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RESEARCH

Oxidative DNA Damage as a Potential Early Biomarker of *Helicobacter pylori* Associated Carcinogenesis

Yasir Raza · Adnan Khan · Amber Farooqui · Muhammad Mubarak · Alex Facista · Syed Shakeel Akhtar · Saeed Khan · Javed Iqbal Kazi · Carol Bernstein · Shahana Urooj Kazmi

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Abstract *Helicobacter pylori* infection is an established risk factor for gastritis, gastric ulcer, peptic ulcer and gastric cancer. *CagA +ve H. pylori* has been associated with oxidative DNA damage of gastric mucosa but their combined role in the development of gastric cancer is still unknown. Here we compare the combined expression of *cagA* and 8-hydroxy-2′-deoxyguanosine (8-OHdG) in normal, gastritis and gastric cancer tissues. Two hundred gastric biopsies from patients with dyspeptic symptoms, 70 gastric cancer tissue samples and 30 gastric biopsies from non-dyspeptic individuals (controls) were included in this study and 8-OHdG was detected by immunohistochemistry (IHC). Histological features and the presence of *H. pylori* infection were demonstrated by Hematoxylin and Eosin (HE), Giemsa and alcian blue-periodic acid-Schiff ± diastase (AB-PAS ± D) staining. DNA was extracted from tissues and polymerase chain reaction (PCR) performed to determine the presence of *ureaseA* and *cagA* genes of *H. pylori*. The results showed the presence of *H. pylori* in 106 (53 %) gastric biopsies out of 200 dyspeptic patients, including 70 (66 %) cases of *cagA + ve H. pylori.* The presence of *cagA* gene and high expression of 8-OHdG was highly correlated with severe gastric inflammation and gastric cancer particularly, in cases with infiltration of chronic inflammatory cells (36.8 % *cagA + ve,* 18 %), neutrophilic activity (47.2 %, 25.5 %), intestinal metaplasia (77.7 %, 35.7 %) and intestinal type gastric cancer (95 %, 95.4 %) ($p \leq 0.01$). In conclusion, *H. Pylori cagA* gene expression and the detection of 8-OHdG adducts in gastric epithelium can serve as potential early biomarkers of *H. Pylori*-associated gastric carcinogenesis.

Y. Raza · A. Khan · A. Farooqui · S. Khan · S. U. Kazmi
Immunology and Infectious Diseases Research Laboratory, Department of Microbiology, University of Karachi, Karachi, Pakistan

Y. Raza
e-mail: yaaasirz@hotmail.com

A. Khan
e-mail: adnankh@uok.edu.pk

A. Farooqui
e-mail: amberfarooqui@hotmail.com

S. Khan
e-mail: saeedkhn@gmail.com

S. U. Kazmi
e-mail: shahanaurooj@yahoo.com

Y. Raza · A. Facista · C. Bernstein
Department of Cellular and Molecular Medicine, College of Medicine, University of Arizona, Phoenix, AZ, USA

A. Facista
e-mail: alexanderfacista@gmail.com

C. Bernstein
e-mail: bernstein324@yahoo.com

M. Mubarak (✉) · J. I. Kazi
Department of Histopathology, Sindh Institute of Urology and Transplantation, Karachi, Pakistan
e-mail: drmubaraksiut@yahoo.com

J. I. Kazi
e-mail: javedikazi@gmail.com

S. S. Akhtar
Department of Surgery and Medicine, Civil Hospital, Karachi, Pakistan
e-mail: dr_aleya@yahoo.com

J. I. Kazi
Department of Histopathology, Ziauddin University and Hospital, Karachi, Pakistan

A. Farooqui
Division of Immunology, International Institute of Infection and Immunity, Shantou University Medical College, 22 Xinling Road, Shantou, Guangdong 515041, China

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Introduction

Gastric pathological disorders such as gastric cancer, gastritis, intestinal metaplasia, and ulcer have been increasingly reported worldwide. Several factors, such as high levels of reactive oxygen species (ROS) are involved in severe damage to the gastric epithelium. While ROS are normal endogenous metabolic end products of cellular metabolism, excessive formation of ROS may be an etiologic factor in causing tissue damage and cancer. ROS directly interact with macromolecules including genomic DNA and cause damage to specific genes responsible for cell proliferation [1, 2] and tumor suppression that lead to tumorigenesis [3]. In the gastric lumen, certain ingested foods, cigarette smoke and other environmental pollutants upregulate generation of ROS, causing severe damage to the gastric epithelium [4]. A role of ROS in the pathogenesis of gastric cancer has also been indicated in several studies [5, 6]. Oxidation of DNA caused by ROS results in the formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a major marker of DNA damage [7] which is responsible for DNA base mutation [8], causing A:T to C:G transversions, as well as misincorporations, while undergoing repair by DNA excision repair enzymes [9]. Hence excess 8-OHdG supports carcinogenesis.

One of the most important factors associated with gastric pathologies is the presence of *Helicobacter pylori* infection. The organism has already been designated as a class I carcinogen for the development of gastric cancer by the World Health Organization despite its presence in the stomach of more than half of the world's population, symptomatically or asymptomatically [10, 11]. The virulence of *H. pylori* is usually determined by the presence or absence of different virulence proteins including the cytotoxin associated gene (*cagA*), vacuolating cytotoxin (VacA) and sialic acid–binding adhesin (SabA) proteins [12]. CagA is the terminal gene product of the *cagA* pathogenecity island that enters the host cell through the type IV secretion system, gets phosphorylated and interrupts various cellular cascades, resulting in epithelial damage [13, 14]. A number of studies have been conducted to elucidate the likely correlation between increased ROS and *cagA +ve H. pylori,* measuring increased oxidative DNA damage in gastric epithelial cells infected with *cagA +ve H. pylori* strains [15, 16]. On the other hand, another study indicated that *cagA +ve H. pylori* infection results in increased production of ROS-scavenging enzymes that prevent ROS DNA injury. However, it is generally thought that *H. pylori* disturbs the balance between oxidants and antioxidants by increasing release of ROS and impairing the levels of scavenging enzymes [17, 18].

Pakistan is among the countries with high rates of dyspepsia and *H. pylori* infection [19–22]. Low socio-economic conditions, overcrowding, poor hygienic status, polymorphism in host genes and frequent carriage of the *cagA* gene by infecting *H. pylori* are important factors contributing to the epidemiology of gastric pathologies in the country [23, 24]. However, statistically robust evidence demonstrating correlation of *H. pylori* and ROS formation in the development of specific gastric pathologies has been elusive previously.

In this study, we analyzed the expression of 8-OHdG by immunohistochemistry (IHC) in gastric tissues obtained from patients suffering from dyspeptic symptoms and gastric cancer. Tissues adjacent to gastric cancers as well as biopsies from patients free from dyspeptic symptoms (controls) were also analyzed. Different degrees of gastritis and gastric adenocarcinoma were semiquantified for 8-OHdG expression to determine whether 8-OHdG systematically increased in tissues with increasing pathology from normal tissue to gastritis to pre-cancerous lesions to gastric cancer. In addition, we evaluated the correlation between the *cagA* status of *H. pylori,* the degree of gastritis and the levels of occurrence of 8-OHdG.

Materials and Methods

Patients

This study involved a total of 300 subjects, divided into three groups. The first group consisted of 200 dyspeptic patients (males = 101, females = 99, mean age = 37.35 years) who underwent eosophago-gastro-duodenal (EGD) endoscopy for upper gastrointestinal symptoms in the endoscopy unit of Dow University of Health and Science, Civil Hospital, Karachi. Two biopsies each from the gastric antrum and the corpus of every individual were collected in 10 % formalin for histological evaluation, DNA extraction and IHC for 8-OHdG. The second group comprised of 70 cases of gastric cancer including diffuse and intestinal type (males = 50, females = 20, mean age = 55.51 years) who reported to Ziauddin University Hospital, Karachi. Fifty small endoscopic and twenty resected gastric tissues were collected from cancerous lesions from these patients. Nine histologically normal tissues near cancerous lesions were also included in this group. Third group consisted of 30 individuals (males = 16, females = 14, mean age = 52.3 years) who did not have dyspeptic symptoms and from whom gastric biopsies were also obtained. These served as a control group. This research was conducted with the permission granted by the ethical review board of the University of Karachi, Pakistan. Written informed consent was obtained from all the participants of this study.

Histology

The histopathological analysis of the gastric biopsies was carried out at the department of Histopathology, Sindh Institute of Urology and Transplantation (SIUT), Karachi. Routinely, 3–4 um sections were cut from paraffin embedded tissues and stained with Haematoxylin and Eosin (HE), Alcian blue-Periodic acid-Schiff ± diastase (AB-PAS ± D), and Giemsa staining. The sections were evaluated for the histological features of gastric inflammation and grading *H. pylori* density according to the updated Sydney system [25]. The infiltration of chronic inflammatory cells, neutrophilic activity, intestinal metaplasia and density of *H. pylori* were graded and evaluated as absent, mild, moderate or severe (0, 1, 2 and 3) respectively.

DNA Extraction

DNA was extracted from 3 to 5 μm sections of formalin fixed paraffin embedded tissues (FFPE). FFPE tissue sections were deparaffinized with xylene, washed with 100 % ethanol to remove xylene traces and air dried. Samples were homogenized and added to a mixture of 20ul 20 % SDS, 80 μl protein kinase buffer (0.375 M NaCl, 0.12 M EDTA, pH 8.0), 40 μl of Proteinase K (10 mg/ml), and sterile water. The mixture was incubated at 55 °C for 24 h for digestion by proteinase K. The next day, 100 μl of 6 M NaCl was added, the mixture was centrifuged at 13,000 rpm and the supernatant was transferred to another sterile tube where the DNA was precipitated by adding 1 ml of 100 % ethanol and the suspension was centrifuged at 14,000 rpm. The DNA pellet was washed with 70 % ethanol, air dried, resuspended in 50 μl of 1× TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) and stored at −20 °C until the polymerase chain reaction procedure (PCR) was employed [26].

Polymerase Chain Reaction (PCR)

DNA samples were used for detection of *H. pylori* by assessing for the presence of the *ureaseA* gene and for *cagA* gene using specific primer sequences [27, 28] (Table 1). A PCR mixture containing 2–3 μl DNA sample, 0.5 μl each of forward and reverse primers, 12.5 μl of 2× master mix (kapaTaq ready mix, Kapa Biosystem, Boston, USA) and nuclease free water was subjected to PCR amplification under the conditions described in Table 1. PCR for human β- globin gene was performed as a control for the quality of sample and DNA extraction [26].

Immunohistochemistry (IHC)

The IHC staining technique was modified from the one described by Papa et al. [29]. Briefly, 4–5 μm sections from FFPE tissues were used for 8-OHdG IHC staining. Sections were deparaffinised in xylene followed by the immersion in ethanol. The endogenous peroxidase blocking step was performed by immersing slides in methanol/H_2O_2 for 20 min. For antigen retrieval, slides were placed in 4 N HCL for 20 min followed by treatment with 0.1 M borax for 5 min. Slides were then loaded into Sequenza racks. 5 % normal horse serum in PBS was used for blocking binding of non-specific proteins and slides were allowed to stand for 60 min. The tissue sections were then incubated with mouse monoclonal anti-8-OHdG antibody (QED-Bioscience, San Diego, CA) at a dilution of 1:400 for 90 min. Mouse monoclonal anti-IgG2a (QED-Bioscience, San Diego, CA) and 2 % BSA in PBS were used as negative controls for 8-OHdG and secondary antibody respectively. Tissue sections were further incubated in biotinylated secondary rabbit anti mouse antibody (Dako corp, Carpinteria, CA) diluted 1:400 in PBS followed by 2 drops of Vectastain ABC reagent for 30 min, and immersed in Diaminebenzidine tetrahydrochloride solution (Sigma, St. Louis, MO) for 4 min. Counter staining was performed with 1:4 dilution of Nancy's hematoxylin for 10 s. Slides were immersed in distilled H_2O and dehydrated in ethanol and xylene. Cytoseal XYL was used to seal the slides with cover slips and slides were evaluated with a Motic BA300 digital photomicroscope at 20× and 40× magnifications for low, medium and high nuclear staining for 8-OHdG positive tissues. The expression of 8-OHdG in the cellular nucleus of gastric surface and glandular epithelium was assessed by brown colored IHC staining. The positive reaction was given a score of 0, 1, 2, 3 or 4 as follows: A total of 200 cells were counted from the area of biopsy with most severe inflammation. Three such areas were assessed and their average labeling index (LI) calculated as: if 0–10 % of the cell nuclei stained positive, the LI was scored as "0." Scores of 1, 2, 3 and 4 corresponded to percent of positive cell nuclei of 11–25 %, 26–50 %, 51–75 % and >75 %, respectively. The designations 0, 1, 2, 3 and 4 were then described as absent, very low, low, medium and high, respectively.

Statistical Analysis

All the data were entered into IBM-compatible SPSS 20 for Windows 7 for statistical analysis. The data were cross tabulated and the mean ± standard deviation (SD) and median with interquartile range (IQR) of the variables were compared. Mann–Whitney U test and Fisher exact test was used for non-parametric data to compare the groups. The association between variables was determined by using the Spearman's test. A p value of less 0.05 was considered statistically significant.

Table 1 Polymerase chain reaction (PCR) primers and other details of PCR conditions used in this study

Target gene	Primer sequence (5′-3′)	Product size (bp)	PCR Conditions	References
β-globin	ACACAACTGTGTTCACTAGC CAACTTCATCCACGTTCACC	110	94ºC, 30 s; 51ºC, 30 s ; 72ºC, 30 s (40 cycles)	[26]
ureaseA	GCCAATGGTAAATTAGTT CTCCTTAATTGTTTTTAC	411	94ºC, 1 min; 45ºC, 1 min; 72ºC, 1 min (40 cycles)	[27]
cagA	TTGACCAACAACCACAAACCGAAG CTTCCCTTAATTGCGAGATTCC	183	94ºC, 30 s; 50ºC, 45 s; 72ºC, 45 s ; (40 cycles)	[28]

cagA cytotoxin associated gene A

Results

Association of *H. pylori* and *cagA* +ve *H. pylori* with Gastric Pathology

In this section, all results were discussed in relation with the findings observed in corpus region because of higher prevalence of *H. pylori* infection in the corpus than the antrum of the stomach. Data related to antral biopsies was not shown and further discussed.

In the first group of tissues, from 200 patients suffering from gastritis, 106 (53 %) were infected with *H. pylori* (Table 2) including 70 cases (66 %) which were found to be positive for the *cagA* gene. A positive association was observed between *cagA +ve H. pylori* and degree of gastric inflammation, particularly in the cases of infiltration of chronic inflammatory cells (36.8 % *cagA + ve*), gastritis with neutrophilic activity (47.2 % *cagA +ve*) and intestinal metaplasia (77.7 % *cagA +ve*), as shown in Table 3. All p values were less than 0.01. *H. pylori* induced gastric lesions are shown in Fig. 1a–d. PCR also proved the presence of *H. pylori ureaseA* gene in 34 (48.5 %) cases of gastric cancer with a statistically significant difference in *cagA +ve* (n=21, 61.7 %, p<0.01) and *cagA −ve H. pylori* (n=13, 38.2 %, p<0.01). It was also significantly noted that intestinal type gastric cancers were more commonly associated with *cagA +ve H. pylori* infection (95 %) as shown in Tables 2 and 3. In the case of control group, 7/30 (23.3 %) were found positive for *H. pylori ureaseA* gene and 2/7 (28.5 %) were found positive for *cagA* gene by PCR. However, none of the controls were positive for *H. pylori* on the histological examination.

Expression of 8-OHdG in *H. pylori* Positive and Negative Gastritis Cases

Gastric tissues collected from corpus of the stomach were observed to show slightly higher immunoreactivity for 8-OHdG in comparison with antral biopsies (data not shown). In gastritis group, *H. pylori +ve* cases were found to show the higher expression (median percent of positive cells with IQR) of 8-OHdG (80.00 (5–95); mean rank = 101.54, p<0.01) (Fig. 2a) than H. pylori negative cases, 10.00 (10–16.25). In particular, 57/70 (81.4 %) of *cagA +ve H. pylori* cases showed high expression of 8-OHdG (85.00 (80–90), mean rank = 165, rs=0.836, p<0.01), while *cagA −ve H. pylori* cases showed low to medium immunoreactivity for 8-OHdG (42.5 (31.25–50), mean rank = 65.75, rs=0.816, p<0.01), illustrated in Fig. 2b. It was further noted that severe infiltration of chronic inflammatory cells, gastritis with neutrophilic activity and intestinal metaplasia were highly associated with increased expression of 8-OHdG (Table 4). Low to medium 8-OHdG expression was more often found in the gastric mucosa of tissues with mild to moderate gastric inflammation. Furthermore, absent or very low nuclear staining was observed in *H. pylori -ve* cases of gastritis and in normal gastric tissues (Table 4).

Table 2 Histologic features on gastric biopsies according to *H. pylori* status

Groups	Histology	*H. pylori* positive, *n* (%)	*H. pylori* negative, n (%)
Gastritis (n=200)	Infiltration of chronic inflammatory cells (1/2/3; n=200)	24(12)/34(17)/48(24)	68(34)/20(10)/6(3)
	Neutrophilic activity (1/2/3; n=86)	8(9.3)/21(24.4)/26(30.2)	17(19.7)/13(15.1)/1(1.1)
	Intestinal metaplasia (1/2/3; n=14)	1(6.6)/3(20)/5(33.3)	2(13.3)/1(6.6)/ 2(13.3)
Gastric Cancer (n=70)	Diffuse Type (n=39)	9(23)	30(76.9)
	Intestinal Type (n=22)	20(90.9)	2(9)
	Adjacent Tissue (n=9)	5(55.5)	4(44.4)
Controls (n=30)	Normal	7(23.3)	23(76.6)

1 = mild; 2 = moderate; 3 = severe (according to Sydney System)

n number. Numbers in parentheses are percentages

Table 3 Histologic features on gastric biopsies according to *cagA* status in 106 patients who were positive for H. pylori infection

Histology (*n*=Hp+)	Mild cagA+/cagA−	Moderate cagA+/cagA−	Severe cagA+/cagA−	*p* value
Infiltration of chronic inflammatory cells (*n*=106)	8(7.54)/25(23.58)	23(21.69)/11(10.37)	39(36.79)/0	<0.01
Neutrophilic activity (*n*=55)	0/8(14.5)	12(21.8)/9(16.3)	26(47.2)/0	<0.01
Intestinal metaplasia [9]	7(77.7)/2(22.2)	–	–	<0.01
Diffuse Type GC (*n*=9)	1(11.1)/8(88.8)	–	–	<0.01
Intestinal Type GC (*n*=20)	19(95)/1(5)	–	–	<0.01
Adjacent Tissue (*n*=5)	1(20)/4(80)	–	–	<0.01
Normal (*n*=7)	2(28.5)/5(71.4)	–	–	<0.01

Numbers in parentheses are percentages

N number, *GC* gastric cancer

Expression of 8-OHdG in *H. pylori* Positive and Negative Gastric Cancer Cases

In gastric cancer group, all cases showed medium to high expression of 8-OHdG. In addition, there was significant difference in the median level of expression of 8-OHdG between *H. pylori -ve* (60 (55–65); mean rank = 21.85, $p<0.01$) and positive cases (85.00 (75–90), mean rank = 33.73, $p<0.01$) (Fig. 2c). Among these, 26 cases had high expression of 8-OHdG including 21 *cagA +ve H. pylori* cases (80.7 %) while 5 *cagA -ve H. pylori* cases (19.2 %) also showed high expression of 8-OHdG (85.00 (80–90), mean rank = 56.86, rs=0.786, $p<0.01$ and 70.00 (62.5–82.5), mean rank = 26.35, rs=0.672, $p<0.01$ respectively), as shown in Fig. 2d. The increased expression of 8-OHdG was more commonly found in tissues from cancerous lesions as well as tissues adjacent to cancerous lesions. Further analysis revealed that intestinal type gastric cancer had high expression of 8-OHdG in 21 of 22 cases (95.4 %), while the diffuse type of gastric cancers and tissues from control subjects had medium and absent occurrence of 8-OHdG respectively, as illustrated in Fig. 3a–c and Table 4.

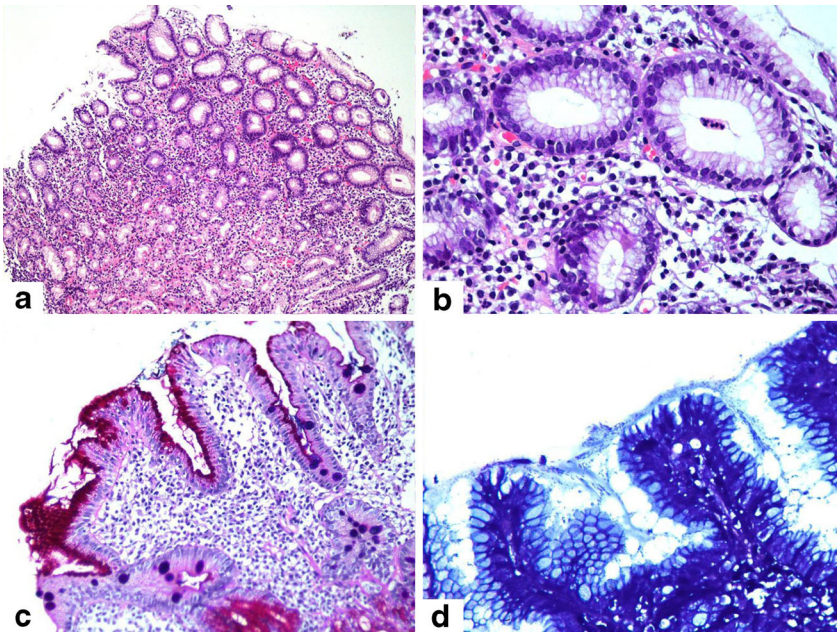

Fig. 1 Representative pictures of *H. Pylori* induced gastric lesions in gastric body mucosal biopsies. **a** Low-power view of gastric body mucosa showing marked inflammatory cell infiltration in the lamina propria (H&E, ×200). **b** Medium-power view of gastric body mucosa showing marked mixed inflammatory cell infiltration in the lamina propria. Pit abscess is also seen in the central gastric pit (H&E, ×400). **c** Low-power view of gastric body mucosa showing moderate chronic inflammatory cell infiltration in the lamina propria and extensive goblet cell metaplasia of the glandular epithelium. (Alcian blue-Periodic acid-Schiff (AB-PAS), ×200). **d** Medium-power view of gastric body mucosa showing heavy *H. Pylori* infection in the gastric pits and on mucosal surface (Giemsa, ×400)

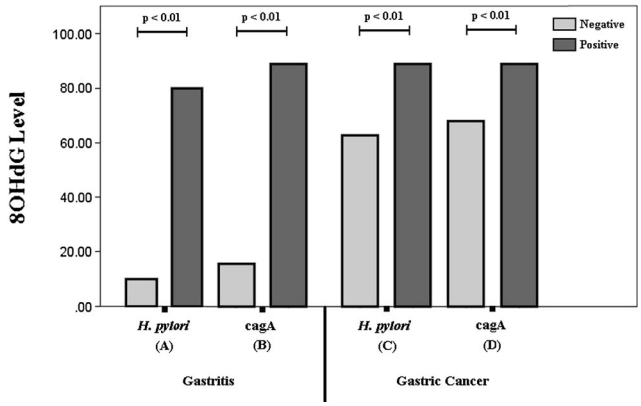

Fig. 2 The median levels of 8-OHdG immunoreactivity in percentage in gastric tissues collected from **a** *H. pylori* -ve and +ve dyspeptic patients; **b** *H. pylori cagA −ve* and+ *ve* dyspeptic patients; **c** *H. pylori −ve* and *+ve* gastric cancer patients; and **d** H. pylori cagA *−ve* and *+ve* gastric cancer patients

Discussion

To the best of our knowledge, this is the largest study from this country on the molecular characterization of *H. Pylori* and the first to describe the association of this organism induced oxidative DNA damage to gastric carcinogenesis from this part of the world. The study samples were collected from two large tertiary care hospitals of Karachi, catering to the health needs of a variety of socioeconomic strata of the society. We believe that the results from this study are fairly representative of the status of *H. Pylori* infection, its genotypes and its association with gastric cancer in the population from Southern Pakistan.

It is well known that *H. pylori* infection is one of the most common causes of gastric and duodenal ulcer. The organism is also considered an important factor in the pathogenesis of gastric cancer [31]. Among several pathogen associated factors, *cagA* gene carriage is specifically referred to as a major trait of ulcerogenic *H. pylori* strains that are able to stimulate gastric epithelium to secrete increased levels of proinflammatory cytokines, resulting in severe gastric inflammation [32]. A previous study also reported that *cagA +ve H. pylori* infection is responsible for increased production of ROS that promote oxidative DNA damage in gastric mucosa [29]. While previous studies explored the possible factors related to the role of *H. pylori* in generating gastric pathology, the mechanism by which *H. pylori* transforms gastric inflammatory lesions into gastric cancer had not been established.

This study was initiated to investigate the roles of *H. pylori* infection, with or without the presence of the *cagA* gene, in causing oxidative DNA damage of gastric mucosa in patients with differing types and severities of gastric inflammatory processes. We observed that *cagA +ve H. pylori* infection is associated with increased expression of 8-OHdG, and this, in turn, is highly associated with the disease severity of gastritis. In the most severe forms of gastritis, high levels of 8-OHdG occur, similar to the high levels in gastric cancer cases. We also observed that *cagA +ve H. pylori* infection is specifically associated with severe levels of infiltration of chronic inflammatory cells, neutrophilic activity, intestinal metaplasia and gastric cancer, particularly intestinal type gastric cancer. These results are similar to those reported in two previous studies [33, 34]. We found that these pathological lesions frequently show high levels of expression of 8-OHdG (Table 4). The findings strongly suggest that high 8-OHdG expression is closely related to *H. pylori* infection combined with *cagA* gene carriage and this combination plays an important role in the development of chronic and severe forms of gastric inflammation. It was previously observed that *cagA +ve H. pylori* disturbs the normal gastric mucosal architecture by injecting cagA protein into the epithelial cells via the type IV secretion system, resulting in a neutrophilic respiratory

Table 4 Histologic features on gastric biopsies and 8OHdG immunoreactivity

Histology; *n*=300	8OHdG Immunoreactivity					*p* value
	Absent	Very low	Low	Medium	High	
Infiltration of chronic inflammatory cells (1/2/3)(*n*=200)	45(22.5)/11(5.5)/1(0.5)	29(14.5)/9(4.5)/0	17(8.5)/9(4.5)/1(0.5)	3(1.5)/9(4.5)/9(4.5)	1(0.5)/20(10)/36(18)	<0.01
Neutrophilic activity (1/2/3)(86)	9(10.4)/7(8)/0	8(9.3)/6(7)/0	7(8)/6(7)/0	1(1)/5(5.8)/5(5.8)	0/10(11.6)/22(25.5)	<0.01
Intestinal metaplasia (1/2/3) [14]	1(7)/1(7)/1(7)	2(14.2)/0/0	0/0/0	0/1(7)/1(7)	0/2(14.2)/5(35.7)	<0.01
Diffuse type GC [30]	0	0	5(12.8)	31(79.4)	3(7.6)	<0.01
Intestinal type GC [22]	0	0	0	1(4.5)	21(95.4)	<0.01
Adjacent tissues [9]	0	1(11)	3(33.3)	3(33.3)	2(22.2)	<0.01
Normal Tissues [31]	30(100)	0	0	0	0	–

1 = mild; 2 = moderate; 3 = severe (according to Sydney System)

n numbers. Numbers in parentheses are percentages

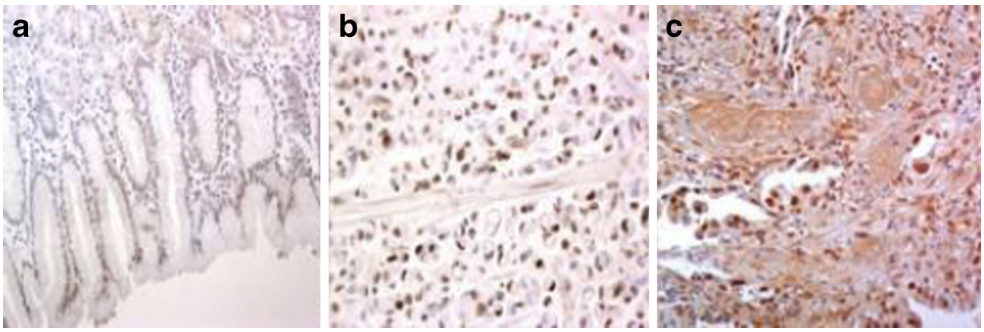

Fig. 3 Representative pictures of immunoreactivity of 8-OHdG in the gastric tissues. **a** Absent 8-OHdG reactivity in normal gastric tissue (IHC, ×20). **b** Medium reactivity of 8-OHdG in diffuse type gastric cancer (IHC, ×40). **c** High reactivity of 8-OHdG in intestinal type gastric cancer (IHC, ×40)

burst [15, 35]. Such a respiratory burst would be expected to create increased ROS, and thereby 8-OHdG, such as we have found. As indicated in previous studies, we also found increased immunoreactivity for 8-OHdG in corpus tissues of our patients compared to those collected from the antral region. This could be related to the fact that bacteria infecting the corpus region have a closer association with the mucosa than bacteria in the antral region [36]. Furthermore, *H. pylori* corpus gastritis is mostly associated with hypochlorhydria, a condition that facilitates the acute phase of infection.

In gastric cancer cases, we observed increased 8-OHdG immunoreactivity with a statistically significant difference in *cagA +ve* as compared to *cagA −ve H. pylori* infected tissues. As shown in Table 4, 8-OHdG immunoreactivity in gastritis cases was correlated with the severity of gastric inflammation. Table 3 shows that *cagA +ve H. pylori* infection and Table 4 shows that 8-OHdG immunoreactivity were considerably more frequently observed in intestinal type gastric cancer than in diffuse gastric cancer. Although the role of *cagA* gene and 8-OHdG expression in the development of intestinal type gastric cancer requires further study, recruiting a high number of gastric cancer cases, our findings are important considering the fact that intestinal type gastric cancer is the most prevalent type of gastric cancer in the Pakistani population. In particular, in future, it would be important to determine the particular *cagA* genotype that is most frequently associated with high expression of 8-OHdG among intestinal type gastric cancer cases.

H. pylori infection with *cagA +ve* strains is the most highly studied factor in the development of gastric cancer. However, in the diffuse type gastric cancer cases, we observed that *cagA -ve H. pylori* infection exhibited a higher (moderate) level of 8-OHdG expression compared to *H. pylori* uninfected cases. The results indicate that in a developing country such as Pakistan, where there are poor environmental conditions with low socioeconomic status, malnutrition and smoking, the majority of individuals will have stomachs with exposure to high levels of ROS formation [30, 37–39]. In this frequent condition, infection with *cagA −ve H. pylori* strains are still able to induce severe DNA damage that contributes to the development of gastric cancer. Many other factors and pathways may be implicated in the pathogenesis of gastric cancer. Our study however, did not address these other variables, which is a limitation of this study.

In conclusion, our findings are consistent with the hypothesis that infection with *H. pylori cagA +ve* strains results in severe degrees of inflammation and oxidative DNA damage leading to progression to gastric cancer. The *cagA* gene expression and the detection of 8-OHdG adducts in gastric epithelium can serve as potential early biomarkers of *H. pylori*-associated gastric pre-cancerous and cancerous lesions.

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Conflict of interest None by any of the authors.

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