p53 Gene Mutations in Gastric Cancer Metastases and in Gastric Cancer Cell Lines Derived from Metastases

Yukishige Yamada, Teruhiko Yoshida, Kenshi Hayashi, Takao Sekiya, Jun Yokota, Setsuo Hirohashi, Katsunori Nakatani, Hiroshige Nakano, Takashi Sugimura, and Masaaki Terada

Genetics Division [Y. Y., T. Y., T. Su., M. T.], Oncogene Division [K. H., T. Se.], Section of Studies on Metastasis [J. Y.], and Pathology Division [S. H.], National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104, and First Department of Surgery, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634 [Y. Y., K. N., H. N.]; Japan

ABSTRACT

Structural alterations of the p53 gene were investigated in tissue specimens of gastric and cervical cancers and in cell lines of gastric, esophageal, and cervical cancers, by polymerase chain reaction-single-strand conformation polymorphism analysis. Two of the four gastric cancer metastases and four of the eight cell lines originally established from gastric cancer metastases were found to have p53 gene alterations in the exon 5 to 11 region; point mutations and amino acid replacements were detected in a liver and an ovary metastasis at exon 7, in the TMK1 and MKN4 cell lines at exon 5, and in the OKAJIMA cell line at exon 10. The normal allele was not found in these cell lines. In the KATO-III cell line, gross deletion and rearrangement of the p53 gene were noted. However, no p53 mutations were identified in 5 in 11 of the p53 gene. During the course of the study, a novel polymorphism in intron 7 of the p53 gene was found, which can be recognized by restriction enzyme digestions of the polymerase chain reaction product.

INTRODUCTION

Although gastric cancer is one of the most prevalent malignancies in the world, little is known yet about genetic changes associated with its development and/or progression. The ras family of oncogenes are the most frequently encountered transforming genes detected by NIH3T3 transfection assay in human cancers. However, in gastric cancer, only one case with the K-ras activation was reported (1) among about 60 cases analyzed, including 37 cases in our laboratory (2). A recent survey using PCR and oligonucleotide hybridization also revealed rarity of the ras mutation in gastric cancer (3). The myc gene amplification was found in several gastric cancer cell lines and in xenografts (4), but its incidence in in vivo tumors is about 10%, the same level as in many other nongastric cancers (5). Nonrandom amplification, however, was noted for two oncogenes coding for receptor-type tyrosine kinases, p-erbB-2 (6) and K-sam/bek (KATO-III cell-derived stomach cancer amplified gene), which is a form of bek-type fibroblast growth factor receptor) (7, 8); it appears that c-erbB-2 and K-sam are specifically amplified in well differentiated and poorly differentiated adenocarcinomas of the stomach, respectively. Specific involvement of tumor suppressor genes was implicated in a variety of cancers (reviewed by Stanbridge (9)), but our previous study by conventional restriction-fragment length polymorphism analysis failed to identify any chromosomal segment deleted at high incidence in gastric cancer (10).

Recently, it was shown that p53, like the product of the Rb gene, may act as a tumor suppressor (11), and its inactivation appears to be one of the most common genetic abnormalities in cancer, even in those without an appreciable incidence of chromosome 17p loss (12). Comparison of the amino acid sequences of human, mouse, and Xenopus laevis p53 proteins revealed five blocks of highly conserved regions (13); 86% of 21 missense mutations were reported to cluster in four “hot spots” on exons 5 to 8, which coincided exactly with the most highly conserved regions (12). However, small deletions as well as point mutations were found to occur in the p53 gene, and the exact positions of the mutated codons were variable. In a previous study on lung cancer (14), the relatively tedious RNase protection assay, which detects only <50% of the mismatched base pairs, was used.

In this article, we investigated structural alterations of exons 5 to 11 of the p53 gene, in gastric, esophageal, and cervical cancers, by the recently developed method of PCR-SSCP analysis (15, 16), a rapid and sensitive way to detect base changes in given sequences of DNA. This method capitalized on the sequence-dependent conformation of the single-strand DNA in a neutral polyacrylamide gel. The differences in the conformation were easily and sensitively detected by gel electrophoresis.

MATERIALS AND METHODS

Tissues, Cell Lines, and DNA Extraction. Twenty-three specimens of gastric cancer, 1T to 19T and 20M to 23M, and five surgical specimens of cervical cancer were obtained at the National Cancer Center Hospital (see Table 1). In seven of these 28 cases, noncancerous tissue was also available. DNA samples from primary tumors, metastases, and noncancerous tissues were represented by the case number followed by T, M, and N, respectively. Eight human stomach cancer cell lines, KATO-III, OKAJIMA, TMK1, MKN1, MKN7, MKN28, MKN45, and MKN74, as well as 13 esophageal cancer cell lines, TE1 to TE13, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. All gastric cancer cell lines, TE3, and TE9 were established from metastatic lesions (Table 1). Four cervical cancer cell lines, SKG-I, SKG-II, and SKG-IIIa were cultured in Ham’s F-12 medium supplemented with 10% fetal bovine serum, and HeLa was cultured in Eagle’s minimum essential medium supplemented with 10% calf serum. These cell lines contained HPV type 16 or 18 DNA sequences (20). High molecular weight DNA was prepared from cell lines and tissues by proteinase K digestion and phenol/chloroform extraction, as described (21).

PCR. The oligonucleotide primers used for PCR of the portions of the p53 gene were designed based on the published sequence (22): PX5LT, GGAATTCCTCCTCGTCAAGCAG; PX6RT, GGAATTCAGGTTGCAAACGGAGCTCAG; PX7LT, GGAATTCCTTGGTGAAGTGTTCCTCGGAC; PX7RT, GGAATTCAGGTTGCAAACGGAGCTCAG; PX8LT, GGAATTCCTATCGTGAAGTGTTCAG; PX-5800

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: PCR, polymerase chain reaction; PCR-SSCP, polymerase chain reaction-single-strand conformation polymorphism; HPV, human papilloma virus.
RESULTS

PCR-SSCP Analysis. The results of the PCR-SSCP analysis of exons 5 to 11 of the p53 gene are summarized in Table 1. The PCR primers PX5LT and PX6RT amplified a 419-base pair fragment spanning from exon 5 to exon 6 of the p53 gene. SSCP analysis showed two bands, each corresponding to one of the complementary strands of a DNA molecule, in all samples except the KATO-III cell line; in this cell line, no PCR product was generated by the primers used in this study, and Southern blot analysis revealed that both alleles of the p53 gene were grossly deleted (data not shown). The mobilities of the two bands in the TMK1 and MKN1 cell lines were different from those of the other samples (Fig. 1A). The other gastric cancer cell lines, 13 esophageal cancer cell lines, 23 gastric cancer tissues, four cervical cancer cell lines, five cervical cancer tissues, and eight noncancerous tissues (liver, two; spleen, one; stomach, three; cervix, one; and placenta, one) all exhibited an identical pattern of bands on SSCP. Exons 7 to 11 were amplified separately, with only a few intron sequences attached on the ends of the amplified fragments. A gastric cancer cell line, OKAJIMA, showed a mobility shift of the band on SSCP gel in the region of exon 10 (Fig. 1B).

The 658-base pair segment containing intron 7 as well as exons 7 and 8 was amplified by PX7LT and PX8RT primers. The SSCP analysis revealed the presence of polymorphism as well as mutations in this segment. With the exception of two metastasis cases, 20M and 22M, in which p53 mutations were identified (see below), the SSCP analysis showed two bands represented by 4T or 8T (Fig. 2A, lane 4 or 8), whereas four bands were observed in four cases, 3T, 9T, 20N, and 21M/21N (Fig. 2A, lanes 3, 9, 18, and 19/20). Furthermore, in the three cases without p53 mutation, 4T, 11T, and 13T, the band migration pattern was identical between noncancerous and cancer tissues.
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Fig. 1. PCR-SSCP analysis of gastric cancer cell lines. PCR-SSCP analysis of DNAs from gastric cancer cell lines was performed as described in the text. A, exon 5–6; B, exon 10 region of the p53 gene. Lane 1, human placenta; lane 2, KATO-III; lane 3, OKAJIMA; lane 4, TMK-1; lane 5, MKN1; lane 6, MKN7; lane 7, MKN28; lane 8, MKN45; lane 9, MKN74.

Fig. 2. PCR-SSCP analysis of gastric cancer tissues. PCR-SSCP analysis of DNAs from gastric cancer tissues was performed as described in the text. A, exon 7–8; B, exon 7 region of the p53 gene. A, lanes 1 to 15, IT to 1ST, respectively; lane 16, human placenta; lane 17, 20M; lane 18, 20N; lane 19, 21M; lane 20, 21N; lanes 21 and 23, 22M; lanes 22 and 24, 22N. Lanes 23 and 24 were exposed for a longer period (5 days) than lanes 21 and 22 (12 h). B, lane 1, 20M; lane 2, 20N; lane 3, 23M; lane 4, 23N.

cancerous tissues from the same patient (data not shown). The band migration variation among individuals disappeared when exons 7 and 8 were amplified separately, to skip intron 7, by the primer pairs of PX7LT/PX7RT (exon 7) and PX8LT/PX8RT (exon 8). Finally, the sequence analysis revealed polymorphic base substitutions in intron 7 (see below).

The metastasis sample 20M showed four extra bands, in addition to the four very faint bands which were observed in the normal tissue of the same patient (20N) on the PCR-SSCP analysis of exon 7 (Fig. 2B). Thus, the single-strand DNA generated from this region of the p53 gene gives rise to two conformations with different mobilities on the gel, resulting in four bands from a pair of complementary strands, as reported previously for the Rb gene (24). A faint abnormal band was detected on the analysis of the exon 7–8 region of another metastasis sample, 22M, only after a long term exposure (5 days) (Fig. 2A, lane 23), and its position was identical to that of sample 20M (Fig. 2A, lane 17). The result was confirmed by a carefully repeated analysis to exclude a possible sample contamination.

In a cancerous tissue, variable degrees of normal cells may be present, and malignant cells themselves are not always genetically homogeneous. Thus, we evaluated the sensitivity of PCR-SSCP analysis using mixed DNA samples of MKN1 and human placenta, at ratios ranging from 1:1 to 1:100 (Fig. 3). The mutated sequence of MKN1 in the exon 5–6 region (Fig. 1A, lane 5) could be identified when it was present in more than one eighth of the total DNA.
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MKN1 1 1 1 1 1 1 1 1
Placenta 0 1 3 7 15 31 100

Fig. 3. Sensitivity of the PCR-SSCP analysis. DNAs with normal (placenta) and mutated (MKN1) exon 5 of the p53 gene were mixed at the ratios indicated in each lane and subjected to PCR-SSCP analysis, as described in the text.

Fig. 4. Point mutation in a gastric cancer metastasis. Analysis of the exon 7–8 region of the p53 gene of specimen 20M showed alteration of sequence from that of 20N. The exon 7–8 region of the p53 gene of specimens 20M and 20N was amplified, cloned, and sequenced as described in the text. A point-mutated codon, a transition from CGG (arginine) to TGG (tryptophan), was identified, in addition to a small amount of a nonmutated codon, CGG; the faint band of the nonmutated codon was visualized after a long exposure (data not shown).

Sequence Analysis. The exon 5–6 region was amplified and cloned from cell lines TMK1, MKN1, and MKN7 and was sequenced. Comparison of the nucleotide sequences of these cell lines and the published sequence of human p53 gene (22) revealed a single point mutation in exon 5 of the TMK1 and MKN1 cell lines, the substitution of valine for methionine at codon 173 (transition from GTG to ATG) of the TMK1 cell line and valine for alanine at codon 143 (transition from GTG to GCG) of the MKN1 cell line. The exon 7–8 region was amplified by the PX7LT/PX8RT pair of PCR primers and cloned from the DNAs of 20M and 20N. In the liver metastasis 20M, a point-mutated codon, a transition from CGG (arginine) to TGG (tryptophan), was identified at position 248, in addition to a nonmutated codon (Fig. 4). The exon 10 region was amplified and cloned from cell lines OKAJIMA and MKN7. The point mutation in exon 10 of the OKAJIMA cell line resulted in generation of a termination codon in place of arginine at codon 342 (transition from CGA to TGA).

The exon 7–8 region was also cloned from 3T, 4T, and 8T. In 8T, two nucleotides positioned 72 bases and 92 bases downstream of the end of exon 7 were C and T, respectively, whereas they were T and G in 4T. The sequences of the samples 3T, 20M, and 20N showed both C and T at the position 72 bases and both T and G at the position 92 bases downstream of the end of exon 7. These polymorphic base substitutions resulted in the differences in the sites of the restriction enzymes AvaII, HaeIII, and MboII (Fig. 5).

DISCUSSION

We analyzed structural alterations of exons 5 to 11 of the p53 gene, in tissue specimens of gastric and cervical cancers and in cell lines established from gastric, esophageal, and cervical cancers, by use of the newly developed method of PCR-SSCP analysis. p53 mutation was found only in cells derived from gastric cancer metastases: two tissue specimens of liver (20M) and ovary (22M) metastases and four cell lines (KATO-III, OKAJIMA, TMK1, and MKN1). Although the mutation in the ovary metastasis, 22M, was not sequenced, the pattern
of migration identical to that of 20M suggested the same base change, CGG to TGG at codon 248. Inasmuch as the same mutation was also reported in two colon cancer cells (12), codon 248 may be a frequent target of the p53 mutation.

TMK1, MKN1, and OKAJIMA cells apparently lost the normal allele of the p53 gene; only two, not four, bands were detected, each representing one of the complementary strands of DNA of a mutated allele; 100 randomly picked up and pooled clones of the relevant p53 region showed only the mutated sequence. Southern and RNA blot analyses, however, did not detect any gross abnormality in these cell lines, with the exception of KATO-III and OKAJIMA. In the KATO-III cell line a major portion of the p53 gene was deleted, and the p53 expression was significantly decreased in the OKAJIMA cell line (data not shown). Loss of normal allele was also suggested for most, if not all, of the tumor cells in the liver metastasis sample 20M, because the intensity of the bands corresponding to the normal allele was far less than half of that of the abnormal bands (Fig. 2B). In the ovary metastasis 22M, the abnormal band was very weak, requiring long exposure for detection. Because we used portions of the tumors that were macroscopically devoid of surrounding normal tissues, the faintness of the mutated band may not be accounted for by the presence of large amounts of noncancerous cells in the sample; rather, it suggests that the mutation occurred in some subpopulation of the tumor cells.

In contrast to the gastric cancer cell lines, none of the 13 esophageal or four cervical cancer cell lines showed any aberration in exons 5 to 11 of the p53 gene. These negative findings, as well as the detection of the p53 mutation in an in vivo metastasis, suggest that the p53 mutations we observed in the gastric cancer cell lines are most unlikely to be developed during culture. The data further suggest that the p53 mutation occurs during the later stage of the carcinogenesis step of gastric cancer in certain histological subsets of human tumors.

All of the nine cervical cancer cell lines and tissues examined in this study contained the HPV type 16 or 18 DNA sequences and did not have changes in the p53 gene. The results are compatible with reports showing that the p53 protein is activated by binding to the E6 protein of HPV type 16 or 18 (25). It should also be noted that the occurrence of the p53 mutation in primary gastric cancer is not totally negated, because (a) exons 1 to 4 were not analyzed in this study, (b) we could not analyze a primary tumor and metastatic lesions in the same patient, and (c) a mutation present in less than one eighth of the cell population appears to be undetectable in this study. We might have underestimated the incidence of p53 mutations in the primary tumors accompanied by contamination of noncancerous cells. It will be necessary for further investigations of primary tumors to subdivide tumors microscopically into regions containing cancer cells and to purify subpopulations of cancer cells by flow cytometric sorting. Nonetheless, the present work suggests that the p53 mutations occur preferentially in the advanced stages of gastric cancer. The rapid and sensitive detection of the mutation by PCR-SSCP analysis will certainly provide essential information for the diagnosis and treatment of gastric cancer in the near future.

We found a novel polymorphism in intron 7 of the p53 gene, which can be identified by alterations of the Avall, HaeIII, and MboII recognition sites on the exon 7–8 PCR product. The incidence of heterozygosity was at least 5 of 28 cases. The polymorphic base changes are located in the midst of the mutation hot spots of the p53 gene (12), and they may be of use for the study of the p53 allelic abnormality in cancer.

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