Next-generation sequencing and biomarkers for gastric cancer: what is the future?

Akihito Kawazoe and Kohei Shitara

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Abstract: Recent years have witnessed an improved understanding of tumour biology and the molecular features of gastric cancer. Remarkable advances in next-generation sequencing technologies have defined the genomic landscape of gastric cancer. In fact, several molecular classifications have been proposed, and distinct molecular subtypes have been identified, which could serve as a roadmap for patient stratification and trials of targeted therapies. At present, clinical trials of new agents, such as receptor tyrosine kinases inhibitors, antibody–drug conjugates and IMAB362 (anti-Claudin 18.2), are ongoing. Furthermore, biomarkers of immune checkpoint inhibitors or combination therapy have been ardently investigated. These developments could facilitate precision medicine for gastric cancer in the near future.

Keywords: Claudin 18.2, gastric cancer, immune checkpoint inhibitors, molecular profiles, receptor tyrosine kinases

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Introduction

Gastric cancer (GC) is the fifth most common cancer and the third leading cause of cancer-related mortality worldwide.[1] Although some chemotherapy (CTx) regimens, including a platinum + fluoropyrimidine combination, trastuzumab [for human epidermal growth factor receptor 2 (HER2)-positive cases], taxanes, irinotecan and ramucirumab, reportedly enhance the survival outcomes of patients with advanced GC (AGC),[2–6] the prognosis remains poor (median survival ~1 year). Although the phase III ATTRACTION-2 trial of anti-programmed death 1 (anti-PD-1) antibody, nivolumab, reported a survival benefit in AGC,[7] the overall response rate (ORR) was approximately 10% and half of the patients exhibited early disease progression. Thus, the establishment of a better selection of patients who might derive greater benefit from PD-1 blockade is warranted. In addition, trifluridine/tipiracil (TAS-102) demonstrated a survival benefit compared with placebo in heavily pretreated patients with AGC.[8] However, until recently, several phase III trials of targeting agents for AGC failed to demonstrate a survival benefit (Table 1). Notably, single-agent activity for AGC is minimal, and a few trials have attempted to identify possible biomarkers before phase III trials; thus, better patient stratification based on molecular profiles is crucial.

This study aims to review the molecular features, promising treatment targets and biomarkers of immune checkpoint inhibitors that could facilitate precision medicine for GC in the near future.

Molecular profiles of GC

The molecular characterization of GC has been rapidly evolving recently. To date, several molecular classifications have been proposed, and distinct molecular subtypes have been identified.[9–14] Reportedly, several receptor tyrosine kinases (RTKs), such as HER2, epidermal growth factor receptor 1 (EGFR), mesenchymal–epithelial transition factor (MET) and fibroblast growth factor receptor 2 (FGFR2), are amplified in GC, and targeted therapies including these molecules have been developed.[15–18] Notably, these amplifications are frequently but not universally mutually exclusive.[15–18] In 2014, The Cancer Genome Atlas (TCGA) network characterized 295 gastric adenocarcinoma cases based on six molecular platforms[9]: somatic copy number analysis, whole-exome sequencing, DNA methylation profiling, messenger RNA sequencing, microRNA sequencing and

Correspondence to:
Kohei Shitara
Department of Gastroenterology and Gastrointestinal Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan
kshitara@east.ncc.go.jp

Akihito Kawazoe
Department of Gastroenterology and Gastrointestinal Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan

Table 1. Recent phase III trials of new agents for gastric cancer.

Target	Trial/authors	Line	Screening	Agent	control	Endpoint	Results	Difference mOS (m) (HR)
HER2	ToGA	1st	HER2	Trastuzumab	(+chemo)	OS	Positive	+2.7 (HR 0.74)
HER2	Logic	1st	HER2 (FISH)	Lapatinib	PBO (+chemo)	OS	Negative	+1.7 (HR 0.91)
HER2	JACOB	1st	HER2	Pertuzumab	PBO (+chemo+Tmab)	OS	Negative	+3.3 (HR 0.84)
HER2	TyTAN	2nd	HER2 (FISH)	Lapatinib	(+chemo)	OS	Negative	+3 (HR 0.84)
HER2	GATSBY	2nd	HER2	T-DM1	Taxanes	OS	Negative	−0.7 (HR 1.15)
EGFR	REAL-3	1st	–	Panitumumab	(+chemo)	OS	Negative	−2.5 (HR 1.37)
EGFR	EXPAND	1st	–	Cetuximab	PBO (+chemo)	PFS	Negative	−1.3 (HR 1.0)
EGFR	ENRICH	2nd	EGFR (IHC)	Nimotuzumab	(+chemo)	OS	Terminated	
mTOR	GRANITE-1	2nd /3rd	–	Everolimus	PBO	OS	Negative	+1.05 (HR 0.9)
mTOR	GRANITE-2	2nd	–	Everolimus	PBO (+chemo)	OS	Negative	+1.0 (HR 0.92)
HGF	RILOMET1	1st	MET (IHC)	Rilotumumab	PBO (+chemo)	OS	Negative	−2.9 (HR 1.36)
MET	METgastric	1st	MET (IHC)	Onartuzumab	PBO (+chemo)	OS	Negative	−0.3 (HR 0.82)
VEGF-A	AVAGAST	1st	–	Bevacizumab	PBO (+chemo)	OS	Negative	+2 (HR 0.87)
VEGFR2	RAINFALL	1st	–	Ramucirumab	PBO (+chemo)	OS	Negative	+0.4 (HR 0.96)
VEGFR2	REGARD	2nd	–	Ramucirumab	PBO	OS	Positive	+1.4 (HR 0.776)
VEGFR2	RAINBOW	2nd	–	Ramucirumab	PBO (+chemo)	OS	Positive	+2.2 (HR 0.807)
VEGFR2	Li *et al*.	3rd	–	Apatinib	PBO	OS	Positive	+1.8 (HR 0.71)

Table 1. (Continued)

Target	Trial/authors	Line	Screening	Agent	control	Endpoint	Results	Difference mOS (m) (HR)
PARP	GOLD	2nd	ATM (IHC)	Olaparib	PBO (+chemo)	OS	Negative	+1.9 (HR 0.79)
STAT3	BRIGHTER	2nd	–	Napabucasin	PBO (+chemo)	OS	Negative	−0.4 (HR 1.01)
PD1	Keynote061	2nd	PD-L1 (IHC)	Pembrolizumab	Paclitaxel	OS	Negative	+0.8 (HR 0.82)
PD1	JAVELIN300	3rd	–	Avelumab	Irinotecan/taxanes/BSC	OS	Negative	−0.4 (HR 1.1)
PD1	ATTRACTION-2	3rd-	–	Nivolumab	PBO	OS	Positive	+1.2 (HR 0.63)

FISH, fluorescence *in situ* hybridization; EGFR, epidermal growth factor receptor 1; HER2, human epidermal growth factor receptor 2; HGF, hepatocyte growth factor; HR, hazard ratio; IHC, immunohistochemistry; MET, mesenchymal–epithelial transition factor; mTOR, mammalian target of rapamycin; OS, overall survival; PARP, poly (ADP-ribose) polymerase; PBO, placebo; PFS, progression-free survival; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

reverse-phase protein array. In addition, microsatellite instability (MSI) testing and whole-genome sequencing were performed. Then, four subtypes of GC were described as follows: (1) tumours positive for Epstein–Barr virus (EBV); (2) MSI-high (MSI-H) tumours; (3) genomically stable (GS) tumours and (4) tumours with chromosomal instability (CIN; Table 2). EBV-positive tumours exhibit recurrent *PIK3CA* and *ARIDIA* mutations, extreme DNA hypermethylation and high amplification of *JAK2, PD-L1* and *PD-L2*. MSI-H tumours exhibit elevated mutation rates, including mutations of genes encoding targetable oncogenic signalling proteins. GS tumours are enriched for the diffuse histological variant and mutations of *CDH1* and *RHOA* or CLDN18–ARHGAP fusion. CIN tumours are frequently observed at the gastroesophageal junction/cardia with recurrent *TP53* mutation and relatively numerous amplifications of RTKs genes. In 2015, The Asian Cancer Research Group (ACRG) proposed four molecular subtypes, including (1) MSI-H, (2) microsatellite stable (MSS) with epithelial–mesenchymal transition features (MSS/EMT), (3) MSS/TP53 mutant (MSS/TP53) and (4) MSS/TP53 wild-type (MSS/TP53^{-}; Table 2).[10] In the MSS/EMT subtype, nearly 70% of recurrences were at the peritoneum, with a markedly poorer prognosis compared with other subtypes, highlighting the need for therapy development for peritoneal dissemination.[10] Recently, Liu *et al.* reported that gastrointestinal tract adenocarcinomas comprised five molecular subtypes, EBV, MSI, hypermutated single-nucleotide variant predominant (HM-SNV), CIN and GS, to distinguish genomic or immunological features.[19] HM-SNV tumours harboured a lower level of CD8 or interferon (IFN)-γ signatures than that of MSI tumours, indicating that indel mutations, which MSI-H tumours often yield, better neoantigens than SNVs. The future clinical trials of targeted and immune therapy in AGC should be designed per differences in genomic or immunological features, as they could affect treatment response and clinical outcomes. Notably, these molecular profiles have been investigated in Japanese AGC. According to GI-SCREEN as the Nationwide Cancer Genome Screening Project, the frequently detected mutations were *TP53* (47.8%), *PIK3CA* (9.2%), *KRAS* (6.0%), *SMAD4* (5.1%), *APC* (4.1%), *TET2* (3.9%), *ERBB2* (3.3%) and copy number variants were *ERBB2* (11.3%), *CCNE1* (11.1%), *KRAS* (3.7%), *FGFR2* (3.3%), *ZNF217* (3.3%), *MYC* (2.7%), *CCND1* (2.3%) and *CDK6* (2.1%).[20] In stage IV AGC, mismatch repair (MMR)-deficient

Table 2. The new molecular-based classification of GC according to The Cancer Genome Atlas (TCGA) 2014 and The Asian Cancer Research Group (ACRG) 2015.

TCGA	Epstein–Barr virus-infected (EBV)	Microsatellite instability (MSI)	Genomically stable (GS)	Chromosomal instability (CIN)
	• EBV-positive • Profound hypermethylation • CDKN2A silencing • 80% PIK3CA mutation • PD-L1/2 overexpression • Immune cell signalling • Frequent *ARID1A* and • *BCOR* mutations • Fundus and body	• Hypermutation • DNA hypermethylation • Silencing of *MLH1* • Elevated somatic mutations (PIK3CA, 42%; ERBB3, 26%) • Older patients • Fundus, body and antrum	• Tumours lacking aneuploidy and elevated rates of mutation or hypermethylation • Somatic *RHOA* and *CDH1* mutations • CLDN18–ARHGAP6 or ARHGAP26 fusions • Mostly diffuse subtype	• Marked aneuploidy • TP53 mutations • Recurrent amplifications of receptor tyrosine kinases (HER2, 24%) • Majority of tumours at the esophagogastric junction
ACRG	**MSS/TP53-**	**MSI**	**MSS/EMT**	**MSS/TP53**
	Intact *TP53* • *MDM2* amplification • EBV infection • Enrichment with *PIK3A* or *ARID1A* mutation and cytokine signature in EBV-positive tumours	• Hypermutation • Silencing of *MLH1* • Frequent mutations in *KRAS, MTOR, PIK3CA, ASL and ARID1A* • Best prognosis	• *CDH1* silencing • Younger patients • Worst prognosis	• *TP53* mutation • Genomic instability • Recurrent amplification (*ERBB2, EGFR, GATA6, MYC, CCNE1 and CCND1*)

(MMR-D) and EBV tumours are identified in 6.2% and 6.2% cases, respectively.[21] These profiles do not largely differ from prior reports mainly conducted outside Japan, supporting the global development of new agents for AGC. Recently, multiplex gene panels, such as NCC Oncopanel and FoundationOne CDx, were approved in Japan to advance personalized medicine, resulting in further genomic profiling in a large cohort of Japanese patients with AGC. Furthermore, the MSI status could be detected by targeted next-generation sequencing (NGS).[22]

Meanwhile, heterogeneity of genomic alterations is one of the main issues in GC,[23,24] which could account for some differences in the incidence of mutations or amplifications and inconsistent molecular characterization across various reports.[9–14] In addition, discordance of dominant oncogenic alterations between primary and metastatic tumour is reported in 32% of AGC tumour samples.[23] Conversely, 87.5% concordance for targetable alterations was noted in metastatic tissue and circulating tumour DNA (ctDNA).[24] Furthermore, other studies suggested the dynamic landscape of the ctDNA profile before and after molecular targeting agents.[25,26] These analyses should also be incorporated into clinical trials of new agents for AGC to elucidate better treatment biomarkers.

Promising treatment targets

Targeting HER2

Approximately 60% of patients with GC belong to the CIN subtype and could depend on RTKs signalling for growth and development.[9,10,27,28] HER2 is a therapeutically relevant RTK in 10–20% of the overall GC population and up to 30% of gastroesophageal junction adenocarcinoma harbouring *HER2* gene amplification or protein overexpression. In the trastuzumab for GC (ToGA) trial, patients treated with trastuzumab (a HER2-directed monoclonal antibody) and CTx exhibited a significant improvement in overall survival (OS; 13.8 *versus* 11.1 months; HR, 0.74; $p = 0.0046$).[29] The OS benefit was the highest in the subset of tumours defined as HER2 immunohistochemistry (IHC) 3+ or IHC2+/fluorescence *in situ* hybridization (FISH) with unprecedented OS of 16 months in the trastuzumab group compared with 11.8 months with CTx alone [hazard ratio (HR) 0.68; 95% confidence interval (CI) 0.5–0.83];[29] thus, it became a standard of care for this patient population.

Several recent studies have demonstrated the potential utility of tissue-based NGS and ctDNA NGS for biomarkers of HER2-tageted therapy.[30–32] Kim *et al*. reported that CCNE1 amplification and low-level HER2 amplification detected by NGS correlated with the lack of response.[30] The cfDNA analysis revealed that detectable ERBB2 copy number amplification in the plasma was predictive to the response, and changes in plasma-detected genomic alterations correlated with the sensitivity and/or resistance of HER2-tageted therapy.[30] In addition, serial ctDNA sequencing illustrated emergences of other genomic aberrations, such as MYC, EGFR, FGFR2 and MET amplifications, at disease progression.[30] Sanchez-Vega *et al*. demonstrated that resistance to the pan-HER inhibitor afatinib correlated with the selection of tumour cells with the loss of EGFR amplification or acquired MET amplification, which might be detected in the plasma cfDNA, in a phase II trial of trastuzumab-refractory HER2-amplified AGC.[33] A phase II trial of margetuximab, an anti-HER2 monoclonal Fc-optimised antibody for antibody-dependent cellular cytotoxicity (ADCC), in combination with pembrolizumab demonstrated that ERBB2 amplification detected by ctDNA progressing on trastuzumab could facilitate identifying AGC patients more likely to respond to the study treatment, especially among PD-L1 positive patients.[34] Furthermore, ctDNA NGS could overcome tissue biopsy errors associated with intratumoural heterogeneity, especially at post-treatment progression.

Lately, antibody–drug conjugates have emerged as a promising strategy in cancer therapy and combine the capability of monoclonal antibodies to precisely target tumour cells with the highly potent killing activity of drugs with payloads too toxic for systemic administration. Nevertheless, trastuzumab-emtansine (T-DM1, an antibody–drug conjugate comprising trastuzumab linked to the cytotoxic agent DM1), which illustrates remarkable efficacy in breast cancer, did not prolong the OS in HER2-positive AGC;[35] this could be, in part, attributed to intratumoural heterogeneity in the HER2 expression and amplification compared with breast cancer.[23,36] Available evidence indicates that most of HER2-positive GCs are heterogeneous with downregulation in HER2 status post-progression on trastuzumab, as well as diverse intratumoural molecular characteristics.[37–39] Thus, the assessment of the HER-2 status just before molecular-targeted therapy could be crucial for attaining therapeutic success.

Trastuzumab deruxtecan (DS-8201a) is an antibody–drug conjugate comprising a humanized antibody against HER2, a novel enzyme-cleavable linker and a topoisomerase I inhibitor payload. A preclinical study demonstrated that DS-8201a exerted a potent bystander effect because of a highly membrane-permeable payload and was beneficial in treating tumours with HER2 heterogeneity that are unresponsive to T-DM1.[40] Indeed, a phase I study of DS-8201a exhibited the antitumour activity in patients with breast cancer and AGC previously treated with T-DM1 or trastuzumab, and in patients with HER2-low tumours.[41] In 44 patients with AGC, the overall response rate (ORR), the disease control rate (DCR) and the median PFS were 43.2%, 79.5% and 5.6 months, respectively.[42] A phase II study (DESTINY-Gastric01) in Japan and South Korea assessing the safety and efficacy of DS-8201a in patients with HER2-positive AGC resistant or refractory to trastuzumab is ongoing (ClinicalTrials.gov identifier: NCT03329690).

Recently proposed novel anti-HER2 therapy, ZW25, is reportedly effective and well tolerated in patients with various HER2-positive tumours.[43] ZW25 is a bispecific antibody that concurrently binds two HER2 epitopes: ECD4, the trastuzumab-binding domain, and ECD2, the pertuzumab-binding domain. Preclinical research indicated that ZW25 exhibits potent antitumour activity at a range of HER2 expression levels and could more effectively silence HER2 signalling than trastuzumab or pertuzumab and stimulates the immune system. A phase I trial reported a response rate of 38% in heavily pretreated HER2-expressing tumours including GC.[44] Further assessment in HER2-positive tumours, including AGC, is ongoing.

In a preclinical study, combining anti-PD-1 and anti-HER2 therapy induce T-cell activation and augment ADCC.[45] A phase II trial of the addition of trastuzumab + pembrolizumab to the first-line CTx exhibited promising results with ORR of 87% and the median PFS of 11.4 months, which warrants further evaluation in an ongoing phase III trial (KEYNOTE811; ClinicalTrials.gov identifier: NCT03615326).

Other RTKs

Up to 60% of GC cases belong to the CIN subtype, which is commonly related to abnormalities on RTKs signalling.[9–15] Various molecular targeting drugs for HER2, EGFR, hepatocyte growth factor (HGF), MET and mammalian target of rapamycin (mTOR) have been assessed; however, most did not exhibit a significant benefit in phase III trials partly because of inappropriate patient selection and molecular stratification (Table 1). Although EGFR, MET or FGFR inhibitors have exhibited antitumour activity for patients with homogenous amplification of these RTKs genes, these cases are relatively rare.[46–48]

Pearson *et al*. reported that AGC patients with high-level clonal FGFR2 amplification exhibit a high response rate to FGFR-1, 2, 3 tyrosine kinase inhibitor (TKI), AZD4547, whereas those with subclonal or low-level amplification did not respond.[49] A randomised phase II trial (SHINE study) of AZD4547 monotherapy *versus* paclitaxel for the treatment of AGC patients with FGFR2 polysomy or gene amplification reported that AZD4547 did not markedly enhance the median PFS compared with paclitaxel.[50] Exploratory biomarker analyses of the SHINE study revealed marked intratumoural heterogeneity of FGFR2 amplification and poor concordance between amplification/polysomy and FGFR2 mRNA expression, indicating that the failure to adequately enrich a clonally amplified population might contribute to the failure of this study.[50] Moreover, AGC patients with high-level FGFR2 amplification attained an objective response for TAS-120, a highly selective covalent FGFR inhibitor.[51] FPA144, an ADCC-enhanced, FGFR2b isoform-specific monoclonal antibody, exhibited antitumour activity in patients with FGFR2b^{+} high (IHC 3+ $\geqslant$10% tumour membrane staining) AGC with an ORR of 19% and a DCR of 57%, respectively. A phase III trial assessing FPA144 and mFOLFOX6 in patients with previously untreated AGC is ongoing. Recently, FGFR2–ACSL5 fusion was identified in an AGC patient with acquired resistance to FGFR inhibition in FGFR2-amplified AGC. Furthermore, JHDM1D–BRAF fusion results in resistance for FGFR inhibitor-resistant cell line, which warrants further research.[52,53]

Although a phase II trial of oral EGFR TKI erlotinib reported an objective response in 4 of 44 patients with gastroesophageal junction adenocarcinoma,[54] no apparent correlation was reported between objective response and tumour biomarkers, such as IHC or FISH. To date, several phase III trials of monoclonal antibodies for EGFR had

been conducted; however, no study has reported a survival benefit of phase III trials (Table 1). A subgroup analysis of the EXPAND study, in which adding cetuximab to first-line capecitabine and cisplatin CTx failed to enhance the clinical outcome in patients with AGC, reported a tendency for enhanced OS, PFS and ORR in a small population of patients with high tumour EGFR IHC scores.[55] Furthermore, Maron *et al*. reported that anti-EGFR treatment attained an objective response in a small population of patients with high-level EGFR amplification detected by both tissue-based NGC and ctDNA NGS.[56]

The addition of HGF and c-MET signalling inhibitors (onartuzumab as anti-MET monoclonal antibody or rilotumumab as anti-HGF monoclonal antibody) to first-line CTx in MET-positive AGC detected by IHC did not exhibit clinical benefit in phase III trials,[57,58] although MET TKIs have exhibited antitumour activity in some patients with high-level MET amplification.[46,47] The lack of appropriate patient selection and molecular stratification could be one of the reasons for the failure in these trials. Considering that the amplification of RTKs does not simply correlate with the protein expression in GC,[17] a comprehensive analysis using both NGC and IHC could be necessary to select patients adequately.

VEGF targeting therapy

Ramucirumab is a human IgG1 monoclonal antibody specific for vascular endothelial growth factor receptor 2 (VEGFR2), which has recently been validated to be effective for AGC by the REGARD and RAINBOW trials.[4,6] In the RAINBOW trial, which compared paclitaxel + placebo with paclitaxel + ramucirumab, patients treated with paclitaxel + ramucirumab exhibited significantly longer OS (median, 9.6 *versus* 7.4 months), longer PFS (median, 4.4 *versus* 2.9 months) and higher response rate (28% *versus* 16%) than those treated with paclitaxel alone. Regrettably, an optimal biomarker for antiangiogenic treatment is still lacking. A recent retrospective exploratory analysis from the REGARD study reported that none of the tested biomarkers (tumour HER2 or VEGFR2 and serum VEGF-C and VEGF-D, and soluble VEGFR1 and VEGFR3) identified a potent predictive biomarker of ramucirumab efficacy.[59] Moreover, per the exploratory plasma analyses from the RAINBOW study, neither VEGF pathway markers nor other markers revealed a predictive correlation with ramucirumab efficacy.[60] Recently, the RAINFALL phase III trial demonstrated that adding ramucirumab to first-line cisplatin/fluoropyrimidine did not exhibit enhanced OS, which also failed to exhibit the utility of plasma biomarkers, such as VEGF-C and VEGF-D.[61] A phase III double-blind placebo-controlled study of regorafenib, multiple VEGF TKIs in patients with AGC is underway, which will also assess predictive biomarkers of this antiangiogenic treatment. Furthermore, apatinib is a multikinase inhibitor that primarily targets VEGFR2 and markedly enhances OS in patients with pretreated AGC, although no biomarkers were identified to predict clinical benefits.[62]

PARP inhibitor

The GC pathogenesis is associated with DNA damage and chronic inflammation from *Helicobacter pylori*[63,64] and EBV infections,[65] to lifestyle factors including obesity and chronic gastric acid reflux. Large-scale genome sequencing of GC suggested that somatic mutations in genes involved in homologous recombination DNA repair are common features.[9,10]

Olaparib is an oral poly (ADP-ribose) polymerase (PARP) inhibitor that blocks DNA base-excision repair and causes synthetic lethality in tumours with homologous recombination repair deficiencies. Ataxia–telangiectasia mutated (ATM) is a gene essential to the cellular double-strand DNA breaks response essential to maintain genome stability levels. In a phase II trial of olaparib combined with paclitaxel *versus* placebo combined with paclitaxel as second-line therapy, a higher OS benefit was noted in AGC patients with ATM-negative tumours (HR 0.35; $p = 0.002$).[66] Unfortunately, the phase III GOLD trial did not report improved OS with olaparib in patients with ATM-negative tumours (HR 0.73; $p = 0.25$) as well as overall population (HR 0.79, $p = 0.026$),[67] indicating that patient selection by the ATM status was not sufficient. Biomarker analysis of the GOLD study revealed that none of the other genetic markers of DNA damage repair, which have proven predictive in other tumour types for full-dose olaparib monotherapy, correlated with the sensitivity to low-dose olaparib combination with paclitaxel in patients with AGC.[68] A phase III trial comparing PARP inhibitor BGB-920 with placebo as maintenance therapy in patients with AGC who responded to first-line platinum-based CTx is under way, which would also assess

the sensitivity to PARP inhibitor monotherapy from tumour specimen. Based on a preclinical study demonstrating that PARP inhibitor upregulates the PD-L1 expression on tumour cells,[69] a phase II trial of olaparib and anti-PD-L1 antibody, durvalumab, was conducted, resulting in an ORR of 10% and a DCR of 25% after 12 weeks because of a high rate of early disease progression following olaparib monotherapy for 4 weeks,[70] which will be explored in combination with CTx.

Targeting therapy for stemness-related pathway or cancer stroma

Cancer stem cells (CSCs) exhibit self-renewal capability and could contribute to malignant tumour growth, disease relapse and metastasis. Signal transducer and activator of transcription 3 (STAT3) activation is one of the hallmarks associated with cancer 'stemness'. STAT3 acts as a transcription factor located downstream of various pro-oncogenic cytokines and JAK. Reportedly, phosphorylated STAT3 activates the transcription of *Nanog* and *Myc* genes.[71] BBI608 (napabucasin) is an orally administered investigational small molecule presumed to affect multiple oncogenic cellular pathways, including the inhibition of STAT3, which has been implicated in providing CSCs with stemness characteristics.[71] Encouraging the antitumour activity of BBI608 and paclitaxel in refractory AGC was observed in a phase Ib and subsequent phase II study with an ORR of 31% and a DCR of 75%.[72] Nevertheless, a phase III trial (BRIGHTER) of BBI608 + weekly paclitaxel *versus* placebo + weekly paclitaxel in patients with AGC failed to improve the OS.[73]

Reportedly, a subpopulation of gastric carcinoma cells expressing EPCAM, CD44, CD44 variant (CD44v), CD133 and CD166 exhibited the properties to generate new heterogeneous tumours *in vitro*.[74] Sulfasalazine is an inhibitor of the cysteine–glutamate exchange transporter, a variant form of CD44v. Sulfasalazin induces a reduction in CD44v-positive cells and intracellular reduced glutathione levels in patients with AGC.[75] However, a phase I trial of the combination of sulfasalazin with cisplatin in patients with CD44v-expressing AGC refractory to cisplatin did not exhibit an apparent antitumour activity.[76]

Recently, Nanki *et al*. illustrated divergent genetic and epigenetic routes to gain Wnt and R-spondin niche independency in phenotype analyses of GC organoids, and a marked correlation between CDH1/TP53 compound mutations, which were also identified in the TCGA 2014 report,[9] and R-spondin independency (Wnt-dependent GC).[77] In this study, xenografting of GC organoids established the feasibility of Wnt-targeting therapy for Wnt-dependent GC.[77] Most recently, a preclinical study reported that the Wnt receptor Fzd7 is a promising target for GC irrespective of the APC mutation status.[78]

Dickkopf-1 (DKK1) is a modulator of the Wnt and PI3K–AKT signalling pathways and contributes to an immunosuppressive tumour microenvironment by activating MDSCs and regulatory T cells (Tregs). DKN-01, a monoclonal antibody against DKK1, acts on innate immune cells, and a preclinical study illustrated the upregulation of both PD-L1 and IFN-γ-related chemokines, indicating a rational for immune checkpoint combination. A phase Ib trial of DKN-01 in combination with pembrolizumab reported encouraging antitumour activity in AGC with an ORR of 23.5% and a DCR of 58.8%, which warrants further investigation.[79]

MMP-9

Matrix metalloproteinases-9 (MMP-9) is an extracellular enzyme involved in matrix remodelling, angiogenesis, tumour growth and metastasis.[80] Chen *et al*. reported a higher expression of MMP-9 in the GC tissue than that in the adjacent healthy tissues.[81] Moreover, its overexpression reportedly correlated with the poor prognosis of GC.[82] Preclinical studies demonstrated that MMP-9 inhibition alters the tumour microenvironment, which correlates with higher CTx penetration and enhanced antitumour immunity. Andecaliximab is a monoclonal antibody that inhibits MMP-9 and has been combined with various CTx regimens. A phase I/Ib trial of mFOLFOX6+ andecaliximab revealed encouraging antitumour activity in AGC patients with the median PFS of 9.9 months in the first-line setting and the ORR of 50%.[83] However, a subsequent phase III study of andecaliximab combined with mFOLFOX6 in the first-line setting for patients with AGC did not markedly improve the OS.[84]

Claudin 18.2

Claudin18.2 (CLDN18.2) is a member of the claudin family of >20 structurally related proteins that form vital components of the tight cell

junctions in epithelia and endothelia;[85] it is not expressed in any healthy tissue, except the stomach mucosa, but broadly expressed in various cancer types including AGC, especially in diffuse-type GCs.[86] Moreover, CLDN18-ARHGAP26/6 fusions have been identified in GCs, with a predominance in GS-type tumours based on the TCGA classification.[9] Reportedly, almost all CLDN18–ARHGAP26/6 fusion-positive GCs expressed CLDN18.2 protein with a higher prevalence of lymphatic and distant organ metastases, especially in the younger age patients.[87] Furthermore, the TCGA data demonstrated that the CLDN18–ARHGAP26/6 fusion was mutually exclusive with driver genes, such as *RHOA* and *CDH1* mutations, which were frequently noted in GS-type tumours.[9]

IMAB362 (zolbetuximab) is a novel chimeric IgG1 antibody highly specific for CLDN18.2; it binds to CLDN18.2 on the tumour cell surface to stimulate cellular and soluble immune effectors that activate antibody-dependent cytotoxicity and complement-dependent cytotoxicity.[88] A phase II study (MONO) demonstrated the efficacy and safety of IMAB362 as monotherapy in patients with metastatic, refractory or recurrent GC.[89] Among 40 patients who received IMAB362 600 mg/m^2, the ORR was 10% and the DCR was 30%. A randomized phase II study (FAST) demonstrated that IMAB362 in combination with first-line CTx exhibited clinically relevant benefit in the PFS and OS in patients with CLDN18.2-positive AGC:[90] IMAB362 + EOX significantly enhanced the PFS (median 7.9 *versus* 4.8 months; HR 0.47; $p = 0.0001$) and OS (median 13.3 *versus* 8.4 months; HR 0.51; $p < 0.001$) compared with EOX alone. A subgroup analysis revealed that the CLDN18.2 expression in ⩾70% of tumour cells correlated with better OS (HR, 0.44), resulting in further patient enrichment (⩾75% of tumour cells) in an ongoing phase III study (Spotlight), which assesses the efficacy of IMAB362 + mFOLFOX6 compared with placebo + mFOLFOX6 as first-line CTx. Reportedly, high claudin18.2 expression (⩾75% of tumour cells) was detected in 36% of patients with AGC.[90]

Recently, a preclinical study indicated that partial or complete tumour elimination was observed in CLDN18.2-positive GC patient-derived tumour xenograft models treated with CLDN18.2-specific CAR T cells.[91] A first-in-class CAR-Claudin18.2 T cell trial for the treatment of gastric and pancreatic cancer is ongoing. Overall, CLDN18.2 is a promising novel treatment target for IMAB362 combined with first-line CTx and CLDN18.2-specific CAR T cells in patients with AGC.

Immune checkpoint inhibitors and its biomarkers

Recently, blockade of immune checkpoint molecules with monoclonal antibodies has emerged as a promising strategy in several malignancies.[92–97] PD-1, which belongs to the CD28 family of proteins, is a negative costimulatory receptor expressed on the surface of activated T cells.[98] The binding of PD-1 and its ligands, PD-L1 and PD-L2 in tumour or immune cells, can inhibit a cytotoxic T-cell response, which leads tumour cells to escape from immune surveillance.[98] Accordingly, the blockade of this interaction restores the antitumour activity of T cells.[98] Clinical trials of anti-PD-1/PD-L1 monoclonal antibodies have reported durable antitumour response and enhanced OS in several malignancies.[92–97]

A phase III ATTRACTION-2 trial of nivolumab, a fully human IgG4 monoclonal antibody against PD-1, for patients with AGC after two or more previous line CTxs demonstrated a survival benefit, resulting in the approval of nivolumab for AGC in Japan as third-line or later-line treatment.[7] However, subsequent randomized trial of anti-PD1/PD-L1 in earlier trials failed to exhibit a survival benefit compared with standard CTx; thus, better treatment selection is warranted to use anti-PD1/PD1 in earlier treatment lines.

PD-L1 expression

An exploratory analysis of ATTRACTION-2 indicated no predictive value of PD-L1 expression on tumour cells.[7] Moreover, in JAVELIN 300, which recently failed to establish a survival benefit for avelumab compared with the investigators' choice of CTx with paclitaxel or irinotecan for patients with AGC, no difference was observed in the OS based on the PD-L1 expression, which was defined as PD-L1 staining on 1% of tumour cells.[99] Results remained the same when PD-L1 was also assessed on immune cells, although the methodology for this assessment is not described. However, a correlation between higher PD-L1 expression [using the combined positive score (CPS), which is a proportional assessment of

PD-L1 staining on both tumour and immune cells] and higher treatment effect was suggested in phase II (KEYNOTE-059) and III trials (KEYNOTE-061) of pembrolizumab.[96,100] In KEYNOTE-059, the ORR in the third-line setting was 22.7% for patients with PD-L1 expression (CPS ⩾1) as determined by 22C3 IHC assay, whereas the ORR was 8.6% for those with PD-L1-negative tumours, resulting in US Food and Drug Administration (FDA) approval of pembrolizumab for PD-L1-positive AGC and PD-L1 22C3 IHC as a companion diagnostic assay.[96] Although KEYNOTE-061 failed to demonstrate improvement in the OS with pembrolizumab in CPS ⩾1 population, patients who expressed high levels of PD-L1 (CPS ⩾10) exhibited a pronounced benefit from treatment with pembrolizumab (HR 0.64; 95% CI 0.41–1.02).[100] The ORRs of pembrolizumab in patients with CPS ⩾10, CPS ⩾1 and CPS <1 (PD-L1-negative) were 25%, 16% and 2%, respectively.[36] The impact of CPS on the efficacy of PD-1 blockade will also be assessed in the ongoing phase III trial (KEYNOTE-062), which compared the efficacy of cytotoxic agents combined with pembrolizumab with that of cytotoxic agents and pembrolizumab monotherapy in patients with untreated AGC (ClinicalTrials.gov identifier: NCT02494583). This study might offer some insights into how to select patients for single-agent immunotherapy or combination with CTx.

In the AGC cohort of CheckMate-032,[101] the ORR was the highest with 1 mg/kg nivolumab + 3 mg/kg ipilimumab (24%), relative to 3 mg/kg nivolumab (12%) or 3 mg/kg nivolumab + 1 mg/kg ipilimumab (8%) cohorts. The ORR seemed numerically higher in patients with PD-L1-positive on tumour cells (⩾1% PD-L1 staining of tumour cell membranes) than PD-L1-negative responses. Other combinations therapy might be necessary to enhance outcomes in PD-L1 negative patients.

MSI-H

As shown in the TCGA 2014 and ACRG 2015 reports, the MSI-H subtype exhibits frequent mutations in multiple genes (including frameshifts or missense mutations) and hypermethylation (including hypermethylation at the MLH1 promoter), which contribute to the enhanced expression of neoantigens. MSI-H/MMR-D colorectal cancer has higher mutation loads than that of MSS/MMR proficient (MMR-P) colorectal cancer, resulting in high infiltration of CD8+ T cells presumably because of the recognition of a high number of tumour neoantigens and its corresponding expression of immune checkpoints in the tumour microenvironment.[102] Indeed, the FDA approved pembrolizumab for patients with MSI-H or MMR-D solid tumours, including AGC based on the durable response in several trials.[103–105]

Recently, pembrolizumab for patients with MSI-H/MMR-D solid tumours was approved in Japan also. In a phase II KEYNOTE-158 trial of pembrolizumab demonstrating that the ORR was 37.2% for 94 patients with MSI-H/MMR-D non-colorectal solid tumours, including patients in Japan, 6 of 13 patients with AGC attained an objective response (ORR 46.2%). Moreover, a subgroup analysis of KEYNOTE-059 and KEYNOTE-061 revealed that the ORR was 57.1% and 46.7% for patients with MSI-H/MMR-D AGC, respectively.[96,100] Based on this evidence, Pan-Asian adapted the ESMO Clinical Practice Guidelines for the management of patients with metastatic GC and recommended that pembrolizumab or nivolumab could be a treatment option for patients with MSI-H/MMR-D AGC in second-line settings.[106]

Other factors to predict response

As patients with MSI-H/MMR-D form a small minority of AGC patients, novel biomarkers to predict response to immunotherapy among MSS/MMR-P are desired. A recent study reported that tumour mutation burden (TMB) correlated with enhanced survival in patients receiving immune checkpoint inhibitors across multiple cancer types.[107] Moreover, Kim *et al.* reported that TMB was a potential biomarker of pembrolizumab for AGC.[108] However, most patients with high TMB had MSI-H/MMR-D status, and not all patients with high TMB attained an objective response.[108] Hence, the precise mechanism regarding the impact of TMB on the efficacy of PD-1/PD-L1 blockade should be investigated in the near future. The TCGA reported that the amplification of the *CD274* gene (which encodes PD-L1) and the *PDCD1LG2* gene (which encodes PD-L2) was frequently observed in EBV-positive GC.[9] Indeed, AGC patients with EBV-positive status reported derived higher benefit from pembrolizumab.[108] Notably, Panda *et al.* reported that a patient with EBV-positive AGC exhibited durable response from treatment with the anti-PD-L1 antibody avelumab, although this tumour had low mutation

burden.[109] A study recently reported that the ORR of nivolumab for AGC after two or more CTx regimens was significantly higher in patients with MMR-D than in those with MMR-P (75% *versus* 13%), PD-L1+ in tumour cells than in those with PD-L1- in tumour cells (57% *versus* 13%) and PIK3CA mutation in those with PIK3CA wild-type (44% *versus* 14%).[110] Remarkably, the ORR was 31% in patients with, at least, one of the following factors: MMR-D, high-TMB, EBV-positive and PD-L1+ in tumour cells *versus* 0% in those without these factors, suggesting that prescreening of these biomarkers could be useful in predicting the clinical benefit of the anti-PD-1/PD-L1 blockade in AGC. Moreover, the diversity and composition of gut microbiome reportedly predicts the effect of PD-1 blockade in patients with AGC.[111] A recent subgroup analysis of ATTRACTION-2 indicated that patients with hyponatraemia along with high neutrophil-to-lymphocyte ratio might exhibit low benefit with nivolumab in terms of early progression and death.[112]

Controversy in hyperprogressive disease

Recently, anti-PD-1/PD-L1 antibodies have anecdotally been reported to cause rapid progression of some cancer types, which is called hyperprogressive disease (HPD),[113–116] although its exact incidence in GC remains unclear. As HPD, perhaps, correlates with poor prognoses, it is imperative to identify predictive factors of HPD. Kato *et al*. identified EGFR mutations and MDM2 amplification as possible molecular predictors of HPD in patients with several solid tumours.[115] Reportedly, FBXW7 mutation or KRAS amplification could be related to HPD in patients with AGC who received nivolumab.[117] Recently, Togashi *et al*. reported an increase in Tregs with proliferative capacity among tumour-infiltrating lymphocytes in AGC patients who exhibited HPD after treatment with an anti-PD-1 antibody.[118] Moreover, an *in vitro* study reported that PD-1 blockade activated not only effector T cells but also Tregs, which promoted tumour progression in a fraction of patients.[118] Further investigations in larger cohorts are warranted to validate HPD-associated biomarkers. Recently, Lo Russo *et al*. illustrated the role of innate immunity in mediating hyperprogression via Fc/FcR triggering on macrophages by anti-PD-1 antibody.[119] A previous *in vivo* study reported that selective inhibition of the VEGF pathway with an anti-VEGF antibody or anti-VEGF TKIs effectively controlled tumour growth and inhibited the infiltration of suppressive immune cells such as Tregs and tumour-associated macrophages, myeloid-derived suppressor cells, while increasing the mature dendritic cell fraction.[120] In addition, heat shock protein 90 (HSP90) inhibitor reportedly enhance antitumour immunity by decreasing Tregs *in vitro* and *in vivo*. Based on these preclinical rational, clinical trials of anti-PD1 antibodies in combination with multiple VEGF TKIs or HSP90 inhibitor are under way, which might not only enhance antitumour activity but also reduce HPD.

Conclusions

Remarkable advances in elucidating molecular profiles of GC have facilitated the development of novel agents such as RTKs inhibitors, antibody–drug conjugates and IMAB362 (anti-Claudin 18.2). In addition, developing appropriate biomarkers for patient selection in early clinical trials could lead to successful results of pivotal clinical trials of new drugs. Considering that the apparent efficacy of PD-1 blockade might be limited to a relatively small subset of AGC patients and some patients exhibit HPD, better biomarkers of immune checkpoint inhibitors or combination therapy should be established in the near future.

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Conflict of interest statement

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