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Association of Myeloperoxidase -463G/A Polymorphism with Duodenal Ulcer disease in the South Indian Context

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ABSTRACT*: Myeloperoxidase (MPO) polymorphism has been linked to gastric cancer, sarcoidosis, Alzheimer's disease and coronary artery disease. MPO plays a key role in bacterial killing and metabolic activation of pro-carcinogens and hence, it is reasonable to propose that MPO polymorphism may harbor a marked impact on gastric cancer risk in Helicobacter pylori (H. pylori) -infected individuals. MPO polymorphism has never been associated with H. pylori -associated gastrointestinal disease heretofore in the Indian context. In a hospital-based case–control study, we investigated if MPO polymorphism had any association with gastroduodenal disease in cases who were H.pylori positive and negative admitted to a tertiary care centre. We enrolled 200 patients admitted with duodenal ulcer (n=100) and gastric cancer (n=100), and 160 healthy control samples. Biopsies obtained during gastroduodenal endoscopies were evaluated for the presence of H.pylori infection. Restriction fragment length polymorphism (RFLP) fingerprinting was employed to characterize the MPO genotypes. We found that the groups markedly differed in regards to distribution of the MPO genotypes investigated. Four individuals carrying MPO A/A genotype were in the duodenal ulcer group. The carriage of MPO allele A is found to be associated with an increased risk for development of duodenal ulcer. The carriage of MPO allele A and H.pylori infection were associated with an increased risk for development of duodenal ulcer. Nonetheless, the GG genotype and H.pylori positivity failed to potentiate each other for development of atrophy.*

KEYWORDS: *Duodenal ulcer; gastric cancer; MPO; polymorphism*

I. INTRODUCTION

Helicobacter pylori (*H. pylori*) is a well-known gram negative bacterial pathogen that chronically infects ~50% of the world's population. Infection often progresses to chronic gastritis, and a subset of individuals develop duodenal (DU), and gastric ulcers (GU), gastric carcinoma (GC) and mucosa-associated lymphoid tissue lymphoma (MAL-Toma) (1-5). The course of infection is affected by bacterial virulence factors, as well as a plethora of host factors viz., genetic predisposition, age, and certain environmental factors (e.g. life style) involving the host.

According to the International Agency for Research on Cancer (IARC)'s report in 2005, gastric cancer is the fourth common cancer and is the second most fatal malignant condition worldwide (6). Several published findings have identified that gastric cancer is associated with gene polymorphisms involved in inflammatory responses, DNA repair, alterations in metabolic enzyme levels and oxidative damage (7, 8). Oxidative stress has long been confirmed to play a key role in determining the host's susceptibility to various diseases and has also been linked to development of cataract and coronary artery disease (9). The polymorphisms in oxidative stress-related enzyme genes have been associated with gastric cancer. MPO, an oxidative stress-related enzyme catalyzes a reaction that produces hypochlorous acid (HOCl), which may cause host DNA damage and could lead to mutation of oncogenes and tumor suppressor genes (10,11). Previously, G–463A polymorphism has been identified in the promoter region of the MPO gene among leukemia patients (12). When compared to G *allele*, *A allele* has been studied to significantly decrease the transcriptional activity owing to disruption of an SP1-binding site in an Alu hormone responsive element (13). Several epidemiological studies have examined the role of MPO G–463A polymorphism in certain cancers involving the lung, breast, esophagus, bladder, liver, larynx and pharynx.(25,26,27,28,29) Notably, a protective effect of the MPO *A allele* has been reported in certain cancers. Recent lines of evidence demonstrated that the MPO genotype was critical in determining the pathogenesis of *H. pylori*–induced atrophic gastritis, and are therefore accepted as a precursor of gastric cancer (14). MPO polymorphism has never been associated to *H. pylori*-associated gastric disease in the south Indian context to the best of our knowledge and therefore, we examined the association between the risk of gastric cancer and MPO G–463A polymorphism in a hospital-based case–control study.

II. MATERIALS AND METHODS

Subjects

Three hundred and sixty individuals (200 cases and 160 controls) admitted to the in-patient departments of the Gandhi Hospital, Hyderabad, AP, India were recruited in the study. The demographic characteristics of the duodenal ulcer, gastric cancer cases and healthy controls considered for the study have been summarized in table 1. The diagnoses of duodenal ulcers and gastric cancer were confirmed by endoscopy and histopathology of biopsy tissue, respectively. Control subjects had no current or previous diagnosis of cancer and genetic disease. Control patients were frequency-matched for sex and 5-year age groups. All subjects were interviewed to obtain information on residence (urban or rural), body weight, smoking status and medical treatment. Smoking was defined as ≥10 cigarettes per day. Approximately, 5 ml venous blood sample was collected from each subject. Leukocyte pellet obtained from each blood sample was stored at −80°C until further analysis.

The subjects were serotyped as *H.pylori* positive and negative based on the Rapid Urease Test. (22). The study was approved by the ethics committee of Osmania University.

MPO genotyping

Genomic DNA was isolated from frozen leukocyte pellet using standard salting out method and the *MPO* G–463 A polymorphism was detected by RFLP fingerprinting method as described elsewhere (15). A 350 bp DNA fragment containing the polymorphic site was amplified by PCR using the forward primer 5′-CGG TAT AGG CAC ACA ATG GTG AG-3′ and the reverse primer 5′-GCA ATG GTT CAA GCG ATT CTT C-3′ (Bioserve Biotechnologies (India) Pvt. Ltd). The PCR reaction was performed in a total volume of 20µl containing 2µl 10× PCR buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.375µM each primer, 200ng of genomic DNA and 1U of *Taq* DNA polymerase (Bioserve Biotechnologies (India) Pvt. Ltd). The PCR conditions were 94°C for 5min, followed by 35 cycles of 60s at 94°C, 60s at 56°C and 60s at 72°C, with a final elongation at 72°C for 10min (Eppendorf Mastercycler). An aliquot of 10µl of the PCR product was digested with 5U of A*ci* (New England BioLabs) in 2µl of 10× NEB buffer 3 (100mM NaCl, 50mM Tris–HCl, 10mM $MgCl_2$ and 1mM dithiothreitol) and 7.5µl dH_2O at 37°C overnight. The DNA fragments were separated on a 2% agarose gel containing 0.5µg/ml ethidium bromide. The G homozygous yields three bands at 169, 120 and 61bp, and the A homozygous produces two bands at 289 and 61bp, where the heterozygote (GA) has four bands at 289, 169, 120 and 61bp. Approximately 10–15% of the samples were randomly selected for repeated assays, and the results were 100% concordant.

Statistical analysis

Statistical evaluations were performed using the SPSS/Windows computer software package (Chicago, IL). Two sample t-tests were used to compare the mean values of variables considered continuous in the DU, GC patients and HCs. The $\chi 2$ test with or without Yate's correction for continuity and Fisher's exact test when appropriate were applied to analyze the categorized variables. Differences were considered to be significant at $p \leq 0.05$. A multivariate analysis with logistic regression was carried out to assess the odds ratios (ORs) of the risk factors of DU and GC.

III. RESULTS

Our study has established that males are prone to duodenal ulcers (p=0.0005) and gastric cancer (p=<0.0001) than female subjects. No significant relationship was seen between the nature of diet and development of duodenal ulcers and gastric cancer. Smoking males were found to be at a higher risk to develop gastric cancer, although no such association was evident in duodenal ulcer. In this context, we also established that the MPO-463G/ A polymorphism was significantly associated with development of duodenal ulcer. Genotypes and allele frequencies of MPO in the study groups are presented in table 2 and the G/G, G/A and A/A genotypes were 72.5%, 27.5% and 0%, respectively in healthy controls and 44%, 52% and 4%, respectively in duodenal ulcer cases. There was no significant relationship between development of duodenal ulcers/gastric cancer and consanguinity* (consanguineous marriages). Notably, we also observed that *H.pylori* infection predisposed to development of duodenal ulcer significantly whereas the onset of gastric cancer was not associated significantly with the infection, though the risk of developing cancer was higher than that of ulcer (table 3). Presence of MPO A alleles were also observed to predispose to duodenal ulcer development based on the frequency but no significance was observed probably due to the small sample size. The two study groups differed in MPO genotype distributions. Interestingly, four individuals carrying MPO A/A genotype were in the duodenal ulcer group, and none of the healthy controls (n=160) had this special genotype. In the present study, we examined MPO genotypes in 100 gastric cancer patients, and interestingly none had the A/A genotypes. When the correlates of MPO polymorphism, smoking and *H.pylori* infection in the development of duodenal

ulcer and gastric cancer among male cases were analyzed (table 4), there were no significant correlation observed.

IV. DISCUSSION

MPO is an important enzyme required to trigger oxidative burst in neutrophils for bacterial killing and the neutrophils are professional phagocytes that manufacture O_2 by the one-electron reduction of oxygen at the expense of NADPH (9,10). Most of the O_2 intermediates formed react with themselves to form H_2O_2. From these agents, a large number of highly reactive microbicidal oxidants are released, including HOCl, ·OH, peroxy nitrite and many others (16). MPO readily oxidizes chloride ions to the strong non-radical oxidant HOCl, which have several cytotoxic effects on bacteria (17). Recent lines of evidence suggest that MPO activity in neutrophils is genetically determined (13). A G-to-A substitution polymorphism in the *MPO* promoter region decrease gene transcription due to the disrupted SP1 binding site, i.e. fewer enzymes would be available to form HOCl (13,15). This could be owing to low MPO activity in *A allele* carriers, whose neutrophils have reduced ability to generate HOCl and other bactericidal reactive oxygen species (ROSs) (13). As observed in the present study, a study by Roe *et al*. (2002) also showed no MPO A/A genotype distributed in 127 Korean gastritis patients investigated. Taken together, these results suggest that the individuals carrying MPO A/A genotype are vulnerable to develop duodenal ulcer.

Recent studies suggest that bacterial load is closely related to the disease outcome. The greater the *H.pylori* bacterial load, worser was the clinical outcome of associated gastritis (18, 19). It has been shown that bacterial densities in duodenal ulcer were relatively higher than that in gastritis. Richter-Dahlfors *et al*. (1998) demonstrated that co-culture of antral epithelial cells with *H.pylori* increased basal gastrin secretion by epithelial cells (20). Furthermore, others revealed that high *H.pylori* density was an independent risk factor of duodenal ulcer (21), thereby proposing that *H.pylori*-infected individuals with higher bacterial loads may stimulate more antral gastrin release, which could lead to excessive acid secretion leading to duodenal ulcer. As described by others, *H.pylori* infection is the most crucial correlate of duodenal ulcer and MALToma pathogenesis.

The present study marks the role of *H.pylori* in causing duodenal ulcer as represented by the statistically significant association, although the risk was lower, probably due to low sample size. The presence of *H.pylori* in causing gastric cancer is also found to have no significant association but an increased risk.

Currently, the host factors affecting the growth of mucosa-associated lymphoid tissues and MALToma remain ambiguous. Whether the low-expression MPO genotype is related to the pathogenesis of MALToma needs to be investigated further. The major paradox in *H. pylori* research is the apparent association of the infection with divergent and mutually exclusive clinical outcomes. The infection increases the risk of duodenal ulcer, a condition characterized by antral-predominant gastritis and high acid secretion while also heightening the risk of gastric cancer, a condition characterized by corpus predominant gastritis and hypochlorhydria. Roe *et al*. (2002), Hsu *et al*. (2008), Jiang Z *et al*. (2012) revealed that MPO genotype is a critical determinant in the pathogenesis of atrophic gastritis subsequent to *H. pylori* infection. A strong positive correlation between the levels of gastric atrophy was found in wild MPO (G/G) genotype but not in low expression (G/A) genotype. This implies that wild MPO genotype is linked with gastric carcinogenesis. Interestingly, we observed that the carriage of MPO *allele A* is related to the development of duodenal ulcer. Aforementioned studies suggest that MPO genotype may be a critical turning factor for the outcomes of *H.pylori*-infected individuals. The carriage of MPO *allele A* and *H.pylori* infection were associated with an increased risk for development of duodenal ulcer. Nonetheless, the GG genotype and *H.pylori* positivity failed to potentiate each other for development of atrophy.

To our knowledge, this study is the first to verify the association of MPO-463G/ A polymorphism with duodenal ulcer disease in the south Indian context. More investigations may be required to clarify the relationship between low-expression MPO genotype, the ROS of neutrophils and fates of *H. pylori*-infected individuals.

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VI. DISCLOSURES

All authors have no conflict of interest.

LIST OF ABBREVIATIONS

AP	Andhra Pradesh
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DU	Duodenal ulcer
EDTA	Ethylene diamine tetra acetic acid
GC	Gastric carcinoma
GU	Gastric ulcer
H.pylori	*Helicobacter pylori*
HOCl	Hypochlorous acid
HRE	Alu hormone responsive element
IARC	International Agency for Research on Cancer
MAL-Toma	Mucosa-associated lymphoid tissue lymphoma
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
OR	Odd's Ratio
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species

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Table 1. Characteristics of duodenal ulcer, gastric cancer and healthy controls

Epidemiological factors		**Duodenal ulcer (n=100)**	**Gastric cancer (n=100)**	**Healthy control (n=160)**
Age (years)	10-20	4	0	8
	20-30	28	4	26
	30-40	34	16	40
	40-50	20	30	46
	50-60	14	50	40
Gender	Males	72 (p=0.0005***)	84(p<0.0001***)	80
	Females	28	16	80
Diet	Vegetarians	24	16	30
	Non-vegetarians	76	84	130
Smoking (Males only)	Smokers	56	72(p=0.0227*)	56
	Non-smokers	16	12	24
Alcohol (Males only)	Alcoholics	58	76	66
	Non-alcoholics	14	8	14
Tobacco chewing (Males only)	Chewers	0	28	48
	Non-chewers	72 (p<0.0001**)	56 (p=0.0009***)	32
Consanguinity	Consanguineous	4	12	26
	Non-consanguineous	96	88	134
H.pylori status	Positive	84	66	102
	Negative	16	34	58

Table 2. Genotypes and allele frequencies of MPO in the study groups

Genotypes	Duodenal ulcer (n=100) (%)	Gastric cancer (n=100) (%)	Healthy controls (n=160) (%)
GG	44 (44)	64 (64)	115 (71.9)
GA	52 (52)	36 (36)	45 (28.1)
AA	4 (4)	0	0

Table 3. MPO polymorphism and *H.pylori* infection in the development of duodenal ulcer and gastric cancer

MPO *allele A* carrier	***H.pylori* infection**	**Healthy controls (n=160, %)**	**Disease condition**	**OR (95%CI)**	***p***
Duodenal ulcer (n=100, %)					
(-)	(-)	56 (35)	23 (23)	--	--
(+)	(-)	2 (1.25)	5 (5)	4.16 (0.89-19.33)	0.0691
(-)	(+)	59 (36.8)	18 (18)	0.38(0.21-0.68)	**0.0012**
(+)	(+)	43 (26.8)	54 (54)	3.1(0.18 -0.53)	**<0.0001**
Gastric cancer (n=100, %)					
(-)	(-)	56 (35)	26 (26)	--	--
(+)	(-)	2 (1.25)	0	--	--
(-)	(+)	59 (36.8)	38 (38)	1.05(0.63-1.76)	0.8552
(+)	(+)	43 (26.8)	36 (36)	1.53(0.90 -2.62)	0.1196

Table 4. Correlates of MPO polymorphism, smoking and *H.pylori* infection in the development of duodenal ulcer and gastric cancer among male cases.

MPO *allele A* carrier	***H.pylori* infection+ Smoking**	**Healthy controls (n=61, %)**	**Duodenal ulcer (n=72, %)**	**OR (95%CI)**	***p***
(+)	(+) Smoking	27 (44.3)	42 (58.4)	1.76 (0.89-3.5)	0.1056
(+)	(+) Non-smoking	8 (13.1)	10 (13.8)	1.07 (0.39-2.9)	0.8965
(-)	(-) Smoking	20 (32.8)	14 (19.4)	0.49 (0.23-1.08)	0.0078
(-)	(-) Non-smoking	6 (9.8)	6 (8.4)	0.83(0.25-2.73)	0.763
MPO *allele A* carrier	***H.pylori* infection+ Smoking**	**Healthy controls (n=61, %)**	**Gastric cancer (n=56, %)**	**OR (95%CI)**	***p***
(+)	(+) Smoking	27 (44.3)	30 (53.6)	1.45(0.7-3.01)	0.314
(+)	(+) Non-smoking	8 (13.1)	6 (10.7)	0.8(0.26-2.45)	0.689
(-)	(-) Smoking	20 (32.8)	16 (28.6)	0.82(0.37-1.8)	0.622
(-)	(-) Non-smoking	6 (9.8)	4 (7.1)	0.27(0.19-2.63)	0.603