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Alteration of *P53*, hMLH1, and HER2 Gene in Bangladeshi Gastric Cancer Patients: Their Association with *H. pylori* Infection and Clinicopathological Factors

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Abstract

Background: Gene alterations are required for the development of gastric cancer, which are influenced by environmental and host factors. We conducted the present study to find the status of *Helicobacter pylori* (*H. pylori*) infection and its association with altered genes *P53*, hMLH1, and HER2 in gastric cancer patients and to analyze their correlation with clinical, pathological, and environmental factors.

Method: This was a cross-sectional study. For genetic (*P53* and hMLH1) study of the gastrectomized tissue DNA extraction and optimization, we performed PCR amplification and DNA sequencing. HER2 was studied by immunochemical technique. The results were matched with tumor status, age and sex, smoking, and *H. pylori* antibody status of the patients to find their association.

Results: The mean age of the patients was 52.91 (±13.94) years. Among the 45 patients selected for genetic tests, 12 aged 40 or more and 33 aged over 40. Among the genes, 33(73.3%) in *P53* and 17(37.7%) in hMLH1 were mutated and 11(24.2%) in HER2 were found to be overexpressed. Chi square and regression analysis showed that they all had associations with *H. pylori* positivity ($P < 0.05$, odds ratio >1). hMLH1 was associated with the location of the tumor, smoking, sex, blood group, and age, and *P53* was found to be affected by extra salt intake, sex, blood group, and age of the patients ($P \leq 0.05$).

Conclusion: Genetic mutation was found in nearly all the patients with gastric cancer, which was significantly associated with *H. pylori* infection. Mass eradication of this organism might play a role in reducing cancer incidence in Bangladesh.

Keywords: Stomach neoplasms, *Helicobacter pylori*, Genes, *P53*, hMLH1, HER2

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Introduction

Gastric cancer has always been one of the most prevalent causes of cancer-related death and continues to be a major public health issue. According to Globocan, over one million new cases were reported in 2018 making it the sixth most common cancer and the second cause of cancer-related death.[1] The relevant literature review expressed that the incidence of gastric cancer varies in different parts of the world, with higher incidence rates documented in eastern Asia, eastern Europe, and South America, while North America and Africa have shown the lowest recorded rates. Since there is the lack of cancer registry, there is no national data base for different cancer incidences in Bangladesh. Institution-based data demonstrate a higher and growing trend of gastric cancer in recent years. Among men, it is the third most prevalent among male cancers, yet based on unpublished data, it has the second place.[2,3]

It is a known fact that gastric cancer is triggered by a combination of environmental factors and accumulation of specific genetic alterations. Environmental factors, for instance smoking, bacterial factors such as *Helicobacter pylori* (*H. Pylori*). Infection, dietary factors such as a high-salt diet, and genetic factors play important roles in gastric carcinogenesis. Certain studies also documented that all the above-mentioned factors play an interactive role for gene alteration leading to human cancer.[4,5] In Bangladesh, in certain areas, particularly the coastal belt, we could observe more prevalence of this deadly disease.[3]

It is speculated that every cancer originates in genetic.[6] Genetic alterations, such as activation of oncogenes K-*ras*, HER2/neo, B-*raf*, and inactivation of tumor suppressor gene *P53* play carcinogenic roles in the process of gastric cancers. Dysfunction of DNA mismatch repair genes, which leads to microsatellite instability (MSI), also has a pivotal role in this regard. The MSI is influenced by MLH1 gene.[6,7] Even though the etiology of gastric cancer is complex and the risk factors vary in different parts of the world, the most important and well-studied risk factors are *H. pylori* infections and host genetic factors, a positive family history for instance.[8] Furthermore, environmental and nutritional factors are considered as important risk factors. Among these factors, diets, smoking, bacterial infections, and a wide range of occupational exposures are expected to have linkage with genetic alterations.[5]

The World Health Organization (WHO) panel in 1994 classified *H. pylori* as a definite human carcinogen among the environmental factors. Several evidence documented that *H. pylori* is one of the most important pathogens for a wide spectrum of gastroduodenal diseases, such as acute and chronic active gastritis, peptic ulcer disease (PUD), gastric mucosa-associated lymphoid tissue lymphoma, and gastric malignancy. Despite the decline in the incidence of gastric cancer in western countries over the last few decades, its incidence is still high in developing country. In Bangladesh, there are fewer studies marking *H. pylori* infection in the community.[9,10] Although several prospective studies have supported that *H. pylori* infection is a risk factor for the development of gastric cancer,[11,12] there is a controversy in some studies; according to them, there could be a positive relationship between *H. pylori* infection and gastric cancer.[13,14]

P53 gene mutation occurs in most of human cancers.[15] Its incidence is about > 30% of gastric cancer. It acts as a tumor suppressor gene, negatively regulates the cell cycle, and requires loss of function of mutations for tumor formation. The gene spans 20 Kb of genomic DNA located at 17 p13 and induces cell cycle arrest or apoptosis.[16] *P53* is also a genomic stabilizer and an inhibitor of angiogenesis.[17] T*P53* mutations are predominantly inactivating and can induce changes in protein conformation. Loss of *P53* function might result in defective DNA replication, genetic instability, and malignant transformation.[18]

HER2 is encoded by a gene located on chromosome 17q21.[19,20] With the increase in the understanding of the molecular biology of HER2 and the availability of genomics and proteomics analyses, it has now been recognized that HER2

Table 1. Primer sequences of *P53* and hMLH1 gene

Primer Name	Sequence
*P53*_1 Forward	5'-ACAAGCAGTCACAGCACATGAC-3'
*P53*_1 Reverse	5'-TTCAGTAGAGAACGGGGTTTCACC-3'
MLH1_5 Forward	5'-GCTCTGACATCTAGTGTGTG-3'
MLH1_5 Reverse	5'-TGAAGACTTAGCAACACGA-3'

has been notified as a severe form of carcinogenic gene, even in gastric cancer.[20,21]

Another area of thought is DNA repair. MLH1 gene contributes to this, whose cytogenetic location is 3p21. The hMLH1 gene, also called gatekeeper, regulates cell proleferation and death. The hMLH1 protein in combination with another protein called PMS2 from a complex are responsible for DNA repair.[21,22]

In spite of the growing trend of cancer in Bangladesh, many of the issues are unknown to us, including cancer incidences, risk factors, and survival rate. Certain environmental factors like diet, smoking, bacterial infections, and a variety of occupational hazards are assumed to be the risk factors for gastric cancer these days.[23]

To date, we do not have any studies on the molecular factors and their association with clinical or environmental factors.

This study aimed to explore the status of alteration of the three genes-*P53*, HER2, and hMlH1 in gastric adenocarcinoa and to find out their association with *H. pylori* infection and clinicopathological factors of the operated patients.

Materials and Methods

The current work was a cross-sectional study. Endoscopically biopsied and histopathologically confirmed as adenocarcinoma stomach patients were admitted for surgical intervention in the department of Surgical Oncology in National Institute of Cancer Research and Hospital Mohakhali, Dhaka, Bangladesh. Every alternate patient was selected as random basis. The case selection was carried out from January 2015 to September 2016. We took written informed consent from each patient. The ethical committee of the institute approved the protocol, who maintains the international code of medical ethics. Throughout history, clinical examination and staging investigations were compiled in the prestructured data sheet. The operative findings and histopathological reports were noted. The blood samples were collected ahead of the surgery and were sent for *H. Pylori* antibody test in the Laboratory of molecular biology department of Bangabandhu Sk Mujib Medical University. Tissue from the tumor and normal looking area of the gastrectomy specimens were collected, preserved and sent for the predefined laboratory.

P53 and hMLH1 gene mutation test

Laboratory facilities were provided by the department of Genetic Engineering and Biotechnology, Dhaka University under Memorandum of understanding (MOU) with department of surgical oncology, NICRH.

Procedure: Normal and tumor tissues were extracted from the specimen immediately after resection. The specimen surface was washed with saline fluid prior to fragment extraction in order to avoid DNA contamination. Areas of tissue extraction from the specimen were demarcated for routine pathologic examination. Only the tissue fragments containing suspected tumor tissue were included for hMLH1 and *P53* analysis. Normal areas of the specimen were used as controls.

DNA Extraction and Optimization

The tissues were stored at -80°C before DNA purification and extraction. Afterwards, they were sent to specialized center for PCR, sequencing, and analysis. The tissues were incubated overnight at 55°C in a buffer containing 100 mM TRIS-HCl (pH 8.5), 5 mM EDTA, 200 µg of proteinase K/mL, and 0.2% sodium dodecyl sulfate. The samples were cooled to room temperature and DNA was then precipitated with isopropanol and dissolved in 500 mL of buffer containing 10 mM TRIS (pH 8.2) and1 mM EDTA.

Table 2. Relationship of gene alteration with Clinicopathological, clinical, and environmental factors (n=45)

Parameters		*P53* (n=33)		hMLH1(n=17)		Her2 (n=11)	
Factors	**Subgroup**	**Number**	***P* value**	**Number**	***P* value**	**Number**	***P* value**
Age	<40 yrs	12	0.123	4	0.711	2	0.464
	>40 yrs	21		13		9	
Sex	Male	23	0.123	10	0.383	8	0.390
	Female	10		7		3	
Tumor status	T1-T3	23	0.520	13	0.395	10	0.05
	T4	10		4		1	
Lymph Node	N0	13	0.520	7	0.478	5	0.398
Status	N1-N3	20		10		6	
Tumor Grade	G1	1	0.744	1	0.787	0	0.512
	G2	18		8		5	
	G3	14		8		6	
Metastasis	M1	2	0.533	1	0.618	1	0.433
	M0	31		16		10	
Location of	Cardia	6	0.285	5	0.05	3	0.201
tumor	Midbody	3		1		0	
	Antrum	24		11		8	
Morphology	Ulcerative	4	0.639	1	0.045	0	0.600
	Proliferative	15		12		11	
	Ulceroproliferative	14		4		4	
Tumor type	Intestinal	25	0.012	11	0.617	10	0.035
	Diffuse	8		6		1	
Smoking	Smoker	23	0.918	12	0.045	7	0.831
	Non Smoker	10		5		4	
Extra salt	Yes	21	0.368	12	0.664	9	0.220
intake	No	12		5		2	

PCR amplification

Oligonucleotide primers for hMLH1 from the long arm of chromosome 18 were designed on the basis of published sequences (D18S55, D18S58, D18S61, D18S64, and D18S69). We carried out PCR-based dinucleotide repeat assays in 96 well plates for 30 cycles; each cycle was carried out at 950°C for 30 seconds, 500°C for 1 minute, and 700°C for 1 minute. Two volumes of stop buffer (95% of formamide, 20 uM sodium hydroxide, and 0.05% bromophenol blue and xylene cyanate) were added at the end of the amplification. Subsequently, the plates were boiled in a water bath for 10 minutes at 1000°C, and the samples were loaded onto 7% polyacrylamide gels containing 32% formaldehyde and 5.6 M urea.

Sequencing

Purified PCR fragments were sequenced directly utilizing a DNA sequencing kit according to Applied Biosystems from USA with Big Dye Terminators on an ABI3700 automated DNA sequences. cDNA of hMLH1(2 484 bp) was sequenced in six overlapping fragments.

Primers for *P53* exon 5 and 6, and hMLH1 exon 7 and 8 were used after adjusting the proper primer designing. Table 1 represents primer sequence.

Following DNA sequencing data analysis, genetic changes were matched with clinicopathological profile, including age and sex, blood group, tumor characters, types, morphology, and location of the tumor. Among the environmental factors, we considered smoking and extra salt intake to find their association with the gene alteration. The data were compiled in written from structured data sheet and later analyzed using Standard software.

HER2/neu test

After receiving the gastrectomy specimen, it was fixed in 10% formaldehyde. Following the fixation, we performed a systemic gross

Table 3. Linking *H. pylori* infection with gene mutation

Genetic factors	Alteration	*H. pylori*		Total	*P* value	Odd ratio
		positive	negative			
P53 mutation	Positive	28/34	5/11	33	0.025	5.6
	Negative	6	6	11		
hMLH1 mutation	Positive	14/34	3/11	17	0.325	1.87
	Negative	20	8	28		
Her2 over expression	Positive	11/34	0/11	11	0.028	4.78
	Negative	23	11	34		

examination. We also submitted an adequate tissue section to fix it in paraffin. Subsequently, the histologic sections with 3-5 micron thickness were obtained from paraffin blocks and stained with haematoxylineosine for histological assessment. Only the gastric adenocarcinoma diagnosed in haematoxylin and eosin sections were selected for immunohistochemical examination. For immunochemical examination of HER2, its antibodies (Hercep Test-Dako, 4B5 cloneventana, CB11 clone-Novocastra) was used. This examination was done in a private laboratory.

Statistics

Our results were calculated and analyzed with standard statistical method and presented in forms of tables. Continuous data were expressed as mean + SD. For analysis of data SPSS for Windows, we utilized IBM SPSS Statistics for Windows, version 22.0, Armonk, NY:IBM Corp software. To study the association among categorical variables Chi squared test (or Fisher's exact test when applicable) was performed. A value of $P < 0.05$ was considered statistically significant in all analyses.

Binomial logistic regression and odd ratio were used to find the association of genetic mutation with *H. pylori* infection, clinical, and environmental factors in gastric cancer patients. If the estimated probability of the association/correlation is greater than or equal to 0.5, SPSS Statistics classifies it as a positive correlation.

Result

The mean age of the patients was 52.91 (±13.94). Among 45 patients selected for genetic tests, 12 were at the age of 40 or below 40 and 33 were above 40 years of age. Male: female ratio was 29:16. *H. Pylori* infection was present in 34(75.5%) of the 45 cases. Among disease profiles, a significant correlation was found between gene alteration for HER2 with tumor type, hMLH1 with location of the tumor, and tumor morphology and *P53* with tumor type ($P<0.05$). A correlation was also observed between extra salt intake and hMLH1 mutated gene (Table-2). Among the genes, 33(73.3%) in *P53* and 17(37.7%) in hMLH1 were mutated and 11(24.2%) in HER2 were found to be overexpressed. Chi square and regression analysis revealed that all had associations with *H. Pylori* positivity ($P < 0.05$, odd ratio >1) (Table 3). We utilized the Wald test in regression analysis ("Wald" column) to determine the statistical significance of each of the independent variables. The statistical significance of the test is found in the "Sig." column. It illustrated that hMLH1 was associated with the location of the tumor, smoking, sex, and age; and *P53* was associated with extra salt intake, sex, and age of the patient ($P\leq0.05$), which is described in tables 4-6.

Discussion

We designed this study in order to investigate the status of gene (*P53*, hMLH1, and HER2) alteration and to observe the association between

Table 4. Regression analysis of *P53* and its association with other variables in the equation

Variables	B	S.E.	Wald	df	Sig.	Exp(B)
location	-2.028	1.323	2.348	1	0.125	0.132
grade	-1.967	1.496	1.728	1	0.189	0.140
staging_T	1.040	0.799	1.693	1	0.193	2.829
staging_N	0.353	1.514	0.054	1	0.815	1.424
Extra salt	-4.409	2.248	3.847	1	0.0500	0.012
smoking	-2.691	1.752	2.358	1	0.125	0.05
morphology	1.017	1.150	0.782	1	0.377	2.764
sex	5.215	2.838	3.375	1	0.05	183.944
type	4.816	3.407	1.998	1	0.157	123.475
Age	6.400	2.975	4.628	1	0.031	601.746
Constant	-30.024	15.114	3.946	1	0.047	0.000

Sig.: Statistical significance; S.E.: Standard error

H. Pylori infection and clinciopathological factors in gastric cancer patients of Bangladesh.

Regarding the association of cytogentics with cancer, so far, there have been no available reports in Bangladesh. Several oncogenes have been reported in the world literature. In our limited report, it was seen that substantial number of patients are carrying mutated genes, which might be associated with carcinogenesis. The present research revealed that the occurrence of *P53* gene mutation in GC patients is quite frequent in Bangladeshi patients. In the present study, the gene mutation was assessed in relation with age, sex, location of tumor, types of tumor, and stage of tumor. Moreover, we investigated the association of *H. pylori*, smoking, and extra salt intake with the altered genes.

Age and sex

It was a common concept that gastric cancer rarely occurs before the age of 40 and that its incidence increases with the advancement of age with the peak incidence occurring in the seventh decade of life. We took several probable reasons into account, including altered carcinogen metabolism and long-term exposure of cancer-causing agents. Carcinogenesis, which is a multistep process, is the result of a part of cumulating effects of the age wise mutations during progression from normal epithelium to carcinoma.[24] Studies have observed that the mutations of several tumor suppressor genes, specifically APC, DCC, and *P53* in the gastric mucosa is higher in older subjects.[25] Meanwhile, other studies have revealed that *P53* mutation is seen in younger subjects of proximal stomach cancers.[26] On the other hand, there are other reports expressing that the rise in mutations of tumor suppressor genes among the older subjects could not be accounted for the presence of cancer in stomach or any other organ.[27] In addition, certain reports have stated that there might be slow progression of pathological changes in different stages of life within stomach. These lesions are thought to be most closely associated with the subsequent development of dysplasia and carcinoma.[28] In this paper, among the younger group (<40 years), it was observed that 100% of the patients showed alteration by any of the genes tested. Accordingly, we obtained different results from the previous facts. There were no gender-associated relationships found in this study.

Tumor profile

We aimed to explore the correlation between gene alteration with the characters of the tumors, like morphology, location, extension, and grading. In this study, though most of the tumors were located within mid body and antrum, there was significant correlation with hMLH1 gene mutation only among three. On the other hand, the extension of the tumor (T) exhibited a correlation with HER2 overexpression. But it was reported that there is no correlation between HER2 and staging of gastric cancer, rather its overexpression might be an early event in gastric carcinogenesis.[7] We found no significant correlation of gene mutation with lymph node involvement. Considering the histopathological variety, majority of the intestinal type were associated with altered *P53* and

Table 5. Regression analysis of hMLH1 and its association with other variables in the equation

Variables	B	S.E.	Wald	df	Sig.	Exp(B)
Location	2.246	0.925	5.902	1	0.015	9.452
grade	-0.145	0.797	0.033	1	0.856	0.865
staging_T	0.000	0.350	0.000	1	0.999	1.000
staging_N	-0.247	0.936	0.069	1	0.792	0.781
extrasalt	0.491	0.853	0.331	1	0.565	1.634
smoking	1.739	0.900	3.734	1	0.05	5.693
morphology	0.804	0.621	1.676	1	0.195	2.235
sex	-1.761	1.043	2.847	1	0.05	0.172
type	0.321	1.739	0.034	1	0.853	1.379
Age	-2.195	1.319	2.769	1	0.05	0.111
Constant	1.164	4.307	0.073	1	0.787	3.203

Sig.: Statistical significance; S.E.: Standard error

overexpressed HER2 gene. Despite the limited number of studies on the clinical relevance with gene alteration in the literature, it has been shown that it has association with advanced stage and higher grade[29,30] though one study found negative association with lymph node status, grading, and metastasis.[31]

Environmental factors

Extra salt intake: It was seen that, in Bangladesh, some areas are prone to have further gastric cancer in their vicinity. It has been also observed that the regular intake of extra salt in the diet is associated with *P53* mutation. A prospective study from a Japanese population suggests that high dietary salt intake (>10 g per day) is a significant risk factor for gastric cancer, which was found to be stronger in the presence of *H. pylori* infection with atrophic gastritis.[32,33]

Smoking is also a common habit among the men in this part of the world. Since gastric cancer is a male dominant disease, smoking is likely to be a pre-disposing risk factor of the disease. In this study, hMLH1 mutated patients had a significant correlation with smoking. Although the role of smoking in several other cancers has long been established, it was not until 2002 that the International Agency for Research on Cancer concluded that there was "sufficient" evidence of causality between smoking and gastric cancer.[34] In a large population-based study in Europe (EPIC), 17.6% of gastric cancer cases were found to be smokers.[35] The mutagenicity and carcinogenicity of tobacco products is well-established for cancer. There is large bulk of works and literatures in this regard. There are good volume works showing the association of *P53* mutation with smoking particularly on lung cancer and bladder cancer.[36,37] In spite of the limited number of studies on stomach cancer, study found smoking had effects on gastric epithelium.[38] Additionally, it was shown that smoking induces DNA damage of *P53* gene in lung mucosal cells.[39] There are not enough works on smoking-induced carcinogenesis concerning gastroentistnal cancer. In a study carried out in northern Iran, smoking was observed to be predominant in the patients having squamous cell carcinoma of eosophagus.[40] In stomach cancer, a mixed pattern of results are found. In a multivariate model, a significant association was found between *P53* mutation and smoking.[41] On the other hand, discordant findings were found in a large Saudi study.[42] In this study, among the three genes, we found mutated hMLH1 gene had association with smoking.

Regarding linkages of smoking with HER2 expression, one study on smokers found positive score 3(+) in 20% of the cases, among whom 32% were former smokers in lung cancer. Similarly, there are few works on hMLH1 gene, particularly in the field of gastric cancer. There is evidence about the relationship of mutation in hMLH1 gene in case of CRC.[43]

In Bangladesh, gastric cancer incidence is on the increase. Regarding *H. pylori* infection, different studies have reported that in the last 20 years, infection has been reducing. A case-control study by Sarkar et al.[8] depicted the relationship of gastric cancer with *H. pylori* infection.

Table 6. Regression analysis of HER2 and its association with other variables in the equation

Variables	B	S.E.	Wald	df	Sig.	Exp(B)
location	-11.479	1021.108	0.000	1	0.991	0.000
grade	-158.962	8728.851	0.000	1	0.985	0.000
staging_T	54.498	2909.615	0.000	1	0.985	46595820469408 4250000000.000
staging_N	-2.498	2.989	0.698	10	0.403	0.082
extrasalt	4.967	2097.301	0.000	1	0.998	143.624
smoking	116.906	7404.063	0.000	1	0.987	5.909E+050
morphology	3.708	3.250	1.302	1	0.254	40.759 sex
	51.467	2964.142	0.000	1	0.986	22481923810393 147000000.000
type	242.008	18815.041	0.000	1	0.990	1.267E+105
Age	-184.491	11237.638	0.000	1	0.987	0.000
Constant	481.107	30888.450	0.000	1	0.988	8.751E+208

According to them, it is well-established that there is also a close association of *H. pylori* infection with gastric cancer.

Conclusion

Bangladesh still has to bear big burden and carry devastating tales of the gastric cancer patients. Even though the disease affects all the socioeconomic group of people, low income people are the most susceptible group. Throughout the treatment process of this disease, on a number of occasions, it is difficult to follow the existing management protocol. This is an obvious fact across the world regarding different environmental risk factors of the disease, emphasizing *H. pylori* as one of the leading causative agent. There are no genetic studies on cancer in Bangladesh. We attempted to find out the association of mutated genes of the three key groups (oncogene, tumor suppressor gene and tumour repair genes) with host, disease and environmental factors. It was found that almost all the patients possessed at least one altered gene. Even though the genes seemed to have no effects on tumor staging and disease assessment, it is speculated that they might have a role in precised medicine; particularly *P53* helps to stratify the disease in the modern treatment. This study indicated that *H. pylori* is strongly associated with genetic mutations for gastric cancer. Consequently, large scale anti-*H. pylori* therapy might play a pivotal role in preventing the steps of carcinogenesis. National measures could be taken in order to prevent the disease. This study explored the relationship of *H. pylori* infection with the genetic changes in cancer patients, which would be a milestone in the cancer research in this country.

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Conflict of Interest

None declared.

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