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OBM Genetics

Review

Epstein-Barr Virus and *Helicobacter pylori* as Two Main Risk Factors in Gastric Cancer

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Academic Editor: Lunawati L Bennett

OBM Genetics
2024, volume 8, issue 4
doi:10.21926/obm.genet.2404272

Received: June 21, 2024
Accepted: November 13, 2024
Published: November 20, 2024

Abstract

Microbial and viral pathogens have emerged as key contributors to cancer development. Research conducted in the last twenty years has significantly enhanced our comprehension of the cancer-causing capabilities of infectious agents. An illustrative instance is gastric cancer (GC), which is closely associated with *Helicobacter pylori* (*H. pylori*) and Epstein-Barr virus (EBV) infections, with approximately 90% of non-cardia GC cases attributed to *H. pylori* infection and around 10% linked to EBV. Despite significant research efforts, GC remains a severe clinical challenge, ranking as the fifth most commonly diagnosed cancer worldwide. In 2020,

an estimated 768,793 people died from GC in the world. The pathogenicity island (PAI), cagA protein, VacA, and other virulence factors in *H. pylori* and several latency factors such as EBNA-1, LMP-1, and LMP2A in Epstein-Barr virus, as well as pattern of gene methylation and EBV and *H. pylori* co-infection are shown as the leading causes of pathogen-related GC. The unique molecular and clinical characteristics associated with EBV and *H. pylori* in GC highlight the importance of further understanding their respective roles in GC development and progression. This knowledge may inform future preventive and therapeutic strategies targeting these infectious agents in the context of GC. This review aims to elucidate the mechanisms underpinning GC's EBV and H. pylori-induced carcinogenesis.

Keywords
H. pylori; Epstein-Barr virus; gastric cancer

1. Introduction

In the last century, gastric cancer (GC) ranked as the fifth most prevalent cancer globally. Approximately 1 million people are diagnosed annually with GC worldwide, resulting in about 738,000 fatalities [1]. Surgery, chemotherapy, radiotherapy, targeted therapies, and immunotherapy have all been used to treat cancer in recent decades. Newer targeted therapies and immunotherapy have been incredibly revolutionary for individualized treatment approaches, potentially significantly improving outcomes, while surgery and chemotherapy have remained traditional approaches [2]. Accordingly, it is established as the second principal reason for cancer-related deaths on a worldwide basis. GC is particularly prevalent in East Asia, with the highest incidence rates observed in East Asia, South America, and Eastern Europe. At the same time, North America and most parts of Africa exhibit lower incidence rates. Developing countries, particularly East Asia, account for approximately 77% of GC cases, while developed nations exhibit a lower incidence rate, comprising approximately 23% of cases [3-5]. Indeed, GC is still one of the most common malignancies diagnosed in China, Japan, and South Korea. Their high incidence may be explained by a very high prevalence of *H. pylori* infection, dietary habits with high salt intake, and some genetic predispositions peculiar to East Asian populations. While *H. pylori* is a dominant risk factor from a worldwide perspective, EBV accounts for approximately 10% of global gastric carcinomas. It affects more minor, more specific subgroups and has a less important role when compared to *H. pylori* in East Asia [4].

The incidence of GC varies between genders and across different regions, with rates typically 2 to 3 times higher in men than in women. The GC manifests as a diverse disease emerging from a prolonged, multifaceted progression influenced by various factors such as bacterial and viral infections, genetic background, and environmental factors [6]. Extensive evidence suggests that GC is attributed to many genetic and epigenetic modifications that influence the functionality of repair genes, tumor suppressor genes, and cell adhesion molecules. In the GC, several genes that act as tumor suppressors, such as p14, p15, p16, hMLM1, GSTP1, CDH1, RASSF1, COX-2, DAP-K, THBS1, CDH4, TIMP-3, RARβ, MGMT, APC, CHFR, DCC, RUNX3, TSLC1, and 14-3-3 sigma, experience silencing as a result of hypermethylation [7-9]. The stomach encompasses various anatomical

sections, such as the cardia (proximal end), the fundus, the body, the pylorus, and the antrum. Tumors situated in gastric cardia generally exhibit a worse prognosis compared to those in the antrum, characterized by survival rates of less than 5 years and higher mortality rates [10].

For a prolonged period, GC persisted without precise detection until the 1950s, when its emergence became suddenly evident. Initially, the cause remained obscure, but it is now recognized that the surge in GC cases correlates with *Helicobacter pylori* (*H. pylori*) infection. This bacterium has cohabited with humans since they migrated from Africa around 58,000 years ago [11]. The carcinogenic mechanism of *H. pylori* has undergone extensive scrutiny over the past two decades. Despite concerted efforts, GC mortality has notably increased across most global regions. However, GC continues to be characterized by a poor prognosis and significant mortality. Generally, countries with higher GC incidence rates tend to exhibit better survival rates than those with lower prevalence, a variance primarily linked to the tumor's location within the stomach. Epstein-Barr virus (EBV), also known as HHV4 and a member of the Herpesviridae family, is another infectious agent associated with GC. EBV has a well-established association with malignancies like nasopharyngeal cancer and post-transplant lymphoma, and more recently, it has been detected in GC samples [12]. The brief mechanism of action for both pathogens is illustrated in Figure 1.

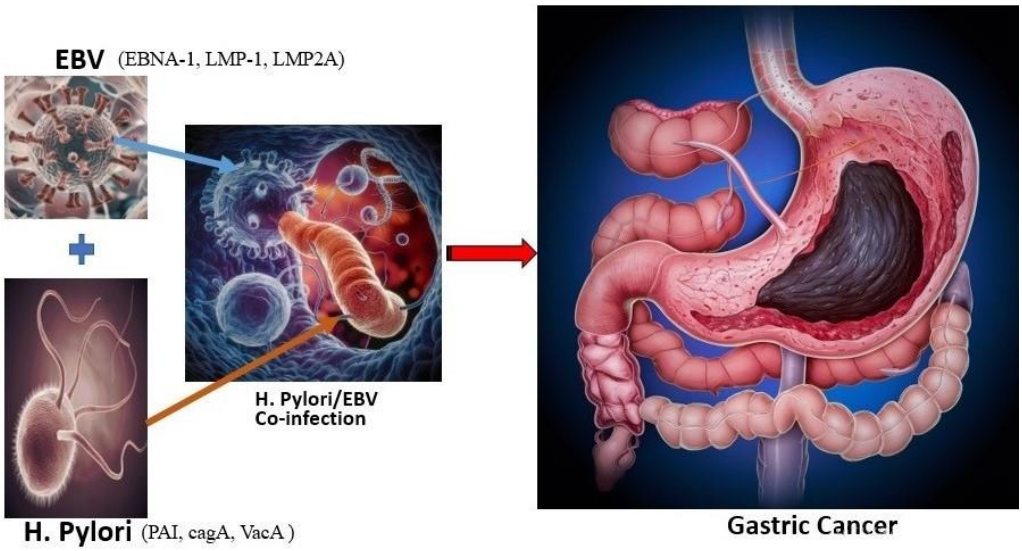

Figure 1 Roles of *H. pylori* and EBV in Gastric Cancer Development and Progression. This figure illustrates the co-infection process of *Helicobacter pylori* (*H. pylori*) and Epstein-Barr virus (EBV) and their combined contribution to gastric cancer (GC) development. *H. pylori* introduces oncogenic factors such as the pathogenicity island (PAI), cytotoxin-associated gene A (cagA), and vacuolating cytotoxin A (VacA), which lead to chronic inflammation and cellular changes conducive to cancer. Meanwhile, EBV expresses latent proteins (e.g., EBNA-1, LMP-1, and LMP2A) that enhance cell proliferation, immune evasion, and epigenetic changes. The interaction of cagA with host cell pathways triggers oncogenic signaling, while EBV's latency-associated proteins further disrupt cellular mechanisms. Together, these factors promote GC progression by manipulating immune response, inducing inflammation, and driving genetic mutations and epigenetic alterations, ultimately leading to malignancy.

2. *H. pylori* Infection and GC

Marshall and Warren's groundbreaking 1984 discovery established the link between *H. pylori* infection and gastric ulcer disease, which paved the way for future research into this infection's significant role in developing GC. This discovery was instrumental in classifying *H. pylori* as a Class 1 carcinogen by the International Agency for Research on Cancer (IARC) due to its established role in progressing from chronic gastritis to GC [13, 14]. Research on this pathogen has spanned over three decades, revealing a clear association between *H. pylori* and non-cardia GC. This comprehension highlights the fundamental importance of chronic inflammation and pathological modifications in cancer progression. Based on this model, GC undergoes a series of sequential changes in the gastric mucosa, beginning with chronic gastric inflammation, followed by atrophic gastric alterations, dysplasia, metaplasia, and ultimately leading to tumor formation [15]. The most common subtype of adenocarcinoma associated with *H. pylori* infection is non-cardia intestinal, primarily due to the bacterium’s capacity to induce chronic inflammation in the gastric mucosa. *H. pylori* is implicated in nearly 90% of non-cardia gastric cancers, with chronic inflammation driving the formation of precancerous lesions. This chronic inflammatory environment facilitates a cascade of histological changes, including atrophic gastritis, intestinal metaplasia, and dysplasia, which ultimately progress to cancer [16]. Consequently, eradicating *H. pylori* offers promise in managing this debilitating disease. Several effective antipyretic treatment regimens targeting the bacterium have been developed and successfully implemented in clinical settings [17]. Examining the impact of eradicating this bacterium on reducing GC prevalence, we can look to developed nations like Australia, where improvements in social and economic conditions have led to a decline in *H. pylori* prevalence and, subsequently, GC rates. However, recent discussions have also brought attention to the role of genetic variations. Clinical trials aimed at reducing cancer incidence through *H. pylori* eradication have controversial effectiveness. Nevertheless, these studies underscore that *H. pylori* triggers this multi-stage disease, and early eradication could effectively prevent its development [18].

Polymorphism in specific genes among susceptible hosts of *H. pylori* influences an individual's risk of developing GC. These gene variants are ATG16L1 in Western populations, NOD1, NOD2, COX-2, and MDM2 in Chinese populations [18], IL-10 in Korean populations [19], MTHFR, and iNOS in Iran [20]. However, the significance of these gene variants as critical risk markers in infected individuals remains unclear, warranting further investigations. Besides genetic variants, epigenetic factors also play a role in the *H. pylori*-induced epigenetic silencing of FOX3, contributing to the premature onset of GC [21]. Moreover, specific gene variants may also reduce the susceptibility of *H. pylori*, such as DNMT-1 in the Chinese population [18], uPA in the Japanese population [22], C7orf10, TSTD2, SMG7, and XPA in the Malaysian population [23]. *H. pylori* exists in two forms in the gastric environment: coccoid and helical. The coccoid form has less antigenicity and produces fewer toxin proteins (cagA, arginase RocF, and tumor necrosis factor-α (TNF-α)), making it easier to escape from the immune system [22].

This bacterium enters the stomach through contaminated food or water and is placed by the flagella in the gastric mucosa cells. In the case of gastric ulcer, it settles on the cells of the gastric epithelium and embeds its flagella there. The microbe needs a small amount of oxygen to survive, and its spiral shape allows the bacterium to enter the gastric lining through the mucous membrane. The bacterium also has receptors that enable it to enter the cell through the mucosa and stick to

the gastric lining. Approximately 20% of *H. pylori* attach to the surface of epithelial cells, while others attach to cell-to-cell junctions [24]. Autotransporter proteins found on the surface of bacteria, such as OipA, SabA, BabA, AlpA, AlpB, HopZ, and others, enhance the attachment of bacteria to the surface of epithelial cells. However, the presence of any of these proteins is not necessary. *H. pylori* is a gram-negative, microaerophilic, helical bacterium 2-4 μm in length and 0.5 to 1 μm in width. It possesses 2 to 6 unipolar flagella, allowing the bacterium to hurry in the mucous solutions lining the gastric epithelial cells. Bacterial infections remain the leading cause of gastric and duodenal diseases. Extensive epidemiological studies reveal that *H. pylori* is associated with chronic gastric, gastric ulcer disease, gastric mucosal lymphoma (MALT), and gastric adenocarcinoma. Typically contracted during childhood, this infection remains entrenched in the host's body for life if untreated [25]. Diverse mechanisms facilitate bacterial adherence to the gastric epithelium, enabling it to endure within the gastric mucosa for prolonged durations. Acid resistance due to urease, bacterial virulence factors, altered host immune response, and induction of signaling pathways are among these mechanisms. Typically, *H. pylori* colonizes in the antrum, the region of the stomach characterized by thick and robust muscles, establishing persistent infection in this area. The only bacterium that can live and grow in the gastric environment in the presence of gastric acid is *H. pylori*. The *H. pylori* can break down urea molecules in tissues and body fluids [18]. This process creates ammonia and carbon dioxide, forming a supermassive sheath around the bacterium that protects it from gastric acid, killing common bacteria. The production of ammonia through urease activity directly causes damage. Lipopolysaccharide toxins may also damage mucosal cells. The Strains of *H. pylori* with specific genes produce toxins, and VacA and cagA are more pathogens. However, even these species do not spread from the gastric membrane to other body parts. This bacterium has evolved to adapt and survive in the human stomach [6].

3. Mechanism of *H. pylori* Carcinogenesis

3.1 Pathogenicity Island (PAI) and CagA Protein

The cagA gene, encoding the cagA protein, is located at the end of 3’ PAI, a 40-kb fragment in genomic DNA, thought to have been obtained by horizontal transfer of genetic material, encoding more than 30 genes. *H. pylori* can be categorized into two groups, cagA+ and cagA-, based on the existence of a specific gene. Notably, cagA+ strains exhibit higher pathogenicity than cagA- strains, with their primary significance in their association with GC. A highly coordinated secretory system called T4SS is established by the cag PAI products, facilitating the transportation of cagA into epithelial cells as part of a complex molecular process. Evidence suggests that lipopolysaccharides, peptidoglycans, and bacterial DNA can also be transported via T4SS [26]. Upon translocation, cagA undergoes phosphorylation by the host tyrosine kinase. The phosphorylated region in this protein is EPIYA (Glu-Pro-Ile-Tyr-Ala), a repeated motif located at the carboxy terminus of the cagA molecule. This motif is responsible for the attachment of cagA to several host proteins and disrupting their function. Four distinct EPIYA types (A-B-C-D) have recently been classified according to amino acid sequences. Geographically, *H. pylori* strains harboring cagA protein with EPIYA type C are predominantly found outside East Asia, whereas EPIYA type D is primarily distributed in East Asia [24, 27]. This geographical distribution plays a critical role in the epidemiology of GC, as EPIYA-D is associated with higher pathogenicity and a greater risk of GC development in East Asian populations. In contrast, EPIYA-C motifs are linked to increased GC risk in other regions, particularly in the U.S.

Studies indicate that the presence of multiple EPIYA-C or EPIYA-D motifs can significantly impact clinical outcomes, as these types contribute to increased cagA phosphorylation levels, enhancing the protein's interaction with host cell signaling pathways and subsequently leading to a more aggressive cancer progression and poorer prognosis [28-30]. Besides EPIYA, the cagA protein has another motif at the carboxy terminus called cagA-Multimerization (CM). The CM motif contains 16 residues responsible for cagA-homodimerization and interaction with PAR1b/MAPkinase, which play a key role in epithelial cell polarity. Polymorphisms in the CM and EPIYA motifs result in variations in the molecular weight of the cagA protein, which can range between 120 and 145 kDa across different *H. pylori* variants [31]. According to our knowledge, cagA is the most important determinant of GC. Numerous human studies have revealed significant links between cagA-positive *H. pylori* infection and increased risk of GC [20, 32]. There is also considerable experimental evidence that cagA acts as an oncoprotein. The oncogenic activity of cagA is facilitated by the inappropriate stimulation of several signaling cascades, which have been shown to alter these signaling pathways in GC and inhibit tumor suppressors. These pathways include JAK/STAT, RAS/ERK, WNT/B-catenin, PI3K/AKT [29, 33]. The bacterial protein CagA is known for its ability to initiate the degradation of the tumor suppressor protein P53 and activate the PI3K/AKT, MDM2/ARF-BP1, and ERK/MDM2 pathways [28]. Until now, only viral proteins like papillomavirus E6 were identified to degrade P53 [31]. cagA alters the expression of P53 isoforms whose N-terminals are shortened, including D133P53 and D160P53 [34]. Interestingly, dysregulated p53 regulation occurs by strain-specific methods, indicating that strains of tumorigenic *H. pylori* possess a greater ability to affect p53 [31]. Research demonstrates that cagA-positive *H. pylori* strains correlate with an increased incidence of p53 mutations in infected individuals relative to non-infected individuals. These mutations diminish DNA repair efficacy and result in heightened epithelial damage in the stomach, which not only facilitates gastric carcinogenesis but also correlates with elevated rates of local recurrence and diminished overall survival in gastric cancer patients [35, 36]. In addition to p53, the *H. pylori* strain reduces the activity of other tumor suppressors such as p14, p27, ARF, SIVA1, etc. Interaction between *H. pylori* and gastric cells leads to oxidative stress and DNA damage via cagA-dependent and non-cagA mechanisms [37]. Double-stranded DNA breaks pose significant harm as they are challenging to repair. *H. pylori*-induced mitochondrial DNA damage may contribute to cellular senescence and the onset of GC. The induction of DNA damage by *H. pylori* is worsened by the suppression of P53 and several DNA repair pathways crucial for appropriate stimulation of the DNA damage response [38]. Acting as an anti-apoptotic factor, cagA induces several prosurvival proteins and pathways, including AKT and ERK kinases, and members of the anti-apoptotic B-cell lymphoma (BCL-2) family such as MCL-2, BCL-2, BCL-X1, among others [39]. Moreover, cagA inhibits pro-apoptotic factors like SIVA1, BIM, and BAD, as well as autophagy regulation and induction of inflammation [23].

Severe inflammation and extensive damage to the gastric tissue are prominent features of human infection caused by cagA-positive strains of *H. pylori*. Studies have indicated that the cagA protein profoundly impacts crucial cellular functions, including the maintenance of epithelial cell barriers, regulation of cell polarity, facilitation of cell proliferation, induction of programmed cell death, modulation of autophagy, regulation of mRNA synthesis, orchestration of inflammatory responses, and modulation of the cellular response to DNA damage. This protein affects the activity of several kinases and cell signaling pathways, such as C-MET, EGFR, CABL, SRC, PI3K, AKT, JAK, FAKP27, P53, RAS, NF-KB, etc. It also affects NF-KB-related pathways [25]. The precise manner in

which a bacterial protein elicits a wide range of effects is not fully understood. One potential hypothesis suggests that cagA may serve as a scaffold protein, engaging with various host regulatory elements to modify their typical functions [40].

The significant difference between GC-positive and GC-negative *H. pylori* is that the former possesses the cagA gene and pathogenicity island, also known as cag PAI. CagA-containing strains, especially those with multiple EPIYA motifs, inject CagA protein directly into gastric epithelial cells via the T4SS. Once inside, CagA interferes with signaling pathways like PI3K/AKT, NF-κB, and JAK/STAT, disrupting normal cellular functions and causing chronic inflammation, reduced apoptosis, and increased cellular motility. In contrast, strains that lack the cag PAI have a limited ability to cause severe inflammation or change host cell signaling. Thereby, cagA-negative strains are more often associated with conditions like gastritis or peptic ulcers but are less strongly associated with cancer development [30, 32, 33]. Additionally, cancer-associated *H. pylori* strains often produce a more active form of the vacA gene, producing the vacA toxin. Strains with the more active s1/m1 version of vacA are linked to more extensive damage to gastric cells and heightened inflammation. In contrast, non-cancerous strains usually carry the less active s2/m2 type, which poses a lower cancer risk [41]. Furthermore, cancer-linked strains typically express higher adhesion proteins such as BabA and SabA, facilitating stronger attachment to gastric epithelial cells and increasing tissue damage and inflammation [42]. Cancer-associated strains are also more adept at inducing severe immune and inflammatory responses, creating a chronic inflammatory environment that promotes tumorigenesis in the stomach. In contrast, non-cancerous strains are less likely to cause intense immune activation and are often associated with milder gastric conditions like gastritis or peptic ulcers [37].

Studies indicate that natural compounds like *Syzygium aromaticum* extract promote immune responses by polarizing macrophages to the M1 phenotype, thereby reducing *H. pylori*-induced inflammation [43]. Similarly, chebulinic acid and 1,3,6-Trigalloylglucose from *Terminalia chebula* have demonstrated anti-adhesive properties by binding to CagA and disrupting *H. pylori* adhesion to gastric epithelial cells, preventing chronic infection and reducing the risk of progression to gastric cancer [44, 45].

3.2 VacA and Other Virulence Factors

The VacA toxin is a significant virulence factor in the tumorigenesis linked to *H. pylori* infection. Its name is derived from its capacity to induce vacuoles in cultured eukaryotic cells. Despite being classified as a pore-forming toxin, VacA's amino acid sequence sets it apart from other toxins known for its pore-forming abilities [46]. VacA biosynthesis involves some serial steps. After the protein is translated, the VacA precursor experiences proteolytic degradation to generate an active 88-kDa toxin, which can either be released into the extracellular spaces or remain bound to the bacterial surface. The released VacA protein binds to the host cell's membrane and creates a specific anionic membrane channel [47]. Several functions associated with cagA activity have been found, such as destruction of gastric epithelial barriers, interference with antigen delivery, inhibition of phagocytosis and autophagy, and suppression of B and T cells that help bacteria to develop persistent infection. VacA enables the cagA to accumulate in gastric epithelial cells by inhibiting lysosomal degradation [48]. The VacA sequence exhibits notable diversity, especially in its three markedly variable regions: the Signal region (S), Intermediate region (i), and Middle region (m). Due

to sequence heterogeneity, the S region is divided into S1 and S2 regions, with additional subdivisions like s1a, s1b, s1c. The I region is classified as i1 and i2, the M region as m1 and m2, and even m2a and m2b [49]. The incidence of GC is higher in individuals infected with variants of s1/i1/m1 type of VacA protein than those infected with s2/i2/m2 types [50]. The s1 and m1 types of VacA alleles-containing strains are associated with gastric and duodenal ulcers. Besides cagA and VacA, *H. pylori* produces various cancer-associated virulence factors. Among them are extracellular proteins (OMPs) crucial for bacterial adhesion, survival, colonization, and stability. These elements contribute to gastric disease by influencing host cell signaling pathways, enhancing T4AA activity, and modifying immune responses [51]. *H. pylori* expresses a diverse set of OMPs categorized into 5 leading families according to sequence identities. The largest is family 1, which includes HOP for (*H. pylori* OMP) and HOR (for HOP-related). Adhesive proteins like BabA (HOPs) and SabA (HOPp) from the HOP subgroup are crucial for binding bacteria to host cells through interactions with fucosylated-lewis B and sialylated-lewis X antigens. This enables binding to the extracellular matrix and gastric epithelial cells. Baba has been demonstrated to boost T4SS activity and is linked to the initiation of double-strand DNA breaks in host cells [52]. SabA, on the other hand, contributes to colonization and inflammation in the human gastric environment [53]. Several studies have examined the association of BabA and SabA expression with clinical outcomes. The presence of BabA in infectious bacteria is associated with metaplasia, gastric adenocarcinoma, and Mucosa-Associated Lymphoid Tissue (MALT). In the same way, SabA in bacteria is related to the risk of malignant lesions and GC [54]. However, some studies have conflicting results [51, 52]. Gastric tumorigenesis has also been associated with other OMPs, namely HOPH, HOPQ, and HOMB [50]. Despite being a highly acidic environment, the human gastric system is not sterile and harbors complex microbial populations that influence tumorigenesis and are vital for maintaining human health. The gastric microbiota is diverse, encompassing proteobacteria, Bacteroides, firmicutes, fusobacteria, and actinobacteria, among others [54].

4. Epstein-Barr Virus Infection and GC

In 1958, the first Burkitt lymphoma in Dennis Burkitt was described as a malignancy primarily affecting the head or neck of African children, displaying one of the fastest growth rates among malignancies [55]. Interestingly, Burkitt's lymphoma was observed in the endemic region of malaria, which has geographically high rainfall and temperatures year-round. This specific geographic pattern of the disease prompted investigations into an infectious agent, leading to the discovery of the Epstein-Barr virus (EBV) by British scientists Anthony Epstein and Yvonne Barr in 1964 [56, 57]. Subsequent research revealed strong associations between EBV and other malignancies, including nasopharyngeal cancer, post-transplant lymphoma, and GC. EBV, a member of the herpes family, also known as HHV4, infects over 90% of the global population [58]. The acquisition of EBV infection commonly occurs in youth, primarily through the saliva, resulting in the infection of oral epithelial cells and various immune cells. While the majority of individuals remain asymptomatic, some may exhibit clinical signs of infectious mononucleosis, which predominantly affects adolescents and young individuals. Additionally, there have been documented cases of gastric complications associated with EBV infection, particularly when co-infected with *H. pylori* [12]. The most severe consequence of EBV infection is the development of malignant cancer. EBV infection has a significant association with various forms of lymphoma and non-lymphoma malignancies, including

Leiomyosarcoma, GC, and nasopharyngeal cancer. Globally, EBV is linked to nearly 1.5% of all cancer cases. Like *H. pylori,* the virus has been categorized as a group I carcinogen by the International Agency for Research on Cancer (IARC) [11]. The induction of EBV-related malignancies is significantly influenced by concurrent infections in addition to underlying conditions. The pioneering work of Burke et al. in 1990 first reported the connection between EBV and gastric lymphoepithelial cancer. Following this, Shibata and Weiss identified the presence of EBV genetic material in 16% of gastric adenocarcinomas two years later [59]. A comprehensive analysis of the genome by The Cancer Genome Atlas Program (TCGA) has uncovered that around 9% of human GCs exhibit positivity for EBV. The EBV-associated gastric cancer, known as EBVaGC, exhibits various molecular abnormalities, including extensive DNA hypermethylation, a high frequency of mutations in genes such as PIK3CA, ARAD1A, and Bcor, and an elevated expression of JAK2, PD-L1, and PD-L2 [60, 61]. These mutations are pivotal in cell proliferation, immune evasion, and tumor aggressiveness, differentiating EBVaGC from other gastric cancer variants [61]. Research indicates that EBVaGC frequently exhibits significant DNA hypermethylation, especially of tumor suppressor genes like p16, p14, and APC, in contrast to reduced hypermethylation rates in EBV-negative gastric cancers [62]. EBVaGC has generally lower rates of lymph node metastasis and usually a better prognosis compared with EBV-negative gastric cancer. This probably came from its distinctive molecular characteristics, with a lower prevalence of aggressive traits in non-EBV-related gastric cancers [60]. Moreover, in EBV, BART (BamHI A rightward transcripts) miRNAs downregulate tumor suppressor genes by directly targeting the 3'UTR of host mRNAs, contributing to immune evasion and cell survival. The BARF1 (BamHI-A rightward frame 1) protein is an oncoprotein that prevents apoptosis and enhances the proliferation of cells; thus, it supports EBV-positive cells for their immune evasion and contributes to gastric carcinogenesis [61]. Persistent EBV infections necessitate infection of B cells, with memory B cells playing a pivotal role in disseminating the virus throughout the lymph nodes. EBV requires several interactions between virus and host proteins to enter epithelial cells. Ephrin receptor A2, avB5-avB6-avB8 integrins, neurophylline 1, Complement Receptor 2 (CR2), and Non-muscle myosin heavy chain IIAs are among the host proteins that facilitate the entry of the virus into cells [63]. Despite the acidic conditions of the gastric environment, it remains unclear how EBV infects gastric epithelial cells. However, EBV is hypothesized to be transmitted to the gastric epithelium via infected B lymphocytes, which infiltrate the gastric mucosa due to inflammation. Thus, inflammatory processes are the prelude to the development of GC [54]. Several studies have demonstrated that the secretion of membrane vesicles by epithelial cells can trigger the virus activation in B lymphocytes harboring latent EBV infection, subsequently resulting in the infection of epithelial cells [60]. Another scenario is that EBV is transmitted in contaminated saliva and is constantly ingested. Under certain conditions, the virus may persist in harsh gastric conditions and affect gastric mucosal cells. One of the particular features of EBV is its ability to infect chronically, lytic, and latently intermittently. Following the entry into the host cell, the virus's double-stranded DNA remains in multiple copies and provides the basis for latent infection. The latent infection is described by considerable variability and flexibility in virus transcription and replication [56]. The EBV requires the latent phase to persist within infected individuals while transitioning to the lytic phase for transmission to new hosts. Upon encountering antigens, B cells differentiate into antibody-producing plasma or memory cells. EBV manipulates specific genes that serve as a differentiation tool, converting infected B cells into a population of long-lasting memory cells shielded from the host's immune system [60]. Through this process of differentiation, EBV expresses

various sets of genes. Each set is considered a different Latency: Latency 0-I-II-III [64]. In stage III Latency, the EBNA1-2-3A-3B-3C-LP and LMP1-2A-2B genes; in stage II Latency, the EBNA1 and LMP1-2A genes; in stage I Latency, EBNAI and sometimes LMP2A are expressed, and in stage 0 Latency no protein or only LMP-2A is expressed. The Latency stage 0 is characterized by the virus residing in memory B cells, where it enters a dormant state and stabilizes within the infected host. EBER I and II are non-coding RNAs present in all Latency forms. Expression of EBV genes in GC was previously considered as Type II Latency but is now considered as Type I Latency in which EBNA1 and sometimes LMP2A are expressed, but LMP-1 has been negatively expressed in many studies. The EBV genome contains a fragment known as BamH1A, which exhibits relatively high expression in EBV-related GCs [65]. Both latent and lytic gene expression are pivotal in gastric carcinogenesis [66]. During this latent phase, EBV expresses a very restricted array of genes that confer an advantage for immune evasion and establishment of a chronic infection. This is characterized by the activation of critical oncogenes with epigenetic alterations, including DNA hypermethylation that silences tumor suppressor genes in facilitating tumor progression. Conversely, the lytic phase, while less prevalent, is essential as it triggers inflammatory responses and modifies immune gene expressions, thereby shaping the microenvironment conducive to tumor growth. It can also, through lytic gene expression, turn on specific immune pathways, including increased PD-L1 expression, which dampens the immune response, promoting an environment favorable for tumor development [66]. Refer to Figure 2 for a detailed illustration of the roles of EBV and host genomes in the oncogenesis of gastric cancer.

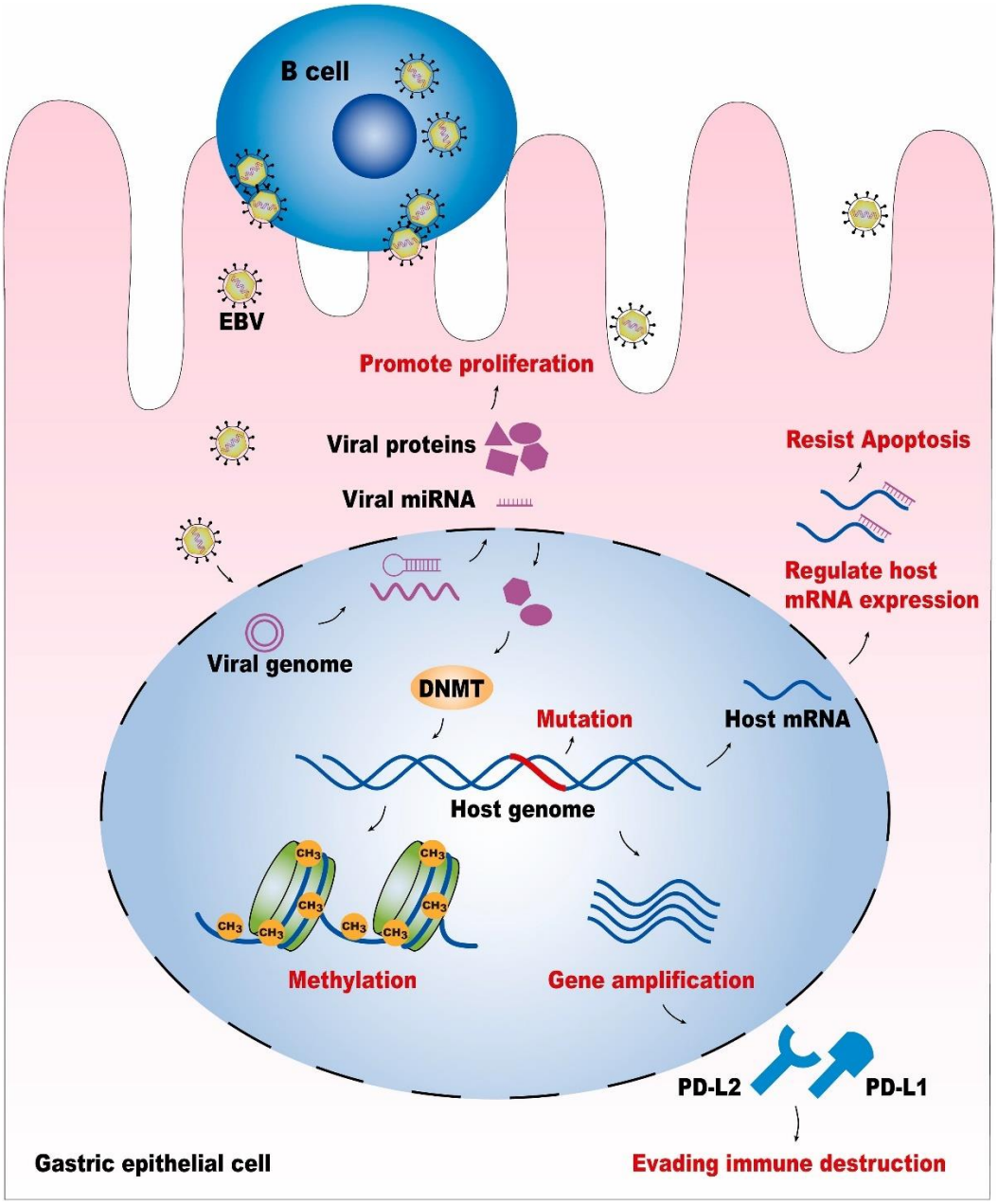

Figure 2 Mechanisms involving EBV and host genome interactions in gastric cancer development. EBV enters gastric epithelial cells primarily through contact with infected B lymphocytes. Once inside, the EBV genome exists as an episome, transcribing elements like EBERs, EBNA1, LMP1, LMP2A, BART miRNAs, and BARF1. Persistent latent infection leads to the expression of specific viral proteins and miRNAs that contribute to oncogenesis by promoting CpG island methylation of tumor suppressor gene promoters. Mutations and gene amplifications in the host genome further drive cancer progression. Viral miRNAs, particularly BART miRNAs, play a regulatory role by targeting the 3'UTR of host mRNA, affecting gene expression. Additionally, immune evasion is facilitated through PD-L1 and PD-L2 upregulation, supporting cancer cell survival and tumorigenesis. Reproduced with permission from reference [61].

5. Several Latency Factors Have Carcinogenic Properties

5.1 EBNA-1

Interfering with EBNA-1 in vitro leads to the loss of the viral episome and the demise of cultured NK/T cell lymphoma, B cell lymphoma, and NPC carcinoma cells [63]. Consequently, EBNA-1 has been suggested as a therapeutic target for EBV-associated malignancies. Despite being expressed in all Latency types (I-II-III), EBNA-1 exhibits weak immunogenicity. It contains a glycine-alanine-rich domain that binds to proteasomal proteins and inhibits their function, preventing the delivery of EBNA-1 peptides to cytotoxic CD8 + T cells by MHC-I. Evidence suggests that EBNA-1 possesses transcriptional activation abilities for both viral and cellular genes, facilitating its transport to the nucleus [67]. EBNA-1 interacts with several cellular proteins to maintain episomal status and execute transcription functions, some of which may augment oncogenic properties. Similar to classical viral oncogenes, EBNA-1 interferes with P53 activation. The promyelocytic leukemia (PML) protein is a tumor suppressor protein that regulates p53. EBV-infected cancer cells significantly express PML nuclear bodies to a lesser extent, and turning off EBNA-1 causes PML levels to return to normal [64]. Although the precise function of PML bodies remains poorly understood, they are targeted during infection by several viral families, likely due to their involvement in antiviral interferon responses. EBVaGCs (EBV-associated gastric cancer) have lower levels of PMLs than EBVnGCs (EBV-negative gastric cancer). EBNA-1, by interfering with PML and P53, increases cell survival after DNA damage [68].

On the contrary, EBNA-1 interacts with USP7, leading to the destabilization of P53. USP7 typically removes ubiquitin from P53, preventing its degradation. However, when EBNA-1 binds to USP7, it disrupts the interaction between USP7 and P53, causing ubiquitination and subsequent degradation of P53. Additionally, P53 is ubiquitinated by MDM2, which further contributes to its instability. In EBVaGC, mutations in the P53 gene are rare, and P53 is directly inhibited by viral proteins [69]. EBNA-1 can also help oncogenesis by modulating various signaling pathways, including TGF-B1, AP-1, NF-KB, and IL-6. EBNA-1 can react with viral and cellular promoters and regulate the transcription of its genes. EBNA-1 increases the transcription of surviving, resulting in cell viability. Another function of EBNA-1 is the induction of reactive oxygen species (ROS) due to increased expression of NOX2, a catalytic subunit of NADPH oxidase. Elevated ROS levels lead to telomere instability and genomic instability [64].

5.2 LMP-1

This protein functions similarly to that of the tumor necrosis factor receptor (TNFR) family and mimics CD40 and TNFR1. However, LMP1 is continuously active without ligand stimulation. The cytoplasmic domains of this protein are associated with several adapter proteins, including JAK-3, TRAF, TRADD, ERK/MAPK, and JAK/STAT, which activate several signaling pathways such as NF-KB, JNK-P38, and PI3K/AKT. Therefore, it has a range of growth-enhancing phenotype effects. Significantly, LMP transformation can alter the morphology of epithelial cells and promote tumor metastasis [65].

5.3 LMP2A

Various studies have shown that LMP2a is expressed in different EBVaGCs. LMP2a and LMP1 mimic the antigen-drive signals that convert infected B cells into memory B cells, creating long-lasting EBV reservoirs. LMP1 and LMP2a act as permanently active receptors at the B cell surface. While LMP2a isn't oncogenic in B cells due to tight regulation of ITAM signaling, epithelial cells lack such controls, leading to the induction of a transformed phenotype upon expression of ITAM Igα/Igβ receptors [68]. PI3K/AKT and RAS/MAPK are essential signaling pathways downstream of ITAM Igα/Igβ, and LMP2a manipulates these pathways in both B cells and epithelial cells. Downstream of these pathways, there are essential mediators of cell survival and proliferation. Thus, LMP2a is a critical survival factor in EBV-infected epithelial cells. LMP2a also induces survivin expression through the NF-KB-dependent signaling pathway. Additionally, LMP2a exerts control over the expression of viral genes through methylation, activating the cell's DNA methyltransferase (DNMT1). This mechanism aids EBV in evading the immune system, ensuring virus latency and stability [70].

6. Pattern of Gene Methylation in *H. pylori* and Epstein-Barr Virus - Associated GC

6.1 Introduction to Therapeutic Targets and Biomarkers in EBV and H. pylori - Associated GCs

Recent studies have identified essential biomarkers and therapeutic targets in various cancers that could apply to EBV- and *H. pylori*-related GCs. Pan-cancer analyses identify genes involved in copper metabolism, mitochondrial DNA repair, and ion channels as therapeutic targets in these contexts. These biomarkers include genes related to cuproptosis, such as ATP7A and ATP7B [71], voltage-gated sodium channels (VGSCs), including SCN1A and SCN11A [72], and the DNA repair gene RAD51 [73], which are highly relevant to cellular metabolism, migration, and genomic stability in oncogenic milieus. Disulfidptosis and mitochondrial DNA repair pathways, particularly involving genes like POLG and PINK1 and broader mitochondrial DNA repair gene set, have been identified as critical in cellular survival [74, 75]. These targets' unique expression and mutation profiles contribute to the distinct progression patterns of EBV- and *H. pylori*-associated gastric cancers, providing a framework for targeted therapeutic strategies.

6.2 Pattern of Gene Methylation in H. pylori

DNA hypermethylation, a change in DNA methylation often found in CpG islands within the promoter regions of tumor suppressor genes in cancer, can result in the silencing of genes that generally modulate cell growth and induce apoptosis. In cancers with CpG island methylator phenotype (CIMP), such a CpG island methylator phenotype can lead to simultaneous silencing of many tumor suppressor genes by extensive hypermethylation and an environment that promotes unlimited cancer cell proliferation. Therefore, in diseases such as *H. pylori* and EBV-associated gastric cancers, methylation profiles establish them both as markers of diseases and active contributors to the disease development and progression processes [76]. Zoridis et al. extensively analyzed methylation profiles in 240 tumor samples and 94 adjacent standard tissue samples, revealing tumor-specific hypermethylation patterns alongside general hypomethylation. Notably, hypermethylation was prevalent in genes associated with stem cells. Intriguingly, cell lines exhibiting the CIMP were sensitive to treatment with DNA methylation inhibitors like 5-Aza-2’-

deoxycytidine, significantly reducing tumor growth [77]. The role of *H. pylori* in increasing the risk of GC by causing epigenetic changes in gastric epithelial cell lines was mentioned [31].

In a study by Cheng et al., an investigation into the methylation pattern of GC samples unveiled that sodium-potassium ATPase regulator (FYXD3) promoter methylation is associated with reduced survival in GC patients. Functional assays investigating FOXD3, a transcription factor, revealed its significant role in GC biology. Specifically, decreased FOXD3 activity correlated with reduced GC cell proliferation and inhibited subcutaneous tumor growth in nude mice while promoting cellular apoptosis [21]. FOXD3 plays a significant role in inhibiting the proliferation and metastasis of gastric cancer cells by actively promoting apoptosis. Such an effect is achieved by suppressing one of the crucial processes in the invasive and metastatic features of cancer cells-EMT. The normalization of the apoptotic pathways and the inhibition of EMT suppress aggressive cell behavior and support the tumor suppressor role of FOXD3, positioning it as a promising therapeutic target for the treatment of gastric cancer. This, therefore, provides an opportunity to target the enhancement of cancer cell sensitivity to apoptosis via the transcription factor FOXD3, which will no doubt be a very useful direction in the future treatment of gastric cancers. Importantly, this effect is achieved through FOXD3's binding to promoters and regulating the transcriptional activity of vital pro-apoptotic genes, such as CYFIP2 and RARB, underscoring its pivotal role in GC pathogenesis. Additionally, diminished FOXD3 expression levels were noted in gastric tumors, emphasizing its pivotal involvement in GC pathogenesis [21, 78].

Improper DNA methylation, catalyzed by the DNMT enzyme, is a hallmark of *H. pylori*-associated GC. Three isoforms of this enzyme, DNMT1, DNMT3A, and DNMT3B, exhibit heightened expression levels in this context [53]. It has been reported that CDH1 (E-cadherin) gene methylation in the *H. pylori*-positive gastric mucosa is higher than the gastric mucosa of negative *H. pylori* [48]. CDH1 is a cell-cell adhesion glycoprotein that is inactive in GC. Elevated levels of inflammatory markers such as COX2, IL1-β, IFN-γ, TNF-α, and inflammation-related genes are observed in *H. pylori*-induced GC [24]. Conversely, gastrokine (GKN1s) expression, known to suppress DNMTs and EZH2, is reduced in this context. In addition, this protein reduces the expression of EZH2 (enhancer of zeste homologue 2), and EZH2 is a potential target in many types of cancer [18]. Another investigation indicates that Forkhead box protein (FOX) expression regulation is disturbed in *H. pylori*-associated GC [20]. Numerous other genes expressed during *H. pylori* infections are associated with the cell cycle and cell proliferation. These include COX 2, RAB40C, FOS, JAK2, MYC, ERBB2, MET, SIRT1, TRAF6, PDCD4, GMNN, FGFR2, ABL1, ECOP, CCNE2, p14, p16, p21, p27, genes involved in apoptosis such as RECK, SMAD4, TRAIL, PDK1, MCL1, BIM, XIAP, as well as genes involved in invasion and metastasis such as WNT 5a, PTEN, EDNRA, EPB41L3, MMP1, MMP10, ROR2, HMGA2, TGF-β, ROBO1, EZH2, casein kinase 2, and ZEB [28, 29, 31, 34]. *H. pylori* can induce oxidative stress, ROS, and RNS, which can cause point mutations in P53. Nitric oxide may induce G: T mismatch during DNA synthesis and, ultimately, G: C to A: T base transversion and epigenetic alterations of oncogenic genes [19].

6.3 Pattern of Methylation in EBV-Related GC

Examining cancer-related signaling pathways revealed that various genes are implicated in EBVaGC. These include cell cycle genes such as IGFBP3, CDKN2A, ID2, HSP70, CCND1, and ID4, cell binding genes like ICAM1, angiogenesis-related genes like HIF1A, and inflammatory genes like COX2. Additionally, three vital tumor suppressor genes, namely CDH1 (E-cadherin), p73, and CDKN2A (P16),

are found to be lost in EBVaGC [62, 79]. Inflammatory genes such as COX2 and HIF1A are pivotal in establishing a tumor microenvironment that facilitates cancer progression. COX2, through the production of PGE2, promotes inflammatory signaling, supporting cell proliferation and immune escape by increasing the expression of PD-L1 and promoting angiogenesis. Moreover, HIF1A, frequently upregulated in reaction to hypoxia or inflammation, stimulates genes that promote vascular development and metabolic adaptation, which are crucial for tumor viability. The differential regulation of COX2 and HIF1A in EBVaGC versus non-EBV gastric cancers highlights the unique inflammatory and immunosuppressive environment in EBVaGC, resulting in differences in clinical progression and therapeutic response [79, 80].

7. *H. pylori* and EBV Co-Infection

The combination of *H. pylori* and EBV acts as a group of carcinogens associated with the development of GC. Individuals with EBVaGC typically harbor EBV DNA, and even patients show high titers of antibodies against EBV before cancer diagnosis. One study found that co-infection with *H. pylori* and EBV in sick children caused gastritis and chronic inflammation more severe than infection with either pathogen alone [81-83]. Both *H. pylori* and EBV contribute to epigenetic alterations in the host cell. A study in the AGS cell line showed that EBV methylates host genes that neutralize cagA in *H. pylori* [69]. Another study found that the association of the Zta gene of EBV with *H. pylori* was directly related to GC [65]. Many genes are methylated in gastric adenocarcinoma due to co-infection with *H. pylori* and EBV. Genes that are most prone to hyper-methylation include COX 2, DAPK, CDH1, and CDKN2A hMLH1. In addition, EBV-positive *H. pylori*-infected individuals were higher in EBV DNA, indicating a role for *H. pylori* in transitioning from the latent phase to the lytic phase [60].

Another study on *H. pylori* and EBV co-infection shows that it induces severe inflammatory responses in individuals and elevates the risk of intestinal GC. Two mechanisms are proposed to explain this phenomenon. Firstly, infection leads to increased inflammatory responses, exacerbating tissue damage caused by both pathogens. This results in a significant increase in IL-1β, IL-8, and TNF-α. The second mechanism is the interaction of gene products, which are more critical in these two pathogens [63]. One study showed that reactivation in EBV occurs via the PLCγ signaling pathway and that the cagA protein in *H. pylori* activates this pathway and several kinases [58].

H. pylori utilizes various mechanisms to circumvent the host immune response, enabling the sustained presence of both *H. pylori* and EBV in co-infected individuals. A major means immune signaling pathways are disrupted involves synthesizing virulence factors such as cagA and vacA proteins. The vacA protein exerts an inhibitory function on the T cells by suppressing their proliferative capacity and cytokine output, thus dampening the adaptive immune response to allow persistent infection. Furthermore, *H. pylori* can stimulate regulatory T cells (Tregs) capable of dampening antimicrobial immunity, allowing EBV to evade immune elimination and establishing latency in B cells [84, 85]. Moreover, *H. pylori* infection enhances the expression of immune checkpoint molecules, including PD-L1, on gastric epithelial cells, thereby suppressing T cell function and facilitating immune evasion. This immunosuppressive milieu not only facilitates the survival of *H. pylori* but also enables EBV to endure by evading immune detection and eradication [86]. Thus, the synergistic effect of these immune modulation mechanisms in coinfected individuals promotes a chronic inflammatory environment and increases the susceptibility to GC.

H. pylori cagA protein and LMP1 and LMP2 of EBV activate NF-KB and MAP kinase, known pathways associated with cell survival and proliferation during carcinogenesis. Moreover, the cagA protein improperly activates the WNT signaling pathway, which triggers the CDX1 gene downstream of this pathway. The product of this gene induces reprogramming and acquisition of stem cell characteristics in gastric epithelial cells by inducing SALL4 and KLF5 factors [65]. Another study shows that *H. pylori* and EBV can transform gastric epithelial cells and play an essential role in carcinogenesis [68]. Both pathogens induce common pathways that ultimately lead to the activation of transforming factors in gastric epithelial cells via the β-catenin/TCF-4 signaling pathway [59].

Szkaradkiewicz et al. discovered that BCL2 expression increased in GC associated with *H. pylori* and EBV alone, but this increase was much more significant in concomitant infections. Additionally, multiple studies have shown that the expression of PCDH10 (protocadherin 10), a calcium-dependent cell adhesion molecule that acts as a tumor suppressor in gastric epithelial cells, is hypermethylated in GC with *H. pylori* and EBV co-infection [87].

A recent study demonstrated that the host SHP 1 protein interacts with the cagA protein, leading to its dephosphorylation and subsequent inhibition of its oncogenic activity. Whereas in co-infection of *H. pylori* and EBV, EBV induces methylation of the SHP 1 gene and inhibits the dephosphorylation of cagA by SHP 1, thereby increasing the oncogenic activity of cagA [69]. Estaji et al. suggested that cagA might contribute to increased EBV lytic gene expression and SHP1 methylation, potentially facilitating the development of GC. Understanding the mechanism underlying EBV-*H. pylori* cagA+ co-infection and host epigenetic changes could prove pivotal in both the diagnosis and prevention of GC [85]. *H. pylori*-positive individuals exhibited an increased anti-EBV IgG titer, indicating that *H. pylori* augments EBV DNA load and elicits more robust immune responses. Co-infection with *H. pylori* and EBV occurs in the early stages of GC in cases of EBVaGC [81].

This study has several limitations that should be considered. First, the available research on this topic involves studies with diverse methodologies, study populations, and geographic locations. This heterogeneity in study designs and populations can make reconciling the findings and drawing generalizable conclusions challenging. Second, most existing studies on the role of *H. pylori* and EBV in GC are cross-sectional or have relatively short follow-up periods. Longer-term longitudinal studies are needed to understand better the temporal relationship between these pathogens and the development of GC. Third, GC is a multifactorial disease, and other factors, such as diet, lifestyle, genetics, and environmental exposures, may also play a role in its development. Disentangling the specific contributions of *H. pylori* and EBV from these other confounding factors can be complex.

8. Conclusion

To date, several clinical findings have confirmed the presence of co-infection with *H. pylori* and EBV in GC. The *H. pylori* and EBV co-infection shows that it induces severe inflammatory responses in individuals and elevates the risk of intestinal GC. This co-infection adds to increased cytokine responses, leading to tissue damage and a chronic inflammatory environment that allows the development of GC. Besides, the interaction of virulence factors, such as CagA and VacA, between *H. pylori* and EBV latency genes presents a complex interplay that affects the signaling pathways of the host toward the promotion of GC. However, significant gaps remain in understanding how these pathogens interact with specific host genes and epigenetic factors that drive GC progression. Future studies must outline the molecular details through which *H. pylori* and EBV interact with host

immune-modulating pathways, including the PD-L1/PD-1 checkpoint. Elucidation of such mechanisms may open new avenues for designing immune-based therapeutic strategies to target these pathogen-host interactions. Furthermore, investigating the reasons behind the selective targeting of a limited number of host cells for infection and elucidating the influence of genetic and epigenetic modifications in promoting chronic infection is essential. This will also serve in providing new approaches toward early diagnosis and personalized therapy and, eventually, preventive measures against GCs in populations at high infection risk. Such research endeavors could elucidate the unique contributions of *H. pylori* and EBV to GC and facilitate the creation of targeted diagnostic biomarkers and therapeutic strategies, thereby improving our capacity to effectively manage and decrease GC incidence.

Author Contributions

E.B conceived of the presented idea and supervised the findings of this work. S.M.H and A.E developed the theory and P.Z, S.S performed the search strategy. E.B point out the virology hints and investigate the search results. H.T, S.N.F critically revised the manuscript. All authors discussed the results and contributed to the final manuscript.

Competing Interests

The authors have no conflict of interest to disclose.

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