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Current status and future potential of predictive biomarkers for immune checkpoint inhibitors in gastric cancer

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ABSTRACT

Immunotherapy is revolutionising cancer treatment and has already emerged as standard treatment for patients with recurrent or metastatic gastric cancer (GC). Recent research has been focused on identifying robust predictive biomarkers for GC treated with immune checkpoint inhibitors (ICIs). The expression of programmed cell death protein-ligand-1 (PD-L1) is considered a manifestation of immune response evasion, and several studies have already reported the potential of PD-L1 expression as a predictive parameter for various human malignancies. Meanwhile, based on comprehensive molecular characterisation of GC, testing for Epstein-Barr virus and microsatellite instability is a potential predictive biomarker. Culminating evidence suggests that novel biomarkers, such as the tumour mutational burden and gene expression signature, could indicate the success of treatment with ICIs. However, the exact roles of these biomarkers in GC treated with ICIs remain unclear. Therefore, this study reviews recent scientific data on current and emerging biomarkers for ICIs in GC, which have potential to improve treatment outcomes.

INTRODUCTION

The prognosis for metastatic or recurrent gastric cancer (GC) remains very poor, making it the third leading cause of cancer-related death worldwide.[1] However, the recent development of immune checkpoint inhibitors (ICIs) targeting the programmed cell death protein-1 (PD-1) and programmed cell death protein-ligand-1 (PD-L1) pathways has produced improved outcomes for GC and already been successfully incorporated into clinical practice.[2] In particular, checkpoint inhibition with anti-PD1 monoclonal antibodies, such as pembrolizumab and nivolumab, has led to durable and significant responses in a minority of GCs.[3–5] As a result, interest has increased in selecting the right patient population to benefit such ICIs, along with further exploration of immunotherapy. Notably, various tumour-related and host-related factors with a critical impact on systemic immune functions may influence the response to ICIs.[6] Moreover, a significant proportion of patients do not respond to these therapies, and there can

also be a threat of unpredictable immune-related adverse events (AEs) and even severe toxicity.[7 8] Therefore, identifying more robust predictive biomarkers is critical to optimise treatment with ICIs, while avoiding unnecessary treatment of patients who could develop life-threatening or life-altering AEs.

GC is a heterogeneous and complex disease.[9] Thus, various approaches, such as molecular classification, have already been proposed for the subhistological exploration of GC as a potential tool for more effective therapeutic strategies. The Cancer Genome Atlas Research Network (TCGA) suggested a comprehensive molecular characterisation of 295 GCs using various platforms, and proposed four distinct subtypes: Epstein-Barr virus (EBV)-positive, microsatellite unstable (microsatellite instability (MSI)), genomically stable and tumours with chromosomal instability.[10] More recently, the Asian Cancer Research Group (ACRG) described four subtypes: MSI, microsatellite stable (MSS)/ epithelial-to-mesenchymal transition, MSS/ TP53-positive and MSS/TP53-negative.[11] While the above subtypings show some overlapping molecular aberrations, MSI was identified as a subtype by both TCGA and ACRG.[29] MSI is a molecular marker of a defective function of the DNA mismatch repair (MMR) system that recognises and repairs the erroneous insertion, deletion and misincorporation of bases that can arise during DNA replication and recombination, as well as repairing some forms of DNA damage.[12] It is also well known that MSI-high (MSI-H) tumours exhibit a high tumour mutational burden (TMB), neoantigen load and immune infiltration, making them respond well to ICIs.[13] Meanwhile, the distinct characteristics of EBV-positive GC is the overexpression of PD-L1 and PD-L2.[14] The driving features of PD-L1 positivity in EBV-positive GC can also be effectively targeted with immunotherapy, similar to the MSI-H subtype. Interestingly, in a recent phase II trial by Kim *et al*,

pembrolizumab showed a promising efficacy in patients with MSI-H and EBV-positive tumours.[15] Accordingly, based on a better molecular characterisation of GC, this review focuses on the current and emerging biomarkers for ICIs that would facilitate precision medicine.

Clinical evidences of ICIs in GC

Several phase II and III trials have recently investigated the PD-1 and PD-L1 blockade in GC, as summarised in table 1.[4 5 16–19] Pembrolizumab is an IgG4 human antibody targeting PD-1, thereby interfering with the interaction between PD-1 and PD-L1.[20] In the phase Ib KEYNOTE-012 trial, 39 patients with PD-L1-positive GC or gastro-oesophageal junction (GEJ) cancer were enrolled in both Asian and non-Asian countries.[21] The objective response rate (ORR) was 22% and durable responses were seen with a median duration of responses of 40 weeks. The subsequent phase II multicohort KEYNOTE-059 trial (cohort 1) enrolled 259 patients with recurrent or metastatic GC and GEJ cancer who received two pretreated lines of chemotherapy.[4] Here, the ORR was 11.6% and median overall survival (OS) was 5.6 months. Following these two trials, the randomised phase III KEYNOTE-061 trial compared pembrolizumb monotherapy with paclitaxel in patients with PD-L1-positive GC that had progressed on first-line flouropyrimidine and platinum doublet chemotherapy.[17] While patients with a PD-L1 status were initially unselected, PD-L1-positive patients with a combined positive score (CPS) of 1 or higher were included in the latter part of the study. The CPS is the number of PD-L1 staining cells, including tumour cells, lymphocytes and macrophages, divided by the total number of viable tumour cells, while a tumour proportion score (TPS) is the percentage of viable tumour cells showing partial or complete membrane staining, relative to all viable tumour cells present in the sample.[22] Approximately 67% of patients were found to have PD-L1-positive tumours using the CPS. Pembrolizumab did not meet its primary end point of a longer OS and progression-free survival (PFS) in patients with PD-L1-positive tumours. Notwithstanding, it is worth noting that patients who expressed PD-L1 CPS of 10 or higher exhibited a better benefit from treatment with pembrolizumab in post hoc analyses, although these subgroup analyses should be interpreted with caution.

Subsequently, the KEYNOTE-062 was a large randomised first-line clinical trial of 763 patients with advanced GC or GEJ cancer who were randomly assigned to one of three arms: pembrolizumab at 200 mg every 3 weeks for up to 2 years, pembrolizumab plus chemotherapy (cisplatin and 5-fluorouracil or capecitabine) or placebo plus chemotherapy.[23] Pembrolizumab was non-inferior to chemotherapy for OS in patients with CPS 1 or higher. No survival benefit was observed with the addition of pembrolizumab to chemotherapy compared with chemotherapy alone in this study.

Nivolumab, also an IgG4 antibody, is very similar in structure to pembrolizumab, except that nivolumab binds to the N-terminal loop on the PD-1 molecule, while pembrolizumab binds to the C'D loop.[24] The phase III ATTRACTION-2 (ONO-4538-12) trial compared nivolumab with a placebo in 493 Asian patients with unresectable or recurrent GC that was refractory to or intolerant of at least two previous standard chemotherapy regimens.[5] The results showed a significantly prolonged OS for the nivolumab group with a 1-year OS rate of 27.3% vs 11.6%. More recently, the long-term survival was reported showing 2-year survival rates of 10.6% for nivolumab and 3.2% for placebo.[25] Nivolumab also showed a significant advantage compared with the placebo in terms of PFS and the radiological objective response. Therefore, these results provide randomised evidence that nivolumab is a valid approach to improving the clinical outcomes for patients with GC in a third-line and subsequent-line setting. However, the overall clinical value of ATTRACTION-02 was partially limited by several important issues.[26 27] The patient population was only Asian and PD-L1 positivity, reported at a low frequency of 14% as this was assessed as TPS rather than CPS, was not associated with the survival outcomes. Plus, there was no comparative data on quality of life. A phase I/II CHECKMATE-032 study also reported that nivolumab and nivolumab plus ipilimumab provide a durable antitumour activity in heavily pretreated Western patients with chemotherapy-refractory GC, GEJ cancer and oesophageal cancer.[19] In particular, the clinical benefit of nivolumab monotherapy was consistent with that reported for Asian patients in the ATTRACTION-02 study. Yet, similar to the ATTRACTION-02 study, there was no association of PD-L1 positivity according to TPS with survival outcomes. Therefore, ongoing randomised controlled trials of ICIs, including pembrolizumab and nivolumab in earlier line treatment need to unify assessment of PD-L1 expression and create more accurate profiles of AEs in GC.

Avelumab is an IgG1 antibody which binds to the front beta-sheet of PD-L1 and possesses PD-1/PD-L1 blockade activity with antibody-dependent cell-mediated cytotoxicity (ADCC).[28] There are some differences between PD-1 inhibition and PD-L1 inhibition, as PD-1 targeting therapeutic antibodies including pembrolizumab and nivolumab block the PD-1/PD-L1 or PD-1/PD-L2 interaction to restore tumor-specific T-cell reactivity without mediating ADCC.[24] The JAVELIN Gastric 300 trial, the first study to compare avelumab with standard chemotherapy in third-line treatment for GC, did not achieve its primary end point of improving OS or the secondary end points of PFS and ORR.[18] This negative finding may be attributed to the usage of the active comparator in the control arm. In addition, there are possible reasons, including the different drug biding sites, heterogeneity of tumour biology and methodology of PD-L1 testing. Notwithstanding, fewer patients experienced AEs with avelumab than with chemotherapy, although researchers found no evidence of clinical benefit compared with commonly used chemotherapy in any of the examined subgroups, including the tumour PD-L1 expression status.

Table 1 Selected phase II and III studies evaluating immune checkpoint inhibitors in gastric cancer

Agents	Target	Study name	Phase	Setting	PD-L1 status	Treatment arms	Patient number	Geographic region	Primary end points	RR (%)	PFS	OS	References
Pembrolizumab	PD-1	KEYNOTE-059 (cohort 1)	II	Third-line or later	Unselected	Pembrolizumab	259*	Global	RR	11.6	2	5.6	4
Pembrolizumab	PD-1	KEYNOTE-061	III	Second-line	Positive	Pembrolizumb	196	Global	PFS, OS	16	1.5	9.1	17
						Paclitaxel	199			14	4.1	8.3	
Nivolumab	PD-1	ATTRACTION-02 (ONO-4538-12)	III	Third-line or later	Unselected	Nivolumab	330	Asian	OS	11.2	1.6	7.5	5
						Placebo	163			0	1.5	5.1	
Nivolumab	PD-1	ATTRACTION-04 (part 1)	II/III	First-line	Unselected	Nivolumab/SOX or CAPOX	40	Asian	RR	65.8	9.5	Not reached	16
Nivolumab	PD-1	CHECKMATE-032	I/II	Third-line or later	Unselected	Nivolumab	59	Western	RR	12	1.4	6.2	19
						Nivolumab1/Ipilimumab3†	49			24	1.6	6.9	
						Nivolumab3/Ipilimumab1†	52			8	1.6	4.8	
Avelumab	PD-L1	JAVELIN Gastric 300	III	Third-line	Unselected	Avelumab	272	Global	OS	2.2	1.4	4.6	18
						Physician's choice‡	133			4.3	2.7	5	

*Among 259 patients, 148 (57.1%) were PD-L1 positive.
†Nivolumab 1 mg/kg plus ipilimumab 3 mg/kg (NIVO1+IPI3) every 3 weeks, nivolumab 3 mg/kg plus ipilimumab 1 mg/kg (NIVO3+IPI1) every 3 weeks.
‡Paclitaxel 80 mg/m² on days 1, 8 and 15 or irinotecan 150 mg/m² on days 1 and 15, each of a 4-week treatment cycle.
CAPOX, capeciabine and oxaliplatin; OS, overall survival; PD-1, programmed cell death protein-1; PD-L1, programmed cell death protein-ligand 1; PFS, progression-free survival; RR, response rate; SOX, S-1 and oxaliplatin.

These results might support the potential of avelumab for combination or maintenance therapy. Notwithstanding, the recent JAVELIN Gastric 100 trial with maintenance avelumab therapy following initial chemotherapy in GC produced disappointing results.[29] Although there was more efficacy seen with avelumab in an exploratory analysis of 137 PD-L1-positive patients with a CPS ≥1 (14.9 vs 11.6 months), no significant difference with respect to OS was observed between the avelumab maintenance and continued chemotherapy groups (10.4 vs 10.9 months, p=0.177).

As discussed above, several clinical trials are currently ongoing with various strategies.[30 31] The phase III ATTRACTION-04 (NCT02746796) trial is evaluating nivolumab plus standard chemotherapy (oxaliplatin plus either S-1 or capecitabine) versus chemotherapy alone in Asian patients.[16] The phase II component (part 1) of this study showed chemotherapy plus nivolumab led to an ORR of 65.8% and median PFS of 9.5 months, while the subsequent phase III trial (part 2) is evaluating the survival outcomes. Another phase III trial, CHECKMATE-649 (NCT02872116), has completed recruitment with over 2000 patients randomising patients to oxaliplatin-based chemotherapy alone or in combination with nivolumab or nivolumab plus ipilimumab.[32] The third arm of this study terminated recruitment early due to early safety signal.

Antiangiogenic treatment, such as ramucirumab and bevacizumab, is also generating recent interest. As several studies have suggested that simultaneously blocking angiogenesis and PD-1 pathways induces synergistic antitumour effects, especially involving the control of the tumour microenvironment (TME).[33 34] Researchers including the current authors noted that ramucirumab in combination with pembrolizumab (JVDF) showed a manageable safety profile with favourable antitumour activity in patients with previously treated GC.[35] Consequently, the phase I/II NivoRam (NCT02999295) trial has evaluated the safety and tolerability of the addition of ramucirumab to nivolumab in patients with GC as second-line therapy.[7] As an alternative antiangiogenic and multi-targeted kinase inhibitor, a phase I trial of regorafenib plus nivolumab is also exploring in GC (NCT03406871).[36] This trial demonstrated an ORR of 44% in GC and ICIs-pretreated patients with GC achieved a partial response (PR) in 11/25 patients. Interestingly, of the seven patients who had received prior anti-PD-1/PD-L1 treatment, three achieved an objective response.

Another interesting phase II trial investigating the role of pembrolizumab plus trastuzumab combined with chemotherapy in patients with previously untreated human epidermal growth factor receptor 2-positive tumours is currently ongoing (NCT02954536).[37] Preliminary results from this study showed 48% of patients experienced reduction in target lesions after one dose of pembrolizumab/trastuzumab before oxaliplatin/capecitabine was introduced in second cycle. The high ORR of 89% was coupled with median PFS of 13 months. This has led to the current phase III KEYNOTE-811 study randomising patients to chemotherapy plus trastuzumab with or without pembrolizumb (NCT03615326).[38] Thus, these therapeutic strategies including combination treatment represent a true opportunity in the contemporary treatment of GC and may produce further success, when considering integrative genomic data.

Biomarkers for ICIs in GC

The identification and validation of reliable biomarkers are important to facilitate precise patient selection and increase the clinical benefit from ICIs. An overview of the predictive roles of biomarkers obtained from tissue or blood and their characterisation in the management of GC is briefly summarised in table 2.

PD-L1 expression

Testing for PD-L1 expression by IHC is the current standard in most solid tumours and several studies have already assessed the clinical outcomes according to the PD-L1 expression status in GC. Nevertheless, different antibodies are being used for IHC to assess with different performance and different scoring criteria for PD-L1 expression. A recent multicentre study (Blueprint PD-L1 IHC Comparison Project) attempted to compare the performance of each IHC PD-L1 assay in lung cancer.[39] Three assays including 22C3 for pembrolizumab, 28-8 for nivolumab and SP263 for durvalumab were found to be comparable to each other in the staining of tumour tissue, whereas SP142 for atezolizumab was found to be less sensitive. However, further study is required to carefully validate these assays in GC.

As noted above, in the KEYNOTE-059 trial, PD-L1 expression was assessed using the PDL1 IHC 22C3 pharmDx assay and measured using a CPS.[4] This trial demonstrated a higher ORR (15.5% vs 6.4%) in patients with high PD-L1 expression, defined as CPS ≥1. Both PFS and OS were also more prolonged in this group.[40] Although pembrolizumab in a second-line trial (KEYNOTE-061) did not significantly prolong OS, greater benefits were seen in tumours with higher PD-L1 expression (CPS ≥10, ORR=25%; CPS ≥1, ORR=16%; CPS <1, ORR=2%).[17] However, the ATTRACTION-02 and JAVELIN Gastric 300 trials showed no clinical improvement for PD-L1-positive tumours as they used TPS rather than CPS.[18 27] These differences may also have been due to the use of different cut-off points and scoring systems, a lack of standardisation of the assays and testing platforms, the heterogeneous nature of PD-L1 expression in tumours, intratumoural/intertumoural heterogeneity and intraobserver/interobserver variability.[41–43] For instance, the ATTRACTION-02 study retrospectively evaluated PD-L1 expression using a PD-L1 IHC 28-8 pharmDx assay, defined as the TPS, while PD-L1 expression was prospectively assessed in tumour cells, tumour-associated lymphocytes and macrophages using a 22C3 pharmDx assay in KEYNOTE-061.[17 27] As such, PD-L1 positivity was only reported in about 15% patients using TPS with nivolumab, whereas it is generally

Table 2 Overview of candidate biomarkers associating with response to immune checkpoint inhibitors in gastric cancer

Biomarkers	Sample source	Methods	Treatment	Recent results in gastric cancer	References
PD-L1	Tumour	IHC	Pembrolizumab	Expression of PD-L1 ≥1 associated with better clinical efficacy (ORR, mRD)	4
			Pembrolizumab	Expression of PD-L1 CPS of 10 or higher associated with better clinical efficacy (OS, ORR)	17
			Pembrolizumab	Expression of PD-L1 associated with higher response rate	15
EBV positivity	Tumour	In situ hybridisation	Pembrolizumab	EBV positivity associated with higher response rate	15
MSI-H	Tumour	MSI testing or IHC	Nivolumb	MSI-H associated with clinical efficacy (ORR, DCR, OS)	19
			Pembrolizumab	MSI-H associated with better clinical efficacy (ORR)	4
			Pembrolizumab	MSI-H associated with better clinical efficacy (ORR, OS)	17
			Pembrolizumab	MSI-H associated with better clinical efficacy (ORR)	15
TMB	Tumour or blood	WES or targeted sequencing	Toripalimab	TMB associated with better clinical efficacy (ORR, OS)	50
TILs	Tumour	Image analysing software or manually counted	ICIs	Presence of TILs associated with better clinical efficacy in various solid tumours, but very limited data in GC	78
GEP	Tumour	Multigene profiling	Pembrolizumab	IFN-gamma (6-gene) signature associated with better clinical efficacy (ORR, PFS)	21
			Pembrolizumab	T-cell inflamed (18-gene) signature associated with better clinical efficacy (ORR, PFS)	4
Gut microbiota	Stool	Culture or molecular technique (sequencing/metagenomics)	ICIs	Various species associated with enhancement and IRAEs of ICIs in various solid tumours	94
NLR	Blood	Complete blood count	ICIs	Increased NLR correlated with DCR and OS*	110
			Nivolumab	Decreased change of NLR associated with better survival	111

*Sixty-seven patients had tumours from the stomach.
CPS, combined positive score; DCR, disease control rate; EBV, Epstein-Barr virus; GC, gastric cancer; GEP, gene expression profiling; ICIs, immune checkpoint inhibitors; IFN, interferon; IHC, immunohistochemistry; IRAEs, immune-related adverse events; mRD, median response duration; MSI-H, microsatellite instability; NLR, neutrophil-to-lymphocyte ratio; ORR, overall response rate; OS, overall survival; PD-L1, programmed cell death protein-ligand 1; PFS, progression-free survival; TILs, tumour-infiltrating lymphocytes; TMB, tumour mutational burden.

between 60% and 70% using CPS ≥1 as the cut-off. More recently, using the CHECKMATE-032 study data, tumours were re-scored using CPS and there was better correlation between nivolumab treatment and survival even at the CPS ≥5 level.[44] Thus, one of the coprimary patient population in the first-line CHECKMATE-649 study, that has recently completed recruitment, is including a subpopulation of patients with PD-L1 CPS ≥5.[32]

EBV positivity
EBV status is also emerging as a potential biomarker for personalised treatment strategies in GC.[14] In situ hybridisation detection of EBV-encoded small RNA in tumour cells is generally recommended to identify EBV-associated GC (EBVaGC).[30] The incidence of EBVaGC varies from 1% to 30% in different regions with an average of 10% worldwide.[45] Nevertheless, this subgroup is associated with better prognosis, thus less frequently found in advanced or metastatic setting. In particular, EBV-positive tumours frequently display PD-L1/2 overexpression, and occasional immune cell signalling activation.[10 14] Several research groups found that the level of PD-L1 expression ranging from approximately 34% to 92% of EBVaGC with

variable results between studies.[20] PD-L1 positivity has also been significantly associated with a poorer prognosis than PD-L1 negativity in EBVaGC. Furthermore, EBV triggers a significantly higher infiltration of CD8+ T cells in TME.[46] In previous studies of 120 patients with EBV-positive cancer, the current authors showed that high levels of tumour-infiltrating lymphocytes (TILs) were associated with a favourable prognosis, while intratumoural PD-L1 positivity with a worse prognosis.[47]

In the phase I JAVELIN Solid Tumour trial where avelumab was shown to be beneficial for a patient with metastatic GC, it is worth noting that EBV-positive tumours with a low mutation burden and MSI tumours with a high mutation burden had statistically significantly higher tumour lymphocytic infiltration when compared with MSS tumours.[48] The strength of immune-mediated signalling signatures in EBV-positive tumours also represents a T-cell-inflamed TME.[10 49] These findings support the concept that ICIs can be used in patients with GC with EBV by suppressing the PD-1 pathway in tumour cells and allowing immune activation. A recent phase II trial by Kim et al demonstrated improved efficacy associated with pembrolizumab in patients with EBV-positive tumours.[15] This study enrolled 61 patients with pretreated GC. In a subgroup analysis, pembrolizumab monotherapy as salvage treatment showed that all six EBV-positive patients with GC attained PR (ORR=100%) with a median duration of 8.5 months. However, in another study, 4 out of 55 patients considered EBV-positive were treated with toripalimab.[50] Only one PR (25%) was observed with two stable and one progressive diseases. Patients with PR was also PD-L1-positive. These contrasting results with pembrolizumab could be due to toripalimab rather than EBVaGC as a predictive biomarker for ICI.

Microsatellite instability-high

MMR deficiency is generally characterised by a failure to repair DNA replication-associated errors, leading to the accumulation of mutations in microsatellite regions of the genome.[51] These phenomena are known as MSI.[52] Currently, two different methods have been validated for detecting MSI-H.[53] The MMR status is assessed by IHC staining to measure the expression levels of the proteins involved in DNA MMR, and a polymerase chain reaction-based exam also tests the length of repetitive DNA that are known as microsatellite in the normal and tumour tissues. While there are discrepancies between the IHC of MMR protein expression and MSI test results, the overall concordance in the two tests is high.[52] The incidence of MSI in GC varies between countries, being relatively high in approximately 5%–30% of Western patients.[51] MSI-H GC is commonly associated with intestinal type, female sex, older age, lack of lymph node metastases and onset in the distal stomach.[54] To date, multiple retrospective studies and limited prospective studies have reported on a positive association between MSI-H and a better prognosis in resectable GC.[55] For example, the MAGIC study reported that patients with MSI-H tumours have

superior survival compared with patients with MSS/MSI-low (MSI-L) tumours when treated with surgery alone and conversely have inferior survival to patients with MSS/MSI-L tumours when treated with perioperative chemotherapy plus surgery.[56] However, similar to EBV status, patients with MSI-H had better prognosis, thus only 4%–5% of patients with metastatic GC would be MMR-deficient. The prognostic and predictive values of the MSI status on the survival of patients with metastatic GC remain a subject of debate.[52]

Theoretically, in the presence of MMR deficiency, undetected DNA replication errors, leading to a tumour with a high mutational burden, reproduce various neoantigens that stimulate T-cell activation and tumour infiltration by immune cells. KEYNOTE-012 trial reported that MSI-H tumours showed a partial response in two out of four patients, regardless of PD-L1 expression.[21] A subgroup analysis of KEYNOTE-059 revealed an ORR of 57.1% for patients with MSI-H GC.[4] In KEYNOTE-061 and more recently reported KEYNOTE-062, there was a substantially enhanced survival benefit in patients treated with pembrolizumab compared with chemotherapy.[17 23] Similar to the results for EBV-positive tumours, the clinical study by Kim et al also showed that MSI-H tumours responded particularly well to pembrolizumab monotherapy (ORR=85.7%).[15] In the CHECKMATE-032 trial that assessed the efficacy of another PD-1 monoclonal antibody nivolumab, the ORR was 29% for the MSI-H group vs 11% for the MSI-L group or MSS group.[19] Therefore, this evidence highlights the potential of MSI-H as a predictor of the response to ICIs in GC.

Of note, whereas MSI-H/MMR deficiency is the most consistent predictor of efficacy to ICIs in GC, a substantial portion of MSI-H GC still has unsatisfactory outcomes even with ICIs. The degree of MSI and resultant mutation load, in part, might explain the variable response to PD-1 blockade in MMR-deficient tumours.[57] Tumours sensitive to PD-1 antibodies showed a loss or a reduction in tumour allele frequency of missense (non-synonymous single-nucleotide variant) and indel mutations after PD-1 treatment suggestive of immune editing of tumour cells

TMB and neoantigen

TMB may be a potential biomarker of outcomes with ICIs in multiple solid tumour types.[41] Generally, cells have a number of repair pathways to maintain their genome stability.[13] The mutational load acquired by defective DNA repair pathways frequently alters protein function and expression, resulting in the formation of neoantigens that serve a source of antitumour immune response. Therefore, it is reasonable to hypothesise that tumours with a high mutational load are more likely to produce neoantigens and increase immunogenicity.[58] In turn, this course of reaction induces a more intensified immune response, resulting in tumours becoming more sensitive to treatment with ICIs.[41] Although tumour-specific neoantigens with high clonality are more predictable and beneficial for the response to ICIs, accurate measurement

of these neoantigens is known to be expensive and time-consuming.[59] In this situation, TMB could be a good approach for indirectly evaluating the neoantigen load. TMB is defined by the total number of somatic non-synonymous mutations per coding area of the tumour DNA.[58] Several studies have already demonstrated the predictive impact of TMB in lung cancer and melanoma. One early study by Yarchoan et al observed a significant correlation between TMB and ORR for anti-PD-1 or anti-PD-L1 therapy.[60] Rizvi et al also reported that patients with TMB >50th percentile exhibited an improved durable clinical benefit rate and PFS versus those with lower TMB.[61] This benefit was also seen in the CHECKMATE-227 study that included 299 patients with advanced non-small-cell lung cancer (NSCLC) who received a combination of nivolumab and ipilimumab as the first-line metastatic setting.[62] A significantly prolonged PFS was reported for the patients with higher TMB treated with the combination treatment, irrespective of the expression of PD-L1. Likewise, a large-scale study across multiple cancer types found a significant association between TMB and the clinical outcome.[63–67] These findings can also provide a novel strategy for subgroups with high TMB, considering that the measurement of the mutational load is a critical factor for therapeutic success.

However, for patients with GC, there is still insufficient evaluation and conflicting results on the utility of TMB as a biomarker of the response to ICIs.[65] Interestingly, Wang et al performed a TMB analysis of 54 patients with chemo-refractory GC who were treated with toripalimab as a monotherapy in a prospective phase Ib/II clinical trial.[50] In this study, TMB-high (TMB-H) patients responded significantly better than the TMB-low patients (ORR 33.3% vs 7.1%, p=0.017). A survival benefit has also been demonstrated for patients with high TMB (OS 14.6 vs 4.0 months, p=0.038). Similar correlation was also found between TMB-H according to circulating tumour DNA and better survival when treated with pembrolizumab in GC.[15] In light of recent approaches, this close relationship between TMB and clinical outcomes also points to the possibility of TMB as a predictive biomarker in patients with GC. However, in the study with regorafenib plus nivolumab, due to small sample size, TMB did not correlate with response or PFS to this combination.[36]

Despite its identified significant predictive role, there are still many challenges in precisely estimating TMB. First, it is difficult to apply the protocol, including whole-exome sequencing or targeted sequencing panels using next-generation sequencing, to clinical practice due to various problems, such as the sample amount, cost, sensitivity, coverage and analysis time.[8 30] Second, a standardised cut-off value for TMB has not yet been clearly established, since many studies have reported a wide range of cutoffs for different tumour types.[58] Thus, given the variety of TMB cutoffs, assays related with TMB in clinical studies should be interpreted cautiously. Moreover, the availability of tumour sampling to detect TMB is commonly limited and TMB may present temporal variability. A novel blood-based TMB approach could be considered as an alternative method, as the advantages of repeating sampling during treatment could provide information of a dynamic immune reaction.[8] Plus, this approach is less invasive and enables investigators to document the evolution of TMB. Interestingly, in a study by Gandara et al, blood-based TMB was correlated with tissue-based TMB and showed a longer PFS in patients with metastatic NSCLC who received treatment with atezolizumab.[68] In summary, TMB could be a novel and independent biomarker that reflects the therapeutic effects of ICIs in GC. However, its accuracy in predicting the efficacy of ICIs varies among studies and still needs to be explored for GC.

Tumour infiltrating lymphocytes

The immune microenvironment of tumours is now recognised as an important determinant for understanding the relationship between a patient's immune system and their cancer, informing prognosis and guiding immunotherapy like ICIs.[69] Tumour cells are typically surrounded by infiltrating inflammatory cells, such as cytotoxic T cells, helper T cell subsets, regulatory T cells, tumor-associated macrophages, dendritic cells and myeloid lineage leucocytes.[70] Among these, differentiated lymphocytes, referred to as TILs, are considered a manifestation of the host immune response against tumour cells and seem to play an important role in various human malignancies.[71] Several studies have already reported the potential of TILs as a predictive parameter.[72] A recent in vitro study proposed that subpopulation of a CD8+ T cells is involved in mediating tumour control and responds to checkpoint blockades.[73] In breast cancer, TILs have been shown to predict patient outcomes and responses to ICIs.[74] Plus, the presence of stromal TILs has been associated with an improved ORR in patients with triple-negative breast cancer receiving pembrolizumab.[75] TILs have also been investigated as a predictive biomarker for breast cancer and could predict the efficacy of atezolizumab.[76] Recent research by Loupakis et al demonstrated a significant correlation between a high number of TILs and clinical responses and survival benefit in a large data set of patients with MSI-H metastatic colorectal cancer treated with ICIs.[77] These clinical benefits were also consistent with another meta-analysis, indicating that TILs are associated with improved prognosis predictions for OS.[78]

Despite such evidence that TILs contribute to determining prognosis, the exact predictive value of TILs in GC treated with ICIs remains unclear.[72] In particular, the detection of TILs could be a key biomarker for the treatment of TIL-rich tumours, such as EBV-positive or MSI-H, considering that ICIs could become an important part of the cancer armamentarium in these GC subsets.[71] However, doubts remain on the methodology of interpreting TILs and the cut-off values for TILs in GC. In contrast to breast cancer, there is no current consensus on estimating TILs in GC specifically.[70] Plus, it is essential to elucidate the precise predictive role of each lymphocyte

subset including, regulatory T cells, which could play a role in immunosuppression and tumour progression.[79] Moreover, the invasive margin or central infiltration could have a different density of T cells, leading to variable results. In malignant melanomas, the density of CD8+ T cells at the tumour edge has been shown to predict the response to pembrolizumab rather than the density at the tumour centre.[80] Galon *et al* recently developed an immunoscore based on the density, location, phenotype and functionality of T cells in colorectal cancer and found these immune infiltrates to be a better predictor of survival than TNM classification.[81] Thus, the application of this approach in clinical practice would seem to be quite feasible for determining the response to ICIs for GC. Therefore, further innovative attempts regarding TILs could assist in discovering an effective biomarker for predicting efficacy of ICIs in GC.

Gene expression signatures

Several studies on gene expression profiling (GEP) are currently attempting to predict the response to ICIs in various types of cancers.[82] In particular, immune gene signatures, such as IFN-gamma signalling and activated T cells, could have potential as predictive markers of ICI responses.[41] Recent GEP revealed that an IFN-gamma-related gene profile obtained from baseline tumour tissue was predictive of the ORR and PFS in patients with melanomas treated with pembrolizumab.[83] Auslander *et al* also reported that a novel immune-predictive score (IMPRES) was significantly correlated with a better response to ICIs, suggesting that GEP could be incorporated in enhancing therapy response.[84] Similarly, additional studies have demonstrated a link between GEP and ICI responses in lung cancer.[85 86] As described above, the KEYNOTE-012 trial for GC investigated the use of a six-gene IFN-gamma signature that was previously identified to predict the response in melanomas. Although this gene signature did not meet significance due to the small number of enrolled patients, a trend was seen associating the responders and the IFN-gamma signature.[21] For further exploration of the association between this gene signature and patient outcomes, the KEYNOTE-059 trial analysed the association of the T-cell inflamed 18-gene signature with response and survival in GC.[4] Higher T-cell inflamed score was associated with an improved likelihood of response to pembrolizumab and improved prognosis for GC, providing a strong rationale for clinical trials using GEP in patients with GC receiving ICIs.

GEP signatures have also been evaluated to correlated response with nivolumab in the CHECKMATE-032 study showing a potential predictive role for response with a 4-gene inflammatory signature incorporating CD274 (PD-L1), CD8A, lymphocyte activating 3 and signal transducer and activator of transcription 1.[19 44] Consequently, it is increasingly evident that GEP is a promising option for selecting patients with GC who could benefit from ICIs. Thus, further well-designed and randomised studies

of large numbers of cases are needed to evaluate the role of GEP as a potential biomarker for ICIs in GC.

Gut microbiota

The relationship between microbiota and clinical responses to ICIs in GC is another ongoing area of research, with several studies exploring how microbiota may affect the therapeutic efficacy of immunotherapy.[6] Gut microbiota play a fundamental role in the maintenance of host physiology and immune homeostasis, interacting with epithelial cells and stromal cells to modulate multiple vital functions.[87] They can also regulate barrier function, pathogen control and cell metabolism. In addition, *Helicobacter pylori* infection can contribute to the establishment of a persistent infection through the creation of an immunosuppressive microenvironment.[88] Chronic *H. pylori* infection results in gastric carcinogenesis and T-cell hyporesponsiveness and induced PD-L1 expression.[89] Interestingly, several studies reported that T cells exposed to *H. pylori* had an impaired ability to proliferate and *H. pylori*-positive tumours showed higher PD-L1 expression, leading to downregulate immune surveillance mechanisms.[90 91] For this reason, there is much speculation that gut microbiota could affect the therapeutic efficacy of immunotherapy, particularly ICIs, and there is already accumulating evidence support this in preclinical studies.[92]

Regarding the effects of ipilimumab, in a mouse model, the antitumour response was found to depend on the gut microbiota including *Bacteroides fragilis* or *Bacteroides thetaiotaomicron*.[93] It was also demonstrated that tumours in antibiotic-treated or germ-free mice did not respond to this ICI. Currently, several clinical studies have reported a link between gut microbiota and ICI responses across multiple human cohorts.[94] Although most studies only included fewer than 50 patients, the results from melanomas showed that the efficacy of anti-PD-1 therapy was influenced by gut microbiota.[95 96] In a recent study conducted by Pinato *et al* that included 196 patients, prior antibiotic therapy and the response to ICI therapy were associated with OS, independent of the tumour site, disease burden and performance status.[97] Another research group analysed the clinical predictors of outcome in 76 patients with GC and 85 patients with oesophageal cancer treated with ICIs.[98] There was no difference in outcomes between patients treated with antibiotics during or in the 2 months preceding ICI treatment versus those who were not. However, decreased OS was observed among those patients who received antibiotics in the 30 days prior to commencing ICIs. This phenomenon indicates that the use of antibiotics may adversely modify the gut microbiota, thereby impairing the antitumour immunity and response to ICIs. In addition to their modulating effects on ICIs, gut microbiota may also be involved in immune-related AEs.[99] Recent evidence found that an abundance of Bacteroidetes was correlated with a low frequency of ipilimumab-induced colitis.[100] Therefore, when taken together, these findings

suggest that gut microbiota may be relevant to the efficacy and toxicity of ICIs. However, the vast majority of studies have been retrospective in nature, with a limited ability to characterise the sample population. Thus, understanding the exact relationship between gut microbiota and the immune response remains limited.

Neutrophil-to-lymphocyte ratio

Besides the above-mentioned biologic and molecular biomarker, laboratory parameters reflecting the condition of systemic inflammation are relatively economical to evaluate, easily measurable, repeatable and ready to use in daily clinical practice.[101] There is increasing evidence that the neutrophil-to-lymphocyte ratio (NLR) can be an effective prognostic marker as well as predictive indicator related to ICIs for various solid tumours.[102 103] In fact, neutrophils are already known to be associated with detrimental outcomes in cancer, participating in different stages of the oncogenic process including tumour growth, invasion and metastases, while lymphocytes might affect a favourable impact on their tumour inhibiting properties.[104 105] Several large studies including melanoma, NSCLC and genitourinary cancer treated with ICIs found that a high NLR resulted in worse OS and PFS across various types of malignancies.[106–109] Recently, Li *et al* prospectively collected data from discovery and validation cohorts among 160 patients with non-colorectal gastrointestinal cancer receiving ICIs.[110] They found that the NLR level was significantly correlated with reduced OS, which is also consistent with other previous studies. In addition, Ota *et al* demonstrated that changes in the NLR values from those at 30 or 60 days after first-dose nivolumab were associated with significantly shorter PFS and OS in patients with GC.[111] Thus, for GC, these findings provide supporting evidence that the NLR may contribute to determining the predictive value of ICIs. Interestingly, some attempts have also been made to assess the correlation between the NLR and pseudoprogression/hyperprogression. A recent retrospective study of 25 patients with NSCLC treated with anti-PD-1/PD-L1 therapy reported that the pre-treatment and post-treatment NLRs were useful in distinguishing between pseudoprogression and true-progression.[112] More recently, another study examined a database of 263 patients with NSCLC and showed that immunophenotyping the peripheral blood CD8+ T lymphocytes was associated with hyperprogression and survival outcomes. Although the studies to date have been small and retrospective, the NLR may be useful for predicting therapeutic effects, especially as an early response marker.[113]

CONCLUSIONS AND FUTURE PERSPECTIVES

Immunotherapy has begun to revolutionise cancer treatment and already emerged as standard treatment for patients with recurrent or metastatic GC. Research has also been focused on finding robust predictive biomarkers for GC treated with ICIs. First, PD-L1 expression by IHC has been widely implemented as a predictive marker for ICIs to identify patients with GC who are more likely to benefit from the therapy. However, the relationship between PD-L1 expression and the therapeutic effect of nivolumab/avelumab still remains unclear, and pembrolizumab requires a clear PD-L1 threshold for effective prediction using a validated CPS score. To obtain convincing data, more precise and standardised methods are also needed to analyse PD-L1 expression. Meanwhile, in the era of molecular classification, accumulating evidence shows that EBV-positive and MSI-H tumours are the most immunogenic GC subtypes and ICIs have achieved an enhanced benefit in these GC subsets. Therefore, in the case of recurrent or metastatic GC, testing for EBV and MSI status should be considered, plus the related impact needs to be addressed in ongoing trials for earlier treatment settings. Third, novel biomarkers, such as TMB, TILs and GEP that exhibit a host cellular immune response against tumours, have also shown encouraging results in GC. However, the challenge remains to apply the data generated from validated clinical trials to clinical practice in order to provide precision immunotherapy. Plus, gut microbiota have been identified as another attractive biomarker for ICIs, with a recognised influence on host immunity and cancer. Clearly, a better understanding of the interaction between the microbial network and antitumour immunity will help to select patients who are more likely to respond to ICIs. Finally, NLR has also been investigated for their potential to be integrated as predictive markers of the response to ICIs, especially as this parameter is relatively more cost-effective and easier to measure. In summary, it is foreseeable that these emerging biomarkers will eventually shift the treatment paradigm of GC. Thus, to optimise this great opportunity, further high-quality evidence with standardised methods and proper patient selection are needed to discover reliable predictive biomarkers for ICIs in GC.

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