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Rate of Epstein-Barr Virus in Gastric Adenocarcinoma in Egyptian Patients in View of the WHO Classification and Correlation with p16 Immunoreactivity

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Abstract

BACKGROUND AND AIM: Gastric cancer (GC) is one of the top causes of cancer-related deaths worldwide. According to the Cancer Genome Atlas, there are four subtypes of GC, with the Epstein-Barr virus (EBV) subtype accounting for about 10% of cases. EBV infection causes EBV-associated GC (EBVaGC). The previous research suggested that the presence of the EBV viral genome in gastric carcinomas could be used as a surrogate marker for targeted therapy and optimal GC treatment.

AIM: We aimed to explore the rate of EBV involvement in gastric carcinogenesis from molecular perspective view and to evaluate the role of the tumor-suppressor protein p16 as a marker for diagnosis in GC Egyptian patients in relation to EBV infection.

METHODS: One hundred-four surgically resected GC cases were analyzed. Two methods including quantitative real-time polymerase chain reaction (qPCR) for detecting EBV-derived latent membrane protein-1 (LMP-1) and Epstein-Barr nuclear antigen-1 (EBNA-1) genes as well as immunohistochemistry (IHC) detection of LMP-1 protein and p16 protein on paraffinized tissue blocks were applied.

RESULTS: Using IHC, p16 protein was presented in 90/104 (86.5%) of the GC cases, and EBV LMP-1 was detected in 4 cases (3.84%). qPCR detected 14 cases positive for EBV (13.46%). In EBV positive cases detected using qPCR, no expression of p16 was detected.

CONCLUSION: EBVaGC has a low incidence in Egypt; loss of p16 expression was recognized in EBVaGC and could be considered as a promising biomarker of EBVaGC. The combination of the two methods IHC and qPCR in addition to p16 is recommended for improving the accuracy of identification of infected cancer.

Introduction

Gastric cancer (GC) is the fifth most frequent malignancy and the second greatest cause of cancer-related death [1]. It is responsible for 6.8% of all cancer cases and 8.8% of total cancer-related death around the world [2]. Still, GC therapy is chemotherapy composed of platinum compounds and fluoropyrimidines [3]. Unfortunately, the 5-year survival rate is expected to be <10% [4]. According to several Egyptian cancer registries, GC is the tenth most prevalent cancer in both sexes, accounting for 2.5% of all cancers. It is the ninth leading cause of cancer death, accounting for 3% of all cancer deaths [5]. The frequency of GC rises with age and 55% of cases are between the ages of 50 and 70 [6].

Cancers caused by human viruses account for up to 20% of all malignancies, with greater rates in developing nations. The International Agency for Research on Cancer has identified pathogens, mostly viruses, as carcinogenic to humans. These include high-risk human papillomavirus (HPV) [7], Epstein-Barr virus (EBV), Kaposi herpesvirus sarcoma herpesvirus, Merkel cell polyomavirus (MCPyV), hepatitis B virus, hepatitis C virus, and human T-lymphotropic virus 1 [8]. EBV was recognized to be involved in GC development [9], [10].

Even though certain remarkable improvements in GC systemic management have occurred, the prognosis of advanced-diseased patients remains poor [11]. According to the Cancer Genome Atlas Research Network (TCGA); several molecular classifications have been recognized as having critical roles in GC therapeutic management. GC is categorized into four molecular subtypes: EBV positive (9%) [12], microsatellite unstable (MSI) tumors (22%), genomically stable tumors (20%), and chromosomal instability tumors (50%) [13]. It is considered that EBV positive and MSI GC s are most likely to respond to immunotherapy drugs. Application of GC patient molecular classification in clinical trials may be critical in choosing the most appropriate GC therapies and avoiding the use of costly drugs [14].

EBV is a human γ herpes DNA virus 4 (HHV-4) that infects immune system epithelial cells and B cells [15]. Some

200,000 cancer cases per year are believed to be linked to EBV, it is described that the frequency of EBV infection in GC ranges from 2 to 20%, with a global average of about 9–10%. These variances in reported frequencies may be due to geographical and environmental features [16], [17]. This form of GC is named EBV-associated GC (EBVaGC) [18]. EBVaGC has distinctive pathological and molecular features, which may also be related to a more favorable prognosis in GC patients [9].

Since the discovery of p16 protein in 1993, it has been known as (cyclin-dependent kinase inhibitor 2A or multiple tumor suppressor 1), a well-known tumor suppressor and a hot spot in the molecular biological research of neoplasm. It regulates cell cycle, senescence, apoptosis, cell invasion, and angiogenesis and is down-regulated in many tumors. Regardless of these well-known tumor-suppressor capabilities, p16 expression can vary depending on the kind of cancer. p16 deletion, mutation, hypermethylation, or overexpression have now been linked to a variety of malignancies, leading to the suggestion that it could be a useful marker for identifying them. As a result, p16 expression is employed as a predictive biomarker for specific cancers [19]. The specificity of p16 has been insufficiently studied in GC, as there is a lack of consensus and clear guidelines or the use of p16 expression in regular clinical management of GC, and its predictive significance remains disputed [19].

Most studies use Polymerase Chain Reaction (PCR) in the detection of EBV nucleic acids [18]. In addition, immunohistochemistry (IHC) is used for the detection and identification of EBV-related proteins, including EBV nuclear antigen 1 (EBNA1) and the latent membrane proteins-1 (LMP-1, LMP2a, and LMP2b) [20]. The objective of this study was to clarify the role of EBV in gastric carcinomas in Egyptian patients with effective selection of reliable lower costs detection methods. This was assessed on paraffinized tissue sections using quantitative PCR (qPCR) and IHC, to demonstrate whether using one of the two techniques alone or in combination is better in identification of EBVaGC. Moreover, we intended to inspect the clinical usefulness and biological significance of p16 expression in EBVaGC.

Materials and Methods

Research study design

The flow chart below indicates the methodology of this research (Figure 1).

Patients and tissue specimens

This study included 104 surgically resected GC cases, where our surgeons involved in the study

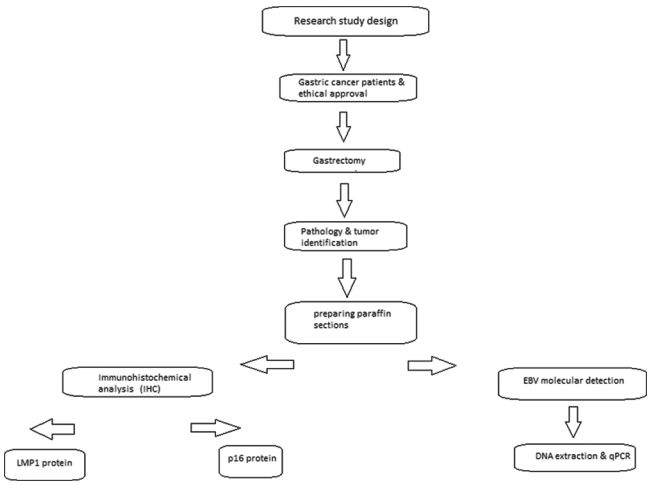

Figure 1: The flow chart of the methodology of this research

participated in these operations (44 partial gastrectomy, 10 subtotal gastrectomy, 40 total gastrectomy, and 10 esophagogastrectomy), Furthermore, 10 normal gastric tissue samples as a control group taken during sleeve gastrectomy from normal gastric wall were included in the study. All specimens were obtained as archived paraffin blocks during a period of 3 years from 2017 to 2020 from Egyptian patients at the Department of Pathology, Theodor Bilharz Research Institute (TBRI), Giza, Egypt and from private hospitals in Cairo, All available demographic data and information were obtained from the patients' pathologic and medical reports: Age, sex, clinical presentation, comorbidities, tumor location, histology, grade and stage, treatment modalities, response, tolerance, relapse/progression, dates of diagnosis, surgery, and relapse/progression, and last follow-up. The inclusion criteria: all untreated GC patients over the age of 18 years. The exclusion criteria: Patients with severe gastritis or atrophic and non-adenocarcinoma malignancy. DNA and proteins destroyed by fixation and preanalytical ischemic time were excluded from the study.

Ethical approval

All of the specimens in the study were coded and patients' names were replaced with codes. The study's protocol was approved by the TBRI institutional review board under Federal Wide Assurance (FWA00010609), and the work was done in accordance with the World Medical Association's Code of Ethics for Human Experiments (Declaration of Helsinki) and its later amendments (GCP guidelines) or comparable ethical standards.

Pathology

A pathologist evaluated hematoxylin and eosin (H and E) stained slides on 3–4 micron slicing of the sample to confirm cancer and select the appropriate paraffin blocks or sections for PCR detection and IHC for all samples.

IHC of p16 protein and EBV LMP-1

Formalin-fixed paraffin blocks were sectioned into 4 μm sections on positively charged slides (Superfrost Plus, Menzel Gläser, Germany), antigen retrieval using CC1 Ultra View Universal DAB Detection Kit (cat no. 760–500, Diagnostics, Roche Tuscon, USA) on Ultra BenchMark automated platform. LMP-1 was detected using Epstein Barr Virus (CSL-4) mouse monoclonal antibody 0.04μg/ml (Cell Marque™, LOT V0001823, USA) and p16 was detected using anti- $p16^{INK4a}$ (E6H4), a mouse monoclonal primary antibody produced against the $p16^{INK4a}$ protein.1μg/ml (VENTANA, CINtec p16 Histology, LOT F11089). As a negative control, the primary antibodies were omitted. As positive controls, known EBV-positive GC patients using IHC and PCR were used.

Two pathologists scored immunohistochemical (IHC) data by counting the percentage of positive cells versus total cells and reporting the staining intensity in at least 10 × 40 fields. For the interpretation of IHC results; the true LMP1 positivity is granular and is localized to the cytoplasm and surface membrane at any percentage even one group of five cells. When nuclear staining and cytoplasmic staining were shown in more than 90% of the tumor cells (block positivity), the staining pattern for the p16 protein was judged positive, and when nuclear staining was seen in <10% of the tumor cells, it was termed negative. Cases of p16 with nuclear and cytoplasmic staining positivity of more than 10% but <90% should be regarded borderline and require further evaluation by *in situ* hybridization (ISH) [21].

DNA extraction and quantitative real-time PCR (qPCR)

Paraffin blocks were cut into10 μm thick slices and DNA sample was prepared according to manufacturer protocol (Genomic DNA Isolation Kit Paraffin-embedded tissue, Cat. No.: SN027-0100, GeneDireX, Inc). Spectrophotometric DNA quantitation was done using Thermo Scientific Nanodrop 2000C spectrophotometer at wavelengths of 260 nm.

Target genes for EBV included EBNA1 and LMP1. qPCRs were performed in triplicates in independent reactions. It was carried out in a total volume of 20 μl, consisting of 10 μl 2× PoweUp™ SYBR™ Green/ROX PCR Master Mix (Applied Biosystems, ThermoFisher SCIENTIFIC), 5 pmol of forward and reverse primers, and 2 μl of 200ng DNA. The primer pairs used are *β*-actin Forward 5'- GGACTT CGAGCAAGAGATG-3', Reverse 5'- CCTTCTGCATCCTGTC-3'(22), EBNA1 Forward 5'- GTCATCATCATCCGGGTC -3', Reverse 5'- TTCGGGTTGGAACCT CCTTG-3', and LMP1 Forward, 5'-CTGCTCATCGCTCTCTGGAA-3′, Reverse, 5′-AGACAAGTAAGCACCCGAAGATG-3′ [22]. qPCR conditions included initial denaturation at 95°C for 15 min, followed by 50 cycles of denaturation (95°C for 30 s), annealing (55°C for 20 s), extension (72°C for 20 s), and melting curve analysis from 60 to 95°C of the PCR products. A positive control sample for EBV was performed during qPCR. Negative controls contained nuclease-free water instead of DNA. The reference gene was *β*-actin to assure good quality of DNA extraction [23].

Statistical analysis

For data analysis, the Statistical Package for the Social Sciences (SPSS) computer application (version 19 windows) was utilized. The Chi-square test or Fisher exact test were used to compare categorical data [n (percent)]. Comparison between numerical data was performed using unpaired t test. Results were expressed as mean ± SD or number (%).

Results

Clinical and pathological data

This study was conducted on 104 gastric adenocarcinoma patients, all tissues of the cases were reviewed by the pathologists who identified the tumor in the block and guided the preparation. Forty (38.5%) of them were males and 64 (61.5%) were females with a gender ratio of 1:1.6, respectively. Their mean ages (± SD) equal to 43.6 ± 10.34 yrs. The mean age (± SD) of females and males was 35.4 ± 3.7 and 48.6 ± 11.5 years, respectively.

In GC patients, the tumors were mainly localized in gastric antrum (44/104 [42.3%]) and whole stomach (Linitis plastica) (30/104 [28.8%]), followed by equal percent distribution in gastric cardia and fundus, gastric body and gastroesophageal junction (10/104 [9.6%]). The gross features of GC were mainly fungating mass (50/104 [48.1%]), diffuse wall thickening (Linitis plastica) (30/104 [28.8%]), and circumferential ulcerating mass (24/104 [23.1%]). Microscopically, they were diagnosed to have gastric adenocarcinoma and the most of the tumors were poorly-differentiated adenocarcinoma (G3) (54/104 [51.9%]) followed by poorly cohesive carcinoma (signet-ring cell carcinoma) (30/104 [28.8%]) and finally well-differentiated adenocarcinoma (G1-G2) (20/104 [19.2%]) (Figure 2).

IHC of p16 protein and EBV-LMP1

The expression of p16 protein was significantly higher in GC patients (90/104 [86.5%]) when compared with those in control group (0/10 [0.0%]) (p = 0.001). As shown in Figure 3, expression of p16 protein was observed in both the nuclei and cytoplasm of cancer cells. In GC patients with positive p16, the site of tumor

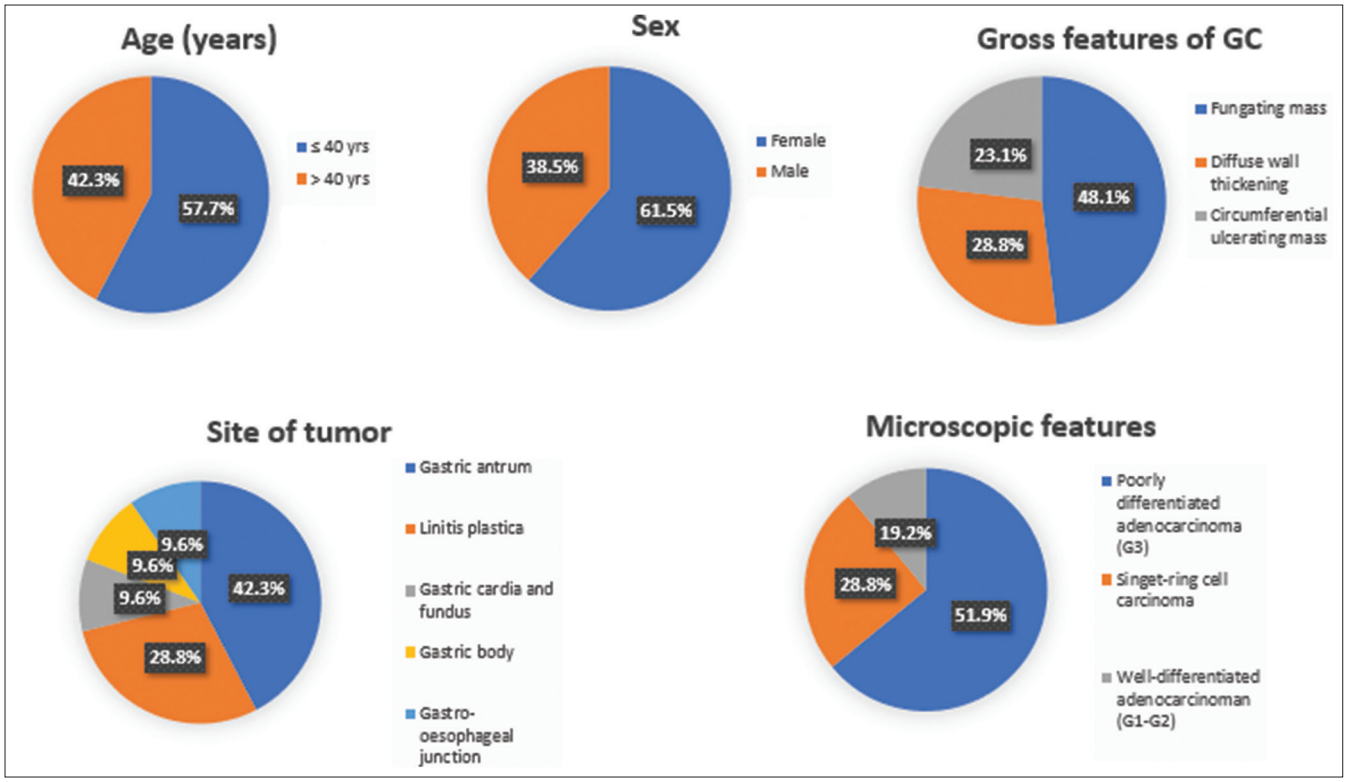

Figure 2: Physical, clinical, and pathological characteristics of the studied GC patients expressed as percentages

was mainly localized in gastric antrum (44.4%), while in case of GC patients negative for p16, the site of tumor was mainly localized in linitis plastica (71.4%) (Chi-square test, x = 15.56, df = 4; p = 0.004). However, both gross and microscopic features are independent in both positive and negative p16 GC patients (x=0.9905, df = 2; p = 0.609) and (x = 3.852, df = 2; p = 0.146, respectively (Table 1). Furthermore, no borderline cases were detected in this study and there was no need for ISH. All positive cases for p16 (90 cases) scored > 90%, that is, nuclear staining and cytoplasmic staining were shown in more than 90% of the tumor cells (block positivity). On the other hand, all p16 negative cases (14 cases),

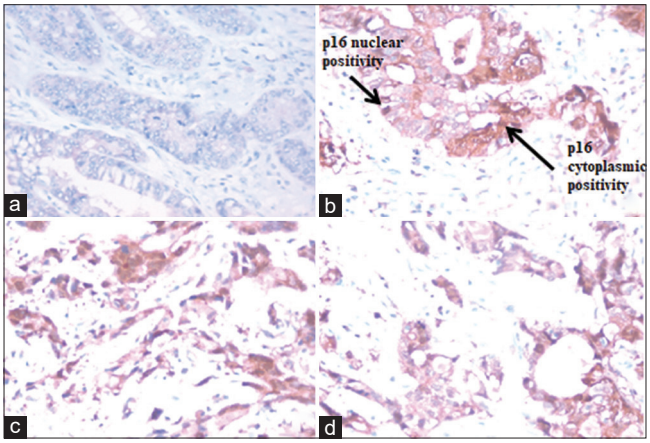

Figure 3: p16 protein expression in gastric carcinoma. (a) A case of infiltrating Adenocarcinoma, Grade II negative nuclear, and cytoplasmic staining for p16. Magnification (400×). Immunohistochemical stain (b-d) shows three cases infiltrating Adenocarcinoma, Grade II showing 90% positive nuclear and cytoplasmic staining for p16. Arrows show the p16 nuclear and cytoplasmic positivity.

nuclear staining was seen in <10% of the tumor cells.

The IHC examination for EBV-LMP1 revealed that only four cases out of the 104 GC patients were EBV-LMP1 positive (3.8%) which was more frequent compared with those in control group (0/10 [0.0%]) (p = 0.528) Figure 4.

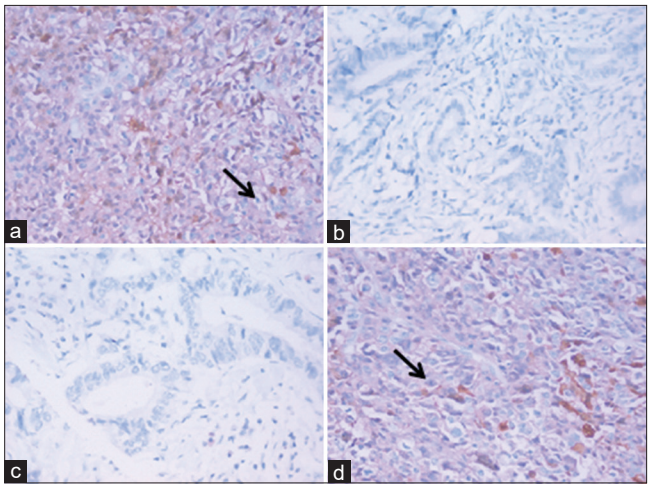

Figure 4: (a and d) show two cases of infiltrating Adenocarcinoma, Grade II positive cytoplasmic staining for EBV LMP-1. Arrows show LMP-1 cytoplasmic positivity. (b and c) show two cases of infiltrating Adenocarcinoma, Grade II negative cytoplasmic staining for EBV LMP-1. magnification (400×)

Quantitative real time PCR

The results of qPCR using primers specific to EBNA1 and LMP-1 genes showed that 14 out of

Table 1: Clinical and pathological characteristics of GC based on the expression of p16 protein

Characteristic	Positive p16 (n = 90)		Negative p16 (n = 14)		p-value
	Frequency	%	Frequency	%	
Site of tumor					
Gastric antrum	40	(44.4)	4	(28.6)	0.004*
Linitis plastica (whole stomach)	20	(22.2)	10	(71.4)	
Gastric cardia and fundus	10	(11.1)	0	(0.0)	
Gastric body	10	(11.1)	0	(0.0)	
Gastroesophageal junction	10	(11.1)	0	(0.0)	
Gross features of GC					
Fungating mass	45	(50.0)	5	(35.7)	0.609
Diffuse wall thickening (Linitis plastica)	25	(27.8)	5	(35.7)	
Circumferential ulcerating mass	20	(22.2)	4	(28.6)	
Microscopic features					
Poorly-differentiated adenocarcinoma (G3)	45	(60.0)	9	(64.3)	0.146
Poorly cohesive carcinoma (signet-ring cell carcinoma)	25	(27.8)	5	(37.55)	
Well-differentiated adenocarcinoma (G1-G2)	20	(22.2)	0	(0.0)	

Data are expressed as number (%), P > 0.05 = not significant. *p < 0.05 = significant.

104 (13.5%) patients were EBV positive. There was no expression of p16 protein in EBVaGC samples detected by both IHC and qPCR, ([0/4] 0.0%) and ([0/14] 0.0%), respectively (p = 0.001) Table 2. Physical, clinical, and pathological characteristics of EBVaGC and non-EBVaGC diagnosed with qPCR technique are shown in (Table 3).

Table 2: Efficacy of both IHC and qPCR techniques in diagnosing GC in p16 protein expression subgroups

Parameters	Negative p16 (n = 14)	Positive p16 (n = 90)	p-value
IHC EBV-LMP1			
EBVaGC (n = 4)	4 (28.4%)	0 (0.0%)	0.001*
Non-EBVaGC (n = 100)	10 (71.4%)	90 (100.0%)	
qPCR EBV (EBNA1 & LMP1)			
EBVaGC (n = 14)	14 (100.0%)	0 (0.0%)	0.001*
Non-EBVaGC (n = 90)	0 (0.0%)	90 (100.0%)	

Data are expressed as number (%),*p < 0.05 = significant.

Discussion

EBVaGC is a distinct molecular subtype of gastrointestinal (GI) cancers. The purpose of GC classification and determining its causes is to assist clinicians in better clinical decision-making and to determine the appropriate intervention that improve patient outcomes. EBV testing has been established in the previous research to aid in correct diagnosis, treatment development, precise prognosis for EBVaGC, and clinical decision-making. EBV-positive state is a promising biomarker for GC immunotherapy, EBVaGC patients treated with immunotherapy showed favorable responses [10]. Furthermore, in the early stages of EBVaGC, less invasive surgery such as endoscopic resection may be indicated. Therefore, it is particularly important to identify EBV status efficiently. The rate of detection of EBV in GC may vary among different methods. For successful clinical management of these individuals, accurate diagnosis is critical [24].

Several techniques, including IHC, ISH, and PCR, are utilized to test EBV-positive stomach cancer. In ISH, the EBER probe detects short mRNA. Fixation of GC tissue in formalin may denature RNA, this sometimes may give weak positive or false negative results. In addition, the EBER probe is expensive [25]. The IHC detection approach is based on the EBV-encoded LMP-1 membrane protein, which cannot identify the virus's location or transcriptional amount. However, as compared to ISH, IHC offers the advantages of simple steps, appropriate protocol, high sensitivity, and low cost, making it a reliable main EBV screening approach. As a screening tool, a viral DNA test using qPCR has been launched; this has proven to be significantly more sensitive than older approaches, suggesting that molecular biomarkers are objective, quantitative, and easily repeatable in all hospitals and laboratories for a wide range of tumor types [13].

The National Cancer Institute and the Adolescent and Young Adult Oncology Progress Review Group (AYAO PRG) defined GC in young adults (GCYA) as tumors diagnosed before age 40 [26]. Our study showed that GCYA was more than in older age. The mean age (± SD) of females and males was 35.4 ± 3.7 and 48.6 ± 11.5 years, respectively. This was in agreement with a study conducted by Moore *et al*., 2020 [27]. In addition, GC in our study was more frequent in females 64 (61.5%) than males 40 (38.5%) with a gender ratio of 1.6:1, respectively. EBV detection using IHC for LMP1 protein was 3.8%, while EBNA-1 and LMP-1 genes of EBV using qPCR were detected in 13.46%. Ten cases that were negative for LMP-1 IHC were positive using qPCR. This can be explained according to the fact that qPCR can amplify the target EBV DNA thousands of times. Although LMP-1 of EBV is a well-known oncoprotein, its expression is extremely low in EBVaGC. Low levels of LMP1, similar to those reported in epithelial infection, were found to be adequate to generate oncogenic characteristics in a number of non-lymphoid cell lines. LMP-1 may be repressed and at the same time can allow tumor cells to grow continually to develop cancer without its expression [28]. This may explain the lower percentage of LMP1 protein detected in our study using IHC compared to the higher percentage of LMP1 gene identified using qPCR. Detection of EBV DNA (using qPCR) is a surrogate method to discriminate EBV-positive cancers from EBV-negative cancers [29], [30]. Tactlessly, qPCR was unable to determine the cellular localization of EBV positive cells, whereas IHC revealed more information about EBV positive cell localization. EBV positive GC was higher in gastric antrum localization, poorly differentiated

Table 3: Physical, clinical, and pathological characteristics of EBVaGC and non-EBVaGC diagnosed with qPCR technique

Characteristic	EBVaGC (n = 14)		non-EBVaGC (n = 90)		p-value
	Frequency	%	Frequency	%	
Age (years)					
≤40 years	10	(71.4)	50	(55.6)	0.263
> 40 years	4	(28.6)	40	(44.4)	
Sex					
Female	10	(71.4)	54	(60.0)	0.414
Male	4	(28.6)	36	(40.0)	
Site of tumor					
Linitis plastica (whole stomach)	5	(35.7)	25	(27.8)	0.364
Gastric antrum	8	(57.1)	36	(40)	
Gastric cardia and fundus	1	(7.1)	9	(10.0)	
Gastric body	0	(0.0)	10	(11.11)	
Gastroesophageal junction	0	(0.0)	10	(11.11)	
Gross features of GC					
Circumferential ulcerating mass	1	(7.1)	23	(25.6)	0.313
Fungating mass	8	(57.1)	42	(46.7)	
Diffuse wall thickening (Linitis plastica)	5	(35.7)	25	(27.8)	
Microscopic features					
Poorly cohesive carcinoma (signet-ring cell carcinoma)	5	(37.55)	25	(27.8)	0.146
Well-differentiated adenocarcinoma (G1-G2)	0	(0.0)	20	(22.2)	
Poorly-differentiated adenocarcinoma (G3)	9	(64.3)	45	(50.0)	

Data are expressed as number (%), P > 0.05 = not significant.

adenocarcinoma (G3) histology and in females more than males.

Variability in molecular and proteomic profiling, which includes immunological biomarkers, might be caused by suboptimal or inconsistent techniques in the collection, handling, and processing of tissues. Both forms of preservation, as well as tissue handling, processing, and storage procedures, can cause damage to proteins and nucleic acids in formalin-fixed, paraffin-embedded (FFPE) tissue specimens. The cold ischemia time (the time it takes for a tissue to become formalin-fixed) is an important aspect in determining whether or not it is suitable for immunoassays. DNA obtained from FFPE samples is susceptible to deterioration, which, in turn, can influence downstream PCR-based detection [31], [32].

Primary EBV infection occurs in the mouth and results in a lifetime virus carrier state known as latent infection [33]. In contrast to other human tumor viruses, such as HPV and MCPyV, which have integrated viral genomes in tumor cells, EBV-associated malignancies, such as Nasopharyngeal carcinoma, have EBV genomes that are maintained as extrachromosomal episomes [31]. The development of EBV-induced malignancies requires latent EBV infection. Infected cells in latent infections express just a small number of viral genes, such as EBNA1 and LMP1 [34]. The latent infection **c**an be distributed into diverse subgroups according to specific viral proteins; in Type I latency of EBV infected cells, EBNA1 gene is expressed, while in Type II latency EBNA1 and LMP1 genes are expressed [35]. Since LMP1 inhibits EBV lytic cycle, detection of LMP1 can distinguish the latent EBV from non-latent lytic infection [36]**,** this is critical for the reason that EBV-directed therapy in clinical trials for EBV-positive GC patients uses lytic induction therapy to convert infected cells from latent to replicative phases of viral infection, which is thought to cause cell death with the risk of bystander killing of adjacent cells [37], [38]. EBVaGC belongs to latency I or latency II EBV infection [39], and detecting LMP1 protein can clarify the type of viral latency.

Results in this study of p16 protein showed that loss of its expression was associated with EBV infection. Leukemia, brain tumors, malignant melanoma, esophageal carcinoma, and lung carcinoma have all been linked to a loss of p16 expression [40]. Furthermore, our results were consistent with the data from the previous studies [41], [42], [43]. The lack of p16 in EBVaGC shows that this kind of gastric carcinoma develops in a different way than other gastric carcinomas that do not have EBV infection and that it may be linked to a p16 abnormality.

Conclusion

This study showed the frequency of EBVaGC in Egypt was relatively low. EBV positive GC was found to have distinct protein expression profile. qPCR can be used to follow-up on IHC findings, and the combination of the two approaches may increase detection accuracy by minimizing the chances of false positives and negatives. p16 protein immunoreactivity can be used in parallel with IHC detection of LMP-1 EBV and qPCR for EBV genes as a screening method for EBVaGC, as there is a significant correlation between p16 positive cases and non EBVaGC. This infers that non-EBVaGC may follow another pathway from EBVaGC. This was a single-institutional study; to confirm the results of this research it is advisable to perform a multi-institutional study with a larger number of cases and from different regions of Egypt.

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