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MISSENSE MUTATIONS AND A DELETION OF THE p53 GENE IN HUMAN GASTRIC CANCER

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Summary: To investigate the molecular pathogenesis of human gastric cancers the p53 gene, a suppressor oncogene, was analyzed in 12 human gastric cell lines. Southern blot and Northern blot analysis revealed a total deletion of p53 gene in KATO-III cells but no major abnormality of p53 gene in other cell lines. By the use of the reverse-transcriptase polymerase chain reaction and direct sequencing 7 cell lines showed point mutations of p53 gene resulting in amino-acid substitutions. Most of them were rare mutations which had not been observed in other types of cancers. One of these mutations was also detected through the use of PCR and oligomer-specific hybridization. Six out of 7 cell lines with mutations of p53 gene also lost one allele of chromosome 17p. Immunoblotting of cell lysates with an antibody specific to p53 demonstrated the absence of p53 protein in KATO-III cell. By contrast, the high levels of the p53 protein were observed in 5 cell lines all of which contained mutations of p53 gene. These results further suggest that the inactivation of p53 gene may play an important role in the transformation of gastric cells to the malignant phenotype. KATO-III cells might be a good model for studying the significance of the loss of p53 gene in cellular transformation.

Human gastric cancer is one of the most frequent tumors worldwide, particularly in Japan (1). Although the molecular mechanism involved in malignant transformation of stomach cells has been explored, little information is available at present. The activation of ras oncogene seems to be infrequent in gastric cancer (2,3). On the other hand, amplification of other oncogenes such as c-erbB gene (4) or K-sam (5) has been observed in gastric carcinoma tissues and established gastric cancer cell lines.

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Abbreviations used are: PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; SDS, sodium dodecyl sulfate.

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There have recently accumulated evidences showing that not only activated oncogenes but also inactivations of tumor suppressor genes play an important role in the development of various types of neoplasms (6). The p53 gene, which initially was thought to be an oncogene, has recently been demonstrated to be capable of acting as a tumor suppressor gene (6). Furthermore, the p53 gene has been shown to be frequently mutated or deleted in a variety of human tumors such as colon (7,8), lung (8,9), and breast cancers (8). Thus, it has been proposed that the inactivation of p53 gene in combination with the activation of other oncogenes such as ras may lead to the transformation of normal cells to the malignant phenotype (6). To better understand the molecular mechanism underlying gastric tumorigenesis, therefore, we have analyzed the p53 gene in 12 human gastric cancer cell lines. We have found that there exist missense mutations of p53 gene in 7 cell lines; most of them are rare mutations and are accompanied by both allelic loss of chromosome 17p and the overexpression of mutated p53 proteins.

MATERIALS AND METHODS

Cell Lines

The gastric cancer cell lines MKN1 (adenosquamous carcinoma), MKN28 (moderately differentiated tubular adenocarcinoma), MKN45 (poorly differentiated adenocarcinoma), MKN74 (moderately differentiated tubular adenocarcinoma), KATO-III (signet ring cell carcinoma) were cultured as suggested by Japanese Cancer Research Resources Bank. AGS (adenocarcinoma) and AZ-521 (adenocarcinoma) were obtained from ATCC. TMK-1 (poorly differentiated adenocarcinoma)(10), KWS (poorly differentiated adenocarcinoma)(11), NUGC-3 (poorly differentiated adenocacinoma)(12) and NUGC-4 (poorly differentiated adenocarcinoma)(12) and JR-1 (signet ring cell carcinoma)(13) were maintained as described previously.

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA was extracted from gastric cancer cells by the standard guanidium thiocyanate/cesium chloride method (14). Complimentary DNA (cDNA) was synthesized by incubating ~2 μg of total RNA with oligo(dT) as a primer and 60 units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) for 60 min at 42 °C as described (15). PCR amplification in total volume of 0.1 ml was performed with one-tenth of the cDNA, 100 pmoles each oligonucleotide primers, 1.25 mM dNTPs and 2.5 units Taq polymerase in PCR buffer (Perkin-Elmer-Cetus) for 30 cycles in a programmable heat block. Each cycle included denaturation at 93 °C for 1 min, annealing at 55 °C for 1.5 min, and primer extension at 72 °C for 2 min. Sense and antisense PCR primers used are 5'-CTTCTGTCCCTTCCCAGAAACC and 5'-CCTCATTCAGCTCTCGGAACATCTCG, respectively. For direct sequencing, a single-stranded DNA was then synthesized by asymmetrical PCR using 1 μl of first PCR product as a template and either a sense or an antisense primer

under the same condition of first PCR but for 25 cycles rather than 30 cycles.

Sequencing of Amplified DNA

For direct sequencing of amplified single stranded DNA, 100 pmole primers were phosphorylated for 30 min at 37 °C by 13.5 unit T4 polynucleotide kinase (Boehringer Mannheim) in a total volume of 42 μl solution containing 10 mM $MgCl_2$, 10 mM dithiothreitol, 50 mM glycine (pH 9.2) and 55 μCi [γ-^{32}P]ATP (6000 Ci/mmol)(New England Nuclear) as described previously (15). The labeled primers were purified by using Sephadex G-25 Quick Spin column, dried and dissolved in 12 μl of water immediately before use in the sequencing reactions. We have used sequencing primers that are different from the oligonucleotides used as primers in the PCR. Primers used for sequencing are follows: 5'-CGGTTTCCGTCTGGGCTTCTTGC and 5'-CTGTCATCCAAATACTCCACACGC for exon 5 and 6; 5'-CCACTGGAGTCTTCCAGTGTG for exon 6 and 7; 5'-TAGTGTGGTGGTGCCCTATGAGCCG and 5'-GGCTGGGGAGAGGAGCTGGTG for exon 7 and 8; 5'-CACGCCCACGGATCTGAAGGGTG for exon 8 and 9. Amplified single-stranded DNA dissolved in 5 μl of water was mixed with 3 μl labeled primer (~25 pmol) and 2 μl of 5 x reaction buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$,and 250 mM NaCl) and the mixture was heated at 95 °C for 10 min for annealing. The mixture (2.5 μl) was then added to each of four tubes, followed by 2 μl of the dideoxy terminator/Sequenase mix (USB). After incubation at 50 °C for 40 min, the reaction was terminated by adding 3 μl of stop solution (95 % formamide/20 mM EDTA/0.05 % bromphenol blue/0.05 % xylen cyanol FF). The samples were analyzed by electrophoresis through a 6 % polyacrylamide/8 M urea gel. All mutations were confirmed by a complete repeat of the experimental procedure; amplification by PCR of stock cDNA, fragment purification, and sequencing of the DNA strand complementary to that sequenced in the initial experiment.

Allele-specific Oligonucleotide Hybridization of Amplified p53 Gene from Genomic DNA by PCR

One μg of genomic DNA extracted from normal stomach, MKN28 or MKN74 was used as a template for PCR to amplify 437 bp fragment containing codon 251. PCR was performed for 30 cycles according to the same protocol as described above. Primers used for PCR are 5'-GCGTGTGGAGTATTTGGATGACAG and 5'-GGCTGGGGAGAGGAGCTGGTG. One-fifth of the amplified PCR product was electrophoresed on 1.8 % of agarose gel and transferred to nylon membranes. The DNA blots were hybridized in a solution containing 5 x SSPE (pH 7.4), 5 x Denhardt's solution, 0.1 % SDS and 100 μg/ml salmon sperm DNA at 37 °C for 6 hr with ^{32}P-labeled synthetic oligonucleotides, which were labeled with [γ-^{32}P]ATP as described above. The wild-type oligonucleotides was 5'-CGGAGGCCCATCCTCAACCATC and the mutant-type oligonucleotide was 5'-CGGAGGCCCCTCCTCAACCATC. The blots were then washed twice at room temperature in 2 x SSPE/0.1 % SDS, followed by a 15-min wash at 65 °C in 5 x SSPE/0.1 % SDS (15).

Southern Blot analysis and Allelic Deletion Analysis

Extracted genomic DNAs (7 μg) from cultured cells were digested with Hind III or Eco RI for 4 hr in the presence of 2 mM spermidine. HinfI was also used for allelic deletion analysis. Digested DNA was separated by electrophoresis on 0.8 % agarose gel

overnight and transferred to Bio-Dyne A Nylon filter (Pall). The p53 cDNA probe which had been originally distributed by Dr. L. Crawford (16) was prepared from plasmid pSP65 (kindly provided by Dr. S. Ishii) by purifying a 2 kbp human p53 cDNA insert. p53 cDNA probe or pYNZ 22 probe (provided by Japanese Cancer Research Resources Bank) for the allelic deletion analysis (17) was labeled with [α-^{32}P]dCTP (3000 Ci/mmol)(New England Nuclear), by using random primer methods (14).

Northern Blot Analysis

Total RNA (20 μg) was electrophoresed on 1.2 % agarose/formaldehyde gel and transferred to nylon filter. The blot was hybridized with ^{32}P-labeled p53 cDNA probe prepared as described above in 50 % formamide, 5 x SSC, 5 x Denhardt's solution, 50 mM $NaPO_4$ (pH 7.0), 0.1 % SDS and 100 μg/ml salmon sperm DNA overnight at 42 ^{o}C. The filter was rehybridized with human β-actin oligonucleotide probe (Oncogene Science) which was labeled by T_4 polynucleotide kinase with [γ-^{32}P]ATP (6000 Ci/mmol)(New England Nuclear) as recommended by supplier.

Western Blotting

Cells harvested were suspended in the lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1 % Triton-X 100, 1 % sodium deoxycholate, 0.1 % SDS, 1 mM phenylmethylsulfonyl fluoride, allowed to incubate on ice for 15 min and then sonicated for 30 s by a probe-type sonicator. The extracted p53 protein were immunoblotted, incubated with anti-p53 monoclonal antibody PAb 1801 (18)(Oncogene Sciences) and visualized with peroxidase-conjugated goat anti-mouse Ig G (Capel) and 3,3'-diaminobenzidine tetrahydrochloride in the presence of H_2O_2.

RESULTS

Recent literatures have demonstrated that most somatic p53 gene mutations in human tumors localized within exons 5-8 (6,7). Thus, exons of 4-9 of p53 gene were amplified by RT-PCR from total RNA of cancer cells and the amplified PCR fragments were directly sequenced. However, no amplified PCR fragment was obtained in KATO-III cells. Furthermore, both Southern blot and Northern blot analysis revealed no band (Fig. 1), suggesting a homozygous deletion spanning coding sequence of p53 gene in KATO-III cell. By contrast, normal Eco RI- and Hind III-digests were observed in MKN1, MKN28 (Fig. 1) and other cell lines (data not shown). The direct sequencing showed that 7 of 12 cell lines contained single point mutations of the p53 gene (Fig. 2)(Table 1). Two cell lines had mutations at codon 251 and three at codon 173 (Table 1). All mutations observed are missense mutations, altering the deduced amino acid sequence of p53. Furthermore, only the altered sequence was observed in every 6 cell line but JR-1, indicating that these 6

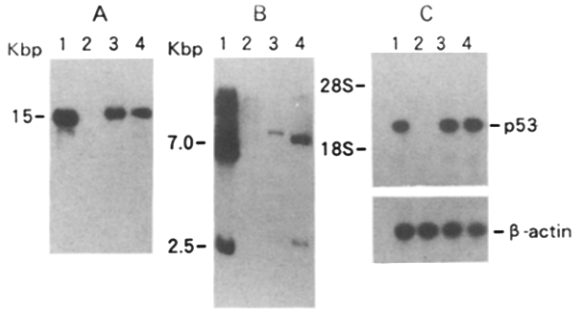

Fig. 1. Southern blot (A,B) and Northern blot (C) analysis of p53 gene in gastric cancer cell lines. Genomic DNAs digested with Eco RI (A) and Hind III (B) or total RNAs were hybridized with either labeled p53 cDNA probe or β-actin probe. Lanes: 1, normal stomach; 2, KATO-III; 3, MKN1; 4, MKN28.

cell lines are functionally homozygous for the mutation and only synthesize mutant p53 mRNA. By contrast, JR-1 showed a point mutation at codon 173 but a wild-type sequence was also present and yielded a signal approximately equal in intensity to the mutant, suggesting the normal allele was retained in this cell line (Fig. 2).

To further confirm the detected mutation of p53 gene, a 437 bp DNA fragment containing codon 251 of p53 gene was amplified from genomic DNA by PCR and hybridized with oligonucleotides specific for either the wild-type sequence or the mutant sequence (Fig. 3). The oligonucleotide specific for the wild-type sequence at codon 251 (ATC) hybridized to DNA from normal stomach mucosa but not to

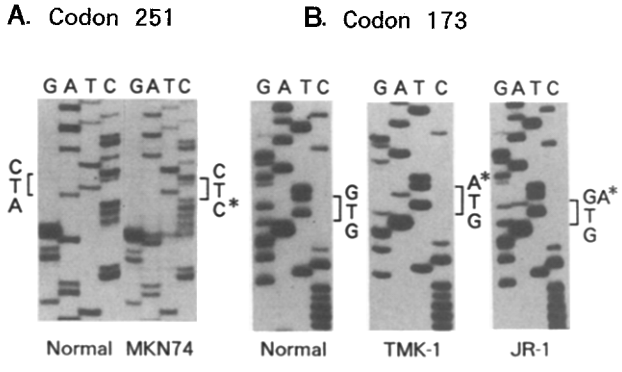

Fig. 2. Missense mutations of p53 in gastric cancer cell lines. Each panel demonstrates the particular point mutation within the codon 251 (A) or 173 (B) relative to its position within the rest of the surrounding sequence. The wild-type sequence autoradiogram shown was obtained from normal stomach tissue. The sequences are read from bottom to top (A) or read from top to bottom (B).

TABLE 1

p53 mutations in gastric cancer cell lines

Cell line	17p alleles	codon	Mutation	Amino acid
MKN1	1	143	GTG->GCG	Val->Ala
MKN28	1	251	ATC->CTC	Ile->Leu
MKN45	1		N.D.	
MKN74	1	251	ATC->CTC	Ile->Leu
KATO-III	1		Deleted	
KWS	1	173	GTG->GCG	Val->Ala
TMK-1	1	173	GTG->ATG	Val->Met
NUGC-3	1	220	TAT->TGT	Tyr->Cys
NUGC-4	2		N.D.	
AGS	2		N.D.	
AZ-521	2		N.D.	
JR-1	2	173	GTG->ATG	Val->Met

N.D., not detected.

DNA from either MKN28 or MKN74. By contrast, the oligonucleotide specific for the mutant sequence at codon 251 (CTC) hybridized to DNAs from both MKN28 and MKN74 but not to DNA from normal stomach. This result further suggests that in both MKN28 and MKN74 codon 251 of p53 gene was mutated in either a homozygous or a hemizygous state.

In order to further analyze the state of p53 gene in these gastric cancer cell line, we examined allelic loss of chromosome 17p, where p53 gene harbors, in these gastric cancer cell lines. We used pYNZ 22, a highly polymorphic DNA sequence on chromosome 17p that identifies variable number tandem repeat sequences (17). When genomic DNA was digested with Hinf I and hybridized with pYNZ 22 probe, only one allele of 17p was observed in 8 of 12 cell lines (Table 1), although non-neoplastic tissues of stomach corresponding to these cancer cells were not examined.

The levels of p53 protein in gastric cancer cells were also analyzed by the Western blotting with the monoclonal antibody PAb 1801, because the overexpression of p53 protein has been often detected in various neoplastic tumors with the mutations of p53 gene (6). As shown in Fig. 4, the presence of trace amount of p53 in normal stomach was demonstrated. As compared with normal stomach, high levels of p53 protein were clearly observed in MKN1, MKN28, MKN74, KWS and TMK-1 (Fig. 4). All these cell line had point mutations of p53 gene (see Table 1). By contrast, a band corresponding to p53 protein was not detectable in KATO-III cell. In other cell lines, the level of p53 protein seems to be slightly higher than or almost equal to that of normal stomach (Fig. 4).

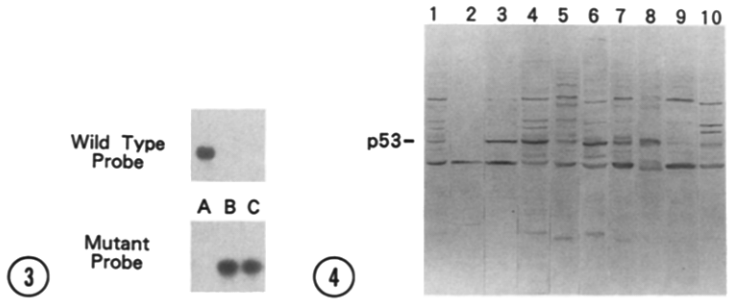

Fig. 3. Allele-specific oligonucleotide hybridization of amplified DNA. The 437 bp fragment of genomic DNA including codon 251 was amplified by PCR from total DNA of normal stomach, MKN28 or MKN74. The DNA blots were then hybridized with ^{32}P-labeled synthetic oligonucleotides. (Upper panel) Hybridization with the wild-type oligonucleotide. (Lower panel) Hybridization with the mutant oligonucleotide. Lane A, normal stomach (control); Lane B, MKN28; Lane C, MKN74.

Fig. 4. Western blot analysis of p53 protein in gastric cancer cell lines. The cellular protein (50 μg) extracted from each cell line was electrophoresed on a 10 % polyacrylamide SDS gel and blotted to a nitrocellulose filter. The filter was then incubated with anti-p53 monoclonal antibody PAb1801, followed by visualization with peroxidase-conjugated goat anti-mouse IgG. Lanes: 1, normal stomach; 2, KATO-III; 3, MKN1; 4, MKN28; 5, MKN45; 6, MKN74; 7, KWS; 8, TMK-1; 9, AGS; 10,NU-GC-4.

DISCUSSION

In the present study, it has been clearly demonstrated that frequent point mutations of p53 gene exist in gastric cancer cell lines. Furthermore, p53 gene is totally deleted in KATO-III cells without the expression of p53 protein. By contrast, overexpression of p53 protein was observed in most of gastric cancer cells which had point mutations of p53 gene. This results are in good accordance with the previous studies demonstrating that the high levels of mutant p53 proteins in established cell lines and primary cancers containing p53 gene mutations (6). All point mutations observed were missense mutations which then alter amino acid sequence of p53 protein. Two were at A -> C substitution at codon 251 and three were at codon 173. During the work of our present study, two other reports showing that mutations and deletions of p53 gene occur in human gastric cancer cell lines (19) and primary gastric cancer tumors (19,20) has recently been published. One has described that p53 gene mutations at codon 251 and codon 173 are also found in primary gastric cancer samples (20), a finding coincides with our present results in gastric cancer cell lines. Of interest is that the p53 gene mutations at codon 251 and codon 173 seems to be rare mutations which have not been observed in

other types of carcinomas (6-9). Although the number of samples examined is small, it is possible, therefore, that these two codon might be hot spots for p53 mutations in gastric cancer. Most of cell lines with p53 mutations have only one allele of chromosome 17p as evaluated with pYNZ 22 probe (Table 1). In these cells, only altered bands in the sequence ladders were observed. Furthermore, allele specific oligonucleotide hybridization of amplified p53 gene by PCR reveled that only oligonucleotide specific for the mutant sequence hybridized to the amplified DNA of either MKN28 or MKN74 but not to amplified DNA from normal stomach. Taken together, results suggest that these gastric cell lines lose one copy of chromosome 17p and have a mutation in the remaining p53 allele, leading to the loss of normal function of the p53 gene product. These results well correspond with recent reports demonstrating that 75-80 % of colon cancer possesses both an allelic deletion of 17p and a point mutation of the remaining allele of p53 gene (7). Although the present study has been performed on stomach cancer-derived cell lines, results suggest that inactivation of p53 gene through mutations and the allelic loss of 17p may play an important role in the development of gastric cancer as seen in other neoplasms.

It has recently been shown that the transfection of human wild-type p53 gene to human carcinoma cells with a deletion or a mutation of p53 can suppress the growth of carcinoma cells in vitro (21,22). By contrast, the transfection of mutant p53 gene at codon 143, the same mutation observed in MKN1, has been shown to be unable to suppress the growth of colon cancer cells (21). Since p53 gene is totally deleted in KATO-III cells, this cell line is a good model for transfection study with either the wild-type or the mutated p53 gene in order to elucidate the possibility that the inactivation of p53 gene is truly involved in tumorigenesis of gastric cells.

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