

## Analysis of *p53*, *K-ras* gene mutation & *Helicobacter pylori* infection in patients with gastric cancer & peptic ulcer disease at a tertiary care hospital in north India

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**Background & objectives:** Mutations in the oncogene and tumour suppressor genes play an important role in carcinogenesis. We investigated the association of *p53* and *K-ras* gene mutation and *Helicobacter pylori* infection in patients with gastric cancer (GC) and peptic ulcer disease (PUD) attending a tertiary care hospital in north India.

**Methods:** In total, 348 adult patients [62 GC, 45 PUD and 241 non-ulcer dyspepsia (NUD)] who underwent an upper gastrointestinal endoscopy were enrolled. *H. pylori* infection was diagnosed by rapid urease test, culture, histopathology and PCR. Mutation in the exon 5-8 of *p53* gene was analyzed by PCR-single stranded conformational polymorphism (SSCP) and confirmed by sequence analysis. *K-ras* gene codon 12 mutation was analyzed by PCR-based restriction fragment length polymorphism.

**Results:** Overall *p53* gene mutation was found in 4.6 per cent of the study population, and its distribution in GC, PUD and NUD was 21, 4.4 and 0.4 per cent, respectively. *p53* gene mutation was significantly higher in patients with GC than PUD ( $P < 0.05$ ) and NUD ( $P < 0.001$ ). No difference in *p53* gene mutation was observed between *H. pylori* infected and non-infected individuals. *K-ras* gene mutation was absent in all the patients.

**Interpretation & conclusions:** Our results show that *p53* gene mutation may be associated with gastric carcinogenesis independent to *H. pylori* infection and absence of *K-ras* gene mutation questions its role in the pathogenesis of GC and PUD in Indian patients.

**Key words** Gastric cancer - gene mutations - *Helicobacter pylori* - peptic ulcer disease

*Helicobacter pylori* has been classified as a major cause of peptic ulcer disease (PUD) and a risk factor for gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma<sup>1-3</sup>. On a global

scale, gastric cancer is the second commonest cancer in the world. There is substantial international variation in gastric cancer incidence with the highest rates reported from China, Japan and other Eastern Asian

countries. Epidemiological studies have proved that *H. pylori* infection is considered as a risk factor for gastric cancer and the International Agency for Research on Cancer (IARC) has classified this bacterium as a definite carcinogen<sup>2</sup>. While the majority of the infected individuals develop no significant clinical disease, others develop two kinds of divergent clinical outcomes – PUD and gastric cancer<sup>4</sup>. The reasons for developing these two extreme phenotypes remain poorly understood and are not explained by bacterial virulence factors alone<sup>4,5</sup>. This highlights the need to explore potential candidate genes of the host involved in the *H. pylori*- associated gastric carcinogenesis.

*p53* protein plays an important role in the maintenance of genomic integrity through the induction of cell growth arrest or apoptosis following DNA damage<sup>6</sup>. Alterations in *p53* gene, leading to a loss of tumour-suppressor function of *p53* protein have been implicated in the aetiology and progression of a variety of human cancers<sup>7,8</sup>. In October 2006, the *p53* database of IARC listed 31.2 per cent gastric cancers with point mutation in the *p53* gene<sup>9</sup>. *K-ras* oncogene encodes a membrane-associated protein, p21<sup>RAS</sup>, with intrinsic GTPase activity involved in cellular signal transduction<sup>10</sup>. It is well known that *K-ras* plays an important role in the pathogenesis of various types of human cancer<sup>11</sup>. Point mutations at codons 12, 13 and 61 of *K-ras* result in a shift of *K-ras* protein toward the activated state, which constitutively activates the mitogenic signal transduction pathway<sup>12</sup>. Frequency of mutated *K-ras* varies among the different tumour types<sup>13</sup>. Point mutations of the *K-ras* are found predominantly in adenocarcinomas. The highest incidence is found in adenocarcinomas of the pancreas, in which approximately 90 per cent of the tumours harbour mutated *K-ras*<sup>10,11</sup>. There were inconclusive data available on *p53* and *K-ras* gene mutational pattern in gastric cancer. It remains unclear that whether mutations in the above mentioned tumour suppressor *p53* gene and *ras*-oncogene are associated with *H. pylori* infection and tumorigenesis. Therefore, this study was undertaken to investigate *p53* and *K-ras* gene mutation in patients with gastroduodenal diseases in addition to *H. pylori* infection attending a tertiary care hospital in north India.

### Material & Methods

**Study population:** A total of 348 consecutive adult patients [62 gastric adenocarcinoma (GC), 45 PUD

and 241 non-ulcer dyspepsia (NUD)] who underwent upper gastrointestinal endoscopy at a tertiary referral center in northern India between September 2002 and May 2007 were enrolled in the study. The diagnosis of gastroduodenal diseases was based on clinical, endoscopic and histopathological examinations. Patients with NUD were considered as controls. The ethics committee of the institute granted approval for the study and the written consent was obtained from all the patients. Subjects who had received anti-microbial therapy, H<sub>2</sub> receptor blockers, proton pump inhibitors and non-steroidal anti-inflammatory drugs in the preceding 30 days prior to endoscopy or anti-*H. pylori* treatment in the past were excluded from this study.

**DNA extraction:** For PCR and mutation detection of *p53* and *K-ras* gene, genomic DNA was isolated from gastric tissues using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) as per the manufacturer's instructions.

**Detection of *H. pylori* infection:** During each endoscopic examination antral biopsies were obtained and subjected to the following tests: rapid urease test (RUT), culture, histopathology and *H. pylori* specific *ureA* PCR following the standard protocol as described earlier<sup>12-15</sup>. *H. pylori* infection was diagnosed if any of the above tests was positive.

**Detection of *p53* gene mutation:** Mutations of the *p53* gene in exon 5-8 were identified by PCR- single stranded conformation polymorphism (PCR-SSCP). Mutations obtained by SSCP in the *p53* gene were finally confirmed by sequence analysis. In brief, PCR was used to amplify exons 5-8 of *p53* gene which are known to be mutational hot spots<sup>16</sup>. PCR was performed in a 50 µl reaction volume containing 100 ng of genomic DNA, 1x PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM each deoxynucleotide, 0.5 µM each specific primers (Table I) and 1.25 U of Taq polymerase (Bangalore Genei, India). The conditions of PCR were as follows: 35 cycles at 94° C for 30 sec, 60° C for 30 sec, 72° C for 30 sec and final extension at 72° C for 10 min were carried out in a thermal cycler (MJ Research, USA) as described previously<sup>16</sup>. A negative control (no DNA template) and positive control (mutated DNA for each exon 5-8 of *p53* gene obtained from Dr Pierre Hainaut, Head, Molecular Carcinogenesis group, IARC, WHO, France, as generous gifts) were run in parallel for each amplification reaction.

Before carrying out SSCP, 10 µl of PCR product was electrophoresed in 2 per cent agarose and visualized

**Table I.** Primer sequences used for *p53* and *K-ras* gene

Gene	Region	Primer sequence	bp	References
<i>p53</i>	E5A	5'-TTCCTCTTCTGCAGTACTC-3' 5'-TCCGTCATGTGCTGTGACTG-3'	152	15
<i>p53</i>	E5B6A	5'-GCCATCTACAAGCAGTCACA-3' 5'-GCCAGACCTAAGAGCAATCA-3'	167	15
<i>p53</i>	E6B	5'-TTAGGTCTGGCCCCCTCTCA-3' 5'-AGTTGCAAACCAGACCTCAG-3'	132	15
<i>p53</i>	E7	5'-TTGTCTCCTAGGTTGGCTCT-3' 5'-CAAGTGGCTCCTGACCTGGA-3'	136	15
<i>p53</i>	E8	5'-TGGTAATCTACTGGGACGGA-3' 5'-CTGCTTGCTTACCTCGCTTA-3'	149	15
<i>K-ras</i>	codon 12	5'-ACTGAATATAAACTTGTGGTAGTTGGACCT-3' 5'-CTATTGTTGGATCATATTCG-3'	106	16

with ethidium bromide stain (0.5 µg/ml) to confirm the absence of contamination and to ensure that the PCR product was a single band of the appropriate size. Ten microlitre of each PCR product was mixed with 10 µl of SSCP dye containing 95 per cent (v/v) formamide, 20 mmol/l di-sodium EDTA, 0.05 per cent (w/v) xylene cyanol and 0.05 per cent (w/v) bromophenol blue. Before electrophoresis, the PCR products were heated at 95°C for 5 min in a water bath. After heat denaturation, the products were immediately placed on ice to prevent renaturation. An aliquot of 20 µl of each denatured sample was loaded onto 0.75 mm thick, 12 per cent (w/v) polyacrylamide gel (29:1 ratio of acrylamide to bisacrylamide) with 5x TBE and EDTA. Electrophoresis of gel was carried out in TBE for 12 h at 18°C with constant current of 200 V. Single stands of DNA were visualized with silver staining. Any extra band(s) and/or change of mobility of bands present in the sample were considered as positive for a mutation. PCR-SSCP was run for all the samples in duplicates to reduce the possibility of false positive results due

to polymerase errors or contamination. The samples that showed consistently aberrant patterns in replicate PCR-SSCP assays were subjected to direct sequencing. Sequencing of the PCR-SSCP positive samples was performed by commercial available sequencing services of Chromous Biotech Pvt. Limited, India.

*Detection of K-ras gene mutation:* Mutation of *K-ras* gene codon 12 was screened by using PCR-restriction fragment length polymorphism (PCR-RFLP) as described previously<sup>17</sup>. In brief, PCR was used to amplify *K-ras* codon 12 region using sequence specific forward and reverse primers (Table I). PCR was performed in a 50 µl reaction volume containing 100 ng of genomic DNA, 1x PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM each deoxynucleotide, 0.5 µM each specific primer and 1.25 U of Taq polymerase. The conditions of PCR were as follows: 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min were carried out in a thermal cycler (MJ Research, USA). A negative control containing no DNA template was run in parallel for each amplification reaction.

**Table II.** Demography of the study populations, *p53* gene mutation and *Helicobacter pylori* infection

Parameter	Gastric adenocarcinoma [GC] (n=62)	Peptic ulcer disease [PUD] (n=45)	Non-ulcer dyspepsia [NUD] (n=241)	Overall (n=348)
Mean age ± SD (yr)	56.60 ± 15.423	49.47 ± 17.216	43.75 ± 14.764	46.78 ± 15.959
Male: female	47:15	31:14	138:103	216:132
<i>H. pylori</i> infection (%)	35 (56.5)	36 (80)*	133 (55.2)	204 (58.6)
<i>p53</i> gene mutation (%)	13 (21)*+	2 (4.4)	1 (0.4)	16 (4.6)

\*P<0.01 compared to NUD and GC; \*\*P<0.001 compared to NUD; +P<0.05 compared to PUD

**Table III.** Details and distribution of *p53* gene mutation (exon 5-8) in patients with gastric adenocarcinoma (GC), peptic ulcer disease (PUD) and non-ulcer dyspepsia (NUD)

Disease	Hp	Exon	codon	Mutation	Nt change	Aa change
GC	+	8	272	Point	GTG - ATG	Val - Met
GC	+	7	245	Point	GGC - AGC	Gly - Ser
GC	+	7	241	Point	TCC - TGC	Ser - Cys
GC	+	7	248	Point	CGG - CAG	Arg - Gln
GC	+	7	241	Point	TCC - TGC	Ser - Cys
GC	+	7	245	Point	GGC - AGC	Gly - Ser
GC	+	7	245	Point	GGC - AGC	Gly - Ser
GC	+	7	249	Point	AGG - AGT	Arg - Ser
GC	+	7	241	Point	TCC - TGC	Ser - Cys
GC	-	8	272	Point	GTG - ATG	Val - Met
GC	-	7	241	Point	TCC - TGC	Ser - Cys
GC	-	5	136	Frame shift	1bp deletion (CAA-CA-)	Frame shift
GC	-	7	245	Point	GGC - AGC	Gly - Ser
PUD	+	6	212	Frame shift	1bp deletion (TTT-TT-)	Frame shift
PUD	-	7	241	Point	TCC - TGC	Ser - Cys
NUD	+	7	245	Point	GGC - AGC	Gly - Ser

Hp, *H. pylori*; Nt change, nucleotide change; Aa change, amino acid change

Before carrying out RFLP, 8 µl of PCR product was electrophoresed in 3 per cent agarose and visualized with ethidium bromide stain (0.5 µg/ml) to confirm the absence of contamination and to ensure that the PCR product was a single band of the appropriate size (106 bp).

Amplified PCR products were digested with *Mva* I (MBI, Fermentas, Vilnius, Lithuania) to distinguish the mutant allele from the wild type allele. Digestion of PCR products with the restriction enzyme was performed at 42°C for 8 h. After digestion, PCR products were electrophoresed on 2.5 per cent agarose gels, followed by ethidium bromide staining. If *K-ras* gene codon 12 was normal, wild type fragments cleaved to yield 77 and 29 bp products. If codon 12 contained a mutation, mutant type fragment yielded a single 106 bp product. The DNA with *K-ras* codon 12 mutation (kindly gifted by Dr Angelina Quintero, University of Mexico city, Mexico) was used as positive control in the study.

**Statistical analysis:** The data were analyzed by Chi square test. The SPSS 12.0 statistical package (Chicago, IL, USA) was used for data management and analysis.

## Results

A total of 348 patients (mean age: 46.78 ± 15.96 yr; 216 male) were enrolled in the study and their distributions were as follows: gastric adenocarcinoma 62 (mean age: 56.60 ± 15.42 yr; 47 male), PUD 45 (mean age: 49.47 ± 17.22 yr; 31 male) and NUD 241 (mean age: 43.75 ± 14.76 yr; 138 male) (Table II). Presence of *H. pylori* infection was seen in 58.6 per cent patients. *H. pylori* infection was significantly higher in patients with PUD than with gastric adenocarcinoma (80 vs 56.5%,  $P < 0.01$ ) and NUD (80 vs 55.2%,  $P < 0.01$ ) (Table II).

PCR-SSCP analysis detected alterations in the *p53* gene in 16 (4.6%) of 348 patients. There were 1, 1, 12 and 2 alterations in exons 5, 6, 7 and 8, of *p53* gene, respectively. *p53* gene mutation in patients with GC, PUD and NUD was 21 per cent (13/62), 4.4 per cent (2/45) and 0.4 per cent (1/241), respectively. *p53* gene mutation was significantly higher in patients with GC than PUD ( $P < 0.05$ ) and NUD ( $P < 0.001$ ). No difference in *p53* gene mutation was observed between *H. pylori* infected and non-infected individuals (Table II). *p53* gene mutation was significantly higher in males



compared to females [87.5% (14/16) vs 12.5% (2/16),  $P < 0.05$ ]. All the mutations obtained by PCR-SSCP were confirmed by sequence analysis (Table III). Amplified products from three tumour samples that yielded normal mobility patterns and from three corresponding adjacent normal tissue samples were sequenced, and no mutations were found in the sequence of the exon 5-8 regions. *K-ras* gene mutation was absent in all the patients with GC, PUD and NUD in our study.

### Discussion

Tumourigenesis occurs when a cell loses its regulated growth cycle and clonally expands beyond control. This requires a series of critical molecular events that cause the cell to divide and escape from normal proliferative control<sup>18</sup>. GC may follow this model. The product of the *p53* gene plays an important role in the negative regulation of cell growth. The wild-type *p53* protein binds to specific DNA sequences as a transcriptional factor that regulates the expression of particular genes in the cell. Consequently, it blocks cell progression through the late G1 phase of the cell cycle<sup>19</sup>. Some mutant proteins fail to block this progression, whereas others can gain a novel function and actually promote cellular proliferation<sup>20</sup>. There are significant geographic differences in the *p53* gene mutation rates in GC. Several investigators have reported *p53* gene mutations in GC around the world. In our study, 21 per cent patients with gastric carcinoma showed *p53* mutations. This falls within a wide range of *p53* mutation frequency of 0 to 82 per cent<sup>21,22</sup> in GC from previous studies that used similar methods for mutation detection.

There is incessant debate on the association of *p53* gene mutation and *H. pylori* infection with GC. Some investigators have reported that *H. pylori* infection induces *p53* gene mutation in gastric carcinogenesis<sup>23,24</sup>. However, others<sup>25,26</sup> and the results of the present study showed that *p53* gene mutations occurred independent to *H. pylori* infection in GC. Hongyo *et al*<sup>26</sup> reported that *H. pylori* infection was present in 55.8 per cent gastric cancer cases and was somewhat more common in cases lacking *p53* gene mutation. In our study *H. pylori* infection was found in 56.5 per cent patients with gastric cancer and *p53* gene mutation was higher in *H. pylori* infected individuals when compared with *H. pylori* uninfected individuals, however, the difference was not statistically significant (69.2%, 9/13 vs 30.8%, 4/13). The role of *p53* gene mutation has not been

studied in patients with PUD and NUD. In the present study, 4.4 and 0.4 per cent *p53* gene mutation was seen in 4.4 per cent patients with peptic ulcer and in 0.4 per cent with non-ulcer dyspepsia. The occurrence of *p53* mutations was higher in *H. pylori* infected individuals than in *H. pylori* non-infected individuals but the difference was not significant [5.4% (11/204) vs 3.5% (5/144)]. Thus, our study provides no evidence that *H. pylori* infection directly induces *p53* gene alterations.

The *K-ras* encodes a membrane-associated protein, p21<sup>RAS</sup>, with intrinsic GTPase activity involved in cellular signal transduction<sup>9</sup>. Point mutations of *K-ras* at specific codons lead to activated oncoprotein, GTP-RAS, with reduced GTPase activity. A mutation at codon 12 alone is sufficient for oncogenic activation<sup>9</sup>. These findings suggest that mutations at codon 12 of *K-ras* may play an important role in gastric carcinogenesis. Of the 348 adult patients enrolled in the study, none had *K-ras* gene mutation.

In literature, variable incidence of *K-ras* gene mutation has been reported in gastric cancer<sup>27,28</sup>. However, some investigators found no evidence for mutation in codon 12 of *K-ras* gene in GC<sup>29,30</sup>, which are concordant to the current finding. Mutations in *K-ras* oncogene do not appear to have any role in gastric carcinogenesis, at least in our population.

In conclusion, we observed *p53* gene mutation in 4.6 per cent of our study population. This mutation was significantly higher in GC when compared with PUD and NUD and it was independent to *H. pylori* infection indicating a role of *p53* gene mutation in gastric carcinogenesis, independent to *H. pylori* infection. Absence of *K-ras* gene mutation in our population questions its role in the pathogenesis of GC and PUD in Indian patients.

We analyzed *p53*, *K-ras* gene mutation and *H. pylori* infection in patients with gastric cancer and peptic ulcer disease and compared it with non-ulcer dyspepsia which is considered as disease control group. Ideally we should have taken asymptomatic healthy controls instead of disease controls but practically that was not possible on ethical ground. Healthy individuals cannot be subjected to endoscopy.

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