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Correlation of p53 over-expression and alteration in *p53* gene detected by polymerase chain reaction-single strand conformation polymorphism in adenocarcinoma of gastric cancer patients from India

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Abstract

AIM: To study the alterations in *p53* gene among Indian gastric cancer patients and to correlate them with the various clinicopathological parameters.

METHODS: A total of 103 gastric cancer patients were included in this study. The *p53* alterations were studied by both immunohistochemical method as well as polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis. We only studied four (exon 5, 6, 7, and 8) of the 11 *p53* exons. The alterations in *p53* were also correlated with respect to various clinicopathological parameters.

RESULTS: Among 103 cases, p53 over-expression and alteration were detected in 37 (35.92%) and 19 (18.44%) cases, respectively. Most of the *p53* alterations were found at exon 5 (31.54%), followed by exon 6 (26.31%), exon 7 (21.04%) and exon 8 (21.04%). A significant correlation of p53 over-expression was found with *p53* alteration (P = 0.000). Concordance between *p53* alteration (as detected by SSCP) and over-expression [as detected by immunohistochemistry (IHC)] was found in 75% cases. We found that IHC-positive/SSCP-negative cases accounted for 21% of cases and IHC-negative/SSCP-positive cases accounted for remaining 4% cases.

CONCLUSION: Our results show that *p53* gene mutations are significantly correlated with p53 protein over-expression, with 75% concordance in over-expression and alteration in the *p53* gene, but 25% disconcordance also cautions against the assumption that p53 over-expression is always associated with a gene mutation. There may be other mechanisms responsible for stabilization and accumulation of p53 protein with no evidence of gene mutation that reflect an accumulation of a non-mutated protein, or a false negative SSCP result.

Key words: Gastric cancer; *p53*; Single strand conformation polymorphism; Gene mutation; Immunohistochemistry

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INTRODUCTION

Gastric cancer is a common disease worldwide[1] and also one of the leading causes of cancer death (5th in male and 6th in female) in India[2]. Its pattern and incidence vary widely between different parts of the world. Costa Rica and Japan have the first and second highest rate in the world with a death rate of 77.7 and 50.5 per 100 000, respectively[1,3]. In the United States, 12 100 deaths from gastric cancer were expected during 2003 with a death rate of 6.8 per 100 000[4]. The overall age-adjusted incidence (world population) of gastric cancer in Jordan is 5.8 per 100 000[5]. The burden of new cancer cases has been estimated in the year 2002 based on the National Cancer Registry Programme in India. The number of

new digestive tract cancer cases was estimated to be approximately 145 000; out of which stomach cancer comprised 23 785 cases in men and 11 890 in women[6]. Although aggressive surgical resection has improved the overall outcome of patients with this carcinoma, results of surgical resection for advanced cancer are still poor, and the search for predictors of disease survival and response to therapy is therefore mandatory[7]. The molecular biology of gastric cancer has been widely studied in the developed world, particularly in Japan[8-11]. However, only a few scattered reports from the developing world have been published[12-16], and there are no similar published data reported from India. The molecular events leading to the development of gastric cancer are largely unknown, but there is now enough evidence to suggest that the functional inactivation of *p53* gene through allelic loss and point mutation plays an important part[17,18].

Several genetic alterations have been shown to play a significant role in tumorigenesis. By and large, the most frequently observed molecular changes occur in the *p53* gene[19]. The *p53* gene encodes a protein involved in control of the cell cycle and acts as a negative regulator in the cell's response to damaged DNA[20]. Functional alteration of p53 protein can occur through several mechanisms: point mutations, deletions, rearrangements in the *p53* gene, binding with viral proteins, binding with cellular proteins, and oligomerization[21]. Wild-type p53 protein has a very short half-life, whereas mutated p53 is stable and can accumulate at high concentration in the nuclei of tumor cells. As a consequence, immunohistochemical staining with specific antibodies can be used to detect mutant p53 protein. Detecting the precise alteration occurring at the genetic level is much more laborious and costly. The most widely used molecular approach is single strand conformation polymorphism (SSCP) analysis of DNA fragment amplified by polymerase chain reaction (PCR)[22], with subsequent sequence analysis. The sensitivity of SSCP is influenced by the experimental conditions, and by the length of the amplified fragment under study.

The aim of present study was to determine *p53* status in Indian gastric cancer patients, who have undergone gastrectomy, and correlate it with various clinicopathological parameters. This study also correlated immunohistochemical staining of p53 protein with SSCP analysis of the PCR-amplified DNA.

MATERIALS AND METHODS

Tumor specimens

From April 2002 to February 2007, cancerous and normal mucosa samples taken from 128 consecutive patients with suspected gastric cancer were enrolled for the study. Cancerous and normal mucosa samples collected from various hospitals after gastrectomy were aliquoted into two sterile tubes. The aliquots for histological and immunohistochemical studies were stored at 4℃ in 10% buffered formalin. On the other hand, aliquots for PCR-SSCP studies were stored at -20℃ in 1X PBS until DNA extraction. The tissue specimen for SSCP analysis was taken from the center of the tumor mass. No patients had undergone previous chemotherapy or radiotherapy. All the clinicopathological data were collected prospectively and registered in the database. A detailed histopathological examination was performed to determine the depth of invasion on the gastric wall and the extent of metastases within regional lymph nodes. Among 128 cases, 25 were found to be histopathologically negative for gastric cancer and thus were excluded from the study. The remaining 103 cases were from West Bengal (n = 60), Jammu & Kashmir (n = 30) and New Delhi (n = 13). Patients included males (n = 78) and females (n = 25) with a mean age of 56 years (range 25-71 years). The clinical stage was determined by the tumor, node, metastasis (TNM) system[23] and histological diagnosis was made according to the classification of Lauren[24].

Immunohistochemical staining

For immunohistochemical analysis of p53 protein, 4-μm-thick tissue sections were cut and stained with anti-p53 monoclonal antibody (DO7, Biogenex Laboratories, San Ramon, USA) by streptavidin-biotin immunoperoxidase technique (LSAB) using the standard avidin-biotin-complex (ABC) method, followed by antigen retrieval by microwave technique as previously described[25,26]. Briefly, 4-μm-thick sections were first mounted on glass slides, treated with methanol (with 4% H_2O_2) for 30 min, and incubated with anti-p53 monoclonal antibody (1:100 dilution; DO1 and DO7, Biogenex Laboratories, San Ramon, USA) overnight at 4℃ in a humid chamber. After washing thrice with PBS, slides were incubated with biotinylated anti-mouse IgG antibody (DAKO-LSAB kit, Peroxidase, M/s Dakopatts, Denmark) for 30 min at room temperature and developed using HRP-streptavidin and diaminobenzidine tetra hydrochloride (Sigma, St. Louis, USA). The slides were incubated at room temperature for 5-10 min under microscopic control till the optimum development of brown-colored product. Finally, sections were counterstained with Harris hematoxylin and mounted in DPX. The reaction was considered as positive when a strong coloration of the cell nucleus was evident. Sections with nuclear staining in less than 10% of the tumor cells were regarded as negative. Positive p53 nuclear staining was categorized into three patterns as follows: (+), only a few scattered positively stained cells (10%-25% of all tumor cells); (++), localized aggregation of positively stained cells (26%-70% of all tumor cells); and (+++), diffuse aggregation of positively stained cells (more than 70% of all tumor cells). Over-expression of the p53 protein was defined when a (++) or (+++) staining pattern was observed. Negative control sections were processed immunohistochemically without incubating with the primary antibody and positive control sections were from a breast cancer tissue known to express high level of p53.

Genomic DNA isolation and PCR amplification of the *p53* exons

Genomic DNA was isolated from tissues of gastric

Table 1 Detail of the primers and cycling parameters

p53 exons	Sequence (5′-3′)	Cycling parameters[1]	PCR product (bp)
5	Sp5F: TGTTCACTTGTGCCCTGACT Sp5R: CAGCCCTGTCGTCTCTCCAG	45 s denaturation at 94℃, 30 s annealing at 54℃, 1 min extension at 72℃	266
6	Sp6F: GCCTCTGATTCCTCACTGAT Sp6R: TTAACCCCTCCTCCCAGAGA	45 s denaturation at 94℃, 30 s annealing at 53℃, 1 min extension at 72℃	160
7	Sp7F: ACTGGCCTCATCTTGGGCCT Sp7R: TGTGCAGGGTGGCAAGTGGC	45 s denaturation at 94℃, 30 s annealing at 56℃, 1 min extension at 72℃	180
8	Sp8F: TAAATGGGACAGGTAGGACC Sp8R: TCCACCGCTTCTTGTCCTGC	45 s denaturation at 94℃, 30 s annealing at 54℃, 1 min extension at 72℃	230

[1]Initial denaturation of 10 min at 94℃ and final extension of 10 min at 72℃ were used in all amplifications. Number of cycles in all amplifications was 35.

Figure 1 SSCP analysis of exons 5, 6, 7 and 8 of *p53* gene. Wild-type and one tumor sample containing mutation are shown for each exon. Only single-strand DNA bands are shown. Heterodimers and double-stranded bands have been omitted.

cancer patients by a modified proteinase K digestion and phenol/chloroform extraction technique. All four exons (5, 6, 7 and 8) were amplified separately and details of the primers and PCR cycling parameters used are mentioned in Table 1. The standard PCR reaction contained 0.5 μg genomic DNA, 200 μmol/L dNTPs, 1X PCR buffer, 1.5 mmol/L $MgCl_2$, 1 U of Taq DNA polymerase and 0.3 μmol/L of each primer. Each PCR reaction contained negative controls, where only water was added in place of genomic DNA. The PCR products were electrophoresed on a 20 g/L agarose gel to check the amplification. Samples with positive amplifications were processed for SSCP analysis.

SSCP analysis

Non-radioactive SSCP was performed as described previously[27] with slight modification. A 2-μL volume of PCR product was denatured in 5 μL of formamide loading buffer by boiling for 10 min. Denatured samples were loaded onto 120 g/L acrylamide non-denaturation gel and electrophoresed at a constant 100 V for 4-6 h. Images of the ethidium-bromide-stained gels were captured using the BioRad Gel Documentation system. Samples with mobility shifts were verified by a second independent PCR-SSCP where silver staining was done for the same.

Statistical analysis

Descriptive statistics i.e. mean, standard deviation and frequency distribution were calculated for all the variables in the study. According to the different categories for the categorized variables, χ^2 test was used to estimate the significance level of the association among them. Student's *t*-test, Pearson correlation and analysis of variance were applied for the continuous variables according to the categories, wherever applicable. $P < 0.05$ was considered statistically significant. SPSS.60 software (SPSS Inc, Chicago, USA) was used for these calculations.

RESULTS

Detection of *p53* gene mutation by SSCP analysis

The *p53* exons 5, 6, 7 and 8 were successfully amplified from all 103 cases which gave expected PCR fragments of 266 bp, 160 bp, 180 bp, and 230 bp, respectively. After SSCP analysis, altered *p53* was identified by the presence of one or two extra bands migrating above or below the normal single-stranded products (Figure 1). Occasionally, mutant bands were also detected between the single and double-strand bands that may have been caused by formation of normal-mutant heterodimers. However, normal *p53* banding pattern was also observed among all cases. SSCP data showed that 17.47% (19/103) of the cases were harboring altered *p53* (Table 2). Out of these 19 cases, six showed alterations in exon 5, five in exon 6, four in exon 7 and four in exon 8. We further observed that the positivity for *p53* mutations was relatively higher in intestinal-type carcinoma (22.44%, 11/49) as compared to diffuse-type (15.66%, 7/45) adenocarcinoma[28] (Table 3).

Detection of p53 protein over-expression by immunohistochemistry (IHC)

We used DO-7 to detect p53 over-expression by IHC

Table 2 Relationship between p53 protein over-expression detected by IHC and *p53* gene mutation detected by SSCP analysis of 103 gastric carcinomas *n* (%)

IHC status	SSCP-negative	SSCP-positive	Total	*P*
IHC-negative	62 (60)	4 (4)	66 (64)	0.000
IHC over-expression	22 (21)	15 (15)	37 (36)	
Total	84 (81)	19 (19)	103 (100)	

Table 3 Correlation between clinicopathological characteristics and *p53* genetic alteration in patients with gastric adenocarcinoma

Factors	*p53* alteration		*n* (%)	*P*[1]
	Negative (%)	Positive (%)		
Age				
< 40 yr	12 (14.29)	1 (5.26)	13 (12.62)	0.533
40-65 yr	55 (65.48)	15 (78.95)	70 (67.96)	
> 65 yr	17 (20.23)	3 (15.79)	20 (19.42)	
Gender				0.407
Male	62 (73.81)	16 (84.21)	78 (75.73)	
Female	22 (26.19)	3 (15.79)	25 (24.27)	
Cell differentiation				0.462
Well	9 (10.72)	2 (10.53)	11 (10.68)	
Moderately	31 (36.90)	8 (42.11)	39 (37.86)	
Poorly	44 (52.38)	9 (47.37)	53 (51.46)	
Histology				0.522
Intestinal	35 (41.67)	10 (52.63)	45 (43.69)	
Diffused	41 (48.81)	8 (42.11)	49 (47.57)	
Undiff/mixed	8 (9.52)	1 (5.26)	9 (8.74)	
Site				0.606
Cardiac	19 (22.62)	2 (10.53)	21 (20.39)	
Fundus	14 (16.67)	2 (10.53)	16 (15.53)	
Body	18 (21.43)	5 (26.32)	23 (22.33)	
Antrum	33 (39.29)	10 (52.63)	43 (41.75)	
Stage				0.813
Ⅰ	26 (30.95)	6 (31.58)	32 (31.07)	
Ⅱ	32 (38.10)	6 (31.58)	38 (36.89)	
Ⅲ	21 (25.00)	6 (31.58)	27 (26.21)	
Ⅳ	5 (5.95)	1 (5.26)	6 (5.83)	

[1]χ^2 test with Fisher's test were used to test the frequency distribution of *p53* alteration. Undiff: Undifferentiated.

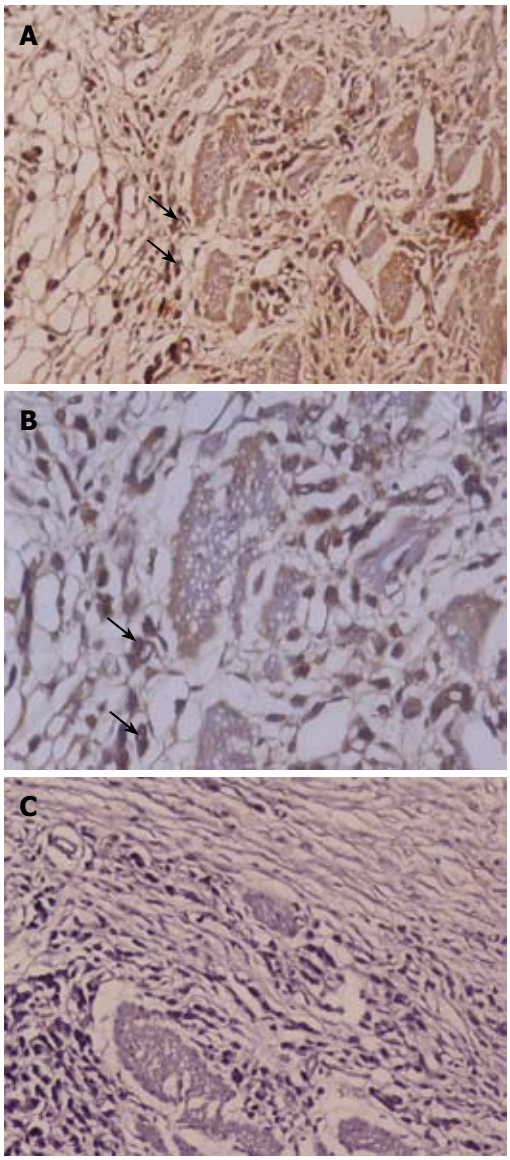

Figure 2 Immunohistochemical staining of p53 protein in diffused type-poorly differentiated gastric cancer. p53 protein over-expression is seen primarily on cell nucleus. A: Positive nuclear staining (× 200); B: Positive nuclear staining (× 400); C: Negative control (× 200).

staining. Overall, 35.92% (37/103) of gastric carcinomas exhibited positive nuclear staining (Figure 2). The concordance of results between SSCP and IHC (i.e. both positive and negative) was 75% (Table 2). In addition, 21% of carcinomas (22/103) stained positively by IHC but failed to display any mutation by SSCP, while the remaining 4% (4/103) displayed mutant bands by SSCP but showed no staining by IHC.

DISCUSSION

The present study describes the status of *p53* gene mutation and over-expression in Indian gastric cancer patients. The association between *p53* abnormalities and pathological characteristics of tumor was also assessed. Mutations of the *p53* gene are the most common genetic alteration, known to occur in a wide range of human cancers[19]. A number of studies have reported the frequency of *p53* alteration in gastric cancer, showing *p53* mutation in 18%-58%[18,29,30] and p53 protein over-expression in 26%-65% of gastric adenocarcinoma[31,32]. Under normal conditions, wild-type p53 protein is rapidly degraded but in carcinomas, the acquisition of a mutant genotype is thought to increase the half-life of the mutant protein , which leads to accumulation and detection by immunohistochemical techniques, an indirect method of screening tumors for mutation within the *p53* gene. However, a number of discordant results have been reported[33,34]. p53 over-expression can also reflect the accumulation of wild-type protein, which is stabilized *via* a mechanism other than mutation-for example, by binding with SV40 T antigen, which greatly increases the half-life of the protein[35]. In the present study, we have attempted to address this issue by comparing *p53* gene mutation and protein over-expression in a large number of gastric tumors. We analyzed here only exons 5 to 8; because most of the *p53* mutations have previously been found to be clustered in

the highly conserved domains that roughly correspond to exons 5-8[36]. Although approximately 90% of the *p53* point mutations have been reported from exons 5-8, the individual incidence rate of *p53* mutations in these exons is not known[37,38]. Although our study is primarily focused on exons 5 to 8, mutations in other exons (13%-22%) could account for some negative SSCP cases[39,40].

Several studies have addressed the relationship between p53 protein accumulation and *p53* alteration in different tumor types[41-43]. The concordance rates between IHC staining and SSCP (both negative and positive) fall within a relatively wide range (50% to 80%), but this probably depends on differences both in methodology and tumor type. In the present study, for establishing a relationship between *p53* gene mutation and p53 protein accumulation, gastric carcinomas were analyzed for exons 5, 6, 7 and 8 of the *p53* gene. We found that p53 protein was over-expressed in 35.92% cases and *p53* gene was mutated in 17.47% of cases. A comparison of PCR-SSCP and immunohistochemical findings showed concordant (i.e. both positive and negative) results in 75% of the cases. In particular, 15 (78.94%) of the 19 cases showing a *p53* gene mutation were also positive for p53 protein accumulation on IHC, while 22 (26%) of the 84 cases without mutation were positive on IHC. In 26 (25%) cases, the immunohistochemical and SSCP results were discordant; negative IHC/positive SSCP in four and positive IHC/negative SSCP in 22. The presence of *p53* gene mutations was significantly correlated with p53 protein over-expression ($P = 0.000$).

Accumulation of p53 protein with no evidence of gene mutation may either reflect an accumulation of a non-mutated protein, or a false-negative SSCP result. The sensitivity of SSCP analysis is still a matter of debate; not only does it depend on the experimental conditions (i.e. temperature, ionic strength and presence or absence of glycerol), but also on the length of the amplified fragment being analyzed[44]. Sheffield *et al*[45] reported that SSCP sensitivity dramatically decreased with increasing fragment size; the percentage of single base substitution detected by SSCP was 90% when the amplified fragment was 135 bp, 70% when it was 200 bp, and 67% as fragment size exceeded 300 bp[46]. As the amplified fragment length we analyzed was 266 bp for exon 5, 160 bp for exon 6, 180 bp for exon 7 and 230 bp for exon 8, SSCP sensitivity for exon 5 and 8 might have been less than 70%. Although our study focused on exons 5 to 8, where the majority of the mutations are thought to be localized[38], mutations in other exons (13%-22%) could account for some negative SSCP cases[39,40]. A false-negative SSCP result may also occur when the tissue is not sufficiently represented in tissue from which the DNA is extracted. The search for *p53* gene mutation by molecular assays is important for defining the functional alterations that are derived from the different mutations. In this regard, it might be advisable to study all of the exons of the *p53* gene. We can justify the inconsistent cases of SSCP and IHC by some possible explanations: first, there may be mutations in exons other than those we examined; second, binding of wild-type p53 protein to a cellular oncogene might have led to an elongated half-life; and third, the proportion of alleles containing *p53* mutations may be relatively low, and not shown up in the analysis[47].

It was reported that wild-type p53 proteins could combine with viral oncoproteins or cellular oncoproteins to enhance their stability and prolong their half-life, leading to p53 protein accumulation in cells. In such cells, IHC staining was still positive even without *p53* gene mutations; furthermore, about 10% of *p53* gene mutations could take place outside of exons 5-8. Therefore, the positive rate of PCR-SSCP targeting only exons 5-8 was usually lower than that of IHC[20,47-49].

In conclusion, PCR-SSCP is a simple and rapid method for the evaluation of *p53* gene mutations and its association with immunohistochemical analysis of the p53 protein expression might provide a more precise use of *p53* as a prognostic marker in gastric cancer.

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COMMENTS

Background

Gastric cancer is a common disease worldwide, including India. The molecular events leading to the development of gastric cancer are largely unknown, but various lines of evidence suggests that the functional inactivation of the *p53* gene plays an important role. The p53 protein is involved in control of the cell cycle. Wild-type p53 protein has a very short half-life, whereas mutated p53 is stable and can accumulate at high concentration in the nuclei of tumor cells, and is detected by immunohistochemical staining with specific antibodies. Polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis was done to screen and detect the presence of any point mutation in *p53* gene.

Research frontiers

In general, the present study is a part of cancer research that is aimed at mutational analysis of the *p53* gene among gastric cancer patients. To the best of our knowledge, this is the first study where an attempt has been made to correlate the *p53* gene mutation with various clinicopathological features among Indian gastric cancer patients.

Innovations and breakthroughs

Authors have analyzed the status of p53 protein expression by immunohistochemistry (IHC) and *p53* gene mutation by PCR-SSCP among gastric cancer cases. This study also provides a significant correlation between immunohistochemical staining of p53 protein and SSCP analysis of the PCR amplified DNA.

Applications

PCR-SSCP in association with IHC of the p53 protein may provide a more precise use of *p53* as a prognostic marker for gastric cancer.

Terminology

SSCP is a technique used to screen and detect the mutational change at

the single nucleotide level. Additional bands of DNA is an indication of gene alteratio. IHC is a technique used to detect the mutated p53 protein in the nucleus of cancer cells. Mutation in the *p53* gene leads to increased half-life and accumulation of p53 protein in the nucleus.

Peer review

The paper describes *p53* alterations among gastric cancer patients in India. The alterations were studied by IHC and PCR-SSCP analyses. A significant correlation of p53 over-expression and alteration in *p53* gene detected by SSCP was found. It is an interesting study and this is also the first such study from an Indian population, including a large sample from a long research period.

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