The Korean Journal of Internal Medicine
Vol. 9, No. 1, January, 1994

Effect of Non-Tumor Cell Contamination on Detection of p53 Gene Mutations in Human Gastric Cancer Cells by Polymerase Chain Reaction Single-Strand Conformation Polymorphism Analysis

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Background: *We have previously studied p53 gene mutations in 25 primary gastric cancer tissues by polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis for exon 4-8 and immunohistochemical staining with anti-p53 antibody. In four cases, the discrepancy of the results was observed between the two methods. In one case, positive by PCR-SSCP but negative by immunohistochemical staining, the mutation was silent. In three cases, the p53 gene mutations were detected only by immunohistochemical staining. This discrepancy may be due to the contamination of the samples by cells without p53 gene mutation, such as non-tumor cells. This study was conducted to investigate the sensitivity of PCR-SSCP analysis to p53 gene mutations when the sample was contaminated with non-tumor cells.*

Methods: *Genomic DNA was extracted by the digestion with proteinase K and phenol-chloroform-ethanol method from two human gastric adenocarcinoma cell lines, MKN-45 and KATO III. To investigate the sensitivity of PCR-SSCP, DNA extracted from cancer cells was mixed with DNA obtained from normal gastric mucosal cells at various ratios. PCR-SSCP analysis for exon 4-8 of the p53 gene was performed with the mixed DNA samples.*

Results: *In KATO III, no PCR products were generated in exon 4-8 of the p53 gene by PCR, suggesting that both alleles from exon 4-8 of the p53 gene were deleted. In MKN-45, the mobility shift was observed in exon 4. Therefore, the effect of non-tumor cell contamination on the detection of p53 gene mutations was conducted using MKN-45 and normal gastric mucosal cells. In the mixed DNA samples of MKN-45 and normal gastric mucosal cells, an extra band with the migration similar to that of MKN-45 was found in the samples of 1:8 dilution or less, while no extra band was grossly detectable in DNA of normal gastric mucosal cells and in the samples of more than 1:16 dilution.*

Conclusions: *These results suggest that the detection of p53 mutations by PCR-SSCP analysis may be underestimated in samples contaminated by a large number of non-tumor cells.*

Key Words: *p53 gene mutation, PCR-SSCP, Gastric cancer cells, Contamination of non-tumor cells*

INTRODUCTION

Stomach cancer is the most common cancer in Korea. However, little is known about the molecular and genetic basis that contributes to the development and progression of stomach cancer[1]. According to the current concept of multistep carcinogenesis, the activation of protooncogenes and inactivation of tumor suppressor genes are involved in the carcinogenesis of a variety of human cancers[2~5]. Recently, using the technique of polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP), genetic alterations in cancer cells have been extensively studied because PCR-SSCP analysis is a highly sensitive

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**This study was supported by a grant from the Research Project for the Development of Atomic Energy, Ministry of Science and Technology, Korea.*

and powerful method for the detection of the genetic aberrations including point mutation[6~12]. The results reported so far suggest that the inactivation of p53 tumor suppressor gene plays an essential role in the certain step of carcinogenesis process, since p53 gene mutations have been frequently observed in a wide range of human cancers.

We have previously examined p53 gene mutations in 25 primary gastric cancer tissues by PCR-SSCP analysis for exon 4-8 and immunohistochemical staining with anti-p53 antibody, DO-7, and found that 12 tissues (48%) among them had gene mutations[12]. In four cases, the discrepancy between the two methods was observed. In one case, which was positive by PCR-SSCP but negative by immunohistochemical staining, the mutation was nonsense mutation. In the remaining three cases, the p53 gene mutations were detected only by immunohistochemical staining. This observed discrepancy may be due to the contamination of cells with normal p53 gene, such as non-tumor cells, because the contamination may decrease the sensitivity of PCR-SSCP analysis.

The purpose of this study was to investigate the sensitivity of PCR-SSCP analysis for p53 gene mutations when the sample was contaminated with normal cells. In the present study, we have examined the p53 gene mutations by PCR-SSCP analysis in the mixed samples of DNAs extracted from a human gastric cancer cell line and normal gastric mucosal cells at various ratios.

MATERIALS AND METHODS

1. Cell Lines

Two human gastric cancer cell lines, MKN-45 and KATO III, kindly donated by Dr. N. Saijo, Japan National Cancer Hospital and Research Institute, Tokyo, Japan, were used in this study. MKN-45 was poorly differentiated adenocarcinoma cells derived from liver metastasis. KATO III was signet ring cell carcinoma cells derived from pleural effusion. These cell lines were maintained as monolayer culture in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat inactivated fetal bovine serum (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 5% CO_2 in a highly humidified incubator at 37℃. Non-tumor gastric mucosal cells were obtained during operation for stomach cancer from the grossly nomral gastric mucosa and used after confirming non-tumor mucosal cells by hematoxylin and eosin staining.

2. PCR-SSCP Analysis

Genomic DNA was prepared from the cell lines by proteinase K digestion, phenol-chloroform extraction and ethanol precipitation, as described previously[13]. SSCP of DNA fragments obtained by PCR was performed by the method described by Orita et al.[14,15] with slight modifications. In brief, oligonucleotide primers were synthesized by the phosphoramidite method using a DNA synthesizer 391 PCRMATE (Applied Biosystems, CS, USA) and purified with PHLC and reverse chromatography. The sequences of oligonucleotides used for the amplification of exon 4-8 of the p53 gene were those reported by Buchman et al.[16](Table 1).

In order to investigate the sensitivity of PCR-SSCP, DNA obtained from MKN-45 was mixed with DNA obtained from normal gastric mucosal cells at ratios ranging from 1:4 to 1:64. PCR was performed with 100 ng of genomic DNA mixed in a total volume of 50 μl of the reaction mixture containing 20 pmol of each primer, 200 μM of dATP, dCTP, dGTP, and dTTP, 0.1 μCi of [^{32}p] dCTP (3,000 Ci/nmol, Amersham, UK) and 0.5 U of Taq polymerase (Perkin Elmer Cetus, USA) by using an DNA Thermal Cycler (Perkin Elmer, USA) with 35 cycles of reaction. Each cycle consisted of denaturation at 94℃ for 1 min, annealing at 62℃ for 1 min and extension at 72℃ for 1 min. After the full reaction, prolonged

Table 1. The Oligonucleotide Primers used to Amplicate the Exon 4-8 of p53 Gene in Polymerase Chain Reaction

exon	Codon	PCR product (bp)	Upstream	Downstream
exon 4	33-125	293	ATCTACAGTCCCCCTTGCCG	GCAACTGACCGTGCAAGTTCA
exon 5	126-201	325	TTCCTCTTCCTGCAGTACTC	GCAAATTTCCTTCCACTCGG
exon 6	179-224	236	ACCATGAGCGCTGCTCAGAT	AGTTGCAAACCAGACCTCAG
exon 7	225-261	139	GTGTTGTCTCCTAGGTTGGC	CAAGTGGCTCCTGACCTGGA
exon 8	262-331	330	CCTATCCTGAGTAGTGGTAA	CCAAGACTTAGTACCTGAAG

extension was performed at 72℃ for 10 min.

The amplified DNA was 5 to 10 fold diluted in a solution containing 0.1% sodium dodecyl sulfate and 10 mM EDTA, denatured at 95℃ for 5 min and electrophoresed on 6% polyacrylamide gel with or without 10% glycerol. Electrophoresis was performed at 1,000 volts for 3 hr under cooling with a fan at room temperature. The gel was dried and exposed to X-ray film at −70℃ for 24hr. PCR-SSCP was repeated twice to ensure the reproducibility.

RESULTS

PCR-SSCP analysis for exon 4-8 of the p53 gene was performed with genomic DNA of two human gastric cancer cell lines, MKN-45 and KATO III. In KATO III, no amplication of the p53 gene was generated by PCR, suggesting that both alleles for exon 4-8 of the p53 gene were deleted (data not shown except for exon 4)(Fig. 1). In MKN-45, the amplified product of the mutated p53 gene was observed in exon 4 by the method of PCR-SSCP analysis. Therefore, the effect of non-tumor cell contamination on the detection of p53 gene mutations was conducted for exon 4 using MKN-45 and normal gastric mucosal cells.

In the mixed DNA samples of MKN-45 and normal gastric mucosal cells, an extra band with migration similar to that of MKN-45 was found (Fig. 2). The extra band was able to be recognized in the samples of 1:8 dilution or less, while no extra band was grossly detectable in the samples of more than 1:16 dilution. This finding suggests that the low-grade contamination should be necessary for the detection of p53 gene mutations by PCR-SSCP particularly in samples contaminated by a large number of cells without p53 gene mutation.

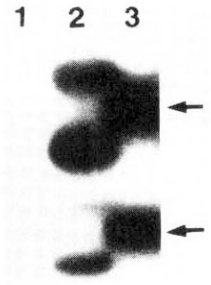

Fig. 1. PCR-SSCP analysis of human gastric adenocarcinoma cell lines, KATO III and MKN-45, for exon 4 of the p53 gene. Lane 1, 2 and 3 are from KATO III, normal gastric mucosal cells and MKN-45, respectively. In KATO III, no amplification of the p53 gene was observed. In MKN-45, the mobility shifted band was detected (arrow).

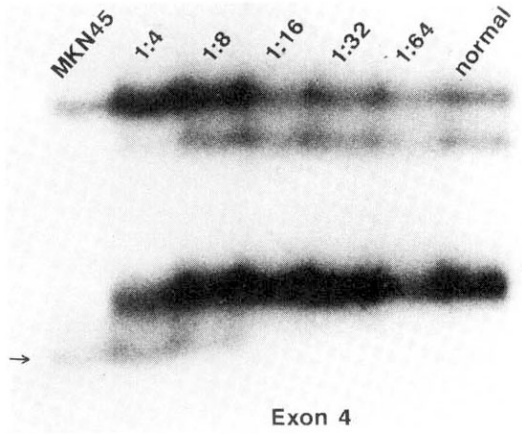

Fig. 2. PCR-SSCP analysis of mixed DNA samples of human gastric adenocarcinoma cells, MKN-45, and normal gastric mucosal cells for exon 4 of the p53 gene at the ratios indicated in each lane. Arrow indicates the mobility shifted band.

DISCUSSION

Since the development of techniques for PCR-SSCP, significant advances have been made in the molecular genetics of human cancers, because of the high sensitivity and rapidity of PCR-SSCP analysis to detect gene aberrations[14,15]. One of the great advantages of PCR-SSCP is to detect the conformational polymorphisms of a particular fragment of DNAs using non-denaturating gel, because conformational change in the single-stranded DNAs develops and induces the band with mobility shift on non-denaturating gel during the electrophoresis while, on denaturating gel, a minute change in DNAs, such as single base substitution, is hardly detected by the electrophoresis. Therefore, PCR-SSCP analysis is very useful for detecting a minute change in a small fragment of DNA, such as point mutation, although conformational change is also affected by the condition of electrophoresis, including electrophoresis buffer, denaturing agents in gel and temperature during electrophoresis.

Recently we have conducted screening for the incidence of the p53 gene mutations in 25 primary gastric cancer tissues surgically removed by using

both PCR-SSCP for exon 4-8 and immunohistochemical staining with anti-p53 antibody, DO-7[12]. As described in the Introduction, four (16%) out of 25 cancer tissues showed the discrepancy of the results between the two methods. In one case, which showed positive PCR-SSCP but negative immunohistochemical staining, the silent mutation was observed on condon 249, AGG-CGG (Arg-Arg). In three cases, which did not show the mobility shift in PCR-SSCP, the p53 gene products were clearly stained by immunohistochemical staining. The exact mechanism by which this discrepancy develops is not clear at present. However, this discrepancy may be explained by the following factors.

If the mutation developed in genes located outside the regions tested, the mutations were not detected by the PCR-SSCP method used in this study, although exons 4 through 8 were known to be the most important regions in cell transformation. The other posibility is the decrease in the sensitivity of PCR-SSCP, when the sample was contaminated by cells with normal p53 gene, such as non-tumor cells or tumor cells with normal p53 gene. This study was aimed to clarify the influence of the contamination of tumor cells, without p53 gene mutation, on the sensitivity of PCR-SSCP analysis and to find the conditions in which the contamination of non-tumor cells, without p53 gene mutation, did not affect the detectability of p53 gene mutation, when the method of PCR-SSCP, widely used for the detection of p53 gene mutations in cancer cells obtained from patients, was employed.

It has been reported by many investigators that the gene amplification and mobility shift in PCR-SSCP are influenced by many factors, such as the primers, conformational variations of the DNA extracted, thermal cyclic conditions, compositions of ingredients of buffers, denaturing agents in gels, conditions of electrophoresis, etc. In this study, we used the PCR-SSCP method usually used for the detection of p53 gene mutations in cell lines and cancer tissues[6~12].

On the other hand, when cancer tissues are obtained from patients, it is inevitable to contaminate non-tumor cells, although the degree of the contamination is dependent on the source of cancer cells, such as primary cancer tissue, lymph node metastasis, pleural effusion, ascites, etc., and the method used for the purification of the cancer cells. In addition, when cancer tissues were collected in the area massed with cancer cells and were proven to have p53 gene mutations, p53 gene mutations usually did not develop in all cancer cells collected. In this study, we used the MKN-45 cell line and normal cells. Therefore, the results obtained are the effect of non-tumor cell contamination on the detection of p53 gene mutations.

Previous investigations revealed that the incidence of the p53 gene mutations in primary gastric cancer tissues did not show consistency, ranging from 10% to 56%[8~12]. The difference in the incidence of p53 mutations may be due to various degrees of contaminating cells with normal p53 gene. Anyway, the contamination of the non-tumor cells is thought to affect the production of PCR products. To clarify the effect of contamination on the results of PCR-SSCP, we mixed DNAs extracted from cancer cells, MKN-45, and normal gastric mucosal cells, instead of mixing the cells, because we thought that the mixing with the genes resulted in a more accurate rate gene mixture than the mixing of the cells followed by DNA extractions.

This study was conducted with two gastric cancer cell lines, at first. However, exon 4-8 of p53 gene was not amplified in KATO III, suggesting that both alleles of exon 4-8 were deleted. In MKN-45, the amplification of aberrant p53 gene was found in exon 4. Therefore, the effect of the contamination was evaluated using DNA of MKN-45. In this study, an extra band corresponding to the mutant p53 gene could be grossly identified if the mutant DNA amount was more than one eighth of the total DNA amount.

It is of great interest that our results described above are in good agreement with the previous report by Yamada et al.[8]. They investigated the sensitivity of PCR-SSCP analysis using mixed DNA samples of MKN-1, human gastric adenosquamous carcinoma cell line and human placenta cells, at ratios ranging from 1:1 to 1:100 and reported that the mutated p53 gene could be identified when MKN-1 gene was present in more than one eight of the total DNA.

Our present and previous studies suggest that the mutation of p53 gene may play an important role in the carcinogenesis of gastric cancer, and PCR-SSCP analysis is a highly sensitive and powerful method to detect the gene aberrations, if cancer cells are not to be contaminated by cells without p53 gene mutation at the ratio of less that one eight. Because we have previously observed the discrepancy between PCR-SSCP analysis and immunohistochemical staining for the detection of p53 gene mutation, further study on the mechanism by which

the discrepancy develops may be needed to interpret the results of PCR-SSCP analysis more reasonably.

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