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Characterizing EBV-associated Gastric Carcinoma (EBVaGC): A deep dive into LMP1 expression patterns

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ABSTRACT

Gastric cancer (GC) is a serious public health issue due to its frequency and severity. It is, for both sexes, one of the most common causes of cancer-related death and is a major contributor to the global burden of disease. Recent data show that Epstein-Barr virus (EBV) has been detected in different histopathological subtypes of gastric carcinoma and that EBV-associated gastric carcinoma (EBVaGC) represents about 10% of all cases. Moreover, the LMP1 protein characterizing the malignant transformation of cells in several cancer models seems to be very rarely expressed in this type of cancer. This study aimed to characterize EBVaGC in our population by detecting LMP1 in gastric carcinomas in about 30 selected patients. The results showed that in our population, nuclear staining predominates, showing that the antrum remains the most sampled site both for these pathologies and for LMP1 positivity (nuclear staining). In general, the LMP1 marking was negative for 22.58%, positive with a nuclear predominance at 64.52%, nuclear and cytoplasmic at 12.90%, and no positive marking for the cytoplasm. Results were not like the different studies on the expression of this oncogenic protein without EBVsCG, probably finding an explanation in the fact that our country is among the endemic regions for this herpes virus. In conclusion, the rate of LMP1 expression among gastric carcinomas does not seem similar to that observed in other countries. This study characterizing EBVaGC in Tizi-Ouzou, Algeria, reinforces the need for further studies to clarify the role of EBV (LMP1) and to explore its potential value as a predictive biomarker for the development of this type of cancer pathology.

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Introduction

Stomach cancer is a common and aggressive malignancy that has a poor five-year prognosis (1). It is the fourth leading cause of cancer-related mortality globally, accounting for nearly 800,000 deaths, or 7.7% of all cancer deaths (2). Environmental and genetic variables, as well as specific medical problems, all impact the development of stomach cancer (3). These include salty diets, smoking, and alcohol consumption (4-10). Notably, according to a meta-analysis (11), lower levels of alcohol intake may increase the risk of stomach cancer and chronic gastritis (12). Age, gender, pernicious anemia, past stomach surgery for benign diseases, and a family history of gastric cancer are other important variables (13-19). Furthermore, research is progressively linking the prevalence of *Helicobacter pylori* to stomach ulcers (Hp) with gastric cancer risk (20-23).

The Epstein-Barr virus (EBV), another pathogen, has received attention due to its link to stomach cancer. EBV, also known as human herpesvirus 4, was isolated from human tumor cells for the first time in 1964 (25). It affects more than 90% of the world's adult population, usually in childhood or early adulthood (26). Although most EBV infections are harmless, EBV is linked to around 1.5% of all cancers worldwide. This virus has been related to several lymphoid cancers, including Hodgkin lymphoma, Burkitt's lymphoma, post-transplant lymphoproliferative illness, and Natural Killer/T-cell lymphoma (27). Furthermore, EBV is linked to epithelial cancers such as nasopharyngeal carcinomas and 10% of stomach cancers (30, 31).

The role of EBV in cancer is yet unclear. EBV is a 172-kb double-stranded linear DNA virus that contains several protein-coding and functional RNA genes (32, 33). It is believed that more than 90% of the world's population has it (34). In cells, EBV may cause two forms of infection: lytic and latent. Its genomic DNA is present in the nucleus as an episome, chromatinized with histones, and only a few latent genes are expressed (36, 37). Because it expresses a narrow set of genes that may avoid the host immune system, this latent infection allows the virus to survive for long periods (38). The expression of dormant

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EBV genes can affect the cell cycle, apoptosis, and immune response, all of which contribute to tumor start and progression. (39). Depending on the gene expression profile, latency can be categorized into three types (I, II, and III), suggesting that EBV can influence cell growth differently (40).

Latency membrane protein 1 (LMP1) is one of the genes that is expressed during latency. Its expression has traditionally been connected to latency types I and II, which have been linked to nasopharyngeal cancer, Hodgkin's lymphoma, and post-transplant lymphoproliferative diseases, respectively. Latency type I, in which LMP1 is not expressed, is linked to Burkitt's lymphoma and gastric cancer (40). This study focuses on LMP1, which is infrequently expressed in GC, according to the literature. LMP1 is detected by immunohistochemistry, a technique often utilized during type I viral latency. This approach not only identifies LMP1 but also offers information on the location of immunological markers inside the cellular compartments of individuals with gastric cancer (GC).

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Materials and Methods

Population selection in the study

In the course of this research, we implemented specific inclusion criteria to carefully choose patients with exclusive stomach tumors and no prior history of nasopharyngeal tumors or any other malignant conditions associated with EBV, such as Hodgkin's lymphoma, or non-Hodgkin's lymphoma. Additionally, we excluded individuals with a history of recent transplantation. Following these exclusions, our study included a total of 31 biopsy samples. We also gathered pertinent clinical information, including age, gender, symptoms, and the reason for their initial medical consultation.

Immunohistochemical detection of LMP-1 protein

We used immunohistochemistry with an anti-LMP-1 antibody to detect EBV on silanized slides. The procedure comprised deparaffinization and heat treatment to restore the epitope. The slides were treated with an H2O2 solution to decrease endogenous peroxidase activity.

The main antibody, Mouse monoclonal [CS 1-4] anti-EBV LMP1, was applied at a 1:5 concentration and incubated at room temperature for 40 minutes. Similarly, the secondary antibody was anti-mouse and conjugated to peroxidase. A substrate solution and the chromogenic solution DAB (3,3-diaminobenzidine tetrahydrochloride) were applied to the slides and incubated for 10 minutes to reveal the development of the antibody-antigen complex.

Tissue samples were counterstained with Mayer's hematoxylin as part of the procedure. To facilitate slide interpretation, we used sections of Hodgkin lymphoma as positive human controls.

Statistical analysis

The statistical analysis was conducted using Excel to create various figures and R software for both descriptive and inferential statistics.

Results

Characteristics of the study population

As previously mentioned, we took care to select a subpopulation of individuals from a population of patients. The purpose of this selection is to randomize our series, based on the inclusion criteria mentioned above.

In this series, the cases were included, represented by 67.74 % male and 32.26 % female patients, with a mean age of 64.74 +/- 6.05 (range 54-79 years). Figure 1 shows the number of patients in each age group, divided by sex.

Gastric cancer is more prevalent in men than in women. This also showed that the majority of cancer patients are over the age of 54.

Gastric pathologies

All gastric pathologies were classified according to the WHO 2017, 8th edition. Several 31 biopsies have been studied after clinical data and information, such as age, sex, symptoms, or reason for the first consultation, were recorded (Table 1).

Regarding tumor location in the stomach, the most predominant sampling site is the antrum region, with 77.42% of patients. This is followed by the tumoral region (16.13%), the intro-fundal region (3.32%), and the oesogastric mucosa (3.23%) (Figure 2).

The most common pathology was differentiated gastric adenocarcinoma (61%), followed by independent cell gastric carcinoma (20%), atrophic gastritis with intestinal metaplasia (10%), mucosal colloid adenocarcinoma (3%), undifferentiated carcinoma of the stomach (3%), and endocrine tumor of the stomach (3%).

Differentiated gastric adenocarcinoma was the most common kind of stomach cancer. The development of abnormal cells in the stomach lining distinguishes it. Other than that, independent cell gastric cancer was unusual. It was identified by the formation of abnormal cells that were not well differentiated, i.e., they did not resemble typical stomach cells. Atrophic gastritis with intestinal metaplasia is a condition that causes the stomach lining to thin and become inflamed. It increases the risk of ac-

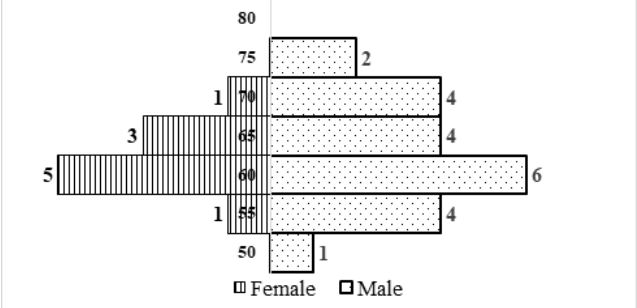

Figure 1. Age pyramid representation of the population studied. The vertical numbers (50 to 80) are patients' ages by years. The side numbers represent the number of patients in each age group.

Table 1. Distribution of patients according to the associated gastric pathology.

Associated Gastric pathology	Rate %
Atrophic gastritis + Intestinal metaplasia	10
Differentiated gastric adenocarcinoma	61
Endocrine tumor of the stomach	3
Independent cell gastric carcinoma	20
Mucosal colloid adenocarcinoma	3
Undifferentiated carcinoma of the stomach	3

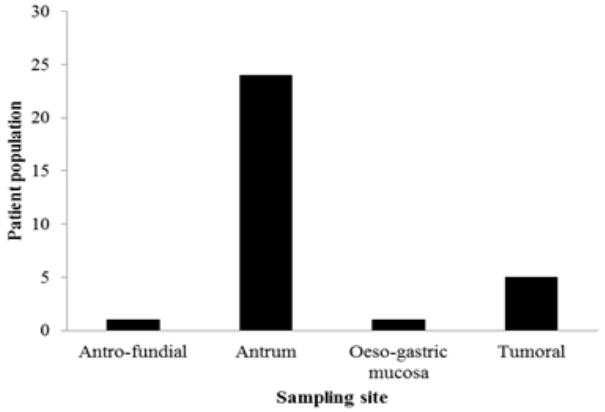

Figure 2. Distribution of patients across sampling sites.

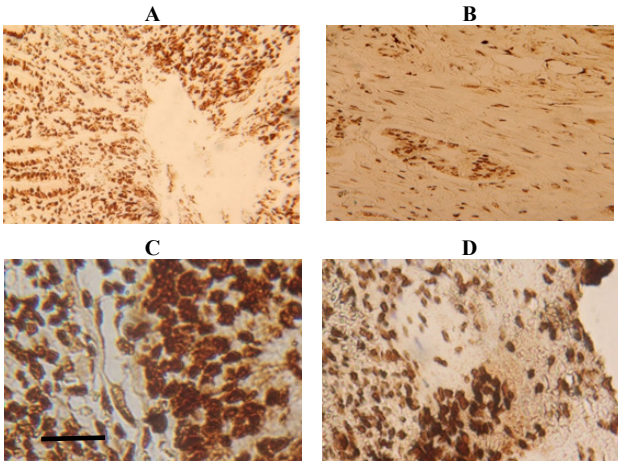

Figure 3. Detection of LMP1 by immunohistochemical labeling in different tissues A: Immunohistochemical section from gastric normal tissue; **B:** Immunohistochemical section from gastric carcinoma (no staining for LMP1); **C:** Positive nuclear staining of LMP1 revealed by immunohistochemical section from a well-differentiated gastric adenocarcinoma; **D:** nuclear and cytoplasmic LMP1 labeling from a well-differentiated gastric adenocarcinoma. Scale bar: 10 µm in C.

quiring stomach cancer. Mucosal colloid adenocarcinoma was distinguished by the formation of a thick, mucus-like substance. Undifferentiated carcinoma of the stomach is a more serious but uncommon kind of gastric cancer. It was distinguished by the development of very aberrant cells that did not resemble any regular stomach cells. Another rare tumor was the endocrine tumor of the stomach, which arose from the hormone-producing cells of the stomach.

Immunohistochemistry staining

According to the marking of LMP1 (Figure 3), the nuclear labeling predominated. This means that the majority of patients in the study had nuclear labeling of their LMP1 (Figure 4).

Regardless of the cellular compartment, LMP1 positivity is more frequent in males than in females. The difference in LMP1 positivity between male and female cells is most pronounced in the nucleus, where LMP1 positivity is 60% in female cells and 40% in male cells. The staining of cells by LMP1 is less strong in the cytoplasm and membrane, although it is still statistically significant.

LMP1 is present mostly in the nucleus, next in the cytoplasm, and finally in the membrane. The proportion of LMP1-positive cells decreases as LMP1 moves from the nucleus to the cytoplasm to the membrane.

Site sampling and cellular localization.

The antrum was the most sampled site for both these pathologies and for LMP1 positivity (nuclear marking) (Figure 5). Other common sampling sites for gastric cancer include the cardia, the part of the stomach that is closest to the esophagus, the fundus (the upper part of the stomach), and the body, the middle part of the stomach.

Relationship between the different factors

Table 2 displayed the results of a Chi-squared test of independence using four categorical variables: Gender, Sampling Location, Pathology, and LMP1 Marking. The entries above the diagonal reflect the Chi-squared statistic for each variable pair, while those below the diagonal provide the related p-value for each test.

The sex and sampling site rows and columns show that the χ^2 statistic for testing the independence of these two variables is 4.92, and the p-value is 0.18. This means that there is an 18% chance of obtaining the two variables are truly independent. If the p-value is less than 0.05, then there is a statistically significant relationship between the two variables. Since all of the p-values in Table 2 are greater than 0.05, we conclude all the variable pairs are independent.

The data in this case does not give significant evidence that Sex, Sampling site, Pathology, and LMP1 marking

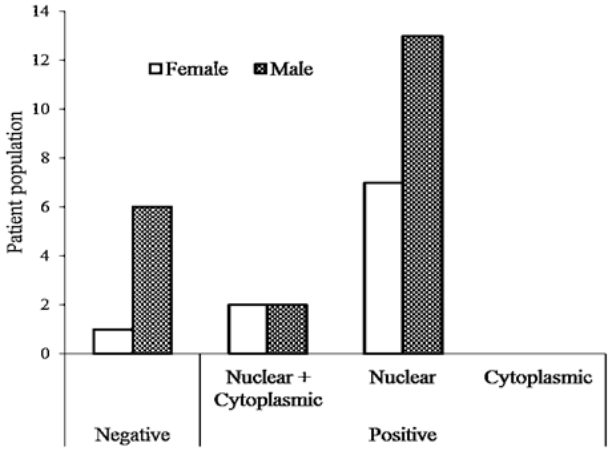

Figure 4. Cellular localization of tissue labeling of LMP1 by gender.

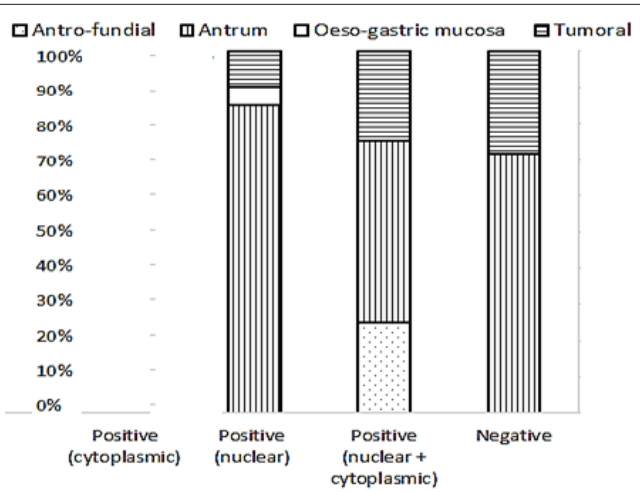

Figure 5. LMP1 marking according to the sampling site.

are connected. It is crucial to remember, however, that a negative result does not always imply that there is no link between the variables. The sample size may have been insufficient to find a significant impact, or the association may be too weak to be identified by the Chi-squared test.

Me an age of patients in four different sampling sites in gastric cancer

Table 3 shows the results of an analysis of variance (ANOVA) test used to compare the mean age of patients in four distinct sampling locations in a stomach cancer research. The F statistic of 0.08 with 3 and 27 degrees of freedom, which was not statistically significant at the 0.05 level ($p = 0.97$), indicated that the mean age of patients at the four sample sites appeared to be the same. However, the mean age difference between the four sample sites in this study is minor but not statistically significant.

Table 4 shows a low, but not statistically significant, difference in mean age between the stomach diseases studied in this investigation. With 5 and 25 degrees of freedom, the F-statistic of 1.57 does not approach statistical significance at the 0.05 level ($p = 0.20$). As a result, the average age of patients in all groups is comparable. The eta-squared statistic of 0.24 indicates a tiny effect size, indicating a slight but detectable difference in mean age across groups. More research is needed to verify this discovery and determine its therapeutic significance.

Gastric LMP1 marking according to age

Within this research, there was a minor and non-significant change in mean LMP1 marking according to age. The F-statistic, which is 0.54 with 2 and 28 degrees of freedom, is not statistically significant at the 0.05 level ($p = 0.59$). This implies that the average LMP1 marking in the three age groups is approximately comparable. The eta-squared score of 0.04 shows a very small effect size, demonstrating very little difference in mean LMP1 marking across the three age groups (Table 5).

Discussion

Our study is the first comprehensive report to characterize EBV-positive gastric pathologies in the North African population. In general, our findings showed a higher

Table 2. Relationships between the different variables studied.

	Sex	Sampling site	Pathology	LMP1 marking
Sex		4.92 (3)	6.11 (5)	1.68 (2)
Sampling site	0.18		11.17(15)	9.20 (6)
Pathology	0.30	0.74		9.63 (10)
LMP1 marking	0.43	0.16	0.47	

NB: The values above the diagonal represent the chi statistic $\chi 2$(Degree of Freedom). The values below the diagonal represent the level of significance.

Table 3. Patients mean age and the different sampling sites.

Sampling site	Antro-fundial		Antrum		Oeso-gastric mucosa		Tumoral		F(3,27)	η^2
	M	SD	M	SD	M	SD	M	SD		
Age	67.00	NA	64.83	6.69	65	NA	63.8	3.83	0.08	0.009

M: Average; **SD:** Standard deviation; **F(df1,df2)**: Fisher statistics with the degrees of freedom; **η2:** eta-square (The effect size).

Table 4. Gastric pathologies of the sample.

Pathology	M	SD	F(5,25)	η^2
Atrophic gastritis + intestinal metaplasia	71.67	2.08	1.57	0.24
Differentiated gastric adenocarcinoma	63.68	6.60		
Endocrine tumor of the stomach	65.00	NA		
Independent cell gastric carcinoma	62.67	2.94		
Mucosal colloid adenocarcinoma	72.00	NA		
Undifferentiated carcinoma of the stomach	69.00	NA		

M: Average; **SD:** Standard-deviation; **F(df1,df2)**: Fisher statistics with the degrees of freedom; **η2:** eta-square (The effect size).

Table 5. LMP1 marking according to age.

LMP1 marking	Negative		Positive (N+C)		Positive (N)		F(2,28)	η^2
	M	SD	M	SD	M	SD		
Age	63.71	5.41	62.5	3.32	65.55	6.68	0.54	0.04

M: Average; **SD:** Standard deviation; **F(df1,df2)**: Fisher statistics with the degrees of freedom; **η2:** eta-square (The effect size).

prevalence of gastric cancer in elderly individuals, with males accounting for 67.4% of the cases and an average age of 64 years. These results are consistent with previous research that also indicates a preference for gastric carcinoma in older male patients. However, it is important to note that due to the size of our study, we cannot definitively determine the prevalence of EBV-positive gastric pathologies. Nonetheless, our results demonstrated an overall positivity rate of 77.2%, involving nuclear, cytoplasmic, or both forms of marking, with a predominance of nuclear marking. It is well-documented in the literature that the prevalence of EBV-positive gastric cancers varies across different geographical regions, ranging from 2% to 20%. The predominance of nuclear localization of the EBV protein LMP1 in our findings suggests a potential interaction with other cellular elements, whether related to cell transformation or not, which warrants further investigation.

Furthermore, the prevalence of EBV-positive gastric malignancies does not follow a predictable trend and is affected by total stomach cancer incidence. According to studies, places with a low prevalence of stomach cancer have a greater rate of EBV-positive cases, whereas areas with a high incidence of gastric cancer have a lower rate of EBV-positive cases. Sousa et al. (46) found in a comprehensive study that North America, which has a low frequency of gastric cancer, has a relationship between EBV and stomach cancer, accounting for 12.9% of cases. In contrast, EBV-positive gastric cancers accounted for only 7.99% of all gastric cancer cases in locations with a high risk of gastric cancer, such as Asia. These geographical differences highlight the complicated interaction between EBV and the epidemiological variables determining stomach cancer incidence.

Our data show that there is no statistically significant relationship between LMP1 marking and the patients' pathological state, sample collection site, or age. This lack of association might be attributed to the high variety across the numerous illnesses and the unique features of each patient.

The location of the tumor would be useful for the characterization of gastric cancer. We found that the antrum is the most common location for the development of gastric cancer. The fact that LMP1 positive is more frequent in the antrum implies that EBV-LMP1 may have a role in the development of gastric cancer. Nonetheless, further studies are needed to validate this link and get a thorough knowledge of LMP1's role in the development of this cancer. Of course, several variables contribute to this disease. The antrum is a vital part of the stomach, located near the pylorus, which serves as the valve that separates the stomach from the small intestine. This area is also particularly vulnerable to the corrosive effects of gastric acid and pepsin, digestive enzymes generated by the stomach. Acid and pepsin exposure can cause stomach lining damage, which can lead to inflammation and aberrant cell development, potentially escalating to cancer. LMP1 is a protein located on the surface of cancer cells that is thought to aid in cell proliferation and dissemination. The presence of LMP1 in the antrum implies that it may have a role in the development of gastric cancer in this part of the body. The sampling site is important since it helps determine the stage of the malignancy and the best treatment method. For example, if the tumor is still in its early stages, surgery may be an option. If the disease has spread to other regions of the body, surgery may not be feasible, and alternate therapies such as chemotherapy or radiation therapy may be prescribed.

Immunohistochemical staining revealed the presence of LMP1 in tissues and pinpointed its location at the cellular level, whether in the nucleus, cytoplasm, or both at the same time. The decision to concentrate on detecting this protein was driven by its low expression in stomach diseases, particularly cancers, which supports a similar viral latency program.

Following the finding of multiple cases of nasopharyngeal carcinoma (NPC) linked to EBV, researchers began to look at additional sites, including the stomach. The capacity of EBV to infect epithelial cells has serious consequences, resulting in malignant transformation and the development of NPCs, a subset of gastric adenocarcinomas, and some salivary carcinomas. NPC is used as a model to study the malignant transformation of epithelial cells in EBV-related carcinomas at many locations (47-50).

Surprisingly, evidence reveals that in gastric adenocarcinoma, EBV may penetrate the gastric epithelium without requiring a particular receptor (51). The most common scenario for EBV infection of epithelial cells includes direct cell-to-cell interaction with B cells (52). This might imply that the presence of LMP1 in this histological kind of gastric cancer is associated with the virus's capacity to easily infiltrate the cells, increasing concerns about directly implicating EBV in this type of cancer. On the other hand, other researchers hypothesized that stomach inflammation would attract EBV-infected B cells to gastric epithelial cells, thus increasing the risk of EBV infection (53).

Another research found that EBV does not integrate into the host genome but persists as an episome in gastric cancer cells (54). According to studies, stomach tissues infected with EBV in epithelial cells contain 3000 times more viral particles than infected B cells (55). To identify infection in gastric epithelial cells, researchers established a limit of 2000 EBV particles/10^5 cells, which was fulfilled in both US and Honduran samples (55).

However, despite being uncommon, EBV infection can be identified in a tiny proportion of non-neoplastic gastric mucosa, often in a single cell or a few glands (54, 56). This shows that EBV infection may occur before clonal proliferation of EBV-infected cells, potentially leading to cancer (57). Indeed, EBV infection has been found in high-grade pre-invasive lesions such as severe dysplasia and nasopharyngeal carcinoma in situ, but not in low-grade illness (58). Similar findings have been reported in EBV-positive gastric cancer with accompanying EBV-negative normal gastric mucosa, inflammatory mucosa, and premalignant lesions (59).

Finally, hereditary factors may contribute to stomach cancers. In the absence of EBV infection, deletions in 3p and 9p have been found in low-grade dysplastic lesions and normal nasopharyngeal epithelium of persons at high risk of developing NPC. These deletions suggest that genetic processes occur early in the pathophysiology of NPC and may predispose individuals to EBV infection in the future (60). As a result, when evaluating the probability of gastric cancer development, it may be argued that EBV requires a changed cellular environment.

Notably, *Helicobacter pylori* infection has been shown to preferentially colonize the antrum (61,62), which cor-

responds to our sample group, where the antrum was the most often impacted location, with LMP1 positive at 64.62% (nuclear marking) and 12.90% (nuclear plus cytoplasmic). This increases the possibility of these two infectious agents interacting. However, expanding the research population would be more dependable in proving any actual collaboration between these widespread and potentially carcinogenic bacteria.

EBV has two life cycles: the lytic cycle and the latency cycle. The former promotes viral particle formation and spread, whereas the latter keeps the virus in the host for a more extended period. EBV is largely found within the host, where a group of oncogenic genes produces EBV-encoded oncoproteins that aid in stomach cell proliferation. The EBV-LMP1 has received a lot of attention. Indeed, LMP1 expression was seen in EBV-associated gastric cancer, suggesting that viral gene expression more closely approximated type I latency, as seen in Burkitt's lymphoma. LMP2A was occasionally expressed at low levels. In more recent EBV investigations, LMP1 gene expression was found at extremely low levels in EBV-associated gastric malignancies, along with LMP2 and other EBV latent genes (63, 64). LMP1's capacity to activate various signaling pathways indicates oncogenic characteristics both in vitro and in vivo.

The discovery of LMP1 protein in gastric epithelial cells in individuals with gastric cancer indicated that the cellular reservoir for EBV maybe B lymphocytes invading the stomach's mucosal lymphoid tissue, allowing viral amplification (65-68). This EBV movement indicates that the virus is switching between epithelial and B host cells. LMP1 is important in cell transformation (69-71). The mechanism requires the LMP1 amino terminus, transmembrane domains, and CTAR1, whereas CTAR2 is necessary for the long-term proliferation of altered B cells (72, 73).

Even though LMP1 is a well-known oncoprotein required for the effective transition of resting primary B cells into autonomously proliferating lymphoblastoid lineages, its expression in EBV gastric carcinomas is exceedingly low and typically undetectable at the protein level. As a result, despite infrequent instances of type II, III, and lytic infections (74-76), stomach malignancies are classed as type I latency.

Furthermore, the role of LMP1 in epithelial cell transition in NPC is clear. It is crucial to note, however, that EBV alone is inadequate to begin carcinogenesis, which normally entails the overexpression of oncogenes and a plethora of faulty tumor suppressor genes. In NPC cells, LMP1 increases the upregulation and phosphorylation of surviving and p53, with p53 facilitating cell cycle advancement without causing apoptosis. These findings provide new directions for research into EBV-positive gastric diseases (77,78).

In summary, there is strong evidence that EBV may have a role in the development of gastric cancer via both direct and indirect processes. These strategies involve infecting epithelial cells and creating a latent program by viral oncogene expression. Furthermore, EBV induces persistent inflammatory responses, which result in tissue damage due to increasing local viral loads.

Scientists are still fascinated by the Epstein-Barr virus. It has been related to a variety of cancers, including stomach cancer. Indeed, many researchers agree that LMP-1 plays a significant role in the regulation of cell proliferation and survival, particularly through the activation of signaling pathways such as NF-B, and thus contributes to oncogenesis as well as tumor cell resistance to conventional treatments.

Our data show that EBV is found in a variety of distinct gastric illnesses, meaning that the virus is more likely to adhere to the fundamental biological and immunological factors known to promote the creation and maintenance of gastric tumors. Exploring the latency characteristics of EBV in the gastric epithelium, and the histological appearance of CG associated with it, as well as providing further molecular and clinical studies, could contribute to a better understanding of its epidemiology, and consequently, help improve the outlook for affected patients. Indeed, EBV could serve as biomarkers for immunotherapy, dedicated to this unique patient population.

EBV has co-evolved with humans for millions of years, and during this close association, the virus has adapted its life cycle to its host, making EBV one of our most common "parasitic" viruses. Today, patients at high risk of treatment failure are candidates for novel treatment regimens, and EBV, a ubiquitous gamma herpes virus, may represent a promising therapeutic target.

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