Current status of immune checkpoint inhibitors for gastric cancer

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Abstract

Recent breakthrough results from immune checkpoint inhibitors (ICI) have paved the way to a new era of cancer immunotherapy. In particular, inhibition of programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) axis with ICI including nivolumab and pembrolizumab has been emerging as a novel treatment strategy for advanced gastric cancers (GC). In a meta-analysis for anti-PD-1/PD-L1 therapy in GC, the objective response rate was 12.0% and the disease control ratio was 34.7%. The ICI treatment in GC provided modest survival benefit and especially, anti-PD-1 treatment could improve the 12-month and 18-month overall survival rate and prolonged the duration of the response. Moreover, it is likely that anti-PD-1/PD-L1 therapy is more effective in subgroups with microsatellite instability-high, Epstein-Barr virus-positive or high mutation burden in advanced GC. The next steps for developing ICI in GC are mainly two challenges as follows. First is the identification of accurate biomarkers that can predict the response to ICI. The second challenge is the clinical development of combinatorial approaches to maximize the efficacy of ICI. In this review, recent advances in ICI for GC are discussed from a viewpoint of translational aspect including biomarkers and tumor microenvironment, and from a viewpoint of clinical aspects including combination therapies.

Keywords Gastric cancer · Immune checkpoint inhibitor · Nivolumab · Pembrolizumab · Biomarker

Introduction

Gastric cancer (GC) is the second leading cause of cancer-related deaths and the sixth most frequent cancers worldwide, and the prevalence is relatively higher in East Asia region in comparison to Western countries (GLOBOCAN 2018 [1]). Currently, curative resection with or without perioperative chemotherapy is a standard treatment for GC and, for unresectable or metastatic advanced GC, chemotherapy using drugs such as platinum compounds, fluoropyrimidines, docetaxel, paclitaxel and irinotecan is a standard therapy [2–5]. In addition, trastuzumab, a monoclonal

antibody (mAb) for human epidermal growth factor receptor 2 (HER2), has been established as a first-line treatment for HER2-positive advanced GC patients [6]. Moreover, ramucirumab, an anti-vascular endothelial growth factor receptor 2 (VEGFR2) mAb, has also been proven efficient for a second-line treatment for advanced GC [7, 8].

Recent breakthrough results from immune checkpoint inhibitors (ICI) such as an anti-cytotoxic T-lymphocyte antigen 4 (CTLA4) mAb (ipilimumab) and anti-programmed death-1 (PD-1) mAbs (nivolumab and pembrolizumab) have paved the way to a new era of cancer immunotherapy, leading to the paradigm shift of cancer treatment [9–12]. In particular, inhibition of PD-1/programmed death-ligand 1 (PD-L1) axis with ICI including nivolumab and pembrolizumab has been emerging as a novel treatment strategy for advanced GC [13, 14]. For example, the ATTRACTION-2 study treated with nivolumab in patients with unresectable advanced or recurrent GC showed an objective response rate (ORR) of 11.2% [13] and proved prolonged overall survival (OS), leading to the establishment of standard options for a third-line treatment for advanced GC in the Japanese guideline. However, although anti-PD-1 mAb is a promising approach for advanced GC patients, the response rate

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still limited and developing novel strategies is necessary to maximize the efficacy of ICI.

In this review, recent advances in ICI for GC are discussed from a viewpoint of translational aspect including biomarkers and tumor microenvironment, and from a viewpoint of clinical aspects including combination therapies.

ICI for GC from clinical points of view

Clinical trials with ICI for GC

We have searched clinical trials as keywords with phase III, gastric cancer and ICI, through the following public database as of 1st March 2020: ClinicalTrials.gov (<https://clinicaltrials.gov/>), World Health Organization (<https://www.who.int/>), EU Clinical Trials Register (<https://www.clinicaltrialsregister.eu/ctr-search/search>), Cancer Council SA (<https://www.cancersa.org.au/>), Japan Registry of Clinical Trials (<https://jrct.niph.go.jp/>), UMIN-CTR (<https://www.umin.ac.jp/>) and Japic Clinical Trials Information (<https://www.clinicaltrials.jp/cti-user/common/Top.jsp>).

As a result, 16 clinical trials were listed up and the trial information is summarized in Table 1. The results from several clinical trials are already reported and others are still under recruiting status. The listed ICI in Table 1 is briefly presented as follows.

Nivolumab

Nivolumab is a human IgG4 mAb that can act against PD-1 and block a co-inhibitory signal on PD-1/PD-L1 and PD-1/PD-L2 axis. In a pivotal phase III trial (ATTRACTION-2), nivolumab significantly prolonged OS in patients with advanced GC in a third-line setting (hazard ratio = 0.63, 95% CI 0.51–0.78; $p < 0.0001$) [13], and the MST was reported to be 5.3 months with nivolumab in comparison to 4.1 months in the placebo group, and 12 month-OS in nivolumab and placebo groups were 26.6% and 10.9%, respectively.

Pembrolizumab

Pembrolizumab is a humanized high-affinity IgG4 mAb that can act against PD-1 and can block interactions on PD-1/PD-L1 and PD-1/PD-L2 axis. In the KEYNOTE-059/ Cohort 1, advanced GC patients treated with pembrolizumab achieved the ORR of 11.6% [15]. However, the phase III trial, pembrolizumab compared with paclitaxel as second-line treatment for advanced GC (KEYNOTE-061, NCT02370498) has failed to meet the primary endpoint to show the clinical efficacy of pembrolizumab monotherapy (OS: hazard ratio = 0.82, 95% CI 0.66–1.03; one-sided

$p = 0.0421$) (PFS: hazard ratio = 1.27, 95% CI 1.03–1.57) [16].

Ipilimumab

Ipilimumab is a human IgG1 mAb that can react against CTLA-4/B7 interaction on mainly antigen-presenting cells/T cells to restore T cell function. In a phase II trial, nivolumab + ipilimumab group showed a higher ORR than the nivolumab alone group (26% vs 14%) (Checkmate-032 [17]). A phase III trial of nivolumab + ipilimumab vs. nivolumab + chemotherapy in patients with advanced GC is ongoing (NCT02872116).

Avelumab

Avelumab is a human IgG1 mAb that can react against PD-L1 and block the interaction of PD-L1 with PD-1 and CD80 molecules. JAVELIN Gastric 300 with avelumab in comparison to chemotherapy in a third-line setting for advanced GC has been reported with a negative result (NCT02625623) (hazard ratio = 1.1, 95% CI 0.9–1.4; $p = 0.81$) [18].

Durvalumab

Durvalumab is a human IgG1 mAb that can react against PD-L1 and block the interaction of PD-L1 with PD-1 and CD80 molecules. A phase Ib/II trial to evaluate the efficacy of durvalumab in patients with metastatic or recurrent GC is ongoing.

Meta-analysis for clinical trials with ICI in GC

Chen et al. performed a meta-analysis for clinical trials with ICI (anti-PD1, anti-PD-L1 and anti-CTLA4) for advanced GC or esophago-gastric junction tumors in a total of 2003 patients from nine clinical trials [19]. As a result, the ORR of all ICI patients, anti-PD-1/PD-L1 subgroup and anti-CTLA-4 subgroup were 9.9%, 12.0% and 2.1%, respectively, and the disease control ratio were 33.3%, 34.7% and 30.1%, respectively. The median progression-free survival of all ICI, anti-PD-1/PD-L1 subgroup and anti-CTLA-4 subgroup were 1.6, 1.6 and 2.9 months, respectively. The median OS of the three groups was 6.0, 5.4 and 7.7 months, respectively.

As for PD-L1 positive tumors in the meta-analysis, the ORR and disease control ratio of anti-PD-1 treatment in PD-L1 positive patients were significantly higher than that of PD-L1 negative patients. As for microsatellite instability (MSI) in the meta-analysis, the ORR and disease control ratio in MSI-high GC was 55.0% and 74.8%, while the ORR and disease control ratio in microsatellite stable patients were 16.6% and 34.4%, respectively. Therefore, the

Table 1 List of phase 2/3 or 3 trials with immune checkpoint inhibitors for Gastric cancer

Traget	Agent	Conditions	Arms	Phase	Line	Number	NCT number	Other identifier
PD-1	Nivolumab	pStage III gastric cancer (including esophagogastric junction cancer)	Nivolumab group: Nivolumab or Chemotherapy (S-1 or CapeOX) Placebo group: Placebo or Chemotherapy (S-1 or CapeOX)	3	Adjuvant	700	NCT03006705	ATTRACTION-5
PD-1	Nivolumab	Unresectable advanced or recurrent gastric cancer (including esophagogastric junction cancer)	Part 1: Nivolumab + SOX Nivolumab + CapeOX Part 2: Nivolumab + SOX or CapeOX Placebo + SOX or CapeOX	2, 3	1st	680	NCT02746796	ATTRACTION-4
PD-1	Nivolumab	Resected esophageal or gastroesophageal junction cancer	Nivolumab Placebo	3	Adjuvant	760	NCT02743494	CheckMate 577
PD-1	Nivolumab	Unresectable advanced or recurrent gastric cancer (including esophagogastric junction cancer)	Nivolumab Placebo	3	3rd	480	NCT02267343	ATTRACTION-2
PD-1	Pembrolizumab	HER2 negative, previously untreated, unresectable or metastatic gastric or gastroesophageal junction adenocarcinoma	Pembrolizumab + FP or CAPOX Placebo + FP or CAPOX	3	1st	1542	NCT03675737	KEYNOTE-859
PD-1	Pembrolizumab	HER2 positive advanced gastric or gastroesophageal junction adenocarcinoma	Pembrolizumab + Trastuzumab + FP or CAPOX or SOX Placebo + Trastuzumab + FP or CAPOX or SOX	3	1st	732	NCT03615326	KEYNOTE 811
PD-1	Pembrolizumab	Gastric and gastroesophageal junction adenocarcinoma	Pembrolizumab + XP or FP Placebo + XP or FP Pembrolizumab + FLOT Placebo + FLOT	3	Neoadjuvant or Adjuvant	1000	NCT03221426	KEYNOTE-585
PD-1	Pembrolizumab	Asian subjects with advanced gastric or gastroesophageal junction adenocarcinoma	Pembrolizumab Paclitaxel	3	2nd	360	NCT03019588	KEYNOTE-063
PD-1	Pembrolizumab	Locally advanced unresectable or metastatic gastric or gastroesophageal junction adenocarcinoma (HER negative and PD-L1 positive)	Pembrolizumab Placebo + SOC	3	1st	763	NCT02494583	KEYNOTE-062
PD-1	Pembrolizumab	Advanced gastric or gastroesophageal junction adenocarcinoma	Pembrolizumab Paclitaxel	3	2nd	592	NCT02370498	KEYNOTE-061
PD-L1	Avelumab	Unresectable, locally advanced or metastatic, adenocarcinoma of the stomach, or of the gastroesophageal junction	Avelumab Oxaliplatin-fluoropyrimidine doublet	3	1st	499	NCT02625610	JAVELIN Gastric 100

Table 1 (continued)

Target	Agent	Conditions	Arms	Phase	Line	Number	NCT number	Other identifier
PD-L1	Avelumab	Unresectable, recurrent, locally advanced or metastatic gastric or gastroesophageal junction adenocarcinoma	Avelumab + BSC Chemotherapy (paclitaxel or irinotecan) + BSC	3	3rd	371	NCT02625623	JAVELIN Gastric 300
PD-1 CTLA-4	Nivolumab Ipilimumab	Unresectable advanced gastric cancer and gastroesophageal junction cancer	Nivolumab + Ipilimumab for four doses, followed by Nivolumab XELOX FOLFOX Nivolumab + XELOX Nivolumab + FOLFOX	3	1st	2032	NCT02872116	CheckMate 649
PD-1 Lag-3	Nivolumab Relatlimab ^a	Unresectable, locally advanced or metastatic LAG-3 positive gastric or gastroesophageal junction adenocarcinoma	Nivolumab + Relatlimab + CapeOX Placebo + CapeOX	3	1st	2420		
PD-1 Lag-3	INCMGA00012 ^b MGD013 ^c	Metastatic or locally advanced, treatment-naïve, HER2-positive gastric or gastroesophageal junction cancer	Margetuximab + INCMGA00012 Margetuximab + INC-MGA00012 + XELOX or mFOL-FOX-6 Margetuximab + MGD013 + XELOX or mFOLFOX-6 Margetuximab + XELOX or mFOL-FOX-6 Trastuzumab + XELOX or mFOL-FOX-6	2, 3	1st	850	NCT04082364	MAHOGANY
PD-1 PD-L1	Nivolumab Pembrolizumab Atezolizumab Durvalumab Avelumab	Advanced solid tumors (including gastric cancer)	Continue treatment with PD-1/PD-L1 inhibitor Discontinue treatment with PD-1/PD-L1-1 inhibitor	3	After 12 months of check point inhibitor treatment	578	NCT04157985	

Relatlimab anti-Lag-3, *INCMGA00012* anti-PD-1 checkpoint inhibitor, *MGD013* anti-PD-1, anti-Lag-3 dual checkpoint inhibitor DART molecule, *BSC* Best Supportive Care, *Margetuximab* Fc-modified anti-HER2 monoclonal antibody

clinical effect of anti-PD-1 therapy in MSI-high GC was markedly better than microsatellite stable, and ORR could be increased at 3.4 times and disease control ratio could be increased at 2.2 times.

Overall, Chen et al. reported that ICI treatment could provide modest survival benefit and especially, anti-PD-1 treatment could improve the 12-month and 18-month OS and prolonged the duration of the response. Moreover, they suggested that anti-PD-1/PD-L1 therapy is more effective in subgroups with PD-L1 positive, MSI-high, Epstein-Barr virus (EBV) positive or high mutation burden in advanced GC. ICI-related biomarkers are discussed in the later parts of this review.

Treatment-related adverse events of ICI in GC

In the meta-analysis by Chen et al. [19] for advanced GC or esophago-gastric junction tumors, the overall incidence of treatment-related adverse events in ICI was 56.8%, and the incidence of $\geq$ grade 3 treatment-related adverse events was 14.6%. The common treatment-related adverse events in the ICI treatment were fatigue (14.1%), pruritus (10.3%), rash (9.8%), diarrhea (8.2%), hypothyroidism (7.0%), decreased appetite (6.1%), nausea (5.7%), and anemia (4.4%). Overall, it is confirmed that treatment-related adverse events with ICI were manageable and less frequent than chemotherapy.

Translational aspect of tumor microenvironment related to ICI in GC

PD-L1 expression by intrinsic factors including genomic alterations, epigenetic regulations and oncogenic signaling activation

Increased expression of PD-L1 in various types of solid tumors has been reported [20], and positive PD-L1 expression has been shown in the range of 25–65% in GC [21]. Meta-analysis including a total of ten studies involving 1901 patients with GC revealed that PD-L1 expression was associated with a shorter OS [22]. PD-L1 expression is recognized to be regulated by various mechanisms including genetic, epigenetic and extrinsic regulations in GC (Fig. 1).

Genomic alterations and epigenetic regulations of *PD-L1* are observed in GC, and these events are closely associated with overexpression of PD-L1. The Cancer Genome Atlas revealed that amplification of the *PD-L1* gene occurs in GC. *PD-L1* resides on chromosome 9p24.1 locus, and GC exhibits copy number gains of 9p24, leading to overexpression of PD-L1 [23]. Especially, 9p amplifications were enriched in EBV-positive GC. In addition to the gene amplification, the structural variation of 3'-UTR in *PD-L1* gene has also been involved in up-regulation of PD-L1 expression

in GC. Kataoka et al. found that the disruption of 3'-UTR of *PD-L1* gene caused aberrant PD-L1 expression in multiple cancers including GC [24]. 3'-UTR of *PD-L1*-disrupted tumor cells attenuated CD8⁺ T-cell reaction, suggesting that PD-L1 overexpression by 3'-UTR truncation is involved in immune evasion of tumor cells [24]. Moreover, genetic polymorphisms of *PD-L1* gene are also associated with increased PD-L1 expression in GC. A polymorphism rs10815225 in the *PD-L1* promoter region locates in the binding-site of Sp1 transcription factor, and thereby increased binding of Sp1 to the region significantly up-regulated expression of *PD-L1* mRNA in GC [25]. A new polymorphisms rs2297136 (AA + AG genotype) in 3'-UTR of the *PD-L1* was also positively correlated with PD-L1 expression in GC [26].

MicroRNAs also contribute to the up-regulation of PD-L1 expression in GC. mRNA expression of PD-L1 is down-regulated by miR-570, and somatic mutation (guanine-to-cytosine mutation) at the 3'-UTR of *PD-L1* led to overexpression of the protein by disrupting miR-570 binding in GC [27, 28]. miR-152 and miR-200b have been reported to be involved in carcinogenesis in GC. Although miR-152 and miR-200b usually function as down-regulators of PD-L1 expression through the binding to the 3'-UTR of *PD-L1*, the expression of these miRNAs was decreased in human GC tissues compared with normal tissues, leading to up-regulation of PD-L1 expression in GC [29, 30]. Similarly, miR-186 was down-regulated in human GC tissues and cell lines [31]. Overexpression of miR-186 inhibited cell proliferation and the expression of PD-L1 by suppressing hypoxia-inducible factor 1 α in human GC cell lines [31].

The activation of oncogenic signaling pathways is closely associated with PD-L1 expression in many cancers [32]. The PI3K/Akt pathway is frequently activated in cancer cells, and its activation triggered by the loss of PTEN expression or mutation of *PIK3CA* gene up-regulates PD-L1 expression in human glioma and triple-negative breast cancer [33, 34]. *PIK3CA* is known as frequently mutated gene in the range of 4 to 25% of GC [35], and Kim et al. reported that *PIK3CA* mutations were associated with increased tumor aggressiveness and activation of Akt pathway in GC [36]. Indeed, GC patients with *PTEN* loss of heterozygosity showed higher PD-L1 expression [37]. Moreover, PD-L1 expression was significantly increased in GC with *PIK3CA* mutation compared GC with *PIK3CA* wild-type [38]. Furthermore, *PIK3CA* mutation was found in 80% of EBV-positive GC, which have a robust expression of PD-L1 [23]. Therefore, activation of the oncogenic PI3K/Akt pathway is involved in PD-L1 expression in GC.

PD-L1 expression by extrinsic factors

Interferon (IFN)- γ produced by mainly tumor-infiltrating lymphocytes is a main extrinsic factor that up-regulates

Fig. 1 The regulation of PD-L1 expression by multiple factors in GC cell. The intrinsic factors (genomic alterations, epigenetic regulations and oncogenic signaling activations) and extrinsic factors (cytokines, chemokines, EBV infection and *H. pylori* infection) induce PD-L1 expression in GC cell. 3'-UTR 3'-untranslated region, *CagA* cytotoxin-associated gene A, *CXCL* CXC-chemokine ligand, *CXCR* CXC-chemokine receptor, *EBNA1* EBV nuclear antigen 1, *EBV* Epstein-Barr virus, *GC* gastric cancer, *HIF-1α* hypoxia-inducible factor-1α,

H. pylori *Helicobacter pylori*, *IFNAR* type I interferon receptor, *IFN-γ* interferon-γ, *IL-8* interleukin-8, *IRF* interferon regulatory factor, *JAK* Janus kinase, *MAPK* mitogen-activated protein kinase, *mTOR* mammalian target of rapamycin, *NOD-1* nucleotide-binding oligomerization domain-1, *PD-L1* programmed cell death ligand-1, *PI3K* phosphoinositide 3-kinase, *PIK3CA* PI3K catalytic alpha, *PTEN* phosphatase and tensin homologue, *STAT* signal transducer and activator of transcription, *T4SS* type IV secretion system

PD-L1 expression in cancer cells [39]. We found that IFN-γ up-regulated PD-L1 expression through the JAK/STAT signaling pathway in GC. PD-L1 expression on tumor cells was positively correlated with IFN-γ level in the GC tissues [40]. Similarly, Moon et al. reported that IFN-γ triggered PD-L1 expression through the JAK2/STAT1/IRF-1 axis in EBV-associated GC [41]. EBV-positive GC expressed EBV

nuclear antigen 1, a transcription factor that maintains the copy number of EBV genomes during cell division, which might be involved in IFN-γ-inducible PD-L1 expression [41].

Other cytokines and chemokines have also been reported to up-regulate PD-L1 expression in GC cells. IL-8 is a pro-inflammatory cytokine and functions as a chemokine to

act on CXCR1 and CXCR2 receptors [42–44]. Sun et al. reported that GC mesenchymal stem cells produced IL-8 and GC mesenchymal stem cells-derived IL-8 triggered PD-L1 expression through activation of the STAT3/mTOR/c-myc signaling pathway in GC cells [45]. IL-8-induced PD-L1 expression caused the resistance of GC cells to the cytotoxic effect of CD8⁺ T cells [46].

Based on The Cancer Genome Atlas dataset, Zhang et al. revealed that chemokine signaling pathway especially the CXCL9/10/11-CXCR3 axis was most likely associated with PD-L1 expression in GC [47]. CXCL9/10/11 belong to the Glu-Leu-Arg-negative CXC chemokine subfamily [48], and all of these chemokines bind the CXCR3 [49]. The expression of CXCR3 was positively associated with PD-L1 expression in GC cell lines and tissues, and treatment of GC cells with CXCL9/10/11 upregulated PD-L1 expression through activation of the STAT and PI3K-Akt pathways [47].

Overexpression of PD-L1 has been observed in *Helicobacter pylori* (*H. pylori*) infected GC. Reyes et al. found that PD-L1 expression was up-regulated on the gastric epithelium during *H. pylori* infection, which inhibited T cell activation and rather enhanced frequency of regulatory T cell phenotype in gastric mucosa [50, 51]. The expression of PD-L1 by *H. pylori* depends on its type 4 secretion system components including the cytotoxin-associated gene A protein and peptidoglycan fragments. Type 4 secretion system components activated the oligomerization domain containing 1-p38 MAPK pathway to increase PD-L1 expression in *H. pylori*-infected gastric epithelial cells [52]. A further investigation by Xie et al. revealed that *H. pylori* also affected miRNA expression which is related to PD-L1 expression in GC cells. Infection of GC cells with *H. pylori* suppressed expression of miR-152 and miR-200b, suppressors of PD-L1 expression in GC as mentioned above, leading to overexpression of PD-L1 [30].

PD-L2 expression on GC

Since PD-1 binds to PD-L2 as well as PD-L1, PD-1/PD-L2 interaction should be involved in the anti-tumor effect of cytotoxic T lymphocyte (CTL) and the clinical response to anti-PD-1 mAb. In fact, several reports showed that PD-L2 expression was independently associated with clinical response to ICI in patients with head and neck squamous cell carcinoma [53], renal cell carcinoma and lung squamous cell carcinoma [54]. However, regulation of PD-L2 expression has not yet been elucidated [53–55], although the mechanism behind PD-L1 regulation has been well demonstrated such as intrinsic and extrinsic factors (Fig. 1). We and Garcia-Diaz et al. reported that PD-L2 expression is induced on tumor cells in the presence of IFN- β and IFN- γ [40, 56, 57]. Furthermore, we have recently reported that 28.4% of GC patients expressed PD-L2 on tumor cells, and

IFN- γ signature significantly correlated with PD-L2 expression, and anti-PD-L2 mAb enhanced the anti-tumor activity of CTL against GC cells expressing PD-L2 [58]. Considering the previous and our results, it is likely that PD-L2 is expressed on GC cells and PD-1/PD-L2 interactions are functionally involved in anti-tumor CTL activities.

The role of tumor-associated macrophage in GC

Tumor-associated macrophages (TAM) are the most abundant immune components in the tumor microenvironment, and they predominantly present as an M2-phenotype (anti-inflammatory phenotype), which associate with tumor progression, invasion and angiogenesis [59]. Indeed, previous reports suggest that high density of TAM predicted a poor prognosis in many types of solid tumors including GC [60]. TAM produce immunosuppressive mediators such as IL-10, tumor growth factor- β , prostaglandins and indoleamine 2, 3-dioxygenase, that suppress functions of CTL and promote the immunosuppressive activity of regulatory T cells [60]. It has been also reported that TAM could generate PD-L1-positive tumor cells via IFN- γ -independent pathway, leading to aggressive cancer [61, 62]. In fact, a significant association between PD-L1 expression in tumor cells and TAM infiltration was observed in GC [63], suggesting that TAM is also likely one of the extrinsic factors inducing PD-L1 expression in tumor cells.

Recent accumulating evidence suggests that TAM often express immune checkpoint molecules including PD-L1, PD-L2, VISTA (V-domain Ig-containing suppressor of T-cell activation) and B7-H4, which tames protective adaptive immunity for tumor progression. The expression of PD-L1 on TAM might be triggered by cytokine stimulation and hypoxia [64, 65] and PD-L1 expression in stroma was significantly associated with dense infiltration of TAM in GC [63]. Lin et al. reported that GC cell-derived colony-stimulating factor (CSF)-2 facilitated secretion of IL-8/CXCL8 by macrophages, and the secreted IL-8/CXCL8 inhibited CD8⁺ T cells function by inducing autonomous PD-L1 expression on macrophages. The CXCL-8-PD-L1 axis in TAM was associated with poor prognosis in human GC [66]. In addition, it has been reported that TAM expressed not only PD-L1 but also its ligand PD-1 in human solid tumors [67]. Wang et al. identified a pro-tumorigenic subset of macrophages that constitutively expressed PD-1 in GC tissues and activation of PD-1 signaling on TAM augmented their immunosuppressive activity [68].

Inhibition of TAM function by chemicals has been shown to reduce its infiltration into tumor [69, 70]. CSF-1 is a crucial mediator of macrophage differentiation and survival, and blockade of CSF-1-signaling by antagonists of CSF-1 receptor could suppress the activity of TAM in the tumor microenvironment [69, 70]. Interestingly, the preclinical

study demonstrated that combination of PLX-3397 (Pexidartinib), a small molecule designed to target CSF-1 receptor, and antagonists of PD-1 and CTLA-4 enhanced productive anti-tumor T-cell responses in pancreatic cancer models [71]. In addition, clinical trials with ICI in combination with CSF-1 receptor inhibitors such as PLX-3397 have been initiated in multiple solid tumors including GC [59]. Inhibition of TAM by CSF-1 receptor inhibitor might synergistically enhance the effects of ICI.

Predictive biomarkers for checkpoint-based immunotherapy in GC

The Cancer Genome Atlas classified GC into four molecular subtypes: EBV positive (9%), MSI-high (22%), genomically stable (20%) and chromosomal instability (50%). Within these subtypes, EBV-positive tumor and MSI-high tumor have shown better response to ICI [23, 72]. In addition, high tumor mutational burden (TMB) [73], T-cell inflamed gene profiling and interferon- γ gene signature [74], and circulating tumor DNA (ctDNA) [75] have been also reported as possible biomarkers predicting clinical outcomes of immunotherapy by ICI (Table 2). The USA Food and Drug Administration has approved the use of pembrolizumab for patients with refractory advanced GC expressing PD-L1, or

patients with unresectable or metastatic MSI-high/mismatch repair deficiency (dMMR) of solid tumors including GC [76, 77]. Thus, PD-L1 expression and MSI-high/dMMR are most likely promising predictive biomarkers of checkpoint-based immunotherapy in GC.

PD-L1 as a biomarker

PD-L1 expression in tumor tissues is measured by immunohistochemistry using FFPA sections, and evaluated by the ratio of PD-L1⁺ tumor cells so-called tumor proportion score (TPS) or by the ratio of PD-L1-stained tumor and immune cells so-called combined positive score (CPS). In the ATTRACTION-2 trial, although approximately 40% of patients were assessed for PD-L1 tumor expression using TPS, survival benefits of nivolumab was shown to be irrespective of PD-L1 status [13]. Moreover, in the CheckMate 032 trial, the responses were shown regardless of tumor PD-L1 expression [17]. On the other hand, in the phase II KEYNOTE-059 trial [76], patients who had PD-L1-positive tumors (CPS ≥ 1) showed an ORR of 22.7% including 2.7% complete response, while patients who had PD-L1-negative tumors (CPS < 1) demonstrated an ORR of 8.6% including 3.4% complete response. In addition, in phase III KEYNOTE-061 trial [16], patients with a PD-L1 CPS of 10 or higher (CPS ≥ 10), but not CPS ≥ 1 , had an

Table 2 Predictive biomarkers of checkpoint-based immunotherapy in GC

Biomarker	Cancer type	Agent	Phase	Significant association	Study or Clinical-Trial.gov, number	References
PD-L1 expression	GC, GEJC	Pembrolizumab	1b	N.A	KEYNOTE-012	[14]
	GC	Nivolumab	3	No (TPS ≥ 1)	ATTRACTION-2	[13]
	GC, EC, GEJC	Nivolumab/ Nivolumab + Ipilimumab	1/2	No (TPS ≥ 1)	CheckMate-032	[17]
	GC, GEJC	Pembrolizumab	2	Better ORR (CPS ≥ 1)	KEYNOTE-059	[76]
	GC, GEJC	Pembrolizumab	3	No (CPS ≥ 1) Better OS (CPS ≥ 10)	KEYNOTE-061	[16]
	GC	Pembrolizumab	2	Yes (CPS ≥ 1)	NCT02589496	[75]
MSI-H/dMMR	GC	Toripalimab	1b/2	Better ORR	NCT02915432	[78]
	GC, GEJC	Pembrolizumab	2	Better ORR	KEYNOTE-059	[76]
	GC, GEJC	Pembrolizumab	3	Better OS	KEYNOTE-061	[16]
	GC	Pembrolizumab	2	Better ORR	NCT02589496	[75]
TMB	GC	Toripalimab	1b/2	Yes	NCT02915432	[78]
EBV positivity	GC	Pembrolizumab	2	Better ORR	NCT02589496	[75]
	GC	Toripalimab	1b/2	No	NCT02915432	[78]
Immune-related gene signatures	GC, GEJC	Pembrolizumab	1b	Better BOR and PFS	KEYNOTE-012	[14]
	GC, GEJC	Pembrolizumab	2	Yes	KEYNOTE-059	[76]
ctDNA	GC	Pembrolizumab	2	Better ORR	NCT02589496	[75]

BOR best overall response, *CPS* combined positive score, *ctDNA* circulating tumor DNA, *dMMR* mismatch repair deficiency, *EBV* Epstein-Barr virus, *EC* esophageal cancer, *EGC* esophagogastric cancer, *GC* gastric cancer, *GEJC* gastroesophageal junction cancer, *ICI* immune checkpoint inhibitors, *MSI-H* microsatellite instability-high, *N.A.* not assessed, *ORR* objective response rate, *OS* overall survival, *PD-L1* programmed death-ligand 1, *PFS* progression-free survival, *TMB* tumor mutation burden, *TPS* tumor proportion score

OS of 10.4 months with pembrolizumab in comparison to 8.0 months with paclitaxel, suggesting that pembrolizumab had a greater effect for patients with higher PD-L1 expression ($\text{CPS} \geq 10$). Taken together, the significance of PD-L1 as a predictive biomarker still remains debatable.

MSI-high as a biomarker

Microsatellites are repeated DNA sequences scattered throughout the human genome, and DNA polymerases more likely to make errors in microsatellites. Because mismatch DNA is usually repaired by the MMR system, dMMR is frequently associated with MSI-high. MSI-high/dMMR are occurred in tumor cells and generate hypermutation and formation of immunogenic neoantigens that could be recognized by immune cells. Thus, tumors with MSI-high/dMMR exhibit dense infiltration of immune cells especially CD8^+ tumor-infiltrating lymphocytes and are thought to be sensitive to ICI. In fact, in the KEYNOTE-059 trial, ORR of pembrolizumab was 57.1% in patients with MSI-high GC, while that was 9.0% in patients with non-MSI-high GC [76]. Moreover, the KEYNOTE-061 trial demonstrated that pembrolizumab monotherapy had better response compared with paclitaxel monotherapy in patients with MSI-high GC [16]. Furthermore, dramatic responses to pembrolizumab were obtained in patients with MSI-high metastatic GC (ORR of 85.7%) [75].

Tumor mutation burden (TMB) as a biomarker

TMB is the total number of mutations per coding area of a tumor genome, and a significant correlation between high TMB and enhanced ORR and progression-free survival for ICI treatment has been observed in certain cancers [73]. A recent phase Ib/II clinical trial (NCT02915432) evaluated the value of TMB as a biomarker for chemo-refractory GC treated with toripalimab, a humanized IgG4 mAb against PD-1 [78]. Patients with high TMB tumors showed significant better responses and longer survival advantage than patients with low TMB tumors (ORR: 33.3% vs 7.1%, OS 14.6 months vs 4.0 months). Samstein et al. broadly analyzed the association between TMB and clinical response to ICI using the clinical and genomic data of 1662 advanced cancer patients including GC ($n = 126$) treated with ICI and 5371 non-ICI-treated patients [79]. A clear trend was observed between high TMB and increasing benefit for ICI treatment across cancer types, and better OS was also observed in most of the cancer types including GC (but not significant, $p = 0.221$). However, further studies are needed for better understating of TMB as a predictive biomarker of immunotherapy with ICI in GC.

EBV-positive as a biomarker

Given that EBV-positive GC exhibits, (1) increased PD-L1 expression in tumor and immune cells (PD-L1 staining in tumor cells in 50% and immune cells in 94%), (2) enrichment of IFN- γ gene signature, and (3) dense infiltration of CD8^+ tumor-infiltrating lymphocytes [72], EBV-positive GC most likely responds to ICI. Surprisingly, Kim et al. reported that all patients who had EBV-positive tumors ($n = 6$) achieved a complete or partial response by pembrolizumab [76], suggesting that EBV-positive tumors might have a strong response to ICI treatment. However, Want et al. recently reported that one partial response, two stable disease, and one progressive disease responses for toripalimab were observed among the four GC patients with EBV-positive ($n = 4$; 72.7%) [78]. Thus, further investigations in larger clinical trials should be needed.

Other factors as a biomarker

Immune-related gene signatures involved in IFN- γ signaling and activated T cell biology have also been proposed to serve as predictive biomarkers for ICI treatment. IFN- γ is produced by natural killer cells and T cells, and it augments cytotoxic function of tumor-infiltrating lymphocytes, recruitment of macrophages, and expression of tumor antigen-presenting MHC molecules in tumor cells, leading to anti-tumor immune response. Six IFN- γ gene signatures including *IDO1*, *CXCL10*, *CXCL9*, *HLA-DRA*, *STAT1* and *IFNG* were significantly associated with improved progression-free survival in pembrolizumab-treated patients with GC [14, 74]. In the KEYNOTE-059 trial, T-cell-inflamed gene expression profiles using 18 genes (*CCL5*, *CD27*, *CD274*, *CD276*, *CD8A*, *CMKLR1*, *CXCL9*, *CXCR6*, *HLA-DQA1*, *HLA-DRB1*, *HLA-E*, *IDO1*, *LAG3*, *NKG7*, *PDCD-1KL2*, *PSMB10*, *STAT1*, *TGIT*) also demonstrated that the profiling score was significantly higher in responders than in non-responder in patients with GC [76]. Moreover, IFN- γ gene signatures were strongly enriched in MSI-high GC and EBV-positive GC, which subgroups showing better response to ICI. However, clinical information related to the predictive value of immune-gene related signatures for ICI treatment in GC is still limited.

Plasma-derived cell-free ctDNA could predict patients likely to respond to ICI in non-small-cell lung carcinoma and urothelial bladder cancer [80]. ctDNA is released from necrotic or apoptotic tumor cells into bloodstream and presents a better correlation of TMB in tumors. Interestingly, Kim et al. demonstrated that ctDNA mutational load score correlated with clinical response to pembrolizumab in GC patients, suggesting that ctDNA might be able to select patients who are most likely sensitive to ICI treatment in GC [36].

Combined with anti-VEGF therapy

Combined with radiotherapy

◀**Fig. 2** Combined therapy with ICI. Combined cancer immunotherapy with anti-VEGF therapy (upper) and radiotherapy (lower). *CTL* cytotoxic T lymphocyte, *HLA* human leukocyte antigen, *HMGB1* high mobility group box 1, *ICI* immune checkpoint inhibitors, *TAM* tumor-associated macrophage, *Treg* regulatory T cell, *VEGF* vascular endothelial growth factor, *VEGFR* VEGF receptor Figure 2 was modified from Nakajima S and Kono K [90] with permission from Hokuryokan Co., Ltd.

Strategies for combined therapy with ICI in GC

To improve the efficacy of ICI, especially anti-PD-1 mAb, in GC, the next promising option is to develop strategies combining anti-PD-1 mAb with radiotherapy, chemotherapy, molecular target agents or other ICI.

Combined with other ICI

T cell exhaustion remains as a critical issue in the immunotherapy and co-expression of multiple immune checkpoints such as T cell immunoglobulin-3 and lymphocyte activation gene-3 is a typical feature of exhausted CD8 T cells in various types of cancers including GC [81, 82]. Therefore, the efficacy of anti-PD-1 mAb is expected to be enhanced by inhibition targeting other co-inhibitory immune checkpoints. Currently, anti-PD-1 mAb combined with ICI targeting lymphocyte activation gene-3 and T cell immunoglobulin-3 are under investigation in several clinical trials (NCT01968109, NCT03662659 and NCT02817633).

Combined with anti-VEGF therapy

In general, it is recognized that tumor-induced angiogenic factors including VEGF can promote tumor neovascularization, and tumor neovascularization is a condition that anti-tumor CTL fail to infiltrate into tumor microenvironment including GC [83]. Therefore, vascular normalization with anti-VEGF therapy have a potential to restore the infiltration of anti-CTL [84].

In addition, within tumor microenvironment in GC, there are several types of immune suppressive cells including regulatory T cells and TAM. It is reported that activity of regulatory T cells and TAM is partially dependent on VEGF/VEGF receptor (VEGFR) axis, and tumor-induced angiogenic factors or hypoxic condition can further promote regulatory T cell and TAM function via VEGF/VEGFR axis, leading to downregulation of anti-tumor CTL activity [85]. Thus, it is likely that inhibition of VEGF/VEGFR axis with chemicals can block the regulatory T cell and TAM function, resulting in enhancement of anti-tumor immunity and synergistic effect is expected in combination of ICI with anti-VEGF therapy (Fig. 2). In fact, Kato et al. reported that anti-VEGFR2 mAb, ramucirumab had a potential to induce

clinical synergism in combination with nivolumab, based on subgroup analysis for ATTRACTION-2 trial [86].

Combined with radiotherapy

In combination of ICI with radiotherapy, radiotherapy is expected to induce immunogenic cell death [87] and the combination can lead to synergistic effect of anti-tumor CTL (Fig. 2) [87]. Irradiation can induce several degree of tumor death and thereafter, some dying components in certain condition could become immunogenic antigen. Then, immunogenic cell death could trigger the uptake of antigenic components by dendritic cells and transfer antigenic signals to T-cell-mediated immunity, resulting in the expansion of anti-tumor CTL. We and others have shown that high mobility group box 1 and calreticulin induced by cytotoxic stresses such as chemoradiation are important mediators to induce immunogenic cell death [87, 88].

Although a promising result from a clinical trial with irradiation followed by anti-CTLA-4 mAbs in lung cancer was reported [89], there is currently no report to prove the synergistic effect of the combination of irradiation with ICI for GC. We are currently performing a phase II clinical trial of irradiation followed by nivolumab for advanced GC (NCT03453164).

Conclusion

The next steps for developing ICI in GC are mainly two challenges as follows. First is the identification of accurate biomarkers that can predict the response to ICI. The second challenge is the clinical development of combinatorial approaches to maximize the efficacy of ICI.

Compliance with ethical standards

Conflict of interest Koji Kono has received research grants from Ono pharmaceutical company, Bristol Myers Squibb and Taiho pharmaceutical company. Koji Kono has received a speaker honorarium from Ono pharmaceutical company, Bristol Myers Squibb and MSD pharmaceutical company. Kosaku Mimura has received a speaker honorarium from Ono pharmaceutical company and Bristol Myers Squibb.

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