *618, 1988*

11) Glover DM, Hames BD: *Oncogenes. New York: Oxford University Press, 1989*

12) Shiraish M, Noguchi M, Shimosato Y, Sikiya T: *Amplification of protooncogenes in surgical specimens of human lung carcinoma. Cancer Res* **49:** *6474, 1989*

13) Levin AJ, Momand J, Finaly CA: *The p53 tumor suppressor gene. Nature* **351:** *453, 1991*

14) Bressac B, Galvin KM, Liang TJ, Isselbacher KJ, Wands JR, Ozturk M: *Abnormal structure and expression of p53 gene in human hepatocaellular carcinoma. Proc Natl Acad Sci USA* **67:** *1973, 1990*

15) Matozaki T, Sakamoto C, Suzuki T, Matsuda K, Uchida T, Nakano O, Wada K, Nishisaki H, Konda Y, Nagao M, Kasuga M: *p53 gene mutations on human gastric cancer: wild-type p53 but not mutant p53 suppresses growth of human gastric cancer cells. Cancer Res* **52:** *4335, 1992*

16) Sommers KD, Merrick MA, Lopez ME, Incognito LS, Schechter GL, Casey G: *Frequent p53 mutations in head and neck cancer. Cancer Res* **52:** *5997, 1992*

17) Wagata T, Shibagaki I, Imaura M, Shimada Y, Toguchida J, yandell DW, Kienaga M, Tobe T, Ishzaki K: *Loss of 17p, mutation of the p53 gene, and overexpression of p53 protein in esophageal squamous cell carcinomas. Cancer Res* **53:** *846, 1993*

18) Vogelstein B, Kinzler KW: *p53 function and dysfunction. Cell* **70:** *523, 1992*

19) Bos J, Fearson E, Hamilton SR, Verlaan-de Vries M, van Boom JH, vander Eb AJ, Vogelstein B: *Prevalence of ras gene mutations in human colorectal cancers. Nature* **327:** *293, 1987*

20) Liu E, Hjelle B, Morgan R, Hecht F, Bishop JM: *Mutations of the Kirstein-ras proto-oncogene in human preleukemia. Nature* **330:** *186, 1987*

21) Quintanilla M, Brown K, Ramsden M, Balmain A: *A carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. Nature* **332:** *78, 1986*

22) Blin AN, Stafford DW: *A general method for isolation of high molecular weight DNA from eukaryotes. Nucl Acids Res* **3:** *2303, 1976*

23) Southern EM: *Detection of specific sequence among DNA fragments separated by gel electrophoresis. J Mol Biol* **98:** *503, 1975*

24) Tsujino T, Yoshida K, Nakayama H, Ito Shimosato T, Tahara E: *Alterations of oncogenes in metastatic tumors of human gastric carcinomas. Br J Cancer* **62:** *226, 1990*

25) Chomczynski P, Sacchi N: *Single-step method of RNA isolation by acid guanidine thiocyanate-phenol-chloroform extraction. Anal Biochem* **162:** *156, 1987*

26) DeBortoli ME, Abou-Issa H, Haley BE, Cho-Chung YS: *Amplified expression of p21 ras protein in hormone-dependent mammary carcinomas of humans and rodents. Biochem Biophys Res Commun* **127:** *699, 1985*

27) Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: *Protein measurement with the folin phenol reagent. J Biol Chem* **193:** *265, 1951*

28) Fruth ME, Davis LJ, Ffleurdelys B, Scolnick E: *Monoclonal antibody to the p21 products of the transforming gene of Harvey murine sarcoma virus and of the cellular ras gene family. J Virol* **43:** *294, 1982*

29) Fujita J, Srivastava SK, Kraus MH, Rhim JS, Tronick SR, Aronson SA: *Frequence of molecular alterations affecting ras protooncogenes in human urinary tract tumor. Proc Natl Acad Sci USA* **82:** *3849, 1985*

30) Orita M, Suzuki Y, Hayashi K: *Rapid and sensitive detection of point mutation and DNA polymorphisms using polymerase chain reaction. Genomics* **5:** *874, 1989*

31) Victor T, Du Toit R, Jordaan AM, Bester AJ, van Helden PD: *No evidence for point mutations in codons 12, 13, and 61 of the ras gene in a high-incidence area for esophageal and gastric cancers. Cancer Res* **50:** *4911, 1990*

32) Correa P, Haenszel W, Cuello C, Tannenbaum S, Archer M: *Model for gastric cancer epidemiology. Lancet* **2:** *58, 1975*

33) Nau MM, Brooks BJ, Carney DN, Gazdar AF, Battey JF, Sausville EA, Minna JD: *Human small-cell lung cancers show amplification and expression of the N-myc gene. Proc Natl Acad Sci USA* **83:** *1092, 1986*

34) Prassolov VS, Sakamoto H, Nishimura S, Terada M, Sugimura T: *Activation of c-Ki-ras gene in human pancreatic cancer. Jpn J Cancer Res* **76:** *792, 1985*

35) Tsuchiya T, Ueyama Y, Tamaoki N, Yamaguchi S, Shibuya M: *Co-amplification of c-myc and c-erbB-2 oncogenes in poorly differentiated human gastric cancer. Jpn J Cancer Res* **80:** *920, 1989*

36) Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishol JM: *Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science* **224:** *1121, 1984*

37) Nau MM, Brooks BJ, Battey J, SAusville E, Gazdar AF, Kirsch IR, McBride OW, Bertness V, Hollis GF, Minna JD: *L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. Nature* **918:** *69, 1985*

38) Libermann TA, Nusbaum HR, Razon N, Kris R Liax I, Soreq H, Whittle N, Waterfield MD, Ullrich A, Schlessinger J: *Amplification and overexpresion of the EGF receptor gene in primary human glioblastomas. Nature* **312:** *144, 1985*

39) Yoshida K, Tsuda T, Matsumura T, Tsujino T, Hattori T, Ito H, Tahara E: *Amplification of epidermal growth factor receptor(EGFR)gene and oncogenes in human gastric carcinomas. Virchows Arch* **57:** *285, 1989*

40) Tahara E: *Oncogenes in human gastric carcinoma. Jpn J Cancer Chemother* **16:** *2149, 1989*

41) Ranzani GN, Pellefata NS, Previdere C, Saragoni A, Vio A, Maltoni M, Amadori D: *Heterogenous protooncogene amplification correlated with tumor progression and presence of metastases in gastric cancer patients. Cancer Res* **50:** *7811, 1990*

42) Koda T, Matsushima S, Sassaki A, Danijo Y, Kakinuma M: *c-myc gene amplification in primary stomach cancer. Jpn J Cancer Res* **76:** *551, 1985*

43) Hupp TR, Meek DW, Midgley CA, Lane DD: *Regulation of the specific DNA binding function of p53. Cell* **71:** *875, 1992*

44) Hollstein M, Sidransky D, Vogelstein B, Harris C: *p53 mutations in human cancer. Science* **253:** *49, 1991*

45) Lane D, Benchimol S: *p53 oncogene or anti-oncogene? Oncogene* **4:** *1, 1990*

46) Fujita K. Ohuchi N, Yao T, Okumura M, Fukushima Y, Kanakura Y, Kitamura Y, Fujita J: *Frequent overexpression, but not activation by point mutation, of ras genes in primary human*

gastric cancers. Gastroenterology **93:** *1339, 1987*

47) Reddy EP, Reynolds RK, Santos E, Barbacid M: *A point mutation is a responsible for the acquisition of transforming properties by T-24 human bladder carcinoma oncogene. Nature* **300:** *149, 1982*

48) Pulcianin S, Santos E, Long LK, Sorrentino V, Barbacid M: *Ras gene amplification and malignant transformation. Mol Cell Biol* **5:** *2836, 1985*

49) Cooper GM: *Cellular transforming genes. Science* **217:***801, 1982*

50) Feinberg AP, Vogelstein B, Droller MJ, Baylin SB, Nelkin BD: *Mutation affecting the 12th amino acid of the c-Ha-ras oncogene product occurs infrequently in human cancer. Science* **220:** *1175, 1983*

51) Varmus HE: *The molecular genetics of cellular oncogenes. Ann Rev Gent* **18:** *553, 1984*

52) Jiang W, Kahn SM, Gullem JG, Lu S-H, Weinstein B: *Rapid detection of ras oncogenes in human tumors: applications to colon, esophageal, and gastric cancer. Oncogene* **4:** *923, 1989*

53) Oda T, Tsuda H, Scarpa A, Sakamoto M, Hirohashi S: *Mutation pattern of the p53 gene as a diagnostic marker for multiple hepatocellular carcinoma. Cancer Res* **52:** *3674, 1992*

54) Suzuki Y, Orita M, Shiraishi M, Hayashi K, Sekiya T: *Detection of ras gene mutations in human lung cancers by single-strand conformation polymorphism analysis of polymerase chain reaction products. Oncogene* **5:** *1037, 1990*

55) Deng G, Lu Y, Chen S, Miao J, Lu G, Li H, Cai H, Xu X, Zheng E, Liu P: *Activated c-Ha-ras oncogene with a guanine to thymine transversion at the twelfth codon in a human stomach cancer cell lines. Cancer Res* **47:** *3195, 1987*

56) Koh EH, Chung HC, Lee KB, Han EK, Oh SH, Min JS, Choi EM, Youn JK, Kim BS: *Point mutation at codon 12 of the c-Ha-ras gene in human gastric carcinoma. J Kor Med Sci* **7:** *110, 1992*

57) O'Hara BM, Oskarsson M, Tainsky MA, Blair DG: *Mechanism of activation of human ras genes cloned from a gastric adenocarcinoma and a pancreatic carcinoma cell line. Cancer Res* **46:** *4695, 1986*

58) Karayiannis M, Yiagnisis M, Papadimitrious K, Spandidos DA: *Expression of the ras oncoprotein in gastric carcinomas and adjacent mucosa. Anticancer Res* **9:** *1505, 1989*

59) Ohuchi N, Hand PH, Merlo G, Fujita J, Mariani-Costantini R, Thor A, Nose M, Callahan R, Schlom J: *Enhanced expression of c-Ha-ras p21 in human stomach adenocarcinomas defined by immunoassay using monoclonal antibodies and in situ hybridization. Cnacer Res* **47:** *1413, 1987*