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PARP1: A potential biomarker for gastric cancer

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ABSTRACT

Gastric cancer (GC) is the third leading cause of cancer mortality worldwide, with an overall 5-y survival rate of 25%. The majority of GCs are caused by infectious agents, including the bacterium *Helicobacter pylori* (*H. pylori)* and *Epstein–Barr virus (EBV)*. Furthermore, inappropriate repair of DNA damage can also result in genomic instability, which has shown to be a key factor in carcinogenesis of different regions including gastric region. Present study was designed to explore the association between base excision repair pathway genes, *PARP1* and *APEX1* and gastric pathology and *H. pylori* infection. Two hundred gastric cancer tissue samples (114 *H. pylori* positive and 86 *H. pylori* negative) and adjacent uninvolved area taken as controls was used for expression analysis of *BER* pathway genes at mRNA level and protein levels using quantitative PCR (qPCR) and immunohistochemistry (IHC) respectively. Oxidative stress and DNA damage was also determined by measuring the level of antioxidant enzymes and comet assay respectively. Significant upregulation in *PARP1* ($p < 0.001$) and *APEX1* ($p < 0.02$) was observed in GC tissue samples compared to controls and this upregulation was more pronounced in *H. pylori* positive cases (HPGC) (*PARP1*, $p < 0.02$: APEX1, $p < 0.04$) than *H. pylori* negative cases (HNGC). Upregulation of *BER* pathway genes in *HPGC* was found correlated with smoking status ($p < 0.0001$), T stage ($p < 0.01$) and lymph node metastasis ($p < 0.03$). Moreover, immunohistochemical staining of *BER* pathway genes was found correlated with a number of clinicopathological characteristics such as tumor type ($p < 0.03$), tumor size ($p < 0.01$) and lymph node metastasis ($p < 0.01$). Expression levels of *APEX1* and *PARP1* gene also correlated with increased oxidative burden ($p < 0.0001$) and DNA damage ($p < 0.001$) in GC patients. Survival analysis showed that upregulation of *PARP1* gene was associated with poor overall survival outcome of gastric cancer patients (HR = 2.04 (95% CI = 1.10–3.76; $p < 0.02$). Univariate and multivariate cox regression analysis showed the upregulated *PARP1* gene (HR = 5.03; 95%CI (2.22–11.35); p = 0.0001), positive smoking status (HR = 3.58; 95%CI (1.67–7.65); p = 0.001), positive status for *H pylori* infection (HR = 4.38; 95%CI (1.82–10.56); p = 0.001) and advance N-stage (HR = 5.29; 95%CI (2.28–12.24); p = 0.0001) were independent prognostic factors for gastric cancer and may serve as a valuable biomarker for the diagnosis and progression of GC and can be helpful in developing individualized treatment strategies for treating GC.

1. Introduction

Gastric cancer (GC) is among the most common malignancies ranked as fifth most common cancer. It accounts for over-all cancer 7% incidence and 9% mortality rate respectively of cancers [11,41]. In Pakistan, incidence rates of GC have been reported as 6.0 per 100,000 in males and 3.2 per 100,000 in females [22,47]. Gastric cancer is a multifactorial disease, including genetic and epigenetic changes in oncogenes, tumor suppressor genes, DNA repair pathway genes, cell cycle pathway genes and *H pylori* infection [34]. Among DNA repair pathway genes, base excision repair (BER) pathway genes constitute the main process for correcting the endogenous DNA damages mainly induced by the action of reactive oxygen species (ROS) [19].

In BER pathway, *APEX1* is considered a rate limiting enzyme, which hydrolyzes 5 backbones of the DNA molecule and generates 3 hydroxyl groups 3 OH and 5 deoxyribose phosphate groups. The role of *APEX1* has been studied in various functional studies. It has been demonstrated that *APEX1* activity significantly increases on exposing the human cells to different amounts of oxidizing agents. Its expression is positively correlated with patient survival and aggressiveness of cancer such as in

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pancreatic and ovarian cancer [3], breast cancer [7], and osteosarcoma [45].

In BER pathway, *Poly (ADP-ribose) polymerase 1 (PARP-1)* gene functions as a DNA double strand break-sensing protein, and its activation is one of the early responses to DNA damage. *PARP1* gene is located inside the nucleus where its main task is to detect and start cellular response against any chemical or metabolic based SSBs. *PARP1* is an effective modifier in case of cellular injuries like DNA lesion formation, strand breakage and particularly oxidative stress [4]. Abnormal expression of *PARP1* gene has been reported in various different cancers, like glioblastoma [33], small cell lung cancer [27] and colon cancer [10].

Variations in BER pathway genes such as *APEX1* and *PARP1* have been reported in different cancers [3,14,25,43,45], but fewer studies have reported expression levels of *APEX1* and *PARP1* gene in gastric cancer with respect to *H. pylori* infection. The aim of this study was to investigate the expression variations of BER pathway gene (*APEX1, PARP1*) in *H. pylori* positive gastric cancer (HPGC) and *H. pylori* negative gastric cancer (HNGC) tissues. The expression levels of selected genes were also correlated with different pathological parameters of GC, oxidative stress and DNA damage.

2. Materials and methods

In present study two hundred gastric tumors and adjacent uninvolved sections taken as controls, were used. Samples were recruited after the surgical procedure from different hospitals of Pakistan including Pakistan institute of medical sciences (PIMS), Holy Family hospital and Military Hospital (MH) Rawalpindi.

The Inclusion criteria for patients were: (i) histologically confirmed gastric cancer; (ii) no previous chemotherapy, radiotherapy or surgery for the tumor; (iii) no previous history of cancer.

The specific tumor core, invasive margins and adjacent healthy mucosa were obtained from these surgically removed tissues and directly stored in RNA later at −80 °C. Tissues were subjected to cryosectioning and stained with hematoxylin and eosin (H/E). Stained slides were then examined and verified by a consultant histopathologist for the presence of cancerous cells. While uninvolved healthy tissue, at a distance of 2 cm away from the tumor was used as controls. The presence of *H. pylori* colonization in each sample was determined by microscopic examination of May-Giemsa stained specimens. Histological diagnosis and tumor classification was based on Lauren's criteria. At patient's recruitment, informed consent was obtained from each subject and each participant was then interviewed to seek detailed information on demographic characteristics and lifetime history of tobacco use. Medical record for tissue donor patients including the TNM stage, differentiation grade, histological type and *H. pylori* infection was also recruited in present study. The study was conducted after prior approval of COMSATS University Islamabad, Pakistan and collaborating hospitals. The demographic parameters of two hundred gastric cancer patients were given in Table 1.

2.1. RNA extraction and quantitative PCR

RNA was extracted from tissue sections using Trizol method and extracted RNA was converted into cDNA using the SuperScript III First Strand Synthesis system (Invitrogen). The synthesized cDNA was stored at 4 °C for further procedure.

Primers specific for selected genes such as *PARP1*, *APEX1* and *β-actin* (internal control) was designed from integrated DNA Technology (IDT). Each qPCR was performed in a 10 µL reaction mixture containing 1 µL cDNA, 1 µL of each forward and reverse primer, 5 µL SYBR Green Master Mix, and 2 µL RNase-free water. qPCR was performed using the StepOnePlus PCR system (Applied Biosystems) at 56 °C under standard conditions. The relative mRNA expression of *PARP1*, *APEX1* was computed using the 2-delta delta Ct analysis method with *β-actin* as the reference gene.

Table 1
Univariate and multivariate analysis of Cox Regression Analysis of overall survival in relation to clinicopathologic characteristics.

Characteristics	N	Univariate analysis HR (95% CI), p-value	Multivariate analysis HR (95% CI), p-value
Age			
< 45	55	1.00	–
> 45	145	0.64 (0.18-2.21), 0.48	–
Gender			
Male	78	1.00	–
Female	122	1.07 (0.55-2.06), 0.84	–
Smoking			
Non-smokers	55	1.00	1.00
Smokers	145	2.96 (1.29-6.76), 0.01	3.58 (1.67-7.65, 0.001)
Tumor type			
Other type	87	1.00	–
Adenocarcinoma	113	1.16 (0.71-1.87), 0.55	–
***H pylori* status**			
H pylori negative	86	1.00	1.00
H pylori positive	114	3.15 (1.32-8.57), 0.01	4.38 (1.82-10.56), 0.001
T-stage			
T1-T2	112	1.00	–
T3-T4	88	1.17 (0.52-2.60), 0.70	
N-stage			
No	165	1.00	1.00
N1-N2	35	3.07 (1.31-7.21), 0.01	5.29 (2.28-12.24), 0.0001
M-stage			
Mo	180	1.00	1.00
M1	20	2.45 (1.15-5.21), 0.02	1.76 (0.91-3.38), 0.09
Grade			
Well-moderately	120	1.00	–
Poorly	80	1.27 (0.75-2.14), 0.37	–
PARP1			
Downregulation	38	1.00	1.00
Upregulation	162	3.97 (1.75-9.03), 0.001	5.03 (2.22-11.35), 0.0001
APEX1			
Downregulation	48	1.00	–
Upregulation	152	1.08 (0.28-4.03), 0.91	–

HR, Hazzard ratio; CI, confidence interval; p-value ≤ 0.05 considered as statistically significant.

2.2. Immunohistochemistry

Expression of *APEX1* and *PARP1* proteins was analyzed by immunohistochemical staining using 200 gastric cancer samples along with adjacent non-cancerous tissues taken as controls. Immuno-histochemical analysis was performed using the DAB chromogen staining kit (Sigma) as described previously [29].

Both tumor and control tissue slides were incubated with mouse anti-*APEX1* (Santa Cruz Biotechnology, Inc. UK) and anti-*PARP1* (Novus Biologicals, Inc. USA) of 1:500 and 1: 1000 dilutions respectively for 1 h at room temperature. Both positive and negative controls were used to validate the procedure and specificity of primary and secondary antibody. Ductal breast carcinoma was used as positive control for *APEX1* and *PARP1*. For negative controls same procedure was used, except for the primary antibody, where phosphate buffer saline (PBS) was added instead of primary antibody.

Three independent histopathologists, unaware of the clinical data, evaluated the immuno-histochemical reactions using light microscopy and the relative intensities. Any disagreement on results was ruled out by mutual consent of the histopathologists. At least ten high power fields were scanned to randomly count the tumor cells and evaluate the immunoreactivity by the following formula:

Immunoreactive score = intensity score × proportion score

Immunoreactivity was scaled on a spectrum of score values from 0 to 12. Score of 0–4 fell in the category of low immunoreactivity, whereas high immunoreactivity was denoted with a score greater than 4. Intensity was graded from 0 to 3 for negative to strong staining.

Intensity score was specified into three levels ranging from (0 to 3); 0 for negative intensity, 1 for weak intensity, 2 for moderate intensity and 3 for strong staining intensity. If there was not a single positive cell the proportion score was valued as 0. Similarly, an average of ≤10%, 11–50%, 51–80% and > 80% positive cells distribution earned the score of 1, 2, 3 and 4 respectively.

2.3. Measurement of oxidative stress

Gastric cancer tissue sample and adjacent controls taken from patients were treated with saline solution to prepare tissue lysate. Two aliquots were prepared, one aliquot was used for the measurement of oxidative stress and second aliquot was stored at −80 °C for the assessment of DNA damage. Oxidative stress was evaluated by quantitative analysis of three antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Enzyme analysis was carried out using human specific ELISA kits purchased from Abcam (UK). Absorbency of enzymes was checked in microplate reader (Platos R496, AMP Diagnostics), and sample concentrations were read from calibration curve.

2.4. Comet assay

Procedure for alkaline comet assay used in this study was adopted by [2] with slide modifications. Gastric cancer tissue and adjacent control tissues were homogenized in saline solution and process for comet assay to detect the overall DNA damage in gastric tissue and adjacent section (taken as controls). Three layered procedure was used and samples were sandwiched between first and second layer of LMP agarose gel. 50 comets were scored for each sample using fluorescent microscope (Leica) equipped with filters and digital cameras. Comets were analyzed using Casp-Labsoftware.

2.5. Statistical analysis

Chi square test and *t*-test are used for the assessment of expression levels of *APEX1* and *PARP1* in gastric tumors compared to adjacent controls. Spearman correlation was used to compare the expression level of *APEX1* and *PARP1* gene with histopathological parameters of gastric cancer. Receiver Operating curves (ROC) were generated and area under ROC curve was calculated to evaluate the diagnostic value of *APEX1* and *PARP1* expression level in discriminating tumor and non-tumor states of the samples. Kaplan Maier analysis, univariate and multivariate cox regression analysis was also performed to assess the survival status of gastric cancer patient with deregulated levels of selected genes. SPSS and Medcalc was used for these statistical tests.

3. Results

Present study was designed to determine the expression levels of *APEX1* and *PARP1* gene in gastric tumors compared to controls. A significant upregulation of *APEX1* gene ($p < 0.03$) was observed in gastric tumors compared with controls (Fig. 1A). This upregulation was more pronounced in advance T stage ($p < 0.001$) and N-stage ($p < 0.001$) compared to early TNM stage of gastric cancer patients as shown in Fig. 1B and C. Expression levels of *APEX1* gene were also correlated with *H. pylori* and smoking status of gastric cancer and significant upregulation was observed in HPGC ($p < 0.01$) compared to HNGC (Fig. 1E). In case of smoking status, expression level of *APEX1* was observed significantly higher in heavy smokers (HS; $p < 0.001$) compared to light smokers (LS) and non-smokers (NS) gastric cancer patients. These results are shown in Fig. 1F.

Significant upregulation of *PARP1* gene was observed in gastric tumor tissues compared to adjacent control sections taken as controls. Significant upregulation of *PARP1* gene was observed in gastric tumors ($p < 0.0001$) compared to controls (Fig. 2A) and this upregulation was more pronounced in advance T-stage ($p < 0.02$), N-stage ($p < 0.04$) and M-stage ($p < 0.001$) compared to early TNM stage of gastric patients (Fig. 2B–D). Expression level of *PARP1* gene was also compared with *H. pylori* status and smoking status of gastric cancer patients. Significant upregulation of *PARP1* gene was observed significantly higher in HPGC ($p < 0.02$) compared to HNGC as shown in Fig. 2E. In case of smoking status, significant upregulation of *PARP1* gene was observed in heavy smokers ($p < 0.02$) compared to light smokers and non-smokers gastric cancer patients as shown in Fig. 2F.

3.1. IHC analysis

Expression level of *APEX1* and *PARP1* genes was also assessed using the immunohistochemistry analysis in gastric tissue sections compared

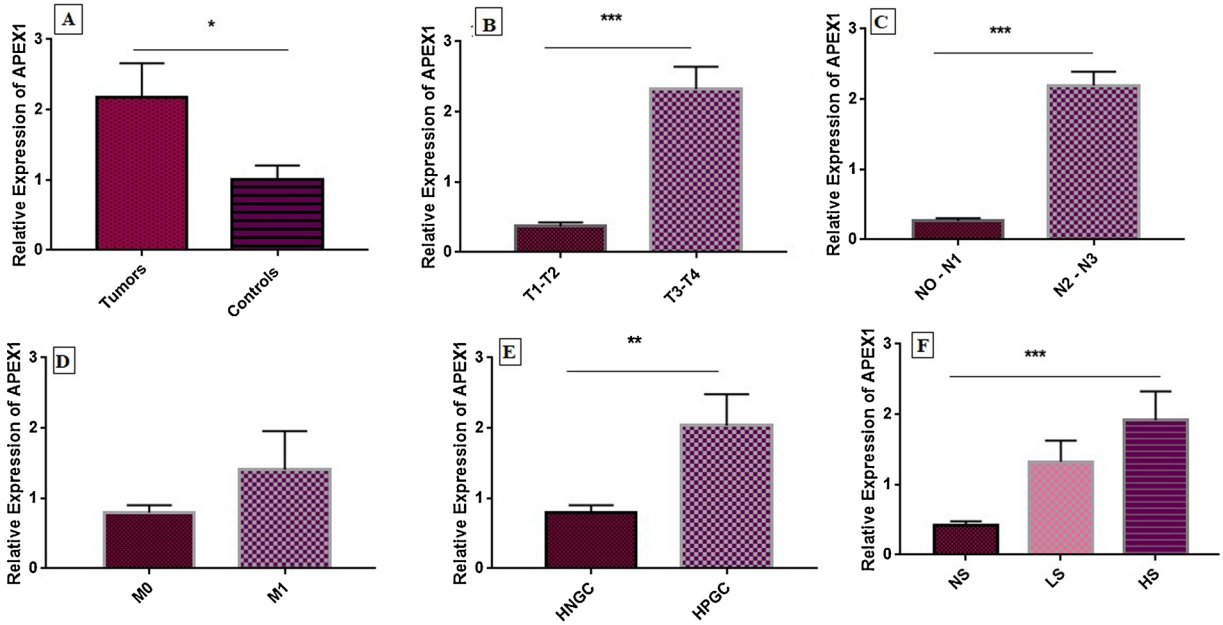


Fig. 1. Relative expression of *APEX1* gene in (A) gastric tumor samples and adjacent healthy sections (B) different T-stage of GC (C) lymph node status of GC (D) metastasis stage of GC (E) *H. pylori* negative and *H. pylori* positive GC (F) smoking status of GC patients. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

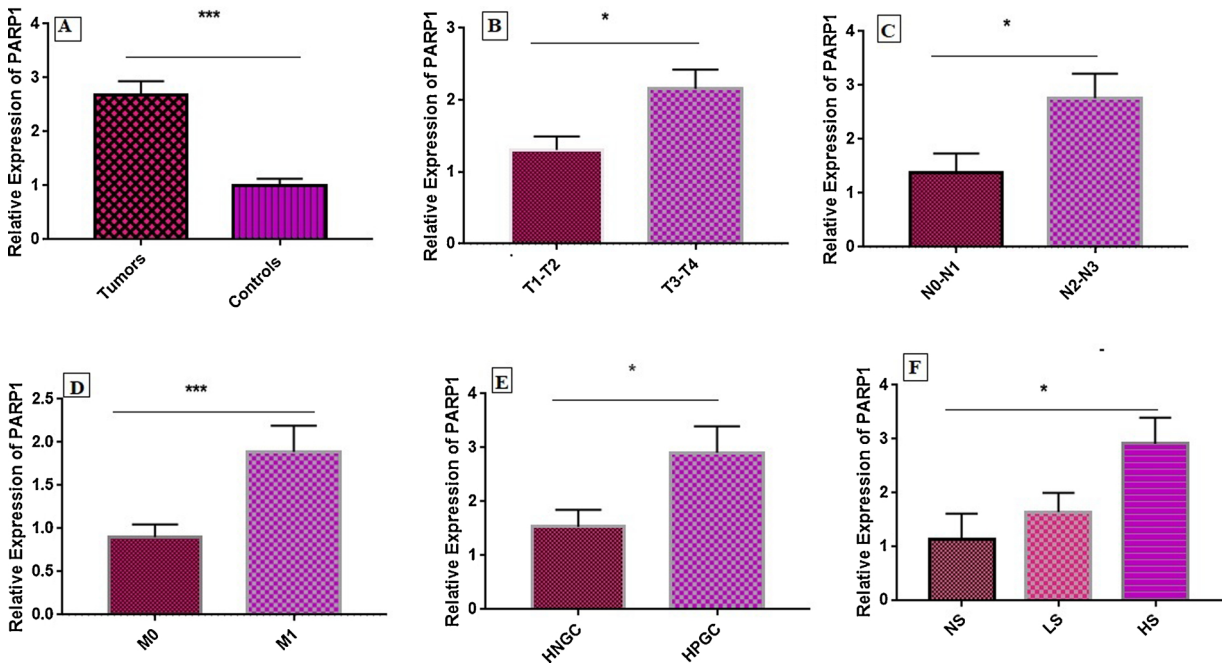


Fig. 2. Relative expression of *PARP1* gene in (A) gastric tumor samples and adjacent healthy sections (B) different T-stage of GC (C) lymph node status of GC (D) metastasis stage of GC (E) *H. pylori* negative and *H. pylori* positive GC (F) smoking status of GC patients. $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

to adjacent uninvolved section taken as controls. Upregulated expression of *APEX1* protein was observed in GC tissue compared to controls and this upregulation was observed higher in different histological types of GC compared to controls as shown in Fig. 3. These histological types are adenocarcinoma (Fig. 3B), signet type (Fig. 3C) and GIST (Fig. 3D). *PARP1* protein also showed the upregulated expression in GC tumor compared to control section as shown in Fig. 4. This upregulated pattern was also observed in different histological type of GC such as adenocarcinoma (Fig. 4B), signet type (Fig. 4C) and GIST (Fig. 4D)

In case of *APEX1* protein expression, 76% cases showed upregulation and 24% cases showed downregulation as shown in Fig. 5A. The relative immunoreactive intensity showed that 60% cases showed strong immunoreactivity, 31% cases showed moderate immunoreactivity and 9% cases showed week immunoreactivity (Fig. 5B).

In case of *PARP1*, 81% cases showed upregulation and 19% cases showed downregulation as shown in Fig. 5C. Immunoreactivity intensity showed that 72% cases showed strong immunoreactivity, 17% cases showed moderate immunoreactivity and 11% cases showed weak

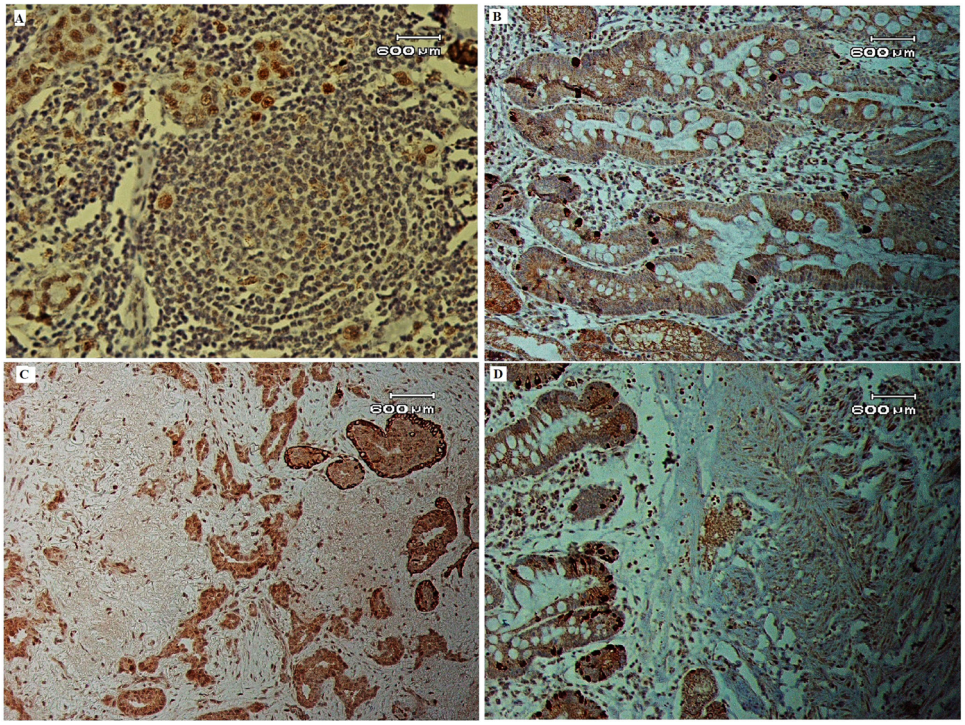


Fig. 3. Immunohistochemistry analysis of *APEX1* expression in gastric cancer tissue samples. Immunohistopathological analysis was performed to examine the *APEX1* expression on (A) control (B) adenocarcinoma type of GC, (C) signet type of GC (D) GIST type of GC. Representative images A, B, C, D (×40) were shown.

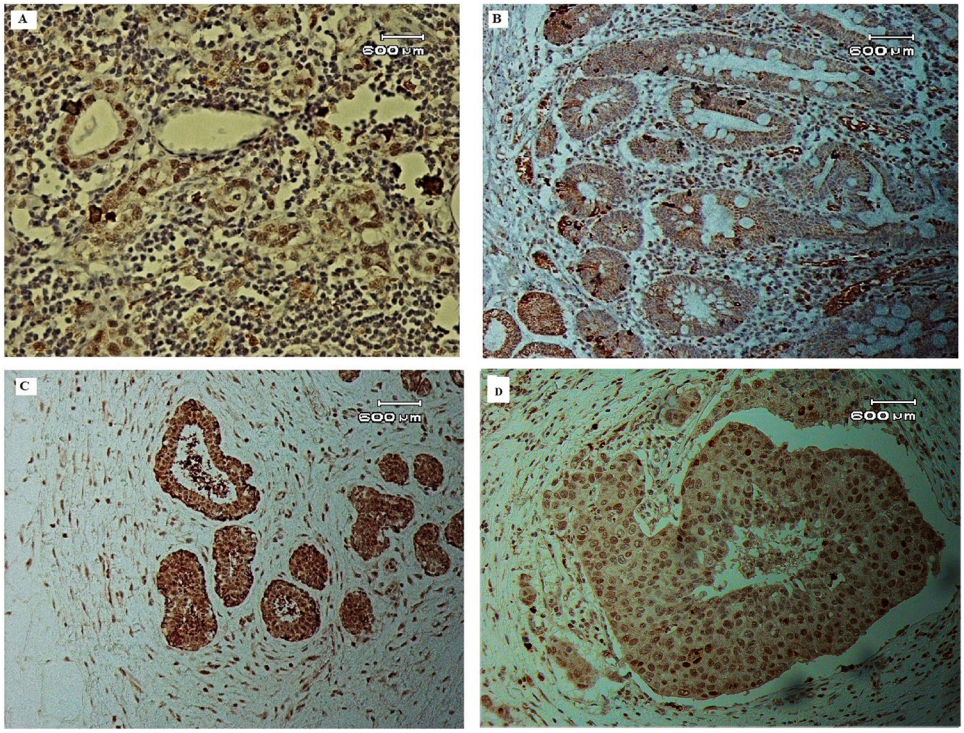


Fig. 4. Immunohistochemistry analysis of *PARP1* expression in gastric cancer tissue samples. Immunohistopathological analysis was performed to examine the *PARP1* expression on (A) control (B) adenocarcinoma type of GC, (C) signet type of GC (D) GIST type of GC. Representative images A, B, C, D (×40) were shown.

immunoreactivity (Fig. 5D).

3.2. Gene to gene and gene to histopathological parameters correlation

Gene to gene and gene to histopathological parameters was correlated using the spearman correlation coefficient analysis and shown in Table 2. In case of correlation at mRNA level significant positive correlation was observed between T-stage vs *APEX1* ($r = 0.479^{**}$, $p < 0.01$), N-stage vs *APEX1* ($r = 0.439^{***}$, $p < 0.001$), grade vs *APEX1* ($r = 0.329^{*}$, $p < 0.04$), T-stage vs *PARP1* ($r = 0.629^{***}$, $p < 0.0001$), N-stage vs *PARP1* ($r = 0.215^{*}$, $p < 0.05$), M-stage vs *PARP1* ($r = 0.630^{***}$, $p < 0.0001$), grade vs *PARP1* ($r = 0.331^{*}$,

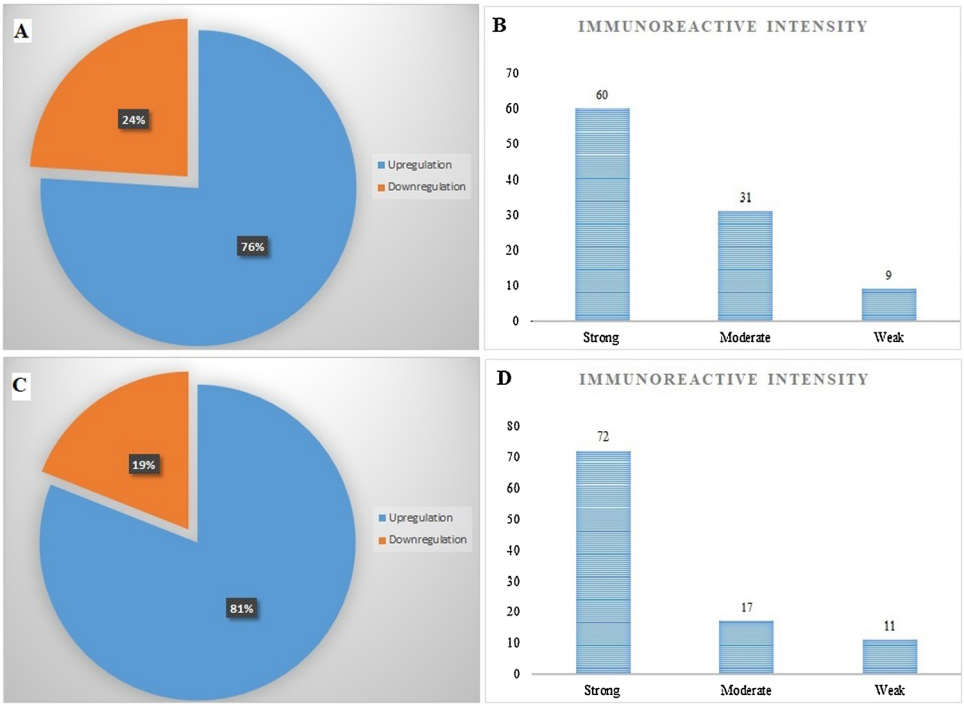


Fig. 5. (A) Changed expressional levels and (B) immunoreactive intensity of *APEX1* protein in gastric cancer tissue samples. (C) Changed expressional levels and (D) immunoreactive intensity of *PARP1* protein in gastric cancer tissue samples.

Table 2
Correlations between BER pathway genes (*APEX1, PARP1*) and histopathological parameters of GC.

T-stage	N-stage	M-stage	Grade	Type	*APEX1*	*PARP1*
T-stage	**0.312***	**0.627*****	**0.279***	0.109	**0.479****	**0.629*****
N-stage		**0.225***	**0.269***	−0.049	**0.439*****	**0.215***
M-stage			**0.519*****	−0.029	0.117	**0.630*****
Grade				**0.219***	**0.329***	**0.331***
Type					0.069	0.088
APEX1						**0.429****
Protein level						
T-stage	**0.312***	**0.627*****	**0.279***	0.109	**0.345***	**0.613*****
N-stage		**0.225***	**0.269***	−0.049	**0.291***	**0.339***
M-stage			**0.519*****	−0.029	**0.266***	**0.413****
Grade				**0.219***	0.039	**0.649*****
Type					0.089	0.048
APEX1						**0.699*****

†Spearman correlation coefficients.
The expression levels of *APEX1* and *PARP1* genes for gastric cancer cases were determined based on mRNA and protein analysis.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$. The p values were computed using one-way ANOVA and $\chi 2$ test.

$p < 0.04$) and *APEX1* vs *PARP1* ($r = 0.429^{**}$, $p < 0.002$) in GC patients as shown in Table 2.

In case of correlation at protein level significant positive correlation was observed between T-stage vs *APEX1* ($r = 0.345^{*}$, $p < 0.04$), N-stage vs *APEX1* ($r = 0.291^{*}$, $p < 0.05$), M-stage vs *APEX1* ($r = 0.266^{*}$, $p < 0.05$), T-stage vs *PARP1* ($r = 0.613^{***}$, $p < 0.0001$), N-stage vs *PARP1* ($r = 0.339^{*}$, $p < 0.04$), M-stage vs *PARP1* ($r = 0.413^{**}$, $p < 0.01$), grade vs *PARP1* ($r = 0.649^{***}$, $p < 0.0001$) and *APEX1* vs *PARP1* ($r = 0.699^{***}$, $p < 0.000a$) in GC patients as shown in Table 2.

3.3. ROC analysis and Kaplan-Meier analysis

Receiver Operating curves (ROC) of *APEX1* and *PARP1* was generated to assess the diagnostic value of selected gene in gastric cancer. Area under the curve (AUC) and 95% confidence interval (CI) was also calculated in this analysis. The area under the curve for *APEX1* gene was 56 (95% CI: 0.45–0.64; $p < 0.94$, Fig. 6A) and for *PARP1* gene was 84 (95% CI:0.76–0.92; $p < 0.0001$, Fig. 6B) as shown in Fig. 6. To further validate the diagnostic value of selected genes, Kaplan-Meier analysis and hazard ratio was calculated using SPSS software. Kaplan-Meier analysis showed that increased expression levels of *APEX1* gene was associated with HR of 0.86 (95% CI = 0.48–1.54; $p < 0.6$) and the increased levels of *PARP1* gene was associated with HR of 2.04 (95% CI = 1.10–3.76; $p < 0.004$). Kaplan–Meier survival curves for the selected genes (*APEX1* and *PARP1*) are displayed in Fig. 6C and D respectively. In case of parameters, positive status for *H. pylori* infection was associated with HR of 2.15 (95%CI = 1.22–3.78; p = 0.0001) and positive smoking status was associated with HR of 1.57 (95% CI = 0.917–2.69; p = 0.03) in gastric cancer patients as shown in Fig. 7A and B.

Overall survival and hazard ratio was calculated using the Cox regression analysis as shown in Table 1. Univariate analysis showed the upregulation of *PARP1* gene was associated with decrease overall survival (HR = 3.97; 95%CI (1.75–9.03); p = 0.001) in gastric cancer patients. However, upregulation of *APEX1* was showed no effect on the overall survival of gastric cancer patients (HR = 1.079; 95%CI (0.28–4.03); p = 0.91) as shown in Table 1. Further analysis showed that positive status of *H pylori* (HR = 3.15; 95%CI (1.32–8.57); p = 0.01) and positive status of smoking (HR = 2.96; 95% CI (1.29–6.76); p = 0.01) was also showed the decrease survival of gastric cancer patients (Table 1). In case of histopathological parameters, advance N-stage (HR = 3.07; 95%CI (1.31–7.21); p = 0.01) and M-stage (HR = 2.45; 95%CI (1.15–5.21); p = 0.01) were associated with worse

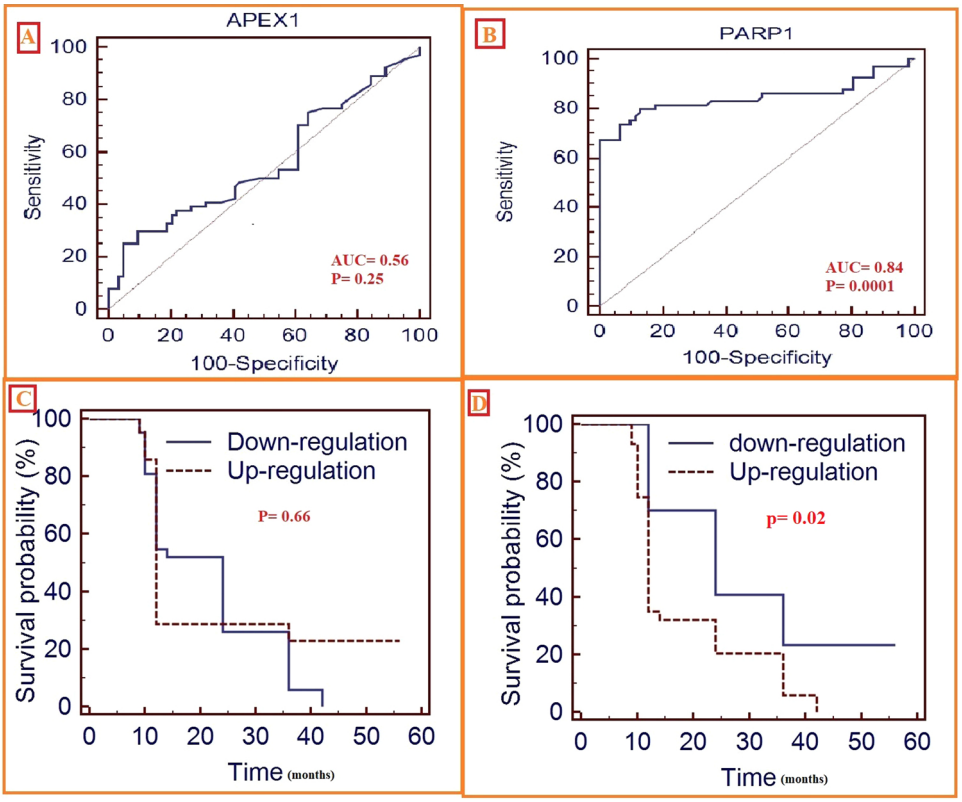


Fig. 6. ROC curve analysis of (A) *APEX1* and (B) *PARP1* in gastric cancer patients. Kaplan–Meier survival curves for (C) *APEX1* and (D) *PARP1* in gastric cancer patients.

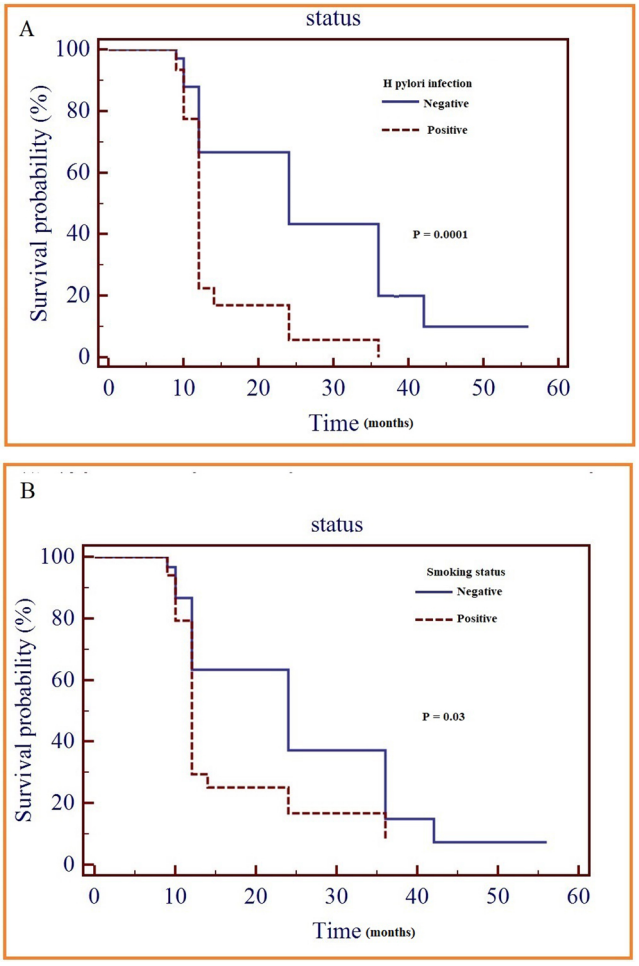


Fig. 7. Kaplan–Meier survival curves for (A) *H. pylori* positive and *H. pylori* negative gastric cancer patients and (B) smokers and non-smokers gastric cancer patients.

overall survival in gastric cancer patients as shown in Table 1.

Multivariate cox regression analysis was also performed and summarized in Table 1. Similar to univariate analysis, upregulation of *PARP1* gene (HR = 5.03; 95%CI (2.22–11.35); p = 0.0001) was independent poor prognostic factor in gastric cancer patients. In case of clinicopathological factors, positive smoking status (HR = 3.58; 95%CI (1.67–7.65); p = 0.001), positive status for *H pylori* infection (HR = 4.38; 95%CI (1.82–10.56); p = 0.001) and advance N-stage (HR = 5.29; 95%CI (2.28–12.24); p = 0.0001) were also independent prognostic factors as mentioned in Table 1.

3.4. Oxidative stress assay

In the present study oxidative stress was also measured by determining the level of antioxidant enzymes such as SOD, Gpx and CAT in gastric tumor section compared to controls, as shown in Fig. 8. A significant lower level of SOD ($p < 0.002$), Gpx ($p < 0.001$) and CAT ($p < 0.001$) was observed in gastric tumor sections compared to controls (Fig. 8A). The levels of these antioxidant enzymes was found significantly lower in HPGC [(SOD, $P < 0.0001$), (Gpx, $p < 0.002$) and (CAT, $p < 0.001$)] compared to HNGC as shown in Fig. 8B. In case of smoking status, significantly lower level of SOD ($p < 0.001$), Gpx ($p < 0.004$) and CAT ($p < 0.002$) was observed in heavy smokers compared to light smoker and non-smoker GC patients as shown in Fig. 8C.

3.5. DNA damage assay

Comet assay was used to assess the numbers of comets and level of DNA damage in gastric tumor sections (Fig. 9A1) compared to adjacent uninvolved section taken as controls (Fig. 9A2). Number of cells with comets was observed significantly higher in gastric tumor section ($p < 0.0001$) compared to adjacent control section of gastric tissues as shown in Fig. 9B. DNA damage was further compared with *H. pylori* status and smoking status of gastric cancer patients. Number of cells with comets was observed significantly higher in HPGC vs HNGC and heavy smokers compared to light smokers and non-smokers gastric cancer patients as shown in Fig. 9C and D respectively.

Furthermore, six comet parameters were also calculated in present study as shown in Fig. 9E. Among these six parameters, comet length ($p < 0.0001$), tail length ($p < 0.002$), %DNA in tail ($p < 0.01$), tail moment ($p < 0.004$) and olive tail moment ($p < 0.001$) were observed significantly higher in gastric tumor sections compared to control sections. These six comet parameters were also correlated with HPGC vs HNGC and heavy smokers compared to light smokers and non-smokers gastric cancer patients as shown in Fig. 9E.

4. Discussion

Gastric cancer is a multifactorial disease and its etiology includes both dietary and non-dietary factors. Smoking [26], alcohol use [28], obesity [15], excessive salt intake [1] and high red meat or processed meat consumption [48] are associated with modest increase in risk of gastric cancer [17]. Infection with *H. pylori* is considered to be an established risk factor in gastric cancer pathogenesis [42]. EBV infection

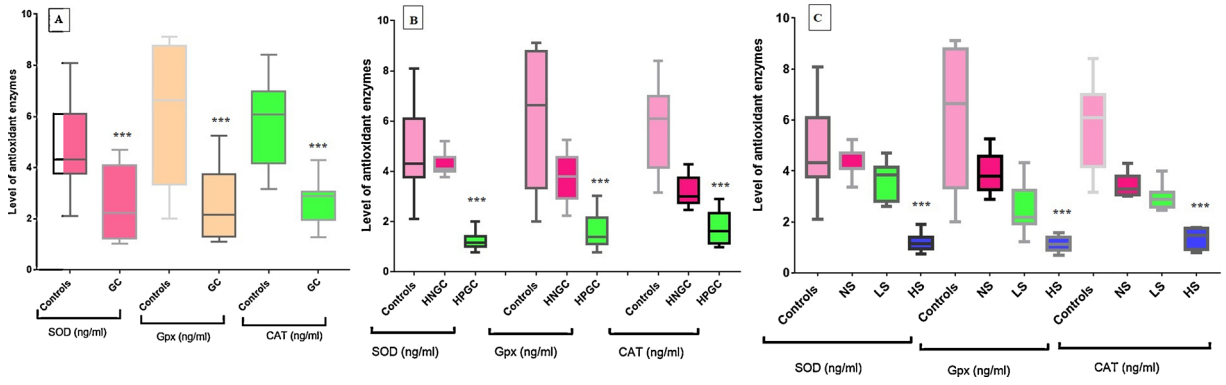
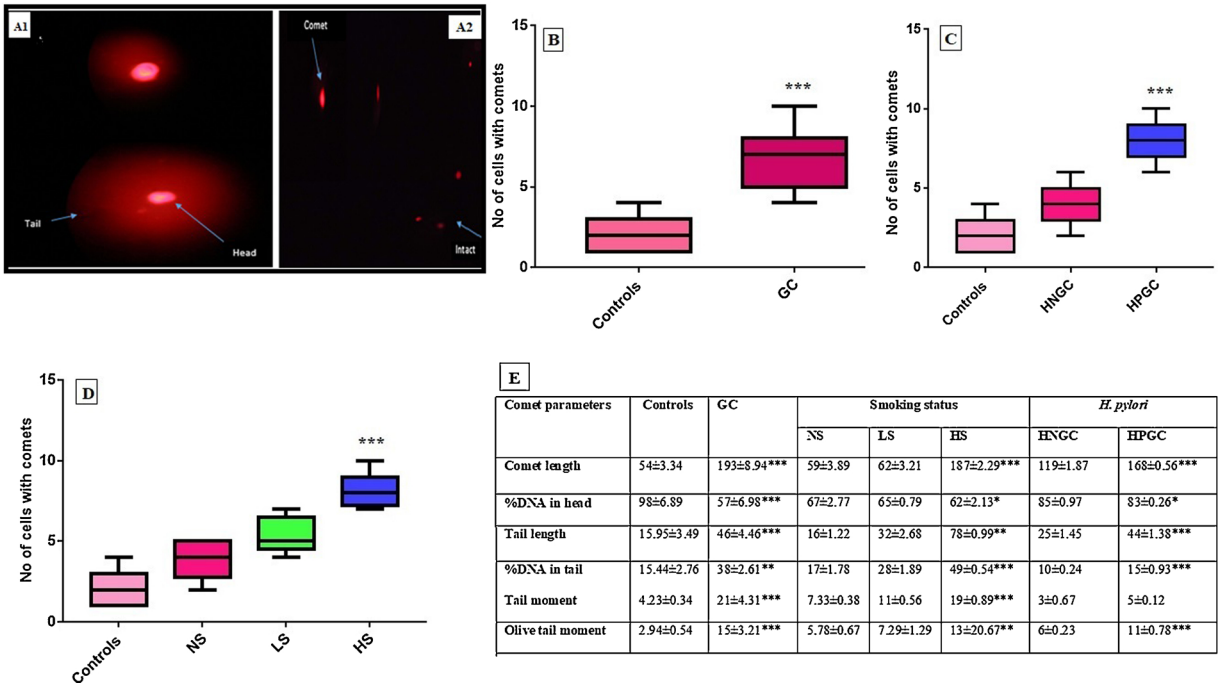


Fig. 8. Measurement of oxidative stress by determining the level of antioxidant enzymes such as SOD, Gpx and CAT in (A)gastric tumor samples vs controls, (B) *H. pylori* positive (HPGC) and *H. pylori* negative gastric cancer patients (HNGC) vs controls, (C) non-smokers (NS), low smokers (LS) and high smokers (HS) vs controls. *p < 0.05, **p < 0.01, ***p < 0.001.



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Comet parameters	Controls	GC	Smoking status			*H. pylori*	
			NS	LS	HS	HNGC	HPGC
Comet length	54±3.34	193±8.94***	59±3.89	62±3.21	187±2.29***	119±1.87	168±0.56***
%DNA in head	98±6.89	57±6.98***	67±2.77	65±0.79	62±2.13*	85±0.97	83±0.26*
Tail length	15.95±3.49	46±4.46***	16±1.22	32±2.68	78±0.99**	25±1.45	44±1.38***
%DNA in tail	15.44±2.76	38±2.61**	17±1.78	28±1.89	49±0.54***	10±0.24	15±0.93***
Tail moment	4.23±0.34	21±4.31***	7.33±0.38	11±0.56	19±0.89***	3±0.67	5±0.12
Olive tail moment	2.94±0.54	15±3.21***	5.78±0.67	7.29±1.29	13±20.67**	6±0.23	11±0.78***

Fig. 9. Comet assay was prformed to measure the comets in (A1) gastric cancer tumors (A2) adjacent uninvolved section taken as controls. Calculation of number of cells with comets in (B) gastric cancer tumors vs controls, (C) *H. pylori* positive (HPGC) and *H. pylori* negative gastric cancer patients (HNGC) vs controls, (D) non-smokers (NS), low smokers (LS) and high smokers (HS) vs controls. (E) Correlation of comet parameters with gastric cancer tumors vs controls, smoking status and *H. pylori* infection status of gastric cancer tumors in comparison with controls. *p < 0.05, **p < 0.01, ***p < 0.001.

also contributes to GC progression either via oncogene expression or inflammation mediated tissue damage mechanisms [31]. Incidence of GC in younger patients is usually associated with familial clustering [39].

In addition to exogenous factors, genetic variations in cell proliferation, antioxidant protection, carcinogen detoxification and DNA repair systems are also critical in GC progression [13,46]. Among these DNA repair pathway, base excision repair (BER) pathway play a significant role in repair of DNA damage produced by ionization radiations and other carcinogens [46]. Any variation in BER pathway genes such as *APEX1* and *PARP1* can alter DNA repair function resulting in an accumulation of DNA damage followed by apoptosis. Abnormalities in the BER pathway genes have been reported in different cancers [14,25,43,45], but few studies have been reported with expression level of *APEX1* and *PARP1* gene in gastric cancer [3]. The aim of this study was to investigate the expression variations of BER pathway gene (*APEX1, PARP1*) in gastric cancer. Expression variation of selected genes were also compared with different causative factors of GC such as presence of *H. pylori* infection and use of tobacco and other kind of smoke. This correlation may helpful to clarify the complicated picture of gastric carcinogenesis. Furthermore, expression level of *APEX1* and *PARP1* gene was also compared with oxidative status and DNA damage of gastric cancer patients.

The expression level of BER pathway gene, *APEX1* was found significantly up regulated in gastric cancer patients compared to controls. Similar findings have been reported in previous studies including pancreatic carcinoma [24], osteosarcoma [45], gastro-oesophageal [3] and colon carcinoma [25]. This upregulated expression of *APEX1* has been observed in a variety of human solid tumors and it is positively interlinked with onset and progression of cancer. However, the role of *APEX1* in tumor advancement is still not well defined [25]. Increased expression of *APEX1* may be associated with mutations [30], methylation of promoter regions, impaired functioning of repair domain [20] and increased oxidative stress [45]. Present study also suggested a positive correlation between *APEX1* transcript level and T-stage, N-stage, tumor grading and metastasis. Similar findings have already been reported in breast cancer [38], colon carcinoma [25] and head and neck cancer [23]. Results of the present study showed that increased level of *APEX1* was significantly associated with aggressive behavior of gastric tumors

Like *APEX1* gene, significant up regulation of *PARP1* gene was also observed in gastric cancer patients when compared to controls. This upregulation was observed more pronounced in advance T-stage, N-stage and M-stage gastric tumors. The findings of present study suggest that *PARP1* may play a role in clinical behavior of gastric cancerogenesis. Our results are found to be concurrent to various previous studies conducted on different carcinomas like in sarcoma [25], colon cancer [10], colorectal cancer [27] and glioblastoma [33]. One possible reason of this upregulation is that *PARP1* overexpression is due to a defective *PARP1* cleavage, reduced apoptosis rate and long term survival of cancer cells [40]. Further Kaplan-Meier analysis showed correlation of decrease 5years survival status of patients with increased *PARP1* gene expression. ROC analysis also confirmed that increased expression of *PARP1* gene can be used as a prognostic marker for prognosis and predisposition of gastric cancer.

Expression levels *APEX1* and *PARP1* gene are also correlated with *H. pylori* infection and significantly higher expression was observed in HPGC compared to HNGC. Several previous reports have suggested that *H. pylori* positive infection resulted in several genetic, epigenetic and different pathway changes [5,37]. This study showed that *H. pylori* infection is associated with high *APEX1* and *PARP1* levels and reduced overall survival. It is possible that *H. pylori* infection may induce the upregulation of selected BER pathway genes, but the mechanism behind this induction need to be explored in further studies. *H. pylori* positive status also results in increased oxidative stress and DNA damage in HPGC compared to HNGC in present study. Although the molecular mechanisms underlying *H. pylori*-induced processes of inflammation and carcinogenesis are unknown, two principal mechanisms have been proposed for these processes: hyper-proliferation of gastric epithelial cells and oxidative damage of stomach mucosa [6]. *Helicobacter pylori* is able to induce polymerphonuclear and mononuclear cell accumulation, which produce reactive oxygen species or reactive nitrogen species that

cause DNA damage to the adjacent epithelial cells [6,18,44]. It has earlier been demonstrated that *H. pylori*-related gastric cancer is accompanied by an increased oxygen free radical formation and peroxidative damage [9,35]. These findings suggest that *H. pylori* infection represents a risk factor triggering the chronic accumulation of DNA damage, leading to degenerative processes of the gastric mucosa, impaired repairing pathway and gastric cancer.

Smoking is another important risk factor of gastric cancer and involvement of smoking in present study was also determined using the criteria of heavy smokers, light smokers and non-smokers gastric cancer patients. Increased level of selected BER pathway genes, oxidative stress and DNA damage was observed in heavy and light smoker HPGC compared to non-smokers HNGC. Previous studies have reported the significant association between DNA repair gene deregulation, smoking status and gastric cancer increased risk [8,16,36]. Tobacco smokers are exposed to many toxic compounds such as nicotine, nitric oxide, carbon monoxide, polyromantic hydrocarbons (PAH), nitrosamines, and aromatic amines [21,32]. Tobacco smoke induces the development of precursor lesions including gastritis, ulceration, and intestinal metaplasia, which ultimately lead to GC [12].

The current study showed significant upregulation of *APEX1* and *PARP1* gene in *H. pylori* positive gastric cancer cases. This upregulation was found associated with increased oxidative stress and DNA damage in gastric cancer patients. Present study also showed the synergistic effect of *H. pylori* infection and use of tobacco and other kind of smoke in deregulation of BER pathway gene and ultimately results in carcinogenesis of gastric region. The study point towards the potential use of these expression variation as marker for use in clinical screening considering the smoking and *H. pylori* virulence. Further replicative studies with larger sample size are warranted to validate our findings.

Conflict of interest

The authors declare that they have no conflict of interests.

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