Detection of Frequent p53 Gene Mutations in Primary Gastric Cancer by Cell Sorting and Polymerase Chain Reaction Single-Strand Conformation Polymorphism Analysis

Gen Tamura, Toshimasa Kihana, Kazuhiro Nomura, Masaaki Terada, Takashi Sugimura, and Setsuo Hirohashi

Divisions of Pathology [G. T., T. K., S. H.], Neurosurgery [K. N.] and Genetics [M. T., T. S.], National Cancer Center Research Institute and Hospital, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan

Abstract

Mutations of the p53 gene were investigated after tumor cell enrichment by cell sorting based on differences in DNA content and polymerase chain reaction single-strand conformation polymorphism analysis in 24 surgical specimens of primary gastric cancer. p53 mutations were detected in exons 4–8 in 64% (9 of 14) of aneuploid tumors but in none of 10 diploid tumors examined. Four of five tumors containing two or three aneuploid subpopulations showed the presence of p53 gene mutations. No correlation was found between the presence of p53 mutations and the degree of histological differentiation of tumors. These findings suggest that p53 gene mutations are related to DNA ploidy alterations as relatively late events of carcinogenesis in gastric cancer. The present method is highly sensitive for detection of genetic abnormalities and is applicable even when various kinds of nontumorous cells are present in tumor samples.

Introduction

The recent progress made in molecular genetics has revealed a consistent set of genetic alterations in various human cancers possibly corresponding to multistep tumor development. Gastric cancer is no exception, and multiple genetic alterations including ras oncogene mutations, gene amplifications, and chromosomal loss of heterozygosity have been detected, although the incidence of these alterations is low compared to those in colorectal carcinoma, another major cancer of the gastrointestinal tract (see Ref. 1 for review). Recently, it has been shown that the p53 gene is a tumor suppressor gene (2) and that its mutations play an important role in the development of many common human malignancies (3). In gastric cancer, however, p53 gene mutations have not been detected in the primary site, having been demonstrated only in metastases and cell lines by use of ordinary DNA extraction procedures and the polymerase chain reaction (1). Although these findings seem to suggest that mutations of the p53 gene are a very late event in gastric carcinogenesis, it is also possible that such mutations could have been underestimated in surgical specimens of primary gastric cancer, which are often heavily contaminated with normal stromal cells and inflammatory cells. Accordingly, we used cell sorting based on differences of DNA content, followed by PCR-SSCP analysis (4), to detect base changes in p53 gene sequences (5).

Materials and Methods

DNA flow cytometry and cell sorting using a FACS-IV (Becton Dickinson, Mountain View, CA) was performed on 24 frozen specimens of primary gastric cancer surgically resected at the National Cancer Center Hospital, Tokyo, Japan. Nuclei were isolated with 0.2% Triton X-100 (Sigma Chemical Co., St. Louis, MO), treated with 0.1% RNase (Sigma), stained with 50 µg/ml propidium iodide (Sigma), and filtered through nylon mesh. Aneuploid tumor cell populations were sorted when determined, and the cells at S + G2M in diploid tumors were sorted for tumor cell enrichment. Then all the exons of the p53 gene were amplified directly from 10² sorted nuclei by the polymerase chain reaction (PCR) using specific oligonucleotide primers as shown in Table 1. The PCR products were subjected to single-strand conformation polymorphism (SSCP) analysis, a newly developed method for detection of structural alterations in DNA including point mutations (4). A second PCR-SSCP analysis was performed to ensure that the results were reproducible in each case which showed mobility shift. For sequencing analysis, exon 5 or 7 of the p53 gene from 4 tumors with mobility shift detected by SSCP analysis was amplified by PCR. The PCR products were cloned into pUC18 vector. About 50–100 mixed colonies were amplified and then sequenced by the dideoxy chain termination method (6). The primers used for sequencing were sense, 5'-TCTTCCTGCGATCTCCCT-3' and antisense, 5'-AGCTGCTCACCATCGCTAT-3' for exon 5, and sense, 5'-ACTGTACCATTGCACCAG-3' and antisense, 5'-GTGGCTCTGACCTGGAGTCTG-3' for exon 7.

Results

DNA flow cytometry proved that 14 (58%) of the tumors were aneuploid and the other 10 (42%) were diploid. Two or three aneuploid subpopulations (multiplody) were detected in a sample in 5 of the 14 aneuploid tumors (Fig. 1). Mutations of the p53 gene were detected in exon 4 in one tumor, exons 5–6 in three and exons 7–8 in five by PCR-SSCP analysis (Fig. 2). As well as two bands with mobility shifts, two bands corresponding to the normal allele were seen in four tumors but not in the remaining five. It could not be determined whether two bands corresponding to the normal allele were from normal cells still present after cell sorting or from the remaining wild-type allele of tumor cells in the four tumors. All the mutations of the 53 gene were detected in aneuploid tumors, but none in diploid tumors (Table 2). Four of five multiploid tumors contained p53 gene mutations, and different aneuploid subpopulations from a tumor sample showed the same mobility shift by PCR-SSCP analysis (Fig. 2) in all four cases. No correlation was found between the presence of p53 gene mutations and the degree of histological tumor differentiation (Table 3). The mutations of the p53 gene, confirmed by sequencing analysis, were point mutations at the third position of codon 173 (GTG–GTA) and at the second position of codon 251 (ATC–AGC) (Fig. 3), a 7-base pair deletion at the first position of codon 137.
Table 1 Oligonucleotide primers (5'-3')

<table>
<thead>
<tr>
<th>Exon</th>
<th>Upstream</th>
<th>Downstream</th>
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<tbody>
<tr>
<td>1</td>
<td>GGAATTGAGATTCTCAGATGATTT</td>
<td>GGAATTCCAGTCAAGGACATACCA</td>
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<tr>
<td>2</td>
<td>GGAATTCTGAGATCTCTTGCAGCATGCA</td>
<td>GGAATTCTGCAATGATCCATCACAG</td>
</tr>
<tr>
<td>3</td>
<td>GCTCTGACTTTCAGACTT</td>
<td>GGAATTCAACCCTTGTCCTTACGAG</td>
</tr>
<tr>
<td>4</td>
<td>GGAATTCATTATTCTCACTCC</td>
<td>GGAATTCCTCAGGAGGCTACCTC</td>
</tr>
<tr>
<td>5-6</td>
<td>GGAATTCCTTCTTCTGCACTGCCA</td>
<td>GGAATTCCTCAGGAGGCTACCTC</td>
</tr>
<tr>
<td>7-8</td>
<td>GGAATTCTGAGATCTCTTGCAGCATGCA</td>
<td>GGAATTCTGCAATGATCCATCACAG</td>
</tr>
<tr>
<td>9</td>
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<td>GGAATTCCCAAGAGTATTGAGATCC</td>
</tr>
<tr>
<td>10</td>
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<td>11</td>
<td>GGAATTCTGAGATCTCTTGCAGCATGCA</td>
<td>GGAATTCCTCAGGAGGCTACCTC</td>
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</tbody>
</table>

Discussion

A combination of cell sorting and molecular genetic analysis is a highly sensitive method for analysis of genetic abnormalities (7) and is applicable even when considerable amounts of non-tumorous cells are present in tumor samples. We have successfully detected mutations of the p53 gene in primary gastric cancers by use of PCR-SSCP analysis, a simple and sensitive method for detecting structural alterations of DNA including point mutations (4), after tumor cell enrichment.

Mutations of the p53 gene were clustered in exons 4-8, the most highly conserved regions, similar to other human malignancies (3), and were frequently detected in aneuploid tumors which had probably arisen from diploid clones during tumor
progression (8); the remaining allele of the p53 gene was considered to have been deleted in at least 56% (5 of 9) of the tumors. In contrast, no diploid tumors contained p53 gene mutations. This is very different from the situation in ras oncogene activation; mutation of the ras oncogene is considered to be an early event in carcinogenesis and is present in diploid cells from which an aneuploid subpopulation arises (7).

Very recently, it has been suggested that mutations of p53 are the rate-limiting step in its inactivation and that once a mutation occurs, loss of the remaining wild-type allele rapidly follows, based on the finding that the great majority of colonic tumors containing p53 gene mutations show loss of this wild-type allele (9). In addition, deletion of chromosome 17p may occur simultaneously with many other chromosomal losses through abnormal mitosis (10). These and our present findings suggest that p53 gene mutations in common malignancies occur as relatively late events in carcinogenesis and that chromosomal instability, which makes cancer cells aneuploid, plays an important role in the selection of tumor cells with p53 gene mutations by causing easy loss of the remaining allele and totally inactivating the normal p53 function.

In multiploid tumors, different aneuploid subpopulations from a tumor sample showed the same mobility shift by PCR-SSCP analysis, indicating the presence of identical p53 gene mutations. Therefore, p53 gene mutations precede the evolution of different aneuploid subpopulations, which are derived from a common aneuploid ancestral clone, caused by additional chromosomal events.

Although no correlation was found between the presence of p53 gene mutations and the histological grade of tumor differentiation in our study, further clinicopathological studies will be necessary in order to elucidate the prognostic significance of p53 gene mutations.

Acknowledgments

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References

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