**p53 Gene Mutations in Human Gastric Cancer: Wild-Type p53 but not Mutant**

**p53 Suppresses Growth of Human Gastric Cancer Cells**

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**ABSTRACT**

To further investigate the role of p53 gene inactivation in gastric tumorigenesis, the mutational status of the p53 gene in primary human gastric cancer samples was examined. Reverse transcriptase polymerase chain reaction and subsequent direct sequencing of the p53 gene from gastric cancer samples revealed frequent point mutations of the p53 gene: some of these coincided with those previously identified in gastric cancer cell lines. In addition, both allelic deletion analysis using pYNZ 22 and polymerase chain reaction-restriction fragment length polymorphism analysis demonstrated an allelic deletion of the p53 gene in cancer tissue which contained a point mutation of the p53 gene in the remaining allele. Transfection of the wild-type or mutant p53 genes into gastric cancer cells showed that the wild-type but none of the mutated p53 genes suppressed the colony formation of gastric cancer cells. Furthermore, the incorporation of thymidine into DNA was reduced in cancer cells expressing the wild-type p53 gene. The glutathione S-transferase wild-type p53 fusion protein bound to simian virus 40 large T antigen in COS-1 cell lysate. None of the p53 fusion proteins containing mutations at codons 143, 175, 248, or 273 bound to simian virus 40 large T antigen. By contrast, two different mutant p53 fusion proteins containing mutations specifically observed in gastric cancer bound to simian virus 40 large T antigen. These results indicate that inactivation of the p53 gene through mutations and the allelic deletion may play an important role in gastric tumorigenesis. These mutations may cause a conformational change in the p53 protein resulting in the loss of suppression by p53 of the growth of gastric cells, partly through disruption of the association of p53 protein with a cellular component.

**INTRODUCTION**

Human gastric cancer is one of the most common tumors worldwide, particularly in Japan (1). Although the molecular mechanism underlying malignant transformation of stomach cells has not yet been well explored, evidence has recently accumulated indicating the involvement of the alterations of multiple genes in gastric tumorigenesis. Amplification of oncogenes such as c-erbB (2) or K-sam (3) has been observed in gastric carcinoma tissues and in established gastric cancer cell lines. On the other hand, RFLP2 analysis has revealed that the allelic loss of chromosome 1 or 17p is frequently associated with primary gastric cancer samples (4). Furthermore, mutations of the p53 gene, which is located at chromosome 17p13 (5–7), have been detected in primary human gastric cancer (8) and gastric cancer cell lines (9–11). The mutation of the p53 gene has been found in conjunction with chromosome 17p allelic deletions in a variety of other types of human cancers such as colon (12–14), lung (14, 15), and breast cancers (14, 16). Since the p53 gene can act as a tumor suppressor gene (17), it has been suggested that its inactivation is involved in gastric tumorigenesis. In the present study, to assess the role of the p53 gene in gastric tumorigenesis, we have further analyzed the mutational status of this gene in primary human gastric cancer samples. We have also determined whether or not the p53 gene mutations detected in gastric cancer impair the function of the wild-type p53 protein in terms of either the suppression of growth of gastric cancer cells or the ability to bind to SV40 LTA.

**MATERIALS AND METHODS**

**Tumor Samples.** Fresh tumor tissues were obtained during surgical resection. Unfixed tumor tissue samples were rapidly frozen in liquid nitrogen or ice-cold ethanol and stored at −80°C. To improve the sensitivity of the detection of p53 gene mutation, sequential frozen sections were mounted on glass slides, stained, and evaluated microscopically to select areas that preferentially contained more tumor and fewer contaminating nontumor cells.

**Reverse Transcriptase PCR and Direct Sequencing.** Reverse transcriptase PCR and direct sequencing were performed as previously described (11). Briefly, total RNA was extracted by the standard guanidium thiocyanate/cesium chloride method (11) from tissue sections which were shown to contain abundant tumor cells by histological examination as described above. cDNA was then synthesized by incubating ~2 µg of total RNA of cancer tissues with oligodeoxythymidine (Boehringer Mannheim) as a primer. PCR amplification was performed in a 100-µl reaction volume using one-tenth of the cDNA and 100 pmol of each oligonucleotide primer for 30 cycles as described (11). Each cycle included denaturation at 94°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 were 5'-CTTCTGTCCCTTCCCAGAAACC and 5'-CCTCCATTCAGCTCTCGGAACATCTCG, respectively. For direct sequencing, a single-stranded DNA was then synthesized by asymmetrical PCR for 25 cycles. We used sequencing primers that are different from the oligonucleotides used as primers in the PCR (11). The sequencing of an amplified single-stranded DNA was carried out as described previously (11). 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.

**Detection of LOH in the p53 Gene.** To detect the allelic deletion of the p53 gene, a highly informative probe, pYNZ 22 (18), mapping near the p53 gene was used after Hinfl digestion of genomic DNA extracted from the tumor specimen and the corresponding normal stomach tissue as described previously (11). Another study to examine the allelic deletion of p53 gene was performed by using PCR-RFLP analysis as described previously (19). Briefly, the DNA fragment (199 base pairs) containing codon 72 of the p53 gene was amplified by PCR from genomic DNA. The PCR amplification was performed using 1 µg genomic DNA as described above. Primers for this PCR were 5'-CTTGC- CGTCCCAACGAAATGGATG (sense) and 5'-CTGGGAAGGGACACCA- GAAGATGAC (antisense). One-tenth of the PCR product was digested with AccI for 2 h at 37°C. The DNA fragments were then separated by electrophoresis on 1.8% agarose gel.

**Plasmids for the Transfection.** The expression vector pCMV-Neo (Invitrogen) used for the transfection study contains a cytomegalovirus promoter-enhancer upstream of the insertion site for cDNA sequences to be expressed together with a second transcription unit containing a
neomycin resistance gene driven by the SV40 promoter. A 1.9-kilobase EcoRI and BamHI fragment, extending from -130 to 1800 relative to the translation initiation site, was isolated from plasmid psp-p53 (kindly provided by Dr. S. Ishii) containing wild-type p53 cDNA, which had been originally distributed by Dr. L. Crawford (20). The fragment was blunt-ended with a Klenow fragment of DNA polymerase, ligated to HindIII linker, digested with HindIII and XbaI, and then cloned into the HindIII-XbaI restriction site of pCMV-neo to generate pCMV-WT. Three different mutant p53 cDNAs containing mutations at codons 143 (mt143), 173 (mt173), or 251 (mt251) were generated by site-directed mutagenesis as described previously (21) and ligated to pCMV-neo, generating pCMV-mt143, pCMV-mt173, and pCMV-mt251, respectively. The mutant p53 cDNA, mt143, contains GCG instead of GTG at codon 173; mt251 contains CTG instead of GTG at codon 173; and mt251 contains CTC instead of ATC at codon 251.

Cell Culture and DNA Transfection. All three gastric cancer cell lines, MKN1 (adenosquamous carcinoma), MKN28 (moderately differentiated tubular adenocarcinoma), and MKN45 (poorly differentiated adenocarcinoma) were cultured in RPMI-1640 supplemented with 10% fetal calf serum at 37°C as described (11). For transfection, 5 × 10⁵ cells were seeded into 10-cm culture dishes 24 h before the transfection. Plasmid DNA (10 μg) dissolved in 50 mM water was mixed with 30 μg of Lipofectin (BRL). The mixture was then added to cells in 5 ml of Opti-minimum essential medium I (Gibco). After 18 h, 5 ml of RPMI-1640 containing 20% fetal calf serum were added to the cells. On the next day, the cells were seeded into three 15-cm culture dishes for the selection of G-418-resistant colonies. Genes for the p53 wild-type and mutant cDNAs were selected in the growth medium containing 600 μg/ml G-418 (Gibco) for 2–3 weeks, and the total number of colonies formed was counted.

To examine the effect of p53 gene expression on DNA synthesis, transfected MKN45 cells were used. The p53 gene expression vector was introduced into MKN45 cells by electroporation as described previously (23, 24). Fresh overnight cultured Escherichia coli (NM 522) transformed with pGEX-3X or one of the pGEX-3X-p53 recombinants were diluted 1:20 in LB medium containing ampicillin (100 μg/ml) and incubated at 37°C. After a 3-4 h incubation, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM, followed by a 20-h incubation. For fusion-protein recovery on glutathione-Sepharose beads (Pharmacia), 30 ml of bacterial culture were centrifuged in 3 ml of NETN containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The bacteria were then briefly lysed on ice by sonication and centrifuged at 10,000 × g for 15 min at 4°C. Aliquots of bacterial supernatant were then rocked for 30 min at 4°C with 50 μl of glutathione-Sepharose (Pharmacia) suspended in NETN (final concentration, 1:1, v/v). After centrifugation, beads were further rocked with 1 ml of whole COS cell lysate prepared as described previously (24) for 2 h at 4°C. The glutathione-Sepharose beads were then washed three times with 1 ml NETN, boiled in 50 μl of 1× SDS sample buffer, and then loaded onto 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were immunoblotted, incubated with anti-p53 antibody Pab 1801 (Oncogene Sciences), followed by visualization with peroxidase-conjugated goat anti-mouse IgG (Amersham) and a 3,3'-diaminobenzidine tetrahydrochloride in the presence of H₂O₂. Slides were then dipped in NTB-2 emulsion (Kodak) and autoradiographed for 1 week at 4°C.

Analysis of the p53 Gene in the Transfected Cells by Southern Blotting and Northern Blotting. In order to determine whether the cells transfected with either wild-type or mutated p53 cDNA retained the transfected sequences, a 465-base pair DNA fragment was generated by PCR for use as a labeled probe in Southern blotting. PCR amplification was performed as described above, and a cDNA synthesized from the total RNA of MKN28 was used as a template. The primers used were 5'-TCCGAATTCTTGTGGCTACTGATCCAC (exon 7 as a sense primer) and 5'-CTCGAGATCCATGGCGGAGGTAGACTGACTGACC (from exon 11 as an antisense primer). The amplified DNA was digested with BamHI and EcoRI, ligated to pUC 19, and then sequenced as described previously (21). This inserted fragment was then excised and labeled with [α-32P]dCTP (3000 Ci/mmol) (New England Nuclear) by random primer methods (21). Using this labeled probe, the transfected p53 gene was easily distinguished from the endogenous p53 gene following the digestion of the genomic DNA with both HindIII and XbaI, resulting in a 5.3-kilobase fragment (20) for the latter and a 1.9-kilobase fragment for the former. Extracted genomic DNAs (7 μg) from transfected cells were digested with HindIII and XbaI for 4 h in the presence of 2 μg spermidine. Digested DNA was separated by electrophoresis on 8% agarose gel overnight, transferred, and hybridized with the labeled probe as described previously (11).

For Northern blotting, total RNA (20 μg) extracted from transfected cells was electrophoresed on 1.2% agarose/formaldehyde gel and transferred to a nylon filter as described previously (11). The p53 cDNA probe was prepared from plasmid pSP65 by purifying a 2-kilobase human p53 cDNA insert. The blot was hybridized with the 32P-labeled p53 cDNA probe in 50% formamide, 5× standard saline citrate, 5× Denhardt’s solution, 50 mM NaPO₄ (pH 7.0), 0.1% SDS, and 100 μg/ml salmon sperm DNA overnight at 42°C. The blots were then washed as described previously (11).

RESULTS

Identification of Missense Mutations of the p53 Gene in Gastric Cancer Samples. In order to analyze the status of the p53 gene in human gastric cancer, we first sequenced the p53 gene in primary gastric cancer samples by reverse transcriptase PCR and direct sequencing. Seven of 14 tumor samples contained single point mutations of the p53 gene (Fig. 1; Table 1). All mutations observed were missense mutations, altering the deduced amino acid sequence of p53. Mutations observed were missense mutations, altering the deduced amino acid sequence of p53. Mutations at codon 251 and codon 173 coincided with those previously observed in gastric cancer cell lines (10, 11). Furthermore, an altered sequence band without a normal sequence band was observed in all five samples, indicating that these tumors are functionally homozygous for the mutations. In the remaining two tumors (no. 7 and no. 14), the wild-type sequence was also present and yielded a single point mutation of the p53 gene (Fig. 1; Table 1). All mutations observed were missense mutations, altering the deduced amino acid sequence of p53. Mutations at codon 251 and codon 173 coincided with those previously observed in gastric cancer cell lines (10, 11). Furthermore, only an altered sequence band without a normal sequence band was observed in all five samples, indicating that these tumors are functionally homozygous for the mutations. In the remaining two tumors (no. 7 and no. 14), the wild-type sequence was also present and yielded a signal approximately equal in intensity to that of the mutant band without a normal sequence band. None of these mutations were observed in normal DNA from the stomach corresponding to these cancer cells. Furthermore, no significant correlation between the occurrence of p53 gene mutations and histological grade of tumor differentiation was observed (p53 mutation-positive: four in well-differentiated, three in undifferentiated) (Table 1).

In order to further analyze the status of the p53 gene in these gastric tumors, we next examined the allelic loss of chromosome 17p, where the p53 gene is harbored (5, 7). When genomic DNA was digested with HindIII and hybridized with pYNZ 22,
p53 GENE MUTATIONS AND GASTRIC CANCER

Fig. 1. Missense mutations of the p53 gene in human gastric cancer. Sequencing of the region spanning exons 5 through 8 was carried out as described under “Materials and Methods.” Each panel shows the particular point mutation within the codon 248 (patient 1) or codon 251 (patient 4) 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.

Table 1 p53 gene mutations in human gastric cancer

<table>
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<tr>
<th>Samples</th>
<th>Pathological diagnosis</th>
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<td>251</td>
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<td>239</td>
<td>AAC — AGC</td>
<td>Asn — Ser</td>
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* Poor, poorly differentiated adenocarcinoma; Tub 1, well-differentiated tubular adenocarcinoma; Tub 2, moderately differentiated tubular adenocarcinoma; Sig, signet ring cell carcinoma.

Transfection of Wild-Type and Mutant p53 Gene into Gastric Cancer Cells. To determine whether point mutations of the p53 gene observed in gastric cancers impair the normal function of the p53 gene product, both wild-type and mutant p53 cDNAs were transfected into the two gastric cancer cell lines, MKN1 and MKN28. As shown previously (11), MKN1 and MKN28 contain point mutations of the p53 gene at codons 143 (GTG—GCG) and 251 (ATC—CTC), respectively. We constructed the wild-type vector pCMV-WT and three different mutant vectors (pCMV-mt143, and pCMV-mt251) as described in “Materials and Methods.” The pCMV-mt143 and pCMV-mt251 contained the same mutations of p53 gene as those observed in MKN1 and MKN28, respectively. pCMV-mt173 contained p53 cDNA mutated at codon 173 (GTG—GCG). These mutations of the p53 gene were also observed in primary gastric cancer (Table 1). These constructs were transfected into MKN1 or MKN28, and geneticin-resistant colonies were counted 3 weeks after transfection. Because the calcium phosphate precipitation method was found to be ineffective for the transfection of vectors into MKN1 and MKN28, we used lipofectin for the transfection. MKN1 cells transfected with the wild-type constructs formed approximately 5-fold fewer colonies than those transfected with either the pCMV vector only or any of the mutant vectors (Fig. 3). In addition, the transfection of wild-type but not mutant p53 suppressed the formation of colonies of MKN28 cells resistant to G-418 (Fig. 3), although the extent of inhibition by the wild p53 of colony formation of MKN28 cells was less than that observed in MKN1 cells. These results suggest that transfection of the wild-type p53 gene into either MKN1 or MKN28 inhibits clonal growth of these cancer cells. We next tried to isolate cell lines that contained exogenous p53 gene and expressed its mRNA. Eight of 24 lines derived from transfection of MKN1 cells with pCMV-mt173 construct contained exogenous mutant p53 cDNA (1.9 kilobases) (Fig. 4A, left). In addition, Northern blotting revealed that these cell lines expressed a second 2.0-kilobase p53 RNA band below the endogenous 2.8-kilobase p53 mRNA (Fig. 4B, left). Six of 24 cell lines of MKN1 cell transfected with pCMV-mt251 also contained exogenous p53 cDNA (Fig. 4A, left) and expressed exogenous p53 mRNA (Fig. 4B, left). By contrast, neither exogenous cDNA nor exogenous mRNA expression was detected in any clonal lines established from MKN1 cells.

Fig. 2. Detection of LOH of the p53 gene in gastric cancers by PCR-RFLP analysis. Genomic DNA was isolated from samples of primary tumors (T) and from normal stomach tissue (N) from patient 4 and patient 6 (see Table 1). PCR was performed using 1 μg of genomic DNA as described under “Materials and Methods.” The amplified DNA fragments were digested with (Lanes 3, 4, 7, and 8) or without (Lanes 1, 2, 5, and 6) AccII and then separated by electrophoresis on 1.8% agarose gel.
transfected with the wild-type p53 gene (Fig. 4, left). Therefore, we next analyzed pooled clones consisting of more than 100 clones 3 weeks after transfection. Southern blotting revealed no detectable exogenous p53 gene sequence in colonies derived from the wild-type p53 transfectants, while the intact p53 cDNA band in colonies derived from the mutant p53 cDNA expression vector (Fig. 4, right). Exogenous p53 mRNA was expressed in pooled clones derived from MKN1 cells transfected with mutant p53 gene but not in pooled clones from cells transfected with the wild-type p55 gene (Fig. 4B, right). Similarly, in transfected MKN28 cells, exogenous p53 DNA was detected in clones derived from mutant p53 gene transfectants but not in cells derived from the wild-type p53 transfectants (data not shown).

Since transfected cells expressing wild-type p53 were not obtained in the stable transfection study, we examined the effect of transient expression of p53 on DNA synthesis. MKN45 cells were chosen for the transient expression study because neither the mutation of the p53 gene nor the overexpression of the p53 gene product was observed in MKN45 cells (11). Seventy-two h after transfection, MKN45 cells were incubated with [3H]thymidine, stained with p53 antibody, and subsequently autoradiographed. Although transfected p53 cDNA was not detected in the stable transfection with the wild-type p53 cDNA, the expression of either the wild-type or mutant p53 protein in the transient transfection of MKN45 cells was detected by immunocytochemical staining (data not shown). As shown in Table 2, the percentage of cells expressing mutant p53 was higher than that of cells expressing wild-type p53. This may reflect the increased stability of mutant p53 as described previously (5). In addition, the percentage of the cells which expressed the wild-type p53 and incorporated labeled thymidine simultaneously

Table 2 Transient expression of p53 and [3H]thymidine incorporation in transfected MKN45 cells

<table>
<thead>
<tr>
<th>Vectors</th>
<th>pCMV-WT</th>
<th>pCMV-mt143</th>
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</thead>
<tbody>
<tr>
<td>No. of cells counted</td>
<td>1200</td>
<td>1200</td>
</tr>
<tr>
<td>No. of cells expressing p53 (% of total cells)</td>
<td>45(3.6)</td>
<td>73(6.1)</td>
</tr>
<tr>
<td>No. of cells incorporating [3H]thymidine and expressing p53 (% of cells expressing p53)</td>
<td>2(0.4)</td>
<td>18 (25)</td>
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Fig. 3. Colony formation of MKN1 and MKN28 cells after transfection with the wild-type and mutant p53 expression vectors. MKN1 cells were transfected with either the wild-type (pCMV-WT) or mutant (pCMV-mt173, pCMV-mt251) p53 expression vectors in 10-cm plastic dishes. MKN28 cells were also transfected with either the wild-type (pCMV-WT) or mutant (pCMV-mt143, pCMV-mt173) p53 expression vectors. The number of total colonies which were resistant to G-418 were counted 3 weeks after the transfection. Each result shown is a representative of three separate experiments.

Fig. 4. Southern blot (A) and Northern blot (B) analysis of transfected MKN1 cells. In A, 7 μg of genomic DNA extracted from either individual (left) or pooled (right) clones of MKN1 cells transfected were digested with HindIII and XbaI, electrophoresed through 0.8% agarose gel and hybridized with 32P-labeled probe generated by PCR as described under "Materials and Methods." The 5.3-kilobase band is derived from endogenous p53 gene and the 1.9-kilobase band from exogenously introduced mutant p53 cDNA (arrows). In B, 20 μg of total RNA extracted from either individual (left) or pooled (right) clones of MKN1 cells transfected was electrophoresed on 1.2% agarose gel/formaldehyde gel, transferred to a nylon filter, and hybridized with a 32P-labeled 2.0-kilobase p53 cDNA probe as described under "Materials and Methods." Each lane indicates pooled clones from transfected MKN1 cells as described in A. The 2.8-kilobase mRNA band is endogenous p53 mRNA, and the 2.0-kilobase band (arrow) is a transcript of exogenously introduced p53 cDNA. Each lane indicates clones from MKN1 cells transfected with the following: Left: Lane 1, CMV, pCMV-neo alone; Lanes 2, 3, and 4, WT, pCMV-WT (wild-type p53); Lane 5, 173, pCMV-mt173; Lane 6, 251, pCMV-mt251. Right: Lane 7, CMV, pCMV-neo alone; Lane 8, WT, pCMV-WT (wild-type p53); Lane 9, 173, pCMV-mt173; Lane 10, 251, pCMV-mt251.

Table 2 Transient expression of p53 and [3H]thymidine incorporation in transfected MKN45 cells

MKN45 cells (5 x 10⁶ cells) were transfected with either pCMV-WT or pCMV-mt143. Seventy-two h after transfection, cells were labeled with 10 μCi/ml [3H]thymidine for 2 h, fixed in formalin, and immunostained with anti-p53 monoclonal antibody PAb 1801. The incorporation of thymidine was evaluated by autoradiography as described in "Materials and Methods." The result shown is representative of two separate experiments.
was greatly reduced when compared with that of cells expressing mutant p53 and incorporating [3H]thymidine. These results suggest that expression of the wild-type but not mutant p53 inhibits DNA synthesis in gastric cancer cells.

Binding of Wild-Type and Mutant p53 Proteins to SV40 Large T Antigen. The wild-type p53 protein forms a specific and stable complex with SV40 LTA (5, 6). By contrast, a murine mutant p53 protein has been shown to be unable to associate with SV40 LTA (5, 6). Since several different mutations of the p53 gene were detected in primary gastric cancer samples, we examined the binding of GST wild-type and mutant p53 fusion proteins to SV40 LTA in COS cell lysate. This experiment as well as the transfection study could assess whether or not p53 gene mutations detected in human gastric cancer impair the normal function of p53 protein. Each pGEX-3X-p53 cDNA recombinant gave rise to a fusion protein that specifically bound to glutathione-Sepharose. The bound proteins were eluted by boiling in an SDS sample buffer and resolved by SDS-polyacrylamide gel electrophoresis (Fig. 5A). Each protein had an apparent molecular weight consistent with the size of the p53 protein encoded by either wild-type p53 cDNA (Fig. 5A, Lane 2) or a mutant cDNA (Fig. 5A, Lanes 3–8) insert plus the M, 26,000 contributed by the GST leader sequence (Fig. SA, Lane 1). The amount of p53 fusion protein recovered on glutathione-Sepharose appeared to be identical for all of the proteins, as shown in Fig. 5A. To determine whether these wild-type and mutant p53 fusion proteins retained the ability to bind to SV40 LTA, a whole cell lysate was prepared from COS-1 cell, an African green monkey kidney cell line transformed with simian virus and known to produce abundant SV40 LTA (22). Aliquots of the cell lysate were equilibrated with glutathione-Sepharose beads bound to GST (Fig. 5A, Lane 1) and glutathione-Sepharose beads bound to each of the GST-p53 fusion proteins (Fig. 5A, Lanes 2–8). Bound proteins were eluted by boiling in SDS sample buffer and resolved by SDS-polyacrylamide gel electrophoresis, and Western blotting was carried out using PAb 419, a monoclonal antibody specific for SV40 LTA. As shown in Fig. 5B (Lane 9), M, 90,000 SV40 LTA in COS cell lysate was specifically recognized by PAb 419. SV40 LTA bound to GST-wild p53 fusion protein (Fig. 5B, Lane 2) but not to GST itself (Fig. 5B, Lane 1). Furthermore, SV40 LTA also bound to mutant p53 fusion proteins containing p53 mutations at codon 173 (Fig. 5B, Lane 4) and codon 251 (Fig. 5B, Lane 7). The amount of SV40 LTA bound to these two mutant p53 fusion proteins appeared to be greater than that bound to the wild-type p53 fusion protein. By contrast, the binding of SV40 LTA to mutant p53 fusion proteins containing p53 mutations at codon 143 (Fig. 5B, Lane 3), 175 (Fig. 5B, Lane 5), 248 (Fig. 5B, Lane 6), and 273 (Fig. 5B, Lane 8) was greatly reduced.

**DISCUSSION**

In the present study, we have demonstrated frequent mutations of the p53 gene in human gastric cancer samples. All point mutations observed are missense mutations which then alter the amino acid sequence of the p53 protein. Of interest is the present study and another (8) also show mutations at codon 173 and at codon 251 in primary gastric tumors. Furthermore, mutations at these two codons has also been observed in gastric cancer cell lines (10, 11). The p53 gene mutations at codons 251 and 173 seem to be rare mutations which have not been observed in other types of cancer (5, 7). Therefore, it is possible that these two codons are hot spots for p53 mutations in gastric cancer, although the number of samples examined is still small. It has been reported that p53 mutations were detected in metastatic lesions of gastric cancer but not in primary lesions of gastric cancer (10). However, frequent mutations of p53 gene have been demonstrated in primary samples of gastric cancer after tumor cell enrichment by cell sorting (8). In the present study, to minimize the contamination by nontumor cells we have used RNA extracted from frozen sections of gastric tumors and successfully detected p53 gene mutations. The advantage of analyses using frozen tissue sections for the detection of genetic alterations of cancers has been previously reported (19, 25). Thus, PCR direct sequencing in combination with tissue sectioning appears to be a more accurate method of detecting genetic changes in clinical samples. No correlation was found between the presence of p53 gene mutations and the histological grade of tumor differentiation in the present study, a result corresponding with the recent report showing mutations of p53 gene in primary gastric cancer (8). It has also been demonstrated that in primary gastric cancer, LOH on chromosome 17p, where the p53 gene is located, is detected regardless of histological type (4). In the present study, most tumor samples with p53 gene mutations have only one allele of chromosome 17p as evaluated with the pYN2 22 probe. This was further confirmed by PCR-RFLP analysis demonstrating the allelic deletion of the p53 gene. In addition, in these tumor samples only altered bands in sequence ladders were observed, indicating that these tumors are functionally homozygous for the mutations and synthesize mutant p53 mRNA. Therefore, present results suggest that these gastric cancer tumors lose one copy of the p53 gene and have a point mutation in the remaining allele.
resulting in the loss of the suppression by the p53 gene product of stomach cell growth.

Thus, the possibility that the expression of the wild-type but not the mutant p53 gene suppresses the growth of gastric carcinoma cells was next tested in the present study. Transfection of the wild-type p53 gene suppressed both the formation of neomycin-resistant colonies of MKN1 or MKN28 cells and the incorporation of [3H]thymidine into MKN45 cells. By contrast, expression of mutant p53 genes, which contain point mutations observed here in gastric cancer samples and in gastric cancer cell lines (10, 11), failed to inhibit colony formation or DNA synthesis. These data indicate that the wild-type p53 inhibits the growth potential of gastric cancer cells. In addition, mutations of the p53 gene may impair the inhibitory effect of p53 gene product on the proliferation of gastric cells, thereby resulting in gastric tumorigenesis. The present results correspond well with recent observations showing that the wild-type but not mutant p53 suppresses the growth of cancer cells (26–29). In the stable transfection, no exogenous p53 gene was detected in the cells transfected with the wild-type p53 cDNA. It is possible that cells expressing the wild-type p53 were not selected for establishing clonal lines. However, this seems unlikely, because no exogenous p53 cDNA was detected even in the pooled clones in which numerous colonies were simultaneously assayed. Therefore, an explanation might be that cells overexpressing wild-type p53 failed to grow and then did not form any colonies during G418 selection. An identical observation has been reported in colon cancer cell lines (26) which were transfected with the wild-type p53 cDNA using an expression vector with the cytomegalovirus promoter. In addition, it has been suggested that genetic alteration during tumorigenesis may increase the sensitivity of cells to p53 inhibition, leading to the growth arrest of cells in which wild-type p53 is overexpressed (26).

The p53 protein forms a tight complex with SV40 LTA (5, 6). By contrast, mutated p53 protein tested so far failed to bind to SV40 LTA (5, 6). Furthermore, the p53 gene mutation observed in human cancers occurs in the p53 domains involved in p53 binding to SV40 LTA (5). Therefore, it has been speculated that there is a cellular LTA-like protein which mediates the suppressor activity of p53 (5, 6). Mutations of p53 gene may impair the association of p53 with this putative p53-binding protein, resulting in the loss of suppressor function of the wild p53. Since different mutant p53 genes, three of which were unable to suppress the growth potential of gastric cancer cells, were observed in gastric cancer, we determined whether these mutated p53s can bind to SV40 LTA. We used a pGEX vector (23) in a bacterial system to produce both the wild and mutant p53s can bind to SV40 LTA with a higher affinity than the wild-type p53. It has also been shown that mutant p53 with an alteration at residue 175 binds tightly to the cellular heat shock protein (5, 30). By contrast, neither the wild-type p53 nor the mutant p53 containing mutations at codon 273 or 281 bind to heat shock protein (5, 30). Thus, different mutations of p53 appear to give rise to various conformational changes of p53 proteins, leading to different biochemical properties of p53. It is possible that there are multiple p53-binding proteins which are involved in the suppressor function of p53. If so, different mutations of p53 would disrupt the association of p53 with different putative p53-binding proteins. It is of particular interest to identify these putative p53-binding proteins; thus the GST-p53 expression system could be a useful tool for this purpose as recently shown in the study of the identification of binding proteins with the retino blastoma gene product (24).

In summary, frequent mutations of the p53 gene in association with the allelic loss of the p53 gene have been demonstrated in primary gastric cancer. Since wild-type but not mutant p53 gene expression inhibits the growth of gastric cancer cells, the inactivation of the p53 gene through allelic loss and mutation in the remaining p53 allele may play an important role in gastric tumorigenesis. Different mutant p53 proteins possess heterogeneous biological properties and fail to associate with cellular components which are involved in the suppressor function of wild p53.

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REFERENCES


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