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ABOUT COVER

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Biomarkers for response to immune checkpoint inhibitors in gastrointestinal cancers

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Abstract

Gastrointestinal (GI) cancers account for a large proportion of cancer deaths worldwide and pose a major public health challenge. Immunotherapy is considered to be one of the prominent and successful approaches in cancer treatment in recent years. Among them, immune checkpoint inhibitor (ICI) therapy, has received widespread attention, and many clinical findings support the feasibility of ICIs, with sustained responses and significantly prolonged lifespan observed in a wide range of tumors. However, patients treated with ICIs have not fully benefited, and therefore, the identification and development of biomarkers for predicting ICI treatment response have received further attention and exploration. From tumor genome to molecular interactions in the tumor microenvironment, and further expanding to circulating biomarkers and patient characteristics, the exploration of biomarkers is evolving with high-throughput sequencing as well as bioinformatics. More large-scale prospective and specific studies are needed to explore biomarkers in GI cancers. In this review, we summarize the known biomarkers used in ICI therapy for GI tumors. In addition, some ICI biomarkers applied to other tumors are included to provide insights and further validation for GI tumors. Moreover, we present single-cell analysis and machine learning approaches that have emerged in recent years. Although there are no clear applications yet, it can be expected that these techniques will play an important role in the application of biomarker prediction.

Key Words: Immunotherapy; Immune checkpoint inhibitor; Biomarker; Predictive response; Gastrointestinal cancer

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Core Tip: Cancer immunotherapy and immune checkpoint inhibitors (ICIs) have recently revolutionized gastrointestinal (GI) cancer treatment, providing unprecedented clinical benefits. However, GI patients treated with ICIs do not fully benefit, and therefore, the identification and development of biomarkers for predicting ICI response have become a pressing issue to be solved now. In this review, we summarize the use of predictive biomarkers for ICI treatment response in GI cancers, and discuss novel biomarkers under development. We also present important biomarkers in other tumors with the aim of providing a cutting-edge reference for GI cancer research.

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INTRODUCTION

Gastrointestinal (GI) cancers are common among all cancer types, and the incidence and mortality rates of GI cancers are increasing year by year, especially in colorectal cancer (CRC), which is also accompanied by a tendency of rejuvenation[1]. GI cancers mainly occur in the GI system and related digestive organs, including the esophagus, stomach, biliary tract system, liver, pancreas, small intestine, rectum, and anus. Among them, hepatocellular carcinoma (HCC) has the highest morbidity and mortality rate. For example, from 2000 to 2016, the mortality rate for HCC increased by 43% (from 7.2 to 10.3 *per* 100000), with a 5-year survival rate of only 18% in the United States[2]. Treatment strategies for GI cancers include surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy, among which immunotherapy is a hot topic in recent years.

Immunotherapy is a relatively new therapeutic strategy that has received widespread attention, mainly including immune checkpoint inhibitors (ICIs), tumor vaccines, and immune cell therapy. Among these, ICIs are most widely used[3]. Immune checkpoints are used by normal cells to regulate immune cytotoxic functions, thus avoiding the destruction of normal tissues. However, this mechanism can also be borrowed by tumor cells to escape the body's immune surveillance and clearance[4]. ICIs can eliminate this inhibitory effect, allowing immune cells to be reactivated to a working state and destroy tumor cells.

The better studied ICIs are CTLA-4 inhibitors and programmed cell death protein 1/programmed cell death ligand 1 (PD-1/PD-L1) inhibitors. Ipilimumab (anti-CTLA-4) was approved by the FDA in 2011 for the treatment of melanoma, followed by the PD-1 inhibitors pembrolizumab and nivolumab for the treatment of melanoma, metastatic non-small cell lung cancer (NSCLC), and DNA mismatch repair-deficient/microsatellite instability-high (dMMR/MIS-H) tumors[5,6]. Although there are many immune checkpoints, not limited to those mentioned above, they have a relatively similar mechanism of action. For example, PD-1 is able to bind to PD-L1 in tumor cells, disabling the ability of T cells to attack cancer cells. Their binding acts as a co-inhibitory signal for T cells and negatively regulates the body's immune response. In turn, tumor cells can upregulate the expression of PD-L1 to inhibit the activation of T cells. This suppression can be abolished after ICI treatment, and in turn, T cells are able to perform their normal functions[7]. In this regard, immunotherapy is now becoming a prospective treatment for GI cancers.

Although immunotherapy has provided sustained clinical benefits, studies have found limitations in the effectiveness of immunotherapy and it is extremely important to study biomarkers to predict more accurate clinical responses[8]. Biomarkers for predicting ICI response have been extensively explored and developed. A variety of biomarkers for GI malignancies have been clinically applied, which can help patients to choose the appropriate targeted therapeutic options. This review highlights biomarkers for predicting the response to ICIs for the treatment of GI tumors. Some biomarkers applied to other tumors are also presented, intending to provide further reference and validation for GI tumors (Figure 1). In addition, we present some new approaches that have emerged in recent years, such as single-cell analysis and machine learning.

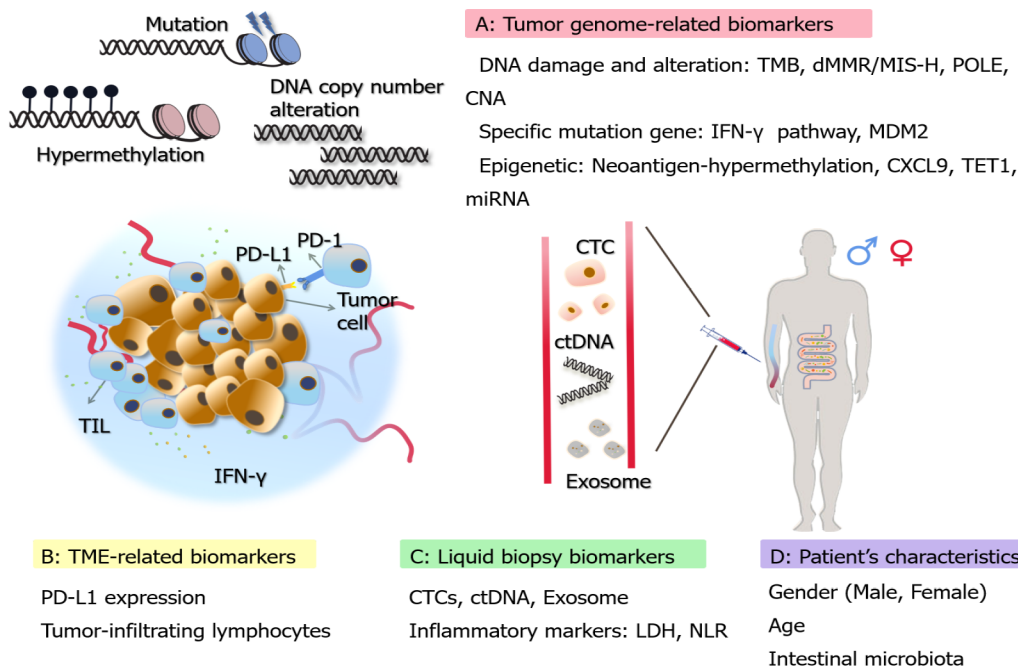


Figure 1 Brief overview of immune checkpoint inhibitor-related biomarkers in gastrointestinal cancers and some novel biomarkers being developed. A: Tumor genome-related biomarkers. The biomarkers in this category are divided into three groups: DNA damage and alteration, including tumor mutation burden, mismatch repair deficiency/high-microsatellite instability (dMMR/MSI-H), POLE, and copy number alteration (CNA); specific mutation genes, including IFN- γ pathway and MDM2; epigenetic alterations, including neoantigen-hypermethylation, CXCL9 epigenetic modification, TET1, and miRNAs; B: TME (tumor immune microenvironment) related biomarkers. PD-L1 expression and tumor infiltrating lymphocytes are involved. In this review, CD8⁺ and CD39⁺CD8⁺ cells are mainly mentioned; C: Liquid biopsy biomarkers. CTCs, ctDNA, and exosomes are grouped into one group. Inflammatory markers taken from peripheral blood are divided into a separate subcategory, including lactate dehydrogenase and neutrophil-to-lymphocyte ratio; D: Patient's characteristics. The patient's gender, age, and intestinal microbiota are classified in this category. ICI: Immune checkpoint inhibitor; GI: Gastrointestinal; POLE: Polymerase gene epsilon; MDM2: Murine double minute 2; CXCL9: Chemokine (C-X-C motif) ligand 9; TET1: Ten eleven translocation 1; PD-1/L1: Programmed cell death-1/Ligand 1; ctDNA: Circulating tumor DNA; CTC: Circulating tumor cells; LDH: Lactate dehydrogenase; NLR: Neutrophil-to-lymphocyte ratio; TIL: Tumor infiltrating lymphocytes.

TUMOR GENOME BIOMARKERS

Tumor mutation burden

The tumor mutation burden (TMB) represents the density of distribution of non-synonymous mutations in the protein-coding region, or simply the number of mutations present in the tumor (Table 1). It is usually defined as the total number of mutations *per* megabase of substitutions and insertions or deletions in the exon coding region of the gene evaluated in the tumor sample and is usually detected as mutations *per* million bases (Mut/Mb)[9]. Traditionally, whole-exome sequencing (WES) has been used to measure TMB, which is considered the standard for TMB determination. However, due to the high cost and relatively slow speed of detection using WES, the accurate determination of TMB by next-generation sequencing (NGS) panels has recently been applied[10]. Quantifying the number of non-synonymous single nucleotide variants (SNVs) by NGS, followed by algorithmic validation and extension to WES, is also one of the feasible approaches in recent years[11].

According to several reports in recent years, increased TMB is associated with the response to ICI therapy, and high TMB was significantly associated with the efficacy of ICIs[12]. There are many data supporting the use of increased TMB as a biomarker for ICI therapy in many pan-cancer treatments. According to a retrospective study that included 27 cancer types, patients with higher TMB were found to have better clinical outcomes and objective response rates (ORR) when treated with PD-1 antibody[13]. In a phase II study of pembrolizumab in Korea, high TMB was defined as more than 400 SNVs in the WES. The results showed that elevated levels of TMB were associated with a high ORR (89%); the moderate TMB group (100-400 SNVs) had an ORR of 20%, while the low TMB group had an ORR of only 7%, indicating a similar positive correlation between high levels of TMB and ICI efficacy, *i.e.*, higher values of TMB represent a higher overall response rate for patients[14].

In another retrospective study, TMB levels of patients with various types of melanoma as well as NSCLC were also classified as low (1-5 Mut/Mb), medium (6-19 Mut/Mb), and high (≥ 20 Mut/Mb). Their analysis indicated that patients with high

Table 1 Summary of biomarkers used or worthwhile in gastrointestinal cancers

Classification	Biomarkers	Tumors	Response	OS	PFS	Others	Ref.
Tumor-genome biomarkers	TMB	Multiple GI	Pos/Neg ¹	14.6/4.0 mo	Unreached/2 mo (CRC)	NA	[19-21]
	dMMR/MSI-H	Multiple GI	Pos	Unreached <i>vs</i> 5.0 mo (CRC)	Unreached <i>vs</i> 2.2 mo (CRC)	Higher DCB (59.1% <i>vs</i> 28.6%, GI tumors)	[30,31]
	CNA	Multiple GI	Neg	Unreached ²	Over 10 mo	NA	[31]
	IFN- γ -related	Multiple GI	Pos	Positive correlation (GC)	Positive correlation (GC and ESCA)	NA	[40,42]
	MDM2	HCC	Neg	NA	NA	Correlated with HPD	[50]
TME biomarkers	PD-L1	Multiple GI	Pos	NA	NA	NA	[53,54]
	TIL	Multiple GI	Pos	Prolonged OS (ESCA)	NA	3-yr RFS 71.6% <i>vs</i> 55.3% (CRC)	[67,78]
Liquid-biopsy biomarkers	ctDNA	Multiple GI	Neg	NA	4.9 mo <i>vs</i> 7.4 mo (GC)	2-yr RFS 66% <i>vs</i> 100% (CRC)	[73,74]
	Exosome	GC	Neg	Reduced OS	NA	High level Exosome	[78]

¹In tumor mutation burden (TMB), the Neg means that immune checkpoint inhibitor treatment response of TMB-L patients may be better by epigenetic modifications.

²Represent a wide variety of gastrointestinal tumors and do not refer to any particular type.

OS: Overall survival; PFS: Progression-free survival; TMB: Tumor mutation burden; dMMR: Mismatch repair deficiency; MSI-H: Highly microsatellite instability; CNA: Copy number alteration; TIL: Tumor infiltrating lymphocyte; ctDNA: Circulating tumor DNA; GI: Gastrointestinal; GC: Gastric cancer; CRC: Colorectal cancer; HCC: Hepatocellular cancer; ESCA: Esophageal cancer; HPD: Hyperprogressive disease; DCB: Durable clinical benefit; RFS: Recurrence free survival; Pos: Positive; Neg: Negative; NA: Not applicable; ICI: Immune checkpoint inhibitor.

levels of TMB had the highest response rate to ICI treatment, reaching 58%, and also had the longest duration of progression-free survival (PFS) at 12.8 mo. The other two treatment groups had a response rate of only 20% and a PFS of only 3.3 mo[15]. Another study detected TMB (cut-off value of 20 Mut/Mb) in 4064 NSCLC patients and found that patients with high levels of TMB (TMB-H) had a significantly higher overall survival (OS) and disease control rate (DCR) when treated with anti-PD-1/L1 agents compared to patients with low levels of TMB (TMB-L)[16]. Similar results were presented in another study showing significantly better durable clinical benefit (DCB) and PFS in the TMB-H population in a cohort with 78 NSCLC patients treated with anti-PD-1/L1 antibodies[17]. Additionally, in a prospective analysis of KEYNOTE-158, Marabelle *et al*[18] assessed the association of pembrolizumab monotherapy in terms of TMB (tTMB) and clinical outcome across ten different advanced solid tumors types, including anal, biliary, *etc*. The results revealed that in terms of efficacy, the ORR (29% *vs* 6%) was better in the tTMB-high group (defined as ≥ 10 Mut/Mb) than in the tTMB-low group (< 10 Mut/Mb), and the median durable response (follow-up of approximately 3 years) was not reached, while the tTMB-low group only reached 33.1 mo[18].

Data from the above-mentioned studies have demonstrated the significant role of high levels of TMB in predicting ICI efficacy, and the results of TMB in GI cancers are no exception to other tumor types. In a phase I study with the anti-PD-1 antibody toripalimib, patients with metastatic gastric cancer (GC) with high TMB (> 20 Mut/Mb) had a better response in survival compared to those with low TMB (15 mo *vs* 4 mo)[19,20]. In patients with advanced GC, patients with high TMB (≥ 12 Mut/Mb) had significantly better efficiency (33.3% *vs* 7.1%) and OS time (14.6 *vs* 4.0 mo) than patients with low TMB (< 12 Mut/Mb)[20]. In a study of metastatic CRC, none of the TMB-H group had achieved PFS (median follow-up > 18 mo), while the TMB-L group had a PFS of only 2 mo and approximately 66% of TMB-L patients developed further disease[21]. In conclusion, high levels of TMB in ICI therapy represent improved patient treatment efficiency and better prognostic outcomes.

Several studies presented at the 2020 American Society of Clinical Oncology meeting confirmed the predictive value of TMB in immunotherapy or combination therapy, although TMB still has limitations as a biomarker. In addition, several general issues deserve further attention, both in the application of GI cancers and in a wide range of other tumor types. First, there is no clear TMB cut-off value as a criterion to

accurately determine which patients can benefit from ICI treatment[22]. Second, testing at the proteomic level may provide a clear picture of the mutational load on the membrane of tumor cells, as some mutations that cause an immune response may originate from only a small subset of genes[23]. Third, factors such as allele frequency might be considered for further and more accurate prediction of ICI efficacy[24].

dMMR/MSI-H

MSI refers to microsatellite instability and MMR refers to mismatch repair function. They are closely related, *e.g.*, when the MMR functions are in a proficient state (pMMR), MSI can be repaired to maintain stability (MSS). In contrast, when the expression of any of the MMR-related proteins goes wrong and the MMR function is in a deficient state (dMMR), it leads to defects in cellular repair functions, allowing DNA to accumulate mutations during replication, ultimately leading to the development of MSI[25]. MSI can be broadly classified as highly unstable (MSI-H), lowly unstable (MSI-L), and stable (MSS). The dMMR and MSI-H can be roughly equated, as can pMMR and MSS[26].

The dMMR occurs in a variety of tumor types, especially common in GI cancers, including colorectum, stomach, small intestine, prostate, *etc.*[27]. It has been shown that dMMR/MSI-H tumors have a much higher somatic mutation rate compared to pMMR tumors and are thought to express a large number of shift-code peptides that act as neoantigens and enhance the immune response[28]. In 2017, the United States FDA first approved the PD-1 inhibitor pembrolizumab for the treatment of patients with solid dMMR/MSI-H tumors[29]. Several clinical trials, including KEYNOTE-012, 016, 028, and 158, which included multiple tumor types, have shown that pembrolizumab has promising durable outcomes in treating patients with dMMR/MSI-H tumors[24].

In the treatment of GI cancers, especially in CRC, dMMR/MSI-H is considered to be a relatively well-established group of biomarkers. In the KEYNOTE-164 clinical trial study, the efficacy of pembrolizumab was evaluated in three cohorts of 11 dMMR-CRC, 21 pMMR-CRC, and 9 dMMR non-CRC patients. An immune-related ORR of 40% and a 20-wk PFS of 78% were observed in the dMMR-CRC cohort, while an ORR of 0 and a 20-wk PFS of 11% were observed in the pMMR-CRC cohort. Median PFS and OS were not achieved in the dMMR-CRC cohort, but were 2.2 mo and 5.0 mo, respectively, in the pMMR-CRC cohort. These results demonstrated that dMMR patients are favorable candidates for treatment with ICIs[30]. Lu *et al*[31] investigated the clinical benefit of ICIs in GI patients. They indicated that the incidence of DCB was significantly higher in dMMR/MSI-H patients (59.1%) than in MSI-L/MSS/pMMR patients (28.6%). In addition, the median PFS time was significantly longer in dMMR/MSI-H patients (7.24 mo) than in MSI-L/MSS/pMMR patients (2.67 mo)[31]. These data reveal that dMMR/MSI-H patients have a more favorable ICI response than the other groups. The dMMR/MSI-H has reliable clinical data as a well-established biomarker in GI cancers, especially in CRC. Its application in other GI cancers also deserves attention and further exploration.

Copy number alteration

Recently, it has also been shown that copy number alterations (CNA), including copy number gain (CNgain) and copy number loss (CNloss), have a predictive role in ICI therapy. In melanoma patients treated with ICIs, CNloss was found to be lower in responders[32]. Some ICI-related immune features were also found to be negatively correlated with CNA in GC and CRC of the Cancer Genome Atlas (TCGA) datasets [33]. Detailed data are presented for elaboration in the study by Lu *et al*[31]. In their study, tumor samples from 93 patients with GI cancers treated with ICIs were tested. CNA load included measures of total CNA, CNgain, and CNloss, while CNgain/CNloss was defined as the total number of genes with CNgain/CNloss present in each sample[31]. They found a significant difference in the CNA burden index between DCB and NDB (no durable benefit) patients treated in the GI group, with DCB patients having a significantly lower CNA burden than NDB patients, suggesting that a low CNA burden may be correlated with better ICIs outcomes. DCB rates were more pronounced in the low and high groups with the same low level of CNgain/CNloss. Further exploration of OS and PFS also led to more favorable data in the low burden group. Based on the study, the group with lower CNA showed a longer median OS (not achieved in all cohorts). For PFS, it was also suggested that the lower CNA group had a longer PFS, all at more than 10 mo[31]. Furthermore, a study by Smeet *et al*[34] on CRC treated with bevacizumab combination therapy also illustrated another perspective on the possibility of CNA as a potential biomarker for ICI treatment. Their study, which also defined three CNA groups, showed that tumors in the low-load

CNA group did not benefit from this combination therapy, while in turn confirmed that ICI therapy is the superior choice. Likewise, the potential of low-load CNA as a predictive biomarker for ICIs was also confirmed[34].

As a noteworthy point, considering the combination of TMB and CNA, a significantly higher proportion of patients with DCB were in the TMB-High/CNA-Low subgroup (12/14) compared to the TMB-Low/CNA-High subgroup (1/28). The median OS (not achieved) was also significantly longer in the TMB-high/CNA-low subgroup than in the other three subgroups (TMB-Low/CNA-Low, 17.3 mo; TMB-High/CNA-High, 12.37 mo; TMB-Low/CNA-High, 6.23 mo)[31]. This result suggests that the combined use of these two biomarkers may have a higher accuracy.

IFN- γ signal and MDM2

Alterations within the tumor-associated signaling pathways also affect the efficacy of ICIs, related to the mechanism of checkpoint inhibitor drugs as well as drug resistance [35]. IFN- γ is a cytokine that stimulates the immune response and is one of the key signals for the activation of immune cells. IFN- γ is also able to trigger a series of events leading to tumor cell death by linking to receptors on the cell surface. Moreover, IFN- γ is able to increase the expression of PD-L1 in tumors and increase the expression of MHC, promoting antigen presentation in antigen presenting cells[36].

Grasso *et al*[37] showed that IFN- γ released by T cells contributes to the amplification of nascent anti-tumor immune response[37]. A study by Karachaliou *et al* [38], which included seven NSCLC patients treated with pembrolizumab, showed that high expression of IFN- γ may be associated with a better PFS and OS in NSCLC patients[38]. Higgs *et al*[39] similarly showed that patients with elevated IFN- γ -associated signaling had a longer median OS (18.1-22.7 mo *vs* 6.5-7.7 mo) and better ORR (6-fold higher) in advanced NSCLC[39]. The above results revealed a trend towards the application of IFN- γ in GI cancers.

KEYNOTE-028 is a phase Ib trial of pembrolizumab in patients with 20 different tumor types, including GI cancers. In the esophageal cohort, 23 patients were enrolled and an IFN- γ signature was detected, showing a trend towards predicting response to ICIs[40]. In GC, Epstein-Barr virus (EBV) is involved in approximately 10% of GC progression, and PD-L1 overexpression is presented as a feature of EBV GC. In addition, IFN- γ signaling was also shown to be involved in a study by Sasaki *et al*[41]. Similarly, in the KEYNOTE-012 clinical trial, which included GC patients treated with pembrolizumab, IFN- γ -related genes were shown to be correlated with OS and PFS [42]. Overall, these results provide useful information revealing the role of IFN- γ in predicting the efficacy of ICIs in GI cancers.

Mutations in genes related to the IFN- γ pathway, such as *IFNGR1/2*, *JAK1/2*, and *IRF1*, also lead to poor outcomes and resistance in patients receiving ICI therapy[35, 43]. The JAKs are key kinases in this pathway, and JAK1/2 shift mutations lead to deficient production of IFN- γ . Shin *et al*[44] indicated that JAK1/2 mutations were associated with resistance to anti-PD-1 therapy in CRC patients[44]. These results suggest that mutations in JAK can lead to poor efficacy of ICIs[44,45].

MDM2 is known as the mouse double minute 2 homolog and is an E3 ubiquitin ligase. When MDM2 is overexpressed due to amplification or improper regulation, it inhibits the activation of P53, which in turn accelerates tumor growth and progression [46]. Kato *et al*[47] analyzed the genomic profiles of 155 patients with multiple tumor types and found that six patients with MDM2 amplification have a time to treatment failure (TTF) less than 2 mo. Four of the six cases (all with MDM2 amplification) showed 2.3 to 42.3-fold hyperprogression compared to ICI pre-treatment[47]. A recent study also showed that cell lines with high MDM2 expression were more potent against T cell-mediated tumor killing, and that targeting MDM2 improve the efficacy of ICIs[48]. These imply that there may be a negative correlation between amplified variants of MDM2 and the efficacy of ICIs, allowing tumors to develop hyperprogression after receiving treatment.

Dysfunction of the MDM2-P53 axis is a major contributor to GI cancers. The main risk factors for HCC include chronic viral infections and metabolic diseases, all of which may contribute to HCC through dysfunction of the MDM2-P53 axis[49]. The results by Wu *et al*[50] on prognostic markers for HCC showed that MDM2 was able to directly act on BIRC5 as well as the downstream transcription factors to regulate its expression, thereby reducing the sensitivity and effectiveness of ICI therapy[50]. Based on the association from the available clinical data, MDM2 is expected to be a more specific negative biomarker for predicting ICIs in HCC, although further prospective studies are needed to corroborate this.

TUMOR IMMUNE MICROENVIRONMENT-RELATED BIOMARKERS

PD-L1 expression

PD-L1 is one of the most studied biomarkers with abundant data in clinical studies [51]. The expression of PD-L1 in tumors measured by immunohistochemistry was one of the first biomarkers developed to predict the benefit of ICIs [52]. In GI cancers such as GC, CRC, and HCC, there is a positive correlation between PD-L1 expression and the efficacy of ICIs [53,54]. Many clinical trials have provided data demonstrating the feasibility of PD-L1 (Keynote-059, Keynote-010, Attraction-02, Checkmate-057, Checkmate-012, *etc.*), and the FDA has approved the application of PD-L1 expression as a biomarker for adjuvant or second-line treatment.

Nevertheless, PD-L1 expression remains limited and somewhat controversial as a comprehensive, stand-alone biomarker. In the trials mentioned above, both Keynote-059 and Attraction-02 did show higher response activity in PD-L1-positive patients, but the data equally showed response activity in PD-L1-negative patients [55]. Concerning the limitations of PD-L1 expression, the following points are noteworthy. First, in the tumor microenvironment, PD-L1 expression displays dynamics and diversity with spatial and temporal heterogeneity [56]. PD-L1 expression detected at a single time point cannot be fully used to assess ICI response [57]. Second, PD-L1 detection criteria are not standardized, with no exact positive scores and thresholds to define [56,58]. Issues such as inconsistent antibody usage and inconsistent detection thresholds make it difficult to standardize staining systems as well [59]. At the molecular level, PD-L1 expression has two components: Tumor cell-associated gene variants and PD-L1 expression induced by IFN- γ secreted by infiltrating T cells. The former has constitutive expression, which is not significantly related to the efficacy of ICIs, while the latter is inducible expression, which is concentrated in the region near the T cells of tumor tissues, and is closely related to the efficacy of ICIs. However, these two types of PD-L1 are not strictly differentiated, which can easily lead to the incorrect conclusion that patients with high PD-L1 expression cannot benefit [60]. Third, the detection methods for PD-L1 expression are not sensitive and precise enough. In an analysis of relevant studies, the response rate to ICIs ranged from 36% to 100% for PD-L1 expression-positive tumors, whereas for PD-L1 expression-negative tumors, the response rate ranged from 0% to 17% [52].

Tumor-infiltrating lymphocytes

Tumor-infiltrating lymphocytes (TILs) represent an effective mechanism of adaptive immunity with anti-tumor potential and have been shown to be associated with prognosis and response to immunotherapy in various types of cancer [61]. TILs originate from areas of tumor tissue, have specific recognition of autologous tumors, and have specific MHC-restricted tumor lysis activity [62]. Among the different types of tumor immune infiltration, the relationship between immune inflammation and ICI treatment is more evident.

Immunoinflammation is characterized by the presence of CD8⁺ and CD4⁺ T lymphocytes in the tumor parenchyma and is accompanied by the expression of immune checkpoint molecules, revealing that ICI treatment may generate a tumor immune response [63]. Analysis of pre-treatment samples showed a relative abundance of CD8⁺ T cells at the infiltrative margins of responders, and serial sampling during treatment showed increased infiltration of CD8⁺ T cells into the tumor parenchyma [64]. Other data showed that patients with high CD8⁺ TIL density achieve a longer PFS and OS compared to those with low density [65]. Similarly, in a retrospective study of a series of patients including some with GI cancers, TILs in tumor biopsy samples were shown to be associated with improved survival [66]. In a study by Xiao *et al* [67] on CRC liver metastases, patients with high CD8⁺ TIL had a significantly longer recurrence-free survival (RFS) than those with low CD8⁺ TIL (median RFS: Unmet *vs* 55.8 mo, 3-year RFS 71.6% *vs* 55.3%) [67]. And the prognostic value of TILs was demonstrated by the higher accuracy of combining with PD-L1 expression. In addition, in esophageal cancer, a cohort with PD-L1 expression combined with high CD8⁺ TILs showed a longer OS [68]. In a peripheral blood analysis of a CRC patient treated with pembrolizumab who had a rapid response, high CD39 expression in CD8⁺ TILs was also found, suggesting that CD39⁺ CD8⁺ TILs may be a promising predictive biomarker in GI cancers [69].

LIQUID BIOPSY BIOMARKERS

Circulating tumor DNA, circulating tumor cells, and exosomes

The non-invasive nature of liquid biopsy reduces patient suffering compared to sampling of surgery, while adding advantages that tissue biopsy does not offer. Liquid biopsy overcomes the inevitable heterogeneity of tissue biopsy, allowing for multiple sampling and providing real-time data on tumor changes and relatively more comprehensive results[70]. Circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and exosomes are commonly promising biomarkers for liquid biopsy.

ctDNA is mainly released by dead cancer cells, or can also be secreted directly by CTCs, reflecting information about the entire tumor genome, and the variability of its data provides the feasibility of dynamic monitoring of tumor progression throughout the treatment regimen[71]. Several studies have shown that high ctDNA mutations are associated with a poor OS and prognosis in patients with different cancer types treated with ICIs[24]. Lee *et al*[72] showed that melanoma patients with persistently elevated ctDNA during anti-PD-1 therapy exhibited less favorable responses with a shorter PFS and OS[72]. Also for GI cancers, among 25 patients with stages I-III CRC, the 2-year RFS was 66% in ctDNA-positive patients compared with 100% in negative patients. In addition, ctDNA showed a negative tendency of recurrence rates, in agreement with the previous result[73]. In a study of 46 advanced GC patients treated with anti-PD-1, the mutational status of baseline ctDNA affected the PFS of patients with a median of 7.4 mo (undetectable ctDNA) *vs* 4.9 mo (detectable ctDNA)[74]. This suggests that ctDNA may serve as a potential negative biomarker for response to ICI therapy in patients with advanced GC. Recent reports have also linked the detection of CTCs to tumor metastasis. The results showed that PD-L1 was overexpressed in CTCs of patients with advanced head and neck cancers, revealing that combined detection of PD-L1 and CTC may have potential as a biomarker for ICI efficacy prediction[75].

Exosomes are extracellular vesicles carrying tumor-associated proteins, metabolites, RNA, DNA, and lipids, which cover most of the information needed for biopsy and can serve as important biomarkers[76,77]. Zhang *et al*[78] found elevated levels of exosomes in GC patients with liver metastases. Serum exosome levels were higher in GC patients than in healthy subjects, and the number of exosomes in serum was positively correlated with the stage of GC[78]. It has been further revealed that the mRNA expression of *PD-L1* in plasma exosomes correlates with the efficacy of ICIs, which may lead to the suppression of effector lymphocytes involved in antitumor immunity, making ICIs less effective[79]. Still in GC, according to Fan *et al*[80], OS was significantly lower in the high exosomal PD-L1 group than in the low group. In their subgroup analysis, this difference was found to be even more pronounced in early GC, suggesting that high exosomal PD-L1 could be used as a predictor of the early stage of GC[80]. The combination of exosome and PD-L1 assays has informative implications in GI cancers; however, it remains to be noted that exosomes still face challenges as biomarkers, and need to be further explored to accurately measure their quantity and purity.

OTHER BIOMARKERS OF WORTH IN GI CANCERS

The details of the above biomarkers that have been studied or applied in GI cancers are summarized in Table 1. And in addition to the biomarkers mentioned above, here we also discuss and summarize some of the biomarkers that appear more frequently in a variety of other tumors, including patient characteristics, neoantigens, inflammatory indicators, and epigenetics (Table 2). These biomarkers deserve further prospective study and development in ICI-treated GI cancers, and provide new ideas for the identification of novel biomarkers as well.

Factors related to the patient's characteristics

The efficacy of ICI treatment is also highly dependent on patient's characteristics, such as gender, age, and the homeostasis of the body's internal environment. The application of these characteristics in GI cancers is not yet supported by a large amount of data, but the correlation of these characteristics with the efficacy of ICI treatment provides a novel idea for future studies, which can be combined with other markers to improve the predictive accuracy. The first point worth mentioning is the possible correlation between the efficacy of ICIs and the gender of the patient. A meta-analysis including 20 randomized controlled trials conducted by Conforti *et al*[81] reported better efficacy of ICIs in male patients than in females[81]. Schreiber *et al*[82]

Table 2 Summary of biomarkers worthy of further development in gastrointestinal tumors

Classification	Biomarkers	Tumor type	Response to ICI	Ref.
Tumor-genome biomarkers	POLE-mutation	Endometrial carcinoma	Pos	[93]
	Neoantigen	Pulmonary adenocarcinoma	Pos	[94]
Liquid-biopsy biomarkers	LDH	Melanoma	Neg	[99]
	NLR	Advanced solid tumors	Neg	[100]
Epigenetic	TET1-mutation	Multiple tumor types	Pos	[105]
	miRNA	Non-small-cell lung cancer	Pos	[107]
Patient characteristic	Gender	NA	Male: Pos; Female: Neg	[81,82]
	Age	NA	Controversial ¹	[84,115]
	Intestinal microbiota	NA	Pos/Neg	[85-87]

¹Age as a marker remains controversial, and there are conflicting cases of relevant data.

POLE: Polymerase gene epsilon; LDH: Lactate dehydrogenase; NLR: Neutrophil-to-lymphocyte ratio; miRNA: Micro RNA; Pos positive; Neg negative; NA: Not applicable; ICI: Immune checkpoint inhibitor.

suggested that women have more effective immunosurveillance mechanisms compared to men, and this immunosurveillance capacity allows women to be less immunogenic in advanced tumors. They further implied that women may have stronger immune escape mechanisms, and thus they may be more resistant to immunotherapy[82].

Age is also an important marker. There is a relationship between aging and restricted immune function, with significant effects on both innate and acquired immune responses[83]. Nishijima *et al*[84] reported an association with better ORR in patients aged less than 75 years treated with ICIs[84]. In addition, the fraction and diversity of the intestinal microbiota were likewise found to be associated with the efficacy of ICIs, where effective patients tend to have high levels of polyphenism and ruminal cocci family[85]. The intestinal microbiota can influence the process of cancer development and progression by altering the host immune system and regulating metabolism[86]. It was evidenced that patients treated with antibiotics for 2 mo before or after ICI treatment had a significantly lower clinical benefit than those without antibiotics, probably because antibiotics disrupted the homeostasis of gut microbiota and certain dominant intestinal flora in patients[87].

POLE and neoantigen

As mentioned above, TMB and dMMR/MSI-H were biomarkers at the tumor genome level, and correspondingly, another one of interest needs to be presented here, which is POLE. Polymerase ϵ (encoded by the *POLE* gene) performs error correction during DNA replication, ensuring the accuracy of the replication process[88,89]. Mutations in *POLE* severely affect the error correction function, leading to the accumulation of a large number of somatic mutations and elevated TMB. CD8⁺ lymphocyte infiltration in tumors is also significantly increased, promoting the production of tumor-specific neoantigens[90-92]. From a retrospective study conducted by Domingo *et al*[90] including 6517 CRC patients, 66 of them (1.0%) were found to have *POLE* mutations with the highest mutational burden, all with MSS[90]. However, it is worth mentioning that even patients with the MSS type carry a highly mutated profile. Howitt *et al*[93] reported that *POLE* mutations in endometrial carcinoma lead to an elevated tumor neoantigen load and PD-1 overexpression in tumor-infiltrating cells [93]. These results indicated that *POLE* mutations have a role as prognostic markers, and the detection of *POLE* can also be applied to GI cancers to predict the survival benefit of ICI therapy.

TMB, dMMR/MSI-H, and *POLE* are all valid indicators as biomarkers, and there is a link between these three. As previously mentioned, mutations in *POLE* can lead to high levels of TMB[11]. Chalmers *et al*[11] indicated that MSI-H can be usually used as a subset of high TMB, and the vast majority of MSI-H samples also had high levels of TMB (83%), with 97% of them having TMB ≥ 10 Mut/Mb. Nevertheless, it depends on the tumor type, and in GI cancers such as gastric, duodenal, and small intestinal adenocarcinomas, MSI-H and high TMB are found almost simultaneously[11]. Both can be used as combined biomarkers to predict the response to ICIs in GI cancers.

Common to all three biomarkers mentioned above is that they all increase neoantigen generation. Higher levels of TMB may increase the chance of immunogenic neoantigens[94]. High levels of somatic mutations in MSI-H and POLE also lead to an increase in neoantigens[30]. It means that these tumor cells are more likely to be recognized by immune cells, in which case the efficacy of ICIs is also more pronounced. It has been suggested that hypermethylation of the neoantigen gene promoter may be important for immune editing and tumor immune escape[95]. Therefore, neoantigens are also in the scope of exploring ICIs biomarkers for GI cancers. Neoantigens are not only highly specific and strongly immunogenic, but are also ideal targets for immunotherapy. The presentation and recognition of neoantigens largely influence the outcome of ICI treatment, making it undoubtedly an important target for predicting the efficacy of ICIs[96]. Studies have shown that in primary pulmonary adenocarcinoma, clonal neoantigen load is associated with a longer OS [94]. The relationship between neoantigens and the clinical benefit of treating GI cancers needs to be supported by additional and more specific data.

Inflammatory indicators

GI cancers are similar to other types of tumors in that tumor-associated inflammatory processes often establish immune tolerance, promote tumor growth and metastasis, and activate oncogenic signal transduction pathways[97]. Some conventional inflammatory indicators, such as neutrophil-to-lymphocyte ratio (NLR) and lactate dehydrogenase (LDH), have been used as ICI response biomarkers for a variety of tumors, which could also serve as promising markers in GI cancers[98]. In a blood test performed on 66 melanoma patients treated with ICIs, baseline values of serum LDH and changes in LDH during ICI treatment were found to correlate with patient response and survival outcomes, with higher baseline serum LDH values and a 10% increase from baseline during treatment likely indicating inferior ICI efficacy[99]. NLR has also been more established as a biomarker. According to the NLR kinetics study in patients with advanced solid tumors treated with PD-1/L1 inhibitors, the median OS of patients with high NLR was 8.5 mo, while the median OS of patients with low NLR was 19.4 mo[100]. Similar results were found by Jiang *et al*[101], showing that high NLR was associated with a poor OS and PFS[101].

Epigenetic markers

Epigenetic alterations are also an area of interest as potential biomarkers. As mentioned above, high levels of TMB tend to be correlated with a better ICI response, but some tumors with low-level TMB may improve the immunogenicity of their tumor neoplastic antigens through epigenetic modifications, when the efficacy of ICIs is instead better[102]. In GC, alterations in the somatic epigenetic promoter have also been described to be associated with immune editing and tumor escape[103]. It has also been shown that the CC family chemokine ligand 9 (CXCL9) is epigenetically modified to suppress its biological function, ultimately blocking effector T cells from infiltrating into the tumor bed for its immune function[104]. In a report examining the relevance of DNA methylation-regulated genes to ICI response, mutated TET1 was significantly enriched among the 21 related genes studied in patients responding to ICIs. Moreover, mutant TET1 was strongly associated with a higher ORR, longer PFS, and better OS and DCB, which could serve as a novel predictive biomarker across multiple cancer types[105].

In addition to modifications such as methylation, miRNAs are also of interest for further development. In epigenetics, miRNA quantification is one of the most accessible markers. MiRNAs can be direct or indirect regulators of PD-L1 expression, as well as of many other immune checkpoints, such as LAG-3, TIM-3, BTLA, or CTLA-4[106]. A study in NSCLC showed that serum miRNA profiles can discriminate responders to ICIs. In that study, Fan *et al*[107] found that increased expression of miR-93, -200, -27a, -28, -424, and other miRNAs were significantly associated with prognosis, highlighting the predictive value of miRNAs[107]. The emergence of TET1, miRNAs, and other epigenetic examples suggests that there are still more possibilities that need to be further explored in the field of GI tumors.

EMERGING TECHNOLOGIES FOR OPTIMIZING BIOMARKERS

Single-cell sequencing analysis

Moreover, with the evolving concept of precision medicine, biomarker research is facing the same trend. Tumors contain different and evolving cell populations, a

property also known as tumor heterogeneity, which is a major driver of resistance to treatment and tumor metastasis and one of the factors affecting the efficacy of ICIs [108]. It is essential to fully understand heterogeneity, especially in the TME. Analysis of TME heterogeneity and the phenotypes of various cell types by single-cell analysis techniques can help optimize existing therapeutic strategies or discover new ones, and improve the efficacy of the currently used biomarkers, although some limitations remain. In uveal melanoma, the single-cell analysis revealed that CD8⁺ T cells predominantly express LAG3 rather than conventional PD-L1, revealing the limited availability of ICIs for treating this type of tumor [109]. It illustrates that the selection of biomarkers in different tumor contexts should be further categorized and considered. In GI cancers, single-cell analysis techniques have also made a notable impact. In the study of GI stromal tumors, Mao *et al* [110] applied single-cell transcriptome analysis to reveal their heterogeneity. They also observed that tumor cell related signatures with high proliferation rates were associated with a high risk of tumor malignancy and metastasis, suggesting that this may serve as a prognostic marker or complement [110]. In a study of CRC by Di *et al* [111], T-cell phenotypes were mapped by single-cell mass cytometry. They identified increased heterogeneity of T cells and immunosuppressive T-cell phenotypes in tumor lesions. Altering this immunosuppressive TME is important to improve the ICI response, and single-cell analysis provides very valuable information to improve the immune response in CRC [111].

Apart from the transcriptomics mentioned above, multi-omics is more noteworthy in single-cell analysis. In a study by Zhou *et al* [112], the percentage of fibroblasts with altered somatic copy number was found to be much higher in CRC than in adjacent normal tissues by using single-cell multi-omics sequencing. Five genes (*BGN*, *RCN3*, *TAGLN*, *MYL9*, and *TPM2*) were also identified as fibroblast-specific biomarkers of poorer prognosis in CRC [112]. This study further explored new CAN-based biomarkers, of which single-cell multi-omics analysis is an essential and important part, which also provides us with new ideas in studying ICI response biomarkers in GI cancers as well.

Machine learning

Along with the growing development of bioinformatics, machine learning, and artificial intelligence, biomarkers will be further improved. For example, in the work of Lu *et al* [113], tumor samples from patients with metastatic GI cancers treated with ICIs were sequenced for immuno-oncology (IO)-related gene targets and combined with the application of linear support vector machine learning strategy to construct an RNA signature (IO score) as a predictive model. Notably, its overall accuracy in discriminating DCB and NDB reached 94% and 83%, respectively, and the IO-score showed superior predictive value with higher odds ratio than the traditional biomarker [113].

CONCLUSION

Research in the field of ICIs has been steadily increasing. In GI cancers, ICI-related studies have also been emerging, addressing the importance of ICIs in tumor immunotherapy from different perspectives. Many recent ongoing studies in GI cancers also highlight the potential for diversification of ICIs, particularly in combination or neoadjuvant therapy, where the utility of ICIs has been further investigated. By combining chemotherapy and targeted agents, these studies provide insight into eradicating micrometastatic GI cancers, overcoming resistance to ICIs, and improving ICI treatment. We summarize in Table 3 a number of clinical studies that are currently ongoing to provide a valuable reference for this purpose. However, it needs to be noticed that these ongoing clinical trials do not specifically target one or more biomarkers to predict response to ICIs. Rather, it is more about the combination of ICI therapy with other therapies, which may have little relevance to our topic. Nonetheless, these clinical trials can provide us with a wealth of useful information that we can use in subsequent data analysis for biomarker identification.

Although many new biomarkers have been identified in GI cancers, there is a relative lack of research compared to other tumor types such as melanoma and NSCLC, and validation from clinical trials is still lacking. In this review, we summarize not only biomarkers that are supported by studies in GI cancers, but also biomarkers that are informed in other tumors, in terms of tumor genomic information, TME, liquid biopsies, and epigenetic and patients' characteristics in relation to ICI response. Among these markers, studies on TMB and PD-L1 need to be further improved, and the delineation of cut-off values is not sufficiently clear, especially for

Table 3 Clinical trials on combination therapy or neoadjuvant therapy being conducted in the immune checkpoint inhibitor treatment of gastrointestinal tumors

Clinical trial ID	Cancer type	Study type	Phase	Number	Strategy	Ref.
NCT02918162	GC; Adenocarcinoma of the GE junction	Interventional	2	40	Pembrolizumab combined with stand of care chemotherapy regimen	[116]
NCT04948125	GC	Interventional	2	20	Camrelizumab combined with Apatinib Mesylate	[117]
NCT04196465	GC, ESCA, HCC	Interventional	2	48	IMC-001 as neoadjuvant therapy	[118]
NCT03841110	GC, CRC	Interventional	1	76	FT500 combined with Nivolumab/Pembrolizumab/Atezulizumab	[119]
NCT02903914	GC, CRC	Interventional	1/2	260	Pembrolizumab combined with Arginase Inhibitor INCB001158	[120]
NCT03259867	HCC, GC, CRC (All have liver lesions)	Interventional	2	80	Pembrolizumab/Nivolumab combined with TATE	[121]
NCT04822103	ESCA	Observational	NA	150	ICIs combined with Neoadjuvant chemotherapy	[122]

GI: Gastrointestinal; GC: Gastric cancer; ESCA: Esophageal cancer; GE: Gastroesophageal; HCC: Hepatocellular cancer; CRC: Colorectal cancer; TATE: Transarterial Tirapazamine Embolization; NA: Not Applicable; ICI: Immune checkpoint inhibitor.

PD-L1 expression, which has been shown in a number of studies to respond to ICIs in PD-L1-negative patients[52]. As a stand-alone biomarker, PD-L1 is still considered to be controversial. In addition, markers associated with patient characteristics also have conflicting data, and current studies are not systematic and not clear enough and need to be confirmed by some large-scale prospective studies[114,115]. Another key point that needs attention is that the current ICI predictive biomarkers for GI cancers are mostly focused on CRC cases, while they have relatively little application in other GI cancers such as GC and HCC, and more research investment is needed.

For the future trend of biomarkers, considering that a single biomarker is mostly insufficient, the strategy of combining two or more biomarkers is noteworthy, such as combining information from epigenetics and tumor genome, TMB and CNA in subgroup analysis, *etc.* The integration of multiple factors is necessary to improve accuracy. And along with the continuous research on ICIs therapy, biomarkers for combination therapy or neoadjuvant therapy also need to keep pace with the development to further promote precision therapy. Meanwhile, with the development of big data and bioinformatics, an increasing number of cutting-edge technologies such as machine learning, artificial intelligence, and single-cell analysis will also be applied for further optimization and refinement, making the efficacy of tumor immunotherapy steadily improved. For the current research, more prospective studies are needed, and more data will help to optimize these computational models. From this point of view, the identification of biomarkers that can be used to accurately predict ICI is just beginning, and much more remains to be done, which could become a major trend and focus in the future.

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