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Research Article

Frequency of p53 Mutations and Epstein-Barr Virus in Pakistani Gastric Cancer Patients and Their Association with the Tumor Grade

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Abstract

Gastric carcinoma (GC) is the fourth most common cause of cancer-associated death worldwide. Our study aimed to find the prevalence of *TP53* mutations and Epstein-Barr virus (EBV) infection in GC and to investigate their association with the tumor grade. A total of 108 formalin-fixed-paraffin-embedded tissue blocks (98 gastric adenocarcinomas and ten controls) were collected, and DNA was extracted from them. The DNA was used to detect the presence of EBV infection by amplifying the Bam HI W region of the EBV genome in a nested polymerase chain reaction (PCR). Exons 5-7 of *TP53* were amplified and sequenced by a commercial sequencer. Immunohistochemistry (IHC) for the expression of caspase-3 was carried out to determine the apoptosis status. Histopathology revealed that of the 98 adenocarcinoma samples, 63.3% (n=62) were poorly differentiated, whereas 36.7% (n=36) were moderately differentiated. Signet rings were present in 30.6% (n=30), and EBV was detected in 18.4% (n=18). Mutation analyses indicated that 76% of the samples were mutated, of which 8% had single nucleotide variations (SNVs) in exon 5 (g.17371G>A and g.17521A>C). Moreover, 76% of the samples had the same SNV (g.18316T>C) in exon 7. We also discovered three novel SNVs; two in exon 5 (g.17371G>A and g.17521A>C) and one in exon 7 (g.18316T>C). No difference in the expression of caspase-3 was observed. The chi-squared test indicated no significant correlation between *TP53* mutations with EBV infection and tumor grade. In our cohort, young males had a higher prevalence of GC. The detection of EBV suggests it might be a risk factor for GC in our population.

Keywords: Gastric adenocarcinoma, *TP53* mutations, EBV, stomach cancer, SNV, Signet ring, caspase, tumor grade

1. Introduction

Gastric carcinoma (GC) is the fifth most common cancer worldwide and the fourth most common cause of cancer-associated death(Sung et al. 2021b).It accounts for approximately 7.7% of all cancer deaths, while globally, it is the second most common cancer seen in men after lung cancer(Daniyal et al. 2015). The incidence of GC varies globally. It is more common in Korea, Japan, and Eastern Asia. In Pakistan, the mortality rate due to GC is 6541 deaths per year. The prevalence of GC is 6 /100,000 in males and 3.6/100,000 in females in Karachi. However, a recent study reported that the incidence of GC is lower in Karachi compared to other Asian countries, despite the high prevalence of *H. Pylori*, a known contributor to its development (Qureshi et al. 2016). Other studies have shown that higher living standards have led to a decline in the prevalence of H. *pylori*. A shift is happening in the prevalence of GC from developed to developing countries, altering the global prevalence of the disease. This explains why low socioeconomic groups are common

victims of GC (Daniyal et al. 2015). Nevertheless, despite the decrease in prevalence, it is a

Table 1. Correlation of TP53 Mutations with tumor grade and EBV infection

		Exon 5 mutations		
		Positive	Negative	
EBV	Positive	1	1	p>0.05
	Negative	13	10	
Signet Ring	Present	1	8	p>0.05
	Absent	1	15	
Tumor Grade	Moderately differentiated	0	12	p>0.05
	Poorly differentiated	2	11	
Gender	Male	2	15	p>0.05
	Female	0	8	
Exon 7 mutation	Present	2	15	p>0.05
	Absent	0	8	

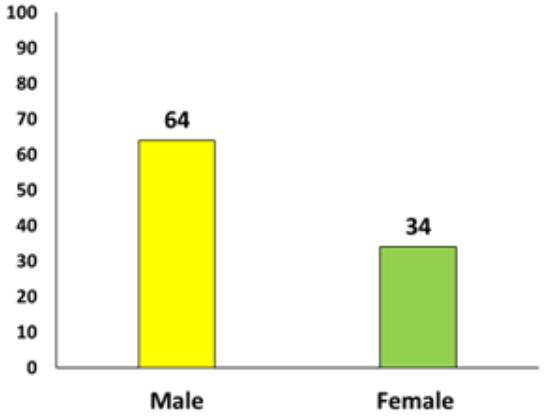
significant cause of cancer-related deaths(Chong et al. 2014).

Several etiologic factors are involved in the development of GC; however, low socioeconomic status, improper diet (salty and spicy food), hereditary factors, and infection with *H. pylori* are the most common. Previous studies have reported that *H. pylori* and inappropriate diet were the main risk factors for distal GC, whereas obesity and gastroduodenal reflux disease commonly contribute to proximal GC (Daniyal et al. 2015, Correa 1992).

Epstein-Barr virus (EBV) has been established as a significant cause of various human malignancies such as Burkitt's lymphoma, nasopharyngeal carcinoma (NPC), Hodgkin's disease, and GC (Crawford 2001, De Aquino et al. 2012). More than 80% cases of lymphoepithelioma-like (LEL) GC, a rare subtype of GC, are EBV positive. Other types of GC have also been identified as EBV-positive even though the ratio is very low (Martin and Green 1995). However, it has been observed that patients with GC and EBV-infected epithelial cells have more viral particles as compared to individuals with only infected B cells, indicating that EBV does, in fact, have some degree of association with GC (Ryan et al. 2009).

TP53 is one of the tumor suppressor genes that control apoptosis and is located on chromosome 17p13 (Matozaki et al. 1992). Any mutation in *TP53* results in the loss of this critical monitoring

function. This will allow cells to progress through the cell cycle with damaged DNA, thus contributing to tumorigenesis(Martin and Green 1995). Mutated *TP53* is more stable compared to



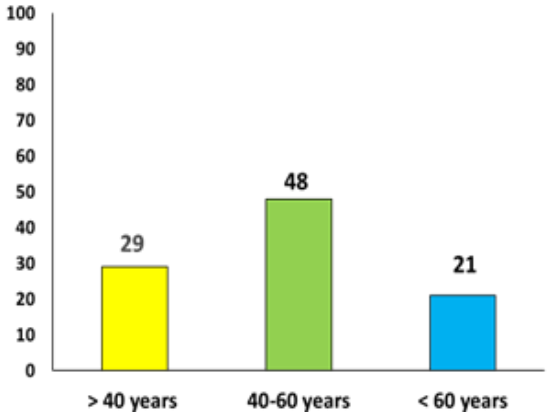


Figure 1a. Gender-wise distribution of samples. Figure 1b. Age-wise distribution of samples

the wild type and accumulates in large amounts in tumor cells (Karim and Ali 2009b). Hence, the development of GC results from a complex interplay between different etiological agents. Another important player is *H Pylori,* a known class I carcinogen and a major risk factor for GC since 1994. However, despite this enhanced understanding of the disease pathogenesis and associated risk factors, cases continue to be a significant cause of mortality(Chong et al. 2014). This means that other factors which might influence its development warrant evaluation. Several studies have also attempted to investigate the histopathological function of the *TP53* gene in the presence of both EBV and *H. pylori* (Lima et al. 2008). Consequently, our study aimed to correlate the occurrence of *TP53* mutation and EBV infection with GC.

2. Materials and Methods

This study was approved by the Institutional Review Board of Dow University of Health Sciences (DUHS) and conducted at the Department of Molecular Pathology, Dow Diagnostic Research, and Reference Laboratory (DDRRL) DUHS. After an expert histopathologist's analysis and approval, 98 formalin-fixed-paraffin-embedded (FFPE) tissue blocks were collected from the DDRRL, DUHS. A non-tumor tissue section at least 10mm apart from the tumor section was collected for the controls. Only those with a minimum of 70% tumor content in GC tissues were included in the cases. These FFPE tissues were screened for the presence of apoptosis and evaluated for EBV genome and *TP53* mutations. All gastric tumors other than adenocarcinoma and all *H. pylori*-positive gastric tumors were excluded.

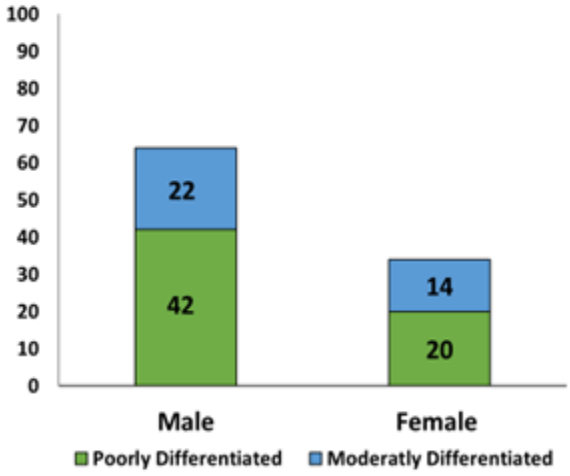
2.1 DNA Extraction from FFPE GC

For the removal of paraffin, all tissue samples were treated with xylene and absolute ethanol according to the standard protocol. A DNA extraction kit (QIAGEN GmbH, Hilden, Germany) was used to extract nucleic acid. As per the manufacturer's instructions, DNA was extracted from all treated samples.

2.2 Detection of EBV by Nested Polymerase Chain Reaction (PCR)

For the detection of EBV in human GC tissues, we amplified the Bam HI-W region of the EBV genome with the help of nested PCR. Primers of Bam HI-W region were F: CCATGT AAGCT-7JGCCTCGAG (F), GCCTTAGATCTGGCTCTTTG (R), CTT TGT CCA GAT GTC AGG GG (F-inner), GCC TGA GCC TCT ACT TTT GG (R-inner). The amplified

PCR product of the Bam HI-W region of EBV was detected on 2% agarose gel with a 100bp ladder and was visualized by ethidium bromide staining using a gel documentation system.



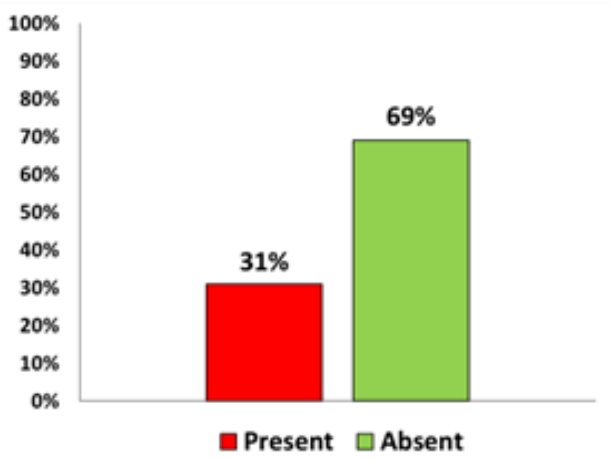


Figure 2a. Histopathological analysis for tumor grade. Figure 2b. Histopathological analysis for presence/absence of signet ring.

2.3 Amplification of *TP53* Gene

The amplification of Exons 5-7 of the *TP53* gene was done by PCR using three pairs of primes. Primers used were TGT TCA CTT GTG CCC TGA CT (F), CAG CCC TGT CGT CTC TCC AG (R) for exon 5, GCC TCT GAT TCC TCA CTG AT (F) TTA ACC CCT CCT CCC AGA GA (R) for exon 6, and ACT GGC CTC ATC TTG GGC CT (F) TGT GCA GGG TGG CAA GTG GC (R) for exon 7. Amplified products were sent for commercial sequencing. Mutation analyses were carried out by aligning the sequences with the reference sequences downloaded from GenBank (NCBI, USA) by using the MEGA7.

2.4 Apoptosis Analysis by Immunohistochemical Staining for Caspase-3

First, the paraffin-embedded slides of tissue samples were deparaffinized. After removing paraffin, slides were put into 400 ml EDTA solution (0.0001 mol/L, pH 6.0). Then the solution was boiled for 2 minutes. Then the slides were allowed to cool, and then all the slides were incubated overnight at 4 Ċ with the caspase-3 monoclonal antibody (from Neo makers). After incubation, all slides were rinsed three times with phosphate-buffered saline (0.01 mol/L, pH 7.4). Then incubated with anti-mouse conjugate having horseradish peroxidase at 37^0 C for 30 minutes. After incubation, slides were rinsed again with phosphate-buffered saline three times. In the last, 3.3-diaminobenzidene was used for staining, and hematoxylin was used for counterstaining. The slides were analyzed by 400-fold magnification.

2.1. GC Grading

The Adenocarcinoma was graded as (1) well differentiated, (2) moderately differentiated, and (3) poorly differentiated according to the guidelines.

2.6 Statistical Analysis

The Chi-squared test was used to investigate the association of *TP53* mutations with EBV infection and tumor grade. The comparison was considered significantly different when the $p>0.05$.

3. Results

Out of the 98 patients that were recruited in the study, a total of 29 (29.5%) were <40 years, 48 (48.9%) were between 41-60 years, and 21

(21.4%) were >61 years. The mean age of patients in our study was 51.4±13.9. There were 64 (65.3%) male and 34 (34.6%) female patients (Figures 1a and 1b).

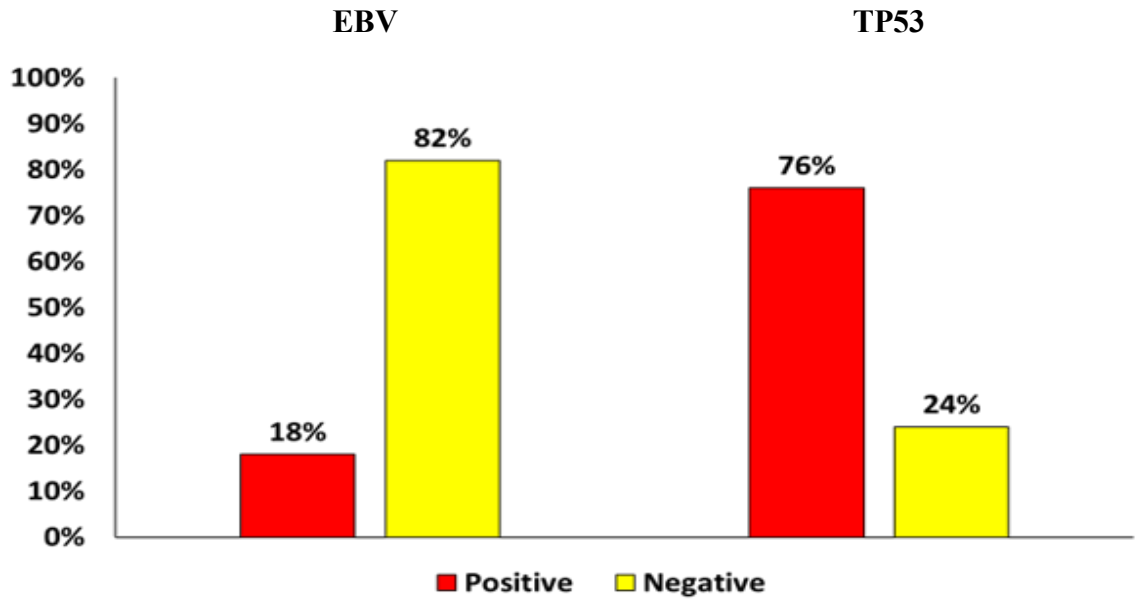


Figure 3. EBV and TP53 positivity of samples.

Of the 98 samples investigated, 63.3% (n=62) of patients had poorly differentiated tumors, while 36.7% (n=36) of the patients had moderately differentiated tumors. Signet ring cells were present in 30.6% (n=30) of samples and absent in 69.4% (n=68) of the samples. Out of 64 (65.3%) male patients, tumors of 42 (65.6%) are poorly differentiated, and 22 (34.3%) are moderately differentiated. Out of 34 (34.6%) female patients, tumors of 20 (58.8%) are poorly differentiated, and 14 (41.1%) are moderately differentiated. (Figures 2a & 2b).

Out of 98 samples, 18 (18.4%) were found to be positive for EBV by PCR, of which 12 (12.2%) were from male patients and 6 (6.1%) were from female patients (Figure 3). Mutational analysis of *TP53* revealed that 76% (n=19) of the samples had mutated *TP53* gene, of which 8% (n=2) of the samples had single nucleotide variation (SNV) in exon 5 i.e., g.17371G>A and g.17521A>C. Moreover, 76% (n=19) of the samples had the same SNV (g.18316T>C) in exon 7. However, no mutations were observed in exon 6 (Figures 3, 4 & 5). The Chi-squared test showed no significant correlation (Table 1) of *TP53* mutations with EBV infection and tumor grade ($p>0.05$).

All samples were stained with α-caspase-3 antibodies to investigate expression patterns of caspase-3 in GC tissues. Controls (normal gastric mucosa) and negative experimental controls (samples without primary antibodies) were also included in the experiment. Results showed no difference in expression patterns of caspase-3 in GC and control tissues (Table 1 & Figure 6).

4. Discussion

GC is an example of a multifactorial disease because it originates via interactions between various environmental and genetic factors. It is the fourth most common cause of cancer-associated death, and its incidence seems to rise (Sung et al. 2021a). Hence, it is very important to investigate new risk factors involved in its development and progression(Bhurgri et al.

2006). Recent studies have indicated an association of EBV and genomic mutations with malignancies such as nasopharyngeal

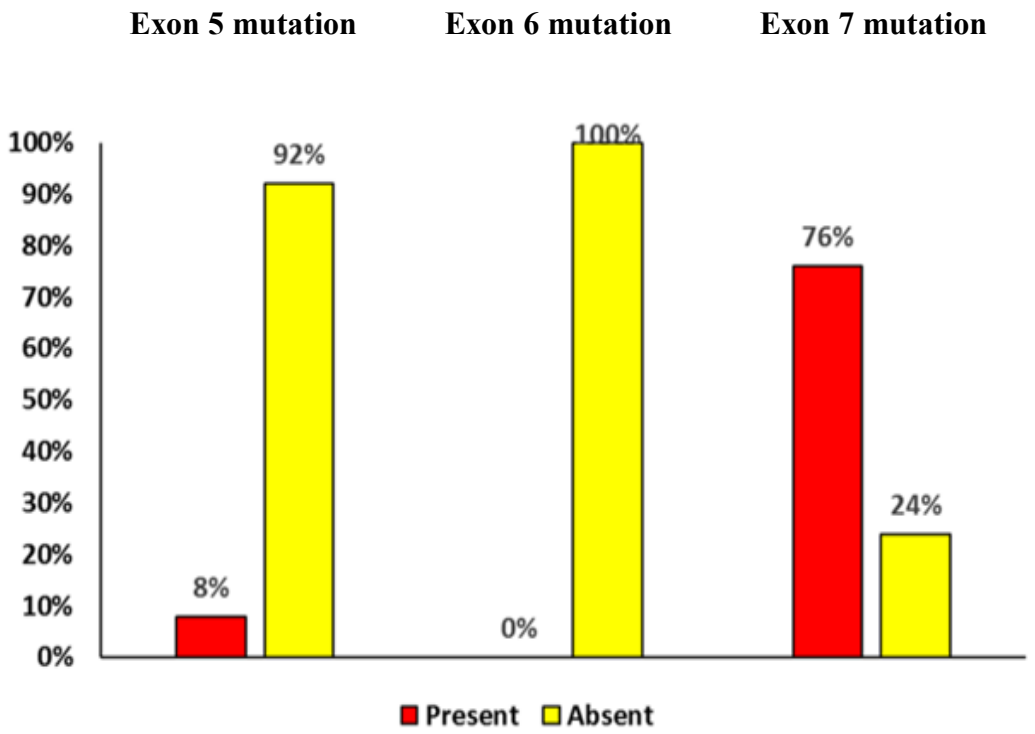


Figure 4. Presence/absence of mutations in exons 5,6,7 of samples.

carcinoma, Burkitt's lymphoma, and GC (De Aquino et al. 2012).

We found that out of 98 tumor samples, 63.3% (n=62) of the patients had a poorly differentiated tumor, while 36.7% (n=36) of the patients had a moderately differentiated tumor. Signet rings were present in 30.6% (n=30) of the samples and absent in 69.4% (n=68). Thus, according to our study, most of the GC in our population are of the poorly differentiated diffuse type. These results are consistent with those of other investigations; Afridi et al. found that 66.7% of their GC samples were of the diffuse infiltrating type and that younger individuals were more likely to develop the diffuse type as compared to the intestinal (Afridi, Bano, and Shafiq ur 2011) .

In many of the previous studies, most cases were diagnosed at or after the age of 70. This is because the incidence rate of GC in most populations rises with age (Roder 2002). This trend has also been previously reported in Pakistan, in which rates of GC peaked in the seventh decade of life (Bhurgri et al. 2006). However, in our study, most of the patients were young males (48.9%) between the ages of 41-60 years, and the mean age of patients was 51.4±13.9. Hence, in Pakistan, compared to other regions of the world, the incidence of GC appears to be higher in younger males. This is also in accord with the findings of some previous studies (Afridi, Bano, and Shafiq ur 2011). This increased incidence of GC in younger patients may be due to improper hygienic conditions and unhealthy dietary

habits. On the other hand, it could be due to some unexplored genetic factors because the diffused type of adenocarcinoma, observed in our study in young males is mostly associated with genetic abnormalities (Hu et al. 2012).
Globally GC is more prevalent in men. Many studies have reported that men are at a higher

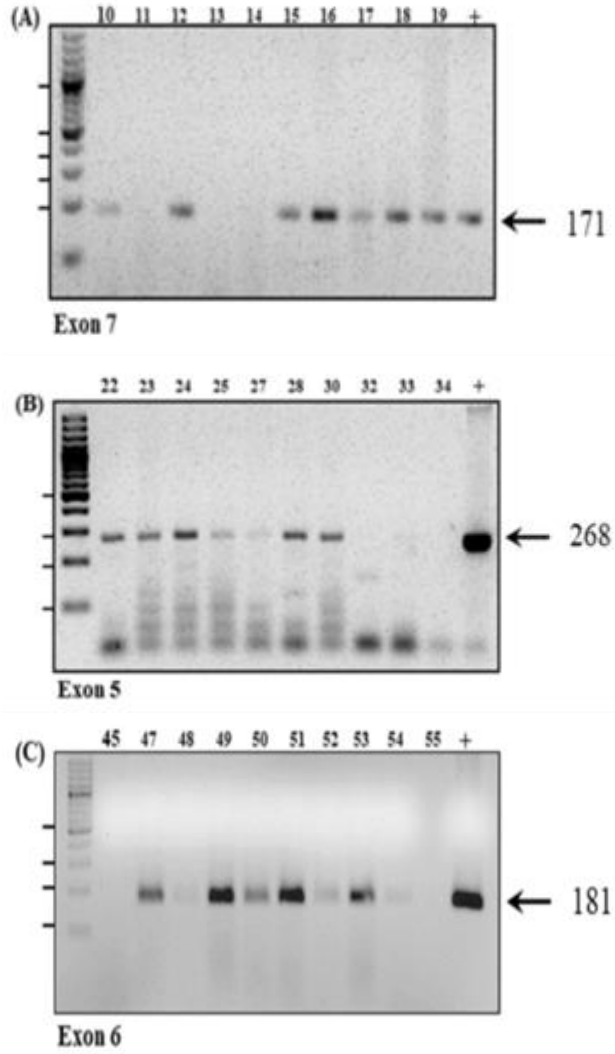


Figure 5. Amplification of Exon 7 (A), Exon 5 (B) and Exon 6 (C).

risk. The incidence of GC is almost twice as high in men as compared to women (Qureshi et al. 2016). Our findings support these observations and show that out of 98 patients, 64 (65.3%) were male, and 34 (34.6%) were female. However, it should be noted that overall, Pakistan has shown a progressive increase in the incidence of GC in females over the past 5 years, indicating that no gender is significantly protected (Bhurgri et al. 2006).

A vast pool of previously published literature already associates EBV with GC. For example, one study found that 22 out of 138 GC FFPE blocks were EBV positive while none of the controls were (Shibata and Weiss 1992). A systematic review of 103 articles concluded that the prevalence of EBV-associated GC is 8.29% worldwide (Sousa et al. 2008). Furthermore, Martínez-López et al. found that more than 80% of the lympho-epithelioma type of GC were associated with EBV, and 10.67% of 75 samples of GC were EBV positive, which further suggests that EBV may have a role in GC development (Martínez-López et al. 2014) . Upon analysis after using nested PCR, we discovered that 18 (18.4%) of our GC samples were positive for EBV; these findings lend further credibility to the notion that EBV is linked to GC development.

Previous studies suggest that the functional loss or inactivation of a tumor suppressor gene known as *TP53* plays a vital role in developing various cancers (Karim and Ali 2009a). Mutations of *TP53* in exons 5-8 have been found in GC samples in previous studies (Karim and Ali 2009a, Tamura et al. 1991). Others also suggested that the base transitions G:C → A:T were the most common genetic changes seen in exons 5-8 of *TP53* and concluded that the mutations in *TP53* are one of the most important genetic changes that occur in the early stages of GC (Renault et al. 1993, Uchino et al. 1993).
To further our understanding of the presence and significance of *TP53* mutations in GC, we amplified three exons (5,6,7) each from 25 samples. The results indicated that 76% (n=19) of the samples were mutated, indicating that they may have an association with GC, as has

been suggested by previous studies. Out of these, 8% (n=2) of the samples had SNV in exon 5 i.e., g.17371G>A and g.17521A>C, while 76% (n=19) of the samples had the same SNV (g.18316T>C) in exon 7. These SNVs are novel and have not been reported before. Meanwhile, no mutations were observed in exon 6. This

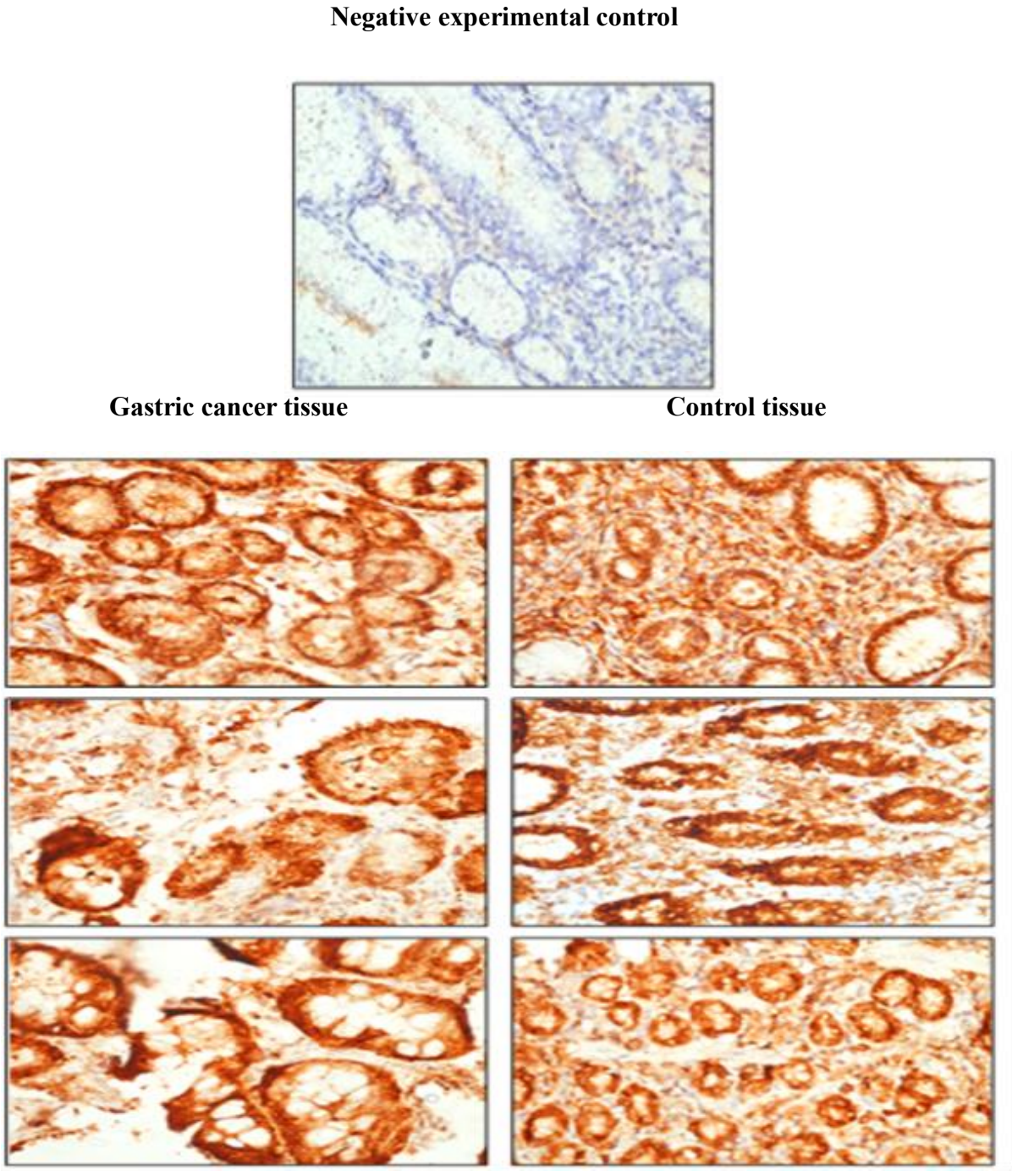


Figure 6. Expression of Caspase-3 in gastric cancer tissues.

finding deviates from the past analyses conducted by other authors, as mentioned above, possibly because of environmental or genetic variation within our population and merits further future evaluation.

Interestingly, although it has been established that *TP53* mutations play a central role in the multistep pathogenesis of GC, it has also been postulated that EBV infection interferes with *TP53* itself. Consequently, EBV-encoded proteins may avoid the need for *TP53* mutations and render them unnecessary for tumorigenesis (Gulley et al. 1996). On the other hand, Lee et al. and Lime et al. discovered no direct relationship between *TP53* mutations and EBV infection in GC(Lee et al. 2004, Lima et al. 2008). In our efforts to analyze a possible link between the two, we found no significant correlation of *TP53* mutations with EBV infection and tumor grade ($p>0.05$). This is in line with the results of past studies and reemphasizes that the presence of EBV does not necessarily influence *TP53*; perhaps EBV uses another pathway to involve itself in the development of GC, but this is just an assumption.

During tumor growth, the apoptosis rate slows or is altered by other mechanisms. Generally, apoptosis can occur via two pathways, and both activate a family of proteins called the caspases that trigger cellular death. It has been observed that there is a decreased expression of caspase 3 in oral squamous cell carcinoma as well as GC (Li et al. 2004, Heshiki et al. 2015). EBV can increase apoptotic resistance in GC via the inhibition of pathways that utilize caspases 8 and 9 (Pattle and Farrell 2006). Given the apparent significance of apoptotic pathways that utilize caspases in the development of tumors, we stained our GC samples with α-caspase-3 antibody. However, we did not observe any significant differences in the expression pattern of caspase-3 in GC and the control tissues.

It should be noted that this study has certain limitations. The sample size of this study was not very large, and samples were only collected from one city in Pakistan, which is not reflective of the entire population. Moreover, due to limited resources, only 25 samples were sequenced. It was also difficult to obtain and utilize accurate data related to GC as no cancer registry is present on a national level, and only a few provincial registries are functional. Besides H. pylori and dietary risk factors, very little work has been done on the other risk factors involved in the development of GC, such as EBV, the frequency of mutations in *TP53*, and the status of apoptosis.

Conflict of Interest

The authors declare that they have no competing interests.

Funding

NA.

Study Approval

This study was approved by the Dow University of Health Sciences, Karachi, Pakistan.

Consent Forms

Consent forms are available with the authors.

Data Availability

All the raw data related to this study is available with the authors.

Authors Contributions

BR and FS conceptualized and organized the study, BR and QTA did the literature search and analysis, BR wrote the initial manuscript, FS wrote the final manuscript and supervised the project.

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