RESEARCH ARTICLE

Helicobacter pylori Infection and a P53 Codon 72 Single Nucleotide Polymorphism: a Reason for an Unexplained Asian Enigma

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Abstract

Aim: P53, the most commonly mutated tumor suppressor gene in all types of human cancer, is involved in cell cycle arrest and control of apoptosis. Although p53 contains several polymorphic sites, the codon 72 polymorphism is by far more common. There are divergent reports but many studies suggest p53 pro/pro SNP may be associated with susceptibility to developing various cancers in different regions of the world. The present study aimed to find any correlation between *H. pylori* infection and progression of carcinogenesis, by studying apoptosis and the p53 gene in gastric biopsies from north Indian population. Materials and Methods: A total of 921 biopsies were collected and tested for prevalence of *H. pylori* by rapid urease test (RUT), imprint cytology and histology. Apoptosis was studied by the TUNEL method. Analysis of p53 gene polymorphism at codon 72 was accomplished by PCR using restriction enzyme BstU1. Observation: Out of 921 samples tested 56.7% (543) were *H. pylori* positive by the three techniques. The mean apoptotic index (AI) in the normal group was 2.12, while gastritis had the maximum 4.24 followed by gastric ulcer 2.28, gastropathy 2.22 and duodenal ulcer 2.08. Mean AI in cases with gastric cancer (1.72) was less than the normal group. The analysis of p53 72 SNP revealed that p53 (Arg/Arg), (Pro /Arg) variant are higher (40.59% & 33.66%) as compared to p53 pro/pro variant (25.74%) inthe healthy population. Conclusions: The North Indian population harbors Arg or Pro/Arg SNP that is capable of withstanding stress conditions; this may be the reason of low incidence of gastric disease in spite of high infection with *H. pylori*. There was no significant association with *H. pylori* infection and AI. However, there is increased apoptosis in gastritis which may occur independent of *H. pylori* or p53 polymorphism.

Keywords: p53 polymorphism - *Helicobacter pylori* - gastric cancer - apoptotic index - North India

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Introduction

Helicobacter pylori (*H. pylori*), is a Gram-negative, microaerophilic bacterium found in the stomach. It discovered in 1982 by Barry Marshall and Robin Warren, in patients with chronic gastritis and gastric ulcers. Since its discovery, there have been many studies suggesting that *H. pylori* increase the risk of gastric cancer (Forman D 1991; Talley et al., 1991; Parsonnet et al., 1991). In 1994, the International Agency for Research on Cancer (IARC) identified *H. pylori* as a class I carcinogen (lARC 1994).

The risk of developing cancer is related to the physiologic and histologic changes induced by a *H. pylori* infection in the stomach (Ferreira et al., 2008). Despite a general decline in the incidence of gastric cancer, it remains the fourth most common cancer and second leading cause of cancer-related deaths worldwide (Yamaoka, 2012).

The integrity of the gastric mucosa is maintained due to a fine balance between cell proliferation and cell death or apoptosis (Kaeffer, 2011). However; this balance can be affected by *H. pylori* infection. *H. pylori* infection has been reported to be associated with increased (Mannick et al., 1996; Moss et al., 1996; Moss, 1998; Peek et al., 1999), unaltered (Peek et al., 1997) and decreased (Zhong et al., 2001) levels of apoptosis in gastric mucosa.

P53 is a tumor suppressor gene, located on chromosome 17p13. It is also known as 'Gatekeeper gene' and has been found to be one of the most commonly mutated genes in all types of human cancer (Zhong et al., 2001). It contains 11 exons, and encodes a 53 kDa phosphoprotein that is a transcription factor for genes that induce cell cycle arrest or apoptosis (Levine, 2012). Although p53 contains several polymorphic sites, only those in exon 4 have been examined in gastric cancer. Exon 4 contains 2 polymorphic sites, 1 at codon 36 and another at codon 72. Of these, the codon 72 polymorphism is by far more common. The polymorphism consists of a single base

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pair change of either arginine or proline which creates 3 distinct genotypes: homozygous for arginine (Arg/Arg), homozygous for proline (Pro/Pro) and a heterozygote (Pro/Arg) (Shepherd et al., 2000). P53 codon 72 polymorphisms have been reported to be associated with cancers of the bladder (Soulitzis et al., 2002), lung (Matakidou et al., 2003), cervix (Sousa H et al., 2011), breast (Tommiska et al., 2005), esophagus (Lee et al., 2000) and colorectum (Koushik et al., 2006). There are divergent reports but many studies suggest p53 pro/pro SNP to be more susceptible for developing various cancers in different regions of the world (Liu et al., 2011; Jing et al., 2012; Liu et al., 2012; Xu et al., 2012).

Studies from different regions of India report that the prevalence of *H. pylori* infection varies from 56 to 89% among gastric cancer cases. A study from North India reported the prevalence of *H. pylori* infection to be 56.5% in gastric cancer patients (Ghoshal et al., 2008). A study from Mizoram reported higher rate of infection by *H. pylori* in stomach cancer patients (Phukan RK 2006 et al). Same study from Northern India reported the prevalence of *H. pylori* as high as 74% in controls as compared to 68% in gastric cancer cases. A study by Misra et al. (2007) showed slightly higher prevalence of *H. pylori* (80%). They also reported that *H. pylori* was more common in diffuse type of cancer than intestinal type (86% *vs* 68%) in contrast to the reports from western countries (Misra et al., 2007). Just like African Enigma there is Asian Enigma showing an increased prevalence of *H. pylori* in normal controls but decreased association with gastric carcinogenesis as compared to west (Misra et al., 2007).

The changes caused by this organism at genetic level are not clear and therefore the present study was aimed to provide experimental evidence of relationship between *H. pylori* infection and gastric carcinogenesis by elucidating its relationship with p53 polymorphism at codon 72 and change in the rate of Apoptosis to elucidate the reasons for decreased prevalence of gastric cancer with *H. pylori* despite high rate of infection

Ther aim of this study was to find the possible explanation of Indian enigma of gastric cancer (Singh and Ghosha, 2006; Pandey et al., 2010; Misra et al., 2014).

Materials and Methods

Nine hundred and twenty one randomly selected subjects coming for endoscopy in Department of Gastroenterology and Hepatology with upper gastrointestinal symptoms were included in the study. 3 biopsies from each subject was collected one was used for RUT and Imprint cytology, other was preserved in PBS for molecular analysis and the remaining was kept in 10% formalin for histopathological examination and study of Apoptosis by TUNEL Method. Patients taking NSAIDs, proton pump inhibitors and antibiotics were excluded.

Rapid urease test (RUT)

An antral mucosal biopsy specimen was placed immediately into a capped Eppendorf tube containing 0.5 ml freshly prepared 10% urea (w/v) in deionised water at a pH of around 6.8 to which had been added two drops of 1% phenol red (freeacid) as a pH indicator. A positive result was recorded if there was a color change from yellow to pink within the first minute (Figure 1) (Thillainayagam et al., 1991).

Imprint cytology

The biopsy smear is prepared on slide by rolling the biopsy with the help of hypodermal needle. Slide was air dried and stained with Loffler's Methylene blue. Bacteria was visualized as blue curves or rods under the microscope (Figure 2) (Misra et al., 1993).

Histology

3-5 um thick sections from paraffin blocks were stained with hematoxylin and eosin and Loefflers methylene

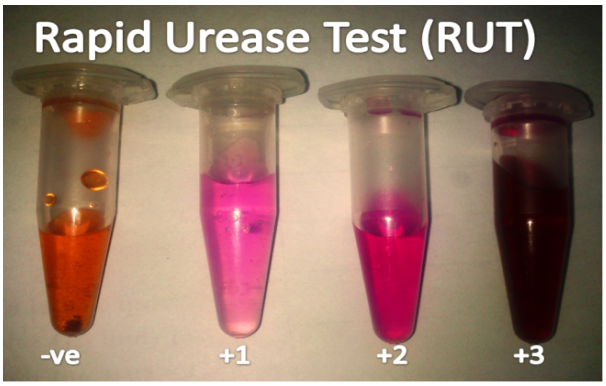

Figure 1. Rapid Urease Test Change in Color Shows Intensity of Infection

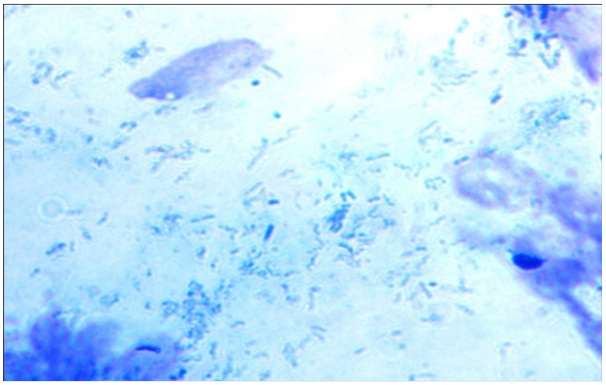

Figure 2. Imprint Cytology Showing Curved Bacteria

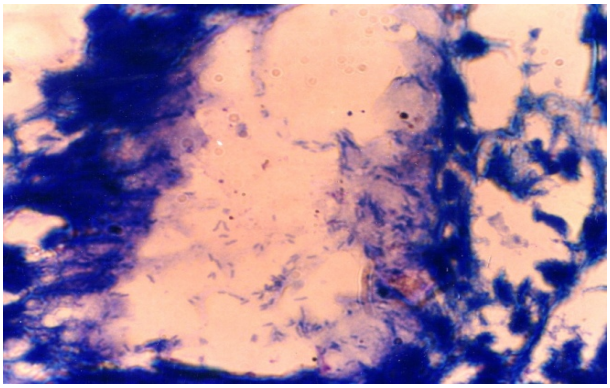

Figure 3. Histology Showing Comma Shaped or Curved *H. pylori*

blue stain and studied for histopathological changes and presence of *H. pylori*. Presence of *H. pylori* was graded as +1, +2, +3 (Figure 3) (Misra et al., 1993; 2000).

P53 polymorphism

It was also studied in 372 biopsies DNA isolation was done by standard Chloroform phenol method followed by quantification and dilution in TE buffer. Study of P53 polymorphism for codon 72 was studied by using PCRwith restirction enzyme BstUI. The primer sequences are according to Chua et al. (2010). Forward:5'GAAGACCCAGGTCCAGATGA-3'and Reverse:5'ACTGACCGTGCAAGTCACAG- 3'

The p53 Pro allele has a unique BstUI site that is absent in the Arg allele, resulting in bands of different sizes as follow:Arg/Arg wild (160 and 119 bp); the Pro/Pro homozygous variant (279 bp) and the heterozygous Arg/Pro variant (279, 160, and119 bp). p53 polymorphism was confirmed by running PCR product on Agarose gel (Pandith AA et. al. 2010) The gel was studied under the Bio-Rad Gel doc,ChemiDoc XRS Figure 4).

Apoptotic index

The Study of Apoptosis was done on 372 samples by TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay using Apoptosis study Kit (Lackzene biosciences), according to the manufacturer's instructions. 5μm thick sections weredeparaffinised in xylene, and rehydrated with a graded alcohol series. After being washed in tris buffer saline (TBS, pH 7.4), the sections were placed in H_2O_2 for 10 min, and the tissues were then digested with proteinase K (20μg/ml in TBS) at 37oC for 10min to enhance nuclear staining of apoptotic cells, Digestion was stopped by washing the sections in TBS. The sections were then treated with terminal transferase enzyme and degoxigenin labelled nucleotides and after words anti-digoxigenin peroxidase solution was applied. The color was developed with DAB, after which the sections were lightly counterstained with Hematoxylin. To confirm staining specificity of the TUNEL method positive control section was prepared. The substitution of equilibrium buffer for TdT was used as negative control.

The Apoptotic Index was calculated as Percentage of TUNEL positive cells in about 500 epithelial cells examined for each sample, under a light microscope (400X magnification). All slides were coded and scored by one observer. Areas that were poorly preserved crushed, folded or retracted were specifically avoided (Figure 5).

Results

The biopsies collected were grouped as Normal(N)-670 (53.34%), Gastric Cancer(GC)- 16(1.28%), Gastric Ulcer (GU)-41(3.26%), Duodenal Ulcer(DU)-26(2.07%), Gastritis (GT)-37(2.94%) and Portal Hypertensive Gastropathy (GP)-131(10.42) on the basis of endoscopic and histological appearances. Majority of the subjects were in the age group of 41 to 50 yrs followed by 21-30 years. Among 921 cases 616 (66.87%) were males and 305 (33.12%) were females. Of the 921 samples observed for *H. pylori* positivity, 56.73% (543) were *H. pylori* Positive and 42.27% (378) were found to be *H. pylori* negative by three techniques RUT, Imprint cytology and Histology. A good correlation was found among the three techniques for identification of *H. pylori*. Positivity of *H. pylori* in different groups was as shown in table. It was maximum in DU followed by GU, gastritis and GC. In gastropathy group the positivity was less than normal controls (Figure 6).

The mean AI in normal group was 2.12. Gastritis had the maximum AI (4.24) followed by GU (2.28), GP (2.22) and DU (2.08). Mean AI in cases with gastric cancer was less than the normal group (1.72).

Percentage of cases showing P53 Pro/Pro, Arg/Arg, and Arg/pro Polymorphism in N, GC, DU, GU, GT and GP cases were as shown in Table 1. The analysis of p53 72 SNP revealed that in North Indian normal population p53 Arg/Arg (40.59) and Pro /Arg variant (33.66) were

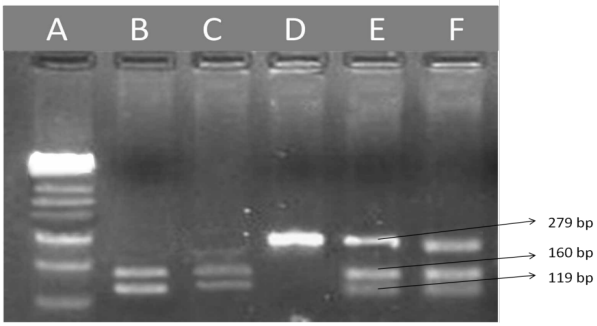

Figure 4. Bands Showing P53 Polymorphism. Arg/Arg wild (160 and 119 bp) shown in lanes B and C; the Pro/Pro homozygous variant (279 bp) in lanes C; and the heterozygous Arg/Pro variant (279, 160, and119 bp) in lane E and F

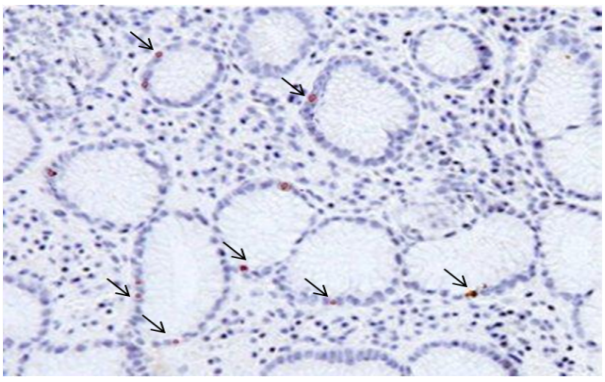

Figure 5. TUNEL Showing Apoptotic Cells

Table 1. Comparison of the *H. pylori* positivity Apoptotic Index (AI) and p53 72 SNP Polymorphism

Variables		N (%)	GT (%)	GP (%)	GU (%)	DU (%)	GC (%)
H. pylori		405/670 (60.44)	17/37(45.94)	53/131 (40.45)	35/41 (85.36)	23/26 (88.46)	10/16 (62.5)
Apoptotic Index		2.12	4.24	2.22	2.28	2.08	1.72
p53 polymorphism	Pro/Pro	26/101 (25.74)	3/14 (21.42)	3/12 (25)	15/41 (36.58)	6/18 (33.33)	5/16 (31.25)
	Arg/Arg	41/101 (40.59)	6/14 (42.85)	5/12 (42.85)	12/41 (29.26)	4/18 (22.22)	4/16 (25)
	Arg/pro	34/101 (33.66)	5/14 (35.72)	12Apr (33.33)	14/41 (34.14)	8/18 (44.44)	7/16 (43.75)

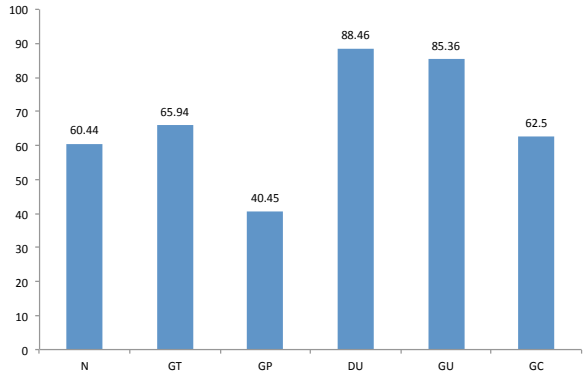

Figure 6. Prevalence of *H. pylori* in Various Gastric Lesions

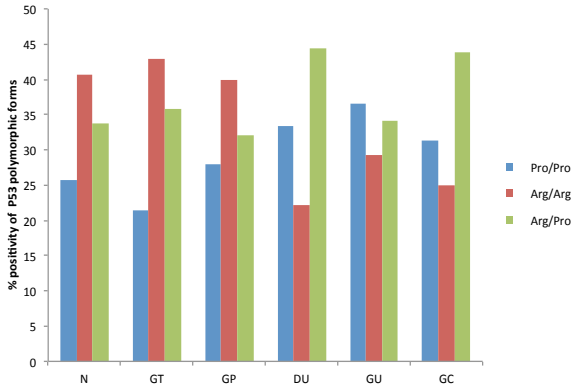

Figure 7. Distribution of Different Polymorphic forms of P53 in Various Lesions

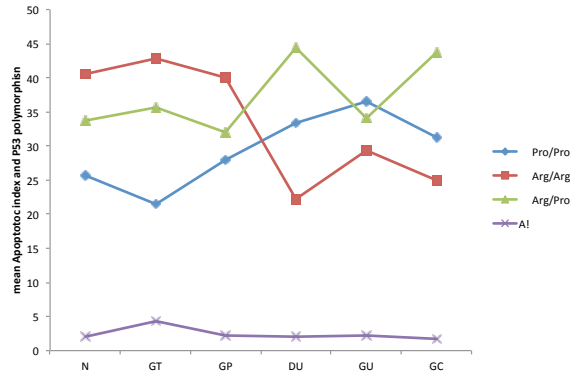

Figure 8. Correlation of AI with Polymorphic forms of P53 in Various Lesions

higher as compared to p53 pro/pro variant (25.75%). Gastritis (GT) and gastropathy (GP) had the similar distribution whereas Gastric cancer, GU and DU cases showed a decrease in Arg/Arg variant and an increase in Arg/Pro and Pro/Pro variant (Figure 7). Variation in P53 polymorphism correlated with changes in AI in various lesions (Figure 8).

Discussion

In the current study *H. pylori* prevalence was determined in different groups of benign gastric lesions and GC using RUT, Imprint cytology and Histology. The study showed a higher *H. pylori* incidences in Normal subjects (60.44%) which was less than that reported earlier from our center (Misra et al., 2007).

Higher incidences of *H. pylori* infections are reported from various other regions of India. The prevalence varies from 56 to 89% among gastric cancer cases. A study from North India reported the prevalence of *H. pylori* infection to be 56.5% in gastric cancer patients (Nath G et. al. 2000). The frequency of Cag A IgG was found to be more common in the healthy controls (89%) compared to gastric neoplasm patients (76%) (Ghoshal et al., 2008). A study by Misra et al. (2007) showed slightly higher prevalence of *H. pylori* (80%) in the control group as compared to the cases (78%). It was also reported that *H. pylori* was more common in diffuse type of cancer than intestinal type (86% *vs* 68%). Another study from Northern India reported the prevalence of *H. pylori* as high as 74% in controls as compared to 68% in gastric cancer cases (Phukan et al., 2006). A study from Mizoram reported higher rate of infection by *H. pylori* in stomach cancer patients (Parkin, 2006). The findings in the study shows a decline of about 20% in the prevalence of *H. pylori* over last 10years (Graham et al., 1991; Gill et al., 1992; 1993; Katelaris et al., 1992; Jain et al., 1999; Misra et al., 2007).

To find the relationship of *H. pylori* with progression of gastric carcinogenesis apoptosis was studied in different gastric lesions and Apoptotic Index (AI) was calculated by TUNEL method. The TUNEL showed no statistically significant difference in AI of different groups ranging between 1.72-2.28 in GC, GU, DU, GP and N. Terga et al. (2007) evaluated the AI and found higher incidences in GT (3.93%) (Targa et al., 2007). Another study by Leite et. al. (2005) showed higher AI in GT (5.2%) compared to N (1.4%). In a Previous study Yoshimura et al. (2000) observed a direct correlation between AI and glandular atrophy. Thus increase in Apoptosis that is not balanced with cell proliferation may increase the risk of GC. We also found lowering of AI (1.72%) in GC as compared to normal group (2.12%), suggesting the progression of disease may be due to decrease the AI of gastric epithelium. Subsequent lowering in AI in gastric cancer is reported (Nakamura et al., 2012). Zhanq et al. (2001) concluded that in the course of the formation of gastric carcinoma, proliferation of gastric mucosa can be greatly increased by *H. pylori*, and *H. pylori* can induce apoptosis in the phase of metaplasia, but in the phase of dysplasia *H. pylori* can inhibit cellular apoptosis.

Apoptosis is regulated by a variety of genes, including p53, which may play an important role to maintain the homeostasis of the gastric tissue (Etienne et al., 2002). p53 gene is key player in the stress responses that preserve genomic stability, responding to a variety of insults, including DNA damage, hypoxia, metabolic stress and oncogene activation (Vogelstein et al., 2002; Vousden and Lane, 2007). Up to 50% of the patients with GC were reported to have p53 alterations (Vousden and Lane, 1997).

Although p53 contains several polymorphic sites, only those in exon 4 have been examined in GC. Of these, the codon 72 polymorphism (rs1042522) is by far the more common, which results in the substitution of arginine (Arg) by proline (Pro) in the transactivating domain.54Changes in its amino acid sequence can alter the ability of p53 to bind to receptors in target genes, alter

recognition motifs for post-translational modifications or alter p53 stability and interactions with other proteins (Walker and Levine, 1996; Thomas, 1999; Shepherd et al., 2002; Bergamaschi et al., 2003; Li and Prives, 2007). Such changes may contribute to tumor progression and a poor prognosis (Katkoori et al., 2009).

Analysis of the p53 codon 72 SNP revealed that the presence of the p53 Pro allele along with decrease in Arg/Arg allele is associated with a small but non-significant increase in risk of gastric lesions, suggesting that p53 (Arg), is more effective in protecting stressed cells from neoplastic development than p 53 (Pro). This finding is in accordance with Mantovani et al. (2007) and Bergamaschi et al. (2006) We also found the p53 (Arg) (Pro /Arg) variant are higher in the normal subjects. Variation in apoptotic index also correlated with the change in the pattern of Arg/Arg allele in various diseases showing that this allele may prevent the carcinogenic changes by stimulating the Apoptosis in the damaged epithelial cells. This may be the reason of low incidences of Gastric cancer in North India in spite of a relatively higher *H. pylori* infection thus possibly providing some explaination to Indian Enigma of Gastric Cancer (Pandey, 2010; Misra et al., 2014).

In conclusion, there was no significant association with *H. pylori* infection and AI. However there is increased apoptosis in gastritis (GT) which may occur independent of *H. pylori* infection or p53 polymorphism. The North Indian population harbors Arg or Pro/Arg SNP that is capable to withstand stress conditions, this may be the reason of low incidences of gastric diseases in spite of high infection of *H. pylori*, the phenomenon called Asian Enigma.

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