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

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

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

\textbf{Results}: Overall \textit{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. \textit{p53} gene mutation was significantly higher in patients with GC than PUD (\textit{P}<0.05) and NUD (\textit{P}<0.001). No difference in \textit{p53} gene mutation was observed between \textit{H. pylori} infected and non-infected individuals. \textit{K-ras} gene mutation was absent in all the patients.

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

\textbf{Key words} Gastric cancer - gene mutations - \textit{Helicobacter pylori} - peptic ulcer disease

\textit{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\textsuperscript{1,2}. 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

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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\(^2\). While the majority of the infected individuals develop no significant clinical disease, others develop two kinds of divergent clinical outcomes – PUD and gastric cancer\(^4\). The reasons for developing these two extreme phenotypes remain poorly understood and are not explained by bacterial virulence factors alone\(^4,5\). 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\(^6\). 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\(^7,8\). In October 2006, the p53 database of IARC listed 31.2 per cent gastric cancers with point mutation in the *p53* gene\(^9\). *K-ras* oncogene encodes a membrane-associated protein, p21\(^{RAS}\), with intrinsic GTPase activity involved in cellular signal transduction\(^10\). It is well known that K-ras plays an important role in the pathogenesis of various types of human cancer\(^11\). Point mutations at codons 12, 13 and 61 of K-ras protein toward the activated state, which constitutively activates the mitogenic signal transduction pathway\(^12\). Frequency of mutated K-ras varies among the different tumour types\(^13\). 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\(^10,11\). 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 tumourigenesis. 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, endoscopical 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\(_2\) 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\(^12-15\). *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\(^16\). PCR was performed in a 50 \(\mu\)l reaction volume containing 100 ng of genomic DNA, 1x PCR buffer, 1.5 mM MgCl\(_2\), 0.2 mM each deoxynucleotide, 0.5 \(\mu\)M 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\(^16\). 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 \(\mu\)l 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 |
|-------|--------|---------------------------------------------------------|-------|------------|
| p53   | E5A    | 5'-TTCTCTTCTTCGAGTCAGA-3'                                 | 152   | 15         |
|       |        | 5'-TCTGGATGCTTGCTGACTG-3'                                 |       |            |
| p53   | E5B6A  | 5'-GGCTCTCAAGCAGTGACCA-3'                                 | 167   | 15         |
|       |        | 5'-GCCAGACCTAAGGCAAAC-3'                                  |       |            |
| p53   | E6B    | 5'-TTGTTGCTCTGCCCTGCCA-3'                                 | 132   | 15         |
|       |        | 5'-AGTTGCTAAACCTGACCTCA-3'                                |       |            |
| p53   | E7     | 5'-TTGGTCTCCTCATTGTTGCTC-3'                               | 136   | 15         |
|       |        | 5'-CAAGTGGCTCCCTGACCTGAG-3'                               |       |            |
| p53   | E8     | 5'-TTGTAATCTACTGGGACGGA-3'                                | 149   | 15         |
|       |        | 5'-CTGGCTTCTCCTGACCTGTA-3'                                |       |            |
| K-ras | codon 12 | 5'-ACTGAAATATAACCTTTGTGATCGGGACCT-3'             | 106   | 16         |
|       |        | 5'-CTATGGTTGGACATATTTG-3'                                 |       |            |

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         |
| H. pylori infection (%)          | 35 (56.5)                          | 36 (80)*                          | 133 (55.2)                        | 204 (58.6)      |
| p53 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
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 (Mbl, 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.

### 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      | -  | 8    | 272   | Point    | GTG - ATG | Val - Met |
| GC      | -  | 7    | 241   | Point    | TCC - TGC | Ser - Cys |
| GC      | -  | 7    | 245   | Point    | TCC - TGC | Ser - Cys |
| GC      | -  | 7    | 241   | Point    | TCC - TGC | Ser - Cys |
| GC      | -  | 7    | 245   | Point    | TCC - TGC | Ser - Cys |
| GC      | -  | 7    | 245   | Point    | TCC - TGC | Ser - Cys |
| GC      | -  | 7    | 249   | Point    | TCC - TGC | Ser - Cys |
| PUD     | +  | 6    | 212   | Frame shift | 1bp deletion (TTT-TT-) | Frame shift |
| PUD     | -  | 7    | 241   | Point    | TCC - TGC | Ser - Cys |
| NUD     | +  | 7    | 245   | Point    | TCC - TGC | Ser - Cys |

Hp, *H. pylori*; Nt change, nucleotide change; Aa change, amino acid change
H. pylori infection was present in 55.8 percent. The role of was not statistically significant (69.2%, 9/13 vs 30.8%, 3.5%). 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 gene plays an important role in the pathogenesis of GC. Some investigators have reported that K-ras gene mutation in gastric carcinogenesis, 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.

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. Healthy individuals cannot be subjected to endoscopy.

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