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Review

Novel Therapeutic Approaches for Epstein-Barr Virus Associated Gastric Cancer

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Abstract. *Epstein-Barr virus (EBV)-associated gastric cancer (GC) (EBVaGC) is classified as one of four GC subtypes by comprehensive molecular characterization. Though the mechanism of tumorigenesis by EBV infection has not yet been fully clarified, EBV infection might contribute to the malignant transformation of GC cells by involving various cellular processes and signaling pathways. EBVaGC has shown the following distinct characteristics in contrast to other subtypes: extreme DNA hypermethylation, recurrent phosphatidylinositol 4,5-biphosphate 3-kinase catalytic subunit alpha isoform (PIK3CA) mutations, overexpression of programmed cell death ligand 1/2 (PD-L1/2), and occasional immune cell signaling activation. Therefore, using these molecular features as guides, targeted agents need to be evaluated in clinical trials for EBVaGC. Accordingly, this review uses the best available evidence to focus on novel therapeutic approaches using the distinct pathologic characteristics of EBVaGC patients.*

Advanced gastric cancer (AGC) remains a major public health problem and the second leading cause of cancer-related deaths worldwide, even though rapid advances in treatment options have improved its prognosis (1, 2). Recently, remarkable progress in tumour biology has led to the development of new therapeutics that target critical aspects of oncogenic pathways or the immune system. In case of AGC, various target agents have already been evaluated in randomized studies, where trastuzumab (anti-human epidermal growth factor receptor 2 (HER2) monoclonal antibody) exhibited anti-tumor activity against 15-20% of HER2-positive AGC (3), while ramucirumab (anti-vascular endothelial growth factor receptor 2 (VEGFR2) monoclonal antibody) and nivolumab (anti-programmed cell death protein 1 (PD-1) monoclonal antibody) were shown to improve survival duration in a second or third-line setting (4, 5). However, most responses to chemotherapy with targeted agents are limited and short in duration, with a median survival of 10~16 months and overall survival at 2 years rarely over 10% (6).

Epstein-Barr virus (EBV)-associated gastric cancer (GC) (EBVaGC) is classified as one of four GC subtypes by the comprehensive molecular characterization (tumors positive for EBV, microsatellite unstable tumors, genomically stable tumors, tumors with chromosomal instability) (7). Though the mechanism of tumorigenesis by EBV infection has not yet been fully clarified, EBV infection might contribute to malignant transformation of GC cells by involving various cellular processes and signaling pathways (8). EBVaGC has shown the following distinct characteristics when compared to other subtypes: extreme DNA hypermethylation, recurrent phosphatidylinositol 4,5-biphosphate 3-kinase (PI3K) catalytic subunit alpha isoform (*PIK3CA*) mutations, overexpression of programmed cell death ligand 1/2 (PD-L1/2), and occasional immune cell signaling activation (7). Importantly, these molecular features can provide a guide for targeted agents that can be evaluated in clinical trials for EBVaGC. Accordingly, this review focuses on novel therapeutic approaches using the distinct pathological characteristics in patients with EBVaGC on the basis of the best available evidence.

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Immuno-oncologic Treatment: Immune Checkpoint Inhibitor

The recent identification of certain targets by The Cancer Genome Atlas (TCGA) Network has improved the accuracy when investigating the application of novel therapies to the four molecular subtypes of GC (7). EBV-positive tumours exhibit *CDKN2A* promoter hypermethylation and recurrent *PIK3CA* mutations. These tumours also display PD-L1/2 overexpression frequently, and occasional immune cell signaling activation (8, 9). In particular, a subgroup of GC with EBV-positivity is characterized by heavy infiltration of lymphoid elements and is associated with inflammatory stroma and rich cytokine milieu (10). In addition, in previous studies of 120 patients with EBV-positive cancer, the current authors showed that high levels of tumor-infiltrating lymphocytes associate with a favorable prognosis and intratumoral PD-L1 positivity with a worse prognosis (11, 12). Therefore, such findings support the concept that immune checkpoint inhibitors can be used as cancer immunotherapy in GC patients with EBV and indicate a pivotal role of immune mechanisms in this GC subset.

To date, most clinical trials on immune checkpoint inhibitors have included GC patients regardless of the subtype. When tested as a treatment option for metastatic or recurrent GC, these inhibitors have been found to be promising drugs in combination or as single agents (Table I) (13). Pembrolizumab and nivolumab are the first two monoclonal antibodies targeting PD-1, thereby interfering with the interaction between PD-1 and PD-L1. Based on the results of the KEYNOTE-059 trial, pembrolizumab monotherapy demonstrated encouraging antitumor activity with acceptable safety in patients previously treated for advanced gastric and gastroesophageal junction cancer (14). The objective response rate and median response duration (range) were reported as 15.5% and 16.3 (1.6 to 17.3) months, respectively, in patients with PD-L1-positive tumors (15). Nivolumab was also clinically explored following the failure of standard management. The ONO-4538-12/ATTRACTION-2 trial compared nivolumab to the placebo in patients with unresectable or recurrent GC, which was refractory to or intolerant of at least two previous standard chemotherapy regimens (5). The results showed a significantly prolonged overall survival (OS) for the nivolumab group with a median OS of 5.26 months (95%CI=4.60-6.37) compared to 4.14 months (3.42-4.86) for the placebo (hazard ratio (HR) 0.63, 95%CI=0.51-0.78; $p<0.0001$). Although another trial (JAVELIN Gastric 300) did not achieve its primary end point of improving OS, avelumab, which is an anti-PD-L1 blocking monoclonal antibody, was still found to exhibit antitumor activity similar to chemotherapy in patients treated with two previous regimens for advanced cancer, and with more favorable safety profiles (16). Notwithstanding, most GC patients only respond temporarily to immune checkpoint inhibitors and then experience disease progression. Moreover, identification of more robust predictive biomarkers for immune checkpoint inhibitors is critical to optimize treatment with these agents (17).

As described above, EBV-positive tumors exhibit genomic amplification of genes encoding PD-L1 that may be potential biomarkers for immune checkpoint inhibitors (18). Interestingly, a recent phase II trial by Kim *et al*. showed a durable survival benefit associated with pembrolizumab in patients with EBV-positive tumors (19). This trial enrolled 61 patients with recurrent or metastatic GC (all sub-types). Twenty-nine patients received pembrolizumab as a third-line treatment, while 32 received pembrolizumab as a second-line therapy for metastatic disease. In a subgroup analysis, pembrolizumab showed a dramatic efficacy for patients with EBV-positive tumors (overall response rate (ORR), 100%). In addition, the ORR was significantly superior for PD-L1-positive GC compared to PD-L1-negative tumors (50.0% *versus* 0.0%; $p<0.001$). Therefore, these findings suggest that EBV-positive GC derives benefit from PD-L1 inhibition and can also be actively considered for up-front pembrolizumab monotherapy.

Meanwhile, accumulating evidence shows that PD-L1 expression could be a potential prognostic and predictive factor for immune checkpoint inhibitors. Therefore, understanding the implications of PD-L1 expression in patients with EBV infection is of critical importance. According to the recent meta-analysis of 1901 GC patients, expression of PD-L1 was identified as a useful predictor of poor OS with final HR for OS of 1.64 (20). However, the evaluation of PD-L1 expression in patients with EBV is still insufficient. Notably, our research group demonstrated that PD-L1-positive EBVaGC patients exhibited a poorer prognosis than PD-L1-negative patients (12). Still, investigating PD-L1 expression has certain limitations that need to be considered (21). The evaluation and interpretation of PD-L1 within tumors and tumor microenvironment have not yet been standardized in GC. A standardized cut-off value for PD-L1 positivity has not yet been clearly established. Plus, conflicting results could also be due to different patient ethnicities and different antibody clones. Therefore, continuous research is needed to recognize additional predictive factors to identify the subgroup of EBV patients who can benefit from immunotherapy.

Molecular Targeted Agents: Phosphoinositide 3-kinase Inhibitors

GC molecular profiling has enabled the TCGA Research Network and Asian Cancer Research Group (ACRG) to classify GC into subtypes (22). For example, EBV-positive tumors have been found to represent 9% of TCGA GC samples with a strong predilection for *PIK3CA* mutation (80%) (7). The

Table I. *Immune checkpoint inhibitors in EBVaGC.*

References	Indication	Study name	Agents	Target	Number	Response rate (%)	Overall survival (Months)	Hazard ratio & *p*-value
(14)	Third-line or later treatment	KEYNOTE-059, Phase II	Pembrolizumab 200 mg every 3 weeks	PD-1	259	11.6 (15.5)†	5.6	-
(5)	Second-line or later treatment‡	ONO-4538-12/ ATTRACTION-2, Phase III	Nivolumab 3 mg/kg every 2 weeks	PD-1	330	11	5.26	0.63 (0.51-0.78) *p*<0.0001
			Placebo		163	0	4.14	
(16)	Second-line or later treatment*	JAVELIN Gastric 300, Phase III	Avelumab 10 mg/kg every 2 weeks	PD-L1	185	2.2	4.6	1.1 (0.9-1.4) *p*=0.81
			Physician's choice (paclitaxel or irinotecan)		186	4.3	5.0	
(19)	Second- or third-line	Phase II	Pembrolizumab 200 mg every 3 weeks	PD-1	61 (6)**	24.6 (100)**	(9.6)***	-

†Response rate in patients with PD-L1-positive; ‡Includes treatments received in the adjuvant setting; *Includes patients who progressed on neo- or adjuvant therapy within 6 months of treatment discontinuation; **patients with EBV-positive; ***median duration of response.

ACRG also found a 6.5% overall occurrence of EBV infection in GC patients and higher occurrence in the microsatellite stable with active tumor protein 53 (MSS/TP53+) subgroup. In particular, the MSS/TP53+ subtype showed a relatively higher prevalence of *PIK3CA* mutations (23). Therefore, these findings suggest that *PIK3CA* participates in several important cellular pathways that potentially play a role in tumorigenesis, warranting further investigation of PI3K inhibition in the case of EBVaGC (Table II).

PI3Ks are involved in various vital functions of cells, such as survival, proliferation, and differentiation (24). Divided into three main classes according to their functions, class I PI3Ks are mainly related with human cancers. Class I PI3Ks are heterodimers and composed of a p85 regulatory subunit and p110 catalytic subunit, where *PIK3CA* is part of the catalytic subunit (25). *PIK3CA* encodes the p110 catalytic subunit and can be up-regulated by activating molecular alterations (26). Essentially, *PIK3CA* mutations are frequently found in exon 1, exon 4, exon 5, exon 9, and exon 20. Among them, the mutations E545K and E542K in exon 9, and H1047R in exon 20 occur frequently in human cancers, while these mutations are more dispersed in EBV-positive tumors (7, 27). When alterations of class I PI3Ks occur, the p85 regulatory subunit loses its ability to inhibit overproduction and excessive release of phosphatidylinositol 3,4,5-triphosphate (PIP3), thereby promoting cell division through an increased release of protein kinase B (AKT) (28-30). Interestingly, AKT overexpression has also been observed in multiple human cancers, including gastric adenocarcinomas (31). Moreover, previous studies have demonstrated that the PI3K/AKT/mammalian target of the rapamycin (mTOR) signaling pathway is frequently altered as a result of *PIK3CA* amplification or overexpression, which has already been shown to play a key role in the initiation and progress of malignant tumors (32, 33).

Although recurrent *PIK3CA* mutations are considered a key parameter in EBV-positive tumors, their clinical significance in GC remains controversial. A recent genomic profiling study reported that activation of the PI3K/AKT/mTOR signaling pathway had a negative effect on OS and progression-free survival (PFS) in AGC (34). In contrast, Fang *et al*. demonstrated that *PIK3CA* amplification had no impact on either survival or the recurrence of GC (35). Another study by Ito *et al*. also found no correlation between *PIK3CA* mutations and prognosis for GC patients (36). Yet, very few studies have reported on the prognostic effects of *PIK3CA* in EBVaGC, warranting further evaluation to determine the clinical implications of *PIK3CA* mutations.

A number of inhibitors to decrease the effect of the PI3K/AKT/mTOR pathway are currently under investigation. When evaluated as a second-line therapy in GC, the AKT inhibitor MK2206 failed to show sufficient impact on survival outcomes (37). Meanwhile, AZD5363 (NCT02451956) and GDC0068 (NCT01896531), also AKT inhibitors, are being evaluated in combination with conventional chemotherapeutic agents (38, 39). Everolimus, an mTOR inhibitor that is an oral formula of a rapamycin analogue, has been investigated in the phase III GRANITE-1 study (NCT00879333) in AGC patients who had progressed after one or two lines of systemic chemotherapy. Although everolimus did improve median PFS, there was no OS benefit (40). Various pan-class I PI3K inhibitors have also been reported, and recent clinical trials

Table II. *List of studies for PI3K and PIK3CA discussed in this review.*

Reference	First author and year of publication	Study design	Summary of findings
(7)	Cancer Genome Atlas Research Network, 2014	Experimental research	The EBV-positive tumors exhibited higher prevalence of *PIK3CA* mutation (80%) which suggests that inhibition of PIK3CA warrants further evaluation in EBV-positive GC.
(35)	Diaz-Serrano *et al*., 2018	Experimental research	PI3K/AKT/mTOR signaling pathway activation could have a differentially negative effect on OS and PFS in AGC.
(41)	Ohtsu *et al*., 2013	Phase III study	Everolimus did not significantly improve overall survival for advanced gastric cancer that progressed after one or two lines of previous systemic chemotherapy, while the safety profile observed for everolimus was consistent with that observed for everolimus in other cancers.
(42)	Hong *et al*., 2012	Phase I study	PX-866, an irreversible small-molecule inhibitor of PI3K was well tolerated and was associated with prolonged stable disease, particularly when using a continuous dosing schedule.

Table III. *Demethylating agents in EBVaGC*.

References	First author and year of publication	Study design	Summary of findings
(60)	Jung *et al*., 2007	Experimental research	Low concentrations of demethylating agents, 5-azacitidine or trichostatin A, induced the expression of EBV lytic genes, such as BMRF1, BZLF1, and BRLF1 in EBVaGC cell line.
(63)	Nakamura *et al*., 2017	Experimental research	Decitabine was found to induce G_2/M arrest, apoptosis, and the expression of E-cadherin in EBVaGC cell line, SNU719 cells.
(64)	Schneider *et al*., 2017	Phase I study	Epigenetic priming with 5-azacytidine prior to chemotherapy in patients with locally advanced esophageal/gastric adenocarcinoma was well-tolerated with significant clinical and epigenetic responses
(65)	Liu *et al*., 2013	Experimental research	Gemcitabine and doxorubicin induced EBV lytic reactivation through up-regulation of EBV immediate-early genes, BZLF1 and BRLF1, in EBV-transformed B cells.

demonstrated that PX-866 (NCT00726583), BYL719 (NCT02551055), and MLN1117 (NCT01219699) showed antitumor activity in patients with incurable cancers including GC (41). Moreover, despite the absence of definitive beneficial effects of PI3K/AKT/mTOR pathway inhibitors, multiple clinical trials involving GC patients are still being carried out due to the possibility of targetable therapy in EBVaGC.

Treatment for Epigenetic Abnormalities: Demethylating Agents

Several studies demonstrated that epigenetic abnormalities, such as promoter hypermethylation, play a crucial role in the development and progression of EBVaGC (42-45). Methylation of both viral and host DNA is important in the carcinogenesis of EBVaGC. Particularly, these methylations control the expression of EBV lytic and latent genes. EBVaGC is known to exhibit type I or II latency, in which EBV-encoded small RNAs (EBERs), EBV-determined nuclear antigen (EBNA) 1, BamHI A region rightward transcripts (BARTs), latent membrane protein (LMP) 2A, and BART miRNAs are all expressed (46). EBV lytic genes, including *BCLF1, BHRF1, BNLF2a,* and *BRLF1*, are also expressed in EBVaGC (47). Ribeiro *et al*. revealed that the most frequently expressed EBV latent proteins are EBNA1 (98.1%) and LMP2A (53.8%), whereas LMP1 and LMP2B are only existed in 10% of cases. Lytic proteins, such as BARF0 and BARF1, and other lytic transcripts are present in approximately 50% of all cases (48). EBV LMP1 and 2A are known to activate cellular DNA methyltransferase which engages in the methylation of the viral and cellular genome (49). DNA methylation is a host defense mechanism against viral DNA to suppress the expression of viral genes, yet also plays an important role in allowing EBV to escape from the

host immune system. The methylation of EBV DNA facilitates latency, which may contribute to the maintenance of EBVaGC.

Virus-induced methylation of the DNA of host cells inactivates tumor suppressor genes and tumor associated antigens (50). Global and non-random CpG island methylation in the promoter regions of many cancer-related genes, which down regulates their expression, is found in EBVaGC (51). The methylation frequencies of several tumor-related genes and DNA loci have been demonstrated to be significantly higher in EBVaGC. EBVaGC also displays promotor hypermethylation in multiple genes related to cell cycle regulation (p14ARF, p15, p16INK4A, and p73), DNA repair (hMLH1, MGMT and GSTP1), cell adhesion and metastases (CDH1, TIMP1, and TIMP3), apoptosis (DAPK and bcl-2), and signal transduction (APC, PTEN, and RASSF1A) (52). Choi *et al*. reviewed published studies that reported DNA methylation in gastric cancers and found that 13 genes, p16 (CDKN2A), COX2, SSTR1, ACSS1, FAM3B, IHH, TRABD, SOX9, WNT5A, FSD1, IRF5, BCL7A, and PTEN, were hypermethylated in both primary EBVaGC and EBVaGC cell lines. Another 11 genes, ICAM1, TFF1, IL15RA, REC8, EPHB6, MDGA2, SCARF2, GKN1, GNN2, NEK9, and SLC7A8, were only hypermethylated in EBVaGC cell lines (53). The mechanism that activates cellular DNA methylation by EBV infection of the gastric epithelial cell is not clearly understood. Hino *et al*. reported that LMP2A can induce the transcription of DNA methyltransferase 1 (DNMT1) through STAT3 phosphorylations (54). However, LMP2A is not present in all cases of EBVaGC. LMP1 can also induce aberrant DNA methylation by activating DNMT1, yet LMP1 is scarcely expressed in EBVaGC (55). Namba-Fukuyo *et al*. suggested that downregulation of TET2 was important for promoting DNA methylation in EBVaGC. TET family genes encode DNA demethylase and TET2 is suppressed by EBV transcripts, such as BARF0 and LMP2A, and TET2-targeting miRNAs (56).

The important feature of epigenetic treatment is the reversibility of epigenetic gene alterations. The therapeutic application of DNA demethylating agents for EBVaGC may be attractive (Table III). Jung *et al*. reported that low concentrations of demethylating agents, 5-azacitidine or trichostatin A, induced the expression of EBV lytic genes, such as BMRF1, BZLF1, and BRLF1, in EBVaGC cell lines (57). As demethylating agents induce lytic EBV infection in latent EBV-infected cells, demethylating agents can induce the lysis of tumor cells (58, 59). Nakamura *et al*. reported that decitabine, a demethylating agent, modulated G_2/M arrest, apoptosis, and the E-cadherin expression in SNU719 cells. The promoters of tumor-suppressor genes, such as p73 and Runt-related transcript factor 3 (RUNX3), were demethylated and their expression upregulated by decitabine treatment (60). Epigenetic agents have also shown antitumor activity against solid tumors in the case of single or combination treatment with standard chemotherapy. Schneider *et al*. carried out a phase I study of 5-azacytidine as a neoadjuvant chemotherapy in patients with locally advanced gastric and esophageal cancer. Epigenetic priming with 5-azacytidine prior to chemotherapy was well tolerated with significant epigenetic and clinical responses, thus a phase II study is underway using the established dose of 5-azacitidine as a priming regimen (61). Liu *et al*. also reported that doxorubicin and gemcitabine activated EBV lytic reactivation through the overexpression of the EBV immediate early genes BZLF1 and BRLF1 in EBV-transformed B cells (62). Therefore, demethylating agents and EBV lytic phase inducing agents can be a useful therapeutic strategy for EBVaGC. However, further studies are needed to clarify the role of demethylating agents and EBV lytic phase inducing agents, along with combination approaches of these agents and standard treatment.

Conclusion

A more profound understanding of the underpinning tumor biology has recently opened a more contemporary clinical approach for AGC. EBVaGC is a distinct subtype of GC as regards both its molecular and clinicopathological features.

For EBVaGC, clinical studies have evaluated the role of molecular targeted agents using the characteristics of extreme DNA hypermethylation, recurrent *PIK3CA* mutations, and PD-L1/2 overexpression. As a result, the driving features of PD-L1 positivity or a high mutational burden can be targeted with immunotherapy as efficaciously as in a microsatellite-instable (MSI) subtype. Moreover, demethylating agents and EBV lytic phase inducing agents have also been shown to be useful therapeutic strategies for EBVaGC. However, further clinical studies are warranted to clarify the roles of these agents and the efficacy of a combination approach with standard treatment for AGC.

Since the identification of new biomarkers is essential for precision medicine, the unique molecular and clinical features of EBVaGC should be utilized to improve the prognosis for patients with this disease. Consequently, a better understanding of the molecular characteristics of EBVaGC and dedicated clinical trials will open new opportunities for personalized therapy.

Conflicts of Interest

The Authors declare that they have no conflicts of interest regarding this study.

Authors' Contributions

All Authors contributed to the conception and design of the study, as well as to drafting and revising the manuscript.

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