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Cancer Res 1991;51:3056-3058. Published online June 1, 1991.

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[CANCER RESEARCH 51, 3056-3058, June 1, 1991]

Advances in Brief

Detection of Frequent *p53* Gene Mutations in Primary Gastric Cancer by Cell Sorting and Polymerase Chain Reaction Single-Strand Conformation Polymorphism Analysis[1]

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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[3]-SSCP analysis (4), to detect base changes in *p53* gene sequences (5).

Received 3/4/91; accepted 4/5/91.

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[1] This work was supported in part by a Grant-in-Aid for a Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare, Japan.

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[3] The abbreviations used are: PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.

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 + G_2M in diploid tumors were sorted for tumor cell enrichment. Then all the exons of the *p53* gene were amplified directly from 10^3 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′-TCTTCCTGCAGTACTCCCT-3′ and antisense, 5′-AGCTGCTCACCATCGCTAT-3′ for exon 5, and sense, 5′-ACTGTACCACCATGCACTAC-3′ and antisense, 5′-GTGGCTCCTGACCTGGAGTC-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 (multiploidy) 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 *53* 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

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Table 1 *Oligonucleotide primers (5'-3')*

Exon	Upstream	Downstream
1	GGAATTCGGATTCCTCCAAAATGATTT	GGAATTCCAGTCAGGAGCTTACCCA
2	GGAATTCTGGATCCTCTTGCAGCAGCCA	GGAATTCCAATGGATCCACTCACAG
3	GCTCTTGACTTTCAGACTT	GGAATTCAACCCTTGTCCTTACCAGAA
4	GGAATTCTTTTCACCCATCTACAGTCC	GGAATTCCTCAGGGCAACTGACCGTGC
5–6	GGAATTCCTCTTCCTGCAGTACTCC	GGAATTCAGTTGCAAACCAGACCTCA
7–8	GGAATTCTCCTAGGTTGGCTCTGAC	GGAATTCCTGCTTGCTTACCTCGCT
9	GGAATTCTTGCCTCTTTCCTAGCA	GGAATTCCCCAAGACTTAGTACCTG
10	GGAATTCCTCTGTTGCTGCAGATC	GGAATTCGCTGAGGTCACTCACCT
11	GGAATTCTGTCTCCTACAGCCAC	GGAATTCTGACGCACACCTATTGC

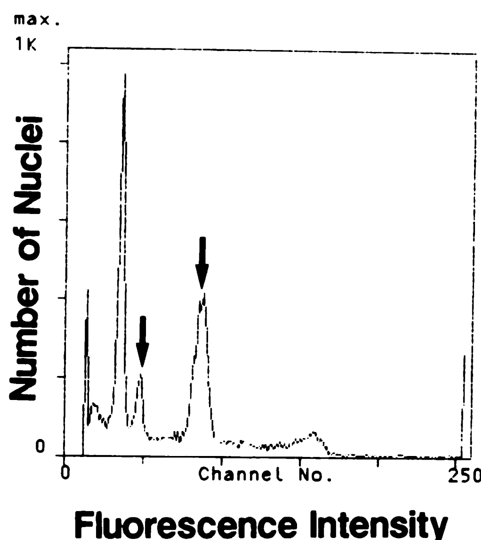

Fig. 1. DNA histogram of a multiploid tumor. Two aneuploid peaks (*arrows*) appear to the right of the normal diploid peak. Aneuploid populations were separately sorted and subjected to PCR-SSCP analysis.

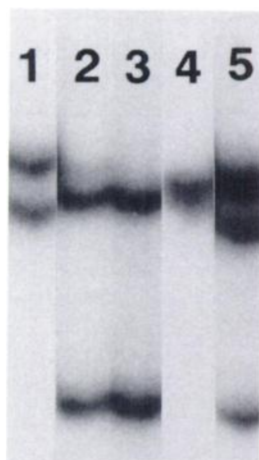

Fig. 2. SSCP analysis of exons 5–6 of the *p53* gene in gastric cancers. *Lane 1*, normal gastric mucosa; *Lanes 2–5*, gastric cancers with mobility shifts. *Lanes 2* and *3* are from different aneuploid subpopulations of a tumor sample.

Table 2 *p53 gene mutation and DNA ploidy in primary gastric cancer*

DNA ploidy	*p53* gene mutation: Positive	Negative	Total	
Diploid	0	10	10	⎤[a] ⎫[a]
Aneuploid	9	5	14	⎦ ⎪
(Multiploid)	4	1	5	⎭
	9	15	24	

[a] $P < 0.01$ by Fisher's test.

Table 3 *p53 gene mutation* and histological type in primary gastric cancer

Histological type[a]	*p53* gene mutation: Positive	Negative	Total	
Well differentiated	5	7	12	NS[b]
Undifferentiated	4	8	12	
	9	15	24	

[a] See Ref. 1.
[b] NS, not significant by Fisher's test.

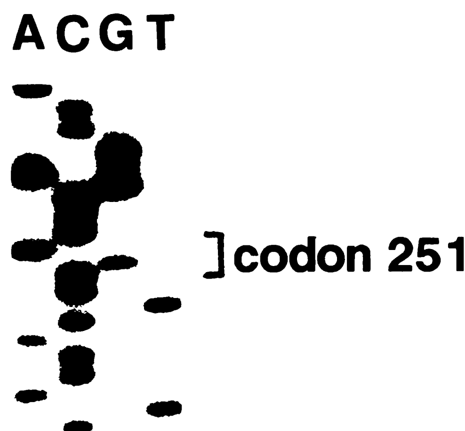

Fig. 3. Sequencing autoradiogram of exon 7 of the *p53* gene. A point mutation, T to G transition, is present at the second position of codon 251 (ATC to AGC).

Table 4 *Mutations of p53 gene in primary gastric cancer*

Exon	Codon	Nucleotide substitution
5	137–139	7-base pair deletion (CTGGCCA)
5	173	GTG to GTA
7	251	ATC to AGC
7	252–253	4-base pair insertion (CTCA)

to the first position of codon 139 (CTGGCCA), and a 4-base pair insertion at the first position of codon 252 to the first position of codon 253 (CTCA) (Table 4).

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

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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

We thank K. Iwasaki, Division of Neurosurgery, National Cancer Center, Tokyo, Japan, for her technical help in cell sorting.

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