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Gastrointestinal cancer immunotherapy: From drug resistance mechanisms to overcoming strategies

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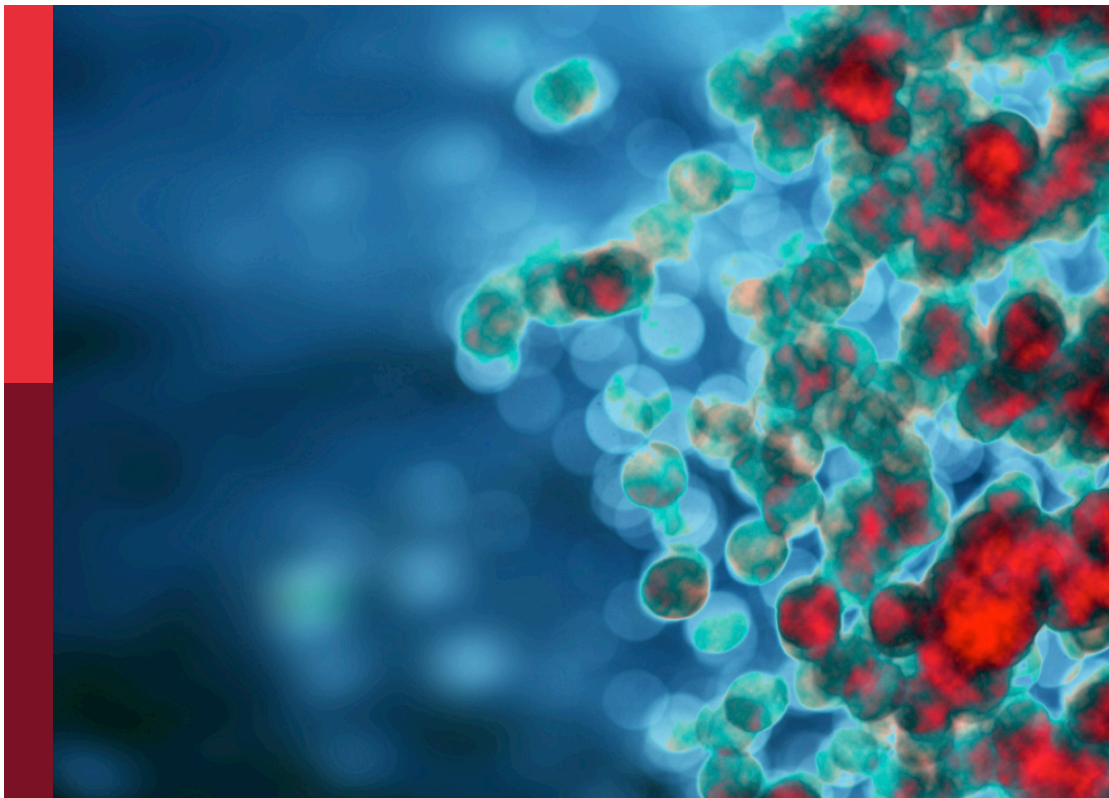

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Gastrointestinal cancer immunotherapy: From drug resistance mechanisms to overcoming strategies

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Editorial: Gastrointestinal cancer immunotherapy: from drug resistance mechanisms to overcoming strategies

Tao Shi[1†], Hanbing Wang[1†], Jia Wei[1†], Jinyan Wang[2*], Yibo Fan[3*], Chunlei Zheng[4*] and Xiaofang Che[5,6,7*]

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Editorial on the Research Topic
Gastrointestinal cancer immunotherapy: from drug resistance mechanisms to overcoming strategies

Gastrointestinal (GI) cancers have long been considered as highly heterogeneous and intractable, with high rates of morbidity and mortality globally (1). Despite the important breakthroughs and clinical success of cancer immunotherapies such as immune checkpoint blockade (ICB) therapy in some cancer types like melanoma (2), the overall response rate (ORR) of ICB therapy in the non-selective GI patients is still not satisfactory (3). About 70-80% of GI patients displayed primary resistance to ICB therapy, while some patients subsequently developed immunotherapy resistance during the treatment process (3). Both tumor-intrinsic factors, such as driver gene mutations or oncogenic pathway activation, and tumor-extrinsic factors, such as the suppressive tumor immune microenvironment (TIME), contribute to the complex drug resistance mechanisms in GI cancers. Thus, current studies aim to develop efficient overcoming strategies to improve treatment responses of immunotherapies (4). In this Research Topic, with the efforts of five guest editors, 15 articles consisting of 6 reviews, 7 original researches and 2 case reports were collected, providing a deep understanding and new comprehensive insight of immunotherapy resistance mechanisms and potential overcoming strategies in GI cancers, including esophageal cancer (EC), gastric cancer (GC), colorectal cancer (CRC) and hepatocellular carcinoma (HCC).

EC is among the deadliest malignancies due to its late-stage diagnosis and escalating worldwide incidence (5). Besides conventional therapies, immunotherapy, represented by ICB, has gained promise in treating patients with EC. To offer an objective and integrated

view of research navigations to promote future advances in ICB, Yang and Wang systemically combed the publication trends and research highlights of PD-1/PD-L1 blockade therapy in EC treatment for the past ten years *via* visualized bibliometric methods. As publication characteristics were displayed varying from countries and time points in the article, the authors pointed out that current research hotspots are focused on neoadjuvant immunotherapy and biomarkers development for esophageal cancer, emphasizing the significance of developing effective biomarkers. Furthermore, Fang et al. reviewed the progress and limitations in immunotherapeutic interventions across-the-aboard, involving ICB, adoptive CAR-T cells and cancer vaccines. Since drug resistance is a crucial threat to satisfactory clinical benefits, the authors discussed resistance mechanisms from two aspects, intrinsic and acquired, and proposed that countermeasures addressing immunotherapy resistance require promising predictive biomarkers and multidisciplinary combination therapies. To find out the immunoregulatory factors related to ICB resistance, Deng et al. compared the transcriptome data of immune cells in the peripheral blood of esophageal squamous carcinoma (ESCC) patients with different responses to PD-1 blockade. They demonstrated that immune checkpoint expression was upregulated in the ICB-sensitive group and identified several genes expression (MT2A, MT1X and MT1E) correlated with ICB resistance. On the other hand, Jin et al. constructed a pipeline, ELISE (Ensemble Learning for Immunotherapeutic Response Evaluation), which incorporates ensemble deep learning and self-attention approaches for accurately predicting responses of patients with esophageal adenocarcinoma (EAC) to ICB therapy. This model based on multi-discipline techniques sheds light on exploiting robust predicting tools to promote efficacies of immunotherapies in EC and other cancers.

GC is another common GI cancer worldwide with high incidence and mortality rates and poor prognosis. Despite immunotherapy (anti-PD-1/PD-L1, programmed cell death protein 1/programmed cell death protein ligand 1) has been approved in advanced GC, the medium overall survival time is still fewer than 24 months (6). Multiple mechanisms, including the aberrant gene/pathway variations of GC cells and the inhibitory immune components in gastric TIME, may contribute to the poor response of ICB therapy. Song et al. constructed a metastasis-related epithelial-mesenchymal transition (EMT) signature (MEMTS) based on differentially expressed genes (DEGs) and EMT gene set from The Cancer Genome Atlas (TCGA) cohort and the Asian Cancer Research Group (ACRG) cohort. They found that high MEMTS predicted poor prognosis and poor response to ICB in GC with an AUC curve of 0.896. Similarly, another bioinformatic analysis based on bulk RNA-seq and single-cell RNA-seq data by Song et al. found that patients with high VCAN expression tended to be resistant to not only adjuvant chemotherapy and adjuvant chemoradiotherapy, but also immunotherapy. Therefore, both MEMTS and VCAN could serve as potential biomarkers for immunotherapy in GC patients. Besides, as for the immune-inhibitory factors in gastric TIME, the review article provided by Liu et al. highlighted the concept of 'tumor immune tolerance', which transforms the TIME from tumor-suppressive to tumor-promoting as the tumor progresses. They summarized that the metabolic and phenotypic changes of both innate immune cells (such as tumor-associated macrophages, neutrophils, and mast cells) and adaptive immune cells (mostly $CD4^+$ and $CD8^+$ T cells) could induce tumor immune tolerance, which subsequently results in the resistance of GC immunotherapy. Moreover, another review article by Kudo-Saito et al. specifically focused on the myeloid villains (including myeloid-derived suppressor cells (MDSCs), regulatory DCs (DCregs), mesenchymal stromal/stem cells (MSCs), macrophages, neutrophils, mast cells and basophils) within the TIME, which all contribute to the tumor immune suppression through different approaches, and can be targeted to improve the clinical outcomes of ICB therapy in GI cancers.

CRC is another major type of GI cancers with a high morbidity rate and poor prognosis (7). Although ICB therapy has achieved important progress in deficient mismatch repair (dMMR)/microsatellite instability-high (MSI-H) colorectal CRC, the majority of CRC patients (approximately 85%) with proficient mismatch repair (pMMR)/microsatellite stability status still respond poorly to immunotherapies (8). In this Research Topic, two reviews provided by Shan et al. and Ding et al. discussed the role and function of immunosuppressive cells (Tregs, TAMs and MDSCs), cytokines (TGF-β, VEGF, IL-4, IL-10, etc.), immune checkpoints, intestinal microbiota and nutrients within microenvironment of CRC that all bring immunotherapy resistance, and summarized the current circumstances of clinical trials that estimate the effects of immunotherapy drugs on CRC patients. As for the impact of tumor-intrinsic factors, Liu et al. identified epigenetic-related gene mutations (Epigenetic_Mut) in 18.35% of MSS-CRC patients from TCGA database and local cohorts. Epigenetic_Mut was also associated with increased infiltration of anti-tumor immune cells and favorable clinical outcomes in MSS-CRC patients receiving PD-1 blockade therapy, indicating that Epigenetic_Mut could be a potential biomarker for ICB therapy in CRC. Also worth noting in this Research Topic are two CRC cases that have opposite treatment outcomes with anti-PD-1 therapy. Although MSI-H status is considered as a favorable biomarker for immunotherapy, Zhang et al. reported a case of LS-associated CRC patient with MSI-H status who failed to benefit from anti-PD-1 therapy. The authors discussed that driver gene mutations like *KRAS* or *PTEN* mutations might be the potential reasons for the poor response of ICB therapy. Interestingly, another case reported by Yang et al. described an advanced MSS/pMMR mCRC patient who had a complete response (CR) after triple-combined therapy (PD-1 inhibitor, radiotherapy and granulocyte-macrophage colony-stimulating factor (GM-CSF)) with progression-free survival (PFS) for more than 2 years so far, thus highlighting the potential value of this triple-combination immunotherapy strategy for MSS/pMMR mCRC patients.

HCC is the most prevalent pathological type of primary liver cancer with dismal prognoses. With the approval of ICB-based therapies as standards of care (9), it is necessary to have an in-depth understanding of the complex TIME which impacts the efficacies of immunotherapy. The establishment of next-generation sequencing methods endows the possibility of analyzing cellular components in the TME and heterogeneous molecular features of HCC (10). Xu

et al. conducted an integrated assessment of transcriptome sequencing, DNA mutation and clinical information in several HCC database-derived cohorts. They discovered that upregulated Wnt/β-catenin signaling signatures are potential predictors for worse-prognosis and ICB-insensitivity, which was linked with poor $CD8^+$ T cell infiltration. Though this WNT-based subtyping still requires *in vitro* or *in vivo* experimental validation, it is of great clinical significance. Meanwhile, because HCC majorly forms under chronic liver inflammation, Yu et al. gave a panoramic view of two crucial components of TIME, tumor-derived exosomes and tumor-associated macrophages, in HCC tumorigenesis and progression. As exosomes play an indispensable role in macrophage polarization, the authors proposed targeting exosomes as a prospective branch of immunotherapy for HCC. Nevertheless, more investigations are needed before clinical applications.

In summary, the 15 articles in this Research Topic explore or discuss the potential drug resistance mechanisms of immunotherapies for GI cancers from different aspects, and provide possible strategies targeting both tumor cells and TIME to improve the treatment efficacies. Unfortunately, the progress of immunotherapy in pancreatic cancer, the recognized immunotherapy-resistant tumor, is not covered in this Research Topic, which is worth further exploring in the future. More research and efforts are required to achieve successful applications of immunotherapy on GI cancers in the future.

Author contributions

TS, HW, and JWe drafted the manuscript. JWa, YF, CZ and XC reviewed and corrected the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Metastasis Related Epithelial-Mesenchymal Transition Signature Predicts Prognosis and Response to Immunotherapy in Gastric Cancer

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Background: Increasing evidence has revealed the effect of epithelial-mesenchymal transition (EMT) on tumor microenvironment and cancer treatment. However, an EMT-based signature to predict the prognosis and therapeutic effect in gastric cancer (GC) has rarely been established.

Methods: Differentially expressed genes (DEGs) between paired primary gastric and ovarian metastatic tumors were identified through comparative RNA-seq analysis, followed by the construction of metastasis-related EMT signature (MEMTS) based on DEGs and EMT gene set. Then, both The Cancer Genome Atlas (TCGA) cohort and the Asian Cancer Research Group (ACRG) cohort were analyzed to explore the potential association between MEMTS and prognosis in GC. Samsung Medical Center (SMC) cohort and two individual immunotherapy treatment cohorts, including Kim cohort and Hugo cohort, were utilized to evaluate the predictive value of MEMTS on the response to adjuvant therapy and immunotherapy, respectively. Finally, the potential association of MEMTS with tumor environment and immune escape mechanisms was investigated.

Results: High MEMTS predicted a poor prognosis in patients with GC. Patients with low MEMTS potentially gained more benefits from adjuvant chemoradiotherapy than those with high MEMTS. MEMTS reliably predicted the response to immunotherapy in GC (area under the curve = 0.896). MEMTS was significantly associated with cancer-associated fibroblasts and stromal score in the aspect of the tumor microenvironment.

Conclusion: MEMTS serves as a potential biomarker to predict the prognosis and response to adjuvant therapy and immunotherapy in GC. MEMTS-based evaluation of individual tumors enables personalized treatment for GC patients in the future.

Keywords: gastric cancer, distant metastasis, epithelial-mesenchymal transition, tumor environment, immunotherapy

INTRODUCTION

Gastric cancer (GC) is the fifth most common malignancy and the third leading cause of cancer-related death globally due to its rapid progression and distant metastasis (1). Despite recent advancements in the comprehensive treatment, metastasis remains as a major hindrance to favorable clinical outcomes (2). Recently, multiple therapeutic modes, especially immunotherapy, have become an essential component of treatment and revealed surprisingly powerful effects on protection against tumors (3). However, drug response varies widely even among patients with comparable clinicopathological features (4, 5), implicating those traditional classifications, pathological TNM staging system in particular, are insufficient for the accurate prediction of therapeutic response. Therefore, the development of a novel molecular signature is urgently needed to precisely identify subgroups of GC patients who are more likely to benefit from therapeutic regimens.

Epithelial-mesenchymal transition (EMT) is a common process during embryogenesis, tissue development, wound healing, and carcinogenesis (6). Generally, in the progression of EMT, epithelial cells undergo loss of epithelial polarity, reorganization of their cytoskeleton, and gain of mesenchymal phenotype with more aggressive properties (7). To date, studies have repeatedly uncovered the impact of EMT on tumor microenvironment and tumor treatment. Xu et al. constructed a risk score model based on EMT-related genes (8) whereas Dai et al. established an EMT-related gene signature for predicting clinical outcomes in GC (9). Furthermore, Oh et al. evaluated two distinct molecular subtypes based on an analysis of genomic and proteomic data to identify therapeutic targets and valuable biomarkers for prognosis and therapy response (10). Although these studies revealed the importance of EMT-related scores in GC treatment, they merely evaluated the predictive value of these scores other than immunotherapy and drug resistance. Therefore, it was necessary to establish a reliable and comprehensive evaluation model possessing of better efficacy.

In the present study, we established a metastasis related epithelial-mesenchymal transition signature (MEMTS) and further analyzed the genomic, transcriptomic, and tumor microenvironmental features of different MEMTS subtypes as well as their responses to adjuvant therapy and immunotherapy. We concluded that the MEMTS was a powerful prognostic biomarker and could reliably predict the response to adjuvant therapy and immunotherapy in GC.

METHODS

Data Sources

Gene expression data in fragments per kilobase million (FPKM) format and corresponding clinical information of gastric cancer in the Cancer Genome Atlas (TCGA) were downloaded from UCSC XENA website (https://xenabrowser.net/datapages/). The FPKM values were converted to transcripts per kilobase millions (TPM) values. The gene expression profiles and corresponding clinical information of Asian Cancer Research Group (ACRG) cohort (GSE66229), SMC cohort (GSE26253), and MD Anderson Cancer Center (MDACC) cohort (GSE28541) were acquired from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). TCGA cohort and ACRG cohort contained 368 and 300 patients with GC, respectively. In SMC cohort, all patients (n = 432) underwent curative gastrectomy and INT-0116 regimen (5-fluoouracil/leucovorin and radiation) as adjuvant treatment (11). All patients in the MDACC cohort (n = 40) received neoadjuvant chemotherapy or chemoradiation therapy (10). For the microarray data from Affymetrix®, the raw "CEL" file was downloaded from GEO database and the microarray data were standardized using the robust multiarray averaging method with the "affy" and "simpleaffy" packages. For the microarray data from other platforms, the normalized matrix files were downloaded directly. The PD-L1 treatment cohorts for gastric cancer (KIM cohort, n = 45) and melanoma (Hugo cohort, n = 26) were obtained from the TIDE database (http://tide.dfci.harvard.edu), which is a computational framework for immunotherapy response prediction. Collectively, TCGA and ACRG cohorts were used to investigate the potential correlation between MEMTS and clinical features and prognosis of GC patients; SMC and MDACC cohorts were used to investigate the predictive role of MEMTS in response to adjuvant therapies; KIM and Hugo cohorts were used to investigate the predictive role of MEMTS in response to immunotherapy. The detailed information of KIM and Hugo cohorts is shown in **Supplementary Tables S1-2**.

RNA Sequencing and Identification of the MEMTS Genes

In this study, we obtained the formalin-fixed paraffin-embedded tissues of primary gastric tumors and matching metastatic ovarian lesions of the patients (n = 4) who received gastrectomy without neoadjuvant chemotherapy or radiotherapy between 2016 and 2020 at Fudan University Shanghai Cancer Center. This study was approved by the Ethics Committee of FUSCC and informed consent was received from all patients. For RNA-seq, RNAstormTM FFPE kit (CELLDATA, CA, USA) was used to isolate total RNA. SMARTer Stranded Total RNA-Seq Kit-Pico Input Mammalian Library preparation kit (Clontech, CA, USA) was used to prepare strand-specific RNA-seq library. Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) and Qsep100 (Bioptic, Taiwan, China) were used to check the quality of library. Illumina sequencing platform (Illumina, San Diego, CA, USA) with 150 bp paired-end run metrics was used for performing RNA-seq. The analysis was carried out by "limma" R package and the significance threshold was set as $|\log2[\text{fold change (FC)}]| > 1$, and False Discovery Rates (FDR) <0.05. The EMT gene set was downloaded from Molecular Signatures Database (http://www.gsea-msigdb.org/gsea/msigdb/). The intersection genes of EMT gene set and upregulated genes in ovarian metastatic tumors were identified as the MEMTS genes, and the average mean of the mRNA expression of MEMTS genes was calculated as MEMTS.

Survival Prognosis and Genetic Alteration Analysis

The overall survival (OS) and progression-free survival (PFS) for patients were compared by Kaplan-Meier curves with the log-rank test, and the cutoff points were selected by "survival" R package. The somatic mutation data of TCGA-STAD were downloaded from UCSC XENA website. The somatic mutation data were analyzed through R package "maftools".

Immune Infiltration Analysis

The immune infiltration among different types of cancers was estimated by CIBERSORT algorithm of "IOBR" R package, which integrated a series of existing algorithms for easy comparison and selection (12, 13). Spearman and distance correlation analysis were used to calculate the correlation of MEMTS and multiple immune cells. Furthermore, the stromal score of each sample was assessed by the R software package "ESTIMATE", and exclusion score of each sample was calculated in TIDE database (http://tide.dfci.harvard.edu) (14).

Gene Set Enrichment Analysis

The gene set enrichment analysis (GSEA) method was applied to study the potential mechanisms of MEMTS in the development of gastric cancer. Firstly, we grouped the STAD samples according to the median of MEMTS in all samples, called the "high" group with the MEMTS greater than the median, and the "low" group with less than the median, compared the differences in gene expression between the two groups, and ranked them according to the value of the foldchange. Then we chose the Hallmarker gene sets which were defined based on prior biological knowledge to analyze all samples in the GSEA method using the R package clusterProfiler (version 4.0.5). The normalized enrichment score (NES) was the primary statistic for examining gene set enrichment results. The false discovery rate (FDR) was the estimated probability that a gene set with a given NES represents a false positive finding. We chose NES and FDR as the indicators of enrichment, (Gene sets with $|NES|>1$ and $FDR<0.25$ were considered to have significant enrichment) and used the R package "enrichplot" (version 1.12.1) to visualize the results.

Drug Sensitivity Analysis

The Gene Set Cancer Analysis (GSCA) database (http://bioinfo.life.hust.edu.cn/web/GSCALite/) integrated transcriptome data from the TCGA database, as well as drug response data, enabling comprehensive analysis of gene sets. The "Drug Sensitivity Analysis" module was utilized to analyze the correlation between gene expression and drug sensitivity. Moreover, we predicted each sample's response to targeted therapy based on the Genomics of Drug Sensitivity in Cancer (GDSC) database through R package "pRRophetic". Half-maximal inhibitory concentration (IC50) was considered as the indicator of drug sensitivity.

Statistical Analysis

All statistical calculations were conducted through R software (version 4.1.1). The comparison of differences between two groups was analyzed using Wilcoxon rank sum test. The comparison of differences between three or more groups was analyzed using the one-way ANOVA or Kruskal–Wallis test. The sensitivity and specificity of MEMTS for immunotherapy response prediction were analyzed through the receiver operating characteristic (ROC) curve, and the area under the curve (AUC) was quantified with pROC R package.

RESULTS

The Landscape of MEMTS in Gastric Cancer

The differential gene expression analysis was conducted between primary gastric tumor and ovarian metastatic tumor in FUSCC cohort **(Figure 1A)**. In view of the significant relationship between tumor metastasis and EMT, we examined the intersection of EMT gene set and DEGs upregulated in ovarian metastatic tumor. Thirteen genes (SNAI2, PFN2, NOTCH2, NID2, MEST, MATN2, LAMA1, ITGB3, GPX7, FBN2, ECM2, DPYSL3, BDNF) were identified as hub genes and subsequent analyses focused on them **(Figure 1B)**. Functional investigation of hub genes revealed that they could activate TSC/mTOR pathway, ER hormone, EMT, RTK pathway, RAS/MAPK pathway, and PI3K/AKT pathway but inhibit cell cycle and apoptosis **(Figure 1C)**, which indicated the predominant role of our hub genes in cancer progression and metastasis. Spearman correlation analysis between hub genes showed that most hub genes were significantly correlated with others **(Figure 1D)**. We further analyzed the distribution of hub genes in the chromosomes and showed that all of them were located in the autosomes **(Figure 1E)**. Considering the critical role of copy number variation and gene mutation in cancer progression (15), we conducted CNV analysis in the TCGA cohort and demonstrated that NOTCH2, MATN2, and MEST had the higher frequency of CNV among the 13 hub genes **(Figure 1F)**. On the other hand, we analyzed the mutation landscape of hub genes in GC patients, which showed that LAMA1 (13%) had the highest mutation frequency, followed by FBN2 (8%), NOTCH2 (6%), NID2 (3%), and DPYSL3 (3%), whereas the lowest mutation frequency was possessed by PFN2 (0%) **(Figure 1G)**. Of note, LAMA1, FBN2, NOTCH2, NID2, and DPYSL3 with a higher mutation frequency were significantly co-occurrent with other genes (**Figure 1H**).

Characterization of Molecular Features of MEMTS-High and -Low Subtypes

We further analyzed genomic alterations in MEMTS-high and MEMT-low subtypes. There was a rough similarity in the kinds of the top 30 genes with the highest mutation frequency between the low and high MEMTS subtypes, while mutation frequency of each gene in the MEMTS-low subtype was almost higher than that in the MEMTS-high subtype (**Figures 2A, B**). Tumor mutation burden has been emerging as a potential immunotherapy biomarker due to the generation of immunogenic neoantigens (16). Hence, we analyzed the differences in tumor mutation burden in

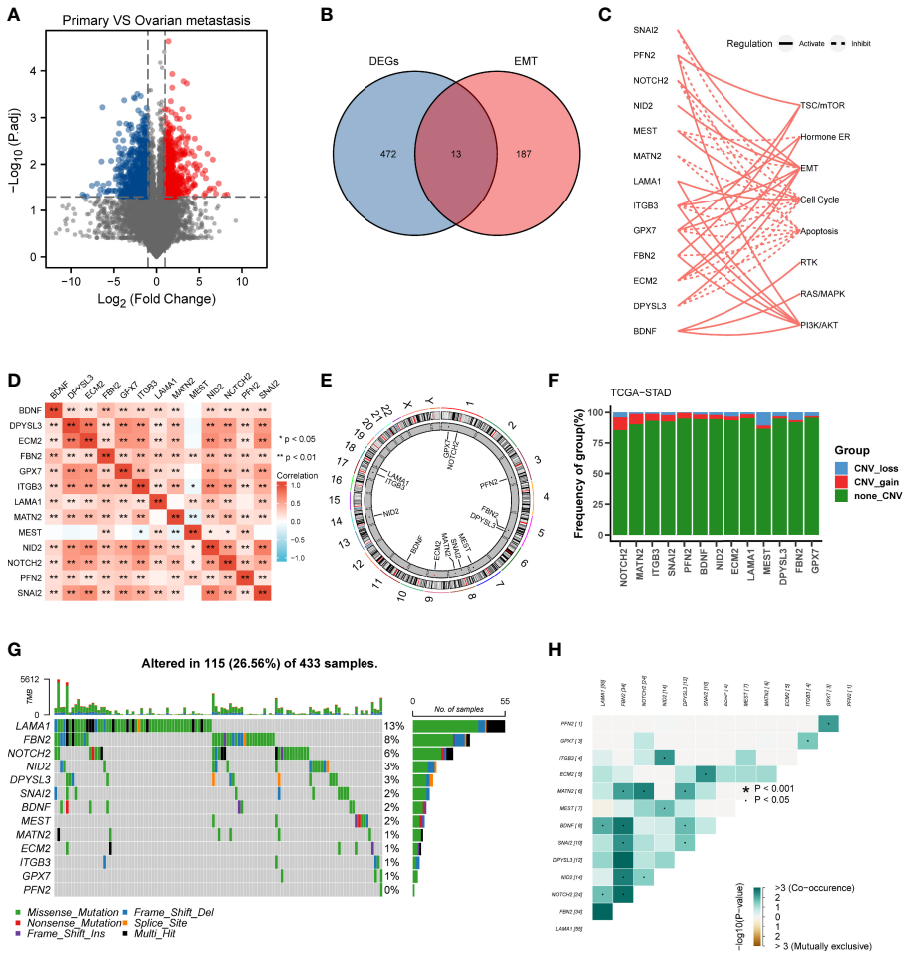

FIGURE 1 | Landscape of genetic variation and correlation of MEMTS in gastric cancer. **(A)** Volcano plot showing DEGs between primary gastric tumor and ovarian metastatic tumor. **(B)** Venn diagram showing 13 genes extracted through taking the intersection of DEGs and EMT hallmark genes. **(C)** The relationships between 13 genes and pathways. The solid and dashed lines denote regulation of activation and inhibition, respectively. **(D)** Spearman's correlation analyzing the correlation among 13 genes. Orange represents positive correlation and the depth of the color represents the size of correlation coefficient. **(E)** The distribution of 13 genes in the chromosomes. **(F)** CNV analysis of hub genes in TCGA cohort. **(G)** Oncoplots showing the mutation landscape of hub genes in GC patients from TCGA database. Each column represents an individual sample. The upper and right barplots denote TMB and the proportion of each variant type respectively while the mutation frequency in each gene is displayed by the numbers on the right. **(H)** The correlation among mutation of 13 genes. Green represents co-occurrence of mutations in two genes.

two subtypes, and the result showed low MEMTS subtype had a higher level of tumor mutation burden compared to MEMTS-high subtype (**Figure 2C**).

Based on different molecular characteristics, gastric cancer was classified into four molecular subtypes respectively in TCGA and ACRG cohort. We found that the MEMTS was the lowest in the Epstein–Barr virus (EBV) subtype and microsatellite instability (MSI) subtypes in the TCGA cohort (**Figure 2D**). In the ACRG cohort, we found that molecular subtype in possession of the highest MEMTS was the epithelial-mesenchymal transition (EMT) subtype, while that in possession of the lowest MEMTS was the microsatellite instability (MSI) subtype (**Figure 2E**). Then we singled out the top 15 mutated genes which positively related to the MEMTS in the TCGA cohort (**Figure 2F**). Considering that high expression of immune checkpoints indicated better response to immunotherapy, we investigated the correlation between these mutated genes and the expression of immune checkpoints. The result showed that the expression of immune checkpoints (CD274, PDCD1, and CTLA4) in patients with PIK3CA or NRXN3 mutation was significantly higher than those in patients with PIK3CA or NRXN3 wild type (**Figures 2G, H**).

The Correlation Between MEMTS and Clinical Features and Prognosis

Next, we compared the clinical features in MEMTS-low and MEMTS-high subtypes in both TCGA and ARCG cohorts. We demonstrated that the proportion of patients at T4 stage was higher in MEMTS-high subtype whereas the proportion of patients at T1 stage was higher in MEMTS-low subtype

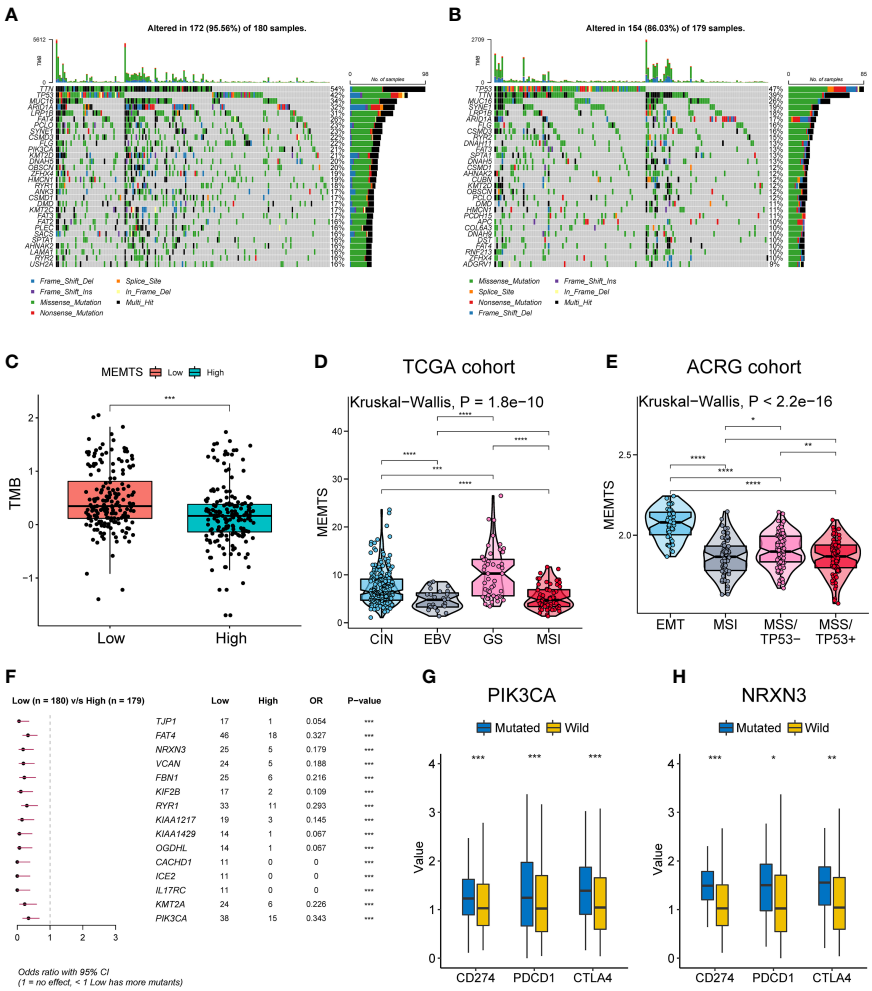

FIGURE 2 | Relationship among the MEMTS, genomic alterations, and molecular subtypes in gastric cancer. **(A, B)** Oncoplots showing landscapes of genomic alterations in low and high MEMTS subtypes, respectively. **(C)** Differences in tumor mutation burden between low and high MEMTS subtypes (***P < 0.001, Wilcoxon test). **(D, E)** The correlations between the MEMTS and TCGA molecular subtypes (Kruskal-Wallis, P = 1.8×10^{-10}), as well as ACRG molecular subtypes of gastric cancer (Kruskal-Wallis, P < 2.2×10^{-16}). The plot shows the median value, the 25th, and 75th percentiles (central lines, bottom, and top of the box), while the whiskers embody 1.5 times the interquartile range (*P < 0.05; **P < 0.01; ***P < 0.001, ****P < 0.0001, Wilcoxon test). **(F)** Top 15 genes with the highest mutation frequency related to the MEMTS in the TCGA cohort. **(G, H)** PIK3CA and NRXN3 mutations distinctly facilitated expression of immune checkpoints (CD274, PDCD1, CTLA4) (*P < 0.05; **P < 0.01; ***P < 0.001, Wilcoxon test).

(Figure 3A). In terms of clinical outcome, the prognosis of patients with high MEMTS was poor when compared to patients with low MEMTS in terms of both progression-free survival (PFS) and overall survival (OS) **(Figures 3B–E)**, which indicated that MEMTS could predict prognosis in patients with GC.

MEMTS Predicted the Response to Adjuvant and Targeted Therapy in GC

It has been demonstrated in various clinical research that adjuvant chemotherapy could improve the prognosis of patients with advanced gastric cancer compared to surgery alone (5, 17). However, drug resistance caused by EMT remained a large obstacle to chemotherapy response. We also investigated the correlation between drug sensitivity and hub genes expression based on the Cancer Therapeutics Response Portal (CTRP) database. The results showed the expression of most of the hub genes was positively related to the IC50 of cancer therapy drugs while the expression of GPX7 was the opposite **(Figure 4A)**. These results could be used to guide the formulation of chemotherapy regimens.

Then, we investigated the correlation between MEMTS and the response to adjuvant chemoradiotherapy in 432 patients who received homogeneous chemoradiotherapy (5-fluoouracil/ leucovorin combined with radiation) after surgery from the SMC cohort. The results showed that patients with low MEMTS gained more benefits in both overall survival and recurrence-free survival than patients with high MEMTS **(Figures 4B, C)**. Analysis aimed at the MDACC cohort showed that the high MEMTS subtype benefited more from neoadjuvant chemoradiotherapy than neoadjuvant chemotherapy **(Figure 4D)**, which suggests that

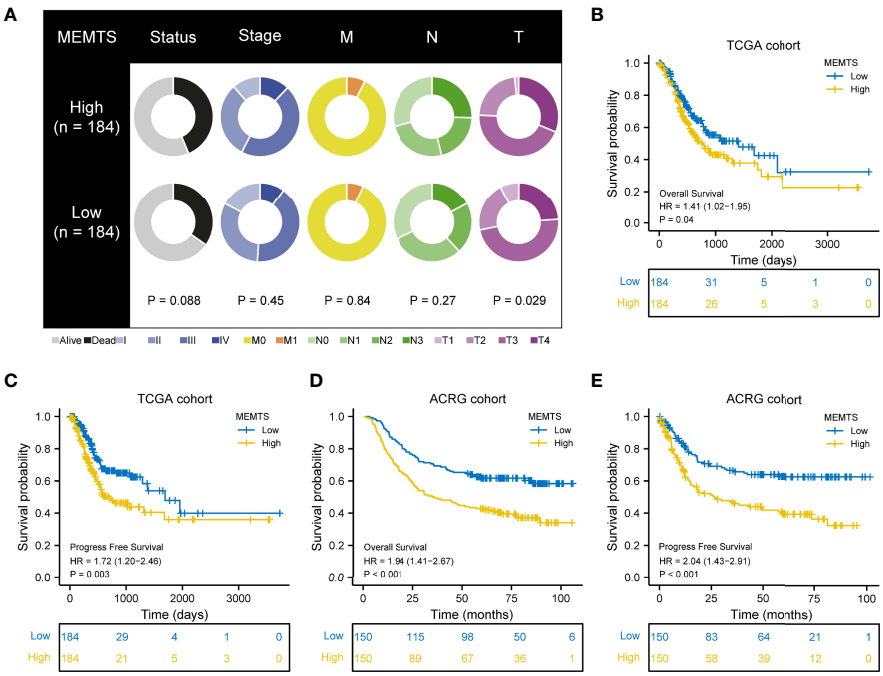

FIGURE 3 | Correlation between MEMTS subtypes and clinicopathological features and prognosis in TCGA and ACRG cohort. **(A)** Different clinicopathological features of high and low MEMTS subtypes in TCGA cohort. **(B–E)** Kaplan–Meier survival plots showing the differences of overall survival and progression-free survival between high and low MEMTS.

neoadjuvant chemoradiotherapy was suitable for patients with high MEMTS. However, the result need to be treated with caution due to the limited number of patients. Interestingly, drug sensitivity analysis uncovered that the IC50 of multiple targeted drugs such as pazopanib, imatinib, nilotinib, and dasatinib in MEMTS-high subtype was significantly lower than those in MEMTS-low subtype (**Figure 4E**), which indicates that patients with high MEMTS are probably more sensitive to targeted therapy. To sum up, MEMTS was instrumental in predicting the response to adjuvant therapy for GC patients. The patients with low MEMTS subtype might benefit from adjuvant chemotherapy while the patients with high MEMTS subtype might benefit from targeted therapy.

MEMTS Predicted the Response to Immunotherapy in GC

The advent of immunotherapy typically represented by PD1/PDL1 checkpoint inhibitors served as an important milestone in tumor treatment. Nivolumab, a monoclonal PD-1 blockade, has recently been approved as first-line treatment in patients with advanced or metastatic gastric cancer in America (18). Considering the promising efficacy of immunotherapy, especially inhibitors of immune checkpoints such as PD-1 and PD-L1, in multiple malignancies including GC, we further evaluated the predictive role of MEMTS in KIM cohort and Hugo cohort. On one hand, we assessed the relationship between MEMTS and immunotherapy responses in KIM cohort, which was made up of advanced GC patients receiving PD-L1 blockade treatment. The MEMTS of patients with different therapeutic effect are shown in **Figure 5A**. The MEMTS of patients in the progressive disease (PD)/stable disease (SD) group was significantly higher than that in the partial response (PR)/complete response (CR) group (**Figure 5B**). Notably, MEMTS-low was the dominant subtype (83%) in the PR/CR group while MEMTS-high was dominant subtype (66%) in the PD/SD group, suggesting that MEMTS was an unfavorable factor for immunotherapy response in GC (**Figure 5C**). We found that MEMTS was negatively correlated with expression of PD-L1 and PD-1 (**Supplementary Figure S1**). Of note, the expression of PD-L1 and PD-1 was reportedly correlated with the response to immunotherapy and clinical outcomes in both major laboratory studies and clinical trials such as Checkmate-649 (19, 20). Furthermore, Both MSI score and EBV status are reportedly the major indicators for the efficacy of immunotherapy (18, 21). Compared with patients with high MSI score or positive EBV status, patients with low MSI score or negative EBV status possessed higher MEMTS (**Figures 5D, E**). Correspondingly, we established the AUC of MEMTS and revealed that the AUC value of MEMTS (0.896) was higher than that of MSI score (0.693) and EBV status (0.708) (**Figure 5F**). On the other hand, we conducted similar analyses in the Hugo cohort consisting of melanoma patients treated with PD1 blockade. Likewise, patients in the response group had lower MEMTS compared to those in the non-response group (**Figure 5G**). In addition, MEMTS was negatively correlated with expression of PDCD1 (**Figures 5H, I**), the protein-coding gene of PD-1, whereas the AUC value of

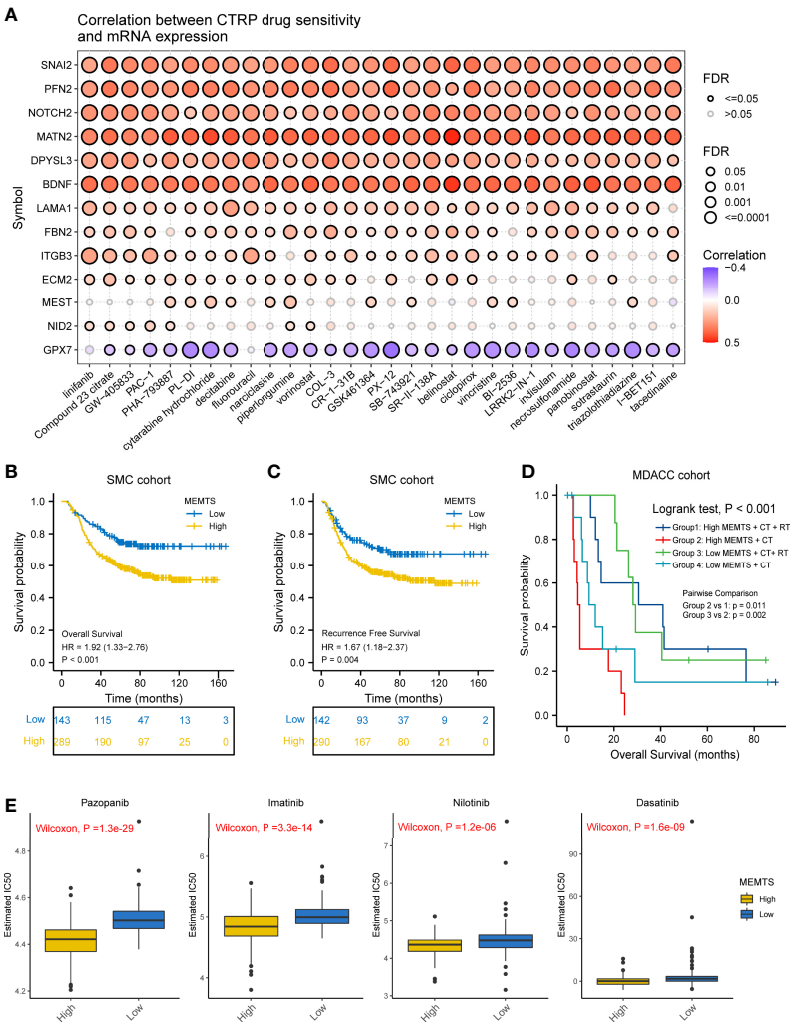

FIGURE 4 | Prediction and correlation of the sensitivity to chemotherapy drugs in gastric cancer. **(A)** The correlation between GDSC drug sensitivity and hub genes expression. **(B)** Kaplan–Meier survival plots showing the differences of overall survival and recurrence free survival between high and low MEMTS. **(C)** Postoperative chemoradiotherapy can significantly improve the prognosis of patients with high and low IMS compared to chemotherapy. **(D)** Kaplan–Meier plot showing the OS of low and high subgroup stratified by MEMTS in patients treated with neoadjuvant chemotherapy or neoadjuvant chemoradiotherapy (MDACC cohort). **(E)** The high MEMTS subtype was related to the lower IC50 of multiple targeted drugs including pazopanib, imatinib, nilotinib, and dasatinib.

MEMTS was higher than that of PDCD1 expression (**Figure 5J**), indicating the potential predictive value of MEMTS in terms of immunotherapy response in patients with multiple malignancies. In summary, as patients of MEMTS-low subtype were more likely to benefit from immunotherapy and vice versa, MEMTS could serve as a promising prediction index of immunotherapy response in GC patients.

MEMTS and Tumor Microenvironment in GC

Tumor microenvironment (TME) played a fundamental role in tumor progression and therapeutic response. To understand the correlation between tumor immune microenvironment and MEMTS subtypes, we used CIBERSORT algorithm to evaluate the distribution of 22 infiltrated immune cells in MEMTS-high and -low subtypes. It was observed that the MEMTS-high subtype exhibited significantly higher infiltration of immunosuppressive cells including T cell regulatory (Tregs) and M2 subtype of macrophages (**Figure 6A**). With respect to non-immune cells, MEMTS was significantly correlated with cancer associated fibroblasts (CAFs) (**Figure 6B**). As CAFs act as stromal cell clusters to exclude T cell infiltration and function (22), we further explored the relationship between MEMTS, stromal score, and T cell exclusion and uncovered that MEMTS was positively associated with both exclusion and stromal score in GC (**Figures 6C, D**). To characterize the function of MEMTS, we assessed the relationship between MEMTS and the known molecular signatures (**Figure 6E**). Of note, stroma-activated pathways such as EMT2, EMT3, Panfibroblast TGF-β response characteristics (Pan-F TBRS),

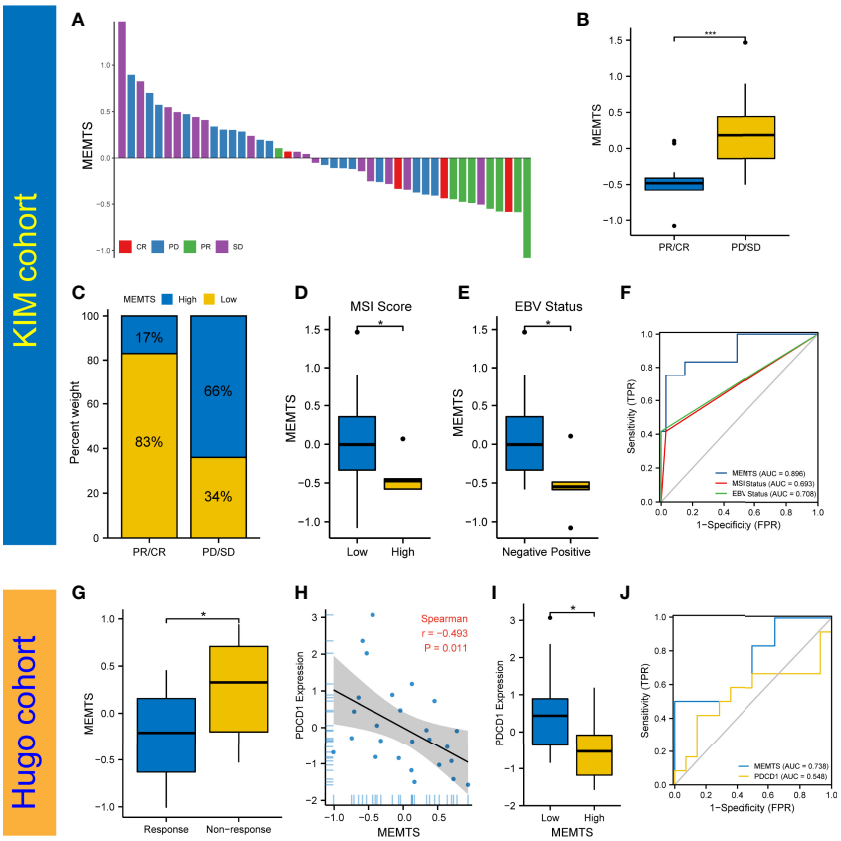

FIGURE 5 | Prediction of the response to immune checkpoint blockade treatment. **(A)** The correlation of MEMTS with response to immunotherapy in KIM cohort. CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease. **(B)** Difference in MEMTS between PR/CR group and PD/SD group (***P < 0.001, Wilcoxon test). **(C)** The proportion of patients with different response to immunotherapy in two MEMTS subtypes. **(D)** Difference in MEMTS between low group and high MSI score group (*P < 0.05, Wilcoxon test). **(E)** Difference in MEMTS between negative and positive EBV status (*P < 0.05, Wilcoxon test). **(F)** The predictive value of MEMTS, MSI status and EBV status in patients treated with immunotherapy (AUC of MEMTS, 0.896; AUC of MSI status, 0.693; AUC of EBV status, 0.708). **(G)** boxplot showing patients with no response to immunotherapy had the higher MEMTS than patients with response to immunotherapy (*P < 0.05, Wilcoxon test). **(H)** Spearman analysis of correlation between MEMTS and PDCD1 expression in Hugo cohort (r = -0.493, P = 0.011). **(I)** Difference in PDCD1 expression between low and high MEMTS subtypes in Hugo cohort (*P < 0.05, Wilcoxon test). **(J)** The predictive value of MEMTS, PDCD1 in Hugo cohort (AUC of MEMTS, 0.738; AUC of PDCD1, 0.548).

and angiogenesis were found to be positively associated with MEMTS while tumor suppressive pathways were negatively related to MEMTS. The oncogenic and immunosuppressive role of MEMTS in GC was illustrated in the heatmap based on ssGSEA analyses (**Figure 6F**). Similarly, the oncogenic pathways such as E2F targets, hypoxia, and EMT were remarkably enriched in MEMTS-high subtype (**Figure 6G**). These results taken together reveal the relationship between MEMTS and TME, which potentially explains the predictive value of MEMTS in adjuvant therapy response in GC.

Recently, single-cell RNA sequencing (scRNA-seq) has emerged as a powerful technology to characterize molecular features of individual cells, which enables the highly accurate understanding of tumor microenvironment (23). To further address the role of MEMTS in TME, we analyzed GSE167297 dataset which was derived from the scRNA-seq analysis of single cells from diffuse-type GC using 10X Genomics. By using UMAP algorithm, 19,765 cells screened out by quality control were divided into eight cell clusters, each of which was annotated with cell lineage based on the cell lineage marker genes. The majority of the annotated cell clusters were immune cells including B cells, T cells, macrophages, NK cells, and dendritic cells (DC) (**Figure 7A**). Apart from the above immune cells, stromal cells such as fibroblasts, endothelial cells, and epithelial cells were the non-negligible ingredients in single-cell atlas. The hub genes of MEMTS were detected mainly in the deep layers of GC tissues (**Figure 7B**). Then we explored the expression of hub genes in eight cell clusters. Intriguingly, most hub genes were highly expressed in fibroblast while NOTCH2, DPYSL3, and MATN2 were also expressed at considerably high levels in endothelial cells or macrophages (**Figure 7C**). In addition, in consistency with previous results, MEMTS was mainly enriched in the fibroblasts, followed by endothelial cells and macrophages

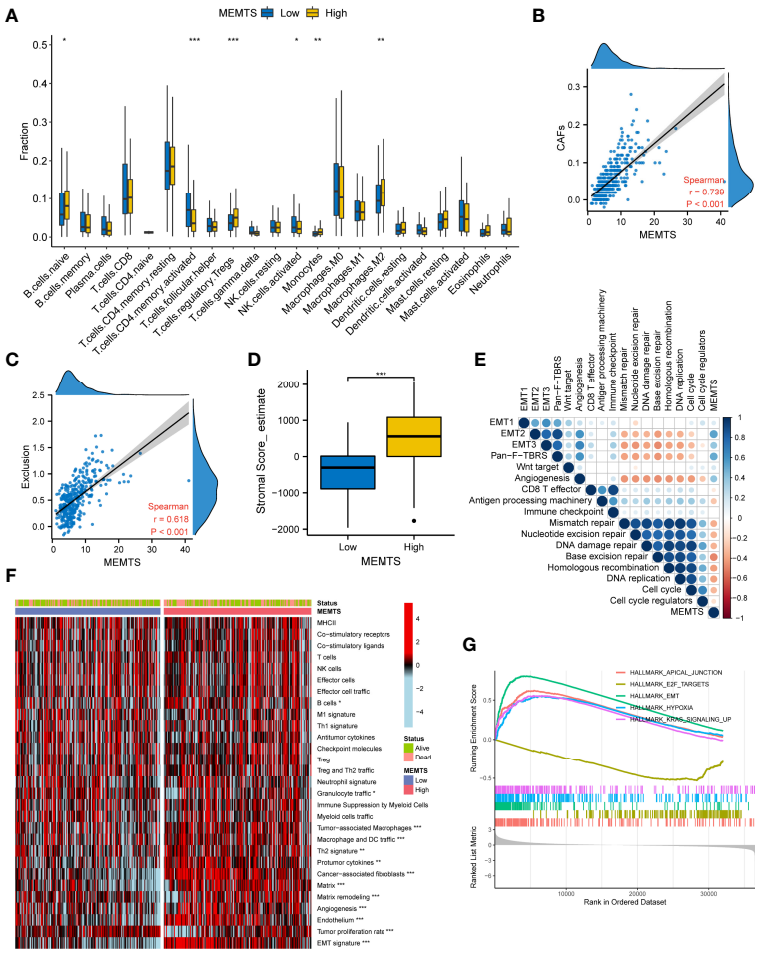

FIGURE 6 | Correlation between the MEMTS and tumor microenvironment in gastric cancer (TCGA cohort). **(A)** Box plots illustrating the relationships between MEMTS subtypes and the infiltration of 22 immune cells (*P < 0.05; **P < 0.01; ***P < 0.001, Wilcoxon test). **(B)** Spearman analysis of correlation between MEMTS and CAFs (Spearman test, r=0.739, P < 0.001). **(C)** Scatter plot depicting a close correlation between MEMTS and T cell exclusion (Spearman test, r=0.618, P < 0.001). **(D)** stromal score calculated by estimate were significantly associated with MEMTS subtypes (Wilcoxon test, ***P < 0.001). **(E)** A corrplot demonstrating correlations among MEMTS and the known gene signatures in TCGA cohort by Spearman analysis. Coefficients are characterized in color and size. Negative and positive correlations are marked with orange and blue, respectively. **(F)** heatmap displaying the relationship among the MEMTS, the status and the pathways related to immunity and tumorigenesis in two subtypes (*P < 0.05; **P < 0.01; ***P < 0.001, Wilcoxon test). **(G)** Curves showing the results of gene enrichment in apical junction, E2F targets, EMT, hypoxia pathways and KRAS signaling.

(**Figure 7D**). Consequently, we further explored the potential association of hub genes with fibroblasts which were classified into inflammatory CAFs (iCAFs) and myofibroblasts (**Figure 7E**). iCAF clusters are featured with high expression of chemokines including CXCL1, CXCL14, CCL2, and interleukin 33 (IL33) while myofibroblast clusters are featured with high expression of ACTA2. A majority (11/13) of hub genes were mainly expressed in iCAFs except for SNAI2 and PFN2 (**Figure 7F**). Notably, MEMTS was primarily manifested in iCAFs rather than myofibroblasts (**Figure 7G**). Taken together, our analyses uncovered the relationship between MEMTS and TME, which not only indicated the significance of MEMTS in tumor immunity and metastasis but also facilitated us to better understand the predictive value of MEMTS in multiple types of adjuvant therapy in GC.

DISCUSSION

GC is a disease accompanied by heavy social economic burden, high incidence, and high mortality (24). Despite great advancements in treatment of GC, tumor recurrence caused by metastasis and drug resistance are still threats to patients with GC (1, 25). As one of the essential processes of tumor metastasis, EMT referred to the transdifferentiation of epithelial phenotypes into mesenchymal phenotypes. EMT enhanced the ability of

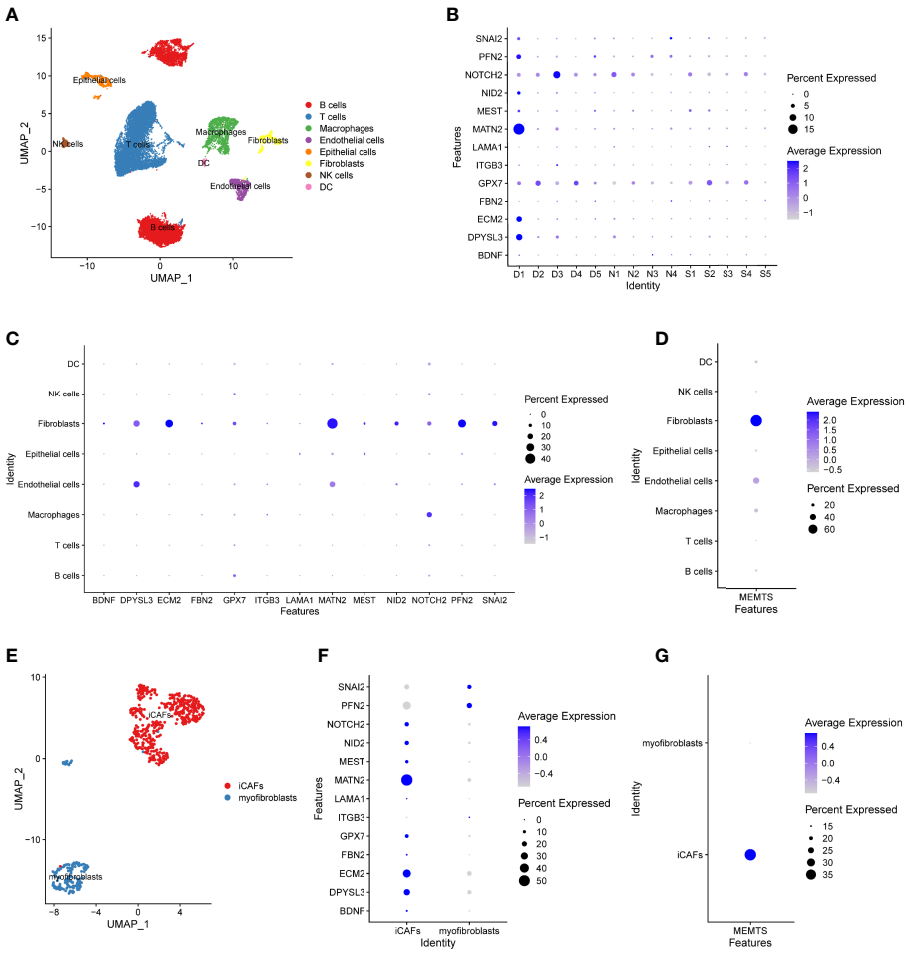

FIGURE 7 | The distribution of the MEMTS in tumor microenvironment. **(A)** UMAP plot showed eight cell types from 19,765 cells. **(B)** The different features of hub genes in deep layer (D1, D2, D3, D4, D5) and superficial layer (S1, S2, S3, S4, S5) of tumor tissues and paired normal tissues (N1, N2, N3, N4). **(C)** The different expression of hub genes in eight cell clusters. **(D)** MEMTS was mainly concentrated in the fibroblasts. **(E)** Fibroblasts were classified into inflammatory CAFs and myofibroblasts on the basis of different molecular characteristics. **(F)** The different features of hub genes in inflammatory CAFs and myofibroblasts. **(G)** MEMTS was primarily manifested in inflammatory CAFs.

tumor cells in migration and invasion, and the expression of certain genes could be used to explore the extent of EMT. In this study, we intersected the gene set associated with EMT and differentially expressed genes between primary gastric tumor and ovarian metastatic tumor. Subsequently we obtained a gene set containing 13 genes and analyzed the correlations and characteristics among them.

Increasing research has revealed the significant relationship between gene mutations and the metastasis of tumor. Tumor mutation burden (TMB) also played a crucial role in improving immunotherapy response in treatment of cancer on account of increased tumor neoantigens expression (16). It has been reported that EMT was negatively related to TMB due to the switch of MLH1 from silence to activation (26). MLH1 was responsible for gene mismatch repair and it was remarkably silenced by methylation of its promoter regions. What's more, MSI molecular subtype of GC was characterized by higher mutation rates and hypermethylation at MLH1 promoter (27). However, it was activated to make DNA repair system intact when EMT occurred, leading to a lower mutation rate. Our results also showed that the high MEMTS subtype showed lower somatic mutation rate and TMB while the low subtype showed the opposite result. Additionally, several studies have demonstrated that patients with the MSI and EBV subtypes of GC were more sensitive to PD1 inhibitors such as pembrolizumab (28). According to our study, MEMTS was significantly lower in the EBV subtype and MSI subtype compared to the other two subtypes. These results further demonstrate that the MEMTS could be used as a robust model for stratifying patients with GC.

Tumor microenvironment (TME) had a noticeable impact on not only progression of tumor but also therapeutic response and clinical outcome. Tumor-infiltrating immune cells and carcinoma-associated fibroblasts (CAFs) within tumor stroma

made the crucial contributions to the activation of EMT progression (29). CAFs in prostate cancer induced EMT *via* secretion of MMPs, which promotes the dissociation of extracellular domain of E-cadherin (30). JAK2/STAT3 pathway was activated by CAFs to promote EMT (31). Stromal cells and altered extracellular matrix also contributed to a sophisticated fiber network in favor of migration and invasion of tumor cells (32). Various molecules originated from CAFs and tumor-infiltrating immune cells such as TGF-β, FGF, EGF, HGF, and IGF1 along with Hedgehog, Notch, and Wnt signaling pathways could promote EMT (33). Apart from promotion of EMT as previously mentioned, TGFβ stimulated tumor development by motivating angiogenesis and fibroblast activation, and attenuated PD-L1 inhibitor response through exclusion of T cells, empowering tumor cells to evade antitumor immune responses (34, 35). HIF-1 downregulated the expression of E-cadherin indirectly by intensifying the expression of ZEB1, ZEB2, and TCF3 and also directly activated the TWIST1 promoter, which demonstrated that hypoxia could contribute to EMT development (36). Fully exploring the alterations of TME characteristics induced by distinct MEMTS patterns, our study showed MEMTS signatures have been investigated to be apparently related to immune infiltration and TME alteration.

Then we explored the role of MEMTS in therapeutic prediction. Aimed at improving antitumor immune response, immunotherapy has revolutionized the paradigm for tumor treatment. Immune checkpoint inhibitors are one of the most profoundly explored immunotherapies for the moment. Improvement in overall survival has been demonstrated in PD1, PDL1, and CTLA4 checkpoint inhibitor strategies compared to conventional chemoradiotherapy (3). However, success of antitumor therapy was usually limited by poor immunotherapy response and the development of drug resistance, which might result from insufficient quantity of infiltrating T cells, absence of checkpoints expression in both tumor cells and T cells, and adapted resistance to checkpoint blockade (37). As to EMT, mesenchymal subtype was widely perceived as a negative role in predicting immunotherapy response and a dominant factor of poor survival in gastric cancer, because it could facilitate immune escape through obstructing drug penetration to the core of tumors by altered TME and insufficient susceptibility to immune effector cells (38). Most recently, it has been explored that EMT simultaneously increased drug resistance through overexpressing ATP-binding cassette (ABC) transporter family and increasing cellular resistance to drug-induced apoptosis (39–41). In our research, more mutation rate in low MEMTS led to immune-checkpoint gene overexpression. Moreover, in KIM cohort, MEMTS showed higher AUC than that of MSI and EBV status. In the Hugo cohort, MEMTS had inverse correlation with PDCD1 expression and AUC of MEMTS was higher compared to PDCD1. All the above results demonstrate MEMTS is an advantageously predictive tool in precision immunotherapy for gastric cancer.

The application of MEMTS must be exercised cautiously as certain limitations of this study are noted. Firstly, the construction of MEMTS is based on the DEGs from transcriptomic analyses of paired primary GC and ovarian metastatic tumors. Therefore, the validity of MEMTS in GC patients with other types of metastases are yet to be investigated even though we tried to address this issue by not only intersecting DEGs with EMT-related gene sets from publicly available databases but also by assessing its predictive value in multiple independent cohorts. Moreover, considering that these cohorts are merely retrospective, further prospective clinical trials are required to validate our findings, especially the predictive role of MEMTS in terms of prognosis and response to various therapeutic types.

To sum up, we constructed an EMT gene set score to stratify GC patients in various cohorts and explored the underlying mechanisms leading to different characteristics between high and low MEMTS samples, which improved our understanding of EMT progression in GC. The results show that the MEMTS could be used to stratify patients and identify those who would benefit more from adjuvant therapy and immunotherapy and to detect brand new strategies and targets for cancer treatment.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE191139.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Clinical Research Ethics Committee of Fudan University Shanghai Cancer Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XL and JG conceived and designed this study. JS and SH performed the experiments. JS and RW analyzed the data and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.920512/full#supplementary-material

Supplementary Figure 1 | The correlation between MEMTS and the expression of immune checkpoints. **(A)** The correlation between MEMTS and the expression of PD-L1. **(B)** The correlation between MEMTS and the expression of PD-1.

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Case report: A colorectal cancer patient with microsatellite instability-high and *MSH2* germline mutation failed to respond to anti-PD-1 immunotherapy

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Lynch syndrome (LS) is characterized by germline mutations in the DNA mismatch repair (MMR) genes. In colorectal cancer (CRC), germline mutations of DNA MMR genes commonly lead to microsatellite instability-high (MSI-H) subtype formation. Recent studies have demonstrated that CRC patients with MSI-H or mismatch repair-deficient (dMMR) status can benefit from anti-PD1 immunotherapy. However, almost 50% of CRC patients with MSI-H status do not respond to it. It is reported that heterogeneity of tumor and abnormal activation of cancer-related signaling pathways contribute to resistance to anti-PD1 therapy. To improve the clinical efficacy of such patients, the underlying mechanisms of resistance to anti-PD1 treatment must be explored. In this case, we describe an LS-associated CRC patient with MSI-H who suffered resistance to anti-PD1 therapy. Here, we attempted to elucidate the potential reasons, and thus appropriate strategies may be derived to overcome this clinical problem.

KEYWORDS

microsatellite instability-high, colorectal cancer, anti-PD1 therapy, lynch syndrome, resistance

Introduction

Lynch syndrome (LS) is caused by germline inactivation of one allele of genes involved in the mismatch repair (MMR) system, namely MLH1, MSH2, MSH6, and PMS2 (1). Inactivation of MLH1 or PMS2 alleles is the most frequent and is associated with approximately 80% of LS cases (2). LS-associated CRC usually presents as a microsatellite instability-high (MSI-H) subtype. Several clinical trials have confirmed CRC patients with MSI-H/dMMR are the beneficiaries of anti-PD1 therapy, and the overall response rate varies from 40% to 60%. Based on these data, anti-PD1 monoclonal antibody (mAb) was approved for first-line treatment of advanced MSI-H CRC, which revolutionized the treatment mode of metastatic colorectal cancer (mCRC) (3). Clinical data have demonstrated the long-lasting and stable antitumor efficacy of anti-PD1 therapy in MSI-H mCRC (4).

Despite the remarkable efficacy of anti-PD1 therapy in advanced MSI-H/dMMR CRC patients, nearly half of MSI-H/dMMR CRC patients do not respond to it. Studies have indicated that the underlying resistance mechanisms and the most straightforward reason are the absence of tumor antigens leads to a lack of recognition by T cells (5). Recently, abnormal activation of multiple tumor-associated signaling has been identified to contribute to the resistance of anti-PD1 mAb. The mitogen-activated protein kinase (MAPK) pathway, the PTEN expression, the PI3K signaling, and the WNT/b-catenin signaling pathway are the main immuneevasive oncogenic signaling pathways. Moreover, loss of interferon-gamma signaling pathways and lack of tumor antigen expression were also involved in tumor immune escape (6–11). With the development of gene sequencing platforms, sequencing analysis of MSI-H/dMMR subtypes of CRC revealed that some specific gene mutations also lead to resistance to PD1 mab therapy. Here, we present a CRC patient with MSI-H and germline MSH2 mutation who failed to respond to anti-PD1 treatment. Immunohistochemical (IHC), PCR, and next-generation (NGS) sequencing assays were used to explore the underlying mechanism of this resistance.

Case description

Case presentation and treatment

A 27-year-old young female patient presented with abdominal pain and with no excrement for 1day and was sent to the hospital for physical examination in July 2017. Colonoscopy examination revealed a space-occupying lesion 30cm from the anal margin. Pathological examination suggested poorly differentiated adenocarcinoma. Computed Tomography (CT) examination showed no metastasis. Then, this patient underwent a radical resection of left colon cancer. The postoperative pathological stage was pT3N2aM0. Postoperative baseline assessment showed no recurrence and metastasis in this patient. We chose the XELOX regimen as postoperative adjuvant chemotherapy. However, this patient had progressive disease (PD) after six cycles of XELOX chemotherapy. ECT and CT examination suggested bone destruction of the right iliac crest, which was considered metastasis. Colonoscopy revealed a neoplasm at the top of the anastomosis, and pathology revealed a moderately differentiated adenocarcinoma (Figure 1A). Since genetic test results suggested a *KRAS* gene mutation in this patient, bevacizumab combined with the FOLFIRI regimen was used as a second-line treatment for her. In addition, we performed local radiotherapy on her

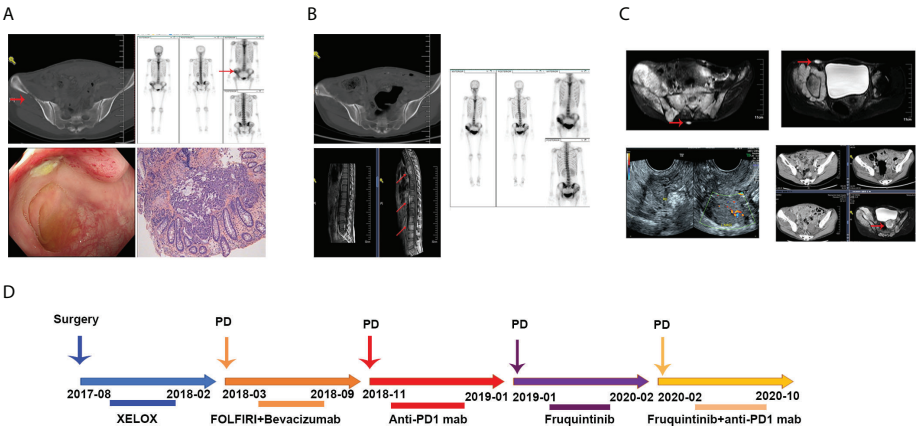

FIGURE 1
(A) Radiologic images of tumor metastases after the XEOLX therapy. **(B)** Radiologic images of tumor metastases after the second-line treatment. **(C)** Radiologic images of tumor metastases after anti-PD1 immunotherapy. **(D)** The entire treatment process of this patient.

right iliac crest metastatic lesion. However, after two cycles of combined targeted therapy with chemotherapy treatment, she developed disease progression again, presenting with enlarged tissue mass shadow around the right iliac crest and multiple metastases in the thoracolumbosacral vertebral body and appendages (Figure 1B). We reviewed her tumor tissue immunohistochemical (IHC) results and found a loss of MSH2 protein (Figure 3B). PCR and NGS tests further confirmed an MSI-H status in this patient. NGS tests also indicated a high tumor mutation burden (TMB) in the patient's peripheral blood and tumor tissue, suggesting that the patient is likely to benefit from PD1 mab treatment. According to it, we chose anti-PD1 therapy (Tislelizumab 200mg every three weeks) as the third-line treatment. Unfortunately, this patient experienced a rapid progression again with widespread metastases including bone, ovary, and retroperitoneal lymph nodes after three cycles of anti-PD1 therapy (Figure 1C). When that, we chose furoquitinib and furoquitinib combined with sindilizumab as the follow-up treatment for her. During this period, this patient's lesions were in a state of slow progression. Due to her poor physical condition, she discontinued therapy in October 2020 and received the best supportive care. She died in April 2021. To intuitively express this patient's treatment process and efficacy, we listed the entire treatment process in Figure 1D.

Family history

Interestingly, when we reviewed her family history, it was worth noting that her grandmother had CRC, three uncles had CRC, and her grandmother's siblings and their children all had CRC. Her family history fulfilled Amsterdam criteria. We presented her family history in Figure 2. NGS tests on blood and tumor tissue showed this patient had an *MSH2* germline mutation. Based on the above clinical and laboratory findings, she was diagnosed as a Lynch syndrome (LS) associated MSI-H CRC patient.

IHC test and gene analysis

We used IHC assay to detect the protein expression of MLH1, MSH2, MSH6, PMS2, CD8, and PD-L1 in the formalin-fixed, paraffin-embedded (FFPE) tumor tissues. For quantitative analysis, we used ipp software to analyze the density of CD8 and PDL1. These data showed a small number of CD8+T cells were infiltrated in the tumor, and a large number of CD8+T cells were infiltrated in the stroma (Figure 3A). And we found a negative protein expression of PDL1 in this patient (Figure 3A).

The tissue DNA was extracted with a QIAamp DNA FFPE tissue kit (Qiagen, Valencia, CA, USA). Circulating tumor DNA (ctDNA) was extracted from plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, CA, USA). The tissue DNA and ctDNA were measured by Qubit 2.0 Fluorometer with a Qubit double-stranded DNA assay kit (Life Technologies, Carlsbad, CA, USA). Capture-based targeted sequencing was performed on tumor tissue and plasma samples using a panel (OncoScreen, Burning Rock Biotech, Guangzhou, China) consisting of 520 cancer-related genes. Sequencing data analysis was performed by OncoScreen Plus™. The result from the peripheral blood test revealed nine gene mutations with high frequency, including *KRAS* p.Ala146, *MSH2* c.793-1G>A, *MSH6* p.lys247fs, *AKT1* p.Glu17Lys, *APC* p.Asn1979fs, *ARID1A* p.phe2141fs, *RB1* p.Ala74fs, *RB1* p.Val654fs and *SLX4* p.Ala938fs. The result from FFPE showed six gene mutations with high frequency, including *KRAS* p.G13D, *MSH2* c.793-1G>A, *APC* p.Asn1979fs, *ATR* p.F153fs, *PTEN* p.K267fs and *PTEN* p.N323fs. These mutations were showed in Figure 3C. In addition, the results of tumor mutation burden from peripheral blood and tumor tissue were 100/Mb and 63.5/Mb, respectively (Figure 3D).

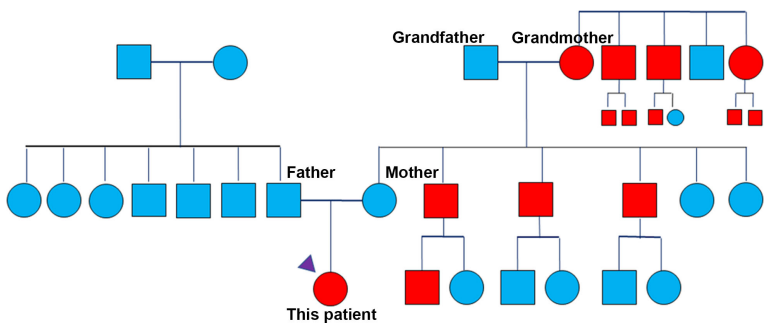

FIGURE 2
The characteristics of her family history.

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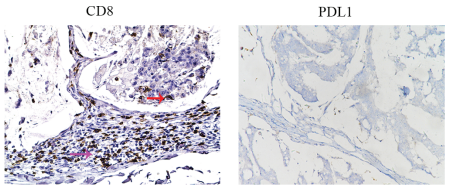
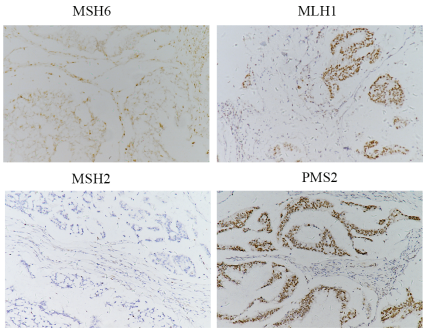
A



Markers	Stroma	Intratumoral
CD8	48 (counts/mm2)	272 (counts/mm2)
PDL1	0 (counts/mm2)	0 (counts/mm2)

B



C

Tissue	Blood
KRAS p.G13D	KRAS p.Ala146
MSH2 c.793-1G > A	MSH2 c.793-1G > A
APC p.Asn1979fs	APC p.Asn1979fs
ATR p.F153fs	AKT1 p.Glu17Lys
PTEN p.K267fs	MSH6 p.lys247fs
PTEN p.N323fs	ARID1A p.phe2141fs
	RB1 p.Ala74fs
	RB1 p.Val654fs
	SLX4 p.Ala938fs

D

	Tissue	Blood
TMB	63.5/Mb	100/Mb

FIGURE 3
(A) The protein expression of CD8 and PDL1 and red arrow represents CD8 positive T cells in the tumor, and the purple arrow represents CD8 positive T cells in the stroma. Quantification results of CD8 and PDL1 expression by IHC with the tumor and tumor stroma. (Microscopic magnification×200) **(B)** IHC images of MMR protein expression in this patient (Microscopic magnification×200); **(C)** NGS detection showed several gene mutations with high frequency from peripheral blood and tumor tissue in this patient. **(D)** Results of TMB detection from peripheral blood and tumor tissue in this patient.

Discussion

This is a case of failure from anti-PD1 therapy in LS-associated MSI-H CRC. This is also a CRC case to describe characteristics such as TMB, tumor immune infiltration factors, and some gene alterations in LS-associated MSI-H CRC patients. In this case, we tried to elucidate the reasons for resistance to ant-PD1 immunotherapy.

Two forms of testing are commonly used in screening MMR or MSI status. IHC was used for detecting MMR proteins and PCR testing for MSI. Due to the development of gene sequencing platforms, NGS has been applied more and more in gene detection. In this case, the IHC assay revealed a loss of MSH2 protein expression. Besides, the PCR test also showed all five single nucleotide sites (BAT-25, MONO-27, CAT-25, BAT-26, and NR-24) were changed. Thus, there is no doubt that this patient is an MSI-H/dMMR CRC patient. As a germline mutation, LS-associated tumors are commonly microsatellite unstable. In this case, the NGS test showed this patient had an MSH2 germline mutation and high TMB. Combined with her family history, she was diagnosed with an LS-associated MSI-H CRC.

Recent studies have demonstrated that solid tumors with MSI-H/dMMR subset commonly obtained a favorable response from anti-PD1 mAb, including CRC (12–14). The excellent efficacy of anti-PD1 therapy in treating MSI-H/dMMR CRCs is associated with the high expression levels of CD8 positive T cells within the tumor tissues (15, 16). The MSI-H CRC exhibits an active immune microenvironment probably due to recognizing many tumor neoantigens (17, 18). In this patient, the IHC assay showed a small number of CD8 positive T cells infiltrated within the tumor tissue, and many CD8 positive T cells were expressed in the tumor stroma, which may have contributed to the failure of immunotherapy.

The keynote-158 clinical trial has confirmed that TMB is a robust biomarker for predicting the efficacy of PD1 mab, with higher TMB indicating better efficacy (19). For TMB detection, peripheral blood and tumor tissues are generally selected. In this case, we found that the TMB in the peripheral blood was higher than in the tumor tissue. We speculated that a large amount of ctDNA in the lesion was released into the peripheral blood after the rapid tumor progression. In contrast, the tumor tissue only represented the TMB in this site.

Previous reports have suggested that MSI-H or immune-infiltrated tumors have evolved mutations that may confer resistance to recognition by the immune system in untreated samples (5). It is observed that *APC* biallelic mutations associate with increased WNT signaling and decreased TILs in MSS and MSI-H tumors (20). Cen et al. reported that mutant *APC* promotes tumor immune evasion *via* PD-L1 in CRC (21). In our case, we found that this patient had an *APC* mutation, which may lead to resistance to anti-PD1 therapy. However, the protein

expression of PDL1 was negative in the tumor tissue of this patient, suggesting that in addition to *APC* gene mutation, there may be other gene dysfunction leading to immune tolerance. It is reported that *PTEN* gene mutation also correlated with response to anti-PD1 therapy. Chida K et al. showed that *PTEN* gene mutations in MSI-H/dMMR gastrointestinal tumors often did not respond to PD1 mab therapy (22). In addition, *KRAS* gene mutations are associated with poor anti-PD1 efficacy (23). In this patient, *PTEN* mutations were detected in the tumor tissue, and *KRAS* mutations were detected in the tumor tissue and peripheral blood. Therefore, the above gene mutations may be related to the failure of PD1 mab treatment. This suggests that the tumor immune microenvironment is very complex, and the factors determining immunotherapy's efficacy still need further exploration. Therefore, the patient's tumor microenvironment characteristics and detailed genomic status must be comprehensively evaluated before anti-PD1 mAb treatment, even for MSI-H tumors. In addition to MSI status, other gene mutations that may affect the therapeutic effect should also be considered comprehensively in screening the population with anti-PD1 treatment advantage among CRC patients.

In summary, this case may have significant clinical implications for MSI-H CRC patients' resistance to anti-PD1 mab therapy, especially those with LS-related MSI-H CRC.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the study involving human participants in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the patient for the publication of any potentially identifiable images or data included in this article.

Author contributions

XQ conceived the idea of the article. QZ composed the manuscript and figure-making. JH and LL supported the clinical data. TW and YZ provided the IHC test. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Immunotherapy resistance in esophageal cancer: Possible mechanisms and clinical implications

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Esophageal cancer (EC) is a common malignant gastrointestinal (GI) cancer in adults. Although surgical technology combined with neoadjuvant chemoradiotherapy has advanced rapidly, patients with EC are often diagnosed at an advanced stage and the five-year survival rate remains unsatisfactory. The poor prognosis and high mortality in patients with EC indicate that effective and validated therapy is of great necessity. Recently, immunotherapy has been successfully used in the clinic as a novel therapy for treating solid tumors, bringing new hope to cancer patients. Several immunotherapies, such as immune checkpoint inhibitors (ICIs), chimeric antigen receptor T-cell therapy, and tumor vaccines, have achieved significant breakthroughs in EC treatment. However, the overall response rate (ORR) of immunotherapy in patients with EC is lower than 30%, and most patients initially treated with immunotherapy are likely to develop acquired resistance (AR) over time. Immunosuppression greatly weakens the durability and efficiency of immunotherapy. Because of the heterogeneity within the immune microenvironment and the highly disparate oncological characteristics in different EC individuals, the exact mechanism of immunotherapy resistance in EC remains elusive. In this review, we provide an overview of immunotherapy resistance in EC, mainly focusing on current immunotherapies and potential molecular mechanisms underlying immunosuppression and drug resistance in immunotherapy. Additionally, we discuss prospective biomarkers and novel methods for enhancing the effect of immunotherapy to provide a clear insight into EC immunotherapy.

KEYWORDS

immunotherapy, esophageal cancer, intrinsic resistance, acquired resistance, biomarker

Introduction

According to a new global report (1), Esophageal cancer (EC) is the ninth most common malignant tumor and the sixth most common cause of cancer-related deaths. The two main pathological subtypes of EC are esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). Unfortunately, because the early symptoms of EC are easily neglected and because the biological characteristics of EC are invasive, patients are often diagnosed at a late stage, with only a 30% five-year survival rate (2). Surgery combined with neoadjuvant chemoradiotherapy (nCRT) remains the first choice of treatment for patients with locally advanced-stage EC. Despite advances in nCRT and surgical therapy, many patients continue to progress to tumor metastases and recurrence. Moreover, side effects limit the use of chemoradiotherapy. Novel therapies against EC are necessary to improve the prognosis of patients with EC (3).

Immunotherapy is a series of treatments aimed at enhancing the strength of the immune system to act against cancer cells by modifying signaling pathways (4). To date, immune checkpoint inhibitors (ICIs) have been applied to treat cancer; they target the suppressed immune system to activate the tumor-cell-killing capacity of immune cells (5). ICIs and autologous T cells expressing chimeric antigen receptors (CAR), the most commonly used immunotherapies, have been evaluated in various cancers (6, 7). In recent decades, immunotherapy has become a prospective option for patients with EC, and increasing evidence has shown that immunotherapy has been successfully used in treating solid and hematologic malignancies and improving patient management. However, immunotherapy resistance has become an extreme challenge that impairs the effects of immunotherapy. Although success has been achieved in the field of immunotherapy for treating patients with EC, most patients do not respond well to immunotherapy, mainly because of both intrinsic and acquired immune resistance. Intrinsic immunotherapy resistance involves innate elements, including normal immune cells and molecules that exhibit mutual interaction during immune progression and inhibit the anti-tumor response. Besides the co-affection of immune cells and molecules, the characteristics of tumor cells also play an important role in intrinsic immunotherapy resistance, although the exact mechanism is still unclear (8). According to recent studies, patients with tumors who were initially responsive to immunotherapy were prone to developing acquired resistance (AR). In particular, in gastrointestinal (GI) cancer, the rate of AR is above 50%. Therefore, clinical researchers need to investigate the potential mechanism of immune resistance in EC and identify novel immunotherapy resistance biomarkers. In this review, we summarize advances in immunotherapy for patients with EC, including ICIs, CAR-T cell treatment, and tumor vaccines that stimulate the immune system and anti-tumor response. In addition, we discuss the mechanism of immunosuppression and drug resistance in EC, prospective biomarkers for predicting immunotherapy resistance, and novel clinical strategies for overcoming immunotherapy resistance.

Immunotherapies for EC

Immune checkpoint inhibitors

Traditionally, antigen-presenting cells (APCs) could submit the major histocompatibility complex (MHC) to T cells. When the T cell receptors (TCR) bind with the submitted MHC, CD8+ T cells are activated and converted into tumor cell killers. Inversely, to protect our system from being harmed by an "overprotective" immune response, the immune checkpoints play a vital role in immunosuppression and act as "inhibitors" to prevent long-lasting inflammation and autoimmunity (9). Programmed cell death protein 1 and programmed cell death ligand 1 (PD-1/PD-L1) are common immune checkpoints in T-cell activation. PD-1 is often expressed on the surface of various immune cells, such as T cells; when it binds to its ligand PD-L1, which is often abnormally highly expressed on tumor cells, the intercellular inhibition signaling pathways of T cells are activated, and the T-cell effect is suppressed (10). Moreover, the PD-1/PD-L1 axis could mediate the process of immune monitoring and play a vital role in tumor progression (11). PD-L1 expression by tumor cells could protect them from lysis mediated by CD8+ T cells (12). When engaged by PD-L1, activated T cells could express CD80, which acts as a receptor delivering a suppression signal, leading to peripheral T-cell tolerance (13). During prolonged exposure to a tumor antigen, T cells upregulate negative regulators such as PD-1, leading to their functional exhaustion (14). Antibody-based immunotherapy that blocks this signaling pathway is a prospective treatment for tumors (Figure 1). Anti-PD-1 antibody development has become a hot spot in the immunotherapy field; this strategy has been proven effective in melanoma, non-small-cell cancer, and renal-cell cancer, exhibiting ideal objective response rates. The combined positive score (CPS) is often used by clinicians to evaluate the expression of PD-L1; the value of CPS is calculated using an immunohistochemical scoring algorithm.

$$\mathrm{CPS} = \left(\text{total number of PD} - \text{L1} - \text{stained cells}/\text{total number of tumor cells}\right) \times 100,$$

where the maximum score is 100 and CPS >1 is considered positive in EC (15). On the basis of the CPS results, patients with PD-L1-negative tumors have been shown to be more likely to exhibit a non-objective response compared with those with PD-L1-positive tumors (16). Recently, clinical trials have evaluated PD-1/PD-L1 inhibitors for EC treatment and have achieved favorable therapeutic effects. Pembrolizumab, a classic high-affinity monoclonal PD-1 antibody, has been shown to result in survival benefits in patients with various tumors. A global

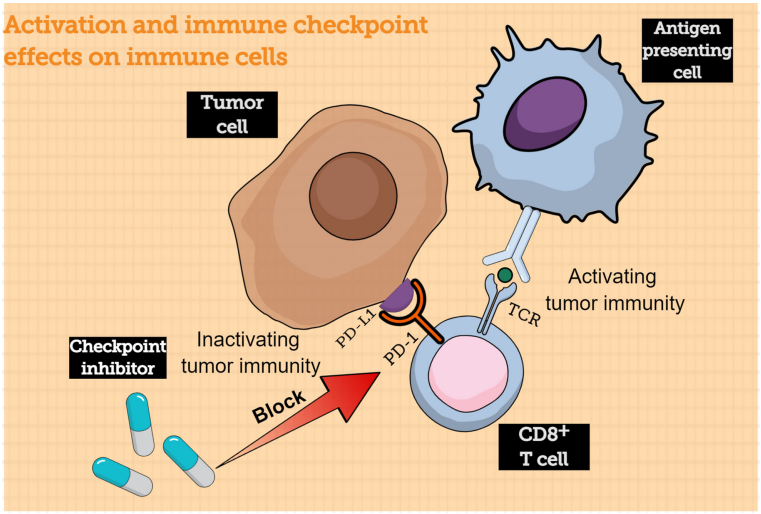

FIGURE 1
Activation of immune-checkpoint effects on immune cells. The CD8+ T cells can be activated by interacting with antigen-presenting cells, following which the CD8+ T cells can acquire the capacity to kill tumor cells. However, tumor cells express immune checkpoint proteins that bind to receptors on the surface of CD8+ T cells to evade immune cells; immune checkpoint inhibitor can block this process, thereby allowing CD8+ T cells to kill tumor cells. TCR, T cell receptor; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1.

multicenter, randomized-control phase III clinical trial enrolled 628 patients with advanced EC who received pembrolizumab therapy; the final trial results showed that patients with PD-L1 CPS ≥10 may benefit more from pembrolizumab than from chemotherapy and that pembrolizumab could prolong the overall survival (OS) of patients with advanced EC (17). Meanwhile, the KEYNOTE-590 clinical trial proved that pembrolizumab combined with chemotherapy could provide superior OS, progression-free survival (PFS), and overall response rate (ORR) compared to chemotherapy alone in patients with advanced EC (18). Pembrolizumab combined with chemotherapy may likely achieve better survival outcomes and may become a new standard treatment for patients with advanced EC. Pembrolizumab, utilized as a second-line therapy for EC in different clinical trials, has shown positive clinical effects in both patients with ESCC and those with EAC (19). A phase II clinical trial included 30 eligible patients with locally advanced or metastatic EC who received camrelizumab (SHR-1210, anti-PD-1) and apatinib (anti-angiogenesis) in combination with chemotherapy. The study results demonstrated the feasible anti-tumor activity of immune checkpoint inhibitors combined with anti-angiogenesis treatment and chemotherapy (20). In the clinical trial ATTRACTION-3, nivolumab, another immune checkpoint inhibitor, was proven to produce an improvement in OS and exhibit better safety compared with traditional chemotherapy in patients with advanced ESCC (21). These extensive clinical trials have demonstrated the superiority of PD-1/PD-L1 checkpoint inhibitors, which exhibit better effectiveness and fewer side effects than conventional chemotherapy. PD-1/PD-L1 checkpoint inhibitors may become a novel prospective therapeutic option for patients with EC. Meanwhile, clinical trials are well underway for various novel PD-1 antibodies, including JS001, durvalumab, and other novel immune drugs against EC, and the results of their therapeutic effects are expected (22–25).

In addition to PD-1/PD-L1, another well-recognized immune checkpoint is T lymphocyte-associated antigen 4 (CTLA4), which is commonly expressed on regulatory T cells (Tregs) and activated T lymphocyte surfaces; it acts as a vital element in T-cell self-tolerance and regulation. Many studies have verified that the overexpression of CTLA4 is associated with T-cell cycle arrest, reduced interleukin-2 (IL-2) expression, and arrested T cell G1 phase (26). Consequently, the function of T cells is reduced, causing the immune evasion of cancer cells. Remarkably, this key immune checkpoint has been used as a therapeutic target in the domain of anti-tumor drugs and immunotherapy (27). A previous study showed that CTLA4 is expressed not only by T cells but also by tumor cells, which indicates that the exact function of CTLA4 is unknown (28). The main representative drugs for CTLA4 target therapy in the clinic are ipilimumab and tremelimumab. CTLA4 checkpoint inhibitor treatment in patients with EC could provide favorable survival benefits and reduce treatment-related adverse events. A phase II clinical trial investigated the CTLA4 inhibitor tremelimumab for patients with gastric cancer (GC) and EAC; a small cohort of patients received a significantly long-lasting benefit and acquired clinical benefit with mild drug-

related toxicity. However, the response rate to tremelimumab was only 5% (29). Owing to the limited number of clinical trials investigating CTLA4 inhibitors in EC, detailed information on the efficiency, safety, and side effects of tremelimumab still need to be determined.

Chimeric antigen receptor T-cell therapy

Tumor cells are highly immunogenic, with the specific expression of tumor-associated antigens (TAA), which are pivotal in activating anti-tumor immune responses. T cells can recognize tumor cells based on TAA molecules and attack tumor cells. Chimeric antigen receptor T-cell (CAR-T-cell) therapy is a type of immunotherapy based on this mechanism. CAR-T-cell therapy refers to the genetic engineering of T-cell antigen receptors. During the process of modification, patient cells are first isolated from peripheral blood and engineered *ex vivo* to generate chimeric receptors that specifically recognize TAAs. Therefore, CAR-T cells possess tumor-recognizing characteristics, and they can be infused back into the blood of patients as an anti-tumor therapy (30). CAR-T cells typically consist of four fragments. The extracellular domain is a variable segment that originates from an antibody that acts as a TAA recognizer. A spacer modulates the distance between tumor and CAR-T cells and connects them to the transmembrane domain. The transmembrane domain can deliver the signal to the intercellular signaling domain, which is mainly composed of CD3ζ, and then activate T cells when engaged with tumor cells through TAAs expressed on the surface of tumor cells (31, 32). CAR-T immune therapy is commonly used in hematologic malignancies and has been proven to be effective in patients with diffuse large B-cell lymphoma (33) and leukemia (34). In the past few years, CAR-T immune therapy has also been explored as a treatment for solid tumors, including EC. According to previous studies, the overexpression of erythropoietin-producing hepatocellular receptor A2 (EphA2) could facilitate carcinogenic effects in various tumors (35); furthermore, EphA2 overexpression has been detected, which is associated with poor prognosis in ESCC (36). Shi et al. constructed EphA2-targeting CAR-T cells that showed a better ability to kill ESCC cells and promote cytokines *in vitro* (37). Another well-known TAA is the human epidermal growth factor receptor 2 (HER2), which is highly expressed in both breast cancer and EC. In an *in vitro* experiment, Yu et al. successfully developed CAR-T cells targeting the HER2 antigen. CAR-T cells showed a strong anti-tumor effect *in vitro*, significantly suppressed tumor growth in xenograft mice, and demonstrated the ability to specifically kill HER2-positive EC cells (38). Additionally, studies have shown that engineered CAR-T cells targeting mucin 1 (MUC1) and CD276 can induce the release of high levels of cytokines, achieving better persistence and durability to regulate a stronger anti-tumor response in a subcutaneous xenograft mouse model of EC (39, 40); this indicates that CAR-T cell therapy merits testing in EC clinical trials in the future. Various preclinical studies have identified novel methods for enhancing the anti-tumor effect of CAR-T cells. Recently, a new generation of CAR-T cells was designed by encoding a truncated cytoplasmic domain that binds to CD3z and CD28 domains together; the modified CAR-T cells showed better persistence and anti-tumor effects than traditional CAR-T cells (41). Zhang et al. designed enhanced CAR-T cells targeting MUC1, which is a complex glycoprotein overexpressed in EC that additionally activates the JAK-STAT signaling pathway. The strengthened MUC1-CAR-T cells survived longer in mice and appeared to exhibit a high treatment efficiency (39). Although many preclinical experiments have proved that CAR-T cells are a prospective therapeutic candidate against EC, no CAR-T-cell therapy has been applied in clinical trials for patients with EC. Additional breakthroughs are of great necessity in the clinical translation of CAR-T-cell therapy.

Tumor vaccines

As described previously, high immunogenicity of TAAs has been identified in EC. Several TAAs are highly expressed in EC, among which the most common TAAs have been confirmed in EC till date, including New York esophageal squamous cell carcinoma 1 (NY-ESO-1), TTK protein kinase (TTK), cancer-testis antigen 2 (CTAG2), and melanoma-associated antigen-A (MAGE-A) (42). Furthermore, the anti-tumor effects or immune-cell reactions to these TAAs could be tested in EC samples from patients. Chen et al. proved that MAGE-A3-specific CD8+ T cells could be isolated from the peripheral blood of patients with EC and that CD8+T cells could react with MAGE-A3 peptide; consequently, these CD8+T cells could specifically lyse certain tumor cells (43). Another study confirmed that the NY-ESO-1 dominant B-cell epitope and NY-ESO-1 antibody could be detected in the serum of patients with various cancers (44). Cancer vaccines, based on immune reactions through specific TAAs, have become a hot topic in cancer therapy; they act by stimulating T cells to exert anti-tumor effects and kill tumor cells. Several peptide vaccines have been tested in clinical trials. Sipuleucel-T, a cancer vaccine, has been shown to exhibit therapeutic effects in prostate cancer by prolonging the overall survival of patients with prostate cancer (45). Additionally, peptide vaccines in patients have shown a good therapeutic effect. Kageyama et al. conducted a clinical trial enrolling 25 patients with advanced EC subcutaneously injected with a cholesteryl pullulan-NY-ESO-1 (CHP-NY-ESO-1) complex vaccine, and no adverse events were observed during the treatment period. The vaccine can induce specific immune responses and provide a better survival benefit in patients with advanced EC (46). Chemoradiation therapy in combination with multiple peptide vaccines (kinase of the outer chloroplast

membrane 1 (KOC1)), upregulated lung cancer 10 (URLC10, TTK, VEGFR1, and VEGFR2) showed a superior effect and a satisfactory level of safety in patients with unresectable ESCC (47). However, tumor vaccines have not been commonly utilized in EC clinical practice thus far, and the mechanism underlying their anti-tumor effect needs further study.

The current advancements in immunotherapy for EC are summarized in Table 1. Immunotherapy has been successfully used in clinics, especially in the field of GI cancer, and it has become a prospective approach against malignancies. Immunotherapy has achieved a significant breakthrough in treating EC, gastric cancer, and colorectal cancer during the past decade, which has brought new hope to cancer patients. Unfortunately, the overall response rate (ORR) of immunotherapy is lower than 30%, and patients who initially respond to immunotherapy are likely to progress to AR (49–51). Moreover, approximately 70% patients appear to exhibit primary resistance to immunotherapy or even develop a hyper-progressive disease, the durability and effect of immunotherapy are extremely reduced. Therefore, clarifying the potential molecular mechanisms involved in immunosuppression is important for selecting preferable strategies for EC immunotherapy.

Potential mechanisms of resistance to immunotherapy in EC

EC cells can abnormally express specific antigens, which can be recognized by immune cells to initiate an anti-tumor immune response. Traditionally, the response of CTLs activated by APCs has been key for eliminating tumor cells. Dendritic cells (DCs), another participant in the immune response, play a vital role in tumor cell antigen delivery, presenting tumor antigens and rendering CTLs capable of killing tumor cells (52). However, EC cells have undergone mutations to evade human immune cells and resist attack by the immune system.

Intrinsic resistance

Several factors are involved in immune resistance in EC. A main strategy used by EC cells to escape the immune response is to upregulate immune checkpoint molecules and downregulate tumor antigens. Immune checkpoints, including PD-1, PD-2, and CTLA-4, are usually expressed on the surface of immune cells. These molecules act as critical molecules to prevent immune cells from inducing inflammation, destruction, and autoimmunity. They can block signaling within T cells when triggered. However, tumor cells may highly express these checkpoint proteins to protect themselves from being lysed by CTLs and escape death (53). To date, studies have verified that many checkpoint inhibitory molecules are upregulated by EC cells. The well-studied inhibitory receptors PD-1 and CTLA-4 are commonly detected in EC (54–56). PD-L1 can even be secreted by tumor cells through exosomes to suppress T-cell immunity, which cannot be restored by ICIs (57). Other inhibitory molecules such as lymphocyte-activation gene 3 (LAG-3) and mucin-domain containing-3 (TIM-3) have been demonstrated to be associated with PD-L1 expression in EAC (56). Recently, indoleamine 2,3-dioxygenase 1 (IDO1), a primary enzyme that produces kynurenine and tryptophan to suppress the immune response, has aroused research interest with respect to EC. Kiyozumi et al. conducted a study involving immunostaining of EC tissues from 305 patients with EC and proved that IDO1 showed an inverse correlation with CD8+ expression, indicating that IDO1 may act as a negative factor in immune regulation (58). In addition to CD8+ T cells, macrophages offer great promise as effectors in the anti-tumor immune response because of their strong ability to perform phagocytosis. CD47 is a critical molecule in the regulation of macrophages, and it acts as an immune checkpoint (59). Early studies described CD47 as a "marker of self," which is a glycoprotein on the surface of red blood cells that protects normal cells from innate immune cells that attack certain hematologic malignancies and solid tumors (60, 61). When activated, CD47 delivers inhibitory signals through signal regulatory protein alpha (SIRPα), a receptor on the surface of macrophages and myeloid cells, impairing the phagocytic activity of macrophages. Thus, the CD47/SIRPα axis serves as a specific myeloid immune checkpoint (62). However, studies have reported that tumor cells can highly express CD47, and abnormal activation of the CD47/SIRPα axis by tumor cells may inhibit the anti-tumor immune response and upregulate the threshold for macrophage phagocytosis (63). Tao et al. demonstrated that the expression level of CD47 is negatively associated with CD8+ T-cell density in ESCC tissues. Additionally, in a preclinical study, they demonstrated that anti-CD47 therapy enhanced the proinflammatory response of immune cells and then CD8+ T cell infiltration density increased in ESCC tissue *in vivo* (63), indicating that the CD47/SIRPα axis might serve as a novel immunotherapeutic target for patients with ESCC. However, the expression of inhibitor molecules on the cancer cell surface has been shown to present high heterogeneity (64). Additionally, the expression level of immune checkpoints could vary among different pathological subtypes (65). Therefore, identifying a reliable immune therapy that targets a certain immune checkpoint remains a severe challenge.

In addition to inhibitory molecule expression, EC cells may secrete cytokines and growth factors to facilitate tumor growth and reduce the anti-tumor immune response. Transforming growth factor-β (TGF-β), a factor secreted by tumor cells (66), plays an important role in immune tolerance by regulating several types of immune cells (67). It is vital for enhancing immune suppression in the tumor microenvironment (TME).

TABLE 1 Current advancements in immunotherapy for EC.

Target	Mechanism	Drug or Treatment	Study type	Reference
PD-L1	Expressed on the surface of EC cells, when binding with PD-1, the activation of T cells is inhibited and cause immune escape	Pembrolizumab	Clinical research	(17–19)
PD-1	The receptor of PD-L1 expressed on the surface of T cells, negatively regulates T cells	Camrelizumab	Clinical research	(20)
		Nivolumab	Clinical research	(21, 48)
		Durvalumab	Clinical research	(24)
		JS001	Clinical research	(25)
CTLA4	Associated with T cell cycle blocked which can lead the T cells G1 phase arrested	Tremelimumab	Clinical research	(29)
		Ipilimumab	Clinical research	(48)
EphA2	Related to poor degree of tumor differentiation and lymph node metastasis in EC	EphA2 targeting CAR-T cells	Basic experiment	(37)
HER2	Highly expressed in EC and associated with poor prognosis	HER2 targeting CAR-T cells	Basic experiment	(38)
MUC1	High expression of MUC1 was associated with tumor size, lymph node metastasis and distant metastasis in EC	MUC1 targeting CAR-T cells	Basic experiment	(39)
CD276	Promotes glucose metabolism in tumor and inhibits the function of CD8+ T cells	CD276 targeting CAR-T cells	Basic experiment	(40)
NY-ESO-1	One of TAAs expressed by EC cells	Tumor vaccines	Clinical research	(46)
KOC1	One of TAAs expressed by EC cells	Tumor vaccines	Clinical research	(47)
TTK	One of TAAs expressed by EC cells	Tumor vaccines	Clinical research	(47)

PD-L1, programmed cell death ligand 1; PD-1, programmed cell death protein 1; CTLA-4, cytotoxic T lymphocyte-associated protein 4; EphA2, hepatocellular receptor A2; HER2, human epidermal growth factor receptor 2; MUC1, mucin 1; NY-ESO-1, New York esophageal squamous cell carcinoma 1; KOC1, kinase of the outer chloroplast membrane 1; TTK, TTK protein kinase; EC, esophageal cancer; CAR-T, chimeric antigen receptor T cell; TAA, tumor-associated antigen.

Previous clinical studies in patients with EC showed that the TGF-β signaling pathway was abnormally hyperactivated (68), and the expression level of TGF-β was significantly associated with the prognosis of patients with EC (69). TGF-β can directly activate regulatory Tregs to inhibit the cytotoxicity of effector T cells, natural killer (NK) cells, and the antigen-presenting function of DCs. Furthermore, TGF-β can block the differentiation of naïve T cells into effector T cells. Therefore, TGF-β has a complex negative impact on the immune system (70). Cancer cells produce TGF-β and use it for tumor growth (71). TGF-β can decrease the level of IL-2, a cytokine that elicits CD4+ T-cell proliferation (72). Li et al. demonstrated that TGF-β signaling can also affect B-cell-mediated immune regulation. When exposed to EC-derived microvesicles (Mvcs), naive B cells are likely to differentiate into TGF-β-producing cells, thereby suppressing the proliferation of CD8+ T cells (73). Several studies have suggested that cancer-associated fibroblasts (CAFs), characterized by high levels of α-smooth muscle actin and fibroblast protein-α, play a prominent role in supporting tumor growth. TGF-β may also be involved in crosstalk between EC cells and CAFs. TGF-β is highly expressed in patients treated with conventional chemotherapeutic medicine, indicating that chemotherapy may upregulate the level of TGF-β and inhibit the immune response (69). As a well-known cytokine, the interleukin (IL) family plays a significant role in immune cellular signal transduction. IL-6 is the principal factor involved in infection and injury reactions (74). Upon binding to its receptors, IL-6 triggers the pathway and activates downstream molecules, such as STAT1 and STAT3, which may enhance the capacity of tumor cells to survive in a highly inflammatory environment and impair immunotherapy effects (75). Because of its inflammatory effects, IL-6 affects immune resistance in EC. IL-6 originates in the TME, and it is involved in various phenotypes of EC *via* different pathways (76). Upregulation of IL-6 can be found in both ESCC and EAC (77). Meanwhile, high levels of IL-6 promote epithelial-to-mesenchymal transition (EMT), clonogenicity, and chemoresistance in EC (78). IL-6 can inhibit the maturation of DCs through the STAT3 signaling pathway, attenuating anti-tumor immunity (79). In addition, elevated levels of IL-6

secreted from CAFs promote the migration of ESCC cells, and the expression of IL-6 is associated with immunosuppressive phenotypes (80). Additionally, elevated levels of IL-10 have been detected in the serum of patients with ESCC, and the IL-10 level has been positively associated with Treg density (81). IL-10 derived from Treg cells can act along with IL-35 to promote the exhaustion of CD8+ tumor-infiltrating lymphocytes (TILs), thus reducing anti-tumor immunity (82).

In addition to the extensive inhibitory molecules, there are robust suppressive cells in the TME within the EC, which remains a major hurdle in immunotherapy efficiency. As a crucial component of the TME, immune cells are necessary for regulating the anti-tumor response. As a subtype of T cells marked by IL-10 and the transcription factor FOXP3, Tregs are crucial for maintaining self-tolerance. When Tregs are activated by an immune response, inhibitory cytokines such as IL-1 and IL-6 are released into the peripheral blood. Thus, Tregs participate in the suppression of anti-tumor immunity (83). Tregs can be selectively recruited by certain factors to infiltrate the tumor stroma (84, 85), and the degree of infiltration of Tregs is associated with poor prognosis in EC (86). The chemokine (C–C motif) ligand 22 (CCL22) has been proposed to act as a key factor in the aggregation of Tregs. CCL22 released by tumor cells and tumor-infiltrating macrophages attracts the recruitment of Tregs through the combination of C–C chemokine receptor type 4 (CCR4) (87). Additionally, Tregs may recognize tumor antigens such as NY-ESO-1 and suppress specific effector T cells (88). Elevated levels of CCL4 and CCL20 were detected in ESCC tissue together with a high density of CD8+ T cells and Tregs, respectively, showing that Tregs and CD8+ T cells may be correlated through selective recruitment *via* specific expression of CCL20 and CCL4 (89). Immunity suppression in ESCC has been shown to occur because of the specific recruitment of CCL20 to Tregs. Other studies have demonstrated that CCL20 may also attract T helper 17 (Th17) lymphocytes in EC (90), thereby recruiting DCs to promote the activation of CD8+ T cells and enhance anti-tumor immunity (91, 92). Th17 is another subtype of T cell associated with immunity regulation and is commonly recognized as a vital mediator in anti-tumor responses and inflammation (93). Th17 cells can be found at elevated levels in the tumor tissues and peripheral blood of patients with EC (90). Th17 cells secrete the inflammatory cytokine IL-17 to enhance the invasiveness of EAC cells through the NF-κB pathway (94). However, IL-17 might also play a protective role by augmenting the expression of cytotoxic molecules to strengthen the tumor-killing effects of NK cells and promote DC infiltration to recruit immune cells in ESCC (92). Therefore, CCL20 and Th17 may play a dual role in tumor immunity and provide a deeper understanding of the role of CCL20 and Th17 in the immune response. Quezada et al. showed that CTLA-4 can be stably expressed by Tregs (95). Meanwhile, anti-CTLA-4 therapy decreased the number of Tregs in tumor tissues, and it was significantly associated with favorable clinical events, implying that Tregs may mutually affect immune checkpoint molecules in immune regulation (96).

As another vital element consists of the immune inflammatory cells in the TME. Macrophages impact the immune system and affect tumor progression. The degree of tumor infiltration by tumor-associated macrophages (TAMs) has been verified to correlate with prognostic outcomes in some malignancies (97). In oncology, TAMs are traditionally divided into two subgroups with different functions in tumor progression. One subtype is tumor-suppressive macrophages (M1), and the other is tumor-promoting macrophages (M2), characterized by the expression of CD163 and CD204 (98). M1 macrophages play a role in tumor inhibition, whereas M2 macrophages facilitate tumorigenesis. M2 macrophages are generally believed to act as negative regulators of the anti-tumor response. However, the underlying mechanisms remain largely elusive. A high density of M2-like TAMs was greatly associated with high levels of PD-L1 expression, and M2-like TAMs secrete TGF-β, indicating the protective function of M2-like TAMs in immune rejection (99, 100). Additionally, the c-Jun NH2 kinase (JNK) signaling pathway has been identified as a key factor in the transition of macrophages from anti-tumorigenic to tumorigenic, activating M2-like TAMs to release CCL17 and CCl22 in Treg recruitment (101).

Accumulated myeloid-derived suppressor cells (MDSCs) have been detected and verified as indicators of poor prognosis in most patients with EC (102). MDSCs accumulate in response to inflammatory regulators and can obstruct both adaptive and innate anti-tumor immune responses (103). MDSCs impact the anti-tumor response mainly by inhibiting T-cell-regulated tumor clearance (104) but may also act through activation of Tregs (105) and impair innate immunity through mutual effects with macrophages and NK cells. In the presence of MDSC, macrophages are prone to converse into M2 macrophages, and MDSC can also combine with M2 macrophages to block immune surveillance driven by IL-13 (106). The crosstalk between macrophages and MDSC facilitates MDSC IL-10 release and reduces IL-12 production by macrophages (107). In EC, IL-6, CCL2, and aldehyde dehydrogenase 1 (ALDH1) stimulated MDSCs (108, 109). Animal experiments have shown that tumor-derived factors such as IL-6, CXCL16, IFNγ, TNFα, and IGFBP-3 positively regulate the expression of CD38, and high expression of CD38 can enhance the immunosuppressive and tumor-promoting capacity of MDSCs (110).

Acquired resistance

Immunotherapy induces an anti-tumor response and has been successfully used as a clinical treatment for EC. However, with broader and more frequent use of immunotherapy, an increasing number of patients with EC have had a prolonged

time to response; this phenomenon is called AR. However, the exact mechanism of AR in EC remains unknown. Traditionally, the main potential mechanisms of AR are believed to be the loss of T-cell effects and recognition through the downregulation of tumor antigens, enhancement of escape mutation variants, interferon-γ (IFN-γ) signaling, and neoantigen depletion. Evidence has shown that these mechanisms could lead to AR during ICI therapy (51).

When the T-cell functional anti-tumor phenotype is changed and their cytotoxic activity is suppressed, patients who exhibit a primary response to immunotherapy might easily develop AR and progress into tumor relapse. As anti-tumor T cells specifically recognize tumor cells that express a certain antigen, tumor cells may likely progress into AR by decreasing the expression or inducing mutation of their antigens. Previous studies have suggested that T cells activated by ICI therapy preferentially recognize mutational antigens (111). The progression of T-cell activation is largely dependent on the antigens recognizing the major histocompatibility complexes (MHCs) of APCs (112) and tumor cell antigens submitted through MHC class I are regulated by various genes. Thus, when genetic deletions, epigenetic changes, or mutations are caused, these neoantigens presented by APCs are also downregulated, which might result in AR to ICI therapy. Hulpke et al. reported a crucial gene, beta-2-microglobulin (B2M), involved in stabilizing the MHC class I molecules at the cell surface (113). Previously, researchers identified that the loss-of-function mutation B2M was associated with MHC class I dysfunction, which indicated the potential molecular pathway of tumor cells escaping immunity. Restifo et al. first proved that in patients with metastatic melanomas who were treated with immunotherapy, B2M was lost, suggesting that the loss of B2M might be a possible factor that facilitates cancer cell acquisition of immunotherapy resistance (114). In addition, Gettinger et al. found in lung cancer that homozygous loss of B2M could lead to the downregulation of MHC class I in cancer cells. They additionally conducted an *in vivo* experiment by injecting knock-out B2M lung cancer cells into immunocompetent mice that received anti-PD-1 therapy. The results showed that B2M knockout cells were less sensitive to PD-1 blockade than the control group. They additionally proved that CD8+ T cells showed considerably lower cytotoxicity than B2M knockout tumor cells, indicating that B2M could mediate tumor cell escape from ICI therapy through MHC class I expression (115). Meanwhile, an early study conducted by Sade-Feldman et al. showed that B2M alterations were enriched in cancer patients insensitive to anti-CTLA4 therapy compared to responders (116). In EC, Wang et al. observed that B2M could be highly expressed through mesenchymal stromal cells (MSCs), which are considered pivotal cells in the tumor microenvironment of EC. The results of their study suggest that stroma-derived B2M might also be involved in EC immunotherapy resistance and might be a potential mechanism of ICI drug resistance (117).

Another pivotal strategy for activating the anti-tumor response is the JAK-STAT pathway. When IFN-γ is secreted by effector T cells, and it binds to the heterodimeric IFNGR1/IFNGR2, the receptor-associated kinases Janus kinase 1 (JAK1) and Janus kinase 2 (JAK2) are activated (118). Recent clinical studies have demonstrated that suppressing mutations in JAK1 or JAK2 may contribute to drug resistance during ICI therapy (119). Zaretsky et al. reported that patients with melanoma treated with ICIs presented loss-of-function mutations in JAK1 or JAK2, which led to resistance to PD-1 blockade. Additionally, they treated cell lines established from patients with AR with ICIs and demonstrated that the downregulation of the JAK protein was significantly associated with tumor sensitivity to IFN-γ (120). In patients who did not respond to CTLA4 inhibitor therapy, the function of IFN-γ was greatly suppressed (119). Li et al. found that IL-18 is usually downregulated, and the expression of IL-18 was positively correlated with IFN-γ. They verified *in vitro* that deficiency of IL-18 could suppress the cytotoxicity of NK cells and CD8+ T cells, indicating that the absence of IL-18 is likely to mediate the IFN-γ pathway during tumorigenesis in ESCC and lead to AR in anti-tumor immunity (121). Others have reported that long noncoding RNAs (lncRNAs) SNHG20 could serve as a carcinogen in ESCC and affect the JAK-PD-L1 pathway to promote ESCC cell progression (122). However, till date, clinical research on these key signal mutations associated with ICI drug resistance in EC is lacking, and whether additional pathways apart from IFN-γ or JAK are involved in AR to ICI therapy remains unclear.

Mutations frequently occur during the progression of tumor growth, some of which produce neoantigens and affect the response to ICI therapy (51). Previous research has shown that in early lung cancer, CD8+ T cells can react with tumor cells that highly express PD-1. Meanwhile, patients with enriched neoantigen expression appear more sensitive to ICI therapy and acquire more clinical benefits. These results suggest that neoantigen expression levels influence ICI therapy effects (123). Therefore, the loss of mutations in neoantigens through the downregulation of copy number or epigenetic repression may result in immune evasion and resistance to ICIs (124). When stimulating the lost neoantigen *in vitro*, T-cell expansion was observed, indicating that neoantigens may play a vital role in reducing AR to immunotherapy in cancer patients. Notwithstanding that such a mechanism has not been verified in EC, depletion of neoantigens has been verified in lung cancer, indicating that similar mechanisms may also be among other malignancies such as EC, which deserves further exploration and elucidation.

Although many potential mechanisms involved in primary or acquired resistance to immunotherapy have been discussed above (Figure 2), elucidating immunotherapy resistance in EC is extremely challenging because not enough clinical trials apply

ICI therapy in EC or to explain the underlying mechanism of immunotherapy and drug resistance. Thus, data from clinical trials and basic experiments are necessary for understanding and overcoming immune resistance and providing more clinical benefits to patients with EC.

Potential biomarkers of EC immunotherapy

The progression of tumors in patients with EC mainly depends on mutual interactions between tumorigenic EC cells, such as EC cell proliferation and invasion capacities, and the interactivity of immune cells induced by various regulators in the TME. Meanwhile, EC resistance to anti-tumor responses is believed to be a consequence of abnormal production of specific molecules, such as stimulatory and inhibitory factors, or an alteration in the effect of T cells and Tregs. Because of this imbalance in the TME and the high expense of immune therapy, it is particularly necessary to identify reliable biomarkers for predicting the prognosis of patients with EC before treatment with immune therapy. To date, genetic alterations in anti-tumor immunity regulation and TILs have been widely reported.

Immune checkpoint proteins

PD-L1, also called CD274 or B7 homolog 1, is a transmembrane protein expressed by DCs and EC cells. PD-1 is often expressed on the surface of T cells as a receptor for PD-L1. When it binds to PD-L1, the anti-tumor effect of T cells can be suppressed. The binding of PD-L1 and PD-1 remains the main mechanism of anti-tumor immunity evasion. Immune checkpoint inhibitors can inhibit their binding and help T cells recognize and kill EC cells. According to previous research, the expression of PD-L1 in ESCC ranges from approximately 40% to 80% (125). Most researchers have suggested that the expression level of PD-L1 in EC cells is a reasonable biomarker for predicting the efficiency of PD-L1/PD-1 inhibitors (16, 126). However, the significance of PD-L1/PD-1 expression in both EC tissues and TILs remains controversial. Hatogia et al. reported that high levels of PD-L1 could be

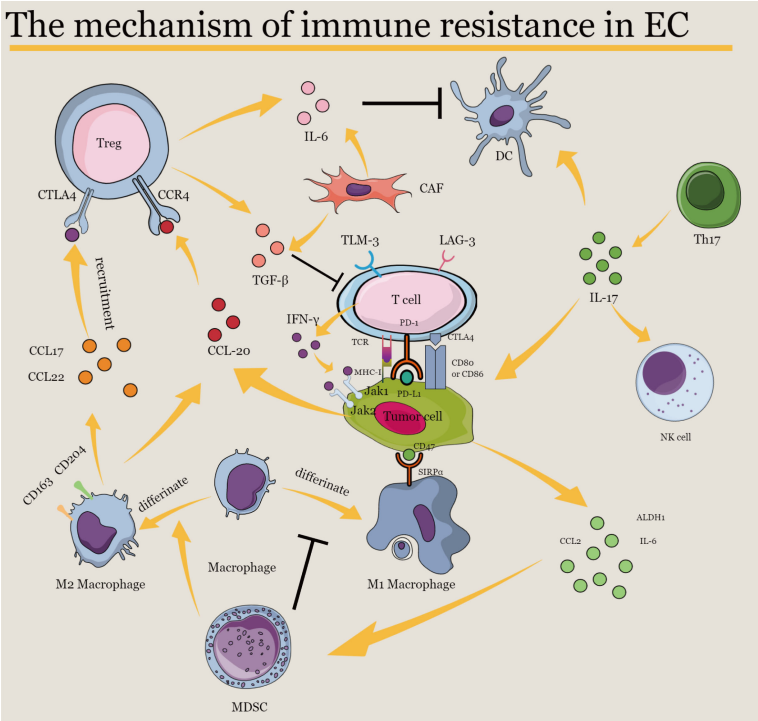

FIGURE 2
The mechanism of immune resistance in EC. IL-6, interleukin-6; IL-17, interleukin-17; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; CTLA-4, cytotoxic T lymphocyte-associated protein 4; CCR4, C–C chemokine receptor type 4; CCL2, C–C motif ligand 2; CCL17, C–C motif ligand 17; CCL20, C–C motif ligand 20; CCL22, C–C motif ligand 22; TGF-β, transforming growth factor-β; TIM-3, T cell immunoglobulin and mucin-domain containing-3; LAG-3, lymphocyte-activation gene 3; IFN-γ, interferon-γ; MHC-I, major histocompatibility complex class I; ALDH-1, aldehyde dehydrogenase 1; SIRPα, signal regulatory protein alpha; JAK1, Janus kinase 1; JAK2, Janus kinase 2; Treg, regulatory T cell; CAF, cancer-associated fibroblast; DC, dendritic cell; NK, natural killer; Th17, T helper 17; MDSC, myeloid-derived suppressor cell.

detected in ESCC cells and TILs, and elevated PD-L1 levels were significantly correlated with survival benefits (127). The results of the clinical trial KEYNOTE-180 revealed that PD-L1 expression level was associated with the therapeutic effect of pembrolizumab. Patients with EC presenting a PD-L1 CPS ≥10 presented more survival benefits than those with a CPS <10 (128). However, in other studies, survival outcomes correlated with PD-L1 expression were the opposite. In a clinical trial of SHR-1210, an anti-PD-1 antibody, Huang et al. showed that PD-L1 expression was not significantly correlated with ORR in patients with EC (129). Hynes et al. verified that in patients with EAC, survival outcomes were worse in patients whose tumors stained positive for PD-L1 than in patients with PD-L1-negative tumors who underwent neoadjuvant chemoradiation therapy (130). In addition, Ohigashi et al. observed that even in patients with ESCC, PD-L1-positive patients exhibited a poorer prognosis, and upregulation of PD-L1 was more pronounced, with worse tumor differentiation, positive lymph node metastasis, and advanced stage of ESCC (131), indicating that PD-L1 status may be a negative predictor of prognosis for patients with EC. These controversial clinical outcomes are mainly due to the heterogeneity of PD-L1 among different samples submitted, different detection methods, and the complex interaction between the anti-tumor immune response and EC cells. In addition, the treatment of patients with EC might considerably affect the outcome, indicating that a high expression of PD-L1 was likely to be a positive biomarker for patients with EC who have undergone immunotherapy but not for patients treated with other therapies. Considering the inconsistency of PD-L1 in EC, ICI therapy might be effective in certain patients with EC presenting low PD-L1 expression, while certain patients with EC presenting high PD-L1 expression might be insensitive to the same treatment. However, the prognostic value of PD-L1 in EC remains unclear. Further clinical research is necessary to confirm this relationship.

CTLA-4 is another transmembrane receptor that shares a B7 ligand with CD28. When CTLA-4 binds to B7, T cells exhibit anergy during the negative regulation of anti-tumor immunity. To date, only one study has investigated the relationship between CTLA-4 expression and the prognosis of EC. Zhang et al. demonstrated that a high density of CTLA-4 in both TILs and EC cells is associated with shortened overall survival (28). Considering that only one study demonstrated the prognostic value of CTLA-4 in EC, the study result may deviate from the true situation, and more prospective studies are needed to determine the exact correlation between them.

Other potential prognostic biomarkers, such as IDO1, IL-8, IL-10, and TGF-β, have been reported to be associated with the therapeutic response and tumor stage in EC (132–134). In the immune microenvironment of EC, anti-tumor cytokines, such as interferon-γ and tumor-killing factors, are generally believed to be insufficient. Immune suppressor factors such as TGF-β and IL-10 are upregulated. Combining immune-promoting and immune-suppressing factors may serve as a better approach for predicting the progression and therapeutic effects of EC. At present, there is a lack of studies investigating EC immune therapy prognosis, and further research is needed to determine the mechanism involved in EC progression and explore more biomarkers with prognostic value.

Tumor-infiltrating lymphocytes

TILs have shown great prognostic value in various solid tumors, such as breast and GI cancers (135). The degree of anti-tumor immunity is largely determined by the degree of infiltration of immune cells into the tumor tissue. Upregulation of both CD8+ and CD4+ TILs in patients with EC is associated with prolonged survival and better therapeutic outcomes of neoadjuvant chemotherapy along with surgical resection (58). Considering the crucial role of TILs in the TME in the immune response, a novel concept called "Immunoscore" was proposed, which incorporates both the TNM stage and TIL degree to serve as an essential parameter for classifying cancers (136). However, the exact mechanism by which TILs are involved in the anti-tumor immune response in EC remains under investigation.

Tumor mutation burden

TMB is commonly defined as the total number of mutations per coding area of the tumor genome. Previous studies have shown that a high mutation burden, especially non-synonymous mutations, is likely to generate neoantigens that can be recognized by T cells to activate anti-tumor immune responses (137). TMB is highly different between various cancers, ranging from 0.001/Mb to above 400/Mb. Early studies have shown that survival outcomes may be prolonged in cancer patients with high TMB who have undergone immunotherapy, indicating that TMB has the potential to act as a predictor of immunotherapy outcome (138). Hellmann et al. conducted a clinical trial using whole exome sequencing to evaluate the influence of TMB in patients with small cell lung cancer. The results showed that patients with high TMB who were treated with ICIs exhibited a higher ORR than those with low TMB (139). Additionally, the efficacy of immunotherapy in combination with ICI therapy was better than that of ICI monotherapy in patients with high TMB. This result was in accordance with the results of early studies in patients with NSCLC treated with nivolumab (140) and patients with melanoma who had received ipilimumab therapy (141), which indicated that TMB might serve as a prognostic biomarker in patients with tumors treated with ICIs. Besides, previous scholars analyzed the association between TMB and clinical outcomes in EC patients who were treated with immunotherapy. The results suggested that EC patients in the

high TMB group obtained more survival benefits (25). However, in the field of EC, few studies have investigated the association between the immunotherapy and TMB, and the number of EC patients included in studies was insufficient. Thus, the reliability of TMB as a biomarker for predicting ICI effects in EC remains unclear. Further prospective clinical studies are needed to clarify this point. Despite the potential prognostic value of TMB in predicting immune response to ICIs, TMB is not without drawbacks. Because of the high heterogeneity among various biological issues even in the same solid tumor, establishing an optimal cut-off value of TMB is challenging. Additionally, the detection of TMB was also faced with strict difficulty, which had not reached a uniform standard. At present, TMB is mainly calculated on the basis of the tumor tissue. However, generally, the number of tumor cells present in one biopsy operation cannot provide an accurate measure of TMB. To overcome this hurdle, some researchers have advocated TMB detection through blood samples. Analyzing the tumor genome from a blood sample has several advantages compared to traditional biopsy, which considers only a specific tumor site. Blood samples can be used for routine diagnosis with less susceptibility to detection bias, and they can be collected using noninvasive methods. Numerous techniques, such as allele-specific PCR and cell-free DNA, can be utilized for blood-based detection (142). Although evaluating TMB from blood samples is a robust approach approved by researchers, blood samples have limited genomic content, and the results need to be verified through clinical validation (143). In general, the correlation between TMB and the response to ICIs has yielded an exciting approach for increasing the precision of immunotherapy in cancer treatment. Nevertheless, several challenges remain. Studies investigating TMB in patients with EC are insufficient to draw convincing conclusions, and the details of the immune mechanism between TMB and ICIs need to be elucidated through prospective clinical studies in the future.

Mismatch repair deficiency

To maintain normal biological physiological activity, regulation of cell differentiation and proliferation, cells must maintain the capacity to protect their innate genome from damage by various adverse factors. When cells are exposed to exogenous and endogenous genotoxic elements, DNA errors are likely to accumulate, which might drive disorderly cell proliferation and convert normal cells into tumor cells with significant heterogeneity; this is a common phenomenon in malignancies. When DNA damage occurs, complex cellular pathways are activated, including apoptosis, cell cycle arrest, and DNA repair, which induce apoptosis and prevent cells from transforming into malignances over time. However, impairment of self-repair capacity renders normal cells sensitive to tumor-inducing factors and gradually results in malignant transformation. In recent years, mismatch repair gene deficiency has been proven to have a high incidence in various malignancies, such as ovarian and GI cancers (144). Deficiencies in mismatch repair, also called microsatellite instability-high (MSI-H) status, have been proven to be caused by mismatch repair genes such as MLH1, MSH2, MSH6, and PMS2; they facilitate the emergence of neoantigens to activate anti-tumor responses (145). In 2015, Le et al. showed that dMMR showed prognostic value in patients with cancer. They discovered that patients with dMMR tumors could possibly benefit from PD-1 blockade therapy, exhibiting prolonged PFS (146). A previous study showed that dMMR levels correlated with the depth of invasion in ESCC (147). In addition, a phase III clinical trial led by Shitara et al. applied whole exome sequencing to analyze samples from both tumor tissue and blood of patients with gastroesophageal junction adenocarcinoma (GEJ) who had been treated with pembrolizumab or paclitaxel. The study results showed that patients in the MSI-H group had a high TMB rate. Meanwhile, patients with MSI-H treated with pembrolizumab were more likely to have better survival outcomes than those who received paclitaxel therapy alone, indicating that MSI-H may serve as a positive predictive factor for the clinical efficacy of immunotherapy (148). At present, the National Comprehensive Cancer Network (NCCN) has recommended the use of pembrolizumab as a subsequent or second-line treatment in EC with dMMR (149); however, the incidence of dMMR in EC is low, only approximately 8% (147).

Unfortunately, till date, the number of studies investigating predictive biomarkers of EC is limited. Therefore, it is important to identify novel biomarkers with prognostic value for evaluating the efficacy of immunotherapy against EC, which can facilitate the selection of eligible patients with EC for immunotherapy and foster the precision of ICI therapy in the future.

Discussion

Future prospects

Establishment of a novel therapeutic standard for EC is anticipated in the future. Multidisciplinary combination therapy has become a hot topic. Immunotherapy combined with surgery, targeted therapy, and chemoradiotherapy has been validated in some malignancies, such as NSCLC and melanoma (150, 151). However, immunotherapy in the field of EC has a long way to go.

Common multimodal immune therapies include PD-1 inhibitors and chemotherapy. A phase III clinical trial, KEYNOTE-590, led by Kato et al., is ongoing among patients with advanced EC treated with pembrolizumab in combination with chemotherapy (152). Kraak et al. showed that GI cell lines treated with 5-fluorouracil (5-FU) chemotherapy usually have increased PD-L1 expression levels. Their results suggest an

alternative mechanism of traditional immune-mediated upregulation and indicate that the combination of 5-FU with a PD-L1 inhibitor may ameliorate the clinical outcomes and improve survival benefits in patients with EC (153).

The CheckMate-032 clinical trial, led by Janjigian et al., enrolled 160 patients with metastatic EC. Patients in the study received the PD-1-blocking nivolumab combined with the CTLA-4 inhibitor ipilimumab. Patients who received combined therapy showed a better clinically meaningful anti-tumor outcome, with higher PFS rates and prolonged durable responses, compared with patients treated with nivolumab alone. However, the adverse event rate of the combination therapy was reported to be more frequent and severe than that of nivolumab monotherapy (48).

Radiotherapy plays a predominant role in the multidisciplinary treatment of ESCC. Radiotherapy can induce tumor cell necrosis and release antigens into the peripheral blood, which is a prerequisite for activating the anti-tumor immune response. Zhang et al. proved that immunotherapy plus radiotherapy had manageable toxicity and antitumor efficacy in patients with ESCC (154). They also demonstrated that combining concurrent chemoradiotherapy and camrelizumab had a promising antitumor effect and manageable safety in locally advanced ESCC patients (155). Interestingly, radiotherapy may partly or completely eliminate tumors outside of the radiation range, and this effect was called the "abscopal effect" (156) (Figure 3). In patients with melanoma, the combination of radiotherapy and CTLA-4 can induce abscopal effects (157). Park et al. conducted preclinical studies to establish EC models. They found that PD-1 inhibitors enhanced the abscopal effects of radiotherapy (157). However, radiation might also elevate PD-L1 levels in tumor cells and lead to radiotherapy resistance (158). The impact of immunotherapy in combination with radiotherapy on EC is largely uninvestigated, and it requires further investigation.

Conclusion

In this review, we describe the current status of immunotherapy for EC and provide a clear depiction of biomarkers with prognostic value in patients with EC who have undergone immunotherapy. We additionally discuss novel strategies based on the immune environment for enhancing the current treatment effect of EC and the underlying molecular mechanisms of immunosuppression. In clinical practice, immunotherapy is commonly used as salvage therapy for patients with late-stage EC. More clinical trials are needed to verify whether immunotherapy can achieve better efficiency in early-stage applications. Because of the divergence among immune environments, it is necessary to elucidate the

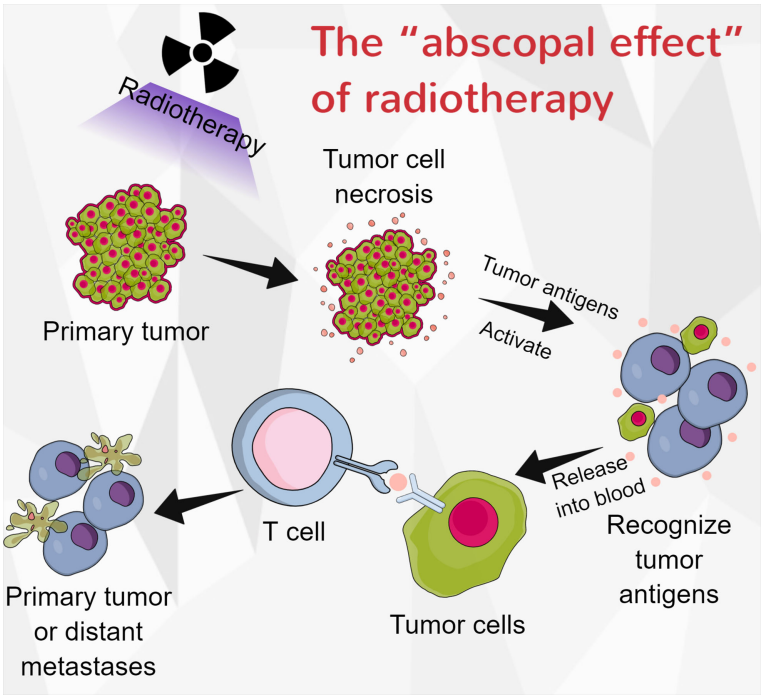

FIGURE 3
The "abscopal effect" of radiotherapy. When tumor-cell necrosis is induced by radiotherapy, the antigens with the cells are released into the blood, and they can be recognized by immune cells. These activated immune cells could then eliminate the primary tumor or distant metastases.

possible mechanisms of immunosuppression in EC so that precise targeted therapies can be developed for overcoming immunotherapy resistance in EC and for improving the prognosis of patients with EC.

Author contributions

PF, JZ, and ZL have contributed equally. PF, conception, manuscript preparation, data collection, manuscript editing, and manuscript review. JZ, conception, manuscript editing and manuscript review. ZL, manuscript editing and manuscript review. YSY, SL, XX, XL, HZ, QS, and XZ manuscript review. YY, conception, manuscript editing and manuscript review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Versican enrichment predicts poor prognosis and response to adjuvant therapy and immunotherapy in gastric cancer

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Background: Increasing evidence has revealed an important role of versican (VCAN) on various aspects of cancer progression. Here, we assessed the impact of VCAN expression on prognosis and the response to adjuvant therapy and immunotherapy in patients with gastric cancer (GC).

Methods: Four independent cohorts containing 1353 patients with GC, were utilized to investigate the effect of VCAN expression on prognosis and response to adjuvant therapy in GC. Two cohorts treated with immune checkpoint blockades were included to assess the predict value of VCAN expression on response to immunotherapy. Moreover, the bulk RNA-seq and single-cell RNA-seq data were analyzed to illustrate the role of VCAN in tumor microenvironment. Clinical outcomes of patient subgroups were compared by Kaplan-Meier curves with the log-rank test.

Result: High VCAN expression was associated with poor prognosis for patients with GC. Compared with patients with high VCAN expression, patients with low VCAN expression benefited more from adjuvant chemotherapy and adjuvant chemoradiotherapy. Moreover, patients with high VCAN expression tended to be resistant to immunotherapy, and VCAN could serve as a promising indicator for predicting the response to immunotherapy. $VCAN^{high}$ tumors showed a specific microenvironment with more cancer associated fibroblasts infiltration and significant enrichment of stromal relevant signaling pathways.

Conclusion: VCAN could predict the response to adjuvant chemotherapy, adjuvant chemoradiotherapy and immunotherapy in GC, and designing new medicine target to VCAN might be an effective way to improve the efficacy of several treatment options for GC.

KEYWORDS

VCAN, gastric cancer, cancer associated fibroblasts, adjuvant chemo therapy, immunotherapy

Introduction

Gastric cancer is one of the most common malignant carcinomas and ranks the fourth leading cause of cancer related death (1). Despite that huge advances in treatment has been achieved in aspects of diagnosis and therapy, patients with GC still have unsatisfactory prognosis (2, 3). Surgery and postoperative adjuvant therapy are the main treatments for GC, and immunotherapy has become an increasingly important part of treatment in the past few years and demonstrated the powerful effect of regressing tumors (4, 5). However, a large number of patients do not respond to these therapies, and it is urgent to explore therapy resistance mechanisms and seek effective biomarkers to better guide clinical treatment.

VCAN, an extracellular matrix proteoglycan, plays an important role in many aspects of organ development and disease (6, 7). VCAN interacts with diverse extracellular matrix (ECM) components like tumor necrosis factor-stimulated gene-6, CD44 and toll-like receptors, all of which are crucial in tissue inflammation caused by infection and injury (8). Increasing studies have shown VCAN is involved in various aspects of cancer progression, including cell proliferation, metastasis, and angiogenesis (9). Moreover, VCAN has been reported to be enriched in chemotherapy-resistant patients with cervical cancer (10). Versican silencing improved the antitumor efficacy of endostatin by alleviating its induced accumulation of myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs) and inflammatory cytokines in the tumor microenvironment (11). However, the impact of VCAN on response to adjuvant therapy and immunotherapy response remains unclear in GC.

In this study, multiple independent cohorts were used to explore the relationship between VCAN expression and response to adjuvant therapy and immunotherapy in GC. Single cell RNA sequencing and bulk RNA sequencing data was utilized to explore the role of VCAN in tumor microenvironment. Through the analysis of multiple omics and independent cohorts, we comprehensively explored the negative effect of VCAN on anti-tumor therapeutic efficacy and its potential mechanisms. We found that patients with low VCAN expression benefited more from adjuvant chemotherapy, adjuvant chemoradiotherapy and immunotherapy, which was associated with cancer associated fibroblasts. Taken together, this study demonstrated the crucial role of VCAN in response to adjuvant therapy and immunotherapy in GC.

Abbreviations: VCAN, Versican; GC, Gastric cancer; CAFs, Cancer associated fibroblasts; OS, Overall survival; RFS, Recurrence free survival; FUSCC, Fudan University Shanghai Cancer Center; SMC, Samsung Medical Center; PBS, Phosphate-buffered saline; ECM, Extracellular matrix; FPKM, Fragments per kilobase million; TCGA, The Cancer Genome Atlas; TPM, Transcripts per kilobase millions; GEO, Gene Expression Omnibus; GSEA, Gene set enrichment analysis; ACRG, Asian Cancer Research Group; MDACC, MD Anderson Cancer Center; TIDE, Tumor Immune Dysfunction and Exclusion; PFS, progressive free survival; IC50, Half maximal inhibitory concentration; ROC, Receiver operating characteristic; AUC, Area under the curve; MSI, Microsatellite instability; GS, Genome stable; EBV, Epstein-Barr virus; CIN, Chromosomal instability; EMT, Epithelial mesenchymal transformation; FAP, Fibroblast activation protein; ACTA2, α-SMA; PDGFRA/B, Platelet derived growth factor receptor α/β; VIM, Vimentin; DC, Dendritic cells; IL33, interleukin 33; ICB, Immune checkpoint blockade; FDA, U.S. Food and Drug Administration; SD, Stable disease; PD, Progressive disease; PR, Partial response; CR, Complete response; Pan-F TBRS, Panfibroblast TGFβ response characteristics.

Methods

Clinical specimens and follow-up

The tissue microarray of FUSCC cohort, comprising 233 samples with gastric cancer who received gastrectomy without neoadjuvant chemotherapy or radiotherapy between November 2008 and June 2010 at the Department of Gastric Surgery, Fudan University Shanghai Cancer Center (Shanghai, China) was used in this study. All GC tissues were collected after received informed consent from patients. The study protocol was approved by the Clinical Research Ethics Committee of Fudan University Shanghai Cancer Center. All patients experienced follow-up every 6 months until November 2015. Overall survival (OS) was defined as the time from surgery to death or the end of follow-up, and recurrence free survival (RFS) was defined as the time from surgery to recurrence/metastasis or the end of follow-up.

Immunofluorescence staining

The automatic immunohistochemical staining machine (Leica, Bond III, Germany) was used for dewaxing and antigen retrieval. After five rinses with phosphate-buffered saline (PBS), tissue array was soaked in hydrogen peroxide solution, incubated at room temperature for 10 min. Then, VCAN antibody (Abcam, ab177480, USA, 1:600) was added to the tissue array, incubated at 37°C for 1 h. After five rinses with PBS, goat anti-rabbit poly-HRP (Leica, DS9800, Germany) was added to the tissue array, incubated at 37°C for 10 min. Dewaxing and antigen repair were performed again. FAP antibody (Abcam, ab207178, USA, 1:250) was added to the tissue array, incubated at 37°C for 1 h. After five rinses with PBS, goat anti-rabbit poly-HRP (Leica, DS9800, Germany) was added to the tissue array, incubated at 37°C for 10 min. The nucleus was stained with DAPI. Finally, 3DHISTECH fluorescence imaging scanner was used for scanning, and HALO platform was used for quantitative analysis of staining

results. The percentage of VCAN positive cells was used to identify the expression level of VCAN protein.

Data sources

Gene expression profiles in the form of fragments per kilobase million (FPKM) and corresponding clinical information of 33 human cancers in the Cancer Genome Atlas (TCGA) were collected from the UCSC XENA (https://xenabrowser.net/datapages/) website. The FPKM values were transformed to transcripts per kilobase millions (TPM) values. Specific information about 33 cancer types could be found in Supplementary Table 1. The gene expression profiles and corresponding clinical information of Asian Cancer Research Group (ACRG) cohort (GSE66229), SMC cohort (GSE26253) and MD Anderson Cancer Center (MDACC) cohort (GSE28541) were gathered in this study for further analysis, which were acquired from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). In SMC cohort, all patients received curative gastrectomy and INT-0116 regimen (5-fluoouracil/leucovorin and radiation) as adjuvant treatment (12). All patients of the MDACC cohort underwent neoadjuvant chemotherapy or chemoradiation therapy (13). For the microarray data from Affymetrix®, we got raw "CEL" file from GEO database and adopted the robust multiarray averaging method with the "affy" and "simpleaffy" packages to standardize the microarray data. For the microarray data from other platforms, we downloaded directly the normalized matrix files. We obtained the PD-L1 treatment cohorts for gastric cancer (KIM cohort) and melanoma (Hugo cohort) from Tumor Immune Dysfunction and Exclusion (TIDE) database (http://tide.dfci.harvard.edu). Processed gastric cancer single-cell gene expression data (GSE167297) was composed of deep layer (D1, D2, D3, D4, D5) and superficial layer (S1, S2, S3, S4, S5) of tumor tissues and paired normal tissues (N1, N2, N3, N4), which were downloaded from GEO database.

Single-cell RNA sequencing data analysis

The single-cell gene expression data was analyzed by the R package "Seurat". Firstly, we eliminated low-quality cells on the basis of the number of genes, RNA and the proportion of mitochondrial genes in each cell. All samples including the rest of cells were integrated into a single profile and batch-effect was adjusted with R package "Harmony". Then, Principal component analysis (PCA) was performed on 1500 genes with significantly different levels of expression after log-normalization and homogenization. Moreover, Uniform manifold approximation and projection (UMAP) algorithm was employed to make further dimensionality reduction and marker genes were figured out through "FindALLMarker" function. Finally, the cell lineage for every cluster was annotated according to the marker genes compared to the cell lineage markers in the CellMarker and PanglaoDB database.

Immune infiltration analysis and gene set enrichment analysis

The immune infiltration among different types of cancers were estimated by multiple algorithms of "IOBR" R package, which integrated a series of existing algorithms for easy comparison and selection (14). Spearman and distance correlation analysis were used to calculate the correlation of VCAN expression and multiple immune cells. The underlying mechanisms of VCAN in the progression of gastric cancer was explored with gene set enrichment analysis (GSEA) method. Firstly, we divided the STAD samples into the high and low group according to the median expression of VCAN in all samples, calculated the differences between the two groups, and arranged differential gene by the value of the foldchange. Then the Hallmarker and Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets on the basis of prior biological knowledge were used to analyze all samples with GSEA method using the R package clusterProfiler (version 4.0.5). We regarded the normalized enrichment score (NES) and false discovery rate (FDR) as the indicators of enrichment, (Gene sets with $|NES|>1$ and $FDR<0.25$ were considered to be possessed with significant enrichment) and utilized the R package enrichplot (version 1.12.1) to visualize the results.

Statistical analysis

All statistical calculations were performed with R software (version 4.1.1). The Wilcoxon rank sum test was employed to analyze the differences between two groups, while the comparison of differences between three groups or more groups was calculated through the one-way ANOVA or Kruskal–Wallis test. The OS, progressive free survival (PFS) and RFS for patients were estimated by Kaplan-Meier curves, the differences were evaluated by log-rank test, and the cutoff points were selected by "maxstat" R package. The receiver operating characteristic (ROC) curve was implemented to analyze the sensitivity and specificity of immunotherapy response prediction of VCAN expression, and the area under the curve (AUC) was assessed using pROC R package.

Result

Landscape of VCAN expression in gastric cancer

Based on "TCGA Pan-Cancer" cohort, we compared the differences of VCAN expression in human pan-cancer and

found that VCAN was widely over-expressed in tumor tissues, such as BRCA, CHOL, COAD, ESCA, GBM, HNSC, KIRC, KIRP, LIHC, LUAD, LUSC, STAD, THCA. In addition, the level of VCAN expression diminished in KICH and PCPG (Supplementary Figure 1A). To further explore the landscape of VCAN expression in gastric cancer, we assessed the mRNA expression level of STAD samples from the TCGA database (TCGA cohort, n = 388) and GSE66229 dataset (ACRG cohort, n = 300). Compared to normal tissues, the level of VCAN expression significantly increased in tumor tissue in STAD (Supplementary Figures 1B, F). Similarly, the level of VCAN expression in tumor tissue was significantly higher than that in surrounding normal tissue from the same sample (Supplementary Figure 1C). Moreover, the level of VCAN expression was correlated with pathological stages and significantly up-regulated level of VCAN expression was observed in advanced gastric cancer in comparison to early gastric cancer (Supplementary Figure 1D).

Molecular subtypes of gastric cancer were established to facilitate the stratification of patients and the implementation of precision therapy (15). In TCGA cohort, the patients were divided into four subtypes including microsatellite instability (MSI), genome stable (GS), Epstein-Barr virus (EBV) and chromosomal instability (CIN). Compared to the CIN and GS subtypes, the MSI and EBV subtypes possessed the lower VCAN expression (Supplementary Figure 1E). The higher VCAN expression was significantly concentrated on epithelial mesenchymal transformation (EMT) subtype in ACRG cohort (Supplementary Figure 1G).

High expression of VCAN was associated with poor prognosis of patients with GC

To further explore the role of VCAN in GC, we constructed tissue microarray of FUSCC cohort containing 233 patients with GC and quantified the expression of VCAN protein using immunofluorescence experiments. Based on the expression of VCAN protein, we divided the patients into the high group (The proportion of VCAN positive cells was more than 19%) and low group (The proportion of VCAN positive cells was less than 19%), and the representative immunofluorescence images of the high group and low group was shown in Figure 1A. The association between VCAN expression and clinicopathologic features in FUSCC cohort was showed in Supplementary Table 2. Survival analysis indicated that high level of VCAN expression was significantly associated with OS and RFS of patients with GC (Figure 1B). Similarly, VCAN expression significantly affected OS and PFS of patients in the TCGA cohort (Figure 1C) and ACRG cohort (Figure 1D). The 5-year survival rate and 5-year progress-free rate of patients in the high VCAN group were significantly lower than those in the low VCAN group (Figures 1B–D). Moreover, we also explored the relationship between VCAN expression and clinical outcome in human pan-cancer. The result showed that the OS of VCAN high expression group was poorer than that in the low expression group in multiple cancers (Figure 1E).

VCAN acted as a promising prognosticator for the response to adjuvant therapy in GC

Multiple clinical studies have shown that adjuvant chemotherapy (ACT) could prolong the survival of patients with advanced gastric cancer compared with surgery alone (16, 17). However, extracellular matrix acted as the physical barrier to hinder the penetration of chemotherapy drugs. Given the strong correlation between VCAN and extracellular matrix, we evaluated whether the expression of VCAN affected the efficacy of ACT. Thus, we conducted survival analysis aimed to patients who received ACT in FUSCC cohort and ACRG cohort. We found that the VCAN could affect OS and RFS of patients who received ACT in FUSCC cohort, and patients in the VCAN high group benefited less from ACT compared with patients in the VCAN low group (Figure 2A). Besides, we assessed the effect of VCAN on the response to ACT of patients in ACRG cohort. High expression of VCAN was associated with poor PFS of patients received ACT in ACRG cohort (Figure 2B). The 5-year survival rate and 5-year progress-free rate of patients received ACT in the high VCAN group were significantly lower than those in the low VCAN group (Figures 2A, B). These results showed that it was feasible to predict the response to ACT through detecting the expression of VCAN.

Though recent studies indicated patients with GC could not benefit more from postoperative chemoradiotherapy than chemotherapy, adjuvant chemoradiotherapy was still considered one of the available treatment options for patients who have undergone less than D2 dissection (18). We evaluated the effect of VCAN expression on the efficacy of adjuvant chemoradiotherapy in 432 patients who received homogeneous chemoradiotherapy (5-fluoouracil/leucovorin and radiation) after surgery from SMC cohort. The results showed that the high level of VCAN expression was significantly related to poorer OS and RFS of the patients and the 5-year survival rate and 5-year recurrence-free rate of high VCAN group were significantly lower than those of low VCAN group (Figure 2C), suggesting that VCAN expression was an unfavorable factor of adjuvant chemoradiotherapy. Then, we assessed the difference of the response to neoadjuvant therapy between high VCAN group and low VCAN group in MDACC cohort, where all patients received neoadjuvant chemotherapy or chemoradiation therapy. The result showed that both the high VCAN group and low VCAN group benefited more from neoadjuvant chemoradiotherapy than neoadjuvant chemotherapy (Figure 2D). However, the effect of VCAN on

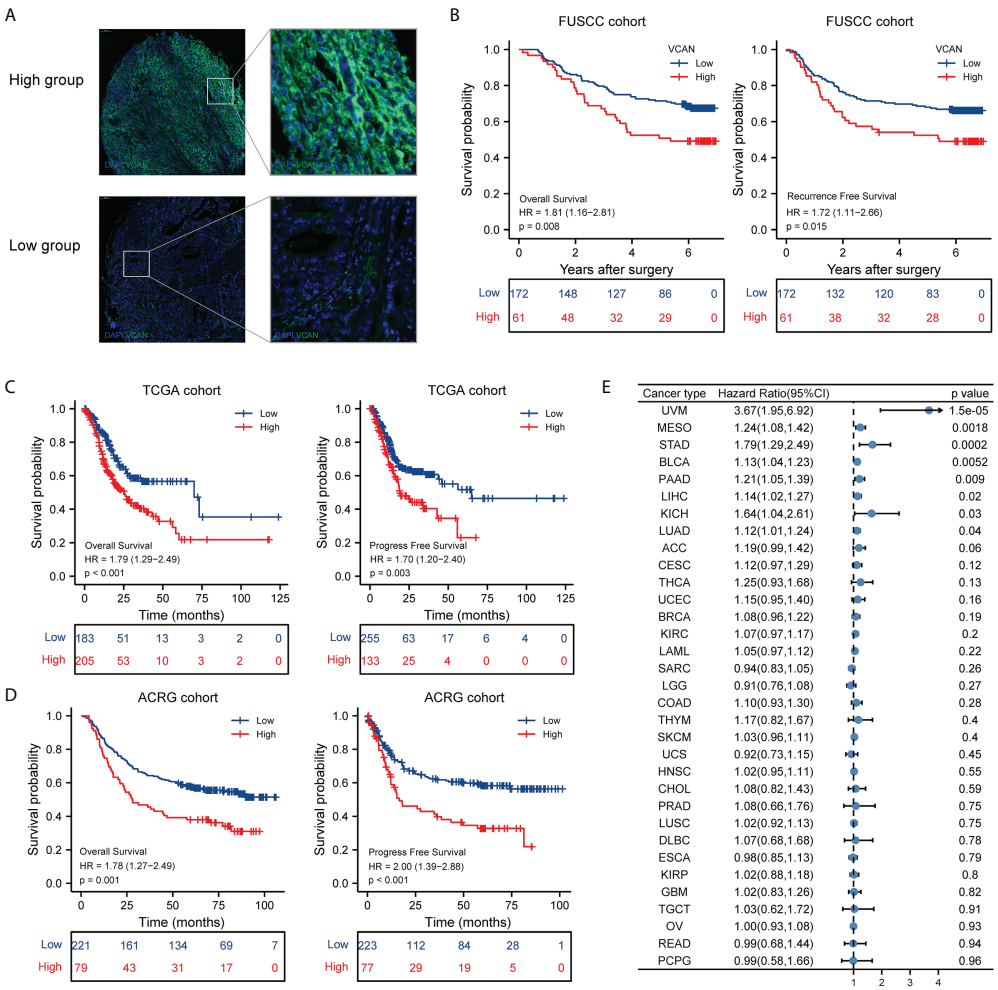

FIGURE 1
Correlation between VCAN expression and overall survival and progressive free survival. **(A)** Representative immunofluorescence images of the low VCAN group and high VCAN group in FUSCC cohort. **(B)** Kaplan–Meier curves of overall survival (OS) and recurrence free survival (RFS) of low VCAN and high VCAN group in FUSCC cohort. **(C)** Kaplan–Meier curves of OS and progress free survival (PFS) of low VCAN and high VCAN group stratified in TCGA cohort. **(D)** Kaplan–Meier curves of OS and PFS of low VCAN and high VCAN group stratified in ACRG cohort. **(E)** The correlation between VCAN expression with OS in human pan-cancer (Pan-cancer Atlas, TCGA).

the efficacy of neoadjuvant chemoradiotherapy was not statistically significant, which needed to be treated with caution due to the limited number of patients. In summary, the expression of VCAN significantly affected the efficacy of adjuvant therapy for GC and targeting to VCAN might be an effective way to improve the efficacy of adjuvant therapy for GC.

VCAN served as an indicator to predict the efficacy of immunotherapy for GC

Immunotherapy that targeted the immune system has revolutionized human cancers treatment, including gastric cancer. Regulation of immune system by immune checkpoint blockade (ICB) led to durable responses in human cancers. Recent clinical trial has shown that nivolumab (the first PD-1 inhibitor) could significantly prolong OS and PFS in patients with advanced gastric, gastro-oesophageal junction, or oesophageal adenocarcinoma (19). Recently, nivolumab (a monoclonal PD-1 antibody) has been approved by the U.S. Food and Drug Administration (FDA) for first-line treatment in patients with advanced or metastatic gastric cancer (20). However, most patients did not respond to immunotherapy, and it was necessary to find specific biomarkers to predict response to immunotherapy for GC (21). We used the KIM cohort (patients with advanced gastric cancer were treated by PD-1 inhibitor) to analyze the relationship between VCAN

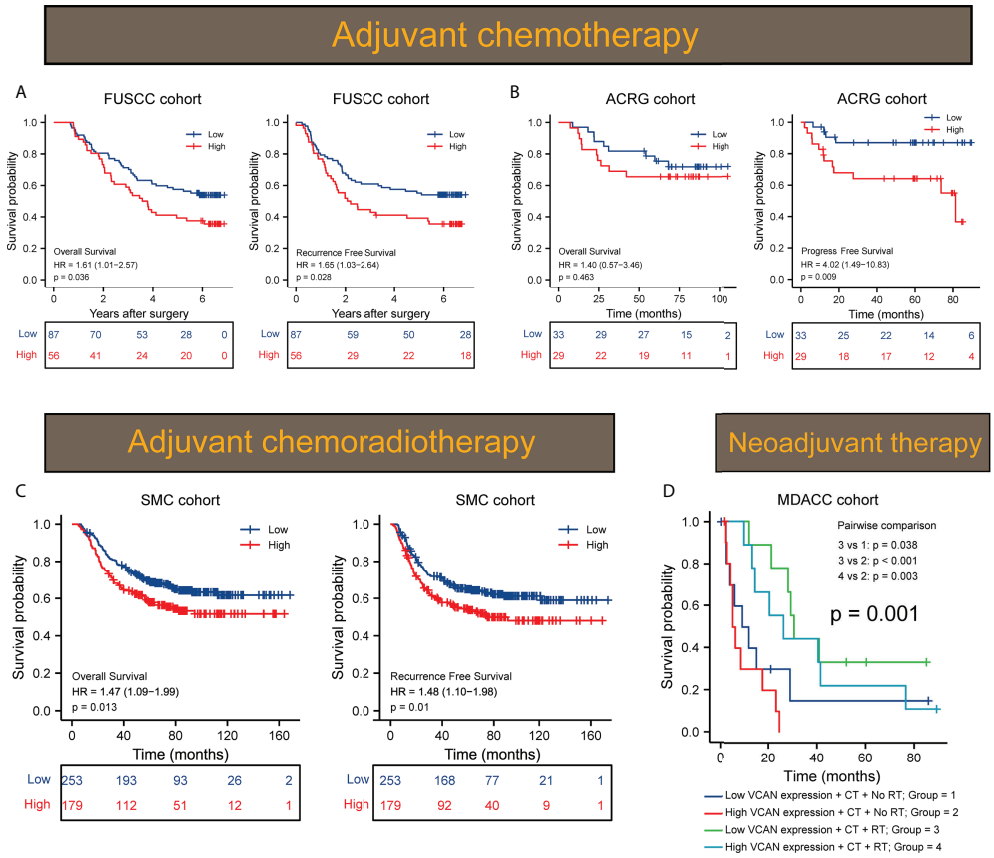

FIGURE 2
VCAN expression predicted response to adjuvant therapy in GC. **(A)** Kaplan–Meier curves of OS and RFS of low VCAN and high VCAN group in patients treated by ACT (n = 143) in FUSCC cohort. **(B)** Kaplan–Meier curves of OS and PFS of low VCAN and high VCAN group in patients treated by ACT (n = 62) in ACRG cohort. **(C)** Kaplan–Meier curves of OS and RFS of low VCAN and high VCAN group in patients treated by adjuvant chemoradiotherapy (SMC cohort, n = 432). **(D)** Kaplan–Meier curves of OS of low VCAN and high VCAN group in patients treated by neoadjuvant chemotherapy or chemoradiotherapy (MDACC cohort, n = 40). CT, chemotherapy; RT, radiotherapy.

expression and immunotherapy responses for GC. According to the RECIST 1.1 guidelines, patients in the CR and PR groups were considered responders and patients in the SD and PD groups were considered non-responders (22, 23). We found that the VCAN expression of patients in the stable disease (SD)/progressive disease (PD) group was significantly higher than that in the partial response (PR)/complete response (CR) group (Figure 3A), and the proportion of PD/SD in high VCAN expression higher than that in low VCAN expression group, indicating that VCAN expression was not conducive to immunotherapy response (Figure 3B). The VCAN expression of patients with different immunotherapy responses was shown in Figure 3D. MSI status and EBV status were found to serve as biomarkers for immunotherapy response (20, 24). Interestingly, we found that VCAN expression in patients with MSI-H subtype and EBV subtype was significantly lower than that in patients with GS and CIN subtypes (Figure 3C). Then, we constructed the ROC curve to assess the predictive value of VCAN in immunotherapy response. We found that the AUC value of VCAN expression for predicting immunotherapy response was higher than that of MSI status and EBV status, and the AUC value as high as 0.985 when VCAN expression, MSI status and EBV status were combined (Figure 3E). Moreover, we also analyzed Hugo cohort (patients with melanoma were treated by PD1 inhibitor) and found patients in non-responding groups had higher VCAN expression than patients in responding group (Figure 3F). The proportion of responder for immunotherapy in the high VCAN group was significantly lower than the low VCAN group (Figure 3G), and the AUC value of VCAN expression was higher than that of PD-L1, PD1 and CTLA4 expression (Figure 3H), which suggested that VCAN might predict immunotherapy efficacy in other cancers. In conclusion, our results showed that patients with high VCAN expression tended to be resistant to immunotherapy, and VCAN could serve as a promising indicator to predict the response to immunotherapy for patients with GC.

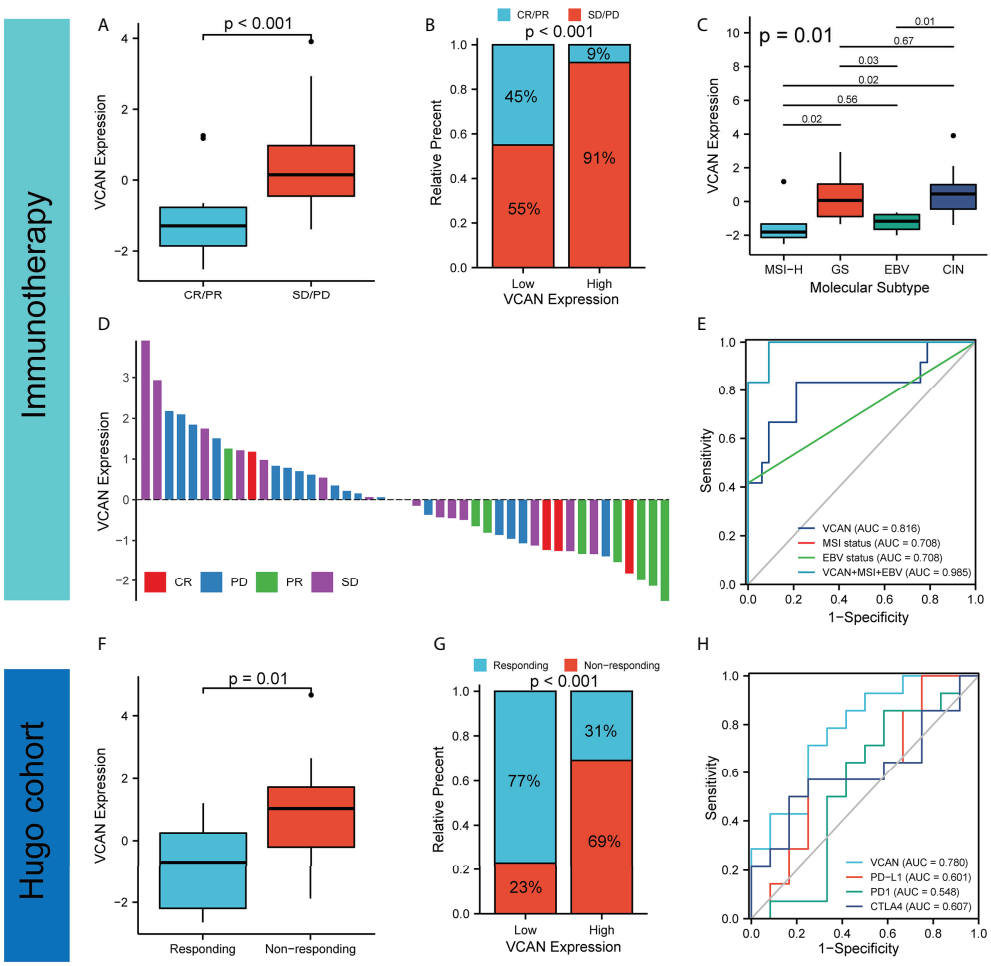

FIGURE 3
VCAN expression predicted response to immunotherapy. **(A)** The expression of VCAN in complete response (CR)/partial response (PR) group and stable disease (SD)/progressive disease (PD) group in KIM cohort. **(B)** Proportion of CR/PR and SD/PD in low VCAN group and high VCAN group in KIM cohort. **(C)** The expression of VCAN in patients with different molecular subtypes in KIM cohort. **(D)** The expression of in patients with different immunotherapy responses in KIM cohort. **(E)** Receiver operating characteristic (ROC) curves of VCAN expression, MSI status, and EBV status in predicting immunotherapy response in KIM cohort. **(F)** The expression of VCAN in responding group and non-responding group in Hugo cohort. **(G)** Proportion of responding and non-responding to immunotherapy in low VCAN group and high VCAN group in Hugo cohort. **(H)** ROC curves of VCAN, PD-L1, PD1 and CTLA4 expression in predicting immunotherapy response in Hugo cohort.

Potential mechanisms by which VCAN affected prognosis and response to therapy for GC

To explore the biological function of VCAN in GC, we quantified the enrichment degree of known biological processes in high VCAN group (the expression of VCAN was higher than the median value) and low VCAN group (the expression of VCAN was lower than the median value) through the "ssGSEA" algorithm in TCGA cohort (25). All of stromal relevant signatures, including EMT1, EMT2, EMT3 and panfibroblast TGFβ response characteristics (Pan-F TBRS), were found to be significantly upregulated in high VCAN group (Figure 4A). Correlation analysis confirmed that stromal relevant signatures and angiogenesis were significantly related to VCAN expression (Figure 4B). GSEA analysis results showed that VCAN significantly improved ECM Receptor Interaction signaling, Epithelial Mesenchymal Transformation signaling and Angiogenesis signaling (Figure 4C). Interestingly, we found that it was in almost human cancers that VCAN expression was positively associated with the enrichment of these signaling pathways (Figure 4D), which demonstrated the potential of VCAN as the common target for cancer treatment.

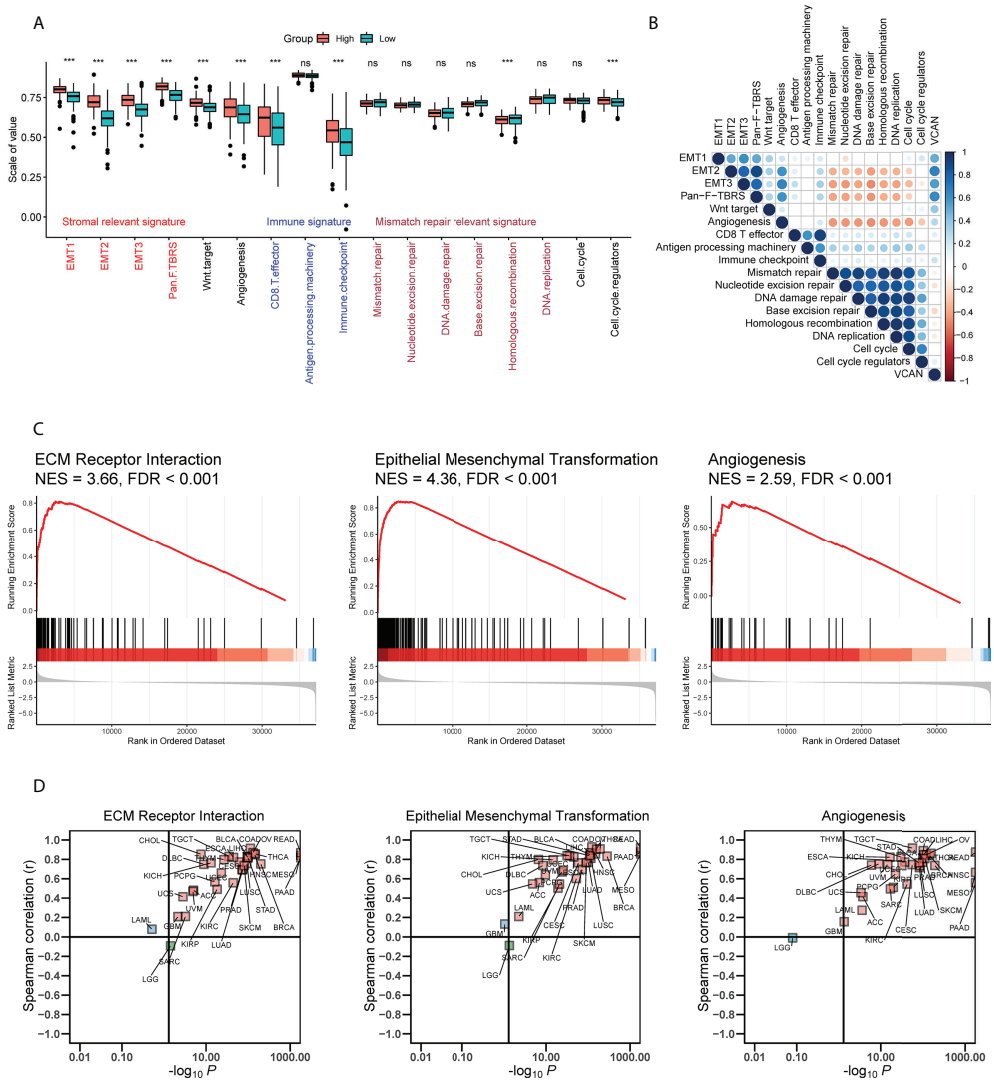

FIGURE 4
Potential mechanisms by which VCAN affected prognosis and response to anti-tumor therapy in GC. **(A)** The enrichment score of known biological processes in high VCAN group and low VCAN group in TCGA cohort. **(B)** The correlation between VCAN expression and the enrichment score of known biological processes in TCGA cohort. **(C)** Gene set enrichment analysis for patients with high VCAN expression and low expression in TCGA cohort. **(D)** The correlation between VCAN expression and the enrichment score of Extracellular Matrix (ECM) Receptor Interaction signaling, Epithelial Mesenchymal Transformation signaling and Angiogenesis signaling in human pan-cancer (Pan-cancer Atlas, TCGA). ***p < 0.001.

VCAN expression was positively correlated with cancer associated fibroblasts in the tumor microenvironment

Tumor microenvironment contained stromal cells and various immune cells, which interacted closely with tumor cells and contributed to tumor progression (26). Multiple algorithms were used to comprehensively assess the landscape of tumor microenvironment in TCGA cohort, including TIMER, CIBERSORT, QUANTISEQ, MCPCOUNTER, XCELL, and EPIC. We found that VCAN was associated with infiltration of a variety of immunosuppressive cells, such as macrophages, T cell regulatory (Tregs), and cancer associated fibroblasts (CAFs) (Figures 5A, C). Recent studies revealed that CAFs was the essential cell for depositing and remodeling the extracellular matrix in human cancers (27, 28). VCAN, an extracellular matrix proteoglycan, played an important role in the extracellular matrix remodeling pathway (8). We assumed that VCAN shaping tumor microenvironment was related to CAFs, and conducted the pan-cancer analysis to further analyze the relationship between VCAN and CAFs. The result showed that

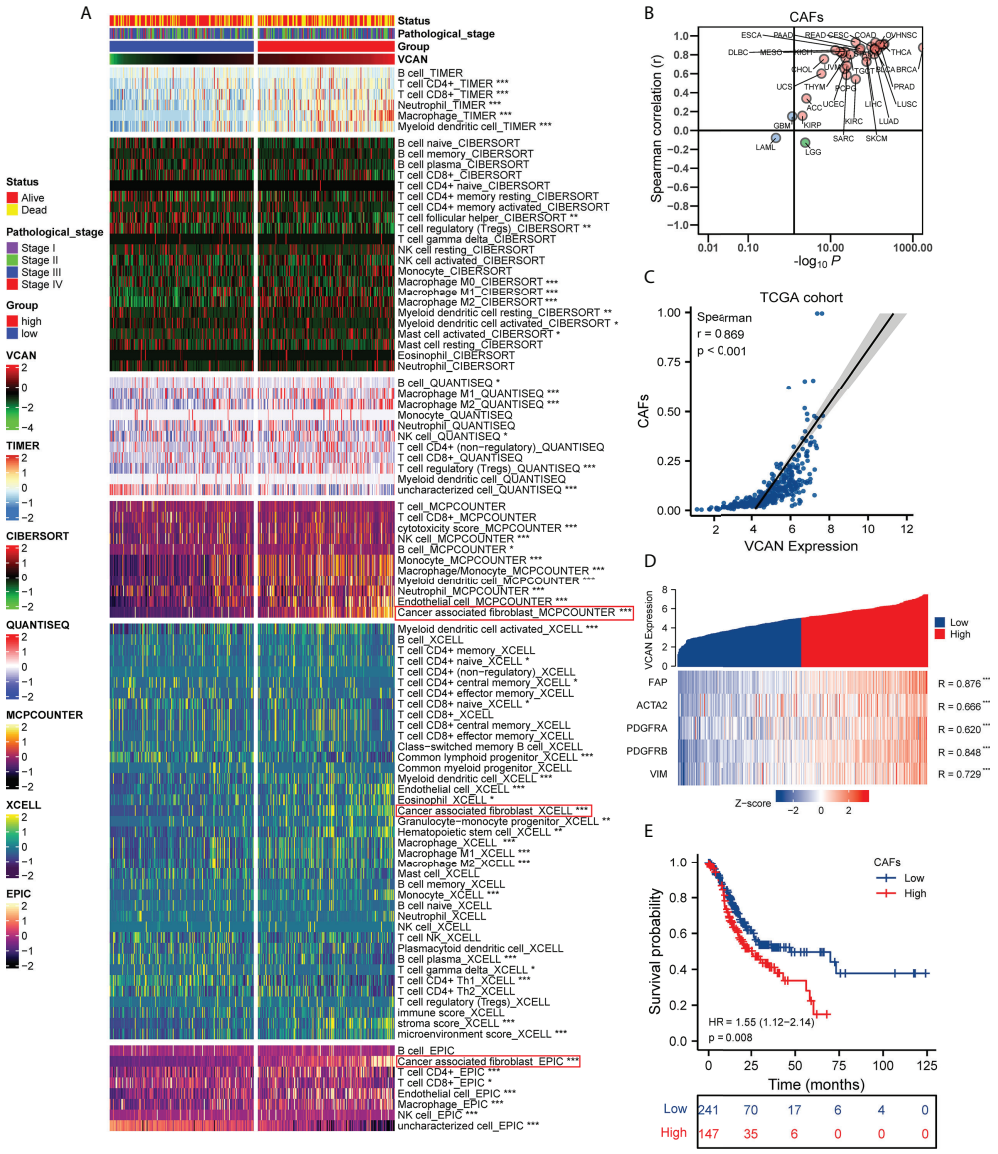

FIGURE 5
The correlation between VCAN expression and the tumor microenvironment. **(A)** The correlation between VCAN expression with the infiltration of immune cells and stroma cells in tumor microenvironment (TCGA cohort). **(B)** The correlation between VCAN expression with the infiltration of CAFs in human pan-cancer (Pan-cancer Atlas, TCGA). **(C)** The correlation between VCAN expression with the infiltration of CAFs in patients with GC (TCGA cohort). **(D)** The correlation between VCAN expression with the expression of CAFs markers in patients with GC (TCGA cohort). **(E)** Kaplan–Meier curves of OS of low and high group stratified by the CAFs infiltration in patients with GC (TCGA cohort). *p < 0.05, **p < 0.01, and ***p < 0.001. GC: gastric cancer; CAFs: cancer associated fibroblasts.

VCAN was positively associated with the level of CAFs infiltration in the vast majority of human cancers (Figure 5B). Fibroblast activation protein (FAP), α-SMA (ACTA2), platelet derived growth factor receptor α/β (PDGFRA/B), and Vimentin (VIM) were widely used as markers to identify CAFs (27). We found that the expression of VCAN mRNA was significantly correlated with the expression of CAFs markers mRNA (Figure 5D). To demonstrate the correlation between VCAN and CAFs at the protein level, we conducted multiple immunofluorescence experiments. The result showed that there were the co-existences of VCAN and FAP in the tumor (Figure S2), which was consistent with the analysis result at mRNA level. Moreover, survival analysis indicated the infiltration of CAFs was an unfavorable factor for the prognosis of gastric cancer patients (Figure 5E). In summary, overexpression of VCAN was often accompanied by the increase in CAFs infiltration in tumor

microenvironment, which was detrimental to the prognosis of patients with gastric cancer.

VCAN was mainly expressed in inflammatory cancer associated fibroblasts

Single-cell technology could characterize the molecular state of each cell, which enables more in-depth research on the tumor microenvironment and tumor heterogeneity, and it has become an indispensable tool in oncology research (29). To further explore the role of VCAN in tumor environment, gastric cancer single-cell dataset GSE167297 containing deep layer (D1, D2, D3, D4, D5) and superficial layer (S1, S2, S3, S4, S5) of tumor tissues and paired normal tissues (N1, N2, N3, N4) was downloaded and analyzed. After quality control, 19765 cells were eventually included in subsequent analysis and a total of 21 cell clusters were identified through UMAP algorithm (Figure 6A). Then, we annotated the cell lineage for every cluster based on the cell lineage marker genes. The single-cell atlas was mainly consisted of immune cells, such as T cells, B cells, Macrophages and dendritic cells (DC). In addition to immune cells, there were non-immune cells (epithelial cells, endothelial cells and fibroblasts) in the single-cell atlas (Figure 6B). We found that VCAN was mainly expressed in fibroblasts and macrophages rather than epithelial cells (Figure 6C), and the expression of VCAN was concentrated in the deep layers of tumor tissues (Figure 6D). Because the bulk RNA-seq analysis revealed the high correlation between VCAN and CAFs infiltration in gastric cancer (Figure 5C), we focused on the expression of VCAN in fibroblasts. Fibroblasts were further divided into three subpopulations with unique genetic signatures. Sub-cluster 0 and Sub-cluster 1 were identified as inflammatory CAFs (iCAFs) based on the enriched expression of chemokines such as CXCL1, CXCL14, CCL2, and interleukin 33 (IL33). Sub-cluster 2 had the high expression of ACTA2, therefore it was identified as myofibroblasts (Figure 6E). Interestingly, VCAN was mainly expressed in iCAFs and was barely expressed in myofibroblasts (Figure 6F). Interestingly, recent study has demonstrated the crucial role of iCAFs in cancer therapy resistance (30). Based on these results, we concluded that VCAN secreted by iCAFs was involved in the activation of stroma related pathways, thereby promoting anti-tumor therapy resistance (Figure 6G).

Discussion

VCAN, known as an extracellular matrix proteoglycan, was mainly constituted by stromal cells (8). Increasing evidence has supported that VCAN overexpression has been implicated in a wide range of malignancies and related to poor prognosis (31). In our study, we found that patients with high VCAN expression displayed worse prognosis in GC. As with our results, it has been documented that VCAN made an impact on the tumor mutation burden and tumor microenvironment of gastric cancer and VCAN lower expression indicated better prognosis and lower grade in GC (32). These reports in combination with our analyses demonstrated the oncogenic part of VCAN in GC. Previous bioinformatic studies also revealed that VCAN was associated with poor prognosis and could serve as a potential independent biomarker for diagnosis and prognosis for patients with GC (33–35). However, whether VCAN expression affected the therapeutic response to anti-tumor treatments remained unclear. Therefore, we mainly focused on the impact of VCAN expression on the efficacy of adjuvant therapy and immunotherapy. Remarkably, this study firstly demonstrated the predictive value of in response to adjuvant chemotherapy, adjuvant chemoradiotherapy and immunotherapy in GC. These results suggested that detection of VCAN expression was conducive to the selection of appropriate treatment and accurate prognostic assessment for patients with GC.

Tumor microenvironment significantly influenced not only tumor progression but also therapeutic response. Tumor microenvironment-mediated therapy resistance resulted from extracellular factors secreted by tumor parenchymal or stromal cells and adhesion of tumor cells to stromal fibroblasts or ingredients of extracellular matrix (36). The development of tumor involved many mechanisms and factors, among which CAFs were regarded as the key components in the tumor microenvironment. CAFs modulated tumor growth, metastasis and therapy responses by remodeling ECM and production of numerous cytokines and chemokines (28). It was reported that the major source of VCAN protein was constituted by CAFs in breast cancer, colon cancer, pharyngeal cancer, ovarian cancer and prostate cancer (31). Similar to previous studies, we found that VCAN was also mainly expressed in CAFs in GC. Upregulation of VCAN in CAFs enhanced ovarian cancer cell motility and invasion potential by activating the NF-κB signaling pathway and upregulated expression of CD44, MMP9 and the hyaluronan mediated motility receptor (37). Specific CAFs clusters could increase PD1 and CTLA4 protein level in Tregs to offer immunotherapy resistance and relevant ECM dysregulation might lead to failure in PD-L1 blockade immunotherapy (38, 39). These studies provided rational explanations for the poor prognosis and the resistance to adjuvant therapy and immunotherapy in the patients with high VCAN expression. Based on the above results, we concluded that targeting VCAN expression in CAFs may be an effective way to inhibit cancer progression and reverse treatment resistance.

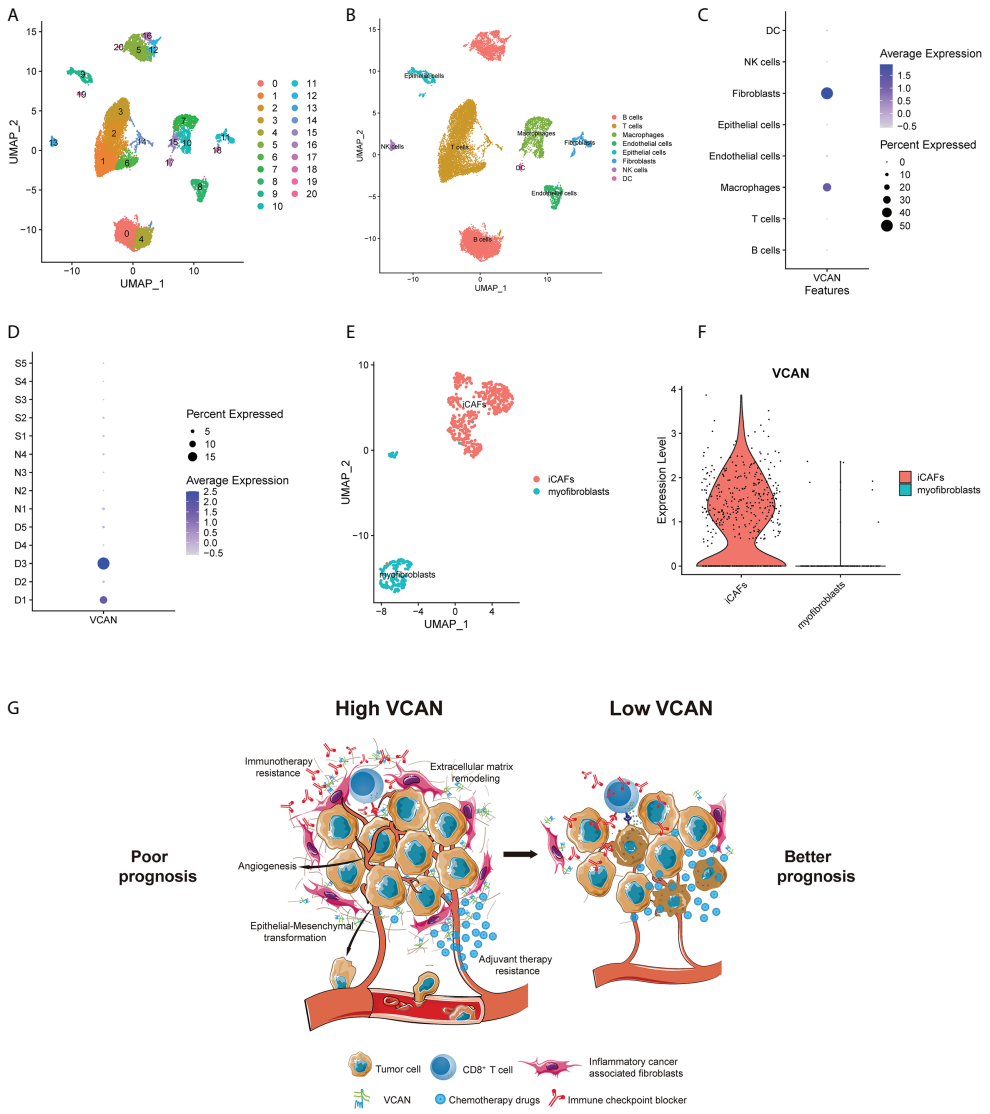

FIGURE 6
The single cell location of VCAN in the tumor microenvironment. **(A)** UMAP plot showed 21 cell clusters from 19765 cells of patients in GSE167297. **(B)** UMAP plot showed eight cell types from 19765 cells of patients in GSE167297. **(C)** Dotplot showed the expression level of VCAN in different cell types in GSE167297. **(D)** Dotplot showed the expression level of VCAN in deep layer (D1, D2, D3, D4, D5) and superficial layer (S1, S2, S3, S4, S5) of tumor tissues and paired normal tissues (N1, N2, N3, N4) in GSE167297. **(E)** Subpopulation analysis aimed to fibroblasts of patients in GSE167297. **(F)** Violin plot showed the expression of VCAN in iCAFs and myofibroblasts in GSE167297. **(G)** Graphic summary of the proposed model. VCAN secreted by iCAFs was involved in the activation of stroma related pathways, thereby promoting anti-tumor therapy resistance. iCAFs: inflammatory cancer associated fibroblasts.

Even though we have consolidated and analyzed multiple independent cohorts, multi-centered and randomized clinical trials were still needed to validate our findings. Synthesizing our findings and previous studies, we proposed the mechanism hypothesis of VCAN influencing prognosis and anti-tumor treatment response. However, the mechanism also needs to be validated by *in vivo* and *in vitro* experiments. Meanwhile, we hope that specific inhibitors for VCAN could be developed to improve the effect of anti-tumor therapy in the future studies.

Conclusions

In conclusion, our findings elucidated that VCAN was correlated with poor prognosis in GC, and patients with high

VCAN expression were more prone to resisting adjuvant therapy and immunotherapy. Given the superior prognostic value and predictive value of response to adjuvant therapy and immunotherapy, VCAN could be used as a biomarker and the new therapeutic target for GC.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Clinical Research Ethics Committee of Fudan University Shanghai Cancer Center. The patients/participants provided their written informed consent to participate in this study.

Author contributions

XL and CL for study concept and design, analysis and interpretation of data, drafting of the manuscript, obtained funding and study supervision. JS and RW for acquisition of data, analysis and interpretation of data, statistical analysis and drafting of the manuscript. SH for technical and material support. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.960570/full#supplementary-material

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Targeting myeloid villains in the treatment with immune checkpoint inhibitors in gastrointestinal cancer

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Despite the clinical outcomes being extremely limited, blocking immune inhibitory checkpoint pathways has been in the spotlight as a promising strategy for treating gastrointestinal cancer. However, a distinct strategy for the successful treatment is obviously needed in the clinical settings. Myeloid cells, such as neutrophils, macrophages, dendritic cells, and mast cells, are the majority of cellular components in the human immune system, but have received relatively less attention for the practical implementation than T cells and NK cells in cancer therapy because of concentration of the interest in development of the immune checkpoint blocking antibody inhibitors (ICIs). Abnormality of myeloid cells must impact on the entire host, including immune responses, stromagenesis, and cancer cells, leading to refractory cancer. This implies that elimination and reprogramming of the tumor-supportive myeloid villains may be a breakthrough to efficiently induce potent anti-tumor immunity in cancer patients. In this review, we provide an overview of current situation of the IC-blocking therapy of gastrointestinal cancer, including gastric, colorectal, and esophageal cancers. Also, we highlight the possible oncoimmunological components involved in the mechanisms underlying the resistance to the ICI therapy, particularly focusing on myeloid cells, including unique subsets expressing IC molecules. A deeper understanding of the molecular and cellular determinants may facilitate its practical implementation of targeting myeloid villains, and improve the clinical outcomes in the ICI therapy of gastrointestinal cancer.

KEYWORDS

gastrointestinal cancer, immune checkpoint, myeloid cells, immunosuppression, inflammation, metastasis, treatment resistance

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1 Introduction

Blocking immune inhibitory checkpoint (IC) pathways, brakes on immune responses, has been in the spotlight as a promising strategy for treating diverse types of cancers, including gastrointestinal (GI) cancer, since the great therapeutic efficacies have been shown in the treatment with IC-blocking antibodies (ICIs) targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA4) (ipilimumab and tremelimumab), programmed cell death protein 1 (PD1) (nivolumab, pembrolizumab, cemiplimab, and spartalizumab), and the PD1 ligand (PDL1) (atezolizumab, durvalumab, and avelumab), even in patients with advanced and metastatic cancer (1). The remarkable achievements have greatly contributed to changing the perception of cancer immunotherapy, and have led to development of a variety of immunotherapeutics, including blocking antibodies targeting other IC pathways or inflammatory pathways, peptide vaccines targeting tumor-associated antigens, and genetically engineered cellular products, for inducing anti-tumor responses in cancer patients (2, 3).

However, adverse events, including autoimmunity (4) and hyperprogression that is a rapid acceleration of the tumor growth and metastasis in patients shortly after treatment (5), are frequently observed in the treated patients. Also, the clinical response rate is relatively low, and most patients eventually show acquired resistance to the treatment even if responding in the beginning of the treatment (6). A reason may be that cancer cells affect numerous immunological components, including stromal cells, vascular cells, and immune cells, which in turn support cancer progression and metastasis. The reciprocal evolution increases heterogeneity and complexity of both tumor cells and the host immunity, leading to creation of refractory cancer (7).

To predict potential responses to anti-PD1/PDL1 therapy, biomarkers have been energetically investigated using advanced technology, and several biomarkers, including the PDL1 expression level as combined positive score (CPS) (8), the frequency of microsatellite instability (MSI) (9), or mutation burden (the number of non-synonymous single nucleotide variants) (10) in tumors have been identified so far. However, these are not necessarily correlated with clinical outcomes, and more precise and accurate biomarkers are still needed in clinical settings. To optimize the clinical efficacies of the ICI therapy, combination regimens with a variety of agents, such as small molecule inhibitors, other ICIs, and vaccines, have also been also evaluated in numerous clinical trials all over the world (3). However, the evaluation is still underway. A distinct strategy is obviously needed for the successful treatment of cancer.

Targeting myeloid cells, such as neutrophils, macrophages (Møs), dendritic cells (DCs), mast cells, may be a promising strategy for fundamentally changing such situation as a breakthrough in cancer immunotherapy. A reason is that myeloid cells are the majority of cellular components in the human immune system, and its abnormality may widely and negatively impact on the entire host, including tumor cells, stroma, and immunity, leading to treatment resistance, whereas the myeloid contents may vary within tumor microenvironment. In clinical settings, many studies have been demonstrated that local and systemic increase of myeloid cells is a poor prognostic marker in GI cancer as described later (11–13). Gut microbiome is known to regulate myelopoiesis, and its homeostasis and recruitment (14). Recent studies have revealed the crucial roles of gut microbiome in maintaining physiological conditions, including nutrient absorption and immune responses, and thus partly but significantly impacts on therapeutic efficacies induced by ICIs, such as anti-CTLA4 mAb (15), anti-PD1 mAb (16), and anti-PDL1 mAb (17). This suggests that elimination and reprogramming of the tumor-supportive myeloid cells facilitate induction of anti-tumor immune responses in the ICI therapy of GI cancer. However, targeting the myeloid villains is not yet practical in clinical settings, because a single/dominant marker that is exclusively and functionally expressed in the villain subsets, such as myeloid-derived suppressor cells (MDSCs), regulatory DCs (DCregs), and mesenchymal stromal/stem cells (MSCs), remain to be defined. To precisely distinguish the myeloid villain subsets is a priority issue for the practical application of myeloid-targeting therapy of cancer. Interestingly, accumulating evidence suggests that IC molecules, which are generally targeted on T cells and natural killer (NK) cells, are functionally expressed in myeloid cells expanded by cancer, and the unique sunsets promote tumor progression and metastasis directly and indirectly *via* inducing immune suppression and exhaustion leading to resistance to anti-PD1 therapy in mouse tumor models (18, 19). These suggest that the increased subsets are promising biomarkers to predict the potential unresponsiveness to anti-PD1 therapy. However, the clinical relevancy of targeting such myeloid subsets remains to be determined.

In this review, we provide an overview of background and current situation of the ICI therapy of GI cancer, and also highlight the oncoimmunological components involved in the mechanisms underlying the treatment resistance, particularly focusing on myeloid cells including the subsets expressing IC molecules. A deeper understanding of the molecular and cellular determinants would contribute to a practical implementation of targeting myeloid villains for improving the clinical effectiveness of the ICI therapy.

2 Background and current situation of the treatment of GI cancer

Development and the success of the ICI therapy surely changed the treatment paradigm for GI cancer in clinical settings (1). However, accumulating evidence suggests a limitation of the treatment due to innate or acquired resistance to the therapy in a majority of patients. To improve the clinical outcome, biomarkers have been explored to predict the potential responses to the ICI therapy, and numerous clinical trials have been conducted by combining a variety of agents for optimizing the therapeutic efficacy (Table 1). We firstly summarize the background and current situation of the treatments for GI cancers, including gastric cancer (GC), colorectal cancer (CRC), and esophageal cancer (EC), in clinical settings.

2.1 Gastric cancer

GC is the sixth most common type of cancer worldwide and ranks third among all causes of death due to malignant disease, while the age-adjusted incidence is decreasing globally (38). The reported risk factors are infection with Helicobacter pylori and Epstein-Barr virus (EBV), smoking, insufficient intake of vegetables and fruit, and alcohol consumption. GC types are histologically classified into two groups, diffuse and intestinal types, and the diffuse type is associated with peritoneal metastasis more frequently, but with hematological metastasis less frequently, as compared to the intestinal type (39). The Cancer Genome Atlas network divides GC into four molecular subtypes: EBV^+ tumors (9%), MSI^+ tumors (22%), tumors with genomic stability(20%), and tumors with chromosomal instability (50%) (40). Local GC can be cured by surgical resection with or without perioperative adjuvant chemotherapy, and systemic chemotherapy is the standard treatment of patients with advanced, unresectable, and recurrent GC (AGC) (41). Since late 1980's and early 1990's, combination of fluoropyrimidine (5-fluorouracil, capecitabine and S-1) and platinum (cisplatin and oxaliplatin) has been commonly and globally used. In late 1990's, docetaxel, paclitaxel, and irinotecan were clinically developed, showing a survival benefit compared with the best supportive care as the second line treatment (42). Recently, trifluridine tipiracil prolonged survival as the third or later line treatment (43).

In 2000's, a door of molecular targeted agents was opened for treating various kinds of malignant diseases. However, there are few options of the agents for treating AGC. For example, survival benefits of anti-HER2 monoclonal antibody (mAb) taruzuaumab in combination with fluoropyrimidine and platinum have been reported as the first-line treatment of $HER2^+$ AGC patients (44), amplification or overexpression of HER2 gene are seen only in 10 - 20% of GC. Anti-angiogenic inhibitor ramucirumab combined with weekly paclitaxel also prolonged survival as the second-line treatment (45). Recently, trastuzumab deruxtecan, which is an anti-HER2 mAb conjugated with containing topoisomerase I inhibitor, showed a higher response rate and longer survival of $HER2^+$ AGC patients as compared to the physician's choice of chemotherapy as the third or later line treatment (46). However, the overall outcome has been low.

A rise of ICIs dramatically changed the situation. The ATTRACTION-2 study that is the first pivotal trial demonstrated a survival benefit of anti-PD1 mAb nivolumab as compared to placebo as the third or later line treatment of AGC (median survival 5.26 versus 4.14 months, hazard ratio [HR] = 0.63, $P < 0.0001$) (47). Recent phase III trials showed positive results as the first line treatment of AGC. For example, the Checkmate-649 study reported nivolumab plus oxaliplatin-based doublet chemotherapy prolonged overall survival (OS) in

TABLE 1 Agents combined with anti-PD1/PDL1 mAbs in ongoing clinical trials (References).

Agents combined	Gastric cancer	Colorectal cancer	Esophageal cancer
Chemotherapeutics	Cisplatin/fluorouracil (20) Cisplatin/capecitabine (20)	Irinotecan/oxaliplatin/leucovorin/fluorouracil/bevacizumab (21) Temozolomide (22)	Fluorouracil/cisplatin (23)
Small molecule inhibitors	MKI Lenvatinib (24) MKI Regorafenib (25) HSP90 inhibitor TAS-116 (26) MMP9 inhibitor Andecaliximab (27, 28)	MKI Cobimetinib (29)	Lenvatinib (NCT04949256) Regorafenib (NCT04704154)
Immune checkpoint inhibitory mAbs	Anti-CTLA4 mAb (30) Anti-LAG3 mAb (31) Anti-TIGIT mAb (32)	Anti-CTLA4 mAb (33–35)	Anti-TIGIT mAb (NCT04732494, NCT04543617, NCT04540211)
Other therapeutics	Peptide vaccine OTSGC-A24 (36)	Anti-EGFR mAb cetuximab (37)	PD1-KO CAR-T targeting MUC1 (NCT03706326) CAR-T targeting EGFRvIII, DR5, NY-ESO-1 and Mesothelin (NCT03941626)

patients with CPS ≥ 5 or ≥ 1 tumors, and all randomized patients (48). The ATTRACTION-4 study conducted in Asian countries also reported the benefit of nivolumab therapy showing significantly longer progression-free survival (PFS) (49). Now, nivolumab has been approved for AGC as the first line treatment in many countries. Anti-PD1 mAb pembrolizumab has been additionally approved for MSI-H AGC as the second or later line treatment, while the incidence of MSI-H is only 5% in AGC (50).

Durable response is a strong point of the ICI therapy. For example, duration of response was as long as 9.5 months in AGC patients even as the third-line treatment with nivolumab in the ATTRACTION-2 study (51), 18.0 months in patients with CPS ≥ 1 tumors as the second-line treatment with pembrolizumab in the Keynote-061 study (52), and 13.7/19.3 months in patients with CPS ≥ 5/≥ 10 tumors, respectively, as the first-line treatment with pembrolizumab in the Keynote-062 study (20). The response durations are longer than cytotoxic agents in AGC. However, the clinical responses are low in the ICI therapy, and more than half of the patients showed progressive disease soon after treatment, suggesting innate and acquired resistance to the treatment (47). The KEYNOTE-061 study reported that pembrolizumab showed no significant survival benefit even in AGC patients with CPS ≥ 1 tumors as compared to weekly paclitaxel as the second-line treatment (52). The Javelin Gastric 300 trial reported that anti-PDL1 mAb avelumab showed slightly inferior survival as compared to the physician's choice of chemotherapy as the third-line treatment (53). In addition, the ATTRACTION-2 study reported that 2- and 3-year PFS rates were only 3.8% and 2.4% in all patients receiving nivolumab as third or later line treatment of AGC (51). Also, the 2-year update analysis of the Keynote-061 study reported that disease progression was seen in 95.4% (377/395) of patients with CPS ≥ 1 tumors, 93.5% (174/186) of patients with CPS ≥ 5 tumors, and 89.8% (97/108) of patients with CPS ≥ 10 tumors as the second line treatment with pembrolizumab (54).

Therefore, biomarkers to predict the therapeutic efficacy have been explored in the ICI therapy, and some factors, including PDL1-CPS score, deficiency of mismatch repair (dMMR), and the frequency of MSI and tumor mutation burden (TMB), have been suggested as diagnostic biomarkers to guide the application of anti-PD1/PDL1 mAbs. PDL1 overexpression in tumor tissues is the first biomarker expected in the anti-PD1/PDL1 therapy. PDL1 overexpression is seen in 25 - 65% of GC patients, and several clinical studies have demonstrated that the high levels of PDL1 are associated with lymph node metastasis, late stage of the disease, and poor prognosis (55, 56). Then, pembrolizumab was approved by the FDA for selectively treating CPS ≥ 1 advanced GC or gastroesophageal junction adenocarcinoma based on the positive results of the KEYNOTE-059 study showing significantly higher response in patients with $PDL1^{+}$ tumors as compared to patients with $PDL1^{-/low}$ tumors (57). Another outstanding biomarker is genomic abnormality that is unable to maintain genomic integrity in tumor cells. The high frequency of MSI (MSI-H) and dMMR are observed in 8 - 37% of GC patients, and TMB is seen in about 11% of GC patients (58). Many clinical studies have demonstrated that the MSI-H/dMMR status is associated with significantly better response and survival outcome in the anti-PD1/PDL1 therapy (59).

However, the conclusions of the clinical significance are still controversial. For example, the Keynote-062 study reported that pembrolizumab monotherapy was not superior to chemotherapy in patients with CPS ≥ 1 tumors, although providing a clinically meaningful benefit in OS of patients with CPS ≥ 10 tumors, and combination of pembrolizumab plus chemotherapy (cisplatin and fluorouracil, or capecitabine) was not also superior to chemotherapy alone in OS of patients with CPS ≥ 1 or ≥ 10 tumors, suggesting the insufficiency of the CPS as a predictive biomarker (20). Thus, combination regimens with other agents, have been alternatively evaluated in many clinical trials for improving the efficacy of the ICI monotherapy of GC. In most cases, anti-PD1/PDL1 mAbs have been combined with other ICIs targeting another IC pathways, such as anti-CTLA4 mAb (30), anti-lymphocyte-activation gene 3 (LAG3) mAb (31), and anti-T cell immunoglobulin and ITIM domain (TIGIT) mAb (32), or small molecule inhibitors targeting the malignant properties of tumor cells (proliferation, differentiation, adhesion, apoptosis, and migration) and angiogenic signaling (60). For example, anti-angiogenic inhibitors, such as regorafenib and lenvatinib, have been clinically evaluated in combination with anti-PD1 therapy. Lenvatinib plus pembrolizumab showed a promising response rate of 66% in 29 patients as the first- or second-line treatment for AGC (24), and regorafenib plus nivolumab also showed a response rate of 44% in 25 AGC patients as the two or more lines of prior chemotherapy in the REGONIVO/EPOC1603 study (25). Now, the LEAP-5 study is underway for evaluation of the combination efficacy of pembrolizumab plus lenvatinib in various solid tumors, including AGC.

However, most clinical trials have shown no synergistic benefits of the combination. For example, no benefits were seen in AGC patients in a phase Ib trial using an inhibitor (TAS-116) of HSP90, which facilitates NLRP3 inflammasome activity during inflammatory responses, in combination with nivolumab (26). Also, no benefits were seen AGC patients in the randomized phase II trial using a matrix metalloproteinase 9 (MMP9) inhibitor andecaliximab in combination with nivolumab (27), although much better responses (5/10 = 50%) were seen in Japanese patients with GC or gastroesophageal junction adenocarcinoma in a phase 1b trial (28). Active immunotherapy has been also clinically evaluated in the treatment of GC. However, most trials have failed. For example, no objective response was observed in a phase I trial with OTSGC-A24 that is an HLA-A*24:02-binding cocktail peptide vaccine targeting multiple tumor antigens (FOXM1, DEPDC1, KIF20A, URLC10 and VEGFR1), although responses of cytotoxic $CD8^{+}$ T cells (CTLs) were enhanced in 75% of AGC patients at 4 weeks after vaccination (36).

2.2 Colorectal cancer

CRC is the third most common primary tumor worldwide and ranks second in terms of mortality (38). Standard conventional treatments for CRC are surgery, chemotherapy and radiotherapy, and these treatments are combined depending on the localization and progression of the disease (61). Complete remission is often unachieved, and > 60% of stage II/III patients require further treatments with irradiation, chemotherapeutics, molecule targeting agents, and/or immunotherapeutics. As described in the GC section, ICI application dramatically changed the treatment paradigm for CRC. PDL1 is overexpressed in about 53% of CRC, but the level is rarely associated with clinical responses to the ICI therapy (62, 63). In contrast, the MSI-H/dMMR status is a strong biomarker to predict potential CRC responders to the ICI therapy. However, the frequency of MSI-H and dMMR varies across tumor types and stages, and the high frequency of the MSI-H/dMMR is observed only in 15 - 19% of CRC (64). The Keynote-177 study reported that pembrolizumab monotherapy showed significantly longer median PFS (16.5 vs. 8.2 months, HR = 0.60, P = 0.0002) than the standard-of-care chemotherapy as the first-line treatment of metastatic MSI-H/dMMR CRC (65). This result led to the FDA approval of pembrolizumab for the first-line treatment of patients with unresectable or metastatic MSI-H/dMMR CRC (66).

The accumulating evidence conversely suggests that the anti-PD1/PDL1 monotherapy is insufficient for treating the rest majority of CRC, microsatellite-stable and MMR-proficient tumors. Therefore, combination regimens with many other agents have been evaluated in numerous clinical trials. For example, the AtezoTRIBE study reported that atezolizumab and chemotherapy (irinotecan, oxaliplatin, leucovorin, fluorouracil, and bevacizumab) significantly prolonged PFS as compared to the chemo-control (21). However, the CheckMate 9X8 phase II/III trial reported at the GI Cancers Symposium 2022 that nivolumab in combination with the standard-of-care chemotherapy (5-fluorouracil, leucovorin, oxaliplatin, and bevacizumab) showed no synergistic effect on PFS in previously untreated patients with metastatic CRC. Molecular targeting small molecule inhibitors have been also combined with the ICIs. For example, Gomez-Roca et al. reported at ASCO 2021 that a multikinase inhibitor lenvatinib synergized with pembrolizumab in producing potent antitumor activity (objective response rate = 22%, and median PFS = 2.3 month) in patients with CPS ≥ 1 tumors in a nonrandomized phase II trial. Many other combination regimens have been now clinically developed: For example, a MAPK signaling inhibitor cobimetinib plus atezolizumab (29), anti-epidermal growth factor receptor (EGFR) mAb cetuximab plus anti-PDL1 mAb avelumab (37), and an alkylating agent temozolomide plus low-dose ipilimumab/nivolumab (22).

The most commonly combined agents are other ICIs targeting another IC pathway. The NICHE study reported that neoadjuvant treatment with a single dose of ipilimumab and two doses of nivolumab showed 100% pathological response in dMMR tumors, and 27% pathological response in MMR-proficient tumors of early-stage CRC patients within 4 weeks after treatment (33). The CheckMate-142 study reported that combination of nivolumab plus low-dose ipilimumab provided robust and durable clinical benefit as the first-line treatment of metastatic MSI-H/dMMR CRC, regardless of the PDL1 expression or the BRAF/RAS mutation status (34). Combination with anti-PDL1 durvalumab and anti-CTLA4 tremelimumab also provided better prognosis (2.5-month improvement of OS) in patients with advanced refractory CRC as compared to the best-supportive-care control in a phase II trial (35). Garralda et al. (abstract #3584) reported at ASCO 2021 that four patients presented partial response and one patient achieved complete response in the phase I first-in-human study using anti-LAG3 antibody MK4280 (favezelimab) and pembrolizumab for 89 patients with advanced microsatellite-stable CRC. The results encouraged the further development of MK4280, and the phase III trial is currently underway.

2.3 Esophageal cancer

EC is ranked as the seventh most common cancer, and is the sixth leading cause of cancer-related mortality in 2020 worldwide (38). EC is characterized by male dominance, geographic variation in incidence, and poor survival in the advanced stage, and is histologically divided into two major subtypes: esophageal squamous cell carcinoma (ESCC) that is the most common subtype (about 85% globally), and esophageal adenocarcinoma (EAC) (67). Profiles of genetic alterations differ between ESCC and EAC. Mutations in NFE2L2, MLL2, ZNF750, NOTCH1, and TGFβR2 are frequently observed in ESCC, but CDKN2A, ARID1A, SMAD4, and ERBB2 in EAC (68). Here, we mainly mention about advanced ESCC with high TMB but low frequency (1.08%) of MSI-H (69), since EAC is treated according to the strategy for GC.

Before the advent of ICIs, cytotoxic agents play crucial roles in the systemic chemotherapy for treating advanced ESCC, providing palliation of symptoms and prolongation of survival. Historically, fluorouracil-based or platinum-based chemotherapy are considered as the standard-of-care chemotherapy as the first line setting, and taxan agents (e.g., paclitaxel) as the second-line or later setting. Molecular targeting inhibitors, such as a small molecule EGFR inhibitor gefinitib (70) and anti-EGFR blocking mAb panitumumab (71), have been evaluated in phase III trials in advanced EC, while no clinical benefit has been shown. The rise of ICIs revolutionarily changed the treatment landscape of advanced EC, and the ICI therapy is now a standard treatment of pretreated

patients with advanced ESCC. The ATTRACTION-3 study that is an international randomized phase III study reported that nivolumab provided significant better prognosis as compared to chemotherapy (docetaxel or paclitaxel) (median OS = 10.9 versus 8.5 months, HR = 0.79, P = 0.0264; 3-year OS rates = 15.3% versus 8.7%) in patients with ESCC refractory to fluoropyrimidine and platinum (23). Other phase III studies using another anti-PD1 mAbs, such as pembrolizumab (72), camrelizumab (73), tislelizumab (74), reproduced the anti-PD1 efficacy in the treatment of pretreated ESCC. Despite the great achievement, however, patients with advanced ESCC mostly experience disease progression after the treatment. Therefore, the response-predictive biomarkers and combination regimens to produce the synergistic effect have been explored for treating EC. However, the MSI/dMMR/TMB status is relatively low in EC (MSH-H in 5 - 10%, dMMR in 3 - 5%, and TMB in 2% of EAC and 0% of ESCC) (75), and no large-scale study has demonstrated the significance of the MSI/dMMR/TMB status in the ICI therapy of EC.

On the other hand, PDL1 expression has been considered as a useful biomarker to predict potential responses to the ICI therapy. PDL1 overexpression is observed in about 20% of EC patients, particularly with ESCC (76), and is significantly associated with lymph node metastasis, later disease stage, and poor prognosis (55). The CheckMate-648 study reported that combination of chemotherapy (fluorouracil and cisplatin) plus nivolumab provided significantly better prognosis (median OS = 15.4 versus 9.1 months, HR = 0.54, P < 0.001) as compared to chemotherapy alone in patients with unresectable advanced, recurrent, or metastatic previously untreated ESCC patients with CPS ≥ 1% tumors (23). In addition, combination of nivolumab plus ipilimumab provided significantly better prognosis (median OS = 13.7 versus 9.1 months, HR = 0.64, P = 0.001) as compared to chemotherapy alone in patients with $PDL1^+$ tumors. Other phase III trials using another anti-PD1 mAbs, such as pembrolizumab (77), camrelizumab (78), sintilimab (79) and toripalimab (80), reproduced the significant anti-PD1 therapeutic efficacy compared to the chemotherapeutic efficacy in patients with advanced ESCC as the first-line settings.

Other agents, such as anti-angiogenic agents and other ICIs, have been clinically evaluated in combination with anti-PD1/PDL1 therapy. For example, there are two studies using small molecule multikinase inhibitors: regorafenib plus nivolumab in a phase II study (NCT04704154), and lenvatinib plus pembrolizumab in a phase III study (NCT04949256). Other ICIs targeting another IC pathway, including T-cell immunoglobulin domain and mucin domain 3 (TIM3), TIGIT, and LAG3, have been mostly combined in clinical trials. The high levels of TIM3 and TIGIT are associated with poor prognosis in ESCC (81), and LAG 3 is upregulated in $CD8^+$ T cells and NKT cells in patients with ESCC (82). These new ICIs are currently under investigation in many clinical trials for EC. For example, the AdvanTIG-203 study is a phase II study using anti-PD1 mAb tislelizumab and anti-TIGIT mAb ociperlimab (NCT04732494). The SKYSCRAPER-07 is a phase III study using atezolizumab plus another anti-TIGIT mAb tiragolumab (NCT04543617). The SKYSCRAPER-08 is a phase III study using chemotherapy with paclitaxel and cisplatin in addition to the immunotherapy with atezolizumab plus tiragolumab (NCT04540211).

To overcome innate and acquired resistance to immunotherapy, cell products with genetically engineered T cells has been clinically developed in cancer therapy. Particularly, T cells transduced with chimeric receptors composed of intracellular domains of immunoreceptors (CD3ζ, CD28 and/or 4-1BB, etc.) and single chain variable domain fragments (scFv) of tumor antigen-specific mAbs, called "chimeric antigen receptor T-cell (CAR-T)", have been clinically developed for treating cancer, including advanced EC. For example, a phase I/II study has evaluated the therapeutic efficacy of MUC1-targeting and PD1-knockout CAR-T cells (NCT03706326). Another phase I/II study has evaluated the therapeutic efficacy of CAR-T targeting multiple tumor antigens (EGFRvIII, DR5, NY-ESO-1 and Mesothelin) (NCT03941626). However, most trials are still underway.

3 Heterogeneity and complexity of the oncoimmunological network

Why is the immune system of cancer patients insensitive to the ICI therapy? A strong reason is enormous heterogeneity and complexity of the oncoimmunological network produced by the interplay between tumor cells and host immunity in cancer patients. Tumor-specific CTLs are generated and activated *via* the immune complexes composed of the T-cell receptor (TCR) and antigen peptide-loading major histocompatibility complex molecule I/II (MHC I/II) expressed on antigen-presenting cells (APCs), such as DCs, B cells, and Møs. Stable engagement with costimulatory molecules, including CD80, CD83, and CD86, is necessary for intensification of the TCR/MHC/peptide stimulatory signals to induce potent CTLs against cancer (83). However, this immune activation cascade is sometimes neglected and interfered by tumor cells. Firstly, tumor cells have an intrinsic potential to evade the immune attack by multiple ways. For example, tumor cells frequently express no or rare MHC I/II due to decrease or inactivation of an oncosuppressor TP53 (84). Also, tumor cells acquire high mobility and cancer stemness, including high self-renewability and anti-apoptotic dormancy contributing to treatment resistance, through a an evolutionarily conserved biological program "epithelial-to-mesenchymal transition (EMT)" in response to various stimuli within the tumor milieu (85). The EMT signaling through the RAS/ERK pathway upregulates PDL1 expression for braking the activation signaling pathways in anti-tumor effector cells by binding to PD1 (86).

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The EMT inducers not only confer aggressive properties on tumor cells, but also create an immune tolerant environment for the successful escape. For example, transforming growth factor-β (TGFβ) stands out as a master regulator of the mechanisms. The canonical TGFβ-SMAD pathway plays a key role in the EMT program in cooperation with other signaling pathways, such as PI3K/AKT, ERK/MAPK, RHOA, and ROCK (87). Alternatively, TGFβ also suppresses cytotoxic functions of CTLs and NK cells directly by reducing the expression of perforin, granzyme B, and NKG2D in these cells, and also indirectly by inducing immunosuppression mediated by regulatory T cells (Tregs) and immature APCs (88). Another key regulator is Wnt5a that is a prototypical activator of the non-canonical Wnt pathway associated with the ROR1/AKT/p65 pathway (89). Wnt5 activates various EMT-governing transcription factors, including the SNAIL family SNAI1 (Snail) and the basic helix-loop-helix factor TWIST, and consequently induces downregulation of adhesion molecules including occludin, ZO1/2, and E-cadherin, but upregulation of mesenchymal molecules including β-catenin, N-cadherin, vimentin, and fibronectin (85). Alternatively, Wnt5a stimulates Møs to secrete immunosuppressive molecule IL10 through the toll-like receptor (TLR)/MyD88/p50 pathway followed by suppression of DC maturation (89). The EMT-undergoing tumor cells further disturb induction and activation of anti-tumor immune responses by orchestrating immunosuppressive and pro-inflammatory cells to build up tolerant and supportive environment for raising the probability of its successful escape (Figure 1). We next summarize the molecular and cellular mechanisms underlying the oncoimmunological network, especially mediated by myeloid cells, which are the major component in the human immune system.

3.1 Immunosuppressors for tumor escape

Snail is an EMT-governing transcription factor. Snail$^+$ tumor cells produce thrombospondin-1 (TSP1) to promote tumor EMT in an autocrine manner, and indirectly through the generation of Treg-inducible regulatory DCs (DCreg) (90). CD47 is a receptor for TSP1, and the significant relationship between its high level and poor prognosis in various types of cancer, including GI cancer. For example, CD47 protein is aberrantly expressed in tumor tissues of GC patients, and the positivity is significantly associated with resistance to fluorouracil-based adjuvant chemotherapy, and the consequent poor prognosis (91). This study also showed that CD47 mRNA expression is especially enriched in GC with MSI and ARID1A mutation. The snail$^+$ tumor cells also produce follistatin-like 1 (FSTL1) to promote tumor EMT in an autocrine manner, and indirectly through the induction of immune exhaustion and dysfunction, and apoptosis in CTLs (92–94). TP53 abnormality (loss, decrease, inactivation, mutation) generates cancer stem cells (CSCs) through the EMT signaling, and induces production of various chemokines, such as CCL2, CXCL1/2, and CXCL10, to recruit immunosuppressive cells, including Tregs and MDSCs (95). CSCs produce a cytosolic heme-containing enzyme

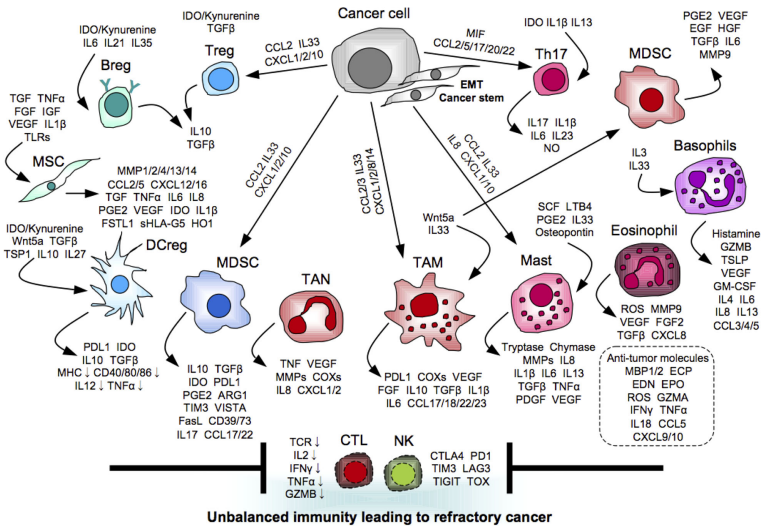

FIGURE 1
Myeloid orchestration leading to refractory cancer. Myeloid cells promote tumor progression and metastasis directly and indirectly *via* providing unbalanced immunity mediated by immunosuppressive and pro-inflammatory molecules to interfere induction and activation of anti-tumor effector cells.

indoleamine 2,3-dioxygenase (IDO) that degrades tryptophan into kynurenine followed by activation of AhR and GCN2 in immune cells (96). Tryptophan is essential for maintaining physiological and immunological homeostasis. The kynurenine-AhR/GCN2 axis widely suppresses cytotoxicity, proliferation, and survival of T cells and NK cells directly, and also indirectly *via* generating various immunosuppressive cells, such as Tregs, regulatory B cells (Bregs), DCregs, and MDSCs (96). IDO also regulates tumor dormancy that is a hallmark of CSCs by triggering G0/G1 cell cycle arrest (96).

Tregs are a heterogeneous population expressing tissue- or function-specific transcription factors, such as GATA3 and STAT3, along with FOXP3 that is a hallmark transcription factor of Tregs, and are the most prominent immunosuppressor that maintain self-tolerance and homeostasis as reviewed elsewhere (97). Bregs are generated *via* suppression of differentiation and maturation of B cells, and/or stimulation with pro-inflammatory cytokines, such as IL6, IL21, and IL35 (98). Bregs highly express and produce immunosuppressive and tumor-promotive molecules, such as PDL1, IL10, and TGFβ, as reviewed elsewhere (98). Here, we highlight immunosuppressive myeloid subsets, including MDSCs, DCregs, and MSCs.

3.1.1 MDSCs

MDSCs are composed of mononuclear (M-MDSCs) and polymorphonuclear myeloid cells (PMN-MDSCs), and an immunosuppressive subset is defined by several markers, such as CD11b, CD14, Ly6C, Ly6G, MHC II, and CD33, in combination, since no specific single marker remains to be identified. MDSCs are expanded and activated particularly by hypoxia in the tumor microenvironment (99). Under the hypoxic condition, hypoxia-inducible factor 1-alpha (HIF1α) induces ectonucleotidases, CD39 and CD73, to transform into MDSCs, and these molecules convert ATP to adenosine that inhibits T-cell functions through the adenosine receptors (100). MDSCs produce various immunomodulatory molecules, such as TGFβ, IL10, IDO, prostaglandin E2 (PGE2), and ARG1, and highly express PDL1 and Galectin-9 that binds T-cell immunoglobulin mucin 3 (TIM3), followed by induction of steady immunosuppression (101). V-domain Ig suppressor of T-cell activation (VISTA) is also upregulated in MDSCs under hypoxic condition, and plays immunosuppressive roles like PDL1 (102). Immunosuppressive Møs called "type 2 Møs (M2-Møs)"are likely a part of MDSCs, since M2-Møs show immunosuppressive activities similar to those of MDSCs. For example, M2-Møs suppress CTL functions not only directly utilizing PDL1 and immunosuppressive cytokines, such as IL10 and TGFβ, but also indirectly *via* production of immunosuppressive cytokines, recruitment of Tregs by CCL23, and polarization of Th2 by CCL17, CCL18 and CCL22 (103).

Increase of MDSCs are strongly associated with accumulation of Tregs in the tumor tissues, probably because MDSCs can expand Tregs directly *via* CD40 expressed on the MDSCs (104), and also indirectly by recruiting Tregs into the tumor milieu *via* producing IL17. The MDSC-derived IL17 induces own production of CCL17 and CCL22 in an autocrine manner, and enhances immunosuppressive activity of the recruited Tregs by upregulating CD39 and CD73 (105). In clinical settings, the high frequency of MDSCs in tumor tissues and peripheral blood is significantly associated with tumor metastasis, higher stages, and poorer prognosis in GC (106, 107), CRC (12), or EC (108–110), suggesting a critical biomarker and possible target in the treatment of GI cancer. In GC, the high levels of M-MDSCs in peripheral blood (106) or PMN-MDSCs in tumor tissues (107) are significantly associated with poor prognosis of patients. The tumor-derived PMN-MDSCs have been shown to highly produce S100A8/A9, which promotes tumor progression directly by upregulating CXCL1 in tumor cells *via* the TLR4/p38-MAPK/NFκB pathway, and also indirectly by suppressing glycolysis, proliferation and tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) production of $CD8^+$ T cells *via* the TLR4/AKT/mTOR pathway, leading to anti-PD1 resistance (107). Also in EC, the PMN-MDSCs have been demonstrated as a predominant myeloid subset in tumor tissues, and the high levels of PMN-MDSCs are significantly associated with advanced staging, low grade, lymph node metastasis, $HER2^-$ status, and poor prognosis of patients (108). M2-Møs has been also noticed in ESCC. For example, infiltration and polarization of M2-Møs are promoted by tumor-derived S100A7, which can directly promote tumor proliferation and migration *via* intracellular binding to JAB1 and paracrine interaction with RAGE receptors (109). This study also showed that the S100A7 positivity in tumor tissues is a poor prognostic factor. Interestingly, a pro-inflammatory cytokine IL32 is highly expressed in ESCC tumor tissues, and the IL32 derived from ESCC extracellular vesicles plays a key role in promoting lung metastasis by inducing M2-Mø polarization *via* the FAK-STAT3 pathway (110).

3.1.2 DCregs

DCregs, alternatively called tolerogenic DCs, are a heterogeneous population. As no specific single marker has been identified, an immunosuppressive subset is defined by upregulation of immunosuppressive molecules (PDL1, IL10, TGFβ, and IDO), but downregulation of MHC II, T-cell co-stimulatory molecules (CD40, CD80, CD86, etc.), and pro-inflammatory cytokines (IL12, TNFα, etc) (111). However, the *in vivo* functions of DCregs, particularly in human, remain unclear. A possible reason is that the number of DCs is small and limited in a body, and DCregs are needed to be induced and be expanded for the analysis by the *in vitro* long-term culture that may modify the phenotypes. In the *in vitro* setting, DCregs can be generated *via* the tolerogenic signaling mediated by

STAT3, AhR and SOCS2 in response to various stimuli, such as IL10, TGFβ, vitamin D3, and/or dexamethasone (112, 113). A pleiotropic cytokine IL27 also generates DCregs accompanied by CD39 upregulation *via* the STAT1/3 signaling (114). Interestingly, DCregs can be generated by stimulation with Helicobacter pylori that is a major cause of GC (115). In GC, DCregs expressing a non-classical and tolerogenic molecule HLA, HLA-G, significantly increase in peripheral blood of patients, and the high levels are significantly correlated with tumor grade, suggesting a critical biomarker in GC (116). HLA-G is also known as a poor prognostic marker in CRC (117). In CRC, tumor cells have been demonstrated to frequently suppress DC maturation, and generate immunosuppressive DCregs and dysfunctional DCs (118, 119). In ESCC, DCregs have been reported as a predominant subset in immune-suppressive cell populations within tumor tissues of patients using single-cell RNA sequencing, albeit few reports showing DCregs in EC so far (120).

3.1.3 MSCs

MSCs with a broad tissue distribution are able to differentiate into a variety of mesenchymal lineages, such as adipocytes, osteocytes, chondrocytes, fibroblasts, and pericytes, suggesting the great and wide impact on the physiological conditions of the host (121). MSCs have been considered as a key player in tumor progression and metastasis leading to treatment resistance in GI cancer (122). As no specific single marker has been identified, human MSCs have been defined using several molecules, such as CD49a, CD73, CD90, CD105, CD146, CD271, and STRO1, in combination with negative expression of CD11b, CD14, CD19, CD34, CD45, CD79a (123). MSCs highly express various chemokine receptors, such as CCR2, CCR3, CXCR4 and CXCR5, and various metalloproteinases, such as MMP1/2/4/13/14 and tissue inhibitors of metalloproteinases (TIMP1/2), and thus promptly migrate into tumor sites in response to chemokines, such as CCL2, RANTES/CCL5, CXCL12, and CXCL16, within the tumor milieu (124). The migrated MSCs are expanded by cytokines, such as TGFβ, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and insulin-like growth factor (IGF). MSCs acquire immunosuppressive and pro-inflammatory properties upon the activation with the microenvironmental cytokines, such as TNFα and IL1β, and/or ligation of the TLRs, such as TLR2, TLR3, and TLR4 (123). The activated MSCs become to promote tumor progression and metastasis directly and indirectly through creating immune tolerant environment by producing numerous immunomodulatory molecules, such as TGFβ, PGE2, VEGF, TNFα, IDO, IL1β, IL6, FSTL1, HO1, and soluble HLA-G5 (125).

However, the *in vivo* functions of MSCs remain obscure despite the numerous studies in the world. As well as DCregs, the number of MSCs are extremely limited in a body, and MSCs are needed to be expanded for the analysis by the *in vitro* long-term culture. In addition, the sources of the MSCs vary depending on the studies. Furthermore, the phenotypes and biological characteristics of MSCs have been demonstrated in regenerative research without cancer. Cancer-associated MSCs must be different from the original MSCs brought up in the absence of cancer. Identification of the precise MSCs in patients with cancer is emergently needed for the practical application of targeting MSCs in cancer therapy.

In clinical settings, cancer-associated fibroblasts (CAFs) rather than MSCs have attracted greater attention as a predominant stromal subset in GI cancer. In CRC, CAFs produce M-CSF that stimulates $CD163^+$ Møs to produce CCL2, HGF, IL6, and CXCL8/IL8 for recruitment and differentiation of monocytes into immunosuppressive Møs like M2-Møs in normal colon, potentially leading to tumorigenesis (126). Single-cell and spatial analysis of CRC tumor tissues also revealed the close relationship between FAP^+ fibroblasts and $SPP1^+$ Møs, and the positivity of both molecules are predictive of less therapeutic benefit from an anti-PDL1 therapy (127). In ESCC, CAFs generate M-MDSCs by the secreted IL6 and exosomal microRNA-21, and the CAF-induced M-MDSCs confer chemoresistance on tumor cells (128). The high levels of CAFs and $CD11b^+$ M-MDSC-like cells are significantly associated with poor prognosis in ESCC.

3.2 Inflammatory facilitators for tumor escape

Persistent and strong stimulation with pro-inflammatory mediators seriously damages the immune system, and facilitate tumor development, progression, and metastasis, leading to treatment failure. Myeloid cells produce a variety of pro-inflammatory molecules, such as cyclooxygenases (COXs), prostanoids, arginase 1, TNFα, IL1β, IL4, IL6, IL10 and IL13, and greatly affect multiple steps of tumor evolution, including genomic instability, metabolic reprograming, stromagenesis, angiogenesis, invasion, dissemination, and modification of host immunity (129). Th17 cells also participate in the inflammatory process for tumor progression and metastasis, albeit partly paradoxical depending on the study condition. Th17 cells are generated by tumor-derived IL1β and IL13, and accumulate in tumor tissues in response to various chemokines, including CCL2, CCL5, CCL20, CCL17, CCL22, and MIF, which are produced from tumor cells (130). Th17 cells highly produce pro-inflammatory molecules, such as IL17, IL1β, IL6, IL23, and nitric oxide (NO), and promote tumor progression directly, and also indirectly *via* inducing angiogenesis. Interestingly, Tregs are converted into Th17 cells by IDO stimulation in tumor-draining lymph nodes (131).

The chronic inflammation induces immune exhaustion and dysfunction by firmly braking the immune activation signals *via* inducing expression of multiple IC molecules, including CTLA4,

PD1, TIM3, LAG3, and TIGIT, in anti-tumor effector cells (132, 133). Consequently, anti-tumor effector molecules, such as IL2, IFNγ, TNFα, and granzyme B (GZMB), is dramatically downregulated in the CTLs and NK cells, and immune exhaustion and dysfunction are provoked locally and systemically in the host. LAG3 suppresses anti-tumor immunity directly by TCR downregulation, and also indirectly by impeding $CD4^+$ T-cell functions *via* competitively binding to MHC II with a higher affinity (134). TIGIT also suppresses anti-tumor immunity by TCR downregulation upon the binding to the ligands, CD155 (PVR) and CD112 (Nectin2), expressed in myeloid cells and tumor cells (135). Exhaustion and dysfunction of NK cells are fear in cancer immunotherapy, since CTLs sometimes miss tumor cells due to the MHC loss on tumor cells as described above. Recently, an HMG-box transcription factor, thymus high mobility group box protein (TOX), was identified as a key regulator of exhaustion of T cells (136). TOX expression is induced by calcineurin and NFAT2, and orchestrates immune inhibitory signals, not only PD1 but also other IC molecules, in $CD8^+$ T cells (137). Interestingly, the TOX binding to PD1 promotes the endocytic recycling of PD1 to maintain abundant PD1 expression on the cell surface, and sustains exhausted status of T cells. CD101 was identified as a marker to distinguish transitionally exhausted T cells, which still exert anti-tumor activities by invigoration, from terminally exhausted and dysfunctional T cells (47).

Here, we summarize pro-inflammatory myeloid subsets, including neutrophils, Møs, mast cells, basophils, eosinophils, which negatively impact on induction of anti-tumor immunity.

3.2.1 Neutrophils

Neutrophils are the most abundant cellular components in the human immune system. Tumor-associate neutrophils, called "TANs", are generated by various cytokines within the tumor milieu, and become to produce a variety of cytokines, such as TNFα, VEGF, and MMPs, and chemokines, such as CXCL1, CXCL2, and CXCL8/IL8, for promoting tumor growth and metastasis, angiogenesis, inflammation, and immunosuppression (138). The significant association between the high levels of neutrophils and poor prognosis has been demonstrated in GC (139–141) and CRC (142). However, the results are sometimes inconsistent potentially due to the high heterogeneity, plasticity, lack of the specific markers, and the short lifespan followed by rapid turnover in the host.

In clinical settings, neutrophil-to-lymphocyte ratio (NLR) in peripheral blood has been noticed as a marker of a systemic inflammatory status in patients, particularly with GC. The elevated NLR is significantly correlated with distant tumor dissemination, such as lymph node metastasis, peritoneal metastasis, osseous metastasis, and hepatic metastasis in GC (139). The elevated NLR is also significantly associated with poor prognosis of AGC patients after anti-PD1 therapy (140). The high levels of $CD66b^+$ TANs at the invasion margin have been reported as another poor prognostic marker in GC (141). This study showed that TANs promote tumor EMT by the secreted IL17a *via* the JAK2-STAT3 signaling pathway. Neutrophils form extracellular fibrous scaffolds constituted of its nuclear and cytoplasmic proteins, called "neutrophil extracellular traps (NETs)", upon the activation, and the NETs have been shown as a pathogenic factor in GI diseases, including GI cancer (143). For example, NETs in peripheral blood and ascites fluids promote tumor extravasation and dissemination into liver and peritoneum leading to metastasis in GC (144).

3.2.2 Møs

Møs with a longer lifespan than polymorphonuclear cells are the most outstanding player in the inflammatory responses linking to cancer progression and metastasis, and have attracted great attention as tumor-associated Møs (TAMs) in cancer. Pro-inflammatory TAMs are recruited by microenvironmental chemokines, such as CCL2, CCL3, CXCL1, CXCL2, CXCL8/IL8, and CXCL14, to tumor tissues, and produce pro-inflammatory and pro-angiogenic molecules, such as cyclooxygenases (COXs), IL1β, IL6, VEGF and FGF, for promoting tumor progression and metastasis in there (103). COXs produce eicosanoids such as prostaglandin E2 (PGE2) and thromboxane 2 (TXA2) from arachidonic acid to cause inflammation (145). COX1 is constitutively expressed in most tissues, but is upregulated in some types of cancer. In contrast, COX2 is induced by pathogenic stimuli not only in tumor cells, but also in other cells, such as fibroblasts, chondrocytes, endothelial cells, and Møs (146). IL1β enhances tumor invasion and dissemination directly, and also indirectly *via* inducing HIF1 expression followed by VEGF production (147). FGF synergizes to promote the VEGF-caused angiogenic process, including migration and proliferation of endothelial cells, and formation of transdifferentiated capillary tubes (148).

Pro-inflammatory properties of MDSCs have been also demonstrated, suggesting a part of the TAMs. MDSCs induce the EMT program by releasing various cytokines, such as PGE2, TGFβ, EGF, and HGF, and strengthen the tumor stemness using IL6 that activates STAT3 and NOTCH pathways (100). The CSCs induce expand and activate MDSCs, and the feedback loop brings up intractable tumors. MDSCs are recruited and activated by IL33, and produce VEGF, FGF, and MMP9 for inducing angiogenesis and tumor invasion in collaboration with other $ST2^+$ cells, including Mø and mast cells (149). The activated MDSCs also promote T-cell differentiation into pro-inflammatory Th17 for facilitating the inflammatory process and consequently tumor progression and metastasis.

In clinical settings, the high level of $CD206^+$ TAMs in tumor tissues has been shown as a significant poor prognostic marker in GC patients with liver metastasis (150). Single cell analysis of

tumor tissues revealed that GC patients with increase of HS6ST2$^+$ tumor cells and SERPINE1$^+$ Møs show unfavorable prognoses (151). These molecules are known to promote tumor growth, adhesion, and migration. In CRC, increase of CD163$^+$ TAMs at the invasive front in tumor tissues is significantly associated with poor prognosis of patients (152). This study also demonstrated that CRC-induced TAMs promote tumor migration and invasion by its secreted IL6 that inhibits expression of a tumor suppressor miR-506-3p followed by production of CCL2 to further recruit TAMs. Another study reported that IL6-prodicing TAMs confer chemoresistance on CRC tumor cells *via* the IL6R-STAT3 signaling pathway that inhibits expression of a tumor suppressor miR-204-5p (153). The CCL2-CCR2 axis is also important in ESCC. CCL2 upregulation and TAM increase are significantly observed in ESCC tumor tissues, and are significantly associated with poor prognosis (154).

3.2.3 Mast cells

Mast cells have pre-formed secretory granules containing classical and non-classical pro-inflammatory molecules, such as histamines, tryptase, chymase, heparin, lysosomal enzymes, and pro-inflammatory cytokines, such as IL6, IL8, TNFα, VEGF, FGF2, and platelet-derived growth factor (PDGF) (155), and are widely known to play a central role in inflammatory pathogenesis, particularly of allergy and cancer (156). Mast cells are recruited by the microenvironmental chemokines, such as CCL2, CXCL1, CXCL8/IL8, and CXCL10, to tumor tissues, and are activated by pro-inflammatory cytokines, such as stem cell facto (SCF), IL33, PGE2, leukotriene B4, and osteopontin, in there (157). SCF stimulates mast cells to produce tryptase and chymase *via* the tyrosine kinase activation signaling of the c-kit receptor, followed by activation of the released MMPs that degrade extracellular matrix components and tissues. The activated mast cells also produce IL1β, IL6, IL8, IL13, TGFβ, TNFα, PDGF, and VEGF for promoting tumor growth and metastasis directly, and also indirectly by provoking angiogenesis and immune chaos (155).

In particular, release of IL33, a member of the IL1 family, from mast cells is a disaster in cancer. IL33 is also released from many other cells, such as endothelial cells, epithelial cells, fibroblasts, and cancer cells, upon cellular stress. IL33 recruits and activates its receptor ST2-expressing cells, including not only pro-inflammatory cells (mast cells, TAMs, basophils, eosinophils, etc.), but also immunosuppressive cells (Tregs, MDSCs, ILC2s, etc.) followed by angiogenesis, immune tolerance, and inflammation in the host (149). IL33 is upregulated in diverse types of cancers, particularly in GC and CRC, and the IL33-mast-TAM axis has been reported as a poor prognostic factor in GC patients (158). In GC, however, IL33 is expressed mainly in epithelial cells, and partly in CD11b$^+$CD64$^+$MHC II$^+$CX3CR1$^+$ Møs, but not in MCPT1/2$^+$ mast cells (159).

Many other studies have demonstrated the significant correlations among the high level of mast cells, angiogenesis, and tumor progression in many cancers, including GC (160) and CRC (161), while the opposite and favorable results have been also reported in several cancers, including EC (162). This inconsistency may partly depend on the proportion of Treg cells and the interaction between mast cells and Treg cells in the host. Because Tregs suppress mast cell functions, such as differentiation, degranulation, IgE-mediated LTC4 production by immunosuppressive cytokines, such as IL10 and TGFβ, and the Treg/OX40-mast/OX40L axis (163, 164), and conversely mast cells confer pro-inflammatory property to immunosuppressive Tregs without losing T-cell-suppressive properties, and promote inflammatory responses (165). Interestingly, a current study using humanized mice (NGS mice transplanted with human CD34$^+$ cells and autologous thymus grafts) has demonstrated that co-localization of mast cells and Tregs in IL33$^+$ tumor tissues is significantly associated with resistance to anti-PD1 therapy (166). They also showed that depletion of mast cells improves anti-PD1 therapeutic efficacy in the tumor models.

3.2.4 Basophils

Basophils have pre-formed secretory granules containing pro-inflammatory cytokines, such as IL4, IL6, IL13, TSLP, GM-CSF and VEGF, and chemokines, such as CCL3, CCL4, CCL5 and CXCL8/IL8, in addition to histamine and granzyme B, and are widely known as a key player in allergy and parasitic infection (167). IL3 and IL33 are potent activators of basophils, and stimulate to produce these molecules. Despite the small number (< 1%) in peripheral blood leukocytes, accumulating evidence suggests that basophils participate in cancer pathogenesis, since basophils are a major source of IL4 that induce Th2 and M2-TAM polarization, and also produce CCL5 to recruit TAMs and Treg cells (168).

In clinical settings, the significant correlation between basophils accumulation in tumor tissues and patient survival has been demonstrated in several types of cancers, including GC (169). Gene expression analysis of patient-derived GC tumors also showed that the high levels of basophil activation signatures (CD123, CCR3, FceRIa, CD63, CD203c, and tryptase) are significantly associated with poor prognosis, while the results are reversal in sarcoma and endometrial cancer (170). In CRC, basopenia (decrease of basophils) in peripheral blood is associated with poor prognosis (171), while the results are reversal in other cancers, including breast cancer and ovarian cancer (168). Thus, the significance of basophils is still controversial in cancer, and should be determined by the further investigations.

3.2.5 Eosinophils

Eosinophils are widely known as a key player in allergy, parasitic and fungal infections, and asthma. However, eosinophils have only recently come to the fore in cancer, albeit still remaining inconsistency. For example, the high levels of eosinophils are significantly associated with poor prognosis in GC and CRC, but with better prognosis in lung cancer and ovary cancer (172). For the priming and expansion of eosinophils, IL5 is an essential molecule. IL5 stimulation induces expression of chemokine receptors for chemoattractant eotaxins (eotaxin1/CCL11, eotaxin2/CCL24, and eotaxin3/CCL26) and many other chemokines (CCL8, CCL7, CCL13, CCL5, CCL15, etc.) rich in tumor microenvironment (172, 173). Eosinophils highly express ST2, RAGE, and Toll-like receptor 4 (TLR4), and are activated by the ligands, including IL33.

Two different types of eosinophils have been reported (172, 173). One is a tumor-promotive type that produces inflammatory and angiogenic molecules (ROS, VEGF, FGF2, MMP9, IL8, etc.), which induce genetic instability, DNA damage, angiogenesis, and EMT of tumor cells. Another is an anti-tumor type that produces the unique acidophilic secondary granules composed of major basic protein 1 and 2 (MBP1, MBP2) and a matrix composed of eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), and eosinophil peroxidase (EPO), and many immunomodulatory molecules (granzyme A, TNFα, IL18, IFNγ, CCL5, CXCL9, CXCL10, etc.). These molecules directly exert cytotoxity on tumor cells, and also induce anti-tumor immunity *via* polarization of M1-TAMs. In clinical settings, the latter anti-tumor type has been implicated in GI cancer, including GC, CRC, and EC, based on the gene expression in tumor tissues, whereas the functions remain to be defined (172, 173).

4 Myeloid subsets expressing IC molecules

The general perception is that IC molecules, such as PD1, CTLA4, LAG3, and TIGIT, are expressed in T cells and NK cells, and the ligands are expressed in the other cells, including tumor cells and myeloid cells. Indeed, there are many reports showing the functions of the ligands expressed in tumor cells and myeloid cells. For example, PDL1 expressed in tumor cells functionally regulates cell proliferation and survival through the ERK/mTOR pathway (174), EMT induction through the RAS/ERK pathway (86), and cell metabolism through the Akt/mTOR pathway (175) in addition to the immune brake in the PD1-expressing cells. PDL1 expressed in myeloid cells induces Treg-inducible DCregs (176), and also suppresses Mø functions, such as proliferation, survival, and activation (177). However, accumulating evidence suggests the significant expressions and functional roles of the IC molecules in myeloid lineages (Figure 2). Most studies demonstrated DCs and Møs expressing expression of CTLA4 or PD1, but several studies reported unique subsets: a MSC subset expressing membrane-bound and soluble CTLA4 that is responsible for the immunosuppressive property (178), and a $LAG3^+CD11b^+$ myeloid subset that induce apoptosis in CTLs (93). This fact opens up new possibilities of indication expansion of ICIs for targeting myeloid cells, which exist and increase much more than T cells and NK cells in cancer patients, while the clinical relevancy of targeting these cells remain to be determined.

4.1 $CTLA4^+$ myeloid subsets

The first report demonstrated CTLA4 expression in human monocytes and myelomonocytic cell lines U937 and THP1 upon the activation with PMA and IFNγ (179). This study also showed that blocking the myeloid CTLA4 partially inhibits its proliferation and T-cell stimulatory molecule expression (CD86, CD54, HLA-DR and HLA-DQ) through the AP1-NFκB signaling pathway. CTLA4 is expressed in monocytes after differentiation. For example, bone marrow monocyte-derived DCs express membrane-bound and soluble CTLA4 upon the maturation with LPS, Poly I:C or inflammatory cytokines, and the CTLA4 ligation with an agonistic anti-CTLA4 mAb enhances IL10 production but suppresses IL8, IL12 and T-cell stimulatory activity (180, 181). The CTLA4 seems to brake the full maturation/activation of DCs. Interestingly, intracellular CTLA4 molecules are packaged in microvesicles of mature DCs, and the microvesicles are transferred to the neighboring DCs for suppressing maturation, suggesting a contagious brake in DCs (182). $CTLA4^+$ TAMs are systemically expanded in mouse and human CRC metastatic settings, and facilitate tumor progression and metastasis directly by generating lipid droplets in tumor cells, and also indirectly by inducing immune exhaustion, leading to anti-PD1 resistance (183). Lipid droplets have been considered as a cellular organelle just for fat storage so far. However, accumulating evidence suggests its important roles in the aberrant lipid metabolism of tumor cells, and the increase of lipid droplets is now gathering attention as cancer stemness (184). Anti-CTLA4 therapy may contribute to alleviation of the inflammatory responses in CRC patients with increased $CTLA4^+$ TAMs.

4.2 $PD1^+$ myeloid subsets

A little later than the CTLA4 discovery, PD1 expression in myeloid cells has been demonstrated. DCs derived from PD1-knockout mice highly produce IL12 and TNFα, which are important for inducing potent CTLs, suggesting an immune brake role of PD1 in DCs (185, 186). $PD1^+$ TAMs highly express

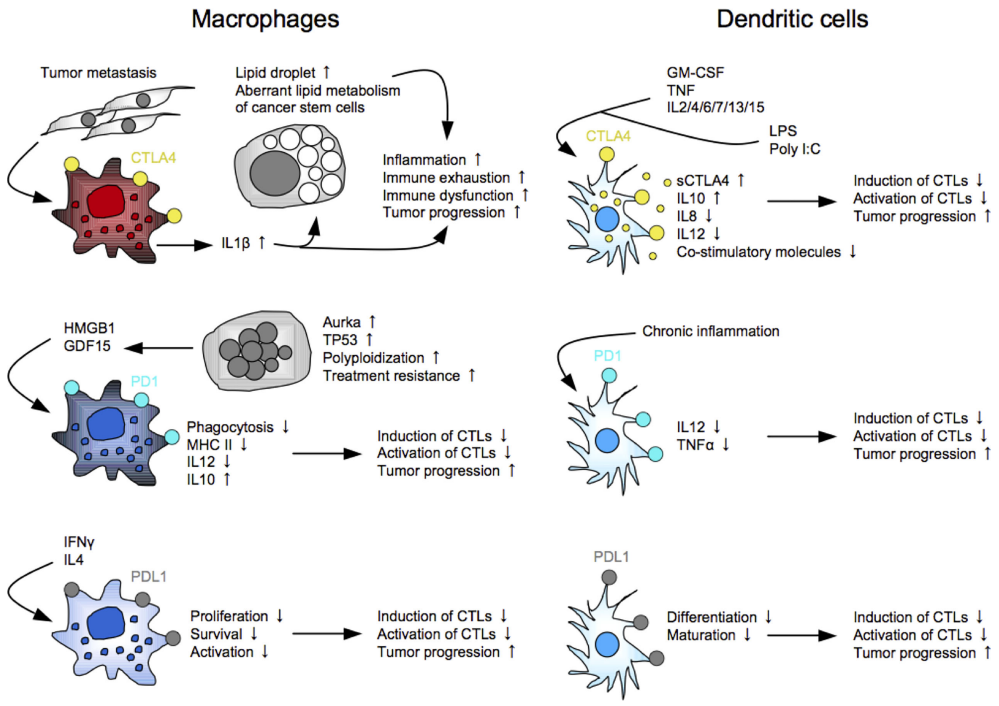

FIGURE 2
Myeloid subsets expressing immune checkpoint molecules. As well as PDL1, CTLA4 and PD1 are functionally expressed in myeloid cells, including macrophages and dendritic cells, and play key roles in induction of immune suppression and exhaustion in the host.

CD206 and IL10, but not HLA-DR, CD64 and IL12, and suppress proliferation of $CD8^+$ T cells (187). This study also showed that $PD1^+$ TAMs are clonally expanded by exosomal HMGB1 derived from EC cells. PD1 ligation is a key component to suppress its phagocytosis of the $PD1^+$ TAMs (188). Interestingly, PD1 is also expressed in Mø in the peritoneal cavity of mice and human. Ozawa et al. reported that $PD1^+$ TAMs with dysfunctional phagocytosis are expanded in the peritoneal cavity with disseminated tumor cells in mouse CRC ascites models and GC patients (189). The peritoneal tumor cells are polyploidy (giant with large nuclei) highly expressing aurora kinase A (AURKA) and GDF15 that is partly involved in the $PD1^+$ TAM expansion. They also showed that treatment with an AURKA inhibitor MLN8237 significantly induced anti-tumor immunity in the anti-PD1-resistant CRC ascites models, providing significant better prognosis. Peritoneal tumor dissemination is frequently seen in GI cancer, and leads to malignant ascites that suddenly and repeatedly relapses even after being drained from the peritoneal cavity, resulting in poor prognosis (190). Despite advances in molecular profiling of the intraperitoneal tumors and immune cells, and many clinical trials using inventive methods, such as cytoreductive surgery and hyperthermic intraperitoneal chemotherapy, therapeutic options for such patients are still extremely limited to palliative treatments of the symptoms (191). These findings may be a ray of light leading to improvement of the present status in the clinical settings. More clinical evidence of $PD1^+$ myeloid cells has been demonstrated. For example, $PD1^+$ DCs increase in tumor tissues and peripheral blood of patients with hepatocellular carcinoma (186), and the high levels of $PD1^+$ TAMs in tumor tissues are significantly associated with poor prognosis in GC (192).

5 Treatment strategy for overcoming the ICI resistance

A promising strategy for successfully treating cancer is breaking the tumor-host interplay for impeding the reciprocal evolution producing oncoimmunological heterogeneity and complexity. Numerous agents, including small molecule inhibitors, antibodies, and genetically modified cells, have been clinically developed for treating cancer, but most clinical evaluations are still underway (7, 193). Targeting immune mediators is the most reasonable approach in cancer immunotherapy. Therefore, in addition to treatment regimens described in the clinical section, we summarize immunotherapeutics, which are likely to optimize the

combination strategy for improving the clinical effectiveness of the ICI therapy, regardless of cancer types.

5.1 Targeting immunosuppressive molecules

As described repeatedly, the clinical efficacies of ICIs targeting CTLA4, PD1, and PDL1 are low in most cases, and thus many inhibitory mAbs targeting other IC molecules, such as TIM3 (TSR-022, MGB-453, INCAGN02390, Sym023, and BGB-A425), LAG3 (Relatlimab, LAG525, REGN3767, MK-4280, Syn-022, and TRS-003), and TIGIT (tiragolumab, BMS-986207, MK-7684, AB154, ASP8374, and COM902), have been pharmaceutically developed. These mAbs have been evaluated in combination with/without other agents, such as chemotherapeutics, molecular targeting inhibitors, and other ICIs, in numerous clinical trials. Bispecific mAbs that simultaneously inhibit two molecular pathways, such as PD1-TIM3 (RO7121661), PD1-LAG3 (RO7247669), and PDL1-LAG3 (FS118), have been also developed, and have been clinically evaluated for advanced and/or metastatic solid tumors, including EC. Anti-TGFβ mAbs (SAR-439459, NIS-793 and fresolimumab), and a small molecule inhibitor of TGFβ receptor I (TGFβRI) kinase for SMAD2 phosphorylation (galunisertib/LY2157299) have been clinically evaluated in combination with anti-PD1/PDL1 therapy in phase I/II trials for advanced solid tumors. M7824 is a bifunctional anti-PDL1-TGFβ trap fusion protein that not only reverts the mesenchymalization of tumor cells, but also activates CTLs and NK cells, and has been clinically evaluated in many trials for advanced solid tumors. Inhibitors targeting IDO1 (epacadostat, GDC-0919, PF-06840003, NLG802, SHR9146, and linrodostat), IDO2 (indoximod), or both (1-MT) have been clinically evaluated in combination with chemotherapy and/or ICI therapy in phase I/II studies for solid tumors and peritoneal cancer. In the ECHO-301/KEYNOTE-252 phase III trial, however, combination of epacadostat plus pembrolizumab showed no synergistic survival benefit as compared to the pembrolizumab monotherapy in patients with unresectable or metastatic melanoma.

Some of the ICIs directly affect myeloid villains expressing the IC molecules described above. However, most of these agents targeting immunosuppressive molecules do not directly affect immunosuppressive myeloid cells. Recently, however, several unique agents targeting immunosuppressive myeloid villains have been clinically developed. For example, anti-VISTA mAbs, including HMBD-002 (NCT05082610) and CI-8993 (NCT04475523), have been evaluated in combination with/without anti-PD1 therapy in phase I trials for advanced solid tumors, as VISTA is a marker of MDSCs, and also plays immunosuppressive roles. Anti-HLA-G mAb TTX-080 has been also evaluated in combination with/without pembrolizumab or cetuximab in phase I trials for advanced solid tumors, including CRC (NCT04485013), as HLA-G is a marker of DCregs and MSCs, and also plays immunosuppressive roles.

5.2 Targeting pro-inflammatory molecules

Basically, inflammatory mediators have been primarily targeted for treating other inflammatory diseases, such as rheumatoid arthritis and pulmonary disease, so far. However, several inhibitory mAbs targeting IL1β (canakinumab), IL6 (tocilizumab, siltuximab, etc.), and IL8 (BMS-986253) have been recently evaluated in combination with/without other agents, such as chemotherapy, anti-HER2 mAb, or anti-PD1 mAb, in many clinical trials for various types of cancers.

COXs are representative of pro-inflammatory molecules in tumor progression mechanisms, and there are a number of *in vivo* therapeutic studies showing the anti-tumor efficacies of a COX1/2 inhibitor aspirin in mouse tumor models (194). The reason may be that aspirin widely suppresses platelet aggregation, endothelial activation, tumor adhesion to the endothelium, recruitment of myeloid cells, and EMT induction in tumor cells. Also, the significant impact of aspirin use has been demonstrated in PDL1^{low} CRC tumors in clinical settings (195). However, aspirin therapeutic efficacy remains to be determined, since most of the clinical studies are retrospective, and COX2-specific inhibitor celecoxib is preferred in clinical therapy. Because COX1 is constitutive expressed in most tissues, whereas COX2 is inducible in pathogenic process, suggesting induction of adverse events by blocking COX1. Blocking COX2, however, may promote tumor metastasis *via* amplifying the COX1-induced events, since it has been shown that COX2 knockout upregulates COX1 that produces TXA2, which induces platelet aggregation to promote cancer metastasis, in mice (145).

5.3 Active immunotherapy

Induction and activation of anti-tumor immune responses is a fundamental strategy in cancer immunotherapy, and thus many immunomodulatory agents, including whole tumor vaccines, DC vaccines, tumor antigen peptides, and viral vectors, have been clinically developed so far, while most clinical trials have failed. Of note, tumor antigens have been recently re-focused as "neoantigens" based on the concept that higher mutations in tumor cells could lead to high immunogenicity that can induces immune responses (196). Numerous neoantigens have been identified using next generation sequencing and advanced bioinformatics technology, and various peptide vaccines (KRAS, DNAJB1-PRKACA, IDH1R132H, AE37, K27M, etc.) and the peptide-pulsed DCs have been clinically evaluated in combination with other treatments, such as chemotherapy and ICI therapy (197). Despite the great expectation, however, most trials have been failing. A potential reason may be the immunological diversity and complexity that can no longer be easily reprogrammed and fixed by the therapy.

5.4 Cell therapy

To elementally raise anti-tumor immunity, genetically engineered T cells and NK cells have been pharmaceutically developed for treating cancer as described previously (198). A great advantage of the CAR-NK therapy is that CAR-NK can be generated using not only autologous but also allogeneic donor cells, whereas only autologous T cells for CAR-T products. However, ex vivo expansion of NK cells is relatively difficult because the lifespan (< 10 days) is shorter than that of T cells (> 10 years) even in normal conditions. NKG2D-transduced CTLs has been recently developed, since NKG2D signaling activates anti-tumor effector cells *via* binding to the ligands (MICA/MICB, ULBP, RAE1, etc.) that are frequently overexpressed in tumor cells. NKG2D-CAR-T cells (CYAD-101, KD-025, NKX101, and NKR-2) have been now clinically evaluated in combination with chemotherapy in phase I/II trials for relapsed or refractory solid tumors (NCT03692429 and NCT04550663).

Three CAR-T products (tisagenlecleucel, axicabtagene ciloleucel, and brexucabtagene autoleucel) have been clinically approved for treating lymphoma, and one CAR-NK product (CellProtect) has been recently approved as an orphan drug for treating multiple myeloma. Despite the success in the treatment of hematological malignancies, however, the therapeutic efficacy is extremely limited in the treatment of solid tumors, and other issues, including serious adverse events, high manufacturing costs needed for the specialized facilities, and a few providers, remain to be solved in the clinical settings. Further improvement of the CAR design is needed for the successful treatment of solid tumors, including GI cancer.

6 Conclusions

Great advances in the profiling of genomic, proteomic, microenvironmental, and immunological approaches have been increasingly clarifying the oncoimmunological landscape underlying the resistance to ICI therapy, and different ICIs targeting other IC pathways and anti-cancer agents targeting multiple signaling pathways have been clinically developed. However, anti-tumor immune responses are not always induced and do not last long in all patients, and a significant proportion of patients acquire resistance to the treatment, possibly because of the oncoimmunological diversity and complexity. Disruption of the reciprocal evolution may successfully repel such refractory cancer. A promising strategy may be elimination and reprogramming of the myeloid villains that are the majority of cellular components in the human immune system. However, a single/dominant marker of the tumor-supportive subset should be identified, and the clinical relevancy of targeting the villain subset should be determined for the practical implementation of targeting myeloid cells in cancer therapy. This will greatly contribute to improvement of clinical outcomes, particularly in the ICI therapy of GI cancer.

Author contributions

CK-S conceptualized, organized the draft manuscript, and wrote the manuscript. NB, HH, and HS drafted the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Mechanism and strategies of immunotherapy resistance in colorectal cancer

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Colorectal cancer (CRC) is the third most common cancer in the world. Although there are standard treatment options for CRC, most patients respond poorly to these treatments. Immunotherapies have gradually emerged due to the increasing awareness and understanding of tumor immunity, exhibiting good therapeutic efficacy in various cancers. Immunotherapies include cytokines, immune checkpoint inhibitors (ICIs), and adoptive cell therapies. In particular, ICIs, which are antibodies against cytotoxic T lymphocyte-associated protein 4 (CTLA-4), programmed cell death 1 (PD-1), or its ligand PD-L1, have been successfully applied clinically for solid tumors, relieving the inhibitory effect of the tumor microenvironment on T cells. However, only a minority of patients with cancer achieve a durable clinical response during immunotherapy. Several factors restrict the efficacy of immunotherapy, leading to the development of drug resistance. In this review, we aimed to discuss the current status of immunotherapy for CRC and elaborate on the mechanisms that mediate resistance to immunotherapy and other potential therapeutic strategies.

KEYWORDS

colorectal cancer, immunotherapy, immune checkpoint inhibitors, drug resistance, potential therapeutic strategies

Introduction

Colorectal cancer (CRC) has a high morbidity rate and poor prognosis. The five-year survival rate for patients with advanced CRC is around 14%, and metastasis occurs in more than 50% of patients with CRC (1, 2). Immunotherapies comprise a novel and effective therapeutic strategy for patients with various cancers. With recent developments in cancer immunotherapy, both hematological and solid tumors respond to this

treatment. In the last decade, immunotherapy has become popular as an alternative to surgery, chemotherapy, and radiotherapy for treating various tumors (3, 4).

Immune checkpoints are a class of molecules expressed on the surface of immune cells that regulate the level of immune activation. They prevent autoimmune abnormalities and launch immune attacks on normal cells. However, in tumors, immune checkpoints, such as programmed cell death 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4), are abnormally activated, resulting in a weakened tumor immune response (5–7). As a result of its ability to interfere with the interaction between immune checkpoints and their receptors, immune checkpoint blockade (ICB) therapy has shown impressive therapeutic effects on a wide variety of tumor types. For CRC, two PD-1-blocking antibodies, pembrolizumab and nivolumab, which are already approved by the Food and Drug Administration (FDA), have shown efficacy in patients with mCRC(metastatic colorectal cancer) that are mismatch-repair-deficient and have high microsatellite instability (dMMR–MSI-H). The ICB drug ipilimumab, a fully humanized monoclonal antibody, blocks the CTLA-4 receptor and has also been approved by the FDA in combination with nivolumab to treat dMMR-MSI-H CRC (8–10). In a study of patients with locally advanced mismatch repair-deficient (dMMR) CRC, 14 patients achieved complete clinical remission after six months of treatment with the anti-PD-1 drug dostarlimab-gxly alone. The exciting result is that dostarlimab has saved all patients in that study from chemotherapy, radiation, or surgery (11). However,immunotherapy is effective in some cases of dMMR-MSI-H CRC but is minimally effective in pMMR-MSI-L CRC. Many colon cancer patients show resistance to immunotherapy. Hence, improving the efficacy of immunotherapy for dMMR-MSI-H and exploring new mechanisms for the treatment of pMMR-MSI-L CRC is key to improving the prognosis of patients with tumors.

Immunotherapy in CRC

Immunotherapy in dMMR–MSI-H and pMMR–MSI-L CRC

Colorectal cancer can be classified through a mismatch repair/microsatellite instability system. Microsatellites represent a kind of tandem repeats including 1–6 nucleotides that frequently occur in nuclear genomes (12). Microsatellite instability (MSI) refers to the insertion/deletion of repeated DNA nucleotide units in microsatellites. During DNA replication, the mismatch repair (MMR) system corrects insertions, deletions, or mismatched bases and identifies and repairs DNA damage (13). MMR deficiency results in the failure to detect and correct microsatellite replication errors, resulting in diffuse MSI. High microsatellite instability usually gives rise to the accumulation of somatic mutations. Of these, frameshift mutations are highly positively correlated with the frequency of neoantigens, MSI, and MMR deficiency. They are usually detected in CRC *via* immunohistochemistry as the loss of MMR proteins (MSH2, MSH6, MLH1 and PMS2) or by testing MSI *via* PCR (14, 15). Based on the mutations of MMR proteins and MSI, CRC can be classified into three main types: dMMR-MSI-H tumors with a higher overall mutation burden (>12 mutations per 10^6 DNA bases), pMMR–MSI-L tumors with a much lower mutation burden (<8.24 mutations per 10^6 DNA bases), and pMMR-MSS tumors lacking MSI features (13, 16). The MSS/MSI-L subtypes occupy a large proportion (85%) of CRC cases, whereas the dMMR/MSI-H patients accounts for only approximately 15% of all CRC cases and 5% of the mCRCs (17).

Immune cell function and classification are key factors in immunotherapy, in which tumor-infiltrating lymphocytes (TILs), as the main force of adaptive immune response, play a pivotal role in defense against tumors (18, 19). And powerful immune cytolytic activity (CYT) badgers deeply with many factors including tumor mutation burden and deregulated immune checkpoint (20, 21). Compared to those harboring pMMR-MSI-L/MSS, dMMR-MSI-H patients usually showed satisfactory prognosis and responded better to ICIs, since they owned a high mutational burden whose accumulation might produce more neoantigens for immune recognition. Because of the higher neoantigen load, dMMR–MSI-H tumors are usually heavily infiltrated by functional TILs, which quickly start it activation program and release a large number of cytokines when receiving stimulus from antigen-presenting cells (4, 22). Furthermore, it proves that MSI-CRC has higher expression levels of immune checkpoints, such as PD-L1, CTLA-4 as well as LAG-3, compared with MSS-CRC, which may explain the positive response of dMMR–MSI-H subtype to immunotherapies (18). A phase 2 clinical study (NCT03206073) confirmed that patients with MSI-CRC show much better progression-free survival (PFS) and objective response rate (ORR) than MSS ones when treated with pembrolizumab. Nevertheless, there are few functional TILs and many immunosuppressive cells, consisting of Tregs, MDSCs, and TAMs, infiltrated in the pMMR-MSI-L tumor microenvironment (Figure 1).

Clinical trials on immunotherapies in CRC

Multiple clinical trials have been conducted to estimate the effects of inhibiting PD-1, PD-L1, or CTLA-4 in dMMR–MSI-H and pMMR–MSI-L CRC. NCT02460198 is a phase II study

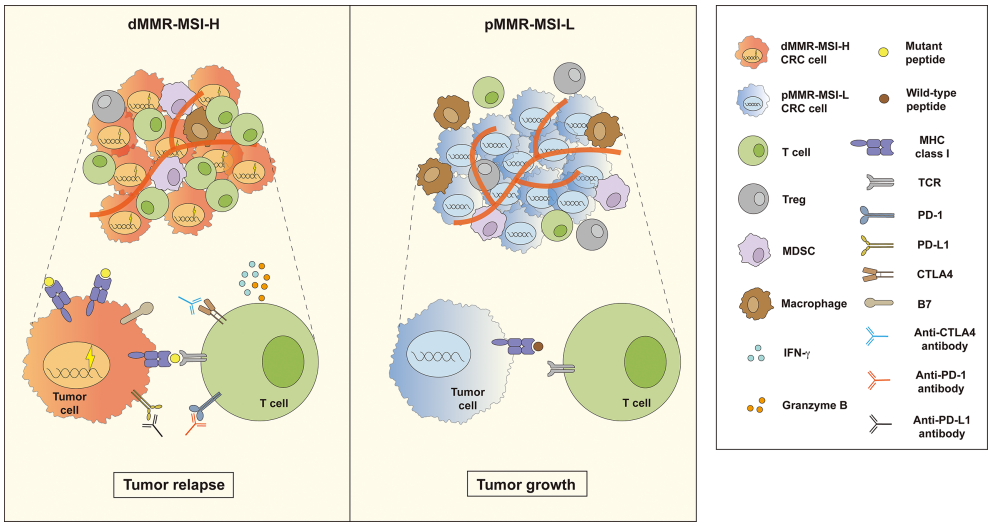

FIGURE 1
Two outcomes of immunotherapy in dMMR–MSI-H and pMMR–MSI-L CRC. Compared with pMMR–MSI-L, patients with dMMR–MSI-H experience better tumor reduction after treatment with immune checkpoint inhibitors (ICIs). Many functional tumor infiltrating lymphocytes (TILs) release a large number of cytokines such as IFN-γ and granzyme B in the dMMR–MSI-H tumor microenvironment (TME). However, the TME of pMMR–MSI-L CRC contains fewer functional TILs and more immunosuppressive cells, such as Tregs, MDSCs, and TAMs, which inhibit TIL function.

conducted on 124 previously treated patients with locally advanced unresectable or metastatic dMMR/MSI-H CRC (cohort A: standard therapies including fluoropyrimidine, oxaliplatin, and irinotecan; cohort B: fluoropyrimidine + oxaliplatin or fluoropyrimidine + irinotecan, with or without anti-VEGF/EGFR monoclonal antibody) (23). Pembrolizumab (200 mg) was intravenously administrated to these patients every three weeks until approximately 52 cycles. ORR was the primary endpoint, and disease control rate, PFS, overall survival (OS), tolerability, safety and response duration were the secondary endpoints. The results showed that the ORR was 32.8% (95% CI, 21.3% to 46%) and 34.9% (95% CI, 23.3% to 48.0%) in cohorts A and B, respectively. The median PFS was 2.3 months (95% CI, 2.1 to 8.1 months) and the median OS was 31.4 months (95% CI, 21.4 to 58.0 months) for cohort A. In contrast, the median PFS was 4.1 months (95% CI, 2.1 to 18.9 months) and the median OS was 47.0 months (95% CI, 19.2 months to not reached) for cohort B. Although most of the patients suffered different adverse events during the trial, only five patients withdrew from either cohort because of adverse events. Finally, pembrolizumab proved effective and safe in dMMR/MSI-H CRCs. Compared with blocking PD-1, the effectiveness of strategies to block PDL1 or CTLA-4 is subtle and seems to be weaker in clinical trials. Another phase II trial (NCT02870920) utilizes durvalumab combined with tremelimumab for treating patients with advanced CRC receiving the best supportive care, the treatment group showed a longer OS (median OS: 6.6 months vs. 4.1 months; P-value = 0.07) than the control group (24). However, superior PFS in treatment group was not observed, with a 95% CI ranging from 1.8 to 1.9 months. In addition, the ORR was only 0.8% (one patient) in the anti-PD-L1/CTLA-4 therapy group. ICIs have a significant impact on tumor treatment, nevertheless, only partial patients benefit from this regimen. This motivated us to deepen understanding of tumor immunity and explore new solutions for this disease. To date, the FDA approved three immunotherapeutic drugs between 2017 and 2018 (pembrolizumab, nivolumab, and ipilimumab) to treat MSI-H/dMMR CRC (25). Pembrolizumab has become the first-line regimen for treating CRC cases owning an MSI-H/dMMR or unresectable phenotype. Nevertheless, due to the immunosuppressive TME and the significantly low ratio of the MSI-H/dMMR subset in all patients with CRC, patients show resistance to immunotherapy and obtain limited improvement from these immunotherapies. Thus, developing new immunotherapeutic targets and combined therapeutic strategies has become a hot topic (Table 1 and Table 2). And numerous trials have focused on these issues through different mechanisms, such as remolding the antibody structure (Tislelizumab, KN035), combining with other targets (anti-VEGF) or plus with chemoradiotherapy, etc. For example, KN035 is a novel anti-PD-L1 antibody with remolded

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TABLE 1 Table.

Target	Checkpoint inhibitor	Phases	Study treatment groups	Trial identifier
Ongoing trials in dMMR/MSI-H CRC				
PD-1	Pembrolizumab	Phase 2	Pembrolizumab+Olaparib	NCT05201612
PD-1	Pembrolizumab	Phase 2	Pembrolizumab	NCT04895722
PD-1	Pembrolizumab	Phase 2	Pembrolizumab	NCT03638297
			Pembrolizumab+cox inhibitor	
			Atezolizumab+Bevacizumab+Mfolfox6	
PD-1	Nivolumab	Phase 3	Nivolumab+Ipilimumab+Fluorouracil	NCT04008030
PD-1	Nivolumab+Ipilimumab	Phase 2	Nivolumab	NCT04730544
			Nivolumab+Ipilimumab	
			Chemotherapy	
PD-1	Tislelizumab	Phase 2	Tislelizumab	NCT05116085
PD-L1	KN035	Phase 2	KN035	NCT03667170
PD-L1	Atezolizumab	Phase 2	Atezolizumab	NCT05118724
			Atezolizumab+IMM-101	
PD-L1	Atezolizumab	Phase 3	Atezolizumab	NCT02997228
CTLA-4	Ipilimumab	Phase 1	Ipilimumab	NCT04117087
			Nivolumab	
			KRAS peptide vaccine	
Ongoing trials in pMMR–MSI-L CRC				
PD-1	Sintilimab	Phase 1|Phase 2	Sintilimab + XELOX + Bevacizumab	NCT04940546
PD-1	Tislelizumab	Phase 2	Tislelizumab	NCT05160727
PD-1	Pembrolizumab	Phase 1|Phase 2	Pembrolizumab+Ataluren	NCT04014530
PD-L1	Durvalumab	Phase 1|Phase 2	Durvalumab+Yttrium-90 RadioEmbolization	NCT04108481
CTLA-4+PD-1	balstilimab	Phase 1|Phase 2	balstilimab+botensilimab	NCT05205330
	botensilimab			
PD-L1+anti-VEGF	Atezolizumab	Phase 2	Atezolizumab+XELOX + bevacizumab	NCT04659382
	Bevacizumab		Bevacizumab+bevacizumab	
			XELOX	
PD-1+anti-VEGF	Pembrolizumab	Phase 2	Pembrolizumab+Bevacizumab+Capecitabine	NCT03396926
	Bevacizumab			

structure empowering it with superior solubility and stability, which makes KN035 the first checkpoint inhibitor administered subcutaneously. A single-arm, phase II study NCT03667170 found that KN035 therapy showed a pretty good ORR of 43.1% (95% CI, 30.8% to 56.0%) in a cohort of 65 advanced CRC patients during a 28-day treatment cycle (26). Since most of the newly admitted clinical trials are still in recruiting status, the results of these strategies are in urgent to see.

Mechanisms of immunotherapy resistance

Multiple mechanisms are involved in the process of immunotherapy resistance. Immunotherapy resistance can divided into Tumor-intrinsic and -extrinsic (Figure 2).

Tumor-extrinsic resistance to immunotherapy in CRC

Immunosuppressive cells in the heterogeneous tumor microenvironment

Intercellular interaction is a well-known immunosuppressive mechanism in the TME, with the classic PD-L1/PD-1 signaling between tumor cells and CD8+ T cells being proven in different cancers. Nevertheless, many suppressive cells other than tumor cells also profoundly inhibit T-cell cytotoxicity, including Tregs, MDSCs, and TAMs. These cell subsets differentiate improperly and upregulate immunosuppressant-associated proteins, which usually form an anti-inflammatory environment and result in CD8+ T cell dysfunction and immune resistance.

Regulatory T cells (Tregs) are CD25+ FOXP3+ CD4 T cells originating from the thymus or peripheral blood (27). Tregs function as a tumor-promoting component in the TME because

TABLE 2 Trials using combination immunotherapies for CRC.

Target	Drugs	Phase	Treatment group	Trial Identifier
PD-L1 CTLA-4	Durvalumab Tremelimumab	Phase 1	Durvalumab+Tremelimumab	NCT01975831
PD-1 IDO1	Pembrolizumab INCB024360	Phase 1|Phase 2	Pembrolizumab+INCB024360	NCT02178722
EGFR PD-L1 RAF VEGF HER2 HER2 MEK	Cetuximab Atezolizumab Vemurafenib Bevacizumab Trastuzumab Pertuzumab Cobimetinib Chemotherapy	Phase 2	5-FU/LV+Cetuximab+Vemurafenib Fluoropyrimidine+Atezolizumab+Bevacizumab Trastuzumab+Pertuzumab Atezolizumab+Cobimetinib Fluoropyrimidine+Bevacizumab	NCT02291289
PD-1	Pembrolizumab Chemotherapy	Phase 2	Oxaliplatin+Leucovorin+5FU+Pembrolizumab	NCT02375672
PD-1 CSF1R	Pembrolizumab AMG820	Phase 1|Phase 2	AMG820+Pembrolizumab	NCT02713529
PD-1 VEGFR	Pembrolizumab Cetuximab	Phase 1|Phase 2	Cetuximab+ Pembrolizumab	NCT02713373
PD-L1 CTLA-4	Durvalumab Tremelimumab	Phase 2	Best Supportive Care Best Supportive Care+Durvalumab+Tremelimumab	NCT02870920
PD-L1 CTLA-4	Durvalumab Tremelimumab Radiotherapy	Phase 2	Radiotherapy+ Durvalumab+ Tremelimumab	NCT03122509
PD-1 CTLA-4 MEK	Nivolumab Ipilimumab Binimetinib	Phase 2	Nivolumab+Binimetinib+ Ipilimumab Nivolumab+Binimetinib	NCT03271047
PD-1 BTK	Pembrolizumab Ibrutinib	Phase 1|Phase 2	Pembrolizumab+Ibrutinib	NCT03332498
PD-1	Pembrolizumab Oncolytic virus	Phase 1	Pembrolizumab+Talimogene Laherparepvec	NCT03256344
PD-1 CCR5	Pembrolizumab Maraviroc	Phase 1	Pembrolizumab+ Maraviroc	NCT03274804
PD-1 CXCR1/2	Pembrolizumab Navarixin	Phase 2	Pembrolizumab+Navarixin	NCT03473925
PD-1 GpA33	MGA012 MGD007	Phase 1|Phase 2	MGD007+MGA012	NCT03531632
PD-1 CCR5	Pembrolizumab Vicrivir	Phase 2	Pembrolizumab+ Vicriviroc	NCT03631407

they inhibit the immune response against CRC through cell-to-cell contact or the secretion of cytokines and metabolites. Studies report that Tregs regulate the immune response mainly through several mechanisms (27–29). First, they can secrete cytokines such as IL-10, TGF-β, and IL-35, which facilitate Treg proliferation and suppress effector T cell activity (29–31). In addition, the surface marker CD25, which is the α-chain of the IL-2 receptor, can deprive IL-2 because of its high affinity (32, 33). Second, Tregs can exert anti-inflammatory effects by catalyzing the conversion of pro-inflammatory adenosine triphosphate to anti-inflammatory adenosine through the CD39-CD73 axis (34). Third, Tregs constitutively express immune checkpoints, such as CTLA-4, which impair APC maturation and cause T cell exhaustion (35). Finally, it has been reported that Tregs utilize granzyme and perforin to lyse DCs or T cells (36, 37). Tregs also inhibit T cells from exerting anti-tumor functions in various ways. Hence, thinking about how to reduce Treg populations or inhibit their function is one of the key methods for improving the effect of immunotherapy.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells that originate from the bone marrow. The immunosuppressive effects of MDSCs mainly depend on two enzymes: inducible Nitric Oxide Synthase (iNOS) and Arginase 1 (ARG1). High ARG1 catalyzes L-arginine to urea and ornithine,causes a depletion of L-arginine in the TME. iNOS uses L-arginine to produce NO. The depletion of L-arginine impairs T cell proliferation and activity by decreasing CD3ζ expression (38).

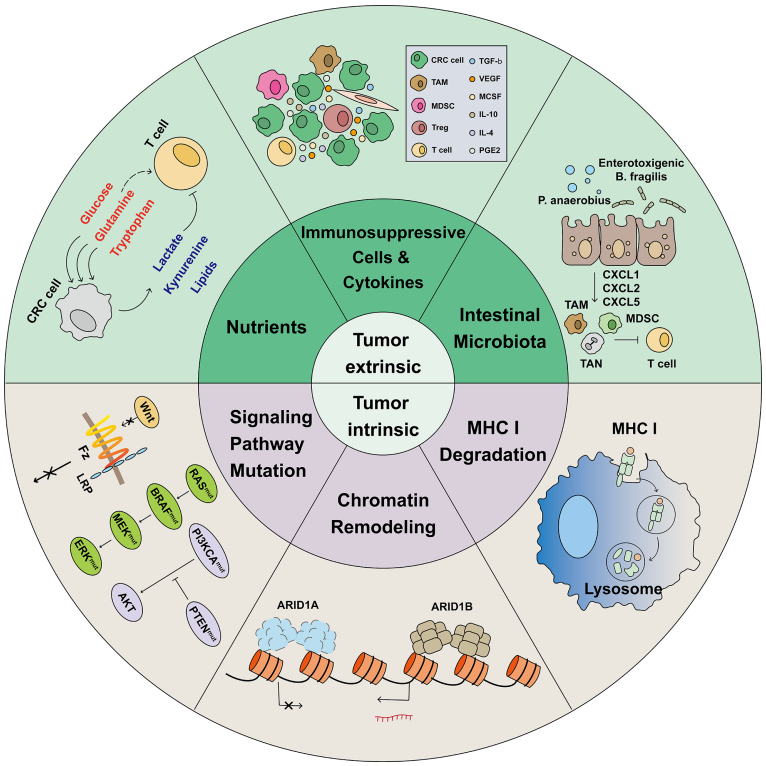

FIGURE 2
Mechanisms associated with immunotherapy resistance. The limited effectiveness of immunotherapy is primarily due to various mechanisms of immunotherapy resistance. In the heterogeneous tumor microenvironment, cells such as Tregs, MDSCs, and TAMs, combined with tumor-released immunosuppressive cytokines, induce tumor infiltrating lymphocytes (TILs) exhaustion. In the TME, tumor cells have a greater ability to compete for nutrients, such as glucose, glutamine, and tryptophan, which are necessary for proper cellular function. Meanwhile, tumor cells release lactate, kynurenine, and Oxidized low density lipoprotein (oxLDLs), which are harmful to TILs. In CRC, intestinal microbiota such as *P. anaerobius* and enterotoxigenic *B fragilis* induce tumor cells to release CXCL1, CXCL2, and CXCL5 and recruit immunosuppressive cells. Before identifying and killing TILs, tumor cells activate the lysosomal degradation pathway of MHC class 1 to escape T cell killing. The switching/sucrose non-fermentable (SWI/SNF) complex has been identified as a tumor suppressor gene in CRC; AT-Rich Interactive Domain-containing protein 1A (ARID1A) is the most frequent target of SWI/SNF mutations. However, ARID1A mutations was found correlated with markedly higher level of immune infiltrates in colon cancer. Gene and signal pathway mutations, such as those in WNT, RAS, BRAF, MEK, ERK, PI3KCA, and PTEN, were reported to be associated with immunotherapy resistance.

In addition, NO, in cooperation with O^{2-}, can result in T cell apoptosis through the tyrosine phosphorylation of the TCR/CD3 complex (39, 40).

Tumor-associated macrophages (TAMs) are key immune components of the TME. Based on their polarization fate, they can be divided into the M1 (pro-inflammatory) and M2 (anti-inflammatory) phenotypes. Interestingly, M1 and M2 cells can repolarize to each other depending on the interventions administered (for example, by targeting mitochondrial metabolic pathways) (41–43). Numerous studies have reported the supportive effects of TAMs on malignant cells in the CRC TME. TAMs can induce tumor angiogenesis by secreting VEGF and MCP-1, MIP-1α, and MIP-2α to promote tumor growth and invasion (44, 45). TAMs impair T cell function in multiple ways, including restricting infiltration, blocking proliferation and activation, and suppressing cytotoxicity. For instance, IL-10 produced by TAMs can decrease CD8 protein levels and impair TCR signaling (46). Other factors such as iNOS, ARG1, and PD-L1 expression also cause T cell dysfunction. Therefore, transforming TAMs from the M2 phenotype to the M1 phenotype is key to restoring T cell function.

Immunosuppressive cytokines in the heterogeneous tumor microenvironment

Cytokines enriched in the TME are another important pathway that interferes with the efficiency of immunotherapy against tumors. Various studies have concluded the vital role of immunosuppressive cytokines (such as TGF-β, VEGF, IL-4, and IL-10) in the disruption of CD8+ T cell function. In brief, immunosuppressive cytokines are well defined to recruit, polarize, or activate immunosuppressive cells and indirectly interfere with T cell function, although some directly inhibit T cell cytotoxicity. These effects restrict the efficiency of immunotherapy.

TGF-β is a multipotent cytokine that inhibits tumor formation at early stages but promotes tumor progression at advanced stages. Most cells in the TME can produce TGF-β, including cancer cells, fibroblasts, TAMs, Tregs, and even platelets (47, 48). Bardeesy et al. reported that TGF-β/SMAD4 signaling results in premalignant pancreatic cell apoptosis harboring KRAS mutations (49). In addition, primary mesenchymal stromal cells in the TME can induce CXCL5 overexpression in CRC, promoting tumor metastasis and angiogenesis *via* the CCL7/CCR1/KLF5 pathway; TGF-β/SMAD4 signaling can reverse this effect (50). However, the pro-tumor and immunosuppressive effects of TGF-β are more important in tumor development and are thought to be potential therapeutic targets. For example, TGF-β enhances EMT in CRC through the USF2/S100A8 axis (51). The TGF-β/SMAD pathway decreases CTL cytotoxicity by decreasing perforin, granzyme A, granzyme B and IFN-γ expression (52). TGF-β is also vital for recruiting suppressive immune cells such as Tregs and TAMs.

Vascular endothelial growth factor (VEGF) is a family of cytokines consisting of five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF. This family of proteins is mainly expressed in CRC tissues and the hypoxic TME and is highly involved in tumor progression and metastasis. The classical function of VEGF is to promote tumor angiogenesis *via* commonly known receptors (VEGFR-1, VEGFR-2, and VEGFR-3) expressed on adjacent vascular endothelia (53, 54). Despite their angiogenic effect, they also strongly influence inflammatory cell infiltration and function. A recent study showed that VEGF-A directly influences T cell exhaustion by inducing the expression of the transcription factor TOX in T cells in MSS CRC (55). Anti-VEGF therapy can also indirectly augment the ability of CD8+ T cells to produce IFN-γ and TNF-α by modulating the hypoxic TME (56).

IL-4 and IL-10 are cytokines classically expressed by Th2 T lymphocytes and are involved in infection and autoimmune diseases. However, they are also highly enriched in multiple cell types in the CRC TME (42, 57–60). Both are important pro-tumor and immune regulatory factors that target IL4R and IL10R receptors, respectively. Koller, F. L. et al. and Liu, H. et al. reported that IL4 could promote HT-29 cell proliferation (61, 62), while Mantilla-Rojas, C. et al. reported that IL-10/SRC contributes to CRC progression (63). IL-4 and IL-10 can suppress CD8+ tumor-infiltrating T cell function by recruiting immunosuppressive components, such as Tregs, M2 TAMs, and MDSCs. However, IL-10 has recently been reported to enhance the anti-tumor effects of CD8+ T cells directly and indirectly (64, 65).

Intestinal microbiota

As luminal tract organs communicate with the outside environment, the colon and rectum are exposed to more than 100 trillion microorganisms, including bacteria, fungi, protozoa, and viruses, from their proximal to distal ends. Physically, the gut microbiota is vital for host homeostasis by providing important nutrients such as vitamins and essential amino acids. However, dysbiosis facilitates many pathological processes such as inflammation, dysplasia, and cancer (66–68). Patients with CRC showed significantly different abundances and reduced diversity of the gut microbiota. For example, *Fusobacterium nucleatum* and *Peptostreptococcus anaerobius* are highly enriched in CRC tissues; however, *Clostridium butyricum* and *Bifidobacterium animalis* are depleted (66, 69). The gut microbiota and its metabolites are widely known to participate in tumor growth and immune responses. *F. nucleatum* promotes HCT116 and LoVo cell proliferation by activating TLR4 signaling (70). It polarizes TAMs to the M2 phenotype through diverse pathways contributing to CRC progression and metastasis (71, 72). The Fap2 protein produced by *F. nucleatum* hinders T cell activation by interacting with TIGIT (73). On the contrary, probiotics, a huge commensal bacterial family in the intestine, are of great importance for tumor regression and improving the effects of immunotherapies. *Clostridium butyricum* releases butyrate into the TME and hinders CRC proliferation by inhibiting HDAC activity (74, 75). Recent studies have shown that the administration of *Lactobacillus rhamnosus GG* and *Lactobacillus casei* can facilitate CD8+ TIL infiltration and cytotoxic cytokine secretion, improving the anti-tumor effect of anti-PD-1 agents (76, 77). As a newly identified TME factor in CRC, the influence of the microbiota on tumor cells and CD8+ T cells is complex and requires further research. Patients with CRC generally show an unbalanced bias towards pro-tumor microbiota enrichment, which indicates that recovering intestinal microbiota homeostasis or the exogenous administration of anti-tumor microbiota and its metabolites is closely related to the prognosis associated with CRC immunotherapy.

Nutrients in the heterogeneous tumor microenvironment

Altered nutrient metabolism is a hallmark of many tumors. As members of the hypoxic and resource-limited TME, tumor cells and CD8+ T cells urgently require large amounts of nutrients to maintain their survival and biological activities. Tumor cells usually exhibit higher glucose consumption and stronger competitive uptake of essential amino acids, exacerbating the shortage of important nutrients and hindering T cell survival and cytotoxicity. In addition, increased lipid production and the generation of transformation products from glucose and some amino acids are also critical immunosuppressive factors that impair T cell immune responses.

Distinct from the normal colorectal epithelium, CRC cells consume large amounts of glucose to maintain rapid expansion. However, they are prone to glycolysis rather than OXPHOS even in ample oxygen conditions, classically called the Warburg effect.

In this process, glucose is converted into pyruvate and ATP together with an important pro-tumor byproduct, lactate, which further contributes to acidosis in the CRC TME (78, 79). Different mechanisms have been reported to regulate glucose uptake by CRC cells. For example, Tang et al. and Wang et al. concluded that the lncRNAs GLCC1 and LINRIS promote tumor glycolysis and facilitate CRC progression by stabilizing c-MYC transcription in mouse or PDX models, correlating with poor prognosis in patients with CRC (80, 81). Moreover, enhanced glycolysis also induced the resistance of CRC cells to chemotherapies such as 5-FU (82). In addition to competitive uptake, malignant cells can directly impair glucose metabolism in T cells by expressing CD155 and combining with TIGIT on the T cell surface, resulting in dysfunction in T cell energy utilization (83–85). The byproduct lactate is also a versatile factor that promotes CRC progression and metastasis through various mechanisms. Deng et al. recently reported that lactate stimulates the tube formation of endothelial cells and eventually angiogenesis in the CRC TME (86). Moreover, several articles have reported the effect of lactate on M2 phenotype polarization in TAMs (87–89). Therefore, eliminating lactate and improving the acidic TME restore CD8+ T cell anti-tumor immunity in many cancers (90–92).

Tryptophan is an essential amino acid that participates extensively in tumor immunity in different cancer types. Three enzymes, IDO1, IDO2, and TDO2, are responsible for tryptophan catabolism, transforming more than 95% of tryptophan to kynurenine, leading to the deficiency of tryptophan in the TME (93, 94). The transformed product, kynurenine, is a profoundly immunosuppressive metabolite. Tumor-derived kynurenine induces PD-1 expression in CD8+ T cells by activating the transcription factor AHR both in a mouse model and in patient samples (95). In addition, kynurenine is negatively correlated with CD8+ T cell infiltration in the CRC TME. The induction of FOXP3 and Treg polarization by kynurenine may contribute to this process (96, 97).

Unlike tryptophan, glutamine is a nonessential amino acid that is highly consumed in many malignancies. It renders many vital biological processes, including nutrient exchange and the biosynthesis of other amino acids, lipids, and nucleotides, by providing nitrogen and carbon (98, 99). As the second most abundant nutrient after glucose in the TME, glutamine catabolism is an indispensable method of energy generation *via* the TCA cycle (99, 100). Because of its role as a substrate for many bioactive elements and ATP production, glutamine is required in malignant cells and other immune components. Turowski et al. first confirmed the ability of glutamine to stimulate the proliferation of human colon cancer cell lines Caco-2 and SW620 (101). Further studies have shown that KRAS mutations heighten glutamine uptake in CRC cells by upregulating SLC1A5 expression. Moreover, it was found that glutaminolysis-derived succinate promotes CRC cell proliferation and stemness by upregulating LRG5 expression and enhancing Wnt/β-catenin signaling (102, 103). Lack of glutamine is seriously affect T cell proliferation after activation (104).

Lipids are a family of hydrophobic or amphipathic molecules that consist of fatty acids, glycerophospholipids, sphingolipids, and sterols. Although high lipid accumulation in the CRC TME has been recognized, their profound impact on tumor-infiltrated CD8+ T cells has only been noticed in recent years. CRC cells can produce various lipids, such as fatty acids, obliging T cells to uptake these lipids, resulting in T cell dysfunction (105–107). Mechanistically, CD8+ T cells in the TME showed the increased expression of CD36, a scavenger receptor for fatty acid uptake. The abnormal accumulation of fatty acids inside T cells further initiates lipid peroxidation, induces T cell ferroptosis, and reduces the production of cytokines, such as IFN-γ and TNF-α (107).

Tumor-intrinsic resistance to immunotherapy in CRC

MHC degradation

The T cell antigen receptor (TCR) is a multimolecular structure expressed on the T cell membrane that recognizes peptide/MHC complexes on the tumor cell surface (108). The expression of MHC class I molecules is crucial for an effective adaptive immune response. A low expression or deletion of MHC class I molecules frequently occurs in colon tumors resistant to immunotherapy (109–111). The oncoprotein SND1 promotes the ER-associated degradation of MHC class I, resulting in disordered CD8+ T cell function and decreased anti-tumor ability (112). A rational combination of systemic chemotherapy and DC i.t. injection has been shown to induce a complete anti-tumor response in MC38 murine adenocarcinoma cells (113). Moreover, increased mitophagy in intestinal epithelial cells can trigger lysosomal membrane permeability, and the subsequent release of proteases into the cytoplasm increases MHC class I presentation and activation of CD8+ T cells *via* cross-modification of dendritic cells (114).

Gene mutations

The occurrence and development of CRC result from multiple gene interactions and the involvement of various signaling pathways. However, gene mutations and defects in signaling pathways also affect the immunotherapy sensitivity and prognosis of patients with colon cancer (115). As a key cascade that regulates cell development and stemness, Wnt/β-catenin pathway plays a prominent role in the occurrence of colon cancer (116). The aberrant activation of the Wnt/β-catenin pathway is a key driver in the maintenance and proliferation of gastrointestinal stem cells (117). The Wnt/β-catenin signaling pathway is also considered an important

carcinogenic signaling pathway related to immune evasion. Activation of Wnt/β-catenin pathway in tumor leads to the decreasing production of CCL4 released by CD103+ dendritic cells. This inhibit CD8+ T cell activation and infiltration (118, 119). The Cancer Genome Atlas Network found that 55% of non-hypermutated tumors had alterations in KRAS, NRAS, or BRAF, which have significant mutually exclusive mutation patterns (12). PIK3CA mutations are some of the most common genetic alterations in solid tumors, inducing defects in the phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway, which is frequently dysregulated. PIK3CA mutations lead to the attenuation of tumor apoptosis and improvement of tumor invasion. Treatment with the PI3K inhibitor LY294002 has been shown to downregulate PIK3CA signaling and inhibit the progression of PIK3CA-mutant colon cancer (120). Meanwhile, mutations of PIK3CA could increase total mutation burden (TMB) which may improve immunotherapy sensitivity (121).

Chromatin remodeling (SWI/SNF complex)

The switching/sucrose non-fermentable (SWI/SNF) complex regulates transcription *via* nucleosome topology modulation and has been identified as a cancer suppressor gene in human malignancies (122, 123). SWI/SNF exhibits a broad mutation pattern associated with the progression and invasion of tumor cells. AT-Rich Interactive Domain-containing protein 1A (ARID1A), as one of the components making up the largest SWI/SNF subunit together with AT-Rich Interactive Domain-containing protein 1B (ARID1B), is the most frequent target of SWI/SNF mutations. mutations in ARID1A impair enhancer-mediated gene regulation and prognosis in patients with colon cancer (124, 125). Interestingly, SWI/SNF complex stability is also vital for tumor cell viability, which might be impaired by ARID1A mutation. In that situation, ARID1B is usually functionally normal and enable to partially compensate for ARID1A function as its homolog. But simultaneous mutation of ARID1A and ARID1B is synthetically lethal for colon cancer. ARID1A mutations was found correlated with markedly higher level of immune infiltrates in colon cancer. MSH2, a Mismatch repair protein, was found to interact with ARID1A. Low expression of ARID1A impair DNA mismatch repair, resulting in increases in TMB and cytotoxic T cell infiltration (126).

Novel strategies to overcome immunotherapy resistance

Immunotherapy resistance of decrease immunotherapy sensitivity in CRC. Targeting these tumor intrinsic and extrinsic resistance mechanisms is the key to improving the effectiveness of immunotherapy (Figure 3).

Chemoimmunotherapy strategy

Chemotherapy was previously thought to be immunosuppressive due to its concomitant severe myelosuppression and leukopenia (127). However, numerous studies have reported its synergistic effects to immunotherapy, which makes the chemoimmunotherapy come back to clinical practice and become standard care for selected

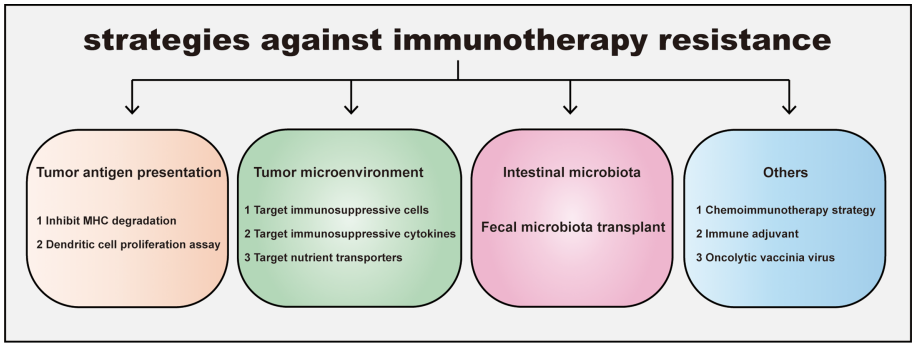

FIGURE 3
Strategies for overcoming immunotherapy resistance in CRC. The resistance leads to an ineffective immunotherapy and tumor progression. Four distinct strategies against immunotherapy resistance are listed: promoting tumor antigen presentation, regulating tumor immunosupprssive microenvironment, manipulating intestinal microbiota and others including combination therapy etc. Inhibiting MHC degradation and boosting dendritic cell proliferation could elevate tumor antigen presentation; Targeting immunosuppressive cells, cytokines in Tumor microenvironment (TME) and nutrient transporters help CD8+T escape the inhibition of TME. In addition, gut microbiota and its metabolite are widely proven to participate in tumor growth and immune response. Consistantly, fecal microbiota transplantation makes patients sensitive to immunotherapy. Other strategies for overcoming immunotherapy resistance, such as the administration of chemoimmunotherapy, immune adjuvant, oncolytic vaccinia virus can also improve the sensitivity of immunotherapy in CRC.

patients (128–130). Indeed, chemotherapy is found to induce tumor immunogenic cell death (ICD), which elicits extensive innate and adaptive immune response. For example, the classical chemotherapy regimen FOLFOX or FOLFIRI is reported to induce DAMPs (damage-associated molecular patterns) in both mouse and human tumor cell lines. And DAMPs is a strong signaling to promote DC maturation and tumor antigen presentation, thus leading to tumor ICD and subsequent enhanced T cell anti-tumor response (131). According to the current conclusions, seeking the dose balance of chemotherapy and immunotherapy may be an attractive strategy to avoid the side effects and boost the therapeutic effects. To illustrate the combined efficacy of immunotherapy and chemotherapy in colorectal cancer, NCT02375672 administrated Pembrolizumab (MK-3475) together with standard-dose mFOLFOX6 regimen to treat 30 advanced CRC patients. And the median PFS is 8.8 months (95% CI, 7.7 to 11.3 months) with ORR being 56.7%, which showed exciting potential of combined medical strategies (132).

Dendritic cell proliferation assay

Before enhancing T cell function with various exogenous elements, the first step for T cells in killing tumor cells is to recognize tumor antigen epitopes, in which endogenous DCs play a key role. However, due to insufficient DC infiltration and abnormal maturation affected by the TME, tumor antigens are usually not well-presented. Thus T cells cannot differentiate and be activated normally. Thus, promoting DC expansion and maturation is a promising method for boosting T cell-based therapy. Fms-like tyrosine kinase 3 ligand (Flt3L) is a growth factor that expands dendritic cells, increasing their number (133). A recent study developed a strategy called an *in situ* vaccine, which combined Flt3L, radiotherapy, and a TLR3 agonist (Flt3L for DC expansion, radiotherapy for loading DCs with the antigen, and TLR3 agonist for DC activation) to test its efficacy in a murine lymphoma model (134). Mechanistically, this strategy expanded and activated intra-tumoral DCs, which further promoted TAA presentation and anti-tumor T cell activation. With an exciting long-term tumor regression of at least 3 months, researchers conducted a clinical trial on patients with low-grade B-cell lymphoma using rhuFlt3L and poly-ICLC combined with low-dose radiotherapy. Preliminary results showed good tolerance and an ORR of 72.7%. This has aroused our interest in the relevance of dendritic cells to ICB therapy and revealed the possibility of developing novel therapies that effectively control drug-resistant CRC, such as the evaluation of combinations of Flt3L and ICIs in clinical trials.

Tumor microenvironment

Targeting the TME is another potential way to boost the effect of immunotherapy. For example, a clinical trial (NCT04126733) that began in 2019 focused on the efficacy of regorafenib, a VEGFR inhibitor targeting TME angiogenesis, in combination with nivolumab in patients with pMMR-MSS CRC. The primary endpoint ORR was only 7.1% (95% CI, 2.4% to 15.9%), and the median PFS was 8.00 weeks (95%CI, 7.86 to 10.57 weeks). In another trial (NCT02713529) that used pembrolizumab combination with AMG820, a CSF1R inhibitor repolarizing TMAs from M2 to M1 type (135), the ORR for patients with pMMR CRC was quite low (4.9%; 95% CI, 0.60% to 16.53%).

Fecal microbiota transplants

Dysbiosis in CRC has multiple influences on the TME, which establishes an environment that favors tumor immune escape. Since patients with tumors always harbor altered microbiota characteristics, rebuilding intestinal microbiota homeostasis may be an attractive way to overcome treatment resistance in these patients. One plausible method is to transplant the microbiota of immunotherapy responders to non-responders. In the clinical trial NCT03341143 that tested this hypothesis, researchers carefully screened two patients with metastatic melanoma whose tumors had completely disappeared after prior PD-1 therapy (136). Then transplant the microbiota of screened two patients to non-responders.Miraculously, the recipients showed tumor reduction or stable disease for more than a year, with an ORR of 20% and a median PFS of 3 months (median follow-up of 7 months). Microbiota transplantation reversed the response to PD-1 drugs in patients with anti-PD-1-refractory melanoma (137, 138). This reveals the potential value and significance of gut microbiota in immunotherapy. In addition, through the adjusted daily diet and the intake of prebiotics, the gut microbiota can influence the pre-existing commensal microbes in the gut. In the future, improving the gut microbiota *via* gut microbiota transplantation or other methods is likely to improve the effectiveness of immunotherapy in patients with CRC.

Oncolytic viruses and engineered bacteria

Due to the low immunogenicity of various tumor cells and the immunosuppressive TME, natural T cells usually have a limited anti-tumor response even in the presence of ICIs. Engineered chimeric antigen receptor T (CAR-T) cells armored with various molecules have recently been developed to resolve this issue (134). Nevertheless, the medical efficacy of CAR-T therapy is still limited because of the low recruitment ratio and the presence of only a few desirable epitopes (139, 140). The use of oncolytic viruses (OVs) and engineered bacteria are promising methods to overcome the deficiencies of CAR-T cells because not only can they get into tumor tissues unimpeded and

directly kill tumor cells, but they can also release or amplify tumor immunogenicity to stimulate immune cell responses, enhance immune cell infiltration, and result in effective anti-tumor immunity (141). A recent study reported that an oncolytic virus called CD19t could induce tumor cells to express CD19 on their surface before killing them, subsequently redirecting CD19-CAR-T cytotoxicity and enhancing the anti-tumor response in mouse models (132). In another research of murine CRC model, a kind of engineered Brucella melitensis (BmΔvjbR) in short of virulence was developed to assist CAR-T efficacy. The team found that intravenous administration of BmΔvjbR could significantly promote intratumoral M1-macrophage polarization and expansion, which further enhanced CAR-T infiltration and activity (142). However, no studies have focused on combining OVs/engineered bacteria and CAR-T cells in clinical patients, which is an urgent need.

Immunoadjuvants

Immunoadjuvants are non-specific non-immunogenic immunostimulatory molecules that can elicit rapid and strong immune responses when administered before or simultaneously with pathogen antigens. However, currently proven clinical immunoadjuvants are usually highly involved in activating humoral immunity instead of cellular immunity, limiting their application mostly in bacterial and some viral infections, like in various vaccines (140). This provoked our interest in seeking adjuvants that prefer the activation of cellular immunity to treat tumors since T cells play a key role in the anti-tumor response. Fortunately, recent studies have shown that manganese salt is an excellent adjuvant in eliciting both NK and T cell anti-tumor responses that mainly promoted DC maturation and antigen cross-presentation in preclinical models, a major breakthrough in the field. In particular, combination therapies comprising anti-PD-1 agents and manganese chloride have shown great preliminary clinical efficacy, with a median ORR of 45.5% in a phase I clinical study, NCT03991559 (143). Thus, manganese salt administration is anticipated for the future development of immunotherapies and tumor vaccines.

Conclusions

The number and function of TILs determine tumor prognosis in tumor immunotherapy. Hence, improving the number and function of TILs is the key to addressing immunotherapy resistance. By further understanding the heterogeneity of the tumor microenvironment and the defense and escape mechanisms of the gut microbiota and tumor cells against the attack of the immune system, we can target these drug resistance mechanisms to reduce immunotherapy resistance and improve the prognosis of patients with cancer. It is believed that with ICIs as the primary therapeutic backbone, targeting the tumor microenvironment and gut microbiota using combination treatments comprising ICIs, radiotherapy, chemotherapy, and various new therapeutic modalities will be continuously carried out, resulting in a higher percentage of patients that would benefit from immunotherapy.

Author contributions

JS and DH contributed to conception and design of the study. JS organized the database. JS, DH and CS wrote the manuscript and created the figures and tables. JS, DH and QL wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Associating resistance to immune checkpoint inhibitors with immunological escape in colorectal cancer

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Colorectal cancer is a common malignant tumor that ranks third in incidence and second in mortality worldwide, and surgery in conjunction with chemotherapy and radiotherapy remains the most common treatment option. As a result of radiotherapy's severe side effects and dismal survival rates, it is anticipated that more alternatives may emerge. Immunotherapy, a breakthrough treatment, has made significant strides in colorectal cancer over the past few years, overcoming specialized therapy, which has more selectivity and a higher survival prognosis than chemoradiotherapy. Among these, immune checkpoint inhibitor therapy has emerged as the primary immunotherapy for colorectal cancer nowadays. Nonetheless, as the use of immune checkpoint inhibitor has expanded, resistance has arisen inevitably. Immune escape is the primary cause of non-response and resistance to immune checkpoint inhibitors. That is the development of primary and secondary drug resistance. In this article, we cover the immune therapy-related colorectal cancer staging, the specific immune checkpoint inhibitors treatment mechanism, and the tumor microenvironment and immune escape routes of immunosuppressive cells that may be associated with immune checkpoint inhibitors resistance reversal. The objective is to provide better therapeutic concepts for clinical results and to increase the number of individuals who can benefit from colorectal cancer immunotherapy.

KEYWORDS

ICI, resistance, colorectal cancer, immune escape, overcome resistance

Introduction

According to the most recent worldwide statistics, the global cancer burden is overwhelming and expanding, with WHO estimates predicting a global cancer burden of 28.4 million cases in 2040. Colorectal cancer (CRC) ranks third in global cancer incidence while second in global cancer death, and colorectal cancer prevention, diagnosis, as well as treatment, are still significant issues that need to be tackled (1). The Chinese Society of Clinical Oncology (CSCO) colorectal cancer recommendations mainly propose surgery, chemotherapy, and radiation, with the combination of these modalities depending on the location, size, grade, and metastasis of colon cancer (2). Tumor cells are not eradicated even with comprehensive therapy, and the outlook for CRC is not exceptional. Furthermore, traditional therapies are indeed imprecise, causing patients to suffer and have a lower quality of life (3–5).

In recent years, immunotherapy has made tremendous progress in solid malignancies such as melanoma and lung cancer (6). Immunotherapy enhances the immune response against cancer cells by improving detection of tumor cell antigens (7, 8). Among them, immune checkpoint inhibitors (ICIs) have achieved the most significant improvements in immunotherapy, achieving rates of durable remission that are unprecedented. The majority of patients, however, have not benefited from treatment, and some in remission have relapsed after a period of remission because they have established medication resistance.

Typically, drug resistance is classified as either primary or secondary. The heterogeneity of the tumor growth process may play a role in the mechanism of primary and secondary ICIs resistance. There is no clear and comprehensive explanation of the immunological medication resistance mechanism. However, regardless of the resistance pattern, immunological escape is the underlying phenomenon. ICIs in CRC are essentially targeted to the MSI-H staging. pMMR is more like a "cold tumor" in the treatment of ICIs and has no more treatment options. In this article, we discuss the immune treatment-related CRC staging, the specific modalities of ICIs treatment, and the tumor microenvironment (TME) and immune escape pathways of immunosuppressive cells that may be associated with the reversal of ICIs resistance. Furthermore, we explore not only MSI-H-related ICIs therapy but also the role of immune escape to inspire the future of inhibiting certain TME or immunosuppressive cells to convert pMMR to "hot tumors" in order for ICIs to be effective. The goal is to obtain more beneficial therapeutic ideas for clinical outcomes and to expand the population benefiting from CRC immunotherapy.

CRC mutation pattern typing

The immune system eliminates highly immunogenic tumor cells in the body. Tumor cells continuously undergo somatic mutations and passively select low-immunogenic variants for proliferation in response to screening for antitumor effects. The MMR/MSI system classification has been significant in the treatment of colorectal cancer.16% of CRCs are hypermutated, of which 75% are connected to MSI-H and 25% have mutations in somatic mismatch repair genes and polymerase E (pole) (9). As a complex enzyme proofreading system, MMR is active during DNA replication, correcting nucleotide pairing mismatches and sliding between the two strands of DNA. Nevertheless, an insertion or deletion mutation during DNA replication cannot be corrected if the MMR mechanism is flawed. A shortened non-functional protein fragment, MSI, is eventually formed as a result of a germ-line mutation in one of the MMR system genes (MLH1, MSH2, MSH6, PMS2, or TACSTD1/EpCAM), or hypermethylation of the MLH1 promoter (10–12). The genome is full of microsatellite sequences, which are polymorphic between individuals yet specific to each tissue of each person.

In order to maintain genomic stability, mismatch repair (MMR) correctly identifies and corrects base mismatches, small base deletions, and insertions that arise during DNA replication or recombination. MMR is subdivided into different mismatch repair (dMMR) and proficient mismatch repair (pMMR). pMMR refers to normal MMR expression. dMMR refers to mutations in genes involved in MMR repair that lead to impairments in gene function and reduced or missing MMR repair competence. MSI is a code-shifting mutation caused by the insertion or deletion of repeating units in tumor cells' microsatellites. pMMR manifests as low-frequency microsatellite instability (MSI-L) or microsatellite stability (MS-S), whereas dMMR manifests as high-frequency microsatellite instability (MSI-H) (13–17). MSI is a crucial component in the progression of CRC (18). The principal method by which CRC can merge with MSI is through the methylation and inactivation of the hMLH1 promoter, which results in mismatch repair mistakes in certified identity management professionals (19), microsatellite sequences are abundant throughout the entire genome; they are polymorphic between individuals but unique in each tissue of each individual (20).

Existing mainstream immunotherapy for CRC

Currently, the chosen clinical treatment technique for colorectal cancer is still predominantly surgery and radiotherapy, despite the unsatisfactory overall effect. To put it another way, immunotherapy has advanced quickly in the field of oncology in recent years and has achieved excellent results in the treatment of solid tumors like melanoma and lung cancer. Therefore, offering novel concepts for the treatment of colorectal

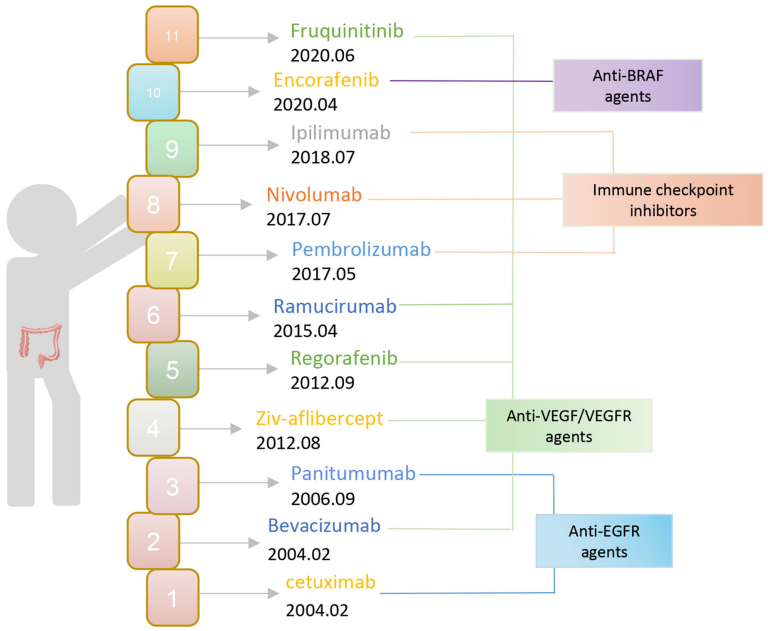

FIGURE 1
The FDA approves immunotherapy for CRC.

cancer and making immunotherapy a well-liked research topic in CRC treatment. The FDA authorized bevacizumab and cetuximab for use as first-line CRC medications in 2004 (Figure 1). Monoclonal antibodies have since entered the CRC immunotherapy arena. The inhibitors of vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) still play an important role as a therapeutic aid today. Then the FDA approved pembrolizumab, an ICI PD-1 monoclonal antibody, in 2017 for the treatment of dMMR-MSI-H. With ICI, immunotherapy is now making the progress in colorectal cancer. Subsequently, the FDA expedited approval of the anti-PD-1 monoclonal antibodies pembrolizumab and nivolumab, in addition to nivolumab and the CTLA-4 monoclonal antibody ipilimumab for the treatment of dMMR-MSI-H. However, ICIs are ineffective against pMMR-MSI-L colorectal cancer almost. Yet patients with dMMR-MSI-H account for less than 5% of all colorectal cancer patients (21). It has been assumed that immunological tolerance and immunotherapy are ineffective in pMMR-MSI-L CRC due to the low number of mutations and the minimal penetration of immune cells (22, 23).

In addition to ICI, the FDA has approved chimeric antigen receptor T-cell (CAR-T) therapy for clinical use. Through attaching the B-cell receptor antigen-binding domain to the T-cell receptor's intracellular area, which is subsequently genetically altered for return to the body, this therapy employs genetically modified immune cells to express autologous T cells that can recognize and destroy tumor cells (24, 25). CAR-T cells are currently restricted to clinical applications targeting the pan-B cell antigen CD19 and are only licensed for the treatment of certain hematological cancers. A significantly higher fraction of activated cytotoxic CD8 TIL in colorectal cancer patients indicated that the degree of T cell penetration into the tumor was directly related to treatment success and suggested that immunological editing could inhibit tumor growth (26). T cells react strongly to antigens from infections but are insensitive to antigens from cells that are unable to exert a comparable cell-killing impact *in vivo*. CAR-T therapy collects T cells from tumors, peripheral blood, or lymph nodes of patients for genetic engineering. Not only because the use of autologous T cells can reduce immune rejection, but also because the use of autologous T cells can promote the identification of tumor cells and increase T cell activation (27). While CAR-T therapy has not yet been approved for the clinical treatment of colorectal cancer, no serious adverse events associated with CAR-T therapy were observed in the 2017 Zhang et al. study (28). CAR-T therapy in patients with carcinoembryonic antigen (CEA)-positive colorectal cancer (CRC) metastases is effective, with 7 out of 10 patients experiencing stable disease within four weeks of CAR-T cell infusion and another 2 patients experiencing tumor shrinkage with the treatment being well-tolerance (28).Aside from the experimental CEA target, there are numerous potential targets for CAR-T cell therapy in colorectal cancer, primarily anti-4-1BB (ANTI-4-1BB), CEA, Guanylate Cyclase 2C (GUCY2C), Tumor Associated Glycoprotein 72(TAG-72), Epithelial cell adhesion molecule(EpCAM), epithelial

glycoprotein 40 (EGP40), NKG2D, human epithelial growth factor receptor-2(HER-2), recombinant human interferon-alpha/beta receptor 1 (IFNAR1), prominin-1 (CD133), epithelial glycoprotein 2 (EGP-2), etc. Active research is also being conducted on the clinical translation of non-traditional immune cells other than T cells in colorectal cancer immunotherapy.

Vaccines are the oldest treatment connected with immunity, and in recent years, tumor vaccine research has been one of the most active fields of study. In 2019, A.E. Snook et al. conducted a phase I clinical trial evaluating the tumor vaccine Ad5-GUCY2C-PADRE(adenovirus vector vaccine) in patients with early-stage colorectal cancer, none of which had an adverse event greater than grade I. GUSY2C antibody responses were seen in 10% of patients, while GUSY2C-specific CD8 cytotoxic T cell responses were seen in 40% of patients (29). Furthermore, the PolyPEPI1018 vaccination (peptide vaccine for conserved cancer antigen expression) combined with first-line maintenance therapy may be the treatment of the future for MSS CRC, for which immunotherapy is rather inefficient. In 11 patients with MSS-mCRC, Joleen M. Hubbard et al. administered subcutaneous injections of PolyPEPI1018 with first-line treatments. Ultimately, there were no serious side effects from the vaccine, five patients got a single dose of PolyPEPI1018 and six patients got up to three doses every 12 weeks. Three patients progressed, five patients were stable, and three patients had partial tumor remission, two of whom had tumors that were small enough to undergo surgery (30). This is an advancement for MSS-CRC.

Bispecific antibodies, a novel immunotherapy concept, are already under investigation for the treatment of CRC. Apart from that, bispecific antibodies, as opposed to monoclonal antibodies, can simultaneously bind to two antigenic epitopes. Bacac et al. created CEA-TCB as a bispecific antibody that binds both CEA expression on cancer cells and CD3 on T cells. This way leads directly to the binding of T cells to cancer cells without the intervention of other immune systems, thereby inducing direct autoimmune destruction of tumor cells (31). As with CRC, which typically has high CEA expression, the progression of CEA-TCB is expected.

In this sense, immunotherapy is more innovative than conventional approaches in that it can produce unique therapeutic results. Nonetheless, the immune system is a part of the individual's system, and excessive interference might result in adverse side effects. ICI, a large class of immunosuppressive pathways present in the body, such as regulate T- cell responses by blocking immune checkpoints (ICs) and increasing T-cell activation. However, over-activation may result in systemic autoimmune disorders (32, 33). At the same time, the widespread use of ICI has led to the development of acquired resistance in many patients who initially responded favorably. The relatively poor scientific and technical understanding of the mechanisms of acquired ICI resistance may hinder the development of immunotherapies for the next generation (Table 1).

Exploration of ICIs' antitumor effects and resistance mechanisms

Immunosuppressive cells

TREG

Regulatory T cells (Treg) are present in both the thymus and the periphery, with natural Treg in the thymus promoting autoimmune tolerance and degenerating with age, whereas peripherally adaptive Treg are antigen-specific suppressor cells that can be converted to Treg by CD4+CD25+ T cells induced by tumor cells, thereby promoting immune escape (34, 35). By lowering the immune response in healthy humans, Tregs can avoid autoimmune disorders. Through a cytokine-dependent or cell-cell contact mechanism, however, Tregs in cancer patients inhibit the immune response to the tumor (36).

Gershon R.K. introduced the concept of immunosuppressive T cells in 1974, demonstrating the crucial role of such T cells in both *in vivo* and *ex vivo* suppressive effects (37). Treg's existence has been demonstrated in recent years by research demonstrating its ability to block tumor rejection (38–41). In 1999, Onizuka, S and Shimizu, J investigated the role of Treg in tumor immunity in mice for the first time. Subsequent studies demonstrated that Treg cells have a negative effect on CTL production as well as the innate immune response, and animal experiments demonstrated that a decrease in the number of Treg cells correlates with a decrease in tumor size. There is a link between a decrease in the number of Treg cells and a diminution in tumor volume (42–45). Besides that, Somasundaram, R. et al. investigated the utility of Treg in CRC in 2002 and discovered that Treg is induced by TGF-β in human colorectal cancer without contact and mediates immune escape to protect tumor cells by inhibiting CTL activation and subsequently acting as a mechanism to inhibit tumor cell destruction (46). The statistical analysis of the case studies revealed that elevated Treg was associated with a poor prognosis for CRC and with recurrent metastases following CRC tumor excision (47). Tumor cells drive the aggregation and synthesis of Treg through a variety of mechanisms throughout tumor progression. In cancer, the release of chemokines CCL17, CCL22, and CCL28 stimulates Treg recruitment (48–50). Autoimmunity exerts an anti-tumor cell effect during the early stages of tumor growth by detecting tumor cell autoantigens and rejecting them. However, as the tumor process advances, CTL-mediated autoimmunity is finally defeated by immunosuppression established through Treg cells. When the number of Treg cells surpasses the number of effector T cells, immunological escape is encouraged (38, 39, 51). Through comparison of a tumor-bearing mouse model constructed by Barbara Valzasina et al. with a tumor-free

TABLE 1 Ongoing clinical trials of immunotherapy for CRC patients.

NCT number	Study Title	Phase	Strategy	Primary outcome measures	genomic stratification
NCT03206073	Pexa-Vec Oncolytic Virus With Immune Checkpoint Inhibition	I/II	PD-L1,CTLA4 Inhibitors and Oncolytic virus	Rate of AEs	MSS
NCT03388190	Anti-tumor IMMunity by OXaliplatin	II	PD-1 Inhibitors and Chemotherapy	PFS	MSS/pMMR
NCT03287427	MYB and PD-1 Immunotherapies	I	Vaccine and PD-1 Inhibitors	Rate of AEs and DLTs	N/A
NCT01885702	Dendritic Cell VacciN/Ation	I/II	Vaccine	Safety and feasibility of vacciN/Ation	MSI-H
NCT04044430	Encorafenib, Binimetinib, and Nivolumab	I/II	MEK,BRAF and PD-1 Inhibitors	Radiographic Response	MSS
NCT03435107	Durvalumab	II	PD-L1 Inhibitors	ORR	POLEmutated/ MSI-H
NCT02437071	Pembrolizumab Plus Radiotherapy or Ablation	II	PD-1 Inhibitors,Radiotherapy and RFA	response rate	N/A
NCT02754856	Tremelimumab and Durvalumab	I	PD-L1,CTLA4 Inhibitors,Laboratory Biomarker AN/Alysis and Surgery	Feasibility	N/A
NCT02983578	Danvatirsen and Durvalumab	II	STAT and PD-L1 Inhibitors	Rate of AEs, SAEs	MMR
NCT03800602	Nivolumab and Metformin	II	Metformin and PD-1 Inhibitors	ORR	MSS
NCT03851614	Inhibitors of DN/A Damage Response, Angiogenesis and Programmed Death Ligand 1	II	PD-L1, PARP and VEGFR2 Inhibitors	Changes in genomic and immune biomarkers	MMR
NCT03639714	PersoN/Alized Neoantigen Cancer Vaccine	I/II	Vaccine	Rate of AEs, SAEs, DLTs	MSS
NCT03436563	M7824	I/II	PD-1 Inhibitors and TGFbetaRII Fusion Protein	ORR	MSI
NCT02873195	Capecitabine and Bevacizumab With or Without Atezolizumab i	II	PD-L1,VEGF Inhibitors and Chemotherapy	PFS	N/A
NCT03290937	Utomilumab, Cetuximab, and Irinotecan Hydrochloride	I	EGER,4-1BB Inhibitors and Chemotherapy	Recommended phase 2 dose of irinotecan hydrochloride	N/A
NCT03228667	CombiN/Ation Immunotherapies	II	N-803,PD-1and PD-L1 Inhibitors	ORR	MSI-H
NCT03186326	Chemotherapy vs Immunotherapie	II	PD-L1, VEGFR, VEGF and EGFR Inhibitors	PFS	MSI
NCT02903914	ArgiN/Ase Inhibitor INCB001158 With Immune Checkpoint Therapy	I/II	PD-1 and ARG I Inhibitors	Rate of AEs	N/A
NCT03610490	MDA-TIL	II	MDA-TIL and Chemotherapy	ORR	N/A
NCT04721301	Ipilimumab, Maraviroc and Nivolumab	I	PD-1,CTLA-4 and CCR5 Inhibitors	Rate of AEs	N/A
NCT03981146	Nivolumab	II	PD-1 Inhibitors	Durable Clinical Benefit	MSS
NCT02888743	Durvalumab and Tremelimumab With or Without Radiation Therapy	II	PD-1,CTLA-4 Inhibitors and Radiation Therapy	ORR	MSS
NCT03712943	Regorafenib and Nivolumab	I	PD-1 and VEGFR Inhibitors	Maximum Tolerated Dose	MMR
NCT03547999	Perioperative CV301 VacciN/Ation With Nivolumab and Systemic Chemotherapy	II	PD-1 Inhibitors, Vaccine and Chemotherapy	OS	N/A
NCT03174405	Avelumab and Cetuximab With FOLFOX in Patients The Phase II AVETUX-CRC	II	PD-L1 Inhibitors	PFS	MSI/MSS
NCT03396926	Pembrolizumab, Capecitabine, and Bevacizumab	II	PD-1 and VEGF Inhibitors,and Chemotherapy	Frequency of treatment-related DLT	MSS
NCT03658772	Grapiprant and Pembrolizumab	I	PD-1 and EP4R Inhibitors	Safety and tolerability of grapiprant alone	MSS
NCT02740985	AZD4635	I	PD-L1, A2AR Inhibitors and Chemotherapy	DLTs	MSS
NCT03867799	iSCORE : Immunotherapy Sequencing	II	PD-1 Inhibitors	DCR	N/A
NCT03289962	Autogene Cevumeran (RO7198457) With Atezolizumab	I	PD-L1 Inhibitors and Vaccine	DLTs	N/A
NCT04208958	VE800 and Nivolumab	I/II	PD-1 Inhibitors, Antibiotics and Microbial Therapy	Safety and tolerability	MSS
NCT03948763	mRN/A-5671/V941 With Pembrolizumab (V941-001)	I	PD-1 Inhibitors and Vaccine	DLTs and rate of AEs	non-MSI-H
NCT03350126	Nivolumab and Ipilimumab	II	PD-1, CTLA-4 Inhibitors	DCR,PFS,ORR	MSI/MMR

(Continued)

TABLE 1 Continued

NCT number	Study Title	Phase	Strategy	Primary outcome measures	genomic stratification
NCT03507699	Immunotherapy and Radiosurgery	I	PD-1,CTLA-4 Inhibitors and TLR9 agonist	DLTs	non-MSI-H
NCT01787500	Vemurafenib, Cetuximab, and Irinotecan Hydrochloride	I	BRAF Inhibitors and Chemotherapy	DLTs	N/A
NCT04513951	AVELUMAB and CETUXIMAB and mFOLFOXIRI	II	PD-1, EGFR Inhibitors and Chemotherapy	Rate of ORR,PFS and Toxicity	N/A
NCT03414983	Nivolumab With Standard of Care Therapy *vs* Standard of Care Therapy	II/III	PD-1 Inhibitors and Chemotherapy	Rate of ORR and PFS	N/A
NCT03170960	Cabozantinib With Atezolizumab	I/II	Tyrosine kiN/Ase and PD-L1 inhibitor	Rate of MTD, ORR and PFS	N/A
NCT03721653	FOLFOXIRI + Bev + Atezo *vs* FOLFOXIRI + Bev	II	PD-1, VEGF Inhibitors and Chemotherapy	Rate of ORR,PFS and Toxicity	N/A
NCT02060188	Nivolumab Alone or Nivolumab CombiN/Ation Therapy	II	PD-1,CTLA-4 and MEK Inhibitors and Anti-Human CD38	Rate of ORR	MSI
NCT03849469	XmAb®22841 Monotherapy With or Without Pembrolizumab	I	XmAb®22841 and PD-1 Inhibitors	Rate of AEs	N/A
NCT03373188	VX15/2503 and Immunotherapy	I	Anti-SEMA4D PD-1,CTLA-4 Inhibitors and Surgery	Evaluate treatment effects,rate of AEs	MSS
NCT02009449	Pegilodecakin (LY3500518)	I	PD-1,VEGF Inhibitors and Chemotherapy	Rate of AEs	N/A
NCT03761914	Galinpepimut-S With Pembrolizumab	I/II	PD-1 Inhibitors and Vaccines	Rate of ORR, CR and TRAEs	N/A
NCT03184870	BMS-813160 With Chemotherapy or Nivolumab	I/II	PD-1 Inhibitors and Chemotherapy	Rate of AEs and DLT'S	N/A
NCT03095781	Pembrolizumab and XL888	I	PD-1 and HSP90 Inhibitors	Rate of AEs and OS	N/A
NCT03239145	Pembrolizumab (Anti-PD-1) and AMG386 (Angiopoietin-2 (Ang-2)	I	PD-1 and VEGF Inhibitors	Rate of ORR,OS,PFS and DLT'S	N/A
NCT04306900	TTX-030 With Immunotherapy With or Without Chemotherapy	I	TTX-030 and PD-1 Inhibitors	Rate of AEs	N/A

N/A, not applicable.

mouse model, it was identified that Treg promotes immune escape by suppressing the proliferation of existing T cells that continuously interact with dendritic cells to maintain the effects of providing autoantigen and costimulation; in addition to favoring the generation of a broader T cell lineage from the circulation to promote immune escape with new Treg (34). The work by Ngiow S F et al. indicated that intra-tumor Tregs are partly responsible for the formation of anti-PD1 resistant tumors and PD1(hi)CD8(+) T cells. Furthermore, the reduction in the CD8+T/Treg ratio can be used to demonstrate the efficacy of an anti-PD-1 monoclonal antibody (52).

MDSCs

After the 1980s, through extensive research on tumor patients, suppressor myeloid cells were identified and characterized. These myeloid cells with suppressive activity were later collectively referred to as myeloid-derived suppressor cells (MDSCs), and there is now abundant evidence that MDSCs play a suppressive role in the immune system. They share a myeloid origin, an immature condition, and a remarkable capacity to inhibit T-cell responses (53–55). MDSCs originate from myeloid cells that failed to differentiate and mature as a result of cancer, inflammation, trauma, autoimmune disorders, etc. Myeloid progenitor cells and immature myeloid cells comprise this diverse cell type (IMCs) (56). The primary manifestation of MDSC immunosuppression is the inhibition of T cell proliferation and the promotion of Treg formation. There is evidence that elevated circulating levels of MDSCs correlate with disease stage, classification, and metastasis development in advanced colorectal cancer (57). MDSCs regulate the metabolism of L-arginine *via* inducible nitric oxide synthase (INOS) and arginase-1 (ARG1), which depletes the microenvironment of L-arginine, inhibits T cell proliferation, cytokine production, and expression of the T cell receptor CD3 zeta chain, converts L-arginine into polyamines, and promotes tumor growth. L-arginine is induced by INOS to create NO and ROS, which lowers CD3 zeta expression and triggers T-cell death (58–63). The synthesis of arginase II by mature myeloid cells such as macrophages does not drain L-Arg from the microenvironment and does not compromise the function of T-cells (64). In 1993, Nakagomi H discovered that T cells isolated from colorectal cancer patients expressed much less CD3 zeta than peripheral blood T cells from the same patients, and that peripheral blood zeta chain levels were significantly lower than T cell zeta chain levels in lymphocytes

(65). Ichihara et al. examined the expression of CD3 zeta in peripheral blood mononuclear cells before and after surgery in 28 patients and found that hydrogen peroxide-mediated stimulation of mononuclear cells decreased the expression of TCR CD3 zeta molecules in peripheral T cells (66). Mizoguchi H had previously hypothesized that T cells from mice with tumors exhibit T cell antigen receptors with little CD3γ and no CD3 zeta, which are substituted by Fc epsilon γ chains. Also diminished was the expression of the tyrosine kinases p56lck and p59fyn. These modifications may be the cause of immunodeficiency in the tumor-bearing host (67). In conclusion, the study of MDSCs is still in its infancy and a great deal of research is still being conducted, but it is already known from current experiments that the level of MDSCs in clinical patients is closely related to the efficacy of immunotherapy and the prognosis of patients. Patients with colorectal cancer who have a poor response to conventional immunotherapy pMMR-MSI-L staging may, potentially, benefit considerably from immunotherapies that specifically target MDSCs. Additionally, the absence of ARG1 activity can reduce the effectiveness with which MDSCs can be inhibited and enhance the sensitivity of PD-1/PD-L1 antibodies (68).

TAMS

Pelka et al. used single-cell RNA sequencing and spatial analysis to compare a large number of colorectal cancer patients' tissues with normal tissues, which had more monocytes and macrophages than normal tissues (69). After specific differentiation, macrophages can be divided into two different polarization states based on their function and level of inflammatory factor secretion: M1 and M2-macrophages (70–73). M1 macrophages boost the Th1 response by ingesting and destroying the target tumor cells. M2 macrophages release anti-inflammatory cytokines that promote angiogenesis and the beginning and progression of tumors (73, 74). In addition, when tumor cells are present, immune cells might connect to them and develop a unique biological phenotype as a result of their interaction. M-MDSCs may then develop into tumor-associated macrophages (TAMs), have an M2-like phenotype, and enhance anti-tumor immunosuppression by promoting tumor angiogenesis or indirectly interfering with the interactions of immune cells in the tumor microenvironment (TME). In the interim, TAMs can attract Tregs by secreting chemokines, allowing Tregs to inhibit T cells through anti-tumor immunological responses (75–78). TAMs are intrinsically inhibitory of CTL cell function, blocking TCR signaling while increasing T cell unresponsiveness and death *via* increased expression of PD-L1 and CTLA-4 in association with the relevant receptors on CTL cells (79). TAMs can also promote tumor development, invasion, metastasis, immunosuppression, angiogenesis, and drug tolerance by secreting cytokines and chemokines that coordinate with inflammatory mechanisms, as demonstrated by the TGF-β, VEGF, PDGF, M-CSF, IL-10, and CXCL (80). In a mouse model of pancreatic cancer already proven, however, inhibiting macrophage CSF-1R (colony-stimulating factor 1 receptor), reducing the frequency of TAM, and increasing IFN production can increase the responsiveness of tumor cells to the treatment. Gemcitabine was much more effective when combined with CSF-1R blockers and PD-1 or CTLA-4 antibodies. It will be worthwhile to wait for equivalent colorectal cancer evidence (81).

The role of immune checkpoints in the treatment of CRC by the mechanism

Immune checkpoint molecules, such as PD-1, PD-L1, and CTLA4, can activate signaling pathways that restrict T-cell function. They are a class of immunosuppressive molecules that are expressed on immune cells to control the level of immune activation. And this type is currently the most frequently targeted immunotherapy agent. James Allison of the United States and Tasuku Honjo of Japan were awarded the 2018 Nobel Prize in Physiology or Medicine for their contributions to the discovery of negative immune regulation, also known as CTLA4 and PD-1, as cancer treatments (82).

CTLA-4 is a protein receptor that inhibits the immunological response in humans. CTLA-4 is expressed on the surface of CD4+ and CD8+ lymphocytes. It competes with the T-cell costimulatory receptor CD28 for interactions with T-cell costimulatory factors and, by binding to CD28 (Figure 2), reduces T-cell proliferation (83–85). These pathways maintain autoimmune tolerance and regulate the duration and magnitude of physiological immune responses induced by peripheral tissues. ICs physiologically prevent autoimmunity by inhibiting immune cells' responses. It is typically initiated by ligand-receptor interactions and can be inhibited by antibodies or by recombinant forms of ligands or receptors (84, 86, 87).Despite this, these molecules are frequently chosen as the primary immune evasion mechanism following the development of tumors. When the FDA approved the CTLA-4 monoclonal antibody Ipilimumab as an immunotherapy for metastatic melanoma in 2011, it was the first time an ICI had been approved for clinical use as a cancer immunotherapy medicine. However, the therapeutic treatment of CRC with the CTLA-4 monoclonal antibody did not demonstrate the anticipated efficacy. Chung conducted a single-arm, multi-center phase II intravenous monoclonal antibody trial utilizing Tremelimumab on 47 patients, with only one patient obtaining a second therapy and reaching a six-month partial remission. The overall survival (OS) median was 19.1 months and the progression-free survival (PFS) median was 2.3 months (88). The trial did not utilize MSI-H in regard to the MMR subgroup,

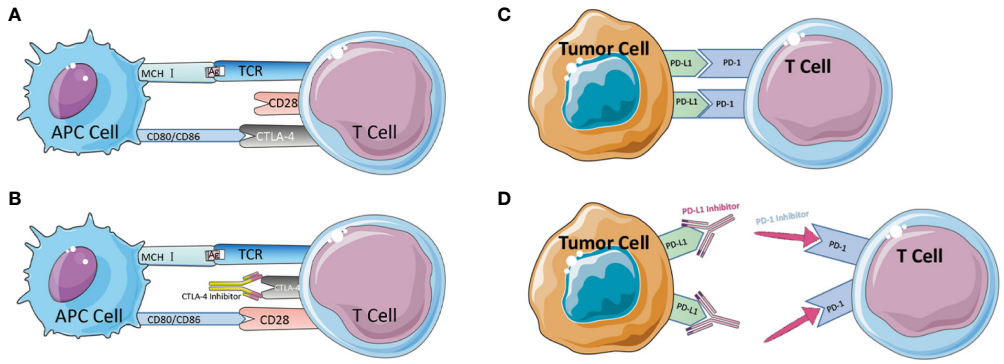

FIGURE 2
(A) Schematic diagram of CTLA-4-mediated immune escape. **(B)** Schematic diagram of CTLA-4 inhibitor to suppress immune escape. **(C)** Schematic diagram of PD-1/PD-L1-mediated immune escape. **(D)** Schematic diagram of PD-1/PD-L1 inhibitor to suppress immune escape.

but the results of this trial imply that CTLA-4 monoclonal antibody may not be suitable for CRC monotherapy.

Besides CTLA-4, the Programmed Death 1 receptor and its ligand (PD-1/PD-L1) are an IC that was discovered in 1992 by Tasuku Honjo in a mouse T-cell hybrid tumor (89). PD-1 is an inhibitory co-receptor expressed on NK cells, B cells, T cells, and TIL cells, indicating that PD-1 has a broader function than CTLA-1. The team of L CHEN released in 1999 an article describing the discovery that the B7-H1 molecule (PD-L1), which can bind to PD-1, co-regulates certain cellular immune responses. In healthy organisms, the interaction between PD-1/PD-L1 restricts T cell effector responses in order to maintain immunological dynamic equilibrium and protect the body against autoimmunity with severe inflammation. PD-L1 is expressed on activated lymphocytes (T cells, B cells, and NK cells), peripheral tissues, and organs. After binding, PD-1 inhibits the kinase that activates T cells *via* the phosphatase SHP250. PD-1 can also inhibit TCR signaling, thereby altering the duration of T cell-APC or T cell-target cell contacts. The combination of PD-1 and PD-L1 induces apoptosis, depletion, and hypofunction in T cells, which in turn inhibits the activation, proliferation, and antitumor activity of CD8+ T cells specific to tumor antigen (79, 89–94). Tumor tissue regulates enhanced PD-1 expression, permitting more PD-1 to bind to ligands, inhibit cytotoxic cells, and limit the release of related cytokines (95). In the absence of a matching therapeutic application, PD-1 can also attach to PD-L2, which is associated with the inhibition of immunological responses and immune tolerance. In 2014, the FDA approved two PD-1 monoclonal antibodies, Nivolumab and Pembrolizumab, for the clinical treatment of metastatic melanoma (Figure 2). In 2010, Julie R. Brahmer completed a single-agent Phase I clinical trial with PD-1 monoclonal antibody involving a total of 14 patients with CRC, one of whom achieved lasting full remission. A subsequent phase II clinical trial that added dMMR status to the evaluation criteria ultimately led to the accelerated FDA approval of pembrolizumab as an option for partial cases in CRC treatment (95). In 2012, 18 out of 207 colorectal cancer patients participated in a multicenter clinical phase I trial of PD-L1 monoclonal antibody. However, no objective reflection (full or partial remission) was detected (96). Although PD-L1 monoclonal antibodies are successful in preventing some solid tumors, they are not very effective in treating colorectal cancer. Later, J. Bendell, J. performed atezolizumab, bevacizumab in combination with FOLFOX, and the combination of MEK inhibitor cobimetinib with atezolizumab in CRC patients, demonstrating improved efficacy, enhanced CD8T cell infiltration, and MHC I expression. Therefore, the PD-L1 therapeutic alliance for CRC has a promising future. Early trial results of PD-1 monoclonal antibodies appear promising, particularly in colorectal cancer patients with the dMMR staging. PD-L1 inhibitors are less active as monotherapy but have enhanced efficacy in combination, potentially extending the indications for ICI to patients with the pMMR staging (53, 97). After the discovery and clinical application of the anticancer capabilities of ICIs, the high occurrence of drug resistance (both primary and acquired) has emerged as a critical concern in the area, limiting their clinical applicability.

Immune escape—the key to drug resistance

ICIs have become a crucial part of the treatment of colorectal cancer. However, not everyone can benefit from it, nor does always benefit. According to R. Cohen et al., five out of 38 mCRC patients treated with ICIs exhibited primary resistance, of which three were dMMR (98). The FDA-approved ORR for mCRC with nivolumab monotherapy is 31%, whereas nivolumab plus ipilimumab investigators evaluated an ORR of 55% (99). These characteristics imply the occurrence of dMMR CRC patients with intrinsic or emerging resistance to immune checkpoint drugs. Two types of resistance to ICIs can be roughly

categorized: (1) Primary resistance, which generally refers to patients who do not respond to ICIs at all from the start and progress quickly or eventually. (2) Acquired resistance, which refers to patients who respond to ICI therapy initially but then progress clinically and/or radiologically (100). The current technique for overcoming primary resistance is to employ combination therapy that mixes immunosuppressive medicines with additional biologics, such as PD-1 inhibitors in combination with tumor vaccines (NCT03289962). In contrast, the mechanism of acquired drug resistance is more complex and has not been studied in detail with precision. Different drug-resistant populations develop resistance at different rates and to varying degrees, but there is no fundamental difference between them. In a nutshell, it is a tumor immune escape mechanism.

Medication resistance major issue—Immune escape hypotheses

In 1909, Paul Ehrlich made the initial discovery and suggestion that tumor formation was caused by an immune system dysfunction and that the immune system itself might limit tumor development through investigations into transplantable breast tumors in mice. At the time, this was not universally accepted by the academic world (101). In the middle of the 20th century, fifty years after Ehrlich's theory, Frank Macfarlane Burnet and Lewis Thomas proposed that mutations in somatic cells were inevitable in the human body but that the body's internal homeostasis could be eliminated by a substance or mechanism in the immune system that could eliminate potentially dangerous mutant somatic cells (102). In the 21st century, Grulich et al. observed a significant incremental increase in cancer risk and a similar pattern in both population groups through a cohort study of AIDS patients and transplant immunosuppressed individuals, indicating a correlation between cancer incidence and immunodeficiency (103). Eventually, Gavin P. Dunn and Robert D. Schreiber proposed a more systematic and comprehensive theory of Cancer Immunoediting to characterize the immune system's defense of the host and its influences on the alteration of tumor disease, ultimately leading to the concept of immunological escape. Three processes are involved in cancer immunoediting: elimination, equilibrium, and evasion. Effective immune evasion will result in the mutation of tumor cells that are insensitive to immunological monitoring, their elimination in the form of genetic or epigenetic alterations, and the initiation of uncontrolled growth that leads to clinically diagnosed cancer (104).

MSI-related immune escape

pMMR is frequently associated with primary immunological resistance, whereas ICIs is more frequently used in dMMR. In 48 percent of all trials, significant tumor shrinkage or no advancement was reported after PD-1 blocking therapy, while in 52 percent of all patients, prompt tumor enlargement after therapy or shrinkage followed by enlargement was observed (105). In MSI-H CRC treatment, ICIs resistance may result from many MSI-H related immune escape symptoms. Insertion or deletion of nucleotides in microsatellite mutations results in translational frameshifts that affect the translational expression of proteins that may express MSI-specific shift peptides (FSP), eliciting intense local and systemic anti-tumor immune responses in the host while evading immune control through various mechanisms (106–109). Nicolas J. Llosa et al. discovered that MSI-H CRC had a high degree of Th1 and CTL activation in the microenvironment (110). Regrettably, MSI-H CRC progression remained brisk. All of this demonstrates that MSI-H has a specific immune escape that contributes to the progression of CRC development (Figure 3). Following immunological monitoring of MSI-H, it was realized that, probably during tumorigenesis, MSI-H colorectal cancer cells chose tumor cells with a defective antigen handling mechanism (APM) to promote their proliferation. Matthias Kloor et al. evaluated the expression of Human Leukocyte Antigen Class I Antigen (HLA-I) subunits in 20 MSI-H CRC and 20 MSS CRC tissues using monoclonal antibodies specific to APM components. Total HLA-I antigen loss was observed in 12 of 20 MSI-H lesions (60%) but in just 6 of 20 MSS colorectal lesions (only 30%). In other words, the MSI-H phenotype of colorectal cancer was associated with a high prevalence of deficient HLA-I antigen presentation (111). HLA-I antigens transport polypeptides from cells to the immune system. When a tumor-specific antigen is present on the cell surface, CD8+ T lymphocytes are able to recognize the antigen and then secrete cytotoxic substances to induce antitumor immunity. Thus, diminished HLA-I antigen presentation is an effective defense against the cytotoxic T cell onslaught (112, 113). The HLA-I complexus is composed of the HLA-I heavy chain, B2M, and a peptide fragment including the molecular chaperone Tapasin, Calnexin, Calreticrin, and ERp57.39, which are assembled in the endoplasmic reticulum in a progressive manner. 14 of 124 CRCs (11%) examined by C.M. Cabrera et al. IHC and Mab analysis exhibited complete deletion of HLA-I. Four cases exhibited inactivation of the 2M biallelic sites and accumulation of intracytoplasmic HLA-I heavy chains, which may result in a failure of T cell identification in the immunological response, as determined by simultaneous MSI-H and RT-PCR analysis (114). 45 β2M mutations have occurred at an advanced stage of carcinogenesis in MSI-H CRC (115). Truncating mutations in the β2M gene, which is involved in the folding and transport of MHC I molecules, can affect the expression of MHC I on the APC surface, leading to reduced antigen presentation and immunotherapeutic resistance. It is believed that abnormal mutations in β2M constitute a key mechanism of tumor resistance to T cell-mediated immune responses and a source of immunotherapeutic resistance (116, 117). Changes to a decrease in β2M and HLA-I may present an opportunity to reverse immunological resistance.

TMEs develop ICIs resistance as a result of immune evasion

The dynamic tumor microenvironment (TME) may be closely related to the mechanism of drug resistance

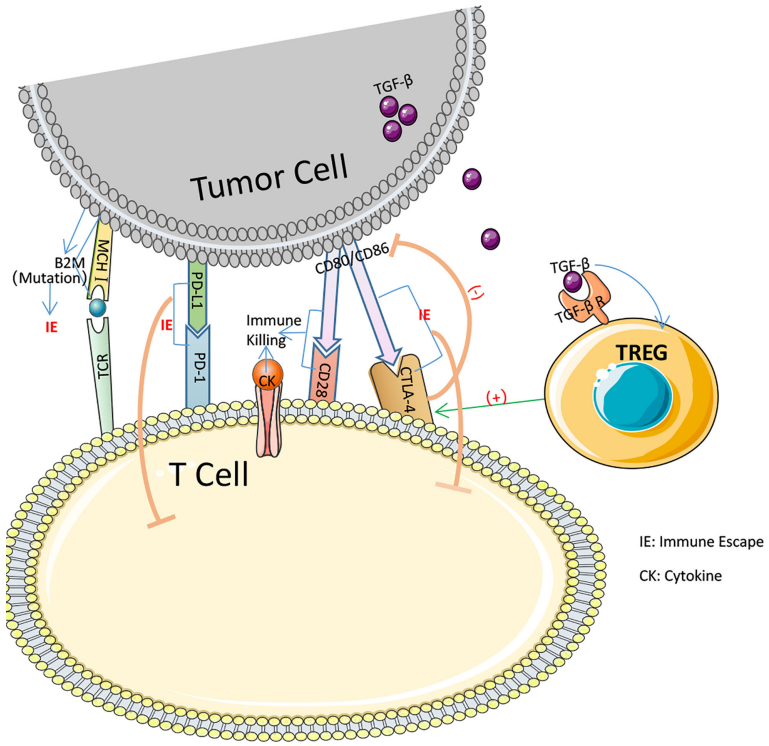

FIGURE 3
Several pathways by which immune escape occurs in colorectal cancer.

development. TME encompasses both anti-tumor immune and pro-tumor growth cells, and the intricate interplay between anti-tumor immunity and immunosuppression alters the balance between tumor growth and tumor elimination on a continuous basis. With the advancement of research and technology, the significance of the relationship between cancer and the immune system is becoming recognized, and in 2011, evading immune destruction was identified as one of the defining characteristics of cancer (118). In addition to cancer cells, the tumor microenvironment now comprises a heterogeneous population of immune cells, interstitial cells, endothelial cells, cancer-associated fibroblasts, and their related secreted factors (119). TME is also a key immune escape and cancer proliferation stimulator (120). Crucial to carcinogenesis is the interaction of malignant cells with diverse cells inside the TME. The TME contains certain immune cells, including T and B lymphocytes, tumor-associated macrophages (TAM), dendritic cells (DC), natural killer (NK) cells, neutrophils, and myeloid-derived suppressor cells (MDSC); also contains stromal cells (such as cancer-associated fibroblasts (CAF), pericytes and mesenchymal stromal cells); the extracellular matrix (ECM) and other secreted molecules like growth factors, cytokines, chemokines and extracellular vesicles (EV); and the network of blood and lymphatic vessels that are co-connected and not only influence each other but are also associated closely with tumor cells (121–124). As described in the theory of cancer immune editing, cancer cells are inactivated by anti-tumor immunity in the tumor microenvironment in the early stages of cancer development, but as the tumor proceeds, the tumor stalemates with the immune system and eventually the clinical manifestations of the tumor cells must undergo immune escape, a process in which TME is accompanied by significant disruption of the cellular immune response. The work of Joel Crespo et al. demonstrates that in the case of late immunosuppressive TME, TIL activation and functional expression are restricted, T cell depletion increases, tumor cells continue to grow meanwhile tumor cells leaving the TME are attacked by other immune cells, thus inferring the existence of immune escape under certain conditions in TME (125).

Continuous angiogenesis, one of the features of tumor development, plays a driving role in the process of TME shift in the direction of immune escape occurrence (118). Rapid tumor proliferation is always accompanied by angiogenesis to meet the needs of tumor cells for oxygen and nutrition (126). Additionally, tumor cells' aberrant angiogenesis is unable to carry enough oxygen. Reduced oxygen levels are present in 50%–60% of solid tumors (127). During the course of the tumor, the high glycolysis rate of the tumor cells generates a significant amount of acidic chemicals,

which causes the TME's weak acidic environment to stand out (128). While this is happening, the structural and functional abnormalities of the tumor's vasculature cause local blood leakage, which raises interstitial fluid pressure (IFP). High IFP then makes it harder for tumor tissue to be perfused, which worsens the tumor's hypoxic, acidic, and high IFP microenvironment (129, 130). In these circumstances, TME stimulates the production of chemokines to encourage the infiltration of immunosuppressive cells, which TME tilts toward immunosuppression (131). Additionally, the hypoxic environment might prevent effector T cells from penetrating. Vascular endothelial growth factor lowers T-cell adhesion molecule expression. As well as causing endothelial cells to express Fas ligands through the Fas/FasL signaling pathway, VEGF-A, IL-10, and prostaglandin E2 (PGE2) decrease T cell mobilization and invasion by killing CD8+ T cells and endothelial cells (132). In addition to significantly reducing the recruitment of immune-suppressive cells to the tumor, blocking intracellular angiogenesis in tumor cells also promotes the infiltration of effector T cells (133). Bevacizumab, as a VEGF monoclonal antibody, received FDA approval in 2004 for the treatment of CRC (134).

From the development of anti-tumor immunity to immune escape, the changes in the TME deserve our research attention. In CRC, there are also dynamic changes in the TME, the mechanisms of which include altered antigenicity of tumor cells and the consequent production of a range of immunosuppressive mediators that modify the interactions between cells in the TME (135). Inhibits the functions that ICIs are supposed to perform.

Conclusion

This review focuses on immunotherapy and immune escape related drug resistance reverse in colorectal cancer, which is an important and rapidly expanding field. Colorectal cancer has long afflicted patients with the danger and uniqueness of being a built-in organ cancer that is not easily identified and treated at an early stage, and at an advanced stage, has a large risk of spreading and is difficult to cure. Colorectal cancer, as a leading cause of death worldwide, will account for approximately 3% of all deaths in 2020, with the incidence rate increasing year by year (1). Traditional cancer treatments, including surgery, chemotherapy, and radiation therapy, have limitations and cannot eradicate the tumor entirely. Moreover, surgery will alter the function of patients' organs; chemotherapy will exacerbate anemia and weakness, and long-term chemotherapy resistance is unavoidable; radiotherapy is radioactive, and white blood cell depletion, hair loss, and even systemic reactions such as radioactive stomatitis and radioactive esophagitis may occur. Immunotherapy has emerged and advanced as a result of the discomfort and side effects during treatment and the bad prognosis following treatment.

More and more relevant clinical studies have been conducted with the debut of ICIs in immunotherapy and the FDA's approval of ICIs as CRC treatment agents. However, we should highlight that not all clinical studies are planned to include a discussion of genotyping concerning ICIs. Perhaps adding more genotyping requirements at enrollment and researching more precise dMMR/pMMR categorization will help us conduct clinical trials more correctly and expand applications with the introduction of ICIs. In addition to the fact that we could not uncover accurate specific biomarkers for CRC, particularly in MSS/pMMR CRC, how to overcome the barriers to make ICIs effective is critical to the success of ICIs in CRC (136, 137). Currently, it is understood to be successful to combine immune checkpoint inhibitors with chemotherapeutic methods (138, 139). 5-Fluorouracil (5-FU) was the first chemotherapeutic drug for CRC that was proven to be successful. In research by Javadrashid et al. (140), it was discovered that 5-FU therapy decreased pancreatic cancer cells' expression of tumor PD-L1. The findings of Afshin Derakhshani et al. showed that capecitabine, a medication that acts as a precursor to 5-FU, significantly reduced CTLA-4 in CRC tumor cells (141). But Van Der Kraak et al. did show that 5-FU therapy led to PD-L1 upregulation in CRC cells (142). How 5-FU functions *in vivo* results in conflicting scenarios with two traditional IC mechanisms. In order to meet our therapeutic goals and increase patient survival rates, more consideration should be given to how to combine the medications to concurrently inhibit PD-L1 and CTLA-4 expression. In the future, we may need to think more about and do more research to see whether combination treatments can reduce the occurrence of immunological resistance and which medications can be used in conjunction with ICIs to provide greater therapeutic results. TME is a similar dynamic *in vivo* mechanism, analogous to the dynamic changes in drug resistance. The pursuit of the potential to reverse drug resistance in TME appears promising. In comparison to typical immune cells, the role of immunosuppressive cells in medication resistance cannot be overlooked. It has been demonstrated that blocking immunosuppressive cells improves the efficacy of ICIs. In addition, specific indicators for determining the success of immunotherapy in patients with colorectal cancer are still unknown. To minimize harmful side effects and maximize the therapeutic efficacy of immunotherapy, particular indices will be selected. In particular, it provides more reliable clinical treatment guidelines for the monitoring of immune-related adverse events (IrAEs).

In future research, it will be essential to comprehend the precise mechanisms and toxicity measurements by which ICIs build resistance. This will promote the development of new diagnostic and therapeutic options to address the limits of the present treatment for ICIs and assist a greater number of CRC patients.

Author contributions

All authors planned and wrote the manuscript and contributed to the article and approved of the submitted version.

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Molecular subtypes based on Wnt-signaling gene expression predict prognosis and tumor microenvironment in hepatocellular carcinoma

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Based on increasing research evidence, hepatocellular carcinoma (HCC) is heterogeneous, and genetic profiling has led to the identification of multiple subtypes of this disease. To advance our knowledge and the ability to use individualized medicine in the treatment of HCC, it is essential to perform a complete and methodical characterization of various molecular subtypes. The canonical Wnt/β-catenin pathway is an evolutionarily conserved complicated signaling mechanism that plays a role in carcinogenesis and progression of HCC. In this study, we acquired RNA sequencing, somatic mutation, and clinical data from 701 patients from The Cancer Genome Atlas and Gene Expression Omnibus databases and stratified patients into two subgroups: WNT-high and WNT-low. In general, the WNT-high subtype is associated with an immunosuppressive microenvironment, poor prognosis, cancer-related pathways, and a low response to immune checkpoint therapy. We also found that WNT3 is negatively linked to $CD8^+$ T-cell infiltration using multiple immunofluorescence assays. Finally, we developed a WNT-related prognostic model to predict the survival time of patients with HCC. In summary, we developed a new classification scheme for HCC based on Wnt signaling signatures. This classification produced substantial clinical effects, both in terms of assessing patient prognosis and immunotherapy administered to patients with HCC.

KEYWORDS

prognosis, tumor microenvironment, Wnt β-catenin signaling, hepatocellular carcinoma, TCGA (The Cancer Genome Atlas Program)

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Introduction

Liver cancer is the fourth leading cause of cancer-related mortality and the sixth most prevalent contributor to cancer morbidity worldwide (1). Hepatocellular carcinoma (HCC) is responsible for the majority of primary liver malignancies. Although its diagnosis has improved owing to advances in imaging techniques, the prognosis is still dismal, with a 5-year survival rate of <20%, and the choices available for treating HCC are limited (1, 2). The development of next-generation sequencing techniques and their widespread availability has given us the opportunity to investigate and record not only the specific genetic alterations of HCC cells but also the specific compositions of the various cell types found in the tumor microenvironment (TME) and their interplay with HCC cells at a certain level that was not previously possible. Therefore, the precise classification of patients with HCC into certain cancer types based on high-sensitivity genetic sequencing may help improve the clinical outcomes.

The canonical Wnt/β-catenin pathway is a complex signaling system that is evolutionarily conserved and affects basic physiological and pathological functions (3). This pathway is implicated in the maintenance of hepatic homeostasis and the development of distinctive hepatic properties, including metabolic zonation and regeneration in a mature healthy liver (3, 4). In HCC, Wnt/β-catenin signaling is often hyperactivated, which subsequently contributes to tumor growth and invasion (5–6). Interestingly, the Wnt/β-catenin pathway was recently characterized as playing a role in modulating the infiltration of immune cells in the TME, which has become a new research interest because of its possible influence on responsiveness to immunotherapy regimens (7–8). The practice of personalized medicine and the creation of innovative treatment strategies may benefit from targeting the Wnt/β-catenin signaling pathway.

In this study, we hypothesized that the molecular subtypes classified by Wnt/β-catenin signaling would exhibit distinct clinical and pathological features, prognostic factors, and TME. This study aimed to (i) identify the molecular subtypes of HCC based on Wnt/β-catenin signaling, (ii) analyze the prognostic value, anti-tumor immunity, and TME among these subtypes, and (iii) construct and validate a WNT-related prognostic model.

Materials and methods

Datasets

The Cancer Genome Atlas (TCGA) database was searched for RNA sequencing, somatic mutations, and relevant clinical data from 365 patients with HCC (https://portal.gdc.cancer.gov/). Similar data were also acquired from 336 patients with HCC in Gene Expression Omnibus (GEO) database to act as verification datasets. The accession number of GEO datasets was GSE14520 and GSE76427 (9, 10).

Integration of protein–protein interaction network

We used the STRING database to create a PPI network, Cytoscape (https://cytoscape.org/), a platform that uses open-source software for the visualization of complicated networks, and the integration of these networks with any type of attribute data. We created a PPI network using Cytoscape and then used this network to examine the interaction relationships of the key genes involved in Wnt signaling-associated genes.

Consensus clustering

Consensus clustering was undertaken to ascertain the molecular subtypes associated with Wnt signaling *via* the "ConcensusClusterPlus" package in R software. Subsequently, the ideal cluster numbers between k = 2 and k = 10 were evaluated, and to ensure that the outcomes would be consistent and easy to reproduce, this method was carried out one thousand times. A cluster map was generated using the pheatmap tool in R.

Principal component analysis

PCA was conducted to evaluate the similarities and differences in transcription patterns across the various types. After loading the gene names together with the associated expression values and sample data, the "limma" package of the R program was employed to perform the analysis. The results were displayed using the "ggplot2" package.

Immune cell type fractions estimation

CIBERSORT was conducted to ascertain the number of 22 different types of immune cells that were present in each HCC specimen. In the CIBERSORT system (https://cibersort.stanford.edu/), the differentiation of 22 different immune cells was accomplished with the use of a leukocyte gene matrix that contained 547 genes. These immune cells comprise resting NK cells, activated NK cells, gamma delta T cells, monocytes, follicular helper T cells, regulatory T cells (Tregs), resting CD4 memory T cells, activated CD4 memory T cells, $CD8^+$ T cells, naïve $CD4^+$ T cells, naïve B cells, memory B cells, plasma cells, macrophages (M0, M1, M2), eosinophils, neutrophils, activated

mast cells, resting mast cells, resting dendritic cells, and activated dendritic cells. To further assess the reliable results of immune score evaluation, we used "immuneeconv" package to estimate immune cell scores based on TIMER and MCP-counter algorithms. The ssGSEA algorithm was completed using the "GSVA" and "GSEABase" packages in R.

Establishment of the WNT prognostic signature

Following the univariate Cox regression analysis, a LASSO Cox regression analysis was conducted on the statistically significant WNT signaling-associated genes to determine the particular coefficient values for each association. The LASSO method of regression analysis is a technique for enhancing the accuracy of predictions and the interpretability of the generated statistical model by performing both variable selection and regularization. Therefore, LASSO Cox regression is an excellent choice for building a prognostic model based on gene expression patterns.

Comparison of the OS rates between the low- and high-risk groups was performed using Kaplan–Meier analysis, which was conducted in R using the survival and Survminer packages.

Prediction of response to immunotherapy

To assess the immune checkpoint blockade (ICB) responsiveness, a tumor immune dysfunction and exclusion (TIDE) investigation was performed. Jiang et al. developed this analytical method (TIDE) that predicts ICB responsiveness by employing the two most important strategies that tumors use to evade the immune system: T-cell dysfunction induced in tumors that have high infiltrating levels of cytotoxic T lymphocytes (CTLs) and suppressed T-cell infiltration in tumors that have a low level of CTLs.

Multiple immunofluorescence

Tissue microarrays of 36 HCC specimens were acquired from Shanghai Outdo Biotech (Shanghai, China) and used to conduct additional research on the link between WNT3 expression and CD8+ T cell presence in the HCC-TME. The multiplex immunohistochemistry (IHC) experiment was performed by employing staining cycles in the following order. Specifically, after deparaffinization, tissue slices of HCC that had been fixed in formalin and embedded in paraffin were subjected to microwave treatment in citrate for antigen retrieval. Next, the sections were blocked in normal goat serum at a concentration of 10% before incubating overnight with primary antibodies: rabbit anti-WNT3 antibody (1:200, ab32249, Abcam) and mouse anti-CD8 antibody (1:100, ab17147, Abcam). The sections were left for a thirty-minute incubation period at ambient temperature with the corresponding horseradish peroxidase-conjugated secondary antibodies (Abcam, CN). The tyramide signal amplification dye was used to display the antigenic binding sites. Each antibody was labeled with cy3-tyramide (1:1,000, G1235, Servicebio) and fluorescein isothiocyanate-tyramide (1:1,000, G1235, Servicebio). The positive percentage of CD8 T cells was calculated using Indica Labs-HighPlex FL module (v3.1.0) of Halo analysis software (Indica Labs, USA). The mean fluorescence intensity of WNT3 was quantified by Indica Labs-Highplex FL (v3.1.0) module of Halo software (Indica Labs, USA). The correlation between WNT3 expression and $CD8^+$ T cell infiltration was calculated by Pearson correlation in R software

Statistical analysis

The overall survival (OS) rates across various groups were compared *via* Kaplan–Meier analysis using Survminer and survival packages in R. The Kruskal–Wallis and Wilcoxon signed-rank tests were used to evaluate potential variations across the subtypes. Univariate Cox analysis was performed to determine the potential prognostic markers. Using the survivalROC R package, a receiver operating characteristic (ROC) curve was plotted to verify the accuracy of the risk model in the prediction of patients' OS. The R software (version 3.5.2) was used for all statistical analyses.

Results

Identification of Wnt signaling-based subtypes by consensus clustering in HCC

We initially investigated alterations in Wnt signaling using TCGA pan-cancer datasets. Among these, CTNNB1 has the highest mutation rate in HCC. These results indicate that the Wnt/β-catenin pathway may play an essential role in carcinogenesis of HCC (Figures 1A, B). Next, we downloaded the Wnt-signaling gene set from the Gene Set Enrichment Analysis (GSEA) (KEGG_WNT_SIGNALING_PATHWAY.v7.5.1). We employed the STRING database to undertake a PPI network analysis to gain a deeper understanding of the mechanism by which these genes are involved in Wnt signaling (Figure 1C). We further determined Wnt signaling-based clusters in HCC using consensus clustering. After k-means clustering, we identified two clusters in TCGA cohort with distinct Wnt signaling-related gene expression patterns (Figures 1D, E). The expression levels of WNT genes varied among the different clusters. Overall, cluster C1 showed the highest Wnt signaling gene expression levels and was

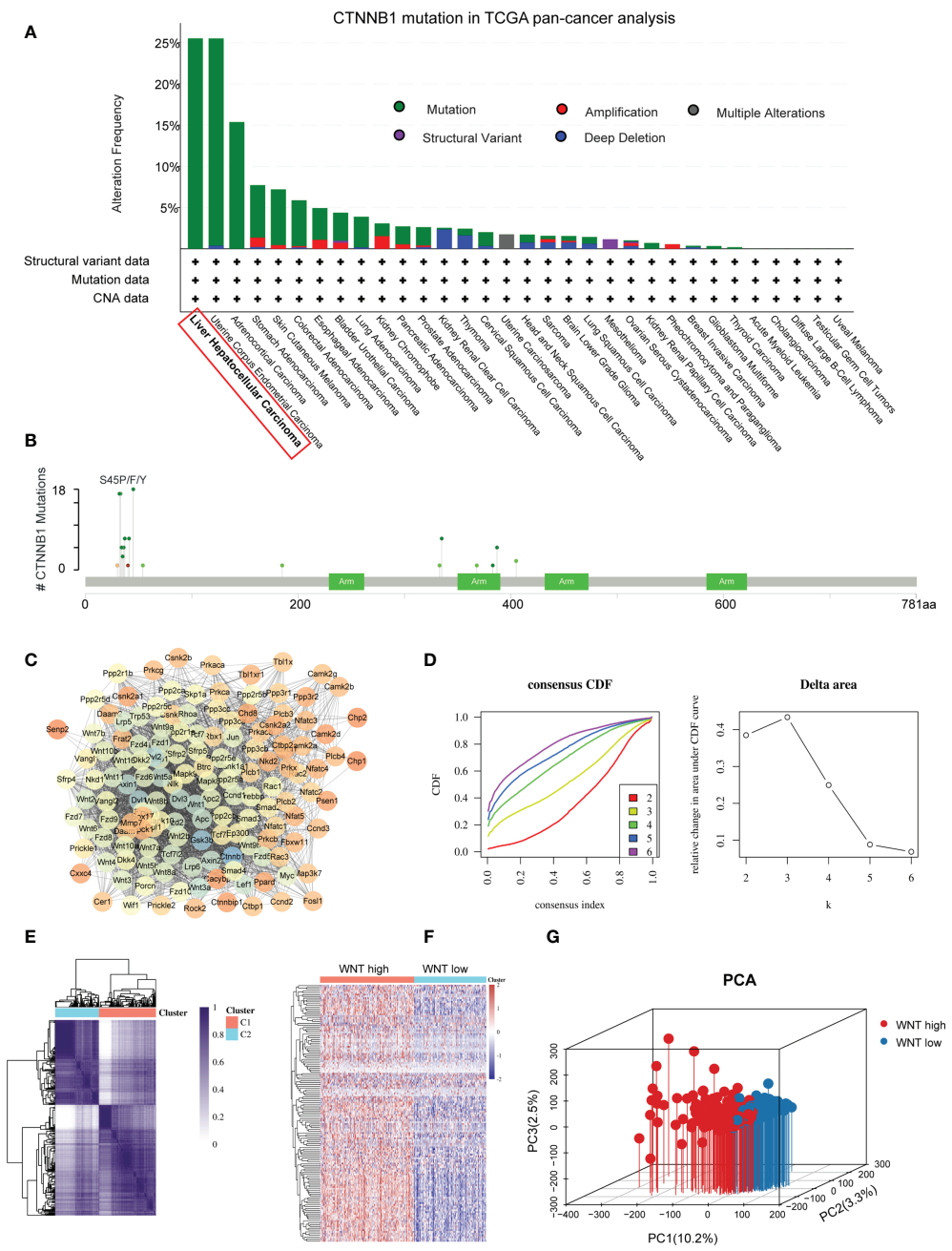

FIGURE 1
Identification of Wnt-based subtypes in HCC. **(A)** The bar plot presenting CTNNB1 mutation in TCGA pan-cancer dataset. **(B)** Amino acid mutation site of CTNNB1. **(C)** Protein–protein interactions of the Wnt signaling genes. **(D)** Delta area curve of consensus clustering. **(E)** Consensus clustering solution (k = 2) for Wnt signaling in HCC samples. **(F)** Heatmap of Wnt signaling gene expression in different clusters. **(G)** Principal component analysis plots.

therefore defined as the WNT-high subtype. In contrast, cluster C2 displayed the lowest expression levels and was hence referred to as the WNT-low subtype (Figure 1F). PCA was performed to compare transcription patterns across various subtypes. In general, the results of PCA revealed that the samples from the two groups were well differentiated from one another, which suggested that both subtypes had unique transcriptional profiles (Figure 1G).

We further validated the repeatability of WNT-based classification in independent sample cohorts (GSE14520). Similarly, patients in the GEO cohort were stratified into WNT-low and WNT-high subtypes (Supplementary Figure 1A).

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Patients stratified into different WNT subtypes presented variant prognosis and clinicopathologic features

Previous studies have shown that WNT signaling performs decisive functions in HCC tumor development. In accordance with these findings, survival studies have demonstrated that patients with different WNT-based subtypes have significantly different clinical outcomes. In general, the WNT-high subtype exhibited an unfavorable prognosis with the shortest OS and progression-free survival (PFS) (Figures 2A, B). In contrast to the WNT-high subtype, the WNT-low subtype was associated with the most satisfactory clinical outcomes. These findings were subsequently confirmed by analyses of the GEO cohort (Figures 2C, D). We further defined 3 or 4 subtypes by Consensus clustering but did not obtain the statistical significance in terms of the prognosis and failed to validate in external cohort GSE14520 (supplementary Figures 2A, B). Therefore, 2 subtypes could be ideal. We next compared the clinicopathological features of the subtypes. Patients stratified into WNT-high subtypes were associated with high grade, stage, and alpha-fetoprotein levels, which is in contrast to WNT-low subtypes (Figure 2E).

WNT-based subtypes present a distinct TME

The newly revealed significance of the Wnt/β-catenin pathway in modulating immune cell infiltration into the TME has sparked fresh interest in this topic. Within the scope of this study, we investigated the TME characteristics across various tumor subtypes. In general, there was no remarkable difference between the WNT-high and WNT-low subtypes in terms of either immune score or tumor purity (Supplementary Figure 3A). The CIBERSORT method was used to determine immune heterogeneity among these subtypes. Supplementary Figure 3B summarizes the landscape of 22 (infiltrating) immune cells. In particular, patients with the WNT-high subtype exhibited substantially elevated proportions of immunosuppressive cells (Tregs, neutrophils, and macrophages), but significantly lower proportions of $CD8^+$ T cells (Figure 3A). Similar to CIBERSORT results, ssGSEA validated a lower proportion of $CD8^+$ T cells in WNT high subtype, and TIMER and MCP-counter verified higher proportions of immunosuppressive cells (neutrophils and macrophages) in WNT high subtype (Figure 3B). WNT3 is a critical molecule involved in WNT signal transduction. We further explored the correlation between WNT3 expression and $CD8^+$ T cell infiltration. In TCGA database, WNT3 expression was negatively correlated with CD8 T cell score (Figure 3C). To further validate the association between WNT3 and $CD8^+$ T cells in HCC, we performed multiplex immunofluorescence analysis. In line with the results from database analysis, multiplexed immunofluorescence analysis showed that high WNT3 expression was associated with low $CD8^+$ T cell levels in the TME (Figure 3D). In addition, most immune checkpoints were elevated in the WNT-high subtype (Figure 4A). Conversely, the WNT-low subtype exhibited an opposite trend. These results illustrate that immunosuppressive cells may drive the immunosuppressive microenvironment of the WNT-high subtype.

The "cancer-immunity cycle" is a conceptualization of anti-tumor immunity that may be broken down into seven sequential processes, which include the following: release of tumor antigens (step 1), tumor antigen presentation (step 2), priming and activation (step 3), trafficking of T cells to tumors (step 4), infiltration of immune cells into tumors (step 5), recognition of tumor cells by T cells (step 6), and killing of tumor cells (step 7). We used TIP (a web-based platform that can resolve tumor immunophenotype profiling issues) to evaluate the anticancer immune activity of the seven-step cancer-immunity cycle among the three subtypes. Although the WNT-high subtype presented the highest activity in steps 1, 2, and 4, great attenuation of steps 5, 6, and 7 was observed (Figure 4B). These results indicate that mitigation of the immunosuppressive microenvironment in the WNT-high subtype may contribute to good clinical outcomes in HCC. In addition, the WNT-high subtype had the greatest number of upregulated genes implicated in the immunosuppressive modulation of immune processes, followed by the WNT-low subtype (Figure 4C).

We subsequently employed TIDE (a computational approach designed to derive the possibility of immune evasion by tumors based on the gene expression patterns of tumor tissues) to investigate the possibility of immunotherapy being effective in clinical settings for certain subtypes. As per the findings of this study, the WNT-high subtype exhibited a decreased response rate in contrast with the WNT-low subgroup, which suggests that patients with the WNT-high subtype are not candidates for immunotherapy (Figures 4D, E). Moreover, we analyzed the underlying pathways that correlated with the subtypes. GSEA revealed that the WNT-high subtype experienced substantial enrichment in the negative modulation of the immune pathway, including TGF-β signaling, hypoxia, glycolysis, and KRAS signaling (Figure 4F).

According to these findings, patients with the WNT-high subtype have a great possibility of developing an immunosuppressive microenvironment as a direct consequence of the up-modulation of immunosuppressive cytokines, the expression of immune checkpoints, and the infiltration of immunosuppressive cell populations, which may ultimately contribute to poor prognosis.

Establishment and verification of the WNT-related prognostic signature

We created a prognostic model based on WNT signaling genes. In univariate Cox analysis, 66 of the 150 WNT genes were

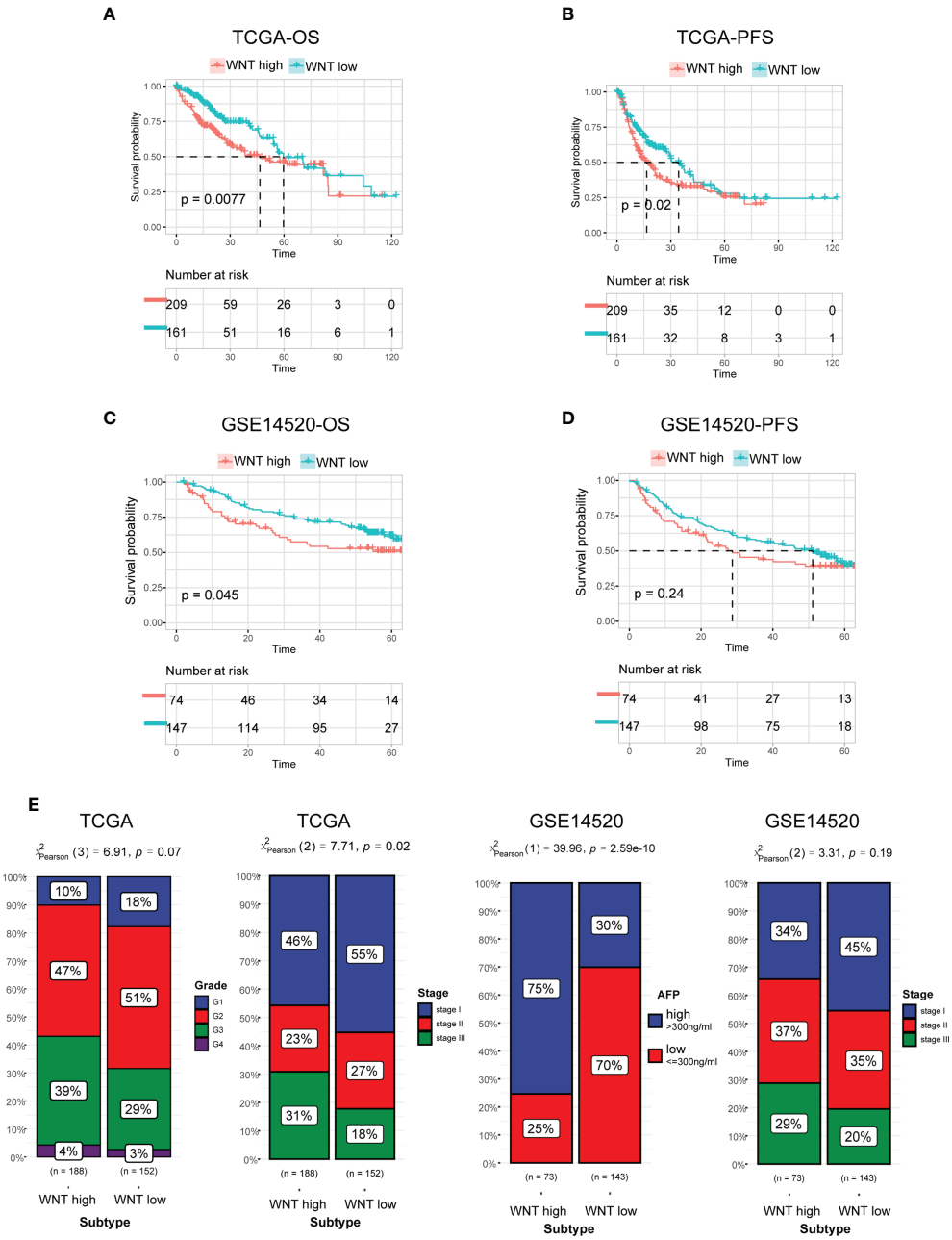

FIGURE 2
Prognosis and clinicopathologic characteristics between the Wnt subtypes. **(A, B)** Kaplan–Meier curves for patients with HCC classified into WNT-low and -high subtypes in TCGA in terms of OS **(A)** and PFS **(B)**. **(C, D)** Validation of Kaplan–Meier curves in the GEO dataset in terms of OS **(C)** and PFS **(D)**. **(E)** Bar plot presenting the clinicopathologic features of these subtypes.

strongly linked to OS. Figure 5A summarizes the top ten genes with the most significant p-values. Subsequently, 66 WNT genes identified by Cox univariate analysis were evaluated and chosen for the prediction model in the LASSO regression analysis. The following equation was used to develop the risk score model: risk score = (0.0116)*RUVBL1 + (0.00454)*CACYBP + (0.01230) *TBL1XR1 + (0.1157)*FZD3 + (0.0004)*RAC1 + (0.0001) *PPP2CA + (0.0113)*PPP2R5B + (0.0015)*AXIN1 + (0.0068) *TCF7L1 + (0.00059)*CUL1 + (0.00371)*FRAT2 + (0.00065) *DVL1 + (-0.0033)*PPP2R1B. Genes included in the final model also showed statistical significance in multivariate Cox analysis (Supplementary Figure 4). Furthermore, we evaluated the

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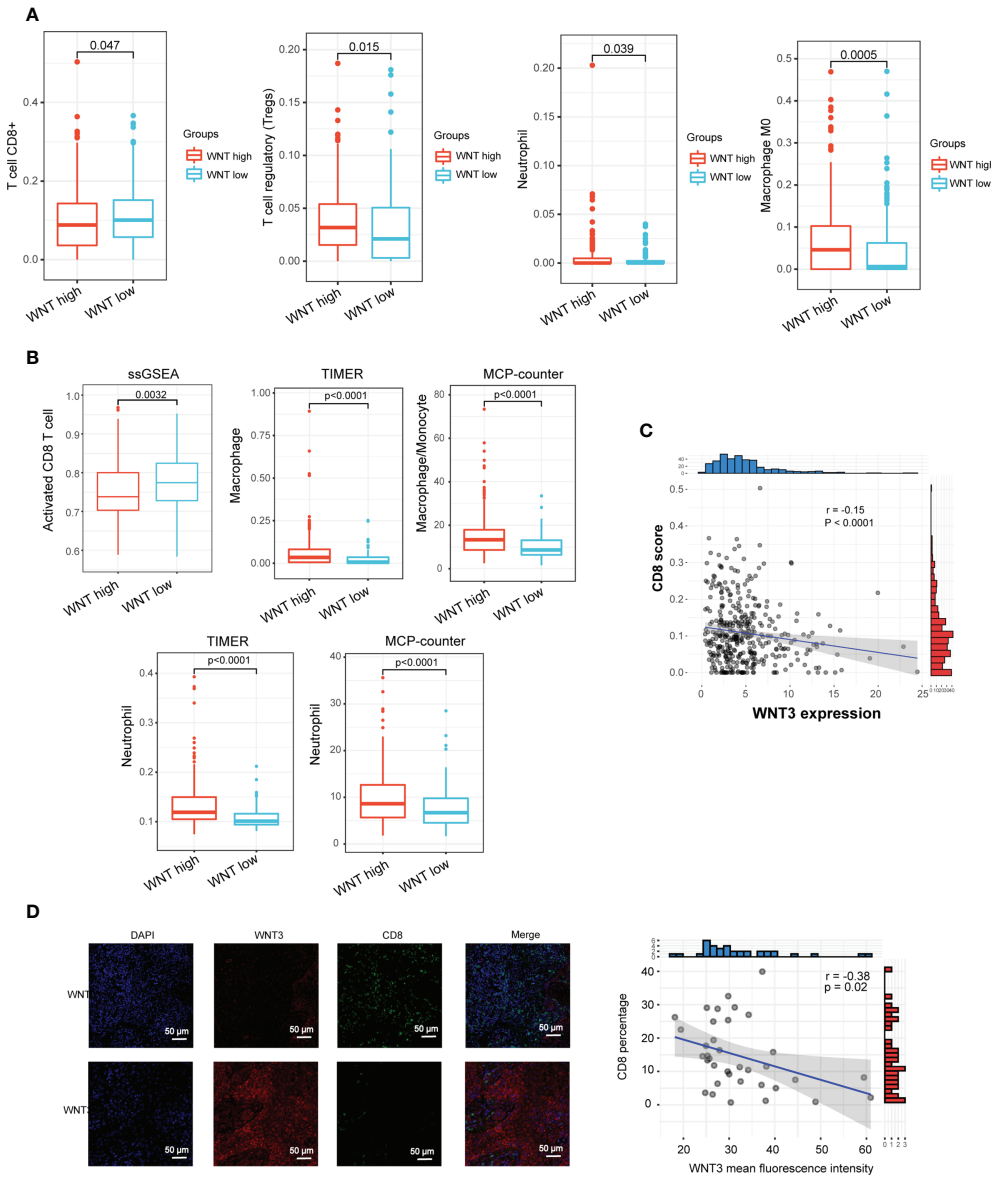

FIGURE 3
WNT-based subtypes are associated with the distinct tumor microenvironment. **(A)** Box plots presenting the infiltration score of CD8 T cells, Tregs, neutrophils, and macrophages. **(B)** Estimation of immune cell type fractions using different algorithms including ssGSEA, TIMER and MCP-counter. **(C)** Correlation between CD8 score and WNT3 expression. **(D)** Multiplex immunofluorescence validated the correlation between $CD8^+$ T cell infiltration and WNT3 expression.

association between the risk score and survival status. As per the findings of our study, the low-risk cohort had a substantially greater number of alive statuses than the high-risk cohort (Figure 5B). The prognostic value of this risk model was additionally evaluated using Kaplan–Meier analysis. Overall, the high-risk score was linked to unfavorable OS and PFS in TCGA training cohort (Figure 5C), which was subsequently verified in the GSE14520 and GSE76427 testing cohorts (Figure 5D).

WNT risk signature demonstrates the high predictive potential for prognostic evaluation

Univariate and multivariate Cox analyses were performed to determine the independent prognostic value of the Wnt signature with regard to OS. As illustrated by the findings of univariate analysis, a high WNT risk score was strongly associated with unfavorable OS (Figure 6A). Other factors associated with

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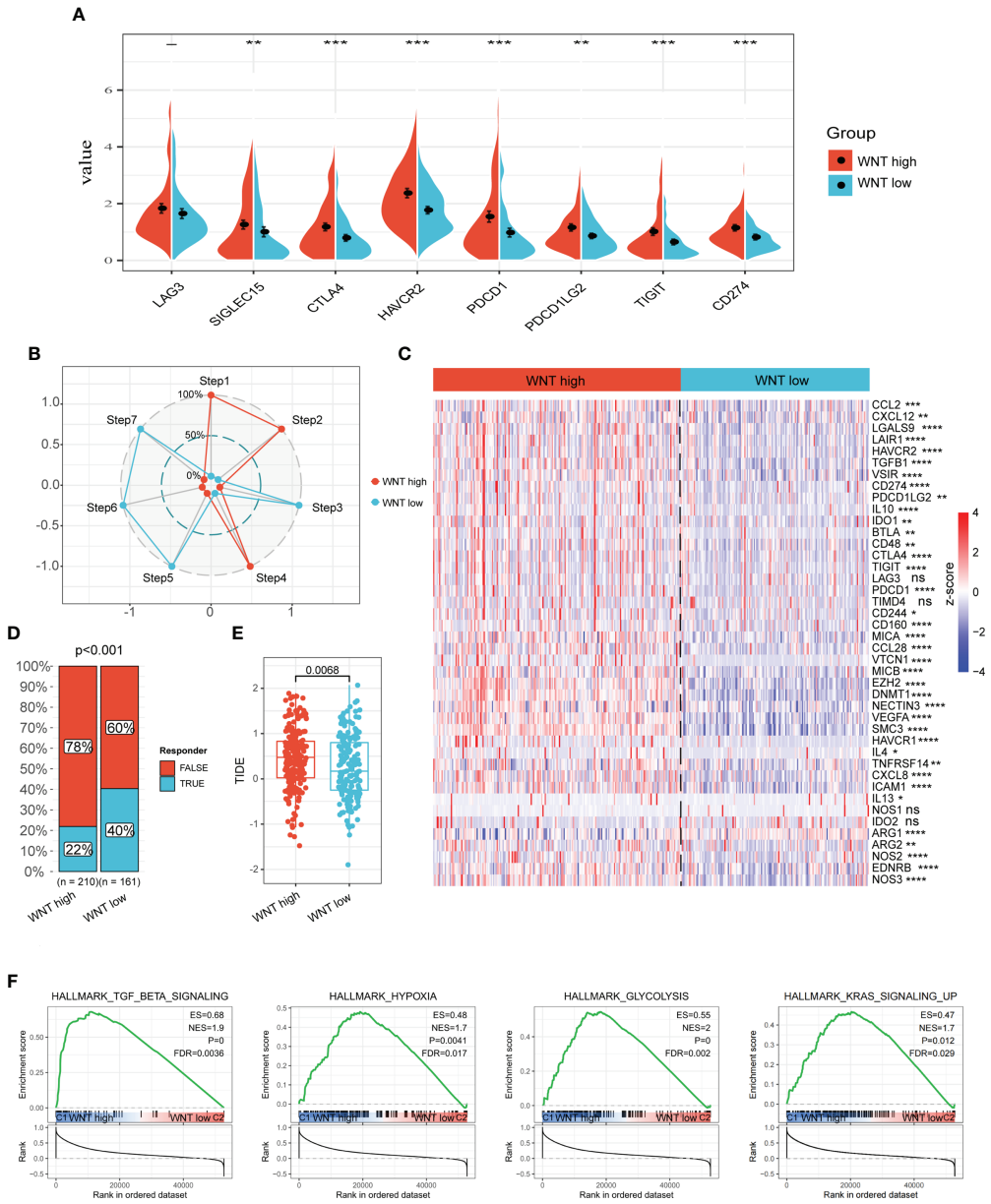

FIGURE 4
WNT-high subtypes are associated with the immune suppressive tumor microenvironment. **(A)** Violin plots of immune checkpoint expression. **(B)** Estimated score of the seven-step cancer-immunity cycle. **(C)** Heatmap of gene expression associated with the negative regulation of the immune processes. **(D)** Bar plot of ICB response rate. **(E)** Box plot of TIDE score. **(F)** GSEA plot of the underlying biological processes associated with WNT subtypes. (ns, p > 0.05; *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001).

unfavorable survival were the T stage and tumor stage. According to the findings of the multivariate study, a high WNT risk score was independently associated with a considerably more unfavorable OS (Figure 6B). This suggests that it may be an independent factor in determining the prognosis of patients with HCC. Subsequently, we performed a ROC curve analysis to examine the degree to which the WNT risk signature was able to accurately predict the survival rates (predictive efficiency) over 1, 3, and 5 years. The area under the ROC curve (AUC) showed strong predictive power, with values of 0.78, 0.7, and 0.66 over 1, 3, and 5 years, respectively (Figure 6C).

Discussion

In this study, our primary objective was to identify different subtypes of HCC based on Wnt signaling. Our results demonstrate

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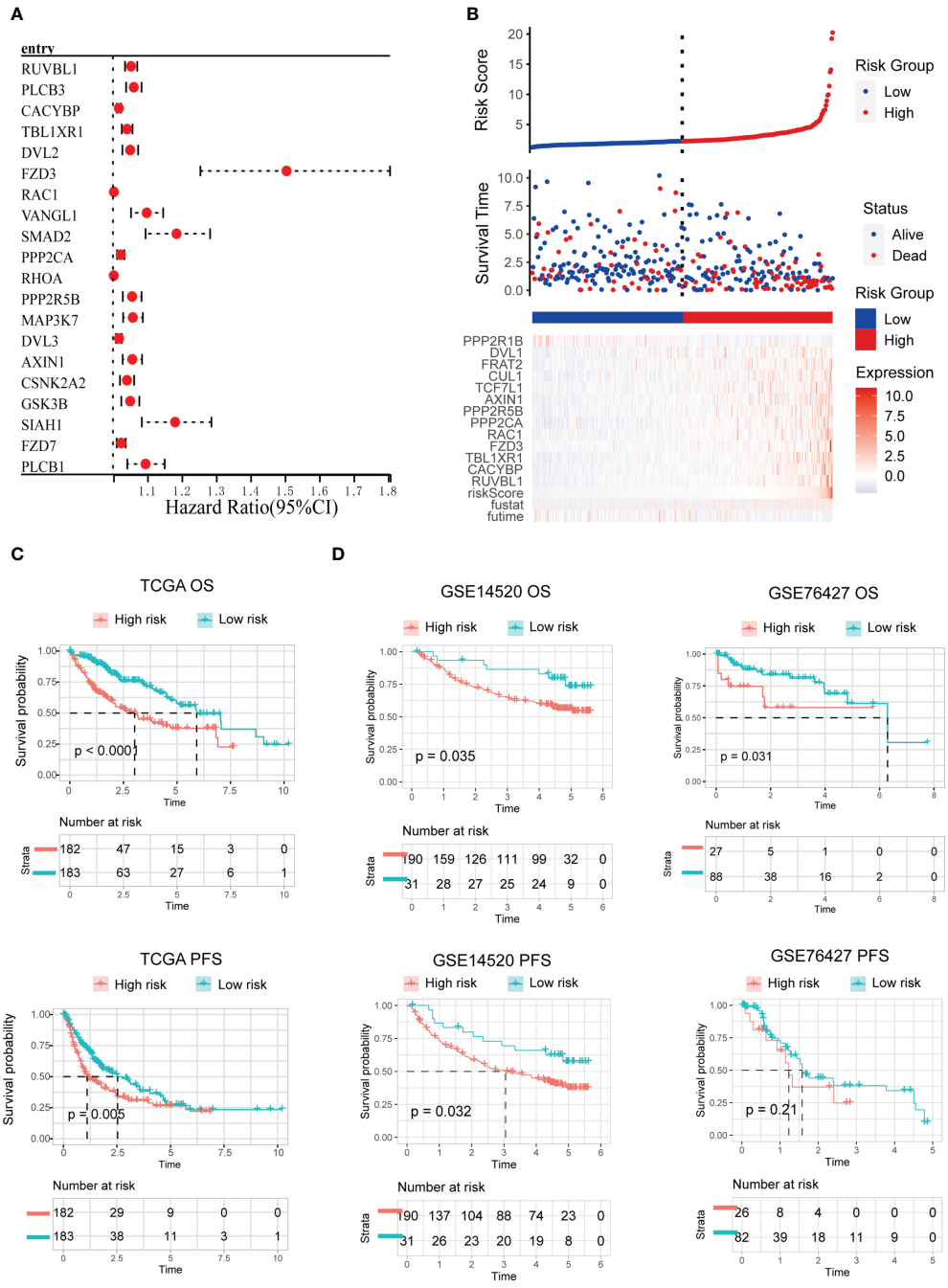

FIGURE 5
Development and validation of the WNT-related prognostic signature. **(A)** Univariate cox analysis of WNT-related genes associated with overall survival. The top ten genes with the most significant p-value are presented. **(B)** Risk scores distribution, survival status of each patient, and heatmaps of prognostic 13-gene risk signature. **(C, D)** Kaplan–Meier curves for patients with high- or low-risk scores in TCGA training cohort **(C)**, GEO testing cohort **(D)**.

that HCC might be classified into WNT-high and -low subtypes with distinct clinicopathological features, prognosis, and TME. We demonstrated that this classification was both predictable and also capable of being reproducible. Collectively, the WNT-high subtype presents a grim prognosis, with an immunosuppressive microenvironment and a high frequency of oncogene mutations. In contrast, the WNT-low subtypes were associated with the most favorable clinical outcomes with the immunoreactive

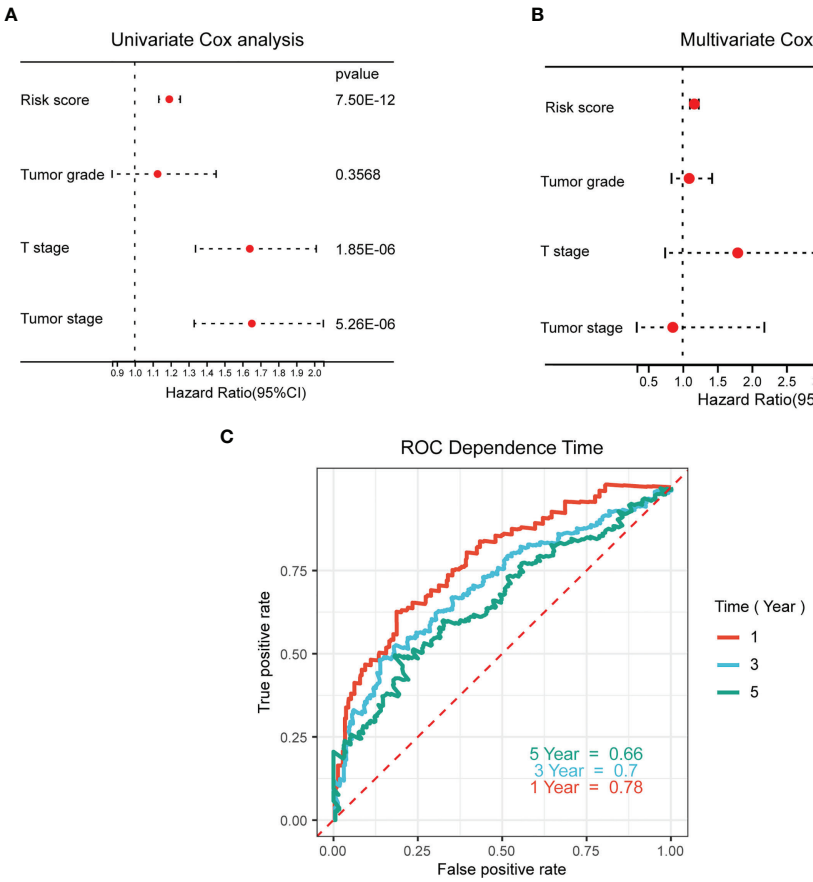

FIGURE 6
Prognostic value of the WNT-associated risk signatures in HCC samples. **(A, B)** Univariate **(A)** and multivariate **(B)** Cox analyses of the independent prognostic value of the WNT-related signature in patients with HCC. **(C)** ROC curves of the predictive efficiency of the WNT risk signature on the 1-, 3-, and 5-year survival rate.

microenvironment among these subtypes. Moreover, we developed and validated a WNT-related prognostic model that presents strong power for prognosis assessment.

Clinical progress has been made in the prediction of patient prognoses and the selection of cancer treatment using molecular classifications in conjunction with gene expression patterns, and the exact classification of oncogenesis has been made possible by recent advances in DNA sequencing and methylation array technology (11–12). In HCC, the discovery of numerous significant molecular markers, the most remarkable of which are *TP53* mutations, has enabled the development of a precise technique for classifying HCC with significant prognostic value (13–14). In addition to the *TP53* mutation, more recent research has uncovered a second significant mutation in these tumors called CTNNB1, which also affects the clinical prognosis of patients. In HCC, the Wnt/β-catenin pathway is often upregulated and linked to the maintenance of tumor-initiating cells, as well as medication resistance, tumor growth, and metastasis (6). In this study, we established WNT-based subtypes that categorized patients with HCC into WNT-low and WNT-high subtypes with distinct clinicopathological features, prognosis, and TME.

Wnt/β-catenin signaling, a highly evolutionarily conserved pathway, functions in multiple cellular processes, including proliferation, differentiation, migration, genetic stability, apoptosis, and stem cell renewal. The recently reported functions of the Wnt/β-catenin pathway in modulating immune cell infiltrates in the TME and immunotherapy have piqued attention (15). Tumor-intrinsic β-catenin signaling suppresses the mobilization of $CD103^+$ DCs in melanoma, preventing antitumor immune function. Mechanistically, active β-catenin signaling causes the transcriptional inhibitor ATF3 to be expressed, which inhibits CCL4 expression (8). Immune evasion, as well as tolerance of anti-PD-1 treatment, is promoted by β-catenin stimulation in hepatocellular carcinoma (8). In addition, high TMB NSCLC tumors activated WNT/β-catenin signaling, which modulated chemokine ligand expression and subsequent immune cell infiltration. Blocking Wnt/β-catenin signaling rescued the effects of anti–PD-1 in high TMB tumors, leading to tumor

clearance. These pieces of evidence highlight the significant influence of this pathway on immunotherapeutic treatment outcomes (16). In line with the evidence, the results of our research indicate that the WNT-high subtype is linked to a lower level of T cell gene expression and lower immunotherapy response. Our evaluations included descriptions of changes in the molecular pathways and gene expression associated with the immune response in these subtypes. Nevertheless, it should be noted that our findings require further validation *in vitro* or *in vivo*. Our findings should be interpreted with this limitation in mind.

The Wnt/β-catenin pathway also play an important role in tumor microenvironment remodel. Interactions between cancer cells and the tumor-associated macrophages (TAMs) have been demonstrated to be mediated by the Wnt/β-catenin signaling pathway. A previous study showed that interleukin-1β, released by TAMs, might enhance the presence of β-catenin through GSK3β phosphorylation in colon cancer cells, thereby preventing the β-catenin destruction complex from performing its normal functions (17). Snail, a soluble component of Wnt target genes, is responsible for stimulating IL-β secretion in macrophages by colorectal cancer cells (18). Moreover, Wnt/β-catenin signaling promotes Treg survival. Similarly, in our study, we identified Treg and macrophage scores enriched in the WNT-high subtype.

In summary, our research sheds light on the links between WNT-based subtypes and prognosis, as well as alterations in the immune TME in patients with HCC. These findings could be useful for developing immune therapy-based treatments for HCC patients in the future. We also developed and verified a WNT-associated prognostic signature that exhibited remarkable value in the prediction of OS in patients with HCC.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by institutional review board of Shanghai Outdo Biotechnology. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

Conceptualization and methodology: WX, CN; writing: WX; statistic calculation and validation: WX, YH, and HL; review and approval of concept/methodology: BC and JW; editing: SW, and JZ; project administration and funding acquisition: XC. All authors contributed to the article and approved the submitted version.

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Our results were generated using some of the basic data from TCGA Research Network: https://www.cancer.gov/tcga.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1010554/full#supplementary-material

SUPPLEMENTARY FIGURE 1
(A) Validation of WNT-based subtypes in GEO dataset.

SUPPLEMENTARY FIGURE 2
(A, B) Kaplan–Meier curves for patients with HCC classified into three **(A)** or four **(B)** subtypes in TCGA in terms of OS and PFS.

SUPPLEMENTARY FIGURE 3
(A) Violin plots of immune score and tumor purity score. **(B)** The relative proportion of immune infiltration in HCC samples.

SUPPLEMENTARY FIGURE 4
(A) multivariate Cox analysis of genes included in the final model.

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H-TEX-mediated signaling between hepatocellular carcinoma cells and macrophages and exosome-targeted therapy for hepatocellular carcinoma

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There is increasing evidence for the key role of the immune microenvironment in the occurrence and development of hepatocellular carcinoma. As an important component of the immune microenvironment, the polarization state and function of macrophages determine the maintenance of the immunosuppressive tumor microenvironment. Hepatocellular carcinoma tumor-derived exosomes, as information carriers, regulate the physiological state of cells in the microenvironment and control cancer progression. In this review, we focus on the role of the exosome content in disease outcomes at different stages in the progression of hepatitis B virus/hepatitis C virus-induced hepatocellular carcinoma. We also explore the mechanism by which macrophages contribute to the formation of hepatocellular carcinoma and summarize the regulation of macrophage functions by the heterogeneity of exosome loading in liver cancer. Finally, with the rise of exosome modification in immunotherapy research on hepatocellular carcinoma, we summarize the application prospects of exosome-based targeted drug delivery.

KEYWORDS

exosomes, hepatocellular carcinoma, liver cancer, hypoxia, TAM, macrophage, therapy, drug resistance

Introduction

Liver cancer has a 5-year relative survival rate of only 20% (1). Liver cancer caused by chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) accounts for approximately 50%–80% of cases (2). Other risk factors include aflatoxin exposure, tobacco and alcohol use, non-alcoholic fatty liver disease, obesity, and diabetes. The distribution of these risk factors varies according to the population, time, and region (1, 3). Therefore, as liver cancer is a chronic inflammation-related cancer, it is crucial to study the role of exosomes during diseaseprogression and in the immune microenvironment.

Macrophages are abundant in the liver and are essential cells in the tumor microenvironment (TME) in liver cancer. During the initial stages of liver cancer at the time of niche formation, hepatic macrophages display an inflammatory phenotype, namely, the M1 type; these cells damage neighboring cells by continuous secretion of reactive oxygen species. In solid tumors that successfully escape immune surveillance, macrophages disproportionately differentiate into the M2 phenotype with anti-inflammatory activity, that is, tumor-associated macrophages (TAMs), which have proangiogenic, matrix remodeling, distal metastasis, and immunosuppressive effects (4). The recruitment of hepatic macrophages in human liver cancer is correlated with disease progression and a poor prognosis (5). Hepatoma cells also play crucial regulatory roles in macrophage proliferation and differentiation during tumor progression (6).

Hypoxia has become one of the most intensively studied features of the TME (7). In this context, in addition to directly secreting cytokines (8), exosome-mediated communication between tumor cells and the stroma is considered an important step in remodeling the TME (9). Multiple studies have demonstrated the adaptive tuning of extracellular vesicle (EV) secretion and contents of liver cancer cells during progression, providing a basis for subsequent remodeling of the surrounding niche (10, 11) and, specifically, for altering TAMs.

In this review, we summarize the roles of exosomes and macrophages during the progression of viral hepatitis to liver cancer, including but not limited to the effects of exosomal contents on macrophages and exosome-based therapeutic prospects for liver cancer.

Involvement of exosomes in hepatocarcinogenesis

Analyses of liver cancer progression associated with chronic inflammation are still needed. Hepatocyte exosomes function as messengers in the formation and evolution of the liver cancer niche (12). Exosomes are released into the intercellular space or enter the hepatic microvasculature to participate in intercellular signal communication or material transport, thereby regulating the pathophysiological state of the liver (13).

In the physiological state, liver parenchymal cells, which make up approximately 80% of the liver volume, secrete exosomes loaded with neutral ceramidase and sphingosine kinase 2 (SK2), which are recognized by recipient hepatocytes and upregulate sphingosine-1-phosphate (S1P) production by target cells, thereby promoting hepatocyte repair and regeneration (14).

In some pathological conditions, liver parenchymal cells, hepatic stellate cells (HSCs), and Kupffer cells (KCs) are the main donor and recipient cells of exosomes and are associated with hepatitis, cirrhosis, and liver cancer (15). The hepatocyte exosomal cargo plays diverse roles in the microenvironment during liver cancer progression (Figure 1).

Viral Hepatitis

Hepatitis caused by HBV/HCV infection is one of the main causes of hepatocellular carcinoma (HCC) (2). Exosomes secreted from hepatocytes infected with HCV carry virus-derived Ago2, heat shock protein 90 (Hsp90), and miR-122, which mediate the stable transmission of HCV in the liver (16–18). Exosome-mediated viral transport helps the virus evade immune system surveillance. MicroRNAs (miRNAs) released from virus-infected hepatocytes inhibit natural killer (NK) cell proliferation and survival and facilitate the evasion of host innate immunity (19). Exosomes containing HCV RNA reduce Toll-like receptor 3 (TLR3) activation and interfere with antiviral interferon-stimulated gene activation (20). T-cell immunoglobulin and mucin domain-containing molecule 3 (TIM-3)/galectin 9 (Gal-9) in exosomes increase HCV-infected hepatocytes, affect monocyte differentiation, and inhibit the immune response (21).

Cirrhosis

During liver cirrhosis, HSCs along with other cells of the liver parenchyma (liver sinusoidal endothelial cells and KCs) play important roles in the development and progression of liver fibrosis (22). Exosomes released by injured hepatocytes are internalized by stellate cells, leading to phenotypic switching of quiescent stellate cells. HSC activation is a major driver of the initiation, progression, and resolution of liver fibrosis (23).

Exosomes released from injured hepatic stellate structures contain abundant fibrotic components that promote fibrosis *via* multiple pathways, such as by stimulating fibroblasts and myofibroblasts to produce collagen from the bone marrow and portal fibrocytes. Connective tissue growth factor (CTGF), a multifunctional heparin-binding glycoprotein, contributes to the promotion of multiple fibrotic processes (24). CTGF, which is widely expressed in activated HSC-derived exosomes, regulates the activation and migration of HSCs and immune responses, whereas exosomes produced by quiescent HSCs are enriched in

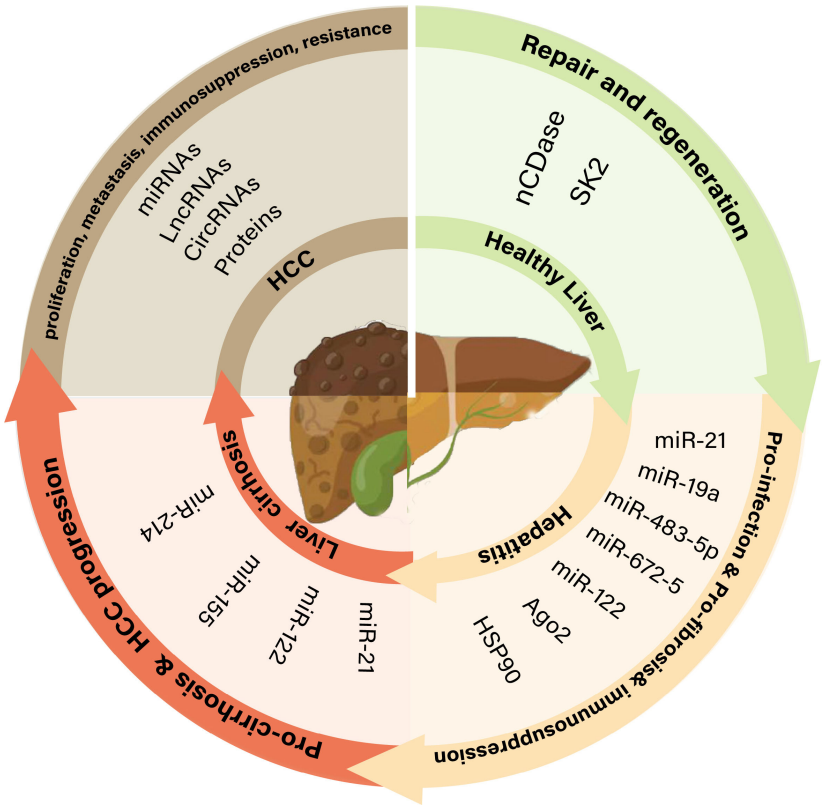

FIGURE 1
The role of exosomes in different pathophysiological states of the liver. nCDase, neutral ceramidase; SK2, sphingosine kinase 2; Ago2, argonaute-2; Hsp90, heat shock protein 90; lncRNAs, long non-coding RNAs; miRNAs, MicroRNAs; circRNAs, Circular RNAs.

miR-214 and twist, attenuating the profibrotic function of activated HSCs (25, 26). Exosomes derived from liver sinusoidal endothelial cells regulate the migratory capacity of HSCs *via* adhesion.

Hepatocellular Carcinoma

HCC is a common malignancy with poor overall survival. The main risk factors for HCC include viral hepatitis, excessive alcohol consumption, and smoking. However, the pathogenesis of HCC is complicated and difficult to determine. Extensive evidence suggests that exosomes derived from cells carry tumor-specific markers, which mediate intercellular communication between cancer cell populations and promote the migration and invasion of recipient cells (27). For non-immune cells, HCC exosomes regulate tumor niche formation by promoting tumor-associated fibroblast transformation and angiogenesis by altering the endothelial vascular phenotype (28, 29). Liver cancer exosomes mainly mediate tumor cell immune escape by inhibiting their maintenance and proliferation, promoting phenotypic transformation, and blocking functional activation (30).

These effects promoting HCC progression depend on proteins and non-coding RNAs (ncRNAs) in exosomes. They are transferred by exosomes and participate in the communication between HCC cells and targeted cells in the TME, thereby affecting tumor angiogenesis, metastasis, and drug and radiotherapy resistance. Therefore, we summarized the current research status of proteins and ncRNAs in HCC exosomes to further emphasize the potential value of these abnormally expressed exosome molecules in HCC as biomarkers for the diagnosis, prognosis, and treatment of HCC (Table 1).

Hypoxia promotes the production and release of exosomes

HCC is a hypermetabolic tumor of the digestive system. Based on the high rate of cell proliferation, the altered blood supply system participates in the exchange of substances within the tumor (66). Therefore, hypoxic signals contribute to liver cancer formation, proliferation, and metastasis (67). In addition to the adaptive changes in cellular components within the TME in response to hypoxia, hepatoma cells transmit post-hypoxic regulatory signals to other cells by secreting EVs (68). Cancer cells with different

TABLE 1 Effects of exosome contents on hepatocellular carcinoma.

Contents	Mechanism	Function	References
Proteins			
LOXL4	Activation of FAK/SRC pathway alters cell matrix adhesion and migration ability	Promotes migration and angiogenesis	(31)
GOLM1	Activated glycogen synthase kinase-3 β / MMPs (GSK-3 β/ MMPs) of the recipient cells signaling axis	Accelerates cell proliferation and migration	(32)
S100A4	Activation of OPN transcription by STAT3 phosphorylation	Promotes tumor metastasis	(33)
HMGB1	Activation of the TLR-MAPK pathway	Promotes TIM-1(+) B-cell proliferation and inhibits CD8(+) T-cell activity	(34)
SMAD3	Enhanced TGF-β-Smad3-ROS signaling	Promotes proliferation and adhesion	(35)
ENO1	Upregulation of integrin αs6β4 expression	Activates the FAK/Src-p38MAPK pathway to promote the growth and metastasis of HCC cells	(36)
CLEC3B	Promotes the phosphorylation of AMPK, thereby decreasing the expression of VEGF	Attenuates migration and invasion of recipient cells and relieves angiogenesis	(37)
CHI3L1	Activation of MAPK and Akt signaling pathways	Promotes tumor metastasis	(38)
EIF3C	Activation of S100A11 expression	Promotes angiogenesis and tumor development	(39)
miRNAs			
miR150	Promotes vascular endothelial growth factor (VEGF) secretion in TAMs	Promotes tumorigenesis	(40)
miR-23a-3p	Upregulation of PD-L1 expression in macrophages *via* STAT3 signaling pathway	Attenuates the anti-HCC immune response	(41)
miR-32-5p	Inhibits PTEN and activates the PI3K/Akt pathway	Induction of multidrug resistance by angiogenesis and EMT	(42)
miR-1247-3p	Downregulation of B4GALT3 and activation β 1-integrin/ NF-κB axis	Promotes tumor status, EMT, chemoresistance, tumorigenicity, and metastasis	(43)
miR-638	By downregulating the expression of VE-cadherin and ZO-1 in endothelial cells	Promotes vascular permeability	(44)
miR-27a-3p	By regulating thioredoxin-interacting protein (TXNIP)	Promotes the stemness of liver cancer	(45)
miR-125b	Disrupted TGF-β1-induced epithelial–mesenchymal transition and TGF-β1/SMAD signaling pathway	Antimetastatic effect	(46)
miR-15a-5p	Inhibits PD1 expression in CD8+ T cells	Inhibits the development of HCC	(47)
miR-210	Entry into endothelial cells inhibits SMAD4 and STAT6	Promotes tumor angiogenesis	(48)
miR-93	Inhibits CDKN1A, TP53INP1, and TIMP2	Promotes proliferation and invasion	(49)
miR-374a-5p	Possibly by regulating GADD45A	Promotes proliferation, migration, and invasion of HCC cells	(50)
miR-92a-3p	By inhibiting PTEN and activating the Akt/Snail signaling pathway	Promotes EMT	(51)
miR-320a	Inhibit PBX3/ERK1/2/CDK2 axis	Inhibits proliferation and metastatic ability	(52)
miR-21	Inhibit PTEN, upregulate PDK1/AKT pathway	Transforms normal hematopoietic stem cells into cancer-associated fibroblasts	(53)
miR-451a	Targeting LPIN1 regulates tumor cell apoptosis and angiogenesis	Inhibits hepatocellular tumorigenesis	(54)
lncRNAs			
TUC339	May be involved in cytokine receptor signaling pathway and CXCR chemokine receptor-binding pathway	Promotes macrophage polarization to M2 (IL-4) phenotype	(55)
lncRNA H19	By upregulating the miR-520a-3p/LIMK1 axis	Promotes the proliferation, migration, and invasion of HCC cells after propofol treatment and inhibits the apoptosis of HCC cells	(56)
SENP3-EIF4A1	Regulation of ZFP36 expression by competitive binding to miR-9-5p	Able to inhibit tumor growth *in vivo*	(57)
FAL1	Upregulation of ZEB1 and AFP by inhibiting miR-1236	Promotes proliferation and migration	(58)
ASMTL-AS1	by activating the YAP signaling pathway	Accelerates tumor progression	(59)
circRNAs			

(Continued)

TABLE 1 Continued

Contents	Mechanism	Function	References
circ-DB	Enhances the expression of USP7 and Cyclin A2 by inhibiting the expression of miR-34a	Promotes tumor growth, inhibits DNA damage	(60)
circ-PTGR1	Activation of MET by interaction with miR-449a	Promotes migration, invasion, and metastasis	(61)
circ-UHRF1	Suppresses NK cell function by degrading miR-449c-5p and upregulating TIM-3 expression	Promotes immunosuppression	(62)
circ-0051443	By upregulating BAK1	Promotes apoptosis and inhibits cell cycle	(63)
circ-CMTM3	Promotes angiogenesis by regulating the miR-3619-5p/SOX9 axis	Promotes HCC tumorigenesis	(64)
circ-TMEM45A	By upregulating the miR-665/IGF2 axis	Promotes the progression of HCC	(65)

LOXL4, Lysyl Oxidase Like 4; GOLM1, Golgi membrane protein 1; A4S100A4, S100 Calcium Binding Protein; OPN, Osteopontin; HMGB1, High mobility group box 1; TLR, toll-liked receptor; MAPK, mitogen-activated protein kinase; Smad3, mothers against decapentaplegic family member3; TGF-β, Transforming growth factor beta ; ENO1, Enolase 1 ; FAK, focal adhesion kinase ; CLEC3B, C-Type Lectin Domain Family 3 Member B ; AMPK, AMP-activated protein kinase ; VEGF, Vascular endothelial growth factor; CHI3L1, Chitinase-3-like protein 1; EIF3C, Eukaryotic Translation Initiation Factor 3 Subunit C; S100A11, S100 Calcium Binding Protein A11; PD-L1, Programmed death-ligand 1; PTEN, Phosphatase and tensin homolog ; B4GALT3, Beta-1,4-Galactosyltransferase 3; ZO-1, zonula occluden-1; Smad4, mothers against decapentaplegic family member4; STAT6, Signal transducer and activator of transcription 6; CDKN1A, Cyclin Dependent Kinase Inhibitor 1A ; TIMP2, Tissue inhibitor of metalloproteinases 2; EMT, Epithelial–mesenchymal transition; GADD45A, Growth Arrest and DNA Damage Inducible Alpha; PBX3, pre-leukemia transcription factor 3; ERK, extracellular signal-regulated protein kinase ; CDK2, Cyclin Dependent Kinase 2; PDK1, Pyruvate Dehydrogenase Kinase 1; LPIN1, phosphatidic acid phosphohydrolase1; CXCR, C-X-C Motif Chemokine Receptor; LIMK1, LIM Domain Kinase 1; ZFP36, zinc finger protein 36 homolog ; ZEB1, Zinc Finger E-Box Binding Homeobox 1; AFP, Alpha-Fetoprotein ; YAP, Yes-associated protein ; USP7, Ubiquitin Specific Peptidase 7; MET, mesenchymal-epithelial transition; BAK1, BCL2 Antagonist/Killer 1; SOX9, SRY-Box Transcription Factor 9; IGF2, Insulin Like Growth Factor 2.

phenotypes communicate *via* exosomes to complete the phenotypic transformation and promote the progression of liver cancer (69). For example, exosomes from highly metastatic MHCC97H cells can communicate with less metastatic HCC cells, increasing their migration, chemotaxis, and invasion (70). Similarly, the EVs of cisplatin-resistant non-small-cell lung cancer cell lines secreted pyruvate kinase M2 (PKM2) under hypoxic conditions. The phagocytosis of these EVs by cisplatin-sensitive non-small-cell lung cancer cell lines induced decreased sensitivity to cisplatin (71).

The adaptive response of tumor cells to hypoxia is mostly regulated by hypoxia-inducible factor 1 (HIF1). HIF1α/2α is also highly expressed in liver cancer (72). Under normoxia, the two proline residues of the HIF-1/2α subunit are hydroxylated by prolyl hydroxylase domain (PHD) enzymes, promoting binding to von Hippel–Lindau (VHL), which mediates the degradation of the hydroxylated HIF-1/2α subunit *via* the ubiquitin-proteasome pathway. However, under hypoxic conditions, the generation and release of EVs are regulated by HIF. During EV biogenesis, RAS superfamily proteins (RABs) are involved in the formation and fusion of membrane buds, and HIF can directly affect the RAS. That is, under hypoxic conditions, HIF is activated to promote the transcription of RABs and finally promote the generation and secretion of exosomes (73, 74). HIF can promote the expression and activation of a series of cell surface receptors, such as epidermal growth factor receptor, glucose transporter receptor, and transferrin receptor, and promote cell internalization and endocytosis (75).

The mechanism by which EV contents (nucleic acids, proteins, etc.) are specifically sorted under hypoxia remains unclear. This process is related to endosomal sorting complex required for transport (ESCRT) complexes and ceramides and may be related to posttranslational processes (76). This modified protein complex is closely related to ubiquitin-like 3 (UBL3)/membrane-anchored Ub-fold protein (MUB). In models of lung injury, proteins and peptides in vesicles were more ubiquitinated under hypoxia (77). This indicates that ubiquitination regulates the loading process of exosome contents under hypoxia.

In addition, HIF1-independent regulation of adaptive responses to hypoxia has been reported, such as phosphoinositide 3-kinase (PI3K), serine-threonine kinase (AKT), mammalian target of rapamycin (mTOR), Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) Rab-GTPase, Wnt/β-catenin, mitogen-activated protein kinases, and oxidative stress (78).

Hepatocellular Carcinoma (HCC)-associated macrophages

Origin and function of macrophages

Under physiological conditions, the liver has a rich blood supply and abundant innate immune cells (such as KCs, NK cells, and T cells). Resident macrophages in the liver are mainly composed of KCs and monocyte-derived macrophages (79). In healthy liver, KCs are the main resident hepatic macrophages. KCs are generally believed to have originated from yolk sac-derived colony-stimulating factor 1 receptor (CSF1R) + erythroid/myeloid progenitors (EMPs), which are present in the fetal liver during embryogenesis. They can maintain liver homeostasis by removing metabolic waste and cell debris, regulating cholesterol homeostasis, maintaining iron homeostasis and iron cycling, mediating immune responses, and promoting immune tolerance (80). Some

circulation-derived monocyte-macrophage populations recognize liver-invading bacteria and recruit neutrophils. In the human liver, hepatic macrophages consist of CD68+macrophage receptor with collagenous structure (MARCO)+KCs, CD68+MARCO-macrophages, and CD14+monocytes. CD68+MARCO+-KCs usually overexpress immune tolerance-related genes and have anti-inflammatory effects, and CD68+MARCO-macrophages and CD14+monocytes have pro-inflammatory effects (5).

From the progression of chronic hepatitis to fully developed tumors, there is a high degree of heterogeneity in the intratumoral microenvironment, with a highly invasive anterior and middle hypoxic and necrotic areas and tumor cells with high and low proliferation (81). There are also different TAM phenotypes in liver cancer, and research on the classification of TAMs and their heterogeneity is still in its infancy (82). In short, TAMs are collections of macrophages, including infiltrating and resident macrophages, originating from various cellular sources. TAM polarization shows plasticity, and cells can exhibit either phenotype. Several studies have provided evidence that the acquisition of an M2-like polarized macrophage phenotype by TAMs promotes tumor progression by promoting angiogenesis, immunosuppression, and growth factor secretion, ultimately leading to metastasis (83).

TAMs secrete excessive proangiogenic factors [e.g., vascular endothelial growth factor (VEGF), platelet-derived growth factor, and transforming growth factor beta (TGFB)] and cell proliferation-stimulating factors (e.g., Interleukin(IL)-1β, IL-6, chemokine (C-C motif) ligand 2 (CCL2), tumor necrosis factor, and VEGF), which strongly promote tumor growth and development (84, 85).

Mechanisms underlying macrophage uptake of exosomes

When exosomal vesicles come in contact with the surface of macrophages, they trigger a functional response (e.g., proliferation and differentiation) *via* membrane surface ligand–receptor recognition signals and/or transport of their contents into the cell, antigen presentation, etc. (86).

Macrophages are initially recognized by protein receptors and adhesion molecules (e.g., tetraspanins, integrins, proteoglycans, and lectins) on the exosome surface. Exosomes are then taken up by activating cell membrane-expressed receptors, fusion with the macrophage plasma membrane, or endocytosis (87). The final contents are delivered to macrophages to exert biological functions. An increasing number of studies have evaluated the mechanism underlying the uptake. For example, exosomes derived from pancreatic cancer preferentially bind to F4/80+ and CD11b+ KCs in the liver, which is promoted by intercellular adhesion molecules and CD11b ligands (88). A study of the liver metastasis of pancreatic cancer cells suggested that endoplasmic reticulum aminopeptidase 1 (ERAP1)-secreting exosomes enhance the phagocytic capacity and NO synthesis activity of macrophages (89). However, some circulating exosomes can protect against phagocytosis by macrophage CD47 enrichment.

The effect of exosomes from non-metastatic K7 and Dunn osteosarcoma cells and the metastatic sublines K7M3 and DLM8 on macrophage phagocytosis was evaluated in a study of osteosarcoma lung metastasis (85). Exosomes secreted by the highly metastatic K7M3 and DLM8 cell lines were incubated with MHS mouse alveolar macrophages, which induced the mRNA expression of *IL-10*, *TGFB2*, and *CCL22* (markers of M2 macrophages). Reduced macrophage phagocytosis, exocytosis, and macrophage-mediated tumor cell killing were also observed. By contrast, exosomes from non-metastatic K7 or Dunn cells failed to inhibit macrophage phagocytosis, exocytosis, and cytotoxicity and did not induce increases in the mRNA expression of *IL10*, *TGFB2*, or *CCL22*.

The uptake of exosomes by macrophages is inseparable from clathrin-dependent endocytosis in which caveolin-1 is essential for the formation of pits (membrane depressions) and accumulates in membrane depressions (90). Clathrin protein heavy chain 1 (Cltc) is encoded by the *cltc* gene and is highly expressed in macrophages (91). When Cltc1 is knocked out, phagocytosis by monocyte-macrophages is inhibited.

HCC Tumor-Derived exosomes are involved in the regulation of the polarization and function of macrophages

Liver macrophages can be activated to M1 and IL-13 *via* the classical activation pathway (bacterial lipopolysaccharide and interferon-gamma secreted by Th1 cells) and alternative activation pathways (cytokines IL-4, IL-10, and IL-13 secreted by Th2 cells). There are two subtypes of M2 macrophages, and these can be further subdivided into M2a, M2b, M2c, and M2d. M1-type macrophages mainly secrete pro-inflammatory factors, such as IL-12, IL-6, IL-18, IL-23, and tumor necrosis factor, and increase the expression of nitric oxide synthase, which is responsible for the defense against pathogen infection. The M2 type expresses high levels of IL-10, IL-1a/b inhibitor, mannose receptor (MRC1), arginase 1 (Arg1), and other anti-inflammatory factors. These two polarization modes are classic models for studies of macrophages (92, 93).

Immunity and metabolism are highly integrated and coordinated. In the initial stage of tissue hypoxia, the anaerobic glycolysis and pentose phosphate pathways of M1 macrophages are activated, whereas M2 macrophages mainly use oxidative phosphorylation and aerobic glycolysis to meet the energy requirements for tissue repair and remodeling. M1 macrophages are considered the most likely precursors of tumor-infiltrating macrophages, and TAMs are frequently M2 macrophages (94).

The long non-coding RNA (lncRNA) TUC339 is highly expressed in HCC-derived exosomes, which can be transferred

across HCC cells to promote tumor growth and metastasis (55, 95). Furthermore, the exosomal long non-coding RNA (lncRNA) TUC339 can be transferred to neighboring macrophages to modulate M1/M2 polarization and suppress antitumor immune responses *in vitro*. Microarray studies have demonstrated that exosomal TUC339 downregulated TLR signaling and Fcγ receptor (FcγR)-mediated phagocytosis pathways in macrophages, and TUC339 knockdown increased the phagocytic activity of macrophages. TUC339 is also involved in cytokine and chemokine receptor signaling, although the exact mechanism is unclear. Tumor cell-derived exosomes also carry miRNAs that regulate the expression of immune response-related genes. miR150 is highly expressed in the plasma of patients with HCC and in HCC-derived exosomes and promotes the growth of vascular endothelial cells by secreting the TAM-derived cytokine factor VEGF (40). VEGF levels are reduced in the plasma and tumor tissues of tumor-bearing mice treated with miR150 inhibitors. HCC exosomal miR-23a-3p upregulates the programmed cell death ligand 1 (PD-L1) expression in macrophages *via* Signal transducer and activator of transcription 3 (STAT3) signaling, which significantly attenuates melatonin-treated HCC cell-derived exosomes (41). PD-L1 expression in phagocytes has been demonstrated *in vivo*. HCC-derived exosomes significantly increased CD11b+F4/80 +CD206+ macrophages, accompanied by upregulation of M2-specific markers, including C-C chemokine ligand 17 (ccl17), C-C chemokine ligand 22 (ccl22), and arg-1. M2 polarization *in vitro* and in HCC-bearing mouse models is driven by miR146a, which is directly regulated by the zinc finger transcription factor Sal-like protein-4 (SALL4) in HCC cells (96, 97). The exosomal lncRNA HMMR-AS1 mediates macrophage polarization *via* the miR-147a/ARID3A axis under hypoxia and affects the progression of HCC (98) (Figure 2).

Therapeutic prospects related to exosomes in liver cancer cells

Role of H-TEXs in liver cancer drug resistance

In the TME, exosomes act as key regulators of the effects of chemotherapeutics by modulating drug efflux, epithelial–mesenchymal transition (EMT), autophagy phenotype, and immunosuppression (Table 2).

Exosome-related drug delivery system based on liver cancer therapy

Additionally, exosomes can assist in the early diagnosis of tumors, monitoring, and prognostic analyses. Because exosomes are endogenous vesicles, they benefit from low immunogenicity,

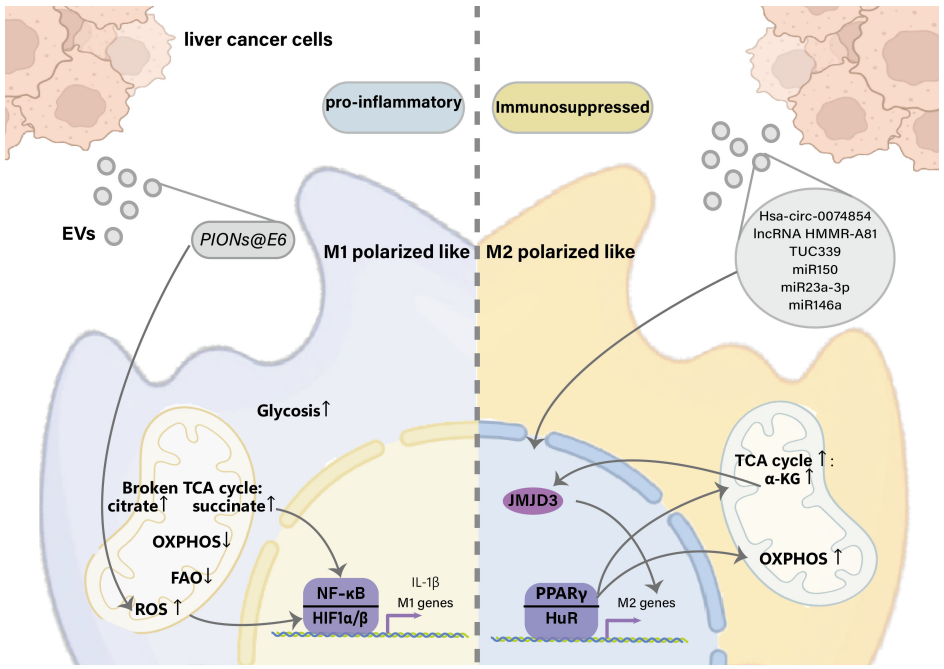

FIGURE 2
Effects of liver cancer-derived exosomes on macrophage polarization. EVs, Extracellular vesicles; TCA, tricarboxylic acid cycle; OXPHOS, Oxidative phosphorylation; FAO, Fatty Acid Oxidation; HIF1, hypoxia-inducible factor 1; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; IL-1β, Interleukin 1 beta; α-KG, α-Ketoglutaric acid; JMJD3, Jumonji domain-containing protein-3; PPARγ, Peroxisome proliferator-activated receptor γ; HuR, Hu antigen R.

TABLE 2 The role of H-TEXs in liver cancer drug resistance.

Donor cells	Contents	Recipient cells	Functions	Mechanism	References
MHCC-97H	HGF	SMCC-7721	Induce sorafenib resistance *in vitro* and *in vivo*	HGF/cMET/Akt signaling	(99)
HepG2	linc-ROR	HepG2	Induce resistance to doxorubicin and camptothecin	Modulate TGF-β/Caspase 3/CD133 signaling	(100)
HepG2	linc-VLDLR	HepG2 and KMBC	Induce resistance to sorafenib and doxorubicin	Enhance ABCG2 expression	(101)
hepa1-6	Tumor-associated antigen	DCs	Increase sorafenib efficacy with PD-1 antibody	Regulate Treg accumulation *via* PD-1/PD-L1 pathway	(102)
HBV-infecte d HepG2	HBX	HepG2	Facilitate OXA resistance	Activate CMA pathway	(103)
AMSCs	miR-199a	HCC cells	Improve HCC chemosensitivity	mTOR pathway	(104)
HCC cells	circUHRF1	HCC cells	Anti-PD1 therapy resistance	NK cell dysfunction by upregulating TIM-3	(62)
HepG2	circ-SORE	HepG2	Induce resistance to sorafenib	Stabilize YBX1	(105)

good biodegradability, low toxicity, and the ability to cross the blood–brain barrier, making them good carriers for drug delivery (106). For example, using doxorubicin, Yong et al. (107) took advantage of the ability of tumor cells to efflux chemotherapeutic drugs through EVs to achieve chemoresistance and encapsulated porous silicon nanoparticles loaded with Adriamycin into tumor cell-derived exosomes. Liang et al. (108) demonstrated that tumor-repopulating cell (TRC)-derived three-dimensional (3D) extracellular microparticles (MPs), which benefit from their softness, accumulate substantially and readily penetrate the liver tumor parenchyma for efficient delivery of chemotherapeutic drugs into TRCs. This results in effective suicide-like TRC killing and favorable therapeutic outcomes. Cytospin-A-related softness of 3D-MPs plays an important role in regulating the *in vivo* transport process. These findings reveal a new aspect of MP biology and provide potentially effective strategies for drug delivery in cancer therapy. This Trojan horse-like nanodrug delivery system using tumor exosomes as a carrier has been shown to have a higher inhibitory effect on tumor cells *in vivo*, and the tail vein injection of tumor-bearing mice is comparable to that of doxorubicin alone. Greater enrichment was observed in the exosome-encapsulated doxorubicin-treated group than in the doxorubicin group. Similarly, the exosome chemotherapeutic drug loading method has been evaluated in research on glioma. However, the liver, the largest solid organ in the human body, contains the most tissue-resident macrophages, and KCs, which account for 80%–90% of all tissue macrophages in the body, are responsible for capturing and removing foreign bodies. Therefore, avoiding the capture of the mononuclear phagocytic cell system and ensuring the delivery of sufficient doses of drugs to the liver tumor area after entering the blood circulation are major issues that need to be resolved in cancer-targeted drug delivery research (109). Belhadj et al. (110) described an "eat/don't-eat" decision switch for macrophages to evade phagocytosis by modifying CD47 outside of EVs. The effectiveness of this switch was verified by Du et al. (111). They engineered an exosome armed with three moieties, surface functionalization with CD47, membrane loading with ferroptosis inducer erastin, and core with photosensitizer RB. The exosomes displayed high delivery efficiency to tumors. Upon irradiation with a 532-nm laser in the tumor region, Erastin (Er) and Rose Bengal (RB) synergistically induced cell death.

Exosome-based immunotherapy in HCC

Cancer immunotherapy reverses the immunosuppressive TME (112). Exosome-targeted immunotherapy of HCC is often associated with dendritic cell (DC)-derived exosomes (DEXs), which have great potential for immunotherapy applications (113, 114).

Lu et al. (115) infected a DC cell line (DC2.4), which was established by transfecting Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Csf2), Myc, and Raf genes into C57BL/6 mice, with a lentivirus-expressing murine α-fetoprotein (AFP). They found that DC-AFP-derived exosomes (DEX-AFP) elicited strong antigen-specific immune responses, resulting in significantly delayed tumor growth and prolonged survival in various HCC mouse models (115). Zuo et al. (116) also used DEX as a carrier for a liver cancer vaccine to initiate a specific immune response against HCC. They decorated DEX with an HCC-targeting peptide (P47-P), an AFP epitope (AFP212-A2), and a functional domain of high-mobility group nucleosome-binding protein 1 (N1ND-N) and demonstrated its potential for the individualized treatment of HCC *via* universal DEX vaccines

(116). Zhong et al. (117) enhanced the antitumor efficacy of a DEX vaccine for HCC using microwave ablation. Zuo et al. (118) demonstrated that alarmin-coated exosomes elicited durable large-scale antitumor immunity in mouse liver tumors. Shi et al. (102) showed that the combination of DC-TEX and a programmed cell death protein 1 (PD-1) antibody (Ab) enhances the efficacy of sorafenib.

However, immunotherapy clinical trials have shown that substantial work is still needed before these findings can be applied to the treatment of cancer in clinical settings. Despite the challenges, DEX remains a promising immunotherapeutic strategy. DEX acts as a stable vesicle with a long shelf life, and its immunostimulatory properties are easily manipulated (through donor DCs). Research advancements have expanded the use of DEX-based cancer treatments in clinical settings (119).

Discussion

In this review, we evaluated the important role of exosomes in HCC progression and immunotherapy. Given that TAMs are a key component of the microenvironment, we summarize the regulatory mechanism by which liver cancer-derived exosomes regulate macrophage polarization, demonstrating that exosomes are a promising tool to target macrophages for HCC immunotherapy.

It should be emphasized that the interaction between the tumor and immunity is dynamic, heterogeneous, and bidirectional, including the immune response to drugs or external stimuli. Furthermore, the tumor cell state and even the genome are altered (120). Even so, the regulatory function of exosomes as messengers cannot be ignored, especially in the treatment of HCC. Chemotherapy resistance has become a major obstacle in improving the prognosis of patients. Targeting exosomes could be a promising strategy for reversing drug tolerance. In addition, the improvement of the efficacy of chemotherapy in patients with HCC by exosome anticancer drug delivery provides a new perspective for clinical treatment.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Tumor microenvironment-mediated immune tolerance in development and treatment of gastric cancer

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Tumor microenvironment is the general term for all non-cancer components and their metabolites in tumor tissue. These components include the extracellular matrix, fibroblasts, immune cells, and endothelial cells. In the early stages of tumors, the tumor microenvironment has a tumor suppressor function. As the tumor progresses, tumor immune tolerance is induced under the action of various factors, such that the tumor suppressor microenvironment is continuously transformed into a tumor-promoting microenvironment, which promotes tumor immune escape. Eventually, tumor cells manifest the characteristics of malignant proliferation, invasion, metastasis, and drug resistance. In recent years, stress effects of the extracellular matrix, metabolic and phenotypic changes of innate immune cells (such as neutrophils, mast cells), and adaptive immune cells in the tumor microenvironment have been revealed to mediate the emerging mechanisms of immune tolerance, providing us with a large number of emerging therapeutic targets to relieve tumor immune tolerance. Gastric cancer is one of the most common digestive tract malignancies worldwide, whose mortality rate remains high. According to latest guidelines, the first-line chemotherapy of advanced gastric cancer is the traditional platinum and fluorouracil therapy, while immunotherapy for gastric cancer is extremely limited, including only Human epidermal growth factor receptor 2 (HER-2) and programmed death ligand 1 (PD-L1) targeted drugs, whose benefits are limited. Clinical experiments confirmed that cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), vascular endothelial growth factor receptor (VEGFR) and other targeted drugs alone or in combination with other drugs have limited efficacy in patients with advanced gastric cancer, far less than in lung cancer, colon cancer, and other tumors. The failure of immunotherapy is mainly related to the induction of immune tolerance in the tumor microenvironment of gastric cancer. Therefore, solving the immune tolerance of tumors is key to the success of gastric cancer immunotherapy. In this study, we summarize the latest mechanisms of various components of the tumor microenvironment in gastric cancer for inducing immune tolerance and promoting the formation of the malignant phenotype of gastric cancer, as

well as the research progress of targeting the tumor microenvironment to overcome immune tolerance in the treatment of gastric cancer.

KEYWORDS

immune tolerance, tumor microenvironment, gastric cancer, immunotherapy, tumor-infiltrating immune cells

Introduction

Gastric cancer (GC) is one of the most common digestive tract malignancies worldwide, ranking fifth in morbidity and fourth in mortality (1). With the development of early diagnosis technology, although the incidence of GC exhibits a certain downward trend, the fatality rate of patients at an advanced stage that is inoperable is very high, and there is no effective treatment plan to date. In recent years, the rise of tumor immunotherapy has fueled the last hope for patients with advanced GC. Currently, the only targeted immunotherapy regimens for GC are Human epidermal growth factor receptor 2 (HER-2) monoclonal antibody, programmed death 1 (PD-1) monoclonal antibody and programmed death ligand 1 (PD-L1) monoclonal antibody. However, in GC patients, only 15–30% of patients are HER-2 positive, and the benefits of the treatment are limited (2). Although the efficacy of the PD-L1 monoclonal antibody is superior to first-line chemotherapy, the overall median survival of patients is only extended by two months. This may be related to the existence of immune tolerance in some patients (3). Chimeric Antigen Receptor T-Cell (CAR-T) therapy for GC is currently limited to clinical trials and a few case reports. An effective anti-tumor immune response includes effective presentation of antigens by dendritic cell (DC) cells, the activation and proliferation of specific T cells, and the maintenance of a lasting immune response. Inhibition of any of these points will lead to immune tolerance of the tumor (4). Therefore, in-depth exploration of the mechanism of immune tolerance in GC will help develop more effective treatment options.

Tumor microenvironment (TME) is the general term for all non-cancer components and their metabolites and secretions in tumor, which includes a large number of immune infiltrating cells, such as immune infiltrating lymphocytes (TILs). These immune cells constitute the immune microenvironment of the tumor. Current studies confirmed that TME has an important impact on malignant phenotypes such as tumor growth, invasion, metastasis, drug resistance, and immune escape. Stomach has a strong acidic environment and a unique endocrine system, which also makes the tumor microenvironment of gastric cancer different. Tumor immune microenvironment has both tumor-promoting and tumor-suppressing effects. In the stage of tumorigenesis, TME has a tumor-suppressing effect. However, as the tumor progresses, components of the tumor-suppressing microenvironment are continuously inhibited, and the tumor-promoting microenvironment is constantly being suppressed, leading to immune tolerance and tumor progression. In the process of tumor progression, on the one hand, tumors inhibit the function, number, and distribution of cytotoxic immune cells in the tumor microenvironment by competing for metabolites, secreting extracellular vesicles and cytokines, reducing the expression of self-antigens, resulting in immune tolerance. A large number of cancer-promoting immune cells continue to dominate tumors, which accelerates tumor progression and further inhibits the function of cytotoxic immune cells. Targeting the tumor microenvironment to inhibit the positive feedback loop of tumor immune tolerance is expected to contribute to a better treatment of tumors. In this article, we summarize the latest mechanisms of cellular components in the tumor microenvironment of GC for inducing immune tolerance, promoting the formation of the malignant phenotype of GC, and targeting the components of the tumor microenvironment to reduce immune tolerance in the research progress on the treatment of GC.

The constitution of GC TME

The tumor microenvironment of GC is composed of extracellular matrix (ECM), fibroblasts, endothelial cells, mesenchymal stem cells, macrophages, lymphocytes, neutrophils and other cell components. The metabolites and cytokines secreted by these cell components (including GC cells) are also important components of TME. These components in GC TME play their own roles in inducing the immune tolerance to promote the GC progress.

Tumor-associated macrophages

Macrophages infiltrating the tumor microenvironment are called tumor-associated macrophages (TAMs), which have two

polarization-activated states, namely, classical M1 polarization with tumor suppressor function and alternatively-activated M2 polarization with tumor-promoting function (5). In GC, M2 polarization of TAM is induced in the tumor microenvironment, and inhibition of M1 polarization is one of the important factors in the formation of immune tolerance (Figure 1A).

Several studies had proved that several molecules participated in M2 polarization in TME, which is closely related to the progress of GC. Pentraxin-3 (PTX3) can inhibit the stemness of GC cells and M2 polarization of macrophages, and prevent the formation of papillary metastases in GC (the early stage of ascites metastasis) (6). ETS-like transcription factor 4 (ELK4) promotes M2 polarization of macrophages by activating lysine-specific demethylase 5A (KDM5A), which inhibits the expression of Praja2 (PJA2) by removing H3K4me3 of the PJA2 promoter, thereby promoting M2 polarization of macrophages (7). Cisplatin induced activation of hypoxia inducible factor 1 alpha subunit (HIF1α) signaling directly drives the transcription of tumor-derived leukemia inhibitory factor, activates the STAT3 signaling pathway, and stimulates M2 polarization of macrophages, thereby promoting the resistance of gastric tumors to chemotherapeutic drugs (8). POU class 1 homeobox 1 (POU1F1) upregulated by High mobility group A 1B/2 (HMGA1B/2) promotes GC metastasis by regulating macrophage M2 polarization in a Chemokine 12 (CXCL12)/CXC motif chemokine receptor type 4 (CXCR4) dependent manner (9). Propionibacterium acnes (*P. acnes*) promotes gastric cancer progression by promoting M2 polarization of macrophages through Toll-like Receptor 4 (TLR4)/phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling (10). Calmodulin 2 (CALM2) in GC promotes M2 polarization of macrophages through the Adenylate kinase 2 (AK2)/Signal transducer and activator of transcription 3 (STAT3)/HIF-1/vascular endothelial growth factor A (VEGFA) axis, thereby promoting GC metastasis and angiogenesis (11). Therefore, these molecules or signal pathway related to the M2 polarization might be the potential targets for treating GC effectively.

In addition, $CD68^{+}$ $CD163^{-}$ M1 macrophages are required for PD-1/PD-L1 monoclonal antibody using in GC treatment (12). Interestingly, the knockdown of STING in THP1 cell line or activation of STING *via* 2'3'-c-GAMP were shown to promote M1 polarization of macrophages and exert an anti-tumor effect, suggesting that the STING pathway has complex and meaningful regulatory roles in macrophages (13).In gastric cancer, macrophages can induce the transformation of mesenchymal stem cells (MSC) cells into fibroblasts, and then participate in the formation of immune tolerance (14).

In fact, macrophages are an emerging tumor therapeutic target, and current therapeutic modalities for TAM include the inhibition of macrophage recruitment in tumors, depletion of macrophages, induction of macrophage reprogramming to the M1 phenotype, and enhanced phagocytosis of macrophages (15). We look forward to future studies that can demonstrate the critical role of TAMs in GC. Currently, CAR-macrophages have entered the phase I clinical trial stage as the latest CAR cells, but their application in GC remains lacking (16).

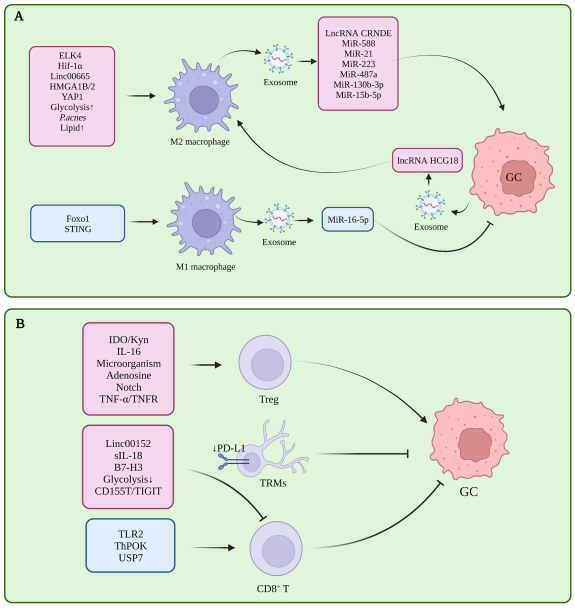

FIGURE 1
(A) Tumor-associated macrophages in gastric cancer immune tolerance. **(B)** Tumor-infiltrating T cells in gastric cancer immune tolerance.

T cells

T cells are highly heterogeneous. In TME, $CD8^+$ T cells assume the role of killing tumor cells, while Treg is the most representative $CD4^+$ immunosuppressive cell. In addition to memory T cells, γδ T cells, Nature killing (NK) T cells, and Th cells have been shown to play an important role in tumor progression and immune tolerance in gastric cancer (Figure 1B).

The decrease in the number and dysfunction of $CD8^+$T cells is one of the reasons for gastric cancer immune tolerance. In GC tissues with high expression levels of B7-H3 (CD276), the density of $CD8^+$ T cells within the tumor was reduced, suggesting that B7-H3 may be involved in the mechanism of tumor evasion of immune responses (17). Toll-like receptor 2 (TLR2) was down-regulated in $CD8^+$ T cells of gastric cancer patients, and TLR2 activation could increase the expression of perforin and granzyme B in $CD8^+$ T cells and enhance $CD8^+$ T cells cytotoxicity (18). The chromatin status of tumor-specific T cells is correlated with their dysfunction (19), and GC patients with high open circulating $CD8^+$ T cell chromatin respond better to pembrolizumab (20). $CD103^+$ $CD4^+$ T cells exhibit an immunosuppressive phenotype and high retention capacity in GC tumor tissues, leading to $CD8^+$ T cell dysfunction, and granzyme B (GZMB), interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α), and perforin (PRF-1) reduction (21).

In an *in vitro* 3D culture model, Treg cells were enriched in early intestinal-type GC and could promote the growth of spheroids by inducing interleukin-2Rα (IL-2Rα) expression and activation of mitogen-activated protein kinases (MAPK) signaling pathway in tumor cells (22). The infiltration level of tumor necrosis factor receptor 2 $(TNFR2)^+$ Tregs increases with the progression of GC. This is a prognostic marker and independent risk factor for GC, and activation of the TNF-α/TNFR2 pathway promotes the immunosuppressive phenotype and function of Tregs (23). Gastric mucosal microbial analysis found that Comamonas and Gaiella were negatively correlated with the number of pDCs and Tregs in GC, and Stenotrophomonas and Selenomonas were positively correlated with the number of pDCs and Tregs in GC, revealing the impact of microorganisms on tumor immunity (24). DAPT, a γ-secretase inhibitor that inactivates Notch signaling, can reduce the immunosuppressive capacity of $CD4^+CD25^+CD127^{dim/-}$ Tregs after DAPT treatment in GC (25). $CD4^+$ T cells in GC can promote the up-regulation of PD-L1 in mesenchymal stem cells through p-STAT3, thereby stimulating the proliferation of GC cells. This further stimulates the proliferation of GC cells. However, this study did not specifically explore the subset of $CD4^+$T functions, and the role of Treg remains to be elucidated (26). Therefore, Treg cells infiltrated in GC tissue play an important role in the progression of the disease by inducing immune tolerance. By targeting the inhibition of Treg production or function, this may relieve the immune tolerance state of GC patients, leading to a more effective delay or treatment of the disease.

The zinc finger and BTB domain containing 7B (Zbtb7b, Alias ThPOK) as transcription factors can upregulate sperm tail PG-rich repeat containing 1 (STPG1) and downregulate Tumor necrosis factor receptor superfamily member 12A (TNFRSF12A) at the transcriptional level, inhibiting the proliferation of gastric cancer cells and promoting the proliferation of T cells (27, 28). The CXXC zinc finger protein 4 (CXXC4) can activate T cells by inhibiting the ETS-like transcription factor 1 (ELK1)/MIR100HG pathway, increase the IFN-γ secreted by $CD3^+$ T cells, and relieve the immune tolerance of GC cells (29). Dexamethasone can inhibit immune evasion by inducing T cell glucocorticoid receptor (GR)/STAT3 pathway-mediated downregulation of PD-L1 and Indoleamine 2,3-dioxygenase 1 (IDO1) (30). In GC, ubiquitin-specific processing protease 7 (USP7) directly interacts with PD-L1 to stabilize it. USP7 inhibitors likewise inhibit tumor proliferation and promote PD-1/PD-L1 expression and immune response (31).

T cells are the executors of tumor immunity, as they directly exercise the tumor-killing function. In the context of inducing immune tolerance in the tumor microenvironment, $CD8^+$ T cells appear dysfunction and exhausted, and immune checkpoint inhibitors against $CD8^+$ T cells appear as an inefficient method. Therefore, reversing the immune tolerance microenvironment in TME and restoring the number, infiltration range, and function of $CD8^+$T cells are the most popular solutions to reduce immune tolerance.

Neutrophils

Tumor-associated neutrophils (TAN) are functionally classified as tumor-suppressing N1 cells and tumor-promoting N2 cells. Transforming growth factor beta (TGF-β) induces N1 to N2 polarization (32). A retrospective study showed that a large number of tumor-associated neutrophils infiltrating GC tissue indicate a greater the risk of lymph node metastasis (33). TANs promote the progression of GC by promoting the polarization of IL-17A producing Th subset cells through the B7-H2-extracellular signal-regulated kinase (ERK) pathway (34). In human neutrophils and GC cells co-culturing experiments, blocking the formation of NETs regulates the expression of Bcl-2, Bax, and nuclear factor kappa B (NF-κB) in GC cells, promoting GC cell apoptosis and inhibiting their invasion (35, 36). Tumor-derived GM-CSF activates neutrophils and induces PD-L1 expression in neutrophils through the Janus kinase (JAK) signaling and activator of STAT3 signaling pathway. Activated $PD\text{-}L1^+$ neutrophils effectively suppress normal T cell immunity *in vitro* and promote human GC growth and progression *in vivo* (37). The FasL $(CD95L)^+$ $PD\text{-}L2^+$ neutrophil subpopulation accounts for more than 20% of all

neutrophils in advanced GC. This conditional neutrophil (TCN) was treated with FasL and/or PD-L2 antibodies. After treatment, the injection of $CD8^+$ T cells into tumor-bearing mice constructed with SGC-7901 can significantly reduce the tumor volume and increase the infiltration of $CD8^+$ T cells, indicating that this subset of neutrophils is involved in gastric cancer. This has a significant immunosuppressive effect against $CD8^+$ T cells (38). *In vitro* co-culture experiments and IHC showed that TAN infiltrates PD-1^+ T cells, inhibits T cell proliferation, up-regulates the expression of PD-L1, and promotes the formation of an immunosuppressive microenvironment (39). Current studies showed that TAN promotes tumor immune tolerance in tumors by remodeling the ECM, promoting angiogenesis, generating NETs, and interacting with other immune cells (40). Currently, there are also some therapeutic regimens targeting neutrophils to relieve immune tolerance. However, reducing the risk of infection caused by neutrophil levels is still the biggest obstacle to this regimen (Figure 2A).

NK cells

NK cells can directly kill target cells and recognize tumor cells that $CD8^+$ T fails to recognize. However, NK cells exhibit dysfunctional behavior in TME (41). The infiltration level of NK cells in tumors and the level in peripheral blood are positively correlated with the prognosis of GC patients, and negatively correlated with the expression level of cyclooxygenase-2 (COX-2) (42, 43). The c-myc of NK cells in the peripheral blood of GC patients is down-regulated at the RNA and protein levels, and mitotic arrest is associated with NK dysfunction in GC patients (44). The expression level of the NK activating receptor NK Group 2 Member D (NKG2D) in GC patients is positively correlated with patient prognosis. Although NK cells in the resting state have little cytotoxicity against GC, NK cells induced by the K562-mb15-41BBL cell line *in vitro* have strong effects on GC cytotoxicity and strong antitumor activity in animal experiments (45). Decreased human leukocyte antigen class I (HLA-I) expression leads to decreased NK cell infiltration in GC and is insensitive to NK cell activity (46). Death-associated protein kinase 1 (DAPK1) downregulates the IKKβ/CSN5 axis in GC, inhibits PD-L1 expression, and activates the killing ability of NK cells (47). Matrix metalloproteinase (MMP)-2, MMP-9, and pan-MMP inhibitors can upregulate the expression of NKG2D ligands in GC, making GC cells more sensitive to NK cells (48). In *in vitro* experiments, prostaglandin 2 (PGE2) secreted by GC cells can inhibit the proliferation of NK cells and induce their apoptosis (42). IL-15 can activate the activity of NK cells and inhibit the formation of liver metastases in a mouse model of GC liver metastasis (49). iNKT cells are involved in the initial steps of anti-tumor immunity. However, the increased frequency of iNKT in peripheral blood of patients with GC does not bring good benefits to the patients. Subsequent experiments have shown that the ability of iNKT cells to degranulate and

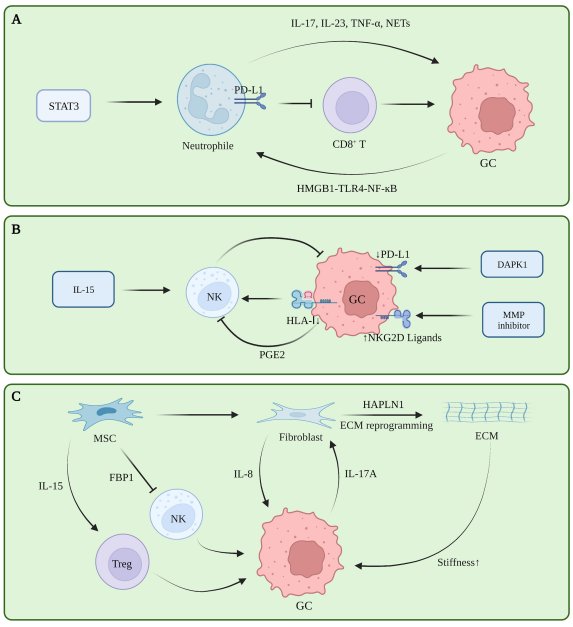

FIGURE 2
(A) Tumor-associated neutrophils in gastric cancer immune tolerance. **(B)** NK cells in gastric cancer immune tolerance. **(C)** ECM, fibroblasts and mesenchymal stem cells in gastric cancer immune tolerance.

produce IFN-γ in patients with GC is impaired (50). Further follow-up studies are required to clarify the heterogeneity of NK cells in GC, and the factors that inhibit NK cell function in GC TME, to find an effective NK cell-based therapeutic regimen for GC (Figure 2B).

ECM

The ECM is a network of collagen, fibronectin, laminin, vitronectin, elastin, as well as growth factors, cytokines, and matrix metalloproteinases that support and maintain the epithelial cell structure (51). These components are mainly secreted by fibroblasts, although other cells in the microenvironment likewise have the ability to secrete these substances (52). In the stage of gastric carcinogenesis, ECM is considered to be an initiating factor of gastric carcinogenesis. Studies showed that different subtypes of GC have different ECM components, and that a lower degree of differentiation indicates a greater abundance of ECM components, higher cell metabolism, and higher degree of metabolic reprogramming (53). Proteomic analysis revealed that ECM components of tumor tissues were no different from normal tissues, whereas their levels varied greatly, which was mainly manifested as increased ECM proteins and decreased basement membrane components that were closely related to tumor angiogenesis, invasion and metastasis, i.e., closely related to the formation of malignant phenotypes (54). During the progression stage of GC, ECM deposits continuously and increases in density, directly interacting with receptors on the surface of tumor cells, reducing E-cadherin/β-catenin, and promoting the proliferation, invasion, and metastasis of GC cells (55). Further, enhanced environmental stress caused by the increased matrix density is likewise an important reason for ECM to promote tumor progression. The researchers cultured GC cells in hydrogels with different stress intensities and found that with the increase of stress, the CD44 expression of tumor cells reversibly became nonfunctional. isomers, promoting the metastasis of GC (56). In fact, the stress role of ECM in breast cancer has been confirmed, and high-strength ECM can promote the epithelial-mesenchymal transition (EMT) process of breast cancer, increase the infiltration of M2 macrophages, and inhibit the function of $CD8^+$ T cells (57, 58). However, this mechanism must be further clarified in GC (Figure 2C).

Fibroblasts

Cancer associated fibroblasts (CAF) are the main cells that secrete and degrade ECM, and also secrete a large number of cytokines, chemokines, and exosomes. In gastric cancer, hyaluronan and proteoglycan link protein 1 (HAPLN1) were the most significantly upregulated genes in fibroblasts. Second harmonic generation imaging with a multi-photon microscope showed that the knockdown removal of HAPLN1 significantly reduces the density, length, width, and number of fibers in the ECM (59). A study on secretomes revealed that a Helicobacter pylori infection can lead to changes in the secretion of fibroblasts in the gastric mucosa, induce metabolic reprogramming and changes in the microenvironment of epithelial cells and tumor cells, and lead to type III EMT changes (especially tumors), where the epithelial-mesenchymal transition is closely related to the occurrence and development of gastric cancer (60, 61). Furthermore, fibroblasts exhibit complex immunomodulatory roles in other tumors. In breast cancer, the Yes-associated protein (YAP) pathway promotes fibroblast-induced ECM hardening, and the hardened ECM activates fibroblasts through YAP again, promoting breast cancer progression and immune tolerance (62). Moreover, various cytokines secreted by fibroblasts have complex regulatory effects on T cells, macrophages, and mast cells. Tumor therapy strategies targeting fibroblasts have also been tested in pancreatic cancer, breast cancer, and other tumors (63), Nevertheless, research in this area on GC remains insufficient (Figure 2C).

Endothelial cells

Angiogenesis provides nutrition and oxygen to the tumor microenvironment and promotes tumor growth. Endothelial cells play an important role in this process (64). Antiangiogenic vascular endothelial growth factor receptor (VGFR) monoclonal antibody and tyrosine kinase inhibitor (TKI) are also one of the treatment schemes for advanced GC. Single cell sequencing revealed the specificity of tumor endothelial cells (TEC) in TME in phenotype and metabolism. Some TECs have the potential to transform into mesenchymal cells in gastric cancer, and these endothelial cells play an important role in angiogenesis (65). Subsequent studies also showed that TEC participated in the formation of tumor immune tolerance under hypoxia (66). TEC can interact with CAF through VEGFA in GC (67).In other tumors, TEC can up regulate the immune checkpoint molecules of T cells and inhibit the activation of T cells (68). TEC expressing FasL can reduce the number of $CD8^+$ T cells and increase the number of Treg (69).

Mesenchymal stem cells

As a type of pluripotent stem cells, mesenchymal stem cells (MSC) can differentiate into fibroblasts in tumors, and further exhibit a tumor suppressor function in some tumors, which is in contrast with the heterogeneity of MSCs and induced differentiation directions in different tumors (70). The heterogeneity of MSC in the tumor microenvironment of GC must be further clarified (Figure 2C).

Endocrine signaling in GC TME

Gastric has endocrine function and can secrete gastrin, cholecystokinin (CCK), secretin and other substances. This is a special characteristic of GC distinct from the other solid tumor, such as lung caner, liver cancer and so on. Which may determine its unique TME for the GC progress. The current research has confirmed that the high expression of gastrin precursor, gastrin, and gastrin downstream receptor CCK2R is an important factor in the occurrence and progression of GC (71). Targeting the gastrin peptide (polyclonal antibody stimulator-PAS) can increase $CD8^+$ T lymphocytes and reduce the number of M2 macrophages in GC (72). Gastric endocrine system can promote gastric cancer, but its role in tumor microenvironment and regulation of immune tolerance of gastric cancer still need further research in the future.

Metabolic heterogeneity GC TME

In gastric cancer TME, the metabolic patterns of various cell components are different. The competition of metabolic substances leads to metabolic reprogramming, thus affecting the function of various cell components in TME, which is one of the reasons for immune tolerance (73).

Glycolysis

TME lacks nutrients, and glucose metabolism is necessary for cell survival. The glucose uptake capacity of gastric cancer cells is significantly higher than other cells in TME. This glucose deficiency will induce other cells in TME to undergo metabolic reprogramming and then lead to their redifferentiation (73). PTEN-induced kinase 1 (PINK1) deficiency in GC causes M2 polarization of TAMs, which is mainly related to the enhanced glycolysis level caused by PINK1 deletion (74). GC cells overexpressing YAP1 can promote M2 polarization by secreting IL-13, which activates the glucose transporter 3 (GLUT3) dependent glycolytic metabolic reprogramming of TAMs (75, 76). While lactate can further promote the M2 polarization of TAM, inhibition of monocarboxylate transporters (MCT) or HIF-1α can significantly reverse this effect (77), hypoxia-induced elevated glycolysis levels can also induce a decrease in M1 macrophages in GC (78), which suggests an important role of glucose metabolism reprogramming in the tumor microenvironment. Polymorphonucler myeloid-derived suppressor cells (PMN-MDSCs) accumulate in GC cells and inhibit $CD8^+$ T cell glycolysis through the S100A8/A9-TLR4/AKT/mechanistic target of rapamycin (mTOR) axis, leading to $CD8^+$ T cell exhaustion, and making GC susceptible to PD-1 therapy immune tolerance (79). In GC, the CD155T/TIGIT signaling pathway can inhibit the uptake of glucose by $CD8^+$ T cells, thereby inhibiting its function (80). MSC can inhibit the glucose uptake and lactate production of NK cells by upregulating FBP1, thereby weakening their glycolytic metabolism and inhibiting the degranulation ability, perforin production, and cytotoxicity of NK cells (81). In GC, inhibition of Glycogen synthase kinase-3beta (GSK-3β) can increase the infiltration of $CD8^+$ T memory stem cells (Tscm) in GC tissue, and promote their differentiation potential and anti-GC ability (82).

High level glycolysis in TME will lead to lactic acid accumulation, induce TME acidification, and inhibit the function of $CD8^+$T cells (83). Increase lactic level not only directly limits the cytolytic function of NK cells, but also indirectly inhibits NK cells by increasing the number of MDSC (84). In addition, high level lactic can also induce M2 polarization of macrophages and enhance Treg function (85).

Lipid

Studies showed that lipid accumulation exists in TAM, and this accumulation of lipids can induce the M2 polarization of TAM, upregulate PD-L1, block the anti-tumor T cell response, and exert an immunosuppressive effect (86). About 30% of tumor-infiltrating lymphocytes (TILs) in the tumor microenvironment of gastric adenocarcinoma are $CD69^+CD103^+$ tissue-resident memory T cells (TRMs) cells. Fatty acid oxidation is necessary for the survival of Trm cells, but the uptake of fatty acids by Trm cells is far less than that of GC cells. The PD- L1 blocker can upregulate the expression of Fabp4/5 in gastric cancer Trm, increase its uptake of fatty acids, and thus enhance its anti-tumor activity (87). $CD8^+CD103^+$TRMs have stronger anti-tumor activity, but exhibit reduced infiltration in gastric cancer, and their cytolytic capacity can be restored by PD-1 blockade and 4-1BB co-stimulation *in vitro* (88).

Amino acid

Glutamine is necessary for tumor cells, and its metabolites can inhibit the proliferation of T cells and the secretion of cytokines (89). The IDO/Kyn pathway is a classic immunosuppressive pathway. Kyn derived from GC cells can increase the level of Treg infiltration and induce Treg cells to secrete IL-10, which further activates the STAT3/BCL2 pathway to induce GC resistance to chemotherapy (90). Adenosine is a key immunosuppressive metabolite in the tumor microenvironment (91), and Treg cells isolated from peripheral blood of GC patients have the ability to promote adenosine production, which in turn inhibits the activity of $CD8^+$ T cells through the A2aR pathway (92).

Cytokines in GC TME

Cytokines are regulators of innate and adaptive immunity, and play an important role in the formation of tumor immune tolerance. GC cells can secrete IL-17A to promote the transformation of normal fibroblasts (NF) into tumor-associated fibroblasts (CAF). MSC in GC can upregulate the ratio of Tregs and increase the expression of PD-L1 by secreting IL-15, promoting the EMT process and immune tolerance in GC (93). Fibroblasts promote the proliferation of GC cells by secreting IL-8, forming a positive feedback to promote the malignant phenotype of GC access (94). Hepatocyte growth factor (HGF) secreted by CAF can promote angiogenesis of GC through PI3K/AKT and ERK1/2 pathways (95). Elevated serum interleukin 8 (sIL-8) levels are closely related to poor prognosis and lymph node metastasis in GC, sIL- 8 can promote GC metastasis by increasing the PD-1 expression of $CD8^+$ T (96). GC-derived TGF-β1 promotes PD-1^- independent $CD8^+$ T cell dysfunction, and the restoration of $CD8^+$ T cell function by combined blockade of PD-1 and TGF-β1 may benefit future GC immunotherapy (97). Cytokines and ECM reorganization in the tumor microenvironment are key factors in the transformation of macrophages from M1 to M2 (98). Studies have shown that TAM can inhibit the function of natural killer (NK) cells in the tumor microenvironment by secreting TGF-β1 (99). M2 macrophage secreted CHI3L1 can play the same role by binding to IL-13Rα2 (100). TAM can activate the NF-κB and STAT3 signaling pathways of GC cells by secreting TNF-α and IL-6, upregulate the expression of PD-L1, and promote the immune escape and proliferation of GC cells (101). The overexpression of secreted acidic cysteine-rich protein (SPARC) in M2 macrophages can inhibit its tumor-promoting effect (102). Studies have also shown that CXCL8 secreted by M2 macrophages can upregulate their own PD-L1 levels (103). IL-4-stimulated EGFR transactivation helps suppress M2 polarization in macrophages, and TAMs in patients with advanced GC have low epidermal growth factor receptor (EGFR) expression, which may be related to the resistance to EGFR monoclonal antibody therapy (104).Tumor-associated neutrophils activate AKT and p38 pathways in MSCs by secreting inflammatory molecules, such as IL-17, IL-23, and TNF-α, inducing their transformation into fibroblasts, and promoting the development of gastric cancer (105). IL-17a produced by TAN can also promote the EMT process of GC through the JAK2/STAT3 pathway (106).

Noncoding RNAs and exosomes in GC TME

Non coding RNA is a kind of RNA without coding function, including miRNA, lncRNA and circle RNA. Non coding RNA can regulate gene expression and protein function, and form a complex regulatory network. In addition, non coding RNA can also affect other cells in the tumor microenvironment through exosomes, which plays an important role in the formation of tumor immunity. The interaction between Linc00665 and BACH1 leads to the activation and binding of BACH1 to the Wnt1 promoter, promoting M2 polarization of TAMs and GC progression (107). LncRNA CRNDE (108), miR-588 (109), miR-21 (110) in M2-polarized TAM-derived exosomes can enhance the resistance of GC cells to cisplatin, while miR-223 enhances GC cell resistance to doxorubicin (111), Meanwhile, miR-487a, miR-130b-3p can promote the progression of GC (112, 113). M2 macrophage-derived miR-15b-5p can promote GC metastasis through the BRMS1/DAPK1 axis (114). M1-polarized TAM-derived exosomes can down-regulate the expression of PD-L1 in gastric cancer cells through miR-16-5p and activate T cell immunity (115). Linc00152 inhibits $CD8^+$ T cell infiltration in GC by binding to enhancer of zeste homolog 2 (EZH2) and regulating the CXCL9, 10/CXCR3 axis (116).

Gastric cancer-derived exosomes can induce the PD-1^+ phenotype in TAMs, most of which will differentiate into M2 macrophages. Furthermore, these exosomes can inhibit the proliferation of $CD8^+$ T cells in the microenvironment promotes the secretion of IFN-γ, which in turn promotes the progression of GC (117). Docking protein-1 (DOK1) downregulates the expression of PD-L1 in TAM (118). Another study showed that gastric cancer-derived exosomes could promote the M2 polarization of TAMs through the lncRNA HCG18-miR-875-3p-Kruppel-like factor 4 (KLF4) pathway (119). The overexpression of lncRNA ANCR promotes GC cell invasion and metastasis by inhibiting the polarization of macrophages toward M1 by downregulating Foxo1 (120). In GC, the low expression of miR-128-3p is closely related to the poor prognosis of patients. The direct target of miR-128-3p is IL-16, which can reduce the infiltration of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs in GC tissue by inhibiting the expression of IL-16 (121). MiR-105-5p is expressed in GC at low levels, and overexpression of miR-105-5p can directly inhibit the expression of PD-L1 and activate $CD8^+$ T cells (122). γδ T cells are a class of T cells recently discovered to have important functions in tumor immune tolerance, Vγ9Vδ2 T cells are the main subset of γδ T, and gastric cancer-derived exosome miR-135b-5p can damage anti-tumor function of Vγ9Vδ2 T cells by targeting SP1 (123). MiR-451 in gastric cancer-derived exosomes can transfer to T cells and activate the mTOR pathway, inducing their differentiation into Th-17 cells (124). Gastric cancer-derived exosomal PD-L1 is upregulated in advanced GC patients treated with 5-FU, leading to systemic immune tolerance (125). Mir-1290 in gastric cancer-derived exosomes can inhibit T cell proliferation through the grainyhead-like 2 (Grhl2)/zinc finger E-box binding homeobox 1 (ZEB1)/PD-L1 axis and participate in immune tolerance (126). PD-L1 in exosomes has an immunosuppressive effect in tumors, and histone lysine-specific demethylase 1 (LSD1) can upregulate

the level of PD-L1 in gastric cancer-derived exosomes and induce T cell immune resistance (127). GC cell-derived exosomes induce neutrophil autophagy and tumor-promoting activation through the high mobility group box-1 (HMGB1)/TLR4/NF-κB signaling pathway, ultimately promoting the proliferation and migration of GC cells (128). Gastric cancer-derived exosomes can upregulate PD-L1 expression in neutrophils by transporting HMGB1, thereby inhibiting T cell function (129).

Immunotolerance targeting therapies in GC

Immune cells and non-immune cell components in the microenvironment induce tumor immune tolerance through a variety of mechanisms, which plays an important role in the occurrence and development of GC. Therefore, targeted intervention in the key links of immune tolerance in the microenvironment of GC is expected to become an effective strategy for its treatment. To date, this treatment mainly includes CAR modified cell therapy, herbal medicines, monomer drugs, oncolytic viruses, and other biological agents (Table 1).

CAR-cell

In recent years, CAR-cell, including CAR-T and CAR-NK had been developed for treating GC. Mesothelin (MSLN)-CAR-T cells can effectively inhibit the growth of GC cells in the Patient-derived tumor xenograft (PDX) model (130). Mesenchymal-epithelial transition factor (cMet)-PD1/CD28 CAR-T is a second-generation CAR. The researchers constructed a PD1/CD28 chimeric switch receptor by fusing the extracellular domain of PD-1 with the transmembrane and intracellular domains of CD28. Converting the inhibitory signal of PD-1 into the activation signal of CD28 can effectively inhibit the growth of GC *in vitro* and *in vivo*, and increase the infiltration of central memory T cells, prolong the long-term anti-tumor effect, and reducing the secretion of inflammatory factor IL-6 (131). The intercellular adhesion molecule 1 (ICAM-1) is expressed in nearly 50% of GC patients. In mouse models, CAR-T cells targeting ICAM-1 can target both primary and metastatic gastric cancers, exhibiting a good therapeutic effect (132). In a mouse model, anti-CD133 chimeric antigen receptor T (CAR-T) can selectively target cisplatin-resistant GC stem cells, and the combined use of cisplatin can improve the therapeutic effect (133). Nanobody VHH1-driven CAR-T (CDH17CART) targeting CDH17 can effectively treat gastrointestinal tumors without affecting normal epithelial cells in mouse experiments (134). Claudin18.2 (CLDN18.2) is a gastric-specific membrane protein, and CLDN18.2-specific CAR-T cells can effectively partially or completely eliminate GC in the PDX model. To date, this treatment passed phase I clinical trials (135, 136). Bispecific trophoblast cell surface antigen 2 (Trop2)/PD-L1-specific third-generation CAR-T cells were developed through lentiviral infection, which can effectively kill GC cells *in vitro* (137). A phase I clinical trial showed that *in vitro* expanded NK cells combined with trastuzumab or cetuximab had a certain therapeutic effect on GC, which was well tolerated by patients (138). A dual-targeting chimeric receptor (DTCR) PD1-DAP10/NKG2D increases the expression of PD1 and NKG2D on the surface of NK92 cells by viral transfection, and has comparable anti-tumor properties in a mouse tumor-bearing model constructed with SGC-7901 activity (139). MSLN-CAR NK cells constructed based on NK-92 cells can effectively kill MSLN-positive GC cells *in vitro* and inhibit tumor growth in the PDX model, with a large number of NK cells infiltrating the tumor (140). CAR cells constructed from various immune cells have shown a certain curative effect in animal models of GC. However, whether these treatments are effective for GC patients, and whether the selection of patients is targeted for their application, still needs extensive research.

Monomer drug

Studies have shown that the antiallergic drug Tranilast can inhibit the secretory function of fibroblasts in peritoneal metastatic GC tissue, effectively improve the tumor microenvironment, increase the infiltration of $CD8^+$ T cells, as well as reduce the infiltration of M2 macrophages and mast cells. This leads to reduced proliferation and fibrosis of GC cells (141). Futibatinib is a novel FGFR1-4 inhibitor that exhibits broad-spectrum antitumor effects in various tumors, including gastric cancer (142). Metformin can promote the secretion of calmodulin-like protein 3 (Calml3) from CAF cells and inhibit the progression of GC (143). In patients with advanced GC, the application of large doses of PPI can inhibit the exosome secretion function of fibroblasts, improve the tumor microenvironment, and inhibit the malignant degree of GC (144). Nevertheless, the effect of PPI on gastric cancer remains controversial. Itraconazole can inhibit the activity of endothelial cells and fibroblasts in GC and alleviate the resistance of GC cells to bevacizumab (145). A selective inhibitor of PI3K-γ isoenzyme, IPI549, restores macrophage function and promotes anti-tumor T cell responses (86). Experiments *in vivo* show that methionine enkephalin (MENK) can promote M1 polarization of macrophages and upregulate the expression of opioid receptor (OGFr) by blocking the PI3K/AKT/mTOR signaling pathway, which inhibits GC cells (146). In $CD8^+$ cells isolated from peripheral blood of tumor patients, the TLR2 agonist Pam3Csk4 enhanced the cytolytic activation of peripheral and tumor-infiltrating $CD8^+$ T cells from GCs (18). *De novo* DNA methylation is acquired by $PD1^+CD8^+$ tumor-infiltrating T cells

TABLE 1 Therapeutic strategies targeting tumor immune tolerance in GC microenvironment.

Drugs	Name	Target	Mechanisam	Reference
CAR-cell	MSLN-CAR-T	GC	Specifically kill MSLN-positive cells and release cytokines	(130)
CAR-cell	cMet-PD1/CD28 CAR-T	GC	Increase the infiltration of central memory T cells	(131)
CAR-cell	ICAM-1-CAR-T	GC	Specifically kill ICAM-1-positive cells	(132)
CAR-cell	CD133-CAR-T	GC	Target cisplatin-resistant gastric cancer stem cells	(133)
CAR-cell	CDH17-CAR-T	GC	Kill tumor cells in a CDH17-dependent manner and do not attack normal epithelial cells	(134)
CAR-cell	CLDN18.2-CAR-T	GC	Specifically kill CLDN18.2-positive cells, Phase I clinical trial	(135, 136)
CAR-cell	Trop2/PD-L1-CAR-T	GC	Specifically kill Trop2 and PD-L1positive cells	(137)
CAR-cell	NK expanded in vitro	GC	Combine with trastuzumab or cetuximab had a certain therapeutic effect on gastric cancer	(138)
CAR-cell	PD1-NKG2D-CAR-NK	GC	Enhance whole blood IFN-γ production and reduced peripheral Tregs, Phase I clinical trial	(139)
CAR-cell	MSLN-CAR-NK	GC	Specifically kill MSLN-positive cells and enhance NK cell infiltration	(140)
Monomer drug	Tranilast	Fibroblasts	Increase the infiltration of CD8+ T cells, and reduce the infiltration of M2 macrophages and mast cells, and reduce proliferation of fibroblasts	(141)
Monomer drug	Futibatinib	Fibroblasts	FGFR1-4 inhibitor, Antitumor effect	(142)
Monomer drug	Metformin	Fibroblasts	Promote the secretion of Calml3, Antitumor effect	(143)
Monomer drug	PPI	Fibroblasts	Inhibit the exosome secretion function of fibroblasts	(144)
Monomer drug	Itraconazole	Fibroblasts	Alleviate the resistance of gastric cancer cells to bevacizumab	(145)
Monomer drug	IPI549	Macrophages	PI3K-γ inhibitor, restores macrophage function and promotes antitumor T cell responses	(86)
Monomer drug	MENK	Macrophages	Promote the M1 polarization, blocking the PI3K/AKT/mTOR signaling pathway	(146)
Monomer drug	Pam3Csk4	T cell	TLR2 agonist, active CD8+ T cells	(18)
Monomer drug	DAC	T cell	Block DNA methylation in activated PD1+CD8+ TILs	(147)
Monomer drug	CCL28 inhibitor	T cell	Inhibit Treg cell infiltration	(148)
Herbal medical	Berberine	Macrophages	enhance the phagocytosis of macrophages and therapeutic effects of CD47 antibody and rituximab	(149)
Herbal medical	Paeoniflorin	Fibroblasts	Inhibit the secretion of IL-6, Antitumor effect	(149)
Herbal medical	Astragaloside IV	Fibroblasts	Inhibit the pathological functions of CAFs	(150)
Herbal medical	Triptonide	Fibroblasts	Abolish the ability of GCAFs to induce epithelial-mesenchymal transition	(151)
Herbal medical	Sophoridine	Macrophages	Inhibit M2 polarization,increase CD8+ T proliferation and cytotoxic function	(152)
Herbal medical	Oleanolic acid	T cell	Promote the balance of Treg/Th17 cells	(153, 154)
Biological agents	Oncolytic virus carrying relaxin relaxin	ECM	Degrade ECM components, increase accumulation of cytotoxic T cells and trastuzumab and PD-1 mAbs	(155)
Biological agents	Fiber-modified hexon chimeric recombinant oncolytic adenovirus	Fibroblasts	Kill gastric CAFs	(156)
Biological agents	Oncolytic herpes simplex virus type 1 virus G47Δ	Macrophages, NK	Decrease M2 macrophages,increase M1 macrophages and NK	(157)
Biological agents	PR-Gel	Macrophages, T cell	Increase CD8+ T-cell and M1 infiltration	(158)

(Continued)

TABLE 1 Continued

Drugs	Name	Target	Mechanisam	Reference
Biological agents	CD137 antibody	T cell	Enhance CD8+ T cell	(159)
Biological agents	iRGD-anti-CD3	T cell	Promote T cell infiltration	(160, 161)
Biological agents	sPH20-IgG2	T cell	Enhance the cytotoxicity of MSLN CAR-T	(162, 163)
Biological agents	m3s193 BsAb	T cell	Enhance activity in T cell recruiting, activation, proliferation, cytokine release, and cytotoxicity	(164)
Biological agents	Hydroxypropyl cellulose photocrosslinked hydrogel incorporating IFN-α2b	T cell	Induce activated T cells into tumor tissue	(165)
Biological agents	αPD1-PEG-PCL	T cell	Target PD1+CD8+ TIL	(166)
Biological agents	DC cell vaccine loaded with MG-7 antigen	Dendritic cell	Activate specific cytotoxic T lymphocytes	(167)
Biological agents	Polylactic-co-glycolic acid nanoparticles encapsulated DC cells and gastric cancer cell soluble lysate	Dendritic cell	Enhance the differentiation of T cells to Th1, enhance the effect of DC vaccine	(168)
Biological agents	Dendritic cells modified by SLC	Dendritic cell	Promote DC maturation, enhance the ability of DCs to T cell chemotaxis and T cell stimulation	(169)
Biological agents	Heat shock protein -glycoprotein gp96	Dendritic cell, NK	Enhance the antigen-presenting ability of DC	(170)
Biological agents	Fusion protein dsNKG2D-IL-15	NK	Recruit and activate NK	(171)
Biological agents	Gastrin Vaccine	Gastrin	Increase CD8+ T lymphocytes and reduce the number of M2 macrophages	(72)

(TILs), which results in graded downregulation of cytokines, such as interferon-γ (IFN-γ), while 5-Aza-2'-deoxycytidine (DAC) *de novo* blocks DNA methylation in activated $PD1^+CD8^+$ TILs (147). The CCL28 blockade inhibits Treg cell infiltration and tumor progression in the mouse model (148). The single drugs mentioned above affect tumor immune tolerance through different mechanisms, thus playing a certain role in the treatment of GC. However, the targeting problem of monomeric drugs *in vivo* may be dangerous, diminishing the therapeutic effect and even causing serious side effects. Improving the targeting precision of their actions, such as by combining them with monoclonal antibodies, may solve this problem.

Herbal medicine

Herbs are widely used as tumor immune regulators and chemotherapeutic sensitizers. Although the therapeutic effect of herbs and their natural compounds is not as significant as that of classical drugs, their advantages of low toxicity and low side effects endow them with potential in tumor treatment (172, 173). In addition, some herbal medicines play an important role in the metabolic regulation of gastrointestinal tumors (174).

Berberine has complex functions in gastrointestinal tumors, including including autophagy, immunity, inflammation, modification of the gut microbiota and miRNA. Berberine is an inhibitor of CD47, which can enhance the phagocytosis of macrophages, and enhance the therapeutic effects of CD47 antibody and rituximab (149). Paeoniflorin improves the immune microenvironment of GC and inhibits the invasion and metastatic ability of GC by inhibiting the secretion of IL-6 in fibroblasts in GC tissue (175). Astragaloside IV and Triptonide can inhibit the cancer-promoting function of fibroblasts in GC (150, 151). Sophoridine inhibits M2-TAM polarization *via* the TLR4/IRF3 axis, increases $CD8^+$ T proliferation and cytotoxic function, and downregulates the expression of $CD8^+$ T cell exhaustion markers PD-1, Tim-3, and Lag-3 (152). Oleanolic acid can promote the balance of Treg/Th17 cells in GC by targeting IL-6 through miR-98-5p, and is a potential drug for the treatment of GC (153, 154).

Other biological agents

Several researchers constructed an oncolytic virus carrying relaxin (RLX), which can degrade ECM components in tumors (155). Results showed that in *in vivo* experiments, the oncolytic virus carrying RLX could effectively degrade the ECM of gastric cancer and increase the activated ECM. The accumulation of cytotoxic T cells and trastuzumab and PD-1 mAbs in gastric cancer tissues yielded significant anti-tumor effects (155). A fiber-modified hexon

chimeric recombinant oncolytic adenovirus targeting CAFs can relatively specifically kill gastric CAFs and inhibit GC cell growth *in vivo* (156). G47Δ, a third-generation oncolytic herpes simplex virus type 1 virus, has passed phase II clinical trials in glioma, and has demonstrated significant anticancer effects in orthotopic tumor models and peritoneal dissemination models of GC. M2 macrophages were decreased, while M1 macrophages and NK cells were increased (157). A research group developed an injectable shear-thinning hydrogel, co-loaded with polyphyllin II (PP2) and resiquimod (R848) (PR-Gel for short), which induces TAM cell M2 in a mouse model of GC. Enhanced repolarization and $CD8^+$ T cell infiltration to M1 exhibited favorable tumor suppressive effects (158). In *in vitro* experiments, the CD137 antibody can effectively induce apoptosis in primary GC cells by enhancing $CD8^+$ T cells *via* activation of NF-κB signaling (159). A novel tumor-penetrating peptide, iRGD-anti-CD3, can immobilize iRGD on the surface of T cells through CD3 binding, promote T cell infiltration, and increase T cell activation and cytotoxicity to target cancer cells in 3D culture models and *in vivo* experiments (160, 161). Replacing the PH20 signal peptide with the tPA signal peptide and linking the IgG2 Fc fragment to construct human hyaluronidase PH20 (referred to as sPH20-IgG2) can enhance the cytotoxicity of MSLN CAR-T against GC in a mouse model (162, 163). Targeting Lewis Y and CD3 (m3s193 BsAb), a formatted novel T cell-binding bispecific antibody with IgG-[L]-scfv exhibited promising anti-GC activity in a mouse huPBMCs/GC co-transplantation model (164). Hydroxypropyl cellulose photocrosslinked hydrogel incorporating IFN-α2b can ensure the activity of IFN-α2b, stably release IFN-α2b to stimulate T cells over a long time, and combined with low-dose radiation of 5 Gy can induce activated T cells into tumor tissue, increasing the immunotherapy effect (165). The conjugation of αPD1 (i.e., nivolumab) to poly(ethylene glycol) (PEG) and poly (ε-caprolactone) (PCL) copolymers with PEG as linker (αPD1-PEG-PCL) by double emulsion solvent evaporation, encapsulating DAC in αPD1-PEG-PCL, this drug can better target $PD1^+CD8^+$ TIL to inhibit and kill GC cells (166). Anti-TGF-β/PD-L1 bispecific antibody YM101 is superior to anti-TGF-β and anti-PD-L1 monotherapies, increasing the numbers of tumor infiltrating lymphocytes and dendritic cells, elevating the ratio of M1/M2, and enhancing cytokine production in T cells (176). As an agonist of STING, bivalent manganese (Mn2+) can cooperate with YM101 to produce more lasting anti-tumor effect and enhance the presentation of tumor antigen (177). M7824 (MSB0011359C) is a bifunctional fusion protein composed of a monoclonal antibody against PD-L1 fused to the extracellular domain of TGF-β receptor II, the dual anti-immunosuppressive function of M7824 resulted in activation of both the innate and adaptive immune systems, which contributed to M7824's antitumor activity relative to monotherapies (178). The above three bioagents had been confirmed that they could suppress multiple tumor cell lines, including colon cancer, lung cancer, breast cancer. Although it is not reported that their roles in GC, it provides potential therapy to GC in future. The DC cell vaccine loaded with MG-7 antigen (MG-7Ag) significantly activates specific cytotoxic T lymphocytes in the GC PDX model (167). Polylactic-co-glycolic acid nanoparticles (NPs) are encapsulated by DC cells, and the GC cell soluble lysate can enhance the differentiation of T cells to Th1 in tumors and enhance the effect of the DC vaccine (168). Dendritic cells modified by the SLC gene can promote DC maturation, enhance the ability of DCs to T cell chemotaxis and T cell stimulation, and induce specific anti-GC cellular immunity (169). The heat shock protein (HSP)-glycoprotein (gp) 96 can enhance the antigen-presenting ability of DC cells and the activity of NK cells *in vitro* (170). The fusion protein dsNKG2D-IL-15 can recruit and activate NK cells and inhibit the growth of GC in a nude mouse model (171).

Conclusion

In fact, there is no lack of immune cells in tumors, including immune cells with tumor suppressor functions. However, the exhaustion and functional inhibition of these cells are fundamental reasons for tumor immune tolerance and immune escape. In-depth research is particularly important for tumors such as GC, which have a large patient base and few treatment options. However, current research on tumor immunity of GC is significantly less than that of lung, colon, and other cancers, and numerous classical mechanisms of action have not been confirmed in GC. This may be related to the unique anatomical characteristics of gastric tissue. As an important part of the digestive system, the stomach is in close contact with various foods ingested from the outside world, thus maintaining its immune tolerance to food. At the same time, due to the long-term high gastric acid environment, Whether it also constitutes a unique immune microenvironment in the stomach may be inseparable from the occurrence and development of GC. The immune escape of gastric cancer is closely related to its tumor microenvironment, especially considering the changes in metabolic patterns and metabolites of each cell component in it, as well as the role of the secretion of each cell component. The dynamic changes in the tumor microenvironment in the tumor-promoting direction during tumor progression are of great significance. Reversing the reprogramming of the tumor microenvironment to counter the tumor-promoting direction can effectively solve the immunosuppression of tumors. Numerous traditional medicines have also been found to exert anti-tumor effects, which suggests that we must pay attention to the possibility of traditional medicines and traditional Chinese medicines as immunomodulators and sensitizers in tumor treatment. Considering the currently popular CAR-T, the main problems involve making CAR-T effectively infiltrate

solid tumor tissue, making CAR-T play a lasting role in the immunosuppressive tumor microenvironment, and finding effective chimeric targets in as many tumor cells as possible, newer modifications and gene targets remain to be developed. NK cells and neutrophils as emerging therapeutic directions in GC must also be further studied.

With the development of omics technology, the heterogeneity of GC and various cellular components in GC TME are well known to us. Their differences are the only way to find solutions to the immune tolerance of GC and achieve precision medicine. Future research must explore the relationship and differences between GC and TME from a holistic perspective, find targets for the overall tumor microenvironment, and determine novel directions for solving GC immune tolerance.

Author contributions

CFL and WY conceptualized the study. YDL drafted the manuscript. YPL and CL conducted the literature review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Research trends on anti-PD-1/PD-L1 immunotherapy for esophageal cancer: A bibliometric analysis

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Objectives: The study aims to summarize publication characteristics of anti-programmed cell death protein 1 (PD-1)/programmed cell death 1 ligand 1 (PD-L1) immunotherapy for esophageal cancer and create scientific maps to explore hotspots and emerging trends with bibliometric methods.

Methods: The publications between 2012 and 2021 were retrieved from the Web of Science Core Collection (WoSCC) on June 20, 2022. Bibliometric tools including HistCite, VOSviewer, and CiteSpace were used for statistical analysis. Data on the trend of the annual output, countries/regions, institutions, journals, authors, subject categories, keywords, and co-cited references were presented in this study.

Results: A total of 552 publications written by 3,623 authors of 872 institutions, 44 countries/regions in 250 journals were included in the bibliometric study. China, USA and Japan were the key countries in this field. Kato Ken, Bang Yung-Jue, *Frontiers in Oncology*, *Journal of Clinical Oncology* and Natl Canc Ctr were the top 1 productive author, co-cited author, productive journal, co-cited journal and prolific institution, respectively. The top 4 most present keywords were esophageal cancer, immunotherapy, esophageal squamous cell carcinoma and PD-L1. Neoadjuvant chemotherapy, response, PD-1 blockade and $CD8^+$ T cell were four latest research frontiers. The keywords reflected the progress from PD-1/PD-L1 expression to the clinical application of PD-1/PD-L1 inhibitors. The current researches mainly focus on neoadjuvant immunotherapy for esophageal cancer and development of biomarkers. Further research is warranted to determine effective predictive biomarkers or models, illustrate the molecular mechanism of combined treatment, and construct the optimal therapeutic strategy.

Conclusions: This study visually analyzed the global trend and hotspots of anti-PD-1/PD-L1 immunotherapy for esophageal cancer over the past decade. The results could guide scientists to comprehensively understand the global frontiers and determine future directions.

KEYWORDS

bibliometrics, anti-PD-1/PD-L1, immunotherapy, esophageal cancer, CiteSpace, HistCite, VOSviewer, Web of Science (WOS)

Introduction

Esophageal cancer is the eighth most common cancer and the sixth major cause of cancer-related death worldwide (1). In 2020, the world witnessed about 604,100 new cases and 544,100 deaths, equaling to the age-standardized morbidity and mortality rates of 6.3/100,000 and 5.6/100,000, respectively (2). Advanced esophageal squamous cell carcinoma (ESCC) is one of devastating tumors with the 5-year survival rate lower than 5% (3, 4). The etiology of esophageal cancer is not completely clear. Recognized risk factors include genetic predisposition, gastroesophageal reflux disease, alcohol consumption, smoking and obesity (5). The alternative clinical treatment for esophageal cancer mainly depends on the stage of the tumor and the specific condition of patients. For esophageal cancer, multidisciplinary approach is an effective strategy for managing this disease, which involves the use of surgery, radiotherapy, chemotherapy, targeted therapy, immunotherapy and other treatments (6). In recent years, the emergence of immunotherapy has brought new hope for esophageal cancer. With the recognition of tumor immunotherapy, the application of immune checkpoint inhibitors (ICIs) has gradually shifted from the back-line and second-line treatments to first-line and even perioperative treatments. PD-1/PD-L1 inhibitors have been approved to be used for the first-line treatment of advanced esophageal cancer, which significantly improves patient prognosis (7). The response rate of ICI alone in esophageal cancer varied from 9.9 to 33.3% in the reported studies (8).

A large number of articles regarding anti-PD-1/PD-L1 immunotherapy for esophageal cancer have been published in the past decade. However, no systematic analysis of the data in the available articles has been performed. The increasing number of publications makes it more necessary to illustrate the state of the development by bibliometric methods (9). Bibliometric analysis consists of the quantification and visualization of the data by applying mathematics and statistics (10). In this way, the research trend of the area can be objectively shown. The bibliometric analysis provides researchers valuable information on the development of a specific field from a macro perspective. The most important data source is the Web of Science Core Collection (WoSCC) database (11).

Up to now, there is no published bibliometric study has systematically evaluated the anti-PD-1/PD-L1 immunotherapy for esophageal cancer from 2012 to 2021. In this work, the research tendency and hotspots of anti-PD-1/PD-L1 immunotherapy for esophageal cancer were visually analyzed using HistCite, VOSviewer, and CiteSpace. The aim was to identify the characteristics of publications, build collaboration networks, present hot words, reveal research frontiers and direct the follow-up work (12, 13).

Materials and methods

Data source and search strategy

The literature retrieval was performed online using the WoSCC database, which is the most influential citation academic document database worldwide, in order to collect publications on anti-PD-1/PD-L1 immunotherapy for esophageal cancer (14). The search was performed on June 20, 2022 to ensure the same conditions and avoid the bias resulting from daily updates (15). Since all data were downloaded from the public database, no ethical approval was needed for this work. The following formula was used to perform the advanced search: TS = ((esophageal neoplasm OR esophagus neoplasm OR esophageal cancer OR esophagus cancer OR esophageal tumor OR esophagus tumor OR esophageal carcinoma OR esophagus carcinoma OR ESCC) AND (programmed cell death 1 receptor OR programmed cell death ligand 1 OR CD274 OR B7H1 OR PD-1 OR PD-L1 OR Nivolumab OR Pembrolizumab OR Lambrolizumab OR Avelumab OR Atezolizumab OR Nivolizumab OR Durvalumab OR Pidilizumab OR Cemiplimab OR Camrelizumab OR Sintilimab OR Tisleizumab OR Toripalimab)). The detailed retrieval process and analysis procedure are shown in Figure 1.

Inclusion criteria and exclusion criteria

The inclusion criteria are as follows: (a) literature on anti-PD-1/PD-L1 immunotherapy for esophageal cancer; (b) literature types include articles and reviews; (c) literature published between 2012 and 2021; (d) literature indexed in WoSCC.

The exclusion criteria are as follows: (a) Unpublished documents; (b) Duplicate reports.

Statistical analysis

WoSCC was used to collect publications for bibliometric analysis and visualization. All the data retrieved from WoSCC were exported in plain text format. HistCite (Clarivate Analytics, Philadelphia, PA, the USA), VOSviewer 1.6.14 (Leiden University, Leiden, the Netherlands) and CiteSpace 5.3.R4 (Drexel University, Philadelphia, the USA) were used for statistical analysis (16). HistCite is a citation analysis software that summarizes and processes data quickly (17). In this study, annual output, language type, document type and total number of citations were analyzed by HistCite. VOSviewer is available for building and viewing bibliometric maps, and displays the results of the cluster analysis, including research characteristics,

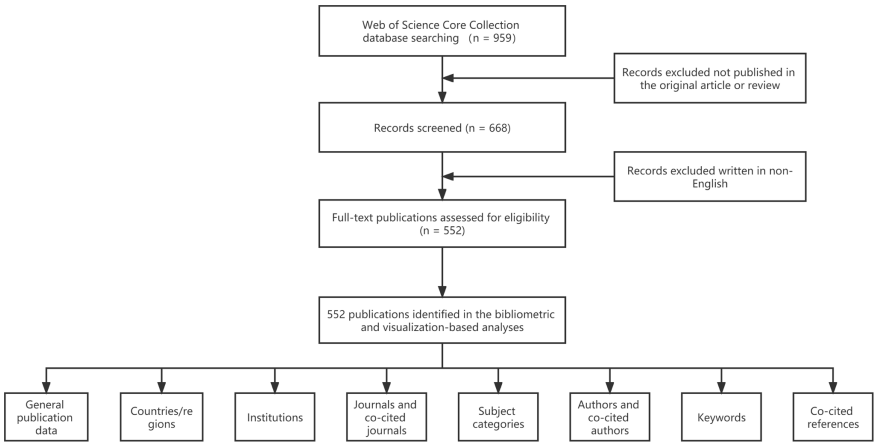

FIGURE 1
Flow Diagram of detailed retrieval process and analysis procedure.

distribution and hotspots (18). The visual maps of countries/regions, institutions, journals, authors, keywords and references were generated by VOSviewer. CiteSpace, a Java application software, was used to explore the collaboration among countries/institutions/authors, identify co-cited authors/references, detect burst keywords and construct visualization maps (19). The software was effective in revealing the trends and dynamics of publications as well as capturing hotspots in a given research field (20). Due to its rich functions, CiteSpace has been widely used for bibliometric analysis. The CiteSpace parameters were as follows: time slicing (2012–2021), years per slice (1), term source (all selection), term type (burst terms), node type (choose one at a time), links (strength: cosine; scope: within slices), selection criteria (top 50 objects), and pruning (pathfinder and pruning sliced networks).

Results

General data and annual output

A total of 552 publications were included in the bibliometric study from 2012 to 2021. These publications were written by 3,623 authors of 872 institutions, 44 countries/regions in 250 journals, with 16,778 citations totally. All publications involved were made up of original articles (n = 410, 74.28%) and reviews (n = 142, 25.72%). The annual output generally maintained an increase trend in the past decade (Figure 2). The most prolific year was 2021 with 216 publications, while the minimum annual output occurred in 2012 with one article. 2014 is the fastest growing year in the past decade. As regard the total citations, the figure peaked at 1,886 in 2018 and bottomed out in 2012.

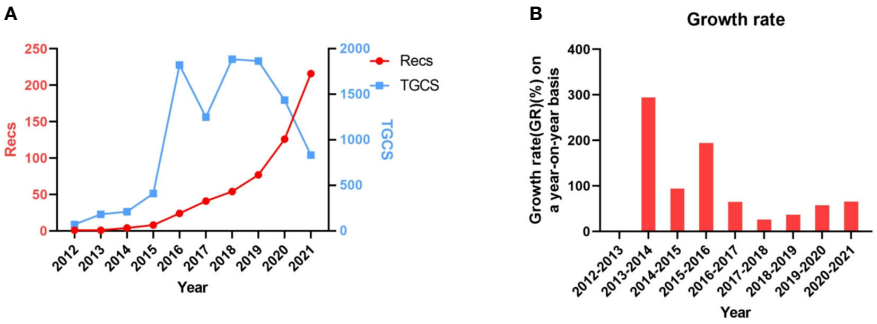

FIGURE 2
(A, B) The annual output, citations and growth rate presented by year from 2012 to 2021.

Countries/regions

A total of 44 countries/regions participated in the publication of anti-PD-1/PD-L1 immunotherapy for esophageal cancer in the last 10 years. Among them, China (n = 227, 32.76%) was the most prolific country/region, followed by the USA (n = 153, 22.08%) and Japan (n = 101, 14.57%). In terms of citations, the USA had the most total citations and France had the highest ratio of Citations/Paper. Table 1 lists the top 8 most prolific countries/regions. A network map was constructed for countries with more than 5 publications. Figure 3 shows that the map had 18 nodes. The 3 largest nodes respectively represented China, the USA and Japan for their huge number of publications. The USA had the most active cooperation with others, with the strongest total link strength (TLS, TLS = 125). The closest cooperation was between China and the USA (TLS = 27).

Institutions

A total of 872 institutions contributed to the research of anti-PD-1/PD-L1 immunotherapy for esophageal cancer. The top 10 most productive institutions from 2012 to 2021 are listed in Table 2. Natl Canc Ctr (Japan, 23 publications) and Zhengzhou Univ (China, 23 publications) tied first place, followed by Sun Yat Sen Univ (China, 21 publications), Natl Canc Ctr Hosp East (Japan, 19 publications) and Fudan Univ (China, 16 publications). The publications of the top 10 institutions accounted for more than 29% of the total publications. As regard the citations, Dana Farber Canc Inst (the USA, 1,108 citations) ranked first. Natl Canc Ctr Hosp East (Japan, 733 citations) and Natl Canc Ctr (Japan, 725 citations) came second and third, respectively. Figure 4 shows the co-authorship network among institutions with 10 or more publications. It is evident that institutions in the same district always closely cooperate with each other. Natl Canc Ctr Hosp East (TLS = 78) had the most active cooperation with others. The closest cooperation was between Natl Canc Ctr Hosp East and Natl Canc Ctr (TLS = 14).

Journals and co-cited journals

All the 552 papers were published in 250 journals. The top 10 prolific journals and co-cited journals are listed in Table 3. The 10 most prolific journals published 164 papers, accounting for 29.71% papers involved in this study. The IF of these journals ranged from 3.111 to 13.801, half of which were higher than 6. Among these journals, *Frontiers in Oncology* (IF = 5.738; 22 publications) had most publications, followed by *Cancer Science* (IF = 6.518; 14 publications) and *Journal for Immunotherapy of Cancer* (IF = 12.469; 13 publications). The top 3 co-cited journals were as follows: *Journal of Clinical Oncology* (IF = 50.717; 1,788 co-citations), *New England Journal of Medicine* (IF = 176.079; 1,232 co-citations) and *Lancet Oncology* (IF = 54.433; 996 co-citations). The co-citations of the 50% of the listed journals were greater than 700 and 7 of the top 10 co-cited journals had IF higher than 50.

Authors and co-cited authors

A total of 3,623 authors contributed to the involved publications. Table 4 shows that the top 10 authors were all from Japan. Among them, Kato Ken with 16 publications and 410 citations was the most productive author, followed by Kojima Takashi (12 publications, 440 citations) and Doi Toshihiko (10 publications, 394 citations). As regard the co-cited authors, Bang Yung-Jue from South Korea ranked first with 197 co-citations, followed by Kato Ken (193 co-citations) and Fuchs Charles S. (184 co-citations).

VOSviewer analyzed the information of authors and co-cited authors, then visualized it in a network map to explore influential researchers and potential collaborators (Figure 5) (21). The 42 authors with more than 5 publications formed

TABLE 1 The top 8 countries according to total publications from 2012 to 2022.

Rank	Country	Number of Publications	Proportion (%)	Total Citations	Citations/Paper
1	China	227	32.76%	3583	15.78
2	USA	153	22.08%	4562	29.82
3	Japan	101	14.57%	2406	23.82
4	England	30	4.33%	1147	38.23
5	Germany	25	3.61%	1145	45.80
6	South Korea	20	2.89%	738	36.90
7	Italy	20	2.89%	480	24.00
8	France	17	2.45%	920	54.12

Data were retrieved from 552 publications with VOSviewer on June 20, 2022.

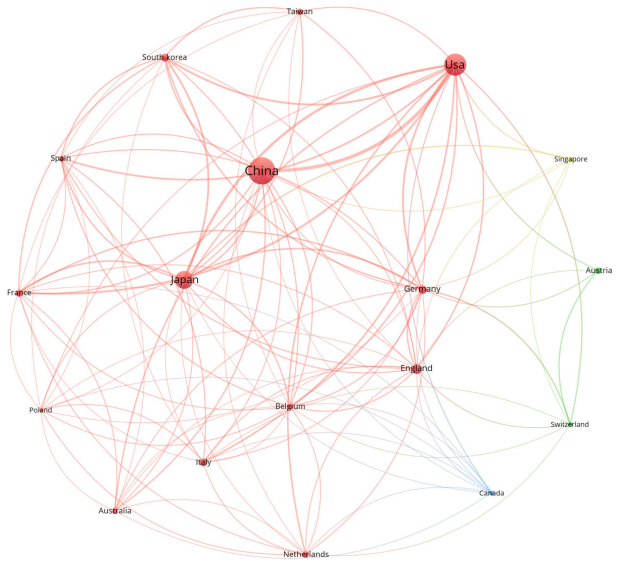

FIGURE 3
The co-authorship network visualization map of countries/regions. Larger nodes represent more publications of the term. Lines between nodes represent the connection between them.

several clusters and almost no collaboration was present among the clusters. The linkages among the authors were clearly less robust. However, the linkages among authors from same cluster were relatively close. When it comes to the co-cited authors, the minimum number of co-citations was set as 60. Unlike authors, the collaborations among 31 co-cited authors were quite active.

Subject categories

In the present study, CiteSpace was used to analyze the information regarding publication categories and construct a knowledge map (Figure 6). The larger node represented more publications of the term. Nodes with high centrality were usually considered as pivotal points in the field (22). In this work, the top 5 subject categories were selected according to the publication number and centrality. Table 5 shows that ONCOLOGY and IMMUNOLOGY ranked first and second, respectively.

Keywords

High-frequency keywords represent the hot topics in a particular field. Fifty-nine keywords with more than 5 occurrences were extracted from 552 publications. The top 4 keywords with most occurrences were listed as follows: esophageal cancer (n = 123), immunotherapy (n = 119), esophageal squamous cell carcinoma (n = 89), and PD-L1 (n = 79). VOSviewer was used to construct the network map of keywords, including esophageal cancer, immunotherapy, esophageal squamous cell carcinoma, PD-L1, PD-1, prognosis and so on (Figure 7A).

CiteSpace was used to identify and analyze keywords with citation bursts, thereby indicating the research hotspots and emerging trends over a period time. The minimum burst duration was set as 1 year. The top 34 keywords with the strongest citation burst are listed in Figure 7B. Among them, clinical significance had the highest burst strength (8.44). Response, PD-1 blockade, $CD8^+$ T cell, and melanoma were the four keywords with the highest burst strength from 2018 to 2019. Neoadjuvant chemotherapy (NCT) was the top 1 keyword with the strongest citation bursts recently.

Co-cited references

Co-cited reference is regarded as one of the most valuable indicators in bibliometrics that displays the key landmark articles of this field (23–25). Table 6 lists the top 10 co-cited references. Among them, 8 articles were clinical trials, 2 were original articles. The article written by Freddie Bray et al. published in *CA Cancer J Clin* ranked first (n = 111) (26), followed by a clinical trial written by Yoon-Koo Kang et al. in *Lancet* (n = 99) (27) and another clinical trial written by Ken Kato et al. in *Lancet Oncol* (n = 97) (28). A co-citation network

TABLE 2 The top 10 most productive institutions from 2012 to 2021.

Rank	The name of institution	Publications	Citations	Location
1	Natl Canc Ctr	23	725	Japan
2	Zhengzhou Univ	23	283	China
3	Sun Yat Sen Univ	21	168	China
4	Natl Canc Ctr Hosp East	19	733	Japan
5	Fudan Univ	16	85	China
6	Mem Sloan Kettering Canc Ctr	15	488	the USA
7	Soochow Univ	13	362	China
8	Dana Farber Canc Inst	12	1108	the USA
9	Chinese Acad Med Sci	12	284	China
10	Chinese Acad Med Sci & Peking Union Med Coll	12	58	China

Data were retrieved from 552 publications with VOSviewer on June 20, 2022.

map was created using articles with more than 40 co-citations and explored the connection among these articles. The map contained 27 nodes, which clearly indicated the scientific relevance among these references. Figure 8A shows that the largest node represented the most co-cited reference. "Yoon-Koo Kang, 2017, *Lancet*, V390, P2461" (TLS = 606) (27) had the most active association with other references, followed by "Charles S Fuchs, 2018, *JAMA Oncol*, V4" (TLS = 529) (32) and "Manish A Shah, 2019, *JAMA Oncol*, V5, P546" (TLS = 480) (30).

References with citation bursts refer to those that are frequently cited during certain a period of time (36). CiteSpace was used to perform references with citation bursts, and the minimum burst duration was set as 1 year. The blue line in Figure 8B represents the timeline in years, while the red line represents the time range in which a reference had citation burst (37). The burst strength of the top 33 references ranged from 4.05 to 14.25. Among them, "Topalian SL, 2012, *New Engl J Med*, V366, P2443 (35)" had the highest burst strength (14.25), which ranked tenth in the list of co-citations, indicating the great influence of this study. The article assessed the antitumor activity and safety of anti–PD-1 antibody in cancer, showing that the adverse-event profile does not appear to preclude its use. "Kudo T, 2017, Lancet Oncol, V18, P631" (29), "Doi T, 2018, *J Clin Oncol*, V36, P61" (38) and "Jiang YB, 2017, *Oncotarget*, V8, P30175" (39) were 3 co-cited references with recent bursts. Toshihiro Kudo et al. conducted a phase II clinical trial and suggested that nivolumab exhibited favorable activity and controllable safety in ESCC (29). Toshihiko Doi et al. reported

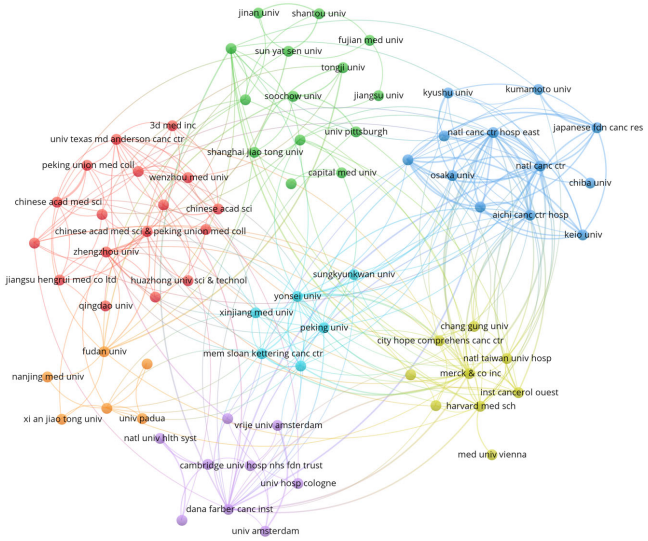

FIGURE 4
The co-authorship network visualization map of institutions.

TABLE 3 The top 10 journals and co-cited journals on anti-PD-1/PD-L1 immunotherapy for esophageal cancer from 2012 to 2021.

Rank	Journal	Publication number	Citation	IF#	Co-cited journal	Co-citation	IF
1	Frontiers in Oncology	22	165	5.738	Journal of Clinical Oncology	1788	50.717
2	Cancer Science	14	434	6.518	New England Journal Of Medicine	1232	176.079
3	Journal for Immunotherapy of Cancer	13	298	12.469	Lancet Oncology	996	54.433
4	Annals of Translational Medicine	11	54	3.616	Clinical Cancer Research	853	13.801
5	Cancers	11	37	6.575	Annals of Oncology	787	51.769
6	Future Oncology	11	207	3.674	Lancet	591	202.731
7	Cancer Immunology Immunotherapy	10	142	6.630	Oncotarget	553	——
8	Clinical Cancer Research	8	812	13.801	Nature	552	69.504
9	Oncotargets and Therapy	8	456	4.345	Cancer Research	502	13.312
10	Oncology Letters	8	80	3.111	Science	352	63.714

Data were retrieved from 552 publications with VOSviewer on June 20, 2022.
#: Abbreviation for Impact Factor.

the results of KEYNOTE-028, a phase Ib study on PD-L1(+) patients with advanced solid tumors (38). Pembrolizumab displayed controllable toxicity and persistent antitumor activity in these patients. Yubo Jiang et al. revealed the prognostic significance of tumor-infiltrating immune cells and PD-L1 expression in ESCC (39).

Discussion

Esophageal cancer has a high degree of malignancy and poor prognosis. As a new therapeutic method, immunotherapy can significantly improve the prognosis of patients (40, 41). The anti-PD-1/PD-L1 antibody is the most commonly used ICI. Therefore, it is important to build an in-depth understanding of publications in this field. In this study, a bibliometric analysis of anti-PD-1/PD-L1 immunotherapy for esophageal cancer from 2012 to 2021 was performed, presenting the research hotspots and trends.

Figure 2 shows that the annual output maintained a rapid growth over the last decade. Literature published between 2012 and 2016 mostly focused on the expression and prognostic value of PD-1/PD-L1. In 2017, results of clinical trials of ICIs for esophageal cancer began to be published. From then on, the annual output increased rapidly, from 40 in 2017 to 216 in 2021. The annual growth rate also increased year by year.

In 2017, Toshihiko Doi (38) and Toshihiro Kudo (29) released their respective clinical trial results, which respectively demonstrated that Pembrolizumab and Nivolumab had certain anti-tumor effect in PD-L1(+) patients who failed second-line or back-like treatment. In 2018, Huang Jing et al. conducted a study on 30 patients with relapsed or metastatic advanced ESCC that showed chemoresistance previously (42). According to their results, the anti-PD-1drug SHR-1210 exhibited definite antitumor activity, with tolerable toxic and side effects. In 2019, the research data from multiple clinical trials were released, including KEYNOTE-180 (30), KEYNOTE-181 (43) and ATTRACTION-03 (28). As revealed by KEYNOTE-180 and

TABLE 4 The top 10 prolific authors and co-cited authors on anti-PD-1/PD-L1 immunotherapy for esophageal cancer research from 2012 to 2021.

	Author				Co-cited authors		
Rank	Name	Publications	Citations	Country	Name	Co-citations	Country
1	Kato Ken	16	410	Japan	Bang Yung-Jue	197	South Korea
2	Kojima Takashi	12	440	Japan	Kato Ken	193	Japan
3	Doi Toshihiko	10	394	Japan	Fuchs Charles S.	184	the USA
4	Baba Hideo	8	222	Japan	Shah Manish A.	163	the USA
5	Yoshida Naoya	8	222	Japan	Le Dung T.	158	the USA
6	Doki Yuichiro	8	114	Japan	Janjigian Yelena Y.	154	the USA
7	Kono Koji	7	264	Japan	Shitara Kohei	134	Japan
8	Ishimoto Takatsugu	7	221	Japan	Kojima Takashi	125	Japan
9	Iwatsuki Masaaki	7	221	Japan	Kang Y.K.	117	South Korea
10	Mori Masaki	7	194	Japan	Topalian Suzanne L.	117	the USA

Data were retrieved from 552 publications with VOSviewer on June 20, 2022.

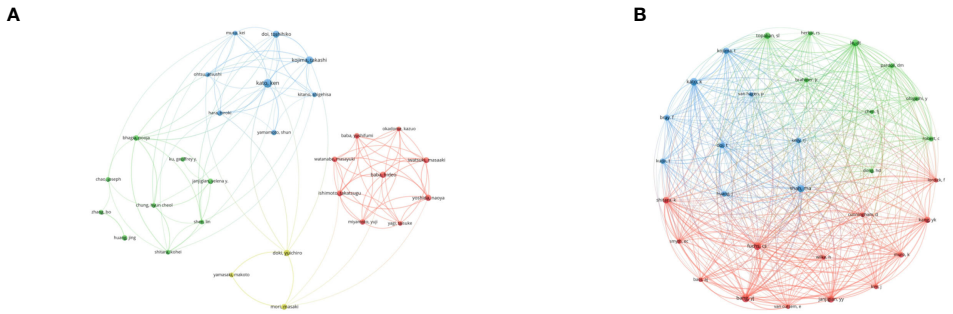

FIGURE 5
(A) Co-authorship network visualization map of authors. **(B)** Co-citation network visualization map of authors.

KEYNOTE-181, Pembrolizumab had remarkable therapeutic effect and favorable safety on patients with PD-L1(+) advanced esophageal carcinoma, supporting the application of Pembrolizumab as the new second-line standard treatment for PD-L1(+) metastatic esophageal carcinoma. In 2019, Pembrolizumab was approved by the USA FDA to be used to treat relapsed, locally advanced or metastatic ESCC patients who had received first-line or later-line systemic treatment, with positive PD-L1 expression in tumor tissues (CPS≥10). In 2020, a breakthrough was made in the immunotherapy for esophageal carcinoma. The preliminary research results from KEYNOTE-590 demonstrated the satisfactory therapeutic effect and safety of Pembrolizumab combined with chemotherapy in the first-line treatment for advanced esophageal carcinoma (44). In the 2020 V5 version of NCCN guidelines, Pembrolizumab combined with platinum-based chemotherapeutic regimens was recommended in the first-line treatment of unresectable, locally advanced, locally relapsed or metastatic esophageal carcinoma with PD-L1 CPS≥10 and negative HER-2 expression. Additionally, CheckMate-577 ushered in the new chapter of adjuvant immunotherapy for esophageal carcinoma, which comprehensively evaluated the therapeutic effect of adjuvant nivolumab on patients with esophageal carcinoma and gastroesophageal junction carcinoma who did not achieve complete pathological remission after neoadjuvant radiochemotherapy (NRCT) (45). Clinical trials of neoadjuvant immunotherapy, such as NICE, KEEP-G 03, and PALACE-1, also reported the preliminary results. In March 2021, based on the KEYNOTE-590 research results, the USA FDA approved the use of Pembrolizumab combined with platinum-based chemotherapy as the first-line treatment for unresectable locally advanced or metastatic esophageal

FIGURE 6
The visualization map of subject categories. The tree ring-shaped nodes represented different subject categories. The lines between two nodes meant co-occurrence. The area of the nodes referred to the number of publications. Nodes with high centrality were deemed as the hot field.

TABLE 5 Top 5 subject categories in terms of publication number and centrality related to anti-PD-1/PD-L1 immunotherapy for esophageal cancer.

Rank	Publications	Category	Centrality	Category
1	539	ONCOLOGY	1.32	ONCOLOGY
2	94	IMMUNOLOGY	0.82	RESEARCH & EXPERIMENTAL MEDICINE
3	74	RESEARCH & EXPERIMENTAL MEDICINE	0.72	CELL BIOLOGY
4	74	GENERAL & INTERNAL MEDICINE	0.66	PATHOLOGY
5	71	GASTROENTEROLOGY & HEPATOLOGY	0.42	SURGERY

Data were retrieved from 552 publications with CiteSpaceV on June 20, 2022.

carcinoma or gastrointestinal junction carcinoma or those not suitable for radical radiochemotherapy, regardless of the PD-L1 expression status. The results of neoadjuvant immunotherapy combined with chemotherapy or radiochemotherapy were also released in 2021. The rapid development of immunotherapy for esophageal cancer suggests the great potential of the field in the future. Given that the feasibility and safety of anti-PD-1/PD-L1 immunotherapy have been confirmed, there might be more publications in the following years. The development prospects of immunotherapy for esophageal cancer could be expected.

China was the top 1 country ranked by total publications, which was consistent with epidemiological status that the incidence rate and fatality rate were high in this country. Although China had a huge number of publications, its citation was not impressive. Germany and France with less publications had high ratio of Citations/Paper, reflecting the high quality of their publications. The USA was the most active collaborator in Figure 3 and played an important role in international cooperation.

As China is one of the high-risk areas of esophageal cancer, 6 of the top 10 institutions are from China. However, the articles published in China were scarcely cited, reflecting the weak influence of these publications. Therefore, Chinese institutions need to find methods to improve the quality of publications.

Natl Canc Ctr and Natl Canc Ctr Hosp East located in Japan were institutions that not only productive but also influential. They were both participating institutions of several important clinical trials, including KEYNOTE-180, KEYNOTE-181 and KEYNOTE-590. Ken Kato from National Cancer Center Hospital together with Toshihiko Doi and Takashi Kojima from National Cancer Center Hospital East were all contributed to the three clinical trials. They were also the top 3 prolific authors. Kato Ken who was the most prolific author ranked second in terms of co-cited authors, and he was a key

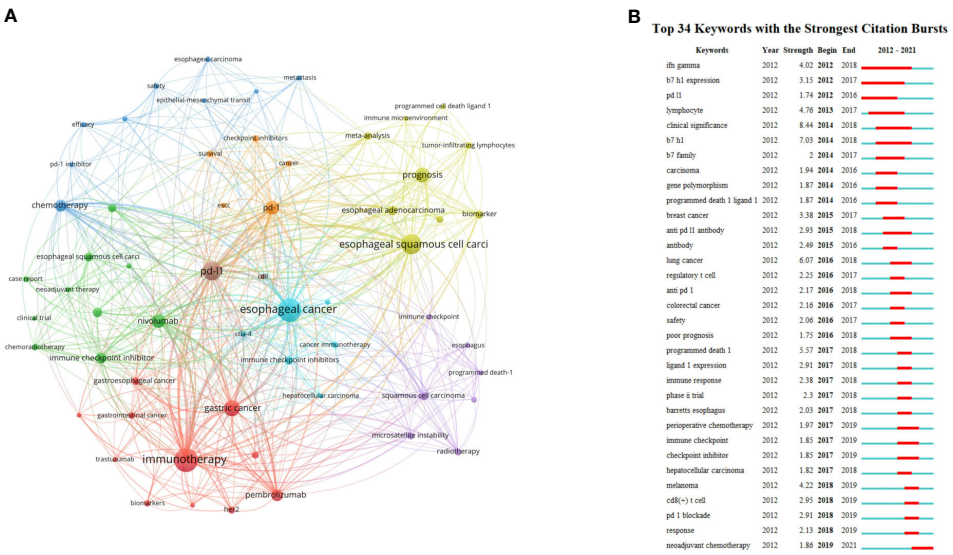

FIGURE 7
(A) The co-occurrence network visualization map of keywords. Keywords in the same color represent were sorted into the same cluster. **(B)** The top 34 keywords with the strongest citation bursts from 2012 to 2021. The red segment of the blue line denoted the burst duration of a keyword.

TABLE 6 The top 10 co-cited reference from 2012 to 2021.

Rank	Co-cited reference	Count
1	Freddie Bray, 2018, CA Cancer J Clin, V68, P394 (26)	111
2	Yoon-Koo Kang, 2017, Lancet, V390, P2461 (27)	99
3	Ken Kato, 2019, Lancet Oncol, V20, P1506 (28)	97
4	Toshihiro Kudo, 2017, Lancet Oncol, V18, P631 (29)	91
5	Manish A Shah, 2019, JAMA Oncol, V5, P546 (30)	83
6	Dung T Le, 2015, New Engl J Med, V372, P2509 (31)	81
7	Charles S Fuchs, 2018, JAMA Oncol, V4 (32)	79
8	Yuichiro Ohigashi, 2005, Clin Cancer Res, V11, P2947 (33)	76
9	Yung Jue Bang, 2010, Lancet, V376, P1302 (34)	74
10	Suzanne L Topalian, 2012, New Engl J Med, V366, P2443 (35)	73

figure of ATTRACTION-3. The top 10 prolific authors were all from Japan, indicating that Japanese scientists made a tremendous contribution in this field.

The analysis of prolific journals guides scientists in identifying core journals for information access and manuscript submissions. Three journals were highly recommended to scientists in the field: *Frontiers in Oncology*, *Cancer Science* and *Journal for Immunotherapy of Cancer*. Moreover, *Journal of Clinical Oncology*, *Lancet Oncology*, and *New England Journal of Medicine*, were the most authoritative journals in this field according to the co-citation amount shown in Table 3. As regard the subject categories shown in Figure 6 and Table 5, ONCOLOGY and IMMUNOLOGY occupied central positions in this field, which were consistent with the analyzing results of the journals.

The current research hotspots were obtained from the high frequency keywords and cited references, which helped researchers to rapidly understand the direction of the research. This work lists the remarkable highlights of the research field as follows.

Clinical significance had the highest burst strength among the 34 keywords. It has always been significant in the field from 2014 to 2018. During this period of time, many studies focused on the prognostic value of PD-1/PD-L1 in patients with esophageal cancer. Numerous studies have suggested that, PD-L1 expression is related to the adverse clinical outcomes of esophageal cancer, supporting its role as a prognostic biomarker (39, 46–49). Further study found that PD-L1 expression in ESCC tumor cells was significantly associated with worse survival while no statistical significance was found between PD-L1 expression in ESCC tumor-infiltrating immune cells and survival (50). Recently, Peipei Wang et al. claimed that increased co-expression of PD-L1 and TIM3/TIGIT was associated with poor overall survival of ESCC (51).

Neoadjuvant chemotherapy was the hottest keywords in the last two years. With the moving forward of immunotherapy,

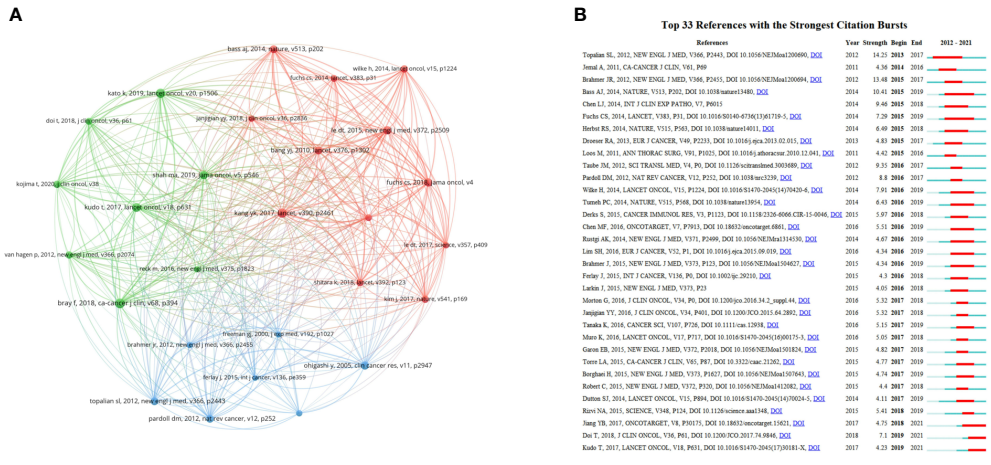

FIGURE 8
(A) The co-citation network visualization map of references from 2012 to 2021. **(B)** The top 33 references with the strongest citation bursts from 2012 to 2021. The red segment of the blue line denoted the burst duration of a keyword.

more and more publications about NCT or NRCT plus immunotherapy are available at present. Multiple studies have evaluated the safety, feasibility and efficacy of neoadjuvant PD-1/PD-L1 inhibitors combined with chemotherapy in treating esophageal cancer patients (52–62). The neoadjuvant treatment of PD-1/PD-L1 inhibitor with chemotherapy produced satisfactory outcomes, indicating its potential as a promising neoadjuvant treatment for esophageal cancer. Besides, Wenqun Xing et al. designed a study to explore the impact of chemotherapy and toripalimab sequence on the pathological complete response (pCR) rate and safety of locally advanced ESCC patients (63). The initial results showed that delaying toripalimab to day 3 in chemoimmunotherapy might achieve a higher pCR rate than that on the same day. In the PERFECT trial, we investigated the feasibility and efficacy of NCRT combined with PD-L1 inhibitor (64). However, most of these studies were single-arm, phase I or II clinical trials. The long-term efficiency of this novel treatment and the validity of the present findings should be confirmed with more large-scale, longer follow-up and prospective comparative trials. In the existing clinical trials on neoadjuvant immunotherapy for esophageal carcinoma, no definite molecular biomarkers are available for selecting the possibly beneficial population. The previous research mainly focused on PD-L1, TMB, EGFR and CD8+ T cells. These studies not only help to identify the molecular biomarkers, but also provide ideas for the design of phase III clinical trials. Further studies should confirm more predictive biomarkers as well as indicators for the selection of a specific treatment.

The application of PD-1/PD-L1 inhibitors in esophageal cancer has achieved unprecedented successes. However, some treated patients exhibit non-response and severe immune-related adverse events. Therefore, immunotherapeutic markers are needed to assist in screening populations who can gain benefits from immunotherapy. At present, PD-L1 expression is used as the major biomarker for efficacy prediction in the application of PD-L1 inhibitors (65). With the deepening of research, DNA mismatch repair-deficient/microsatellite instability (dMMR/MSI) (66), tumor mutational burden (TMB) (67), copy number variation (CNV), polymerase epsilon (POLE) (68, 69), circulating tumor DNA (ctDNA) (70), inflamed gene expression profile (71, 72), tumor-infiltrating lymphocytes (TILs) (73), and immune gene signatures (74) have been suggested to show certain potential in predicting efficacy, which deserve further verification. Park R et al. Mentioned that, for esophageal cancer, there is no highly sensitive or specific marker apart from dMMR/MSI-H (75). Consequently, it is necessary to develop biomarkers for immunotherapy.

For the time being, the sensitivity and specificity of single biomarkers are not high enough. As a result, they may not be used as biomarkers alone. The combination of multiple biomarkers contributes more to predicting the immunotherapeutic efficacy in esophageal cancer. Moreover, when immunotherapy is used in combination with other treatments, whether a specific biomarker can maintain its prediction ability should be further analyzed. Whether predictors verified in advanced tumors can be applied in perioperative treatment is another important problem to be answered by on-going and future trials. In the future, it is promising to develop more precise tools to predict the anti-PD-1/PD-L1 therapeutic efficacy in esophageal cancer patients by standardizing and normalizing diverse biomarkers, intensively investigating the relations of different biomarkers, and applying computer technologies and medical databases, which is of great significance for individualized immunotherapy.

As far as we know, this is the first bibliometric study regarding anti-PD-1/PD-L1 immunotherapy for esophageal cancer in the past decade. The article hopes to guide scholars select research direction, references, cooperative institutions and authoritative journals. The data analysis was relatively objective and comprehensive, clearly displaying the research status visually. However, here are some limitations as follows.

1. The study included articles and reviews retrieved from WoSCC. Articles of other types or from other databases could not be involved in our study, thus limit the comprehensiveness of the study.
2. Papers published from 2012 to 2021 were retrieved on June 20, 2022. However, the database is still updating the data. Therefore, some recent publications could not be included. Besides, the number of citations of recent literature might be affected.
3. All of the publications included were in English, which might lead to a linguistic bias. Languages like Chinese, Japanese, French, German, Polish, Hungarian, Portuguese, Rumanian and Korean were not involved in the database. Therefore, it is likely that our results may not be applicable to publications in other languages.
4. Although analysis process was performed by software objectively, the method to explain these results had inherent subjective bias by individuals.

Still, it is believed that this article provides the overall situation and research trend of anti-PD-1/PD-L1 immunotherapy for esophageal cancer. The results could provide readers a general overview of the landscape, especially to those without in-depth knowledge. The information could also be used to explore possible collaboration partners, potentially relevant publications, and promising research directions. Our study not only exhibits important milestones of esophageal cancer immunotherapy but also offers a better guide to the future. We sincerely hope that bibliometric and visual analyses will give us more ideas in this field.

Conclusion

To conclude, this article provides a comprehensive understanding of publications on anti-PD-1/PD-L1 immunotherapy for esophageal cancer from 2012 to 2021, providing valuable information to researchers in this field. This article presents data on the trend of annual output, countries/regions, institutions, journals, authors, subject categories, keywords, and co-cited references obtained using bibliometric analysis. Neoadjuvant chemotherapy, response, PD-1 blockade and CD8+ T cell were four latest research frontiers. Further studies and more cooperation are needed worldwide. Overall, our results could help the discovery of new perspectives and determine future directions.

Data availability statement

Data were retrieved from 552 publications with VOSviewer on June 20, 2022. The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ supplementary material.

Author contributions

Study conception, design and data analysis: YY. Paper writing: YY. Language polishing, paper review and editing: FW. All authors read and approved the submitted version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Single cell sequencing revealed the mechanism of PD-1 resistance affected by the expression profile of peripheral blood immune cells in ESCC

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Background: Esophageal squamous carcinoma (ESCC) is a highly lethal malignancy with poor prognosis. The effect of transcriptome characteristics of patient immune microenvironment (TME) on the efficacy of immunosuppressive agents is still poorly understood.

Methods: Here we extracted and isolated immune cells from peripheral blood of patients with PD-1 monoclonal antibody sensitivity and resistance, and conducted deep single-cell RNA sequencing to describe the baseline landscape of the composition, lineage, and functional status of infiltrating immune cells in peripheral blood of patients with esophageal cancer.

Results: The transcriptome characteristics of immune cells were comprehensively analyzed, and the dynamic changes of cell percentage, heterogeneity of cell subtypes and interactions between cells were explained. Co-expression and pedigree tracking based on T-cell antigen receptors revealed a significant proportion of highly migratory intertissue-effector T cells. GO and KEGG enrichment pathway Analysis of $CD8^+$ effect-T cells ESCC_S group and ESCC_D1,2 group, found that in the up-regulated enrichment pathway, ESCC_S group enriched more PD-L1 and PD-1 checkpoint pathways expressed in tumors (JUN/NFKBIA/FOS/KRAS/IFNG), which also exist in T cell receptor signaling pathways. MT2A, MT1X and MT1E were differentially expressed in ESCC patients with PD-1 monoclonal antibody resistance, which may be related to the resistance of PD-1 mMAB.

Conclusions: This study has an in-depth understanding of the influence of peripheral immune cell infiltration on the sensitivity of monoclonal antibody

PD-1 in patients with esophageal cancer, which is helpful to promote the immunotherapy of patients with esophageal cancer.

KEYWORDS

single cell sequencing, PD-1 mMAB, peripheral blood, immune cells, ESCC

Introduction

Esophageal squamous carcinoma (ESCC) is one of the most challenging gastrointestinal tumors with high mortality (1). It was estimated to be the ninth most common cancer and the fifth leading cause of cancer death globally (2, 3). Current treatment options for patients with esophageal cancer include multidisciplinary management of local regional and locally advanced disease, and chemotherapy for palliative treatment of metastatic disease; however, esophageal cancer has a poor prognosis, with a low 5-year survival rate (4). With the widespread use of immune checkpoint inhibitors (ICIs), immunotherapy has made significant advances in the treatment of cancer patients. ICIs has several approved indications for the treatment of metastatic gastrointestinal malignancies, including gastric, esophageal, colorectal and hepatocellular carcinomas (5, 6).

PD-1 (programmed cell death -1) is a member of the CD28 cell surface receptor family that is expressed on activated T cells, B cells, NKT cells, monocytes, and macrophages, is crucial in regulating T -cell activation and tolerance (6, 7). The programmed death-ligand 1 (PD-L1)/PD-1 pathway is one of the most studied mechanisms of tumor immune escape (8). The successful development of PD-1 and PD-L1 monoclonal antibodies (mMAB) has led to significant advances in immunotherapy. Despite the clinical promise of ICIs, only a small proportion of patients with CI-responsive cancer subtypes benefit from treatment with anti-PD-1, PD-L1, and CTLA4 antibodies because of the high heterogeneity and complexity of cancer and the multiple mechanisms it uses to evade immune surveillance in addition to inhibiting anti-tumor T cell responses (9). It is of great significance to explore the mechanism of influencing the sensitivity of patients with esophageal cancer to PD-1 mMAB and to find effective biomarkers for predicting efficacy.

Various types of immune cells are present in peripheral blood, and studies have reported that they can predict the treatment response and clinical efficacy of anti-immune checkpoint inhibitors in patients with advanced cancer (10). The PBMC (Peripheral blood mononuclear cell) cell model, which includes T and B cells (~80%), natural killer cells (~10%), and monocytes (~10%), plays an important role in immune responses (11). Due to the availability and reproducibility of peripheral blood, it is more simple and feasible to study biomarkers of anti-tumor immunity by detecting and analyzing blood components compared with tissue samples (12). Therefore, the analysis of immune cells in peripheral blood of esophageal cancer patients is of great significance to explore the mechanism of PD-1 mMAB resistance in esophageal cancer.

Based on single cell data analysis, the expression of MT2A, MT1X and MT1E decreased in ESCC patients resistant to PD-1 monoclonal antibody, which may be related to PD-1 mMAB resistance. Metallothioneins (MTs) are small proteins rich in cysteine, which play an important role in metal homeostasis and prevention of heavy metal toxicity, DNA damage and oxidative stress. In humans, there are four main subtypes of Metallothioneins (MTs) (MT1, MT2, MT3, and MT4), which are encoded by a gene located on chromosome 16q13 (13). New evidence suggests that MTs play a key role in tumor formation, progression and drug resistance. However, MTs expression is not universal in all human tumors and may depend on tumor type and state of differentiation, as well as other environmental stimuli or genetic mutations.

MT2A stability triggers the apoptosis switch in stress response. XAF1 interacts directly with MT2A and promotes its lysosomal degradation, leading to elevated levels of free intercellular zinc, followed by p53 activation and XIAP inactivation. XAF1 is activated as a unique transcription target of metal-regulated transcription factor-1 (MTF-1) in signaling apoptosis, and its protein is unstable in the lysosomal pathway of MT2A induced by MTF-1 under cellular quiescent stress, indicating mutual antagonism between XAF1 and MT2A. Clinically, XAF1 and MT2A expression levels are negatively correlated in primary colon cancer and multiple cancer cell lines (14). Direct interaction of MT2A with BARD1 and BRCA1, co-localization of MT2A and BARD1 was detected by immunofluorescence. MT2A knockdown enhances oxaliplatin sensitivity in HT29 OR cells MT2A interacts with BARD1/BRCA1 and positively regulates and promotes oxaliplatin resistance in colorectal cancer cells (15). MT1X is considered as a tumor inhibitor, and is involved in the progression and metastasis of HCC (16). Expression and survival analysis showed that MT1X mRNA expression level was higher in normal tissues,

which was associated with better prognosis of HCC patients (17). MT1E inhibits cell growth *in vitro* and *in vivo*, and MT1E can induce apoptosis of HCC cells and inhibit their metastasis. MT1E epigenetic silencing caused by promoter methylation may play an important role in HCC (18).

In this study, we performed single-cell RNA sequencing (scRNA-seq) to analyze peripheral blood immune cells from 4 patients with esophageal cancer who were treated with PD-1 mMAB. By comparing the peripheral blood immune cells of PD-1 mMAB sensitive and resistant patients, we comprehensively analyzed the transcriptomic characteristics of immune cells, and deciphered the dynamic changes of cell percentage, the heterogeneity of cell subtypes, and the interactions between cells, providing new knowledge for the biological basis of immunotherapy for esophageal cancer.

Methods

Human tissues dissociation and preparation

The tumor tissue samples of ESCC patients were obtained from Tianjin Medical University Cancer Institute and Hospital. As determined by clinical specialists, patients with PR (partial response, partial response was achieved with a reduction of ≥30% in the sum of maximum diameters of target lesions for at least 4 weeks) or SD (stable disease, the disease was stable, and the sum of maximum diameters of target lesions was not reduced to PR or enlarged to PD) after immunotherapy for more than half a year were classified as sensitive group, and patients with PD (progressive disease, disease progression, an increase of at least 20% in the sum of the maximum diameters of target lesions, or the appearance of new lesions) after immunotherapy for less than half a year were classified as drug-resistant group. We collected patients with advanced esophageal squamous cell carcinoma who were unresectable, sensitive or resistant to PD-1 mMAB, including two patients in each group. Their peripheral blood was collected and PBMCs were isolated for single-cell transcriptome sequencing. All patients provided informed consent, and Tianjin Medical University Cancer Institute and the hospital ethics Committee approved all aspects of the study (Ethics Approval Number: E2020169).

Single cell RNA sequencing

The single-cell suspension with the concentration of 1×10^5 cells/mL was prepared in PBS (HyClone). Single-cell suspensions were then loaded onto microfluidic devices and scRNA-seq libraries were constructed according to Singleron GEXSCOPER protocol by GEXSCOPER Single-Cell RNA Library Kit (Singleron Biotechnologies) (19). Individual libraries were diluted to 4nM and pooled for sequencing. Pools were sequenced on Illumina HiSeq X with 150 bp paired end reads.

scRNA-seq quantifications and statistical analysis

The original reads are processed using an internal pipeline to generate gene expression profiles. Briefly, after filtering read one without poly T tails, cell barcode and UMI (unique molecular identifiers) was extracted. Adapters and poly A tails were trimmed (fastp V1) before aligning read two to GRCh38 with ensemble version 92 gene annotation (fastp 2.5.3a and featureCounts 1.6.2) (20). Reads with the same cell barcode, UMI and genes are grouped together to calculate the number of UMI per gene per cell. Using the same cell barcode, combine UMI and genes, and calculate the number of UMIs for each gene in each cell. Use the UMI count table of each cell's barcode for further analysis.

Further analysis was performed using the UMI counting table for each cell bar code.

Reads with the same cell barcode, UMI and gene were grouped together to calculate the number of UMIs per gene per cell. The UMI count tables of each cellular barcode were used for further analysis. Cell type identification and clustering analysis using Seurat program (21, 22). The Seurat program (http://satijalab.org/seurat/ , R package,v.3.0.1) was applied for analysis of RNA-Sequencing data. UMI count tables were loaded into R using read.table function. Then we set the parameter resolution to 0.6 for FindClusters function to clustering analyses. Differentially expressed genes (DEGs) between different samples or consecutive clusters were identified with function FindMarkers. GO function enrichment analysis was performed on the gene set using the clusterProfiler software to find biological functions or pathways that are significantly associated with the genes specifically expressed (23).

Primary analysis of raw read data

Raw reads from scRNA-seq were processed to generate gene expression matrixes using an internal pipeline. Briefly, raw reads were first processed with fastQC (24) v0.11.4 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and fastp (25) to remove low quality reads, and with cutadapt (26) to trim poly-A tail and adapter sequences. Cell barcode and UMI were extracted. After that, we used STAR (27) v2.5.3a to map reasds to the reference genome GRCh38 (ensembl version 92 annotation). UMI counts and gene counts of each cell were acquired with featureCounts (20) v1.6.2 software, and used to generate expression matrix files for subsequent analysis.

Quality control, dimension-reduction and clustering

Before analyses, cells were filtered by UMI counts below 30,000 and gene counts between 200 to 5,000, followed by removing the cells with over 20% mitochondrial content. After filtering, the functions from Seurat v2.3 (22) was used for dimension-reduction and clustering. Then we used NormalizeData() and ScaleData funcitons to normalize and scale all gene expression, and selected the top 2000 variable genes with FindVariableFeautres function for PCA analysis. Using the top 20 principle components, we separated cells into multiple clusters with FindClusters. Batch effect between samples was removed by Harnomy (28). Finally, UMAP algorithm was applied to visualize cells in a two-dimensional space.

Differentially expressed genes (DEGs) analysis

To identify differentially expressed genes (DEGs), we used the Seurat FindMarkers function based on Wilcox likelihood-ratio test with default parameters, and selected the genes expressed in more than 10% of the cells in a cluster and with an average log(Fold Change) value greater than 0.25 as DEGs. For the cell type annotation of each cluster, we combined the expression of canonical markers found in the DEGs with knowledge from literatures, and displayed the expression of markers of each cell type with heatmaps/dot plots/violin plots that were generated with Seurat DoHeatmap/DotPlot/Vlnplot function. Doublet cells were identified as expressing markers for different cell types, and removed manually.

Multi-label immunofluorescence assay

(1) Put the tissue chips into the oven, set the temperature to 63 degrees, and bake for one hour.

(2) Dewaxing is completed in the automatic dyeing machine, and the dewaxing time is as follows:

Two cylinders of xylene, each 15 minutes; 2 jars of 100% alcohol, 7 minutes each; 90% alcohol 1 jar, 5 minutes; One jar of 80% alcohol, 5 minutes; One jar of 70% alcohol, 5 minutes. (3) Antigen repair: Dilute 10 repair solution to 1× working solution, microwave oven to high heat for 3min and boil, then put in the glass slides, microwave oven power to low heat to continue repair for 15-20min (ensure that the tissue is immersed in liquid during the whole process), cool naturally at room temperature, and soak the glass slides in pure water. (4) Remove endogenous peroxidase:

The slides were removed, placed in a wet box, treated with commercial H2O2 for 10min, and cleaned with TBST. (5) The slides were taken out and placed in a wet box, then blocking buffer was dropped and incubated for 10min. (6) Primary antibody incubation: Blocking buffer was removed, diluted primary antibody working solution was dropped, incubated at room temperature for 1h, and the slides were cleaned by TBST. (7) The slides were removed, placed in a wet box, dropped secondary antibody, incubated at room temperature for 10min, and cleaned by TBST. (8) The slides were removed and placed in a wet box. Opal dye diluent (dilution ratio: 1:100) was dropped and incubated at room temperature for 10min. The slides were cleaned by TBST. (9) Dilute 10× repair solution to 1× working solution, microwave oven to high heat for 3min and boil, then put in glass slides, microwave oven power to low heat to continue repair for 15-20min (ensure that the tissue is immersed in liquid during the whole process), cool naturally at room temperature, TBST clean the glass slides. (10) DAPI dyeing and sealing.

Pathway enrichment analysis

To investigate the potential functions of DEGs, the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were used with the "clusterProfiler" R package (23). Pathways with p_adj value less than 0.05 were considered as significantly enriched. Gene Ontology gene sets including molecular function (MF), biological process (BP), and cellular component (CC) categories were used as reference. Protein-protein interactions (PPI) of DEGs in each cluster were predicted based on known interactions of genes with relevant GO terms in the StringDB v1.22.0.

Gene regulatory network inference

To analyze transcription factor regulatory networks, we performed SCENIC R toolkit (29) using scRNA expression matrix and transcription factors in AnimalTFDB. Regulatory networks were predicted by the GENIE3 package based on the co-expression of regulators and targets. We used the RcisTarget package to search for transcription factor binding motifs in the data. Genes involved in a predicted regulatory network were defined as a gene set, whose auc value was calculated by the AUCell package to assess the activity of the regulatory network in cells.

Trajectory analysis

Cell differentiation trajectory was reconstructed with the Monocle2 (30). Differentially expressed genes were used to sort cells in order of spatial-temporal differentiation. We used DDRTree to perform FindVairableFeatures and dimension-reduction. Finally, The trajectory was visualized by plot_cell_trajectory() function.

Results

Single-cell transcriptome analysis of peripheral immune microenvironment in ESCC

We collected 4 patients with advanced esophageal squamous cell carcinoma who were surgically unresectable and treated with PD-1 mMAB. According to their sensitivity to PD-1 mMAB, they were divided into sensitive and drug-resistant groups, abbreviated as ESCC-S and ESCC-D, respectively, and then replaced them with the abbreviation (Table 1). Their peripheral blood was collected and PBMCs were isolated for single-cell transcriptome sequencing to explore the cellular characteristics of TME (Figure 1A). After initial quality control assessment and dual body removal, we obtained single-cell transcriptomes totaling 19878 cells, total number of genes identified ranged from 24274 to 30512 per cell, with an average of 27099 for the detected genes. The single-cell map of immune cell transcriptome in peripheral blood of ESCC was characterized, and the differential characteristics of subsets and gene expression in sensitive and drug-resistant groups were analyzed. By graph-based uniform manifold approximation and projection (UMAP), 9 high-confidence cell clusters were identified to show main cell-types based on the expression of known marker genes. In particular, they were as follows: neutrophils, classical monocytes, T cells, platelets, plasma, nonclassical monocytes, B cells, basophils, dendritic cells (DCs). Cell clustering is manually annotated based on marker genes of each cell type, as shown in the Table 2.

The dimension reduction UMAP by cell type and cell source is shown in the Figures 1B, C. The bar chart shows the proportion of each cell type (Figure 1D). According to the bar chart, it can be seen that the proportion of each cell type in the samples of the two sensitive groups is similar. Therefore, for the convenience of subsequent data analysis and comparison, we combined the sensitive group data into one group (Figure 1E). However, the proportion of all types of cells in D1 and D2 was quite different, so they were not combined in order to find out the difference.

According to the proportion of cells after cell clustering, three distinct cell subpopulations were selected, including T cells, monocytes and neutrophils (Figure 1F). Heatmap showing the relative expression level of genes across cells which were used to identify cell types (Figure 2A).

A single-cell atlas of T cells in peripheral blood of esophageal carcinoma

According to previous literature reports and cell marker gene annotation database, T cells were subdivided into four cell types, including effector T (Te), effector memory T (Tem), initial or central memory T and nature kill (NK) cells (Figures 2B–D, Table 3). Through the analysis of differential genes, no corresponding cell subgroup was found, so we defined it as the unkown group (Figures 2B–D). In addition, we wanted to know whether there were exhausted T cells in the peripheral blood. As shown in the Figure 2E, we compared the marker of exhausted T cell, and found that marker genes TIGIT and TIM3 were less expressed, and the other 4 markers were basically unexpressed (Figure 2E).

Pseudo time sequence analysis of T cell subsets

Based on the changes in gene expression levels of peripheral blood T cell subsets over time, the cell lineage development was constructed (Supplementary Figures 1A). There are two developmental branches on the pseudo-temporal sequence of quasi-temporal analysis, from naive T cells and memory T cells to effector T cells (Supplementary Figures 1B). The distribution of each cell type alone on a quasi - time trajectory is also shown in Supplementary Figures 1C. The distribution diagram of each sample in the pseudo timing trajectory, in which different colors represent the cell types in each sample, and the results of pseudo timing analysis of confluent cells (Supplementary Figures 1D). With the dynamic change of pseudo time, gene expression changes with pseudo time (Supplementary Figures 1E). The expression of the first 8 genes in reverse order of Q value varies with the change of pseudo time (Supplementary Figures 1F)

The results of pseudo time sequence analysis of T cell subsets showed the differentiation of three T cell subsets (Supplementary Figures 1A), the differentiation and development trajectories of T cell subsets simulated according to time change, and the development trajectories of T cells from different sample sources (Supplementary Figures 1C). We observed that most of Naive_T_or_Tcm cells differentiated into $CD8^+$ effector T cells (CD8_Te cells) and only a small part differentiated into (CD8_Tem cells) in peripheral blood of ESCC. The ESCC_D1

TABLE 1 Basic information of patients with ECSS.

Number	Sample Name	Species	Age	Gender	TNM Stages	Therapeutic Regimen	Sensibility
1	ESCC_S1	human	65	Male	IIIB	Paclitaxel + cis-platinum + PD-1 mMAB	Yes
2	ESCC_S2	human	55	Male	IVB	Paclitaxel + cis-platinum + PD-1 mMAB	Yes
3	ESCC_D1	human	54	Male	IVA	Paclitaxel + cis-platinum + PD-1 mMAB	No
4	ESCC-D2	human	60	Male	IVA	Paclitaxel + cis-platinum + PD-1 mMAB	No

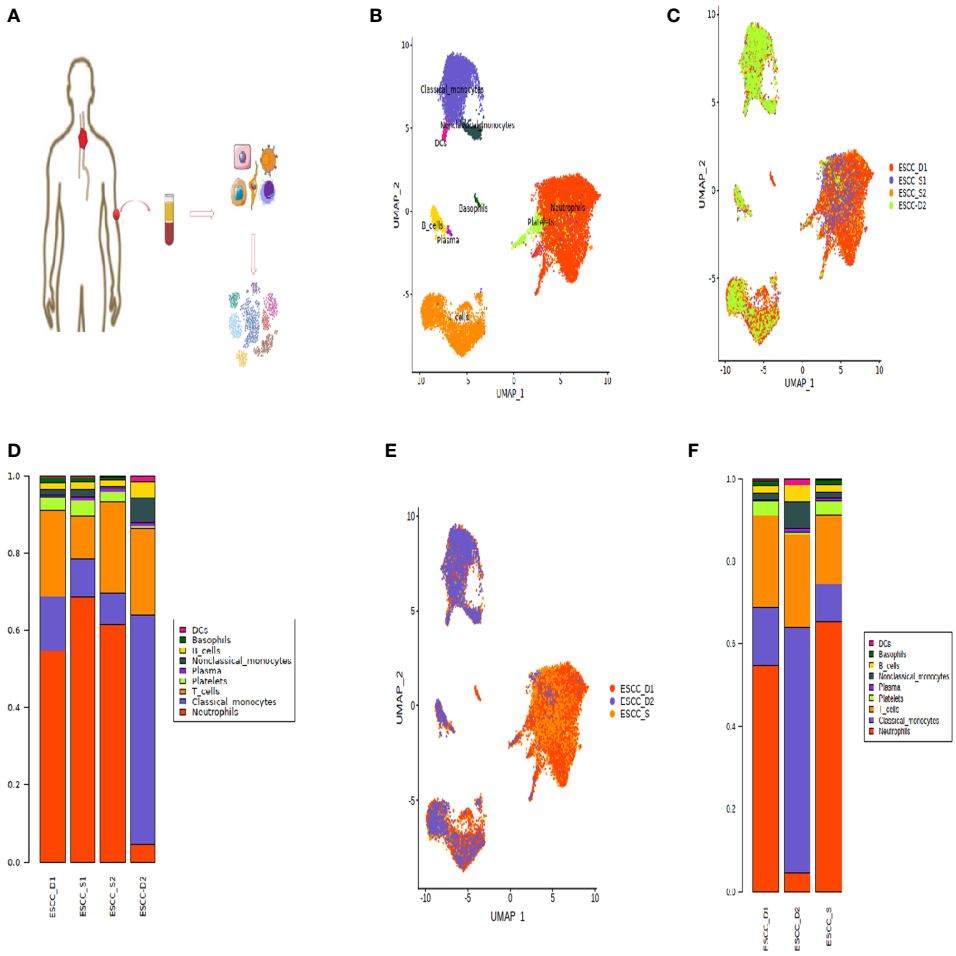

FIGURE 1
Peripheral blood single cell atlas of patients with esophageal cancer. **(A)** Workflow showing the scRNA-seq experimental design and initial data exploration. **(B, C)** UMAP plot of 18,121 high-quality immune cells to show nine main cell-types based on the expression of known marker genes, colored by cell type and cell origin respectively. **(D)** The proportion of cells that contributed to each cluster by each sample, colored by cell types. **(E)** UMAP plot of 18,121 high-quality immune cells to show nine main cell-types based on the cell sources. **(F)** The proportion of cells that contributed to each cluster.

TABLE 2 Cell types and corresponding markers.

Cell type	Abbreviation	Marker
T cells	T cells	CD3D,NKG7,IL7R
B cells	B cells	MS4A1,CD79A
Classical monocytes	Classical_monocytes	CD14,VCAN,FCN1
Nonclassical monocytes	Nonclassical_monocytes	FCGR3A,FCN1,IFITM3
Dendritic cells	DCs	CD1C,FCER1A
Neutrophils	Neutrophils	CSF3R,CXCR2,FCGR3B
Basophils	Basophils	CLC,CPA3
Platelets	Platelets	PPBP,PF4

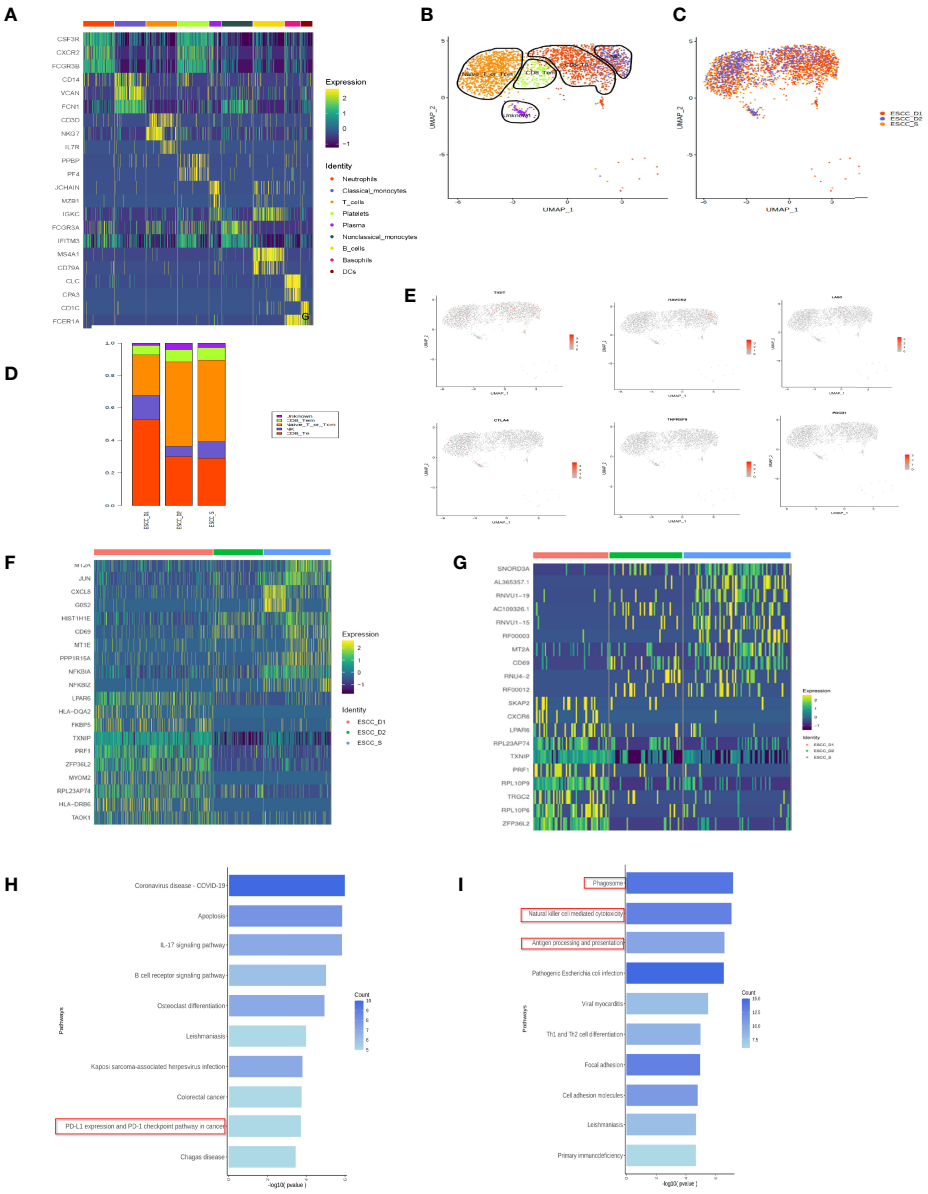

FIGURE 2
T cell subsets in peripheral blood of esophageal carcinoma. **(A)** Heatmap showing the relative expression level of genes across cells which were used to identify cell types. **(B)** UMAP showing the clustering of T cell subsets based on the expression of marker genes. **(C)** UMAP showing the merge T cells from ESCC patients. Each dot corresponds to one single cell colored according to cell cluster. **(D)** The proportion of cells that contributed to each cluster. **(E)** The expression of signature genes in exhausted T cells. T cells are less or do not express the marker genes of exhausted T cells. **(F)** Heatmap showing the relative expression level of genes between PD-1 mMAB resistant and sensitive ESCC patients in $CD8^+$ T effective cells. **(G)** Heatmap showing the relative expression level of genes between PD-1 mMAB resistant and sensitive ESCC patients in $CD8^+$ T memory cells. **(H)** GO and KEGG enrichment analysis of ESCC_S group and ESCC_D1, 2, $CD8^+$ effector T cell group up-regulated differential gene enrichment pathways. **(I)** GO and KEGG enrichment analysis of ESCC_S group and ESCC_D1, 2, $CD8^+$ effector T cell group down-regulated differential gene enrichment pathways.

group mainly differentiated into $CD8^+$ effector T cells in the middle and late stage of differentiation, and the ESCC_D2 group mainly differentiated into $CD8^+$ effector T cells in the early stage of differentiation. In the ESCC_S group, Naive_T_or_Tcm cells differentiated into $CD8^+$ effector T cells in the whole process.

The dynamic change trend of gene expression (Supplementary Figures 1F) shows three gene expression patterns in the process of T cell differentiation. Cluster1 represents the gene group with decreasing expression along with the process of T cell differentiation, Cluster2 represents

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TABLE 3 T cell subsets and corresponding markers.

Cell type	Abbreviation	Marker
CD8+effector T cells	CD8_Te	CD3D,CD8A/B,NKG7,GNLY
CD8+ effector memory T cells	CD8_Tem	CD3D,CD8A/B,GZMK,KLRG1,IL7R
Naïve T cells/ Central memory T cells	Naive_T_or_Tcm	CD3D,CCR7,TCF7,IL7R
NK cells	NK	CD3D-,TRDC,KLRD1,KLRF1

the gene group with increasing expression. Cluster3 indicates the presence of both up-regulated and down-regulated gene groups. In addition, we show the dynamic changes in the expression of some genes during differentiation. As shown in Supplementary Figures 1G, chemokine CCL5 was gradually up-regulated when Naive_T_or_Tcm cells began to differentiate, and then its expression gradually became stable after differentiation into $CD8^+$ effector T cells. Genes related to cytotoxicity, such as NKG7, GNLY and CST7, began to be up-regulated in $CD8^+$ effector T cells at the early stage of differentiation, and gradually increased with the extension of differentiation time. Other genes related to cytotoxic status, such as GZMB and PRF1, were up-regulated at late differentiation stage.

Differences of peripheral blood T cells between patients with sensitivity and drug resistance to PD-1 mMAB in esophageal cancer

The proportion of $CD8^+$ effector T cells in ESCC_D1 group was the highest, compared to ESCC_S group. The proportion of $CD8^+$ memory T cells was the lowest, and there was no significant difference between ESCC_D1,2 group and ESCC_S group. ESCC_S group had the highest proportion of primary/juvenile T cells, which was higher than ESCC_D1,2 group (Figure 2D). Heatmap showing the relative expression level of genes between PD-1 mMAB resistant and sensitive ESCC patients in $CD8^+$ T effective cells and $CD8^+$ T memory cells (Figures 2F, G). GO and KEGG enrichment pathway Analysis of $CD8^+$ effect-T cells ESCC_S group and ESCC_D1,2 group, found that in the up-regulated enrichment pathway, ESCC_S group enriched more PD-L1 and PD-1 checkpoint pathways expressed in tumors (JUN/NFKBIA/FOS/KRAS/IFNG), which also exist in T cell receptor signaling pathways. In the down-regulated enrichment pathway, the genes related to phagocytic NK cell-mediated cytotoxicity and antigen presentation were enriched in group ESCC_D1,2. $CD8^+$ memory T cells ESCC_S group and ESCC_D1 group were also enriched in the down-regulated enrichment pathway of NK cell-mediated cytotoxicity and antigen presentation and other related genes. KEGG pathway enrichment analysis showed that compared with ESCC_D1 group, ESCC_S group was enriched in more PD-L1 and PD-1 checkpoint pathways expressed in tumors (JUN/NFKBIA/FOS/KRAS/IFNG), as well as B-cell receptor signaling pathways (Figure 2H).

In addition, genes with enriched up-regulated pathways in the ESCC_D1 group were mainly related to phagocytic and NK cell-mediated cytotoxicity and antigen presentation (Figure 2I).

MT2A, MT1E and MT1X were differentially expressed in PD-1 mMAB resistant ESCC patients

KEGG enrichment analysis of ESCC_S group and ESCC_D1 group showed that decreased gene expression in D1 group was related to cell apoptosis and PD-L1 expression (Figure 3A). ESCC_D2 compared with ESCC_S group and neutrophils activated to participate in the immune response, and the expression of immunoantigen presentation related genes decreased. ESCC_D2 compared with ESCC_S-enriched B cell receptor signaling pathway related pathways decreased (Figure 3B). In order to explore the role of differential genes in promoting PD-1 mMAB resistance in ESCC, we compared the differential genes in ESCC_D1 and ESCC_D2 contrast sensitive patients (Figures 3C, D), and detected the differentially expressed genes in both resistant patients compared with sensitive patients by Venn map (Supplementary Figures 1H, I).

Among these differential genes, MT2A, MT1E and MT1X have attracted our attention. Compared with the sensitive group, the expression of MT2A, MT1E and MT1X in ESCC_D1 and ESCC_D2 patients was decreased, with statistical significance ($p<0.001$, $p<0.05$, $p<0.05$). These three molecules belong to the Metallothioneins family, and previous studies have shown that they play a tumor suppressor role in some malignant tumors, but there are few studies in esophageal cancer. GEPIA database analysis, MT1E, MT1X molecule transcription levels in esophageal cancer patients and normal control group MT2A, MT1E, MT1X molecule (Figure 3E). The expression of MT2A, MT1E and MT1X in esophageal cancer patients and normal persons was detected by TCGA database, and it was found that the expression of MT2A, MT1E and MT1X in esophageal cancer patients was significantly reduced (Figures 3F–H). TCGA database was used to analyze the relationship between expression of MTA2, MT1E and MT1X in esophageal cancer

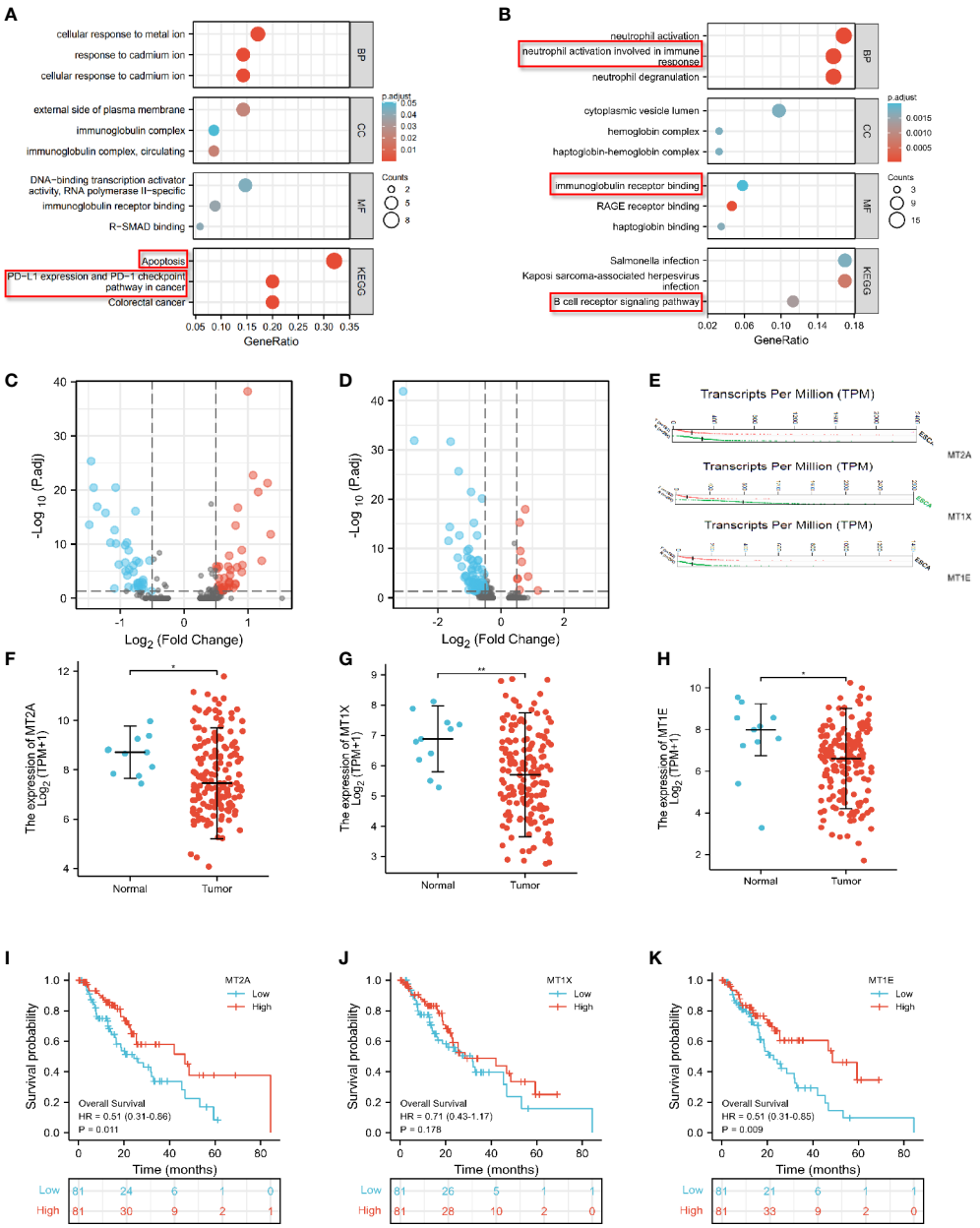

FIGURE 3
Differential expression analysis of MT2A, MT1X and MT1E. **(A)** GO and KEGG enrichment analysis of genes with significantly lower expression in PD-1 mMAB resistant patients ESCC-D1 compared with sensitive patients ESCC-S. **(B)** GO and KEGG enrichment analysis of genes with significantly lower expression in PD-1 mMAB resistant patients ESCC-D2 compared with sensitive patients ESCC-S. **(C)** The volcano map shows the differentially expressed genes in ESCC_D1 patients compared with ESCC_S patients. **(D)** The volcano map shows the differentially expressed genes in ESCC_D2 patients compared with ESCC_S patients. **(E)** GEPIA database analysis, MT2A, MT1E, MT1X molecule transcription levels in patients with esophageal cancer and normal controls. **(F–H)** TCGA database analysis, MT2A, MT1E, MT1X molecule expression levels in patients with esophageal cancer and normal people. **(I–K)** TCGA database was used to analyze the prognosis and survival of patients with high and low expression of MTA2, MT1E and MT1X in esophageal cancer patients. *p < 0.05, **p< 0.01, respectively.

and prognosis and survival (Figures 3I–K, Supplementary Figure 1J), and the analysis results showed that low expression of MT2A, MT1E and MT1X was associated with poorer overall survival.

Next, the correlation between MT2A, MT1E, MT1X expression and $CD8^+$ T cells and other immune cells in various malignant tumors was analyzed by bioinformatics database (Figures 4A–C).

MT2A, MT1E, MT1X expression is associated with $CD8^+$ T cell infiltration in malignant tumors including esophageal cancer. TIMER database was used to analyze the relationship between the expression of MT2A, MT1E and MT1X in ESCC and the infiltration of T cells, B cells, natural killer cells, dendritic cells, macrophages and other immune cells (Figures 4D–F). The expression of MT2A, MT1E and MT1X in esophageal carcinoma and their relationship with $CD8^+$ T cell infiltration were detected (Figures 4G–I).

Sting database analysis of molecular interactions of MT2A, MT1E and MT1X showed that interactions with other molecules in the Metallothioneins family (Figures 5A–C). GeneMANIA database analysis of MT2A, MT1E, MT1X analysis of the interaction of molecules, and showed correlations with detoxification of inorganic compound,stress response to copper ion and metal ion (Figures 5D–F). In order to further identify the key factors related to drug resistance of PD-1 mMAB in esophageal cancer, we performed Multi-label immunofluorescence assay validation on tissue sections of PD-1 mMAB sensitive and PD-1 mMAB resistant samples of ESCC patients after anti-PD-1 treatment. The results of Multi-label immunofluorescence assay showed that compared with the tissues of sensitive patients, the proportion of $CD8^+$ T cells positive for MT2A, MT1E and MT1X was less in the esophageal cancer tissues of resistant patients, which were consistent with the results of single cell sequencing (Figure 5G, Supplementary Figures 1K, L).

These results indicated that the expression of MT2A, MT1E and MT1X in $CD8^+$ T cells of PD-1 mMAB resistant ESCC patients is decreased, which may be related to the poor sensitivity of PD-1 mMAB.

Analysis of single cell subsets of monocytes in peripheral blood of esophageal carcinoma

Monocytes were subdivided into 4 cell clusters in single-cell analysis, which could not be defined according to existing regulations, so they were temporarily named as Cluster 1-4 (Figure 6A). The proportion of Cluster 1 and Cluster 2 was relatively high, and the proportion of Cluster 3 cells in the resistant group was lower than that in the sensitive group (Figures 6B, C). We also identified specific gene sets for these cell subpopulations to allow more in-depth analysis of regulatory pathways. The list of differential genes in each cluster of monocytes was selected and sorted in descending order of avg_logFC, and the top 10 genes were selected for heat map drawing, in which duplicate genes would be removed (Figure 6D). GO enrichment analysis and KEGG pathway enrichment analysis were performed for each cluster. The Cluster 1 enrichment analysis showed that the differential genes with decreased expression in the resistant group compared with the sensitive group were related to type I interferon signaling interferon, neutrophil activation, neutrophil degranulation (Figure 6E, Supplementary Figures 2A). Cluster 2 is related to protein targeting to the membrane, localization to the endoplasmic reticulum and mRNA decomposition (Figure 6F,Supplementary Figures 2B). Cluster 3 is mainly enriched in type I interferon signaling pathway and associated with virus infection. Significantly enriched genes included MX1 or 2/ISG15/OAS3/IFI6/IFIT2 or 3/XAF1/OAS1/IFI35/IFITM3 (Figure 6G, Supplementary Figures 2C). Cluster 4 is related to mRNA/RNA splicting (Supplementary Figures 2D, E).

Type I interferon is key driver of inflammation and immune suppression in chronic infections, providing essential inflammatory signals, however, initiate feedback suppression in immune cells and cancer cells (31). The enrichment pathway of monocytes subsets in PD-1 mMAB resistant patients is related to type I interferon, which may be related to the reduction of PD-1 mMAB sensitive.

Heterogeneity analysis of single cell subsets of neutrophil in peripheral blood of esophageal carcinoma

Neutrophils were further subdivided into 5 clusters for further analysis (Figure 7A). Neutrophil Cluster 1-4 accounted for a higher proportion, while Cluster 5 accounted for a smaller proportion (Figures 7B, C). The list of differential genes in different subpopulations of monocytes was sorted in descending order of avg_logFC, and the top 10 genes were selected for heat mapping, in which duplicate genes would be removed (Figure 7D). The proportion of Cluster 1 neutrophils was the highest and the differential genes with reduced expression in the resistant group compared with the sensitive group were significantly correlated with the antigen presentation process of MHC class II molecules in the immune response, the cellular response of type I interferon and the type I interferon signaling pathway (Figure 7E, Supplementary Figure 3A). The enrichment function of Cluster 2 is mainly related to the cellular response of type I interferon and the type I interferon signaling pathway (Figure 7F, Supplementary Figure 3B). This suggests impaired anti-tumor function such as neutrophil antigen presentation recognition in patients with PD-1 mMAB resistance. GO enrichment analysis of Cluster 3 showed that the gene with reduced expression in the resistant group was associated with defense response to virus and type I interferon signaling pathway (Figure 7G, Supplementary Figure 3C). The results of differential gene enrichment analysis in Cluster 4 is related to chemotaxis of neutrophils and DCs, cytokine release and T cell-mediated cytotoxicity (Figure 7H, Supplementary Figure 3D). Cluster 5 is involved in protein targeting to membranes, endoplasmic reticulum, and ribosomal assembly (Supplementary Figures 3D, E).

The enrichment pathway of neutrophil subsets in the resistant group was also related to type I interferon, and was related to the chemotaxis of neutrophils and DCs, which may play a synergistic role with monocytes in the PD-1 mMAB resistant of esophageal cancer.

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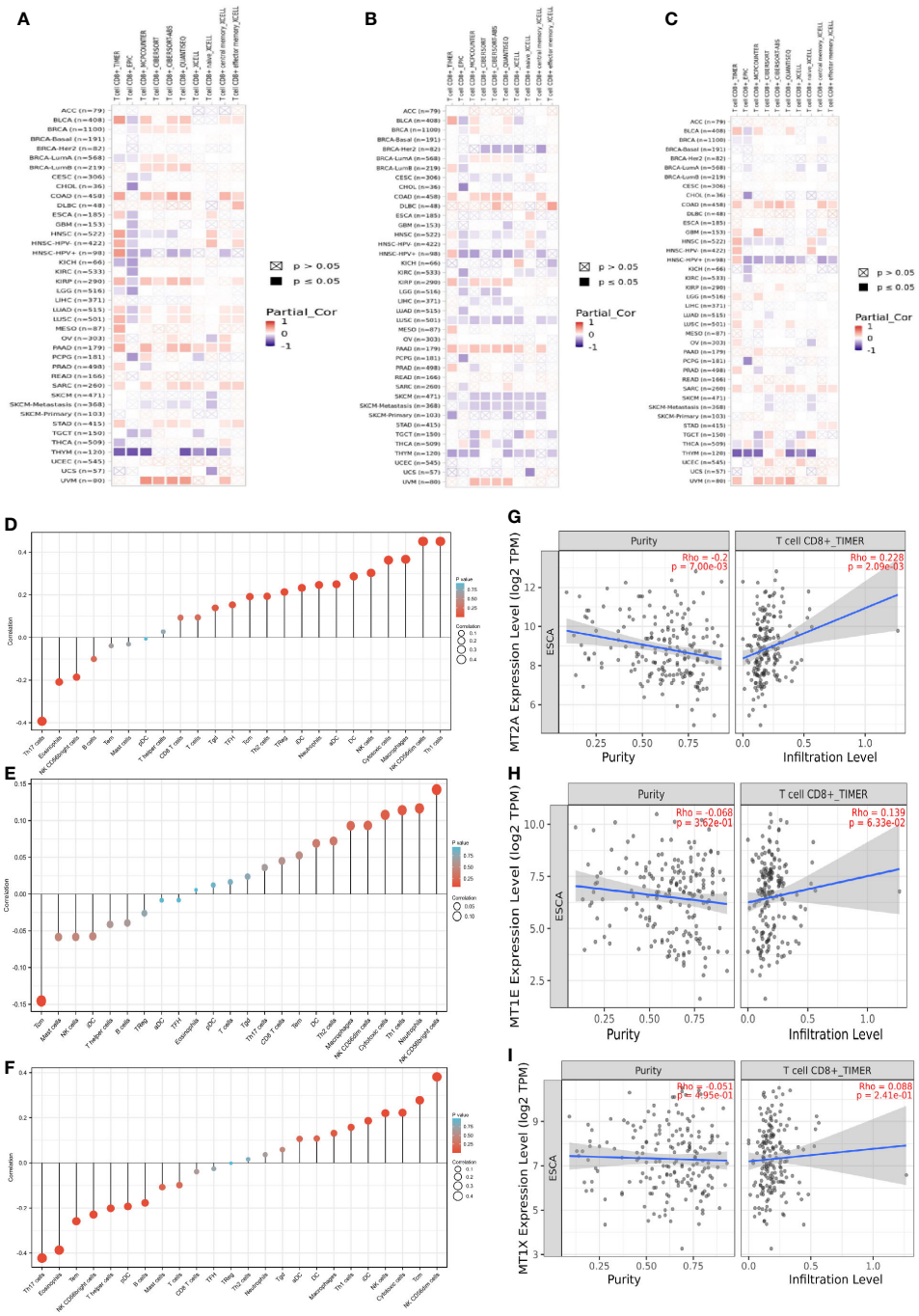

FIGURE 4
Prognostic correlation analysis of MT2A, MT1X and MT1E. **(A–C)** The correlation between MT2A, MT1E, MT1X expression and CD8$^+$ T cells in various malignancies was analyzed by various databases. **(D–F)** TIMER database was used to analyze the relationship between MT2A, MT1E, MT1X expression and various immune cell infiltration in ESCC. **(G–I)** The relationship between MT2A expression and CD8$^+$ T cell infiltration in ESCC was analyzed by TIMER database.

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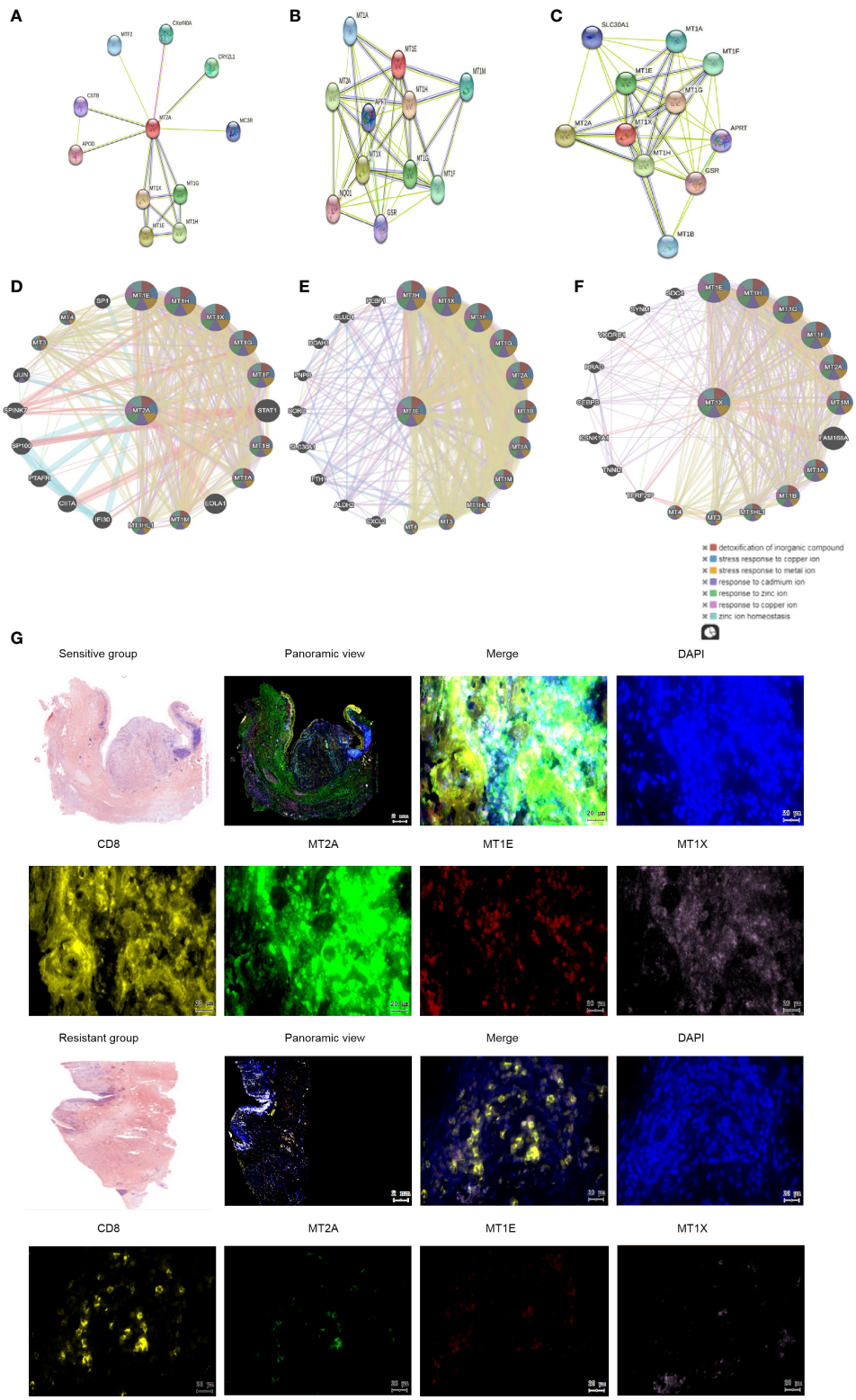

FIGURE 5
Molecular interactions of MT2A, MT1X and MT1E. **(A–C)** String database analysis of genes interacting with MT2A, MT1E and MT1X. **(D–F)** GeneMANIA database was used to analyze the interaction between MT2A, MT1E and MT1X. **(G)** The expression of MT2A, MT1E and MT1X in PD-1 mMAB sensitive and resistant esophageal cancer tissues was detected by multicolor immunohistochemical assay (n=3).

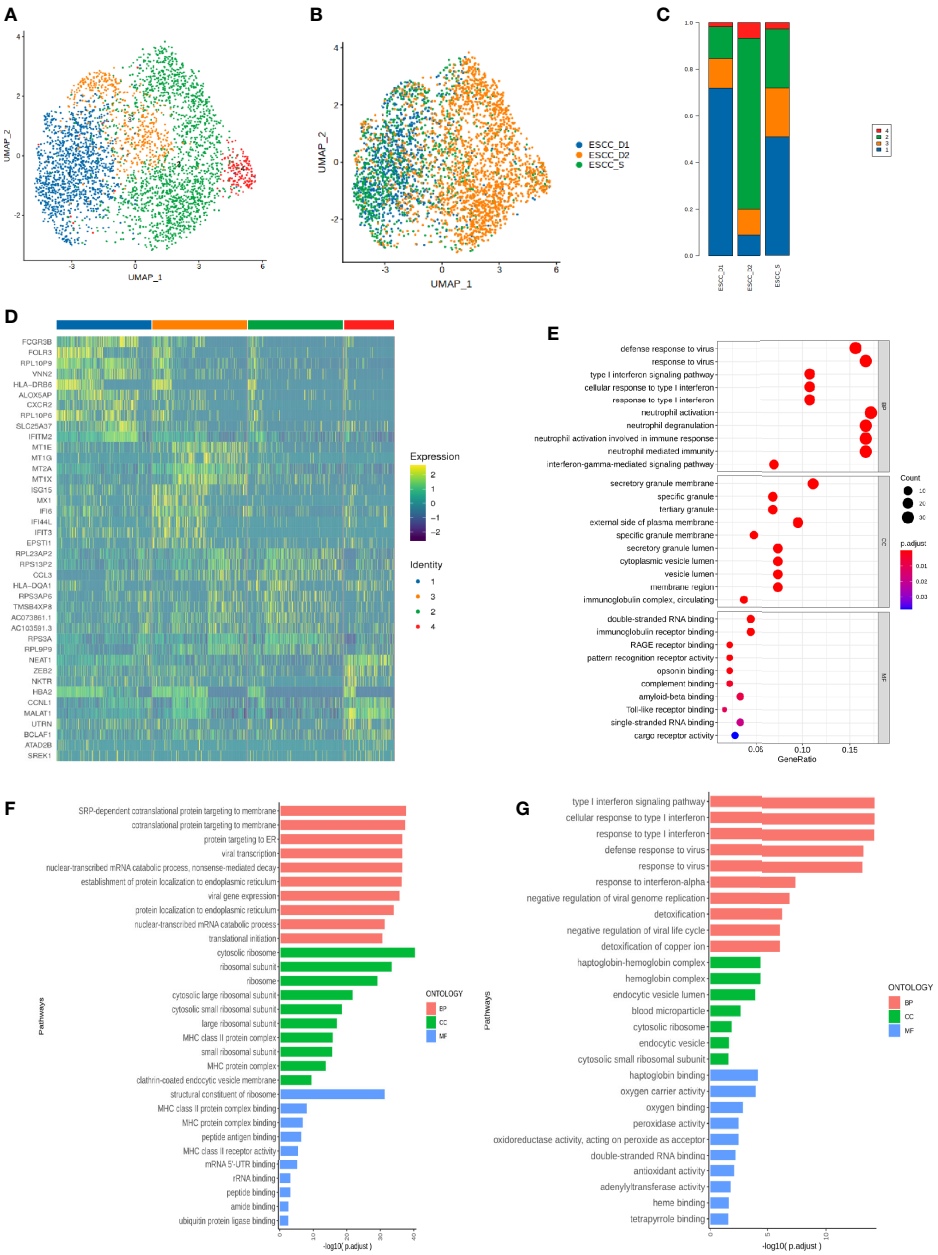

FIGURE 6
Single-cell subsets of mononuclear cells in peripheral blood of ESCC. **(A)**UMAP showing the clustering of monocyte subsets based on the expression of marker genes. Each dot corresponds to one single cell colored according to cell cluster. **(B)** UMAP showing the merge monocyte cells from ESCC patients. **(C)** The proportion of cells that contributed to each cluster by each sample. **(D)** The heat map shows the relative expression levels of differential genes in each subtype of monocytes. **(E–G)** GO enrichment analysis of monocyte Cluster 1-3.

Regulation analysis of single cell subsets of transcription factors

The regulation of transcription factors on gene expression of single cell subsets was further explored, AUC scores of transcription factors and target gene sets were shown in the heat map (Supplementary Figures 4A). Top10 Regulon's AUC matrix clustering heat map in each cell is shown in Supplementary Figures 4B. Taking effector T cells as an example, regulon-specific scatter plots of sensitive and drug-resistant patients were shown, highlighting the highest top 10 Regulon (Supplementary Figures 4C-E). According to the Venn diagram results, ESCC_D2 had four transcription factors specifically expressed higher than the other two samples (Supplementary Figures 4F).

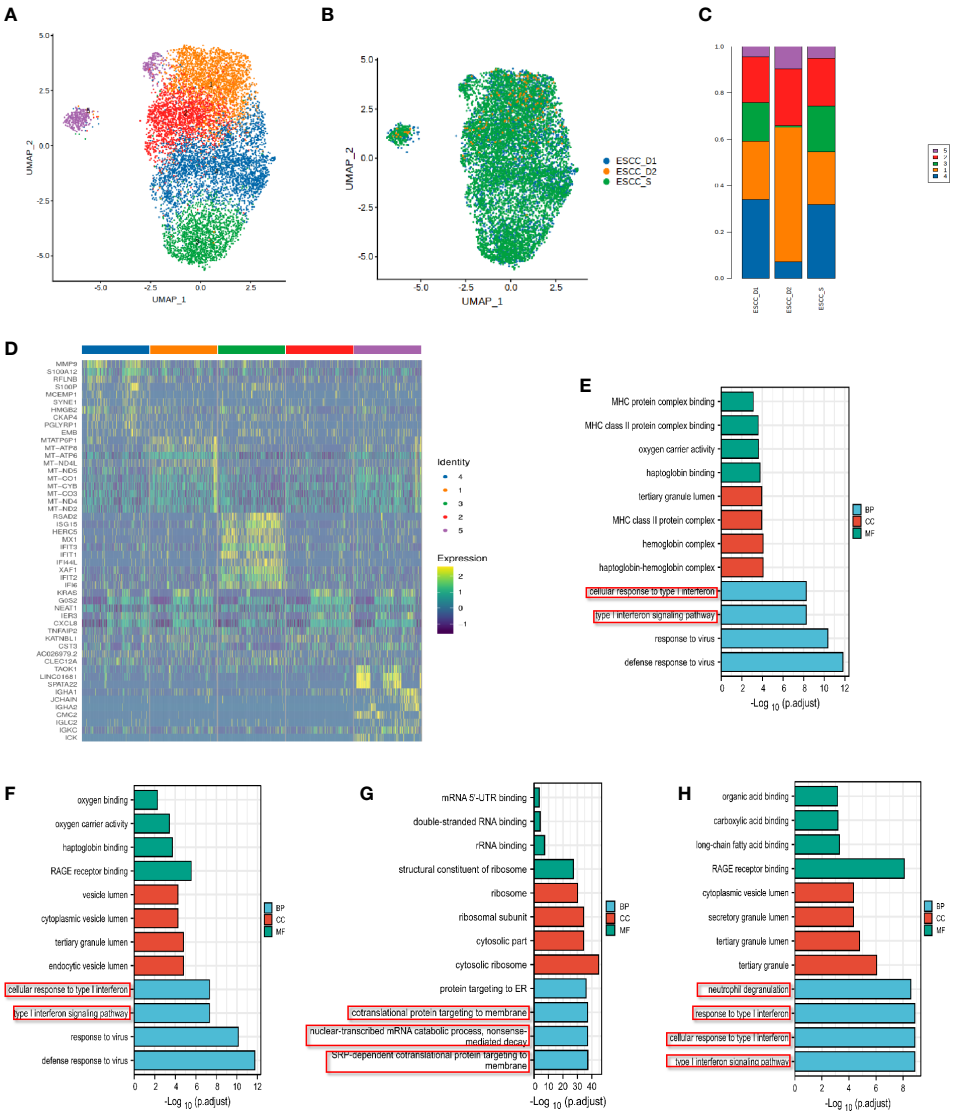

FIGURE 7
Analysis of single cell subsets of neutrophils in peripheral blood of ESCC. **(A)** UMAP showing the clustering of neutrophils subsets based on the expression of marker genes. **(B)** UMAP showing the merge neutrophils from ESCC patients. **(C)** The proportion of cells that contributed to each cluster by each sample. **(D)** The heat map shows the relative expression levels of differential genes in each neutrophil cluster. **(E)** Neutrophil cluster 1 GO enrichment analysis showed that it was related to MHC class II molecular antigen presentation process. **(F–H)** GO enrichment analysis of neutrophil Cluster 2-4.

Among them activated T nuclear factor (NFAT) has been identified for the first time as a major stimulus-responsive DNA binding factor and transcriptional regulator in T cells (32). NFAT transcription factors are assumed to play a central role in the carcinogenesis of pancreatic cancer (33). Array analysis showed that NFATc2 was the c-REL target gene among the 12 trail inducing genes that were the strongest in apoptotic resistant cells (34). NFAT may play an important role in resistance to PD-1 mMAB in esophageal cancer.

Disscusion

ESCC is the leading malignant tumor worldwide, accounting for about 572,000 new patients and 508,000 deaths annually (35). ESCC usually contains extensive genomic changes, and although there are exceptions, a high mutation load is associated with a better response to checkpoint blockade. A number of large phase II/III clinical trials targeting the first and second lines of advanced esophageal cancer have confirmed that immunotherapy brings

significant clinical benefits to patients with advanced esophageal cancer (36, 37).

PD-1 belongs to the CD28 cell surface receptor family and is expressed on activated T cells, B cells, NKT cells, monocytes and macrophages. Its ligand, PD-L1, is upregulated in many cancers and is an important target in immunotherapy of tumors (38, 39). Anti-programmed death 1 (PD-1)/programmed death ligand-1 (PD-L1) therapy shows antitumor activity in patients with metastatic esophageal cancer (40). In a randomized Phase III study of Keynote-181, embrolizumab extended overall survival (OS) in patients with advanced esophageal cancer compared with chemotherapy as second-line treatment, compared with programmed death ligand 1 (PD-L1) combined positive score (CPS)≥10 (41). Nivolumab was associated with a significant improvement in overall survival and favorable safety compared to chemotherapy in previously treated patients with advanced esophageal squamous cell carcinoma, and may represent a new standard second-line treatment option for these patients (42). While in Keynote-590, drug K combined with first-line chemotherapy for the whole population brought significant OS improvement regardless of PD-L1 expression (43). Other studies have also found that immunotherapy has a better effect on patients with PD-L1 CPS≥ 10, but some patients can still benefit from PD-L1 CPS < 10. Therefore, how to screen immunotherapy population is very important. In other words, PD-L1 is not the only population screening marker, and more markers need to be found to guide immunotherapy, so as to develop individualized and precise treatment plans.

Recently, scRNA-seq has been developed to untargeted quantification of the transcripts present in individual cells (44). Advances in molecular biology, microfluidics and bioinformatics have empowered the study of thousands or even millions of individual cells from malignant tumors at the single-cell level of resolution (45). The use of single-cell sequencing in cancer research has revolutionized our understanding of the biological features and dynamics within cancer lesions (46). The ability to find and characterize abnormal cells in the population has potential implications for further understanding of drug resistance and recurrence in cancer therapy (47). At present, an increasing number of tumor studies, including a variety of solid tumors, such as malignant melanoma (48), lung cancer (49, 50), breast cancer (51), straight colon cancer (52), gastric cancer (53), esophageal cancer (37) and pancreatic cancer (54), have used single-cell sequencing technology to map tumor immune cells. The interaction between tumor and immune system can be comprehensively evaluated by visual method, which can be used to predict the efficacy of immunotherapy.

We sequenced peripheral blood of 4 patients with esophageal cancer by single-cell sequencing technology to explore the influence of immune cell gene differences on cancer PD-1 sensitivity of esophageal patients. Compared with ESCC_S group, ESCC_D1 group had the highest proportion of $CD8^+$ effector T cells. Analysis of $CD8^+$ effect-T cells ESCC_S group and ESCC_D1 group showed that among the up-regulated enrichment pathways, ESCC_S group enriched more PD-L1 and PD-1 checkpoint pathways (JUN/NFKBIA/FOS/KRAS/IFNG) expressed in tumors. These pathways are also present in T cell receptor signaling pathways. MT2A, MT1E and MT1X were differentially expressed in ESCC patients resistant to PD-1 mMAB. The expression of MT2A, MT1E and MT1X in esophageal cancer patients and normal controls was detected by TCGA database, and it was found that the expression of MT2A, MT1E and MT1X in esophageal cancer patients was significantly reduced, which was associated with poor prognosis. Metallothionein is a cysteine rich cytoplasmic protein with low molecular weight (6-7 kDa), which plays an important role in metal ion homeostasis and detoxification (55). In recent years, many studies have shown that MTs expression is different in different tumors, suggesting that MTs may play an important role in tumorigenesis (13, 56). MTs expression is not universal in all human tumors, and may play different or even opposite roles in different tumors. A Japanese researcher found that MT2A was highly expressed in CAF cells constructed by them. Knockdown of MT2A inhibited the expression and secretion of insulin-like growth factor binding protein 2 (IGFBP2), and recombinant IGFBP2 promoted the migration and invasion of ESCC cells through NF-κB, Akt and Erk signaling pathways (57). The opposite effect of MTs in different tumors may be related to tumor type and differentiation, other environmental stimuli or gene mutations (13).

MT2A, MT1E, MT1X are identified as potential novel therapeutic targets in ESCC. Compared with the sensitive group, MT2A, MT1E, MT1X expression were down-regulated in immune-resistant patients, and were correlated with the infiltration of various immune cells, including $CD8^+$ effector T cells, in tumor tissues. Therefore, MT2A, MT1E, MT1X may be potential predictors of anti-PD-1 therapy in patients with advanced esophageal squamous cell carcinoma.

Our results also found that differences in T cell subtype characteristics between immunotherapy response and nonresponse groups could not be determined solely by the proportion of cell subtypes, but circulating $CD8^+$ effector T cells were the dominant cell subsets in both response and nonresponse groups. Similar findings have been reported in other solid tumors. For example, a study reported that after anti-PD-1 inhibitor treatment in melanoma patients, the frequency of CD8 effector memory T cells in the circulating blood of responders increased, while the frequency of CD4 effector memory T cells and CD8 naive T cells decreased (58). In recent years, more and more researchers have discovered the disturbance of ICIs on tumor microenvironment and circulating immune cells in peripheral blood by single cell sequencing technology. Overall, the difference in immune cells is mainly between T cells. Among T lymphocytes, cytotoxic $CD8^+$ T cells

are usually affected by ICIs treatment, and they play a huge role in tumor monitoring, editing and control (59).

Monocytes and neutrophils are also important immune cells in peripheral blood. Reduced PD-1 expression on peripheral blood T cells and reduced monocyte populations in the glioblastoma tumor microenvironment were more frequent in the neoadjuvant group than in patients treated only in the neoadjuvant group (60). Studies have shown that the endogenous microbiome in pancreatic cancer promotes tumorigenesis by differentially activating Toll-like receptors selected in monocytes to generate tolerance immune programs (61). Interleukin-17 in pancreatic cancer recruits neutrophils, triggers neutrophil extracellular traps (NETs), and excludes cytotoxic CD8 T cells from the tumor, reducing the sensitivity of immune checkpoint blockade (PD-1, CTLA4) (62). Elevated serum interleukin-8 and enhanced intratumoral neutrophil infiltration are associated with poorer prognosis in advanced cancer and reduce the clinical benefit of immune checkpoint inhibitors (63). We found that the enrichment pathway of monocyte and neutrophil subsets in PD-1 mMAB resistant patients was related to type I interferon, which may also be one of the influencing factors of reduced PD-1 mMAB sensitivity in esophageal cancer patients.

At present, the detection of PD-L1 is still mainly by immunohistochemistry, but there is a certain difficulty in tissue detection in specimen collection. Tumor cells can achieve immunotherapy resistance through a variety of mechanisms, such as T cell depletion leading to PD-1 blockade treatment resistance, or induction of tumor cells expressing PD-L1 leading to adaptive immune resistance. ESCC has entered the era of immunity. Biomarkers still need to be explored to screen the beneficiaries of immunotherapy. A large number of studies have reported that peripheral blood immune cells participate in the body's anti-tumor immune response.

In this study, the effect of peripheral immune cell infiltration on the sensitivity of monoclonal antibody PD-1 in patients with esophageal cancer was thoroughly investigated by single cell sequencing technology, which provides a new idea for immunotherapy and effective biomarkers for esophageal cancer.

Conclusion

In summary, we investigated the effect of peripheral blood immune cell infiltration on the sensitivity of PD-1 mMAB in ESCC by single cell sequencing. Through comprehensive analysis of the transcriptome characteristics of immune cells, the dynamic change of cell percentage, heterogeneity of cell subtypes and interactions between cells were explained to provide new understanding and potential therapeutic targets for the biological basis of ESCC immunotherapy.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Tianjin Medical University Cancer Institute and the hospital ethics committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

TD, HW, CY, and MZ performed most of the experiments, analyzed data, and wrote the manuscript. ZJ, MB, TN, RL, JW, SG, LZ, and YB reviewed and edited the manuscript. HZ designed the experiments and edited the manuscript. HZ is the guarantor of this work and, as had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1004345/full#supplementary-material

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Epigenetic-related gene mutations serve as potential biomarkers for immune checkpoint inhibitors in microsatellite-stable colorectal cancer

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Background: Combination therapy with immune checkpoint inhibitors (ICIs) may benefit approximately 10-20% of microsatellite-stable colorectal cancer (MSS-CRC) patients. However, there is a lack of optimal biomarkers. This study aims to understand the predictive value of epigenetic-related gene mutations in ICIs therapy in MSS-CRC patients.

Methods: We analyzed DNA sequences and gene expression profiles from The Cancer Genome Atlas (TCGA) to examine their immunological features. The Harbin Medical University Cancer Hospital (HMUCH) clinical cohort of MSS-CRC patients was used to validate the efficacy of ICIs in patients with epigenetic-related gene mutations (Epigenetic_Mut).

Results: In TCGA, 18.35% of MSS-CRC patients (78/425) had epigenetic-related gene mutations. The Epigenetic_Mut group had a higher tumor mutation burden (TMB) and frameshift mutation (FS_mut) rates. In all MSS-CRC samples, Epigenetic_Mut was elevated in the immune subtype (CMS1) and had a strong correlation with immunological features. Epigenetic_Mut was also associated with favorable clinical outcomes in MSS-CRC patients receiving anti-PD-1-based therapy from the HMUCH cohort. Using immunohistochemistry and flow cytometry, we demonstrated that Epigenetic_Mut samples were associated with increased anti-tumor immune cells both in tumor tissues and peripheral blood.

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Conclusion: MSS-CRC patients with epigenetic regulation impairment exhibit an immunologically active environment and may be more susceptible to treatment strategies based on ICIs.

KEYWORDS

immune checkpoint inhibitor therapy, biomarker, microsatellite-stable, colorectal cancer, epigenetic-related gene mutations

Introduction

Immune checkpoint inhibitor (ICI) therapy has achieved impressive success in deficient mismatch repair (dMMR)/microsatellite instability-high (MSI-H) colorectal cancer (CRC). ICI has been considered a standard therapy by the FDA, including the use of programmed death receptor 1 (PD-1) monoclonal antibodies and CTL-associated protein 4 (CTLA-4) monoclonal antibodies (1–3). However, the vast majority of CRC cases (approximately 85%) are characterized by proficient mismatch repair (pMMR)/microsatellite stability (MSS) tumors that do not respond to ICIs (4). Recent studies suggest that a subgroup (approximately 10-20%) of MSS-CRC patients might benefit from combination regimens of ICIs (5–7). Therefore, predictive biomarkers for screening these patients are urgently needed.

Current clinical and investigational studies of screening MSS-CRC patients who would benefit from ICIs treatment are limited. PD-L1 expression is a classic biomarker, but the Keynote-028 study demonstrated that PD-L1$^+$ MSS-CRC patients could not benefit from ICI therapy (8). *POLD1*/*POLE* mutations are predictive but occur in only 1% of MSS-CRC patients (9). Biomarkers such as tumor mutation burden (TMB), tumor-infiltrating lymphocytes (TIL), neo-antigen load (NAL), and immune-regulatory gene expression profiling (iGEP) may allow the selection of clinical patients for ICIs. However, the lack of uniform detection methods and validated cutoffs limit the use of these methods (5, 10–12). Several emerging biomarkers, such as gut microbiota and T-cell-receptor (TCR) sequencing, have also shown predictive value, although they are not yet clinically applicable (7, 13). DNA damage response (DDR) gene mutations may induce a hypermutational phenotype (14), and recent studies have shown that patients with MSS-CRC and mutations in the DDR system have better immune responses and outcomes following ICI therapy (15, 16). However, the pathogenicity of different DDR gene mutations in MSS-CRC remains unclear, and their incidence is significantly lower than in endometrial, ovarian, or biliary tract cancers (17).

Epigenomic alterations can affect tumor immunogenicity and anti-tumor responses by regulating genome stability and chromatin accessibility (18). Additionally, several epigenetic-related gene mutations have been shown to exhibit predictive functions in ICI therapy for multiple types of tumors. ARID1A, an AT-rich interactive domain-containing protein 1A, is a component of the switching defective/sucrose non-fermenting (SWI/SNF) complex that plays a role in chromatin remodeling (19), and increasing evidence suggests that ARID1A alterations are correlated with better outcomes after ICI therapy for bladder cancer, nonsmall-cell lung cancer (NSCLC), and gastric cancer (20, 21). ARID1A mutation is defined as an immunologically active subgroup in MSS-CRC patients with abundant intratumoral T-cell infiltration (22). Lysine methyltransferase 2 (KMT2) family members facilitate transcription and gene accessibility by methylating lysine 4 on histone H3 (H3k4) (23), and *KMT2* family mutations have also been linked to a favorable response to ICIs in multiple cancers (24). Furthermore, as identified using clustered regularly interspaced short palindromic repeats (CRISPR), KMT2D mutant tumors exhibit an increased mutation burden, IFN-γ-stimulated antigen presentation, and a higher sensitivity to ICIs. Moreover, disruption of DNA methylation signatures has been identified as a marker of anti-PD-1 therapy efficacy in NSCLC (25), and TET1, a DNA demethylase, enhances the immunotherapeutic effect (26). Although this evidence points to the role of epigenetic regulation in anti-tumor immune responses, there is no clinical data on the association between comprehensive epigenetic-related gene mutations (mutations in genes that are involved in epigenetic modifications) and the clinical benefit of ICIs in MSS-CRC.

Given the proposed role of epigenetic regulation impairment in predicting the response to ICIs, we hypothesize that epigenetic-related gene mutations in MSS-CRC cause hypermutation and improve the expression of immune response gene sets. As a result, we conducted this study to clarify the value of epigenetic-related gene mutations as an indicator of immunotherapy efficacy in patients with MSS-CRC. For this purpose, we analyzed whole-exome sequencing (WES) data from TCGA to study TMB, frameshift mutation (FS-mutation), and immune characteristics of Epigenetic_Mut and Epigenetic_Wt groups of MSS-CRC samples. Additionally, in a Chinese clinical MSS-CRC cohort of 89 patients who received PD-1-based treatment, we found that Epigenetic_Mut

was associated with favorable clinical outcomes. Here, we report the relationships between epigenetic-related gene mutations and TMB, FS-mutation, immunomodulatory mRNA expression signature, and ICI therapy efficacy in patients with MSS-CRC.

Materials and methods

Patient information and sample collection

To determines the incidence of epigenetic-related gene mutations in MSS-CRC, we analyzed DNA sequencing and gene expression profiles of 514 MSS-CRC patients from two cohorts: (1) a TCGA cohort consisting of 425 MSS-CRC patients and (2) a HMUCH cohort comprising 89 Chinese patients with annotated response and mutational data from Harbin Medical University Cancer Hospital (the inclusion and exclusion criteria are shown in Supplementary Figure 1). This study was approved by the Ethics Committee of the Harbin Medical University Cancer Hospital (No. KY2022-20).

Epigenetic-related gene status definition

Epigenetic-related gene status (Epigenetic_Wt or Epigenetic_Mut) was defined based on the presence of a loss-of-function (LOF) variant in 68 genes that have been proposed as core genes of epigenetic regulation (18). Supplementary Table 1 presents a detailed description. Nonsense, frameshift, and splice site changes within consensus regions and start lost/gained variants were considered to be LOF variants. Missense and in-frame variants were excluded from the analysis.

DNA extraction and sequencing

For the TCGA cohort, gene mutation data were acquired using the GDC Data Portal. We assessed the mutational status of epigenetic-related genes in CRC using exome-sequencing data from HMUCH. For analysis, DNA was extracted using a DNA Kit (Applied Biosystems, Foster City, CA, USA), from whole blood samples or formalin-fixed paraffin-embedded (FFPE) tissues of each patient. The lymphocytes from the whole blood samples were isolated by centrifugation at 1,600 × g for 10 min in red cell lysis buffer (Tiangen, RT122, Beijing, China) at 25°C, and DNA was extracted using a genomic DNA kit (Tiangen, DP304, Beijing, China). We sheared the DNA into fragments of 150-200 bp using an ultrasonicator and used a KAPA Kit (KAPA Biosystems, Wilmington, MA, USA) to prepare DNA fragment libraries for the Illumina platform (Illumina HiSeq X-Ten, Illumina, USA). Probe hybridization capture technology and Illumina high-throughput sequencing were used to detect the exonic regions and some intronic regions of 825 tumor-related genes (Genetron Health Co., Ltd. Beijing, China) (Supplementary Table 2).

Analysis of MSI status, TMB, and FS-mutation in the TCGA and HMUCH cohorts

MSI status for the TCGA cohort was determined using the MSI sensor (version 0.5). In brief, for MSI sensor scores < 3.5, samples were considered to be MSS; otherwise, they were considered MSI (27). Published studies using the TCGA cohort provided FS-mutation and TMB data (28–30), and MSI status for the HMUCH cohort was determined using a 3730 sequencer (Life Technologies, Carlsbad, CA, USA). For this purpose, whole blood samples or prepared FFPE tissue were diluted to 2 ng/μL or 20 ng/μL, respectively, followed by the addition of 2.8 μL of ddH_2O, 4 μL of 2.5× Buffer A, 2 μL of 5× MSI Primer Mix, and 0.2 μL of Taq DNA Polymerase I. PCR amplification was carried out as follows: pre-denaturation at 95°C for 5 min; followed by 30 cycles at 94°C for 30 s, 60°C for 1 min, and 70°C for 1 min; and then a final extension at 60°C for 30 min. Finally, the temperature was reduced to 15°C, and the samples were centrifuged at 3,000 × g for 1 min. NR-21 and BAT-26 were labeled with blue fluorescent dye, BAT-25 with green dye, and NR-24 and MONO-27 with yellow dye. Finally, tumors were classified as MSI-H if two or more markers showed instability; otherwise they were classified as MSS.

Analysis of the consensus molecular subtypes (CMSs) in the TCGA cohort

Consensus molecular subtypes (CMSs) are classification systems for CRC and include immune (CMS1), canonical (CMS2), metabolic (CMS3), and mesenchymal (CMS4) subtypes. These subtypes were identified through a large-scale analytical study and have unique molecular and metabolic characteristics (31).

Immune-related signature analysis

Our study compared the RNA expression of patients with Epigenetic_Mut and Epigenetic_Wt using gene signatures for the IFN-γ pathway and other immunological responses (Supplementary Table 3) (12, 32). We obtained TCGA transcriptome profiles from the GDC data portal, and used transcripts per kilobase million (TPM) normalization to normalize gene expression. The geometric mean of gene expression levels in the $\log_2$ (TPM + 1) format was used to evaluate immune signatures.

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Clinical outcomes

The objective response rate (ORR), disease control rate (DCR), progression-free survival (PFS), and overall survival (OS) were the main clinical outcomes of interest. The Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 was used for the assessment of ORR and divided into complete response (CR) and partial response (PR). DCR was defined as CR, PR, or stable disease (SD) lasting more than six months. PFS was evaluated from when immunological therapy was initiated until progression or death, and patients who did not progress were examined at the last scan. OS was evaluated from the start of ICI therapy until patient death or the end of the trial, and the patients with whom we lost contact were classified based on the date of last contact.

Immunohistochemistry (IHC)

Primary tumor paraffin sections of 4 μm were processed for immunochemistry to evaluate $CD8^+$ and $FOXP3^+$ lymphocytes according to the following protocol: roast, deparaffination, and rehydration before performing heat-mediated antigen retrieval with EDTA buffer (pH 9.0), inactivation of endogenous peroxidase activity with 3% H_2O_2, incubation with antibody against CD8 (ab101500, 1:500; Abcam, Cambridge, UK) or against FOXP3 (ab200334, 1:500; Abcam) at 4°C overnight, exposure to a DAB IHC Detection Kit after incubation with biotinylated secondary antibodies, and counterstaining with Mayer's hematoxylin solution. An open-source platform for biological-image analysis (Fiji/ImageJ) was used to estimate the densities of $CD8^+$ and $FOXP3^+$ lymphocytes.

Flow cytometry analysis

The peripheral blood mononuclear cells (PBMC) of CRC patients were isolated by centrifugation with erythrocytes lysate and were used to analyze $PD1^+CD8^+$T cells and $CD3^-CD56^+CD16^+$NK cells by flow cytometry. The PBMC were stained for 30min on ice using the following antibodies: FITC anti-human CD8 (344704, Biolegend), PE anti-human PD1(367404, Biolegend), APC anti-human CD3 (300312, Biolegend), PE anti-human CD56 (985902, Biolegend), and PerCP anti-human CD16 (302030, Biolegend). Stained cell suspensions were analyzed using the BD flow cytometer (BD Accuri C6 Plus), and data analysis was performed using FlowJo_v10.8.1.

Statistical analysis

Fisher's exact test was used to analyze the relationship between epigenetic-related gene mutations and the ORR or DCR, and the Kaplan–Meier method and log-rank test were employed to examine the PFS and OS probabilities of the Epigenetic_Mut and Epigenetic_Wt CRC groups. Based on the Mann–Whitney U-test, TMB, FS-mutation, tumor-infiltrating lymphocytes, expression of immune-related genes, and immune signatures were compared between the Epigenetic_Mut and Epigenetic_Wt CRC groups. Statistical analysis was conducted using two-sided tests with a nominal significance level of 0.05 using R version 3.5.2.

Results

The mutational landscape of epigenetic-related genes of MSS-CRC in the TCGA cohort

A total of 68 epigenetic-related genes involved in 13 different pathways were included in the current research, including genes involved in modifying DNA, histones, and protein complexes that reshape chromatin structure (Supplementary Table 1). In the TCGA cohort, MSI-H and MSS-CRCs had epigenetic-related gene mutation frequencies of 66.67% (50/75) and 18.35% (78/425), respectively. The three most frequently mutated pathways in the MSS-CRC cases from TCGA were SWI_SNF, Histone_methylase, and CHD (Figure 1A), and the epigenetic-related genes ARID1A, KMT2C, and RSF1 had the highest mutation rates in the TCGA cohort (Figure 1B).

Epigenetic-related gene mutations are linked with the TMB, FS-mutation, and molecular subtype of CRC

High levels of TMB and FS-mutations (FS_mut) reflect a high degree of genomic instability and potential immunogenicity of a tumor, and both of these are therefore potential biomarkers of immune checkpoint inhibitor responsiveness. Hence, we examined the relationships between TMB, FS_mut, and epigenetic-related gene mutation status. In TCGA cohort, epigenetic-related gene mutations were associated with an increased incidence of TMB in MSS-CRC (median mutation rate of 4.76/mb vs. 4.99/mb in Wt and Mut cases, respectively; p = 7.4e-05; Figure 2A). A higher rate of FS_mut was also linked with epigenetic-related gene mutations in MSS-CRC (median frameshift mutation rate of 1.39/mb vs. 1.79/mb in Wt and Mut cases, respectively, p = 3.5e-06; Figure 2B). Molecular subtypes of CRC (CMS) are currently a highly recognized classification method for CRC that can accurately guide patient treatment and prognosis. CMS1, also known as the immune subtype, has better immune activity and high reactivity to ICIs. Here, we analyzed the distribution of Epigenetic_Mut samples based on

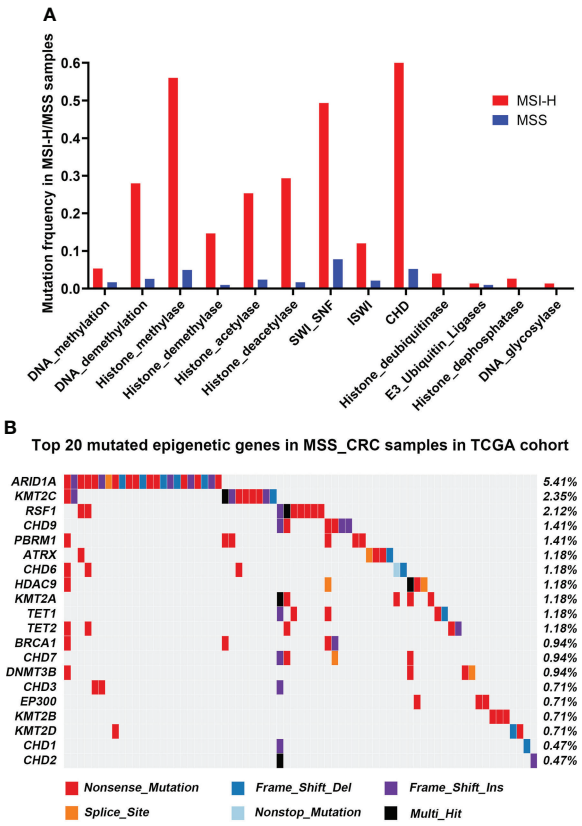

FIGURE 1
Mutational landscape of Epigenetic-related genes associated with MSS-CRC cases from the TCGA and HMUCH cohorts. **(A)** The frequency of epigenetic regulatory pathway alteration in MSS-CRC cases and MSI_H samples from the TCGA cohort. **(B)** The top 20 mutated epigenetic-related genes in MSS-CRC samples from the TCGA cohort.

molecular subtype in all CRC and MSS-CRC cases (Figures 2C, D). Among the CMS1-CRC cases, 74.12% were Epigenetic_Mut samples (74.12%, 63/85), but in MSS CMS1-CRC cases, this rate was 40% (12/30). Both for all samples and MSS CRC specifically, Epigenetic_Mut samples were enriched in the CMS1 (immune subtype) group.

Epigenetic_Mut is related to increased immune activity in MSS CRC

To identify the tumor immune microenvironment, we compared Epigenetic_Mut and Epigenetic_Wt for immune signatures, tumor-infiltrating lymphocytes, and expression of immune checkpoints and key genes. We demonstrated that epigenetic-related gene mutations increased the expression of immune response genes, including those involved in the IFN-γ pathway, antigen presentation, and cytotoxic T-cell function (Figure 3A). In addition, the expression of NK cell-related genes was increased in the Epigenetic_Mut group. Other immune cells also showed an upward trend, but no statistical difference was observed due to the limited cohort size (Figure 3B). Finally, we compared the expression of immune checkpoints and key genes between the two groups. In line with the immune response pathway, several immune checkpoints and key genes were upregulated in the Epigenetic_Mut group. In particular, the expression of *LAG3* and HAVCR2 was significantly elevated, and elevated levels of *TNFRSF4*, *PDCD1*, and *IL4I1* were very nearly statistically significant (Figure 3C).

Epigenetic_Mut predicts favorable clinical outcomes following ICI therapy

Next, to validate the function of epigenetic-related gene mutations further in predicting responsiveness to ICI therapy in MSS-CRC, we collected a clinical cohort of 89 MSS-CRC patients who had received PD-1 mAb-based treatment. Table 1

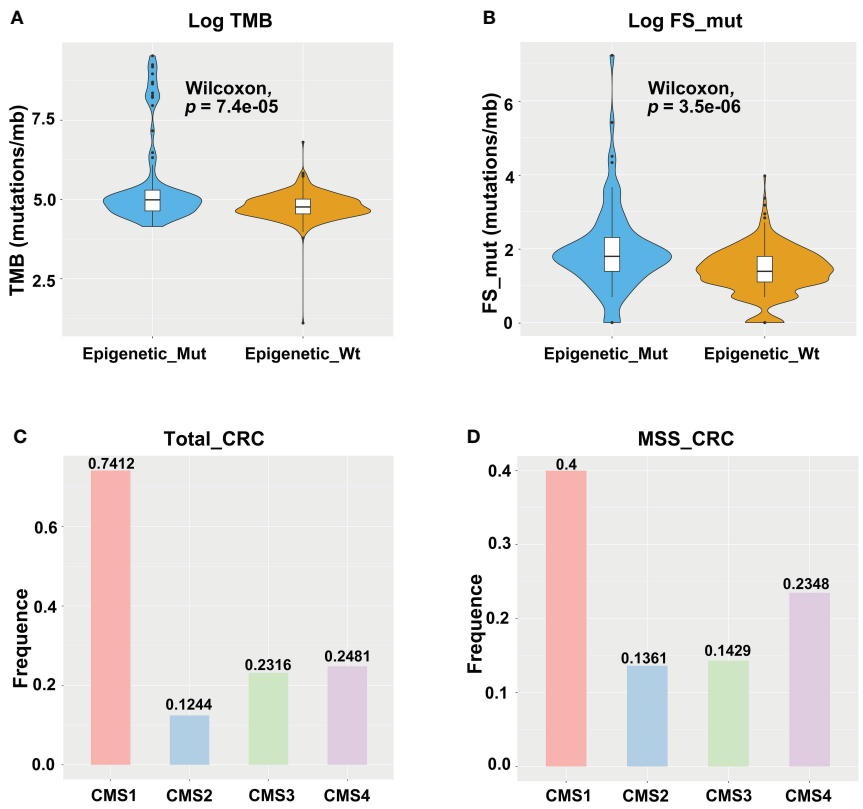

FIGURE 2
Epigenetic-related gene mutations are linked with the TMB, FS-mutation, and molecular subtypes of CRC. **(A)** TMB violin plot of Epigenetic_Mut and Epigenetic_Wt from MSSCRC samples. **(B)** FS-mutation rate violin plot of Epigenetic_Mut and Epigenetic_Wt from MSS-CRC samples. **(C)** Molecular subtype-specific fold enrichment of epigenetic-related genes mutation in all CRC cases (MSI-H/MSS). **(D)** Molecular subtype-specific fold enrichment of ARID1A mutation in MSS-CRC.

shows the baseline patient characteristics based on epigenetic-related gene status. Of the 89 patients, 24 had Epigenetic_Mut, and 65 had Epigenetic_Wt. Using RECIST version 1.1, we evaluated the patients' best overall responses. Compared to Epigenetic_Wt, Epigenetic_Mut had a significantly higher ORR (Figure 4A, 37.50% (9/24) vs. 15.38% (10/65), Fisher's exact test $P = 0.039$). As for DCR, the rate was 66.67% (16/24) in patients with epigenetic-related gene mutations from ICI treatment but only 36.92% (24/65) in patients without epigenetic-related gene mutations (Figure 4B, Fisher's exact test $P = 0.017$). As expected, PFS was greatly improved in patients with epigenetic-related gene mutations compared to those without epigenetic-related gene mutations in this cohort (Figure 4C, mPFS:6.00 vs. 3.17 months, Log_rank $P = 0.002$, HR = 0.4778), and ICI treatment also had a greater benefit on OS in the Epigenetic-Mut group than that in the Epigenetic-Wt group. (Figure 4D, mOS: 10.80 vs. 6.07 months, Log_rank $P = 0.003$, HR = 0.4279). In addition, we screened 9 genes with high mutation frequency from all epigenetic-related genes, whose predictive value has been demonstrated in other solid tumors, including ARID1A, ATRX, KMT2A/B/C/D, and TET1/2/3. The results showed that MSS-CRC with these gene mutations had more considerable ORR (Supplementary Table 4, 8/16, 50%) and DCR (Supplementary Table 4, 13/16, 81.25%).

The abundance of immune cells in tumor tissue and peripheral blood of patients with or without epigenetic-related gene mutation

We explored the densities of $CD8^+$ and $FOXP3^+$ cells in MSS-CRC samples with different epigenetic-related gene statuses using IHC. Of the 34 MSS-CRC samples, 10 had epigenetic gene mutations. Further, we captured representative images of $CD8^+$ cells and $FOXP3^+$ cells from three samples. The first patient had an *ARID1A* mutation (*ARID1A Frame_Shift_Del*), and the second patient had a *KMT2D* mutation (*KMT2D Nonsense_mutation*). Both samples showed increased $CD8^+$ cell density and decreased $FOXP3^+$ cell density in tumor tissues (Figures 5A, B). However, in

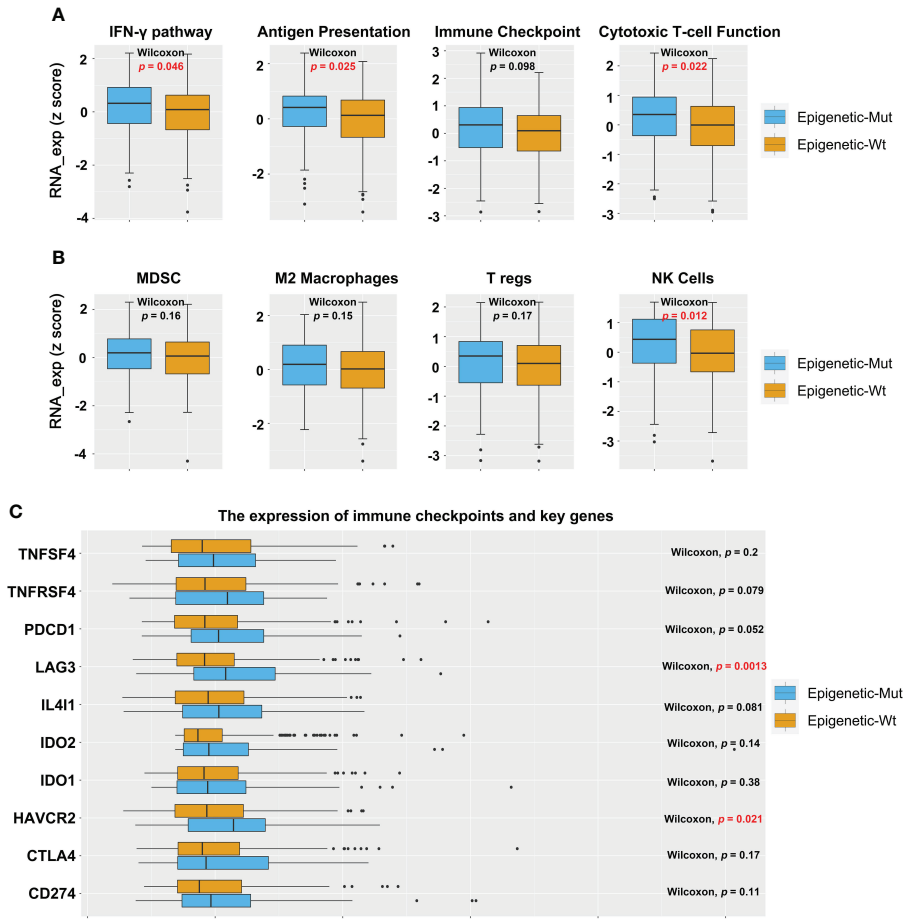

FIGURE 3
Epigenetic_Mut is associated with increased immune activity in MSS-CRC. **(A)** The RNA expression of immune response gene sets in MSS-CRC based on the epigenetic-related genes' mutational status. **(B)** The RNA expression of immune cells gene sets in MSS-CRC based on the epigenetic-related genes' mutational status. **(C)** The RNA expression of a single immune response gene in MSS-CRC based on the epigenetic-related genes' mutational status.

the third patient, who did not present any epigenetic-related gene mutations, the density of $CD8^+$ lymphocytes was lower, and the density of $FOXP3^+$ lymphocytes was higher than that of the other two (Figure 5C). $CD8^+$ and $FOXP3^+$ cell densities were counted in 38 patients (Epigenetic_Mut, N = 10; Epigenetic_Wt, N = 28), and from this we discovered that $CD8^+$ cell density increased in the Epigenetic_Mut group (Figure 5D) and that the $FOXP3^+$ cell density decreased in the Epigenetic_Mut group (Figure 5E). Furthermore, the ratio of CD8/FOXP3 cells in the Epigenetic_Mut group was significantly higher than that in the Epigenetic_Wt group (Figure 5F). Next, we collected peripheral blood from 12 patients with MSS-CRC (3 with epigenetic-related gene mutations) and measured the proportion of $CD8^+PD1^+$T cells and $CD3^-CD56^+CD16^+$NK cells by flow cytometry. We found that both the proportion of $CD8^+PD1^+$T cells and $CD3^-CD56^+CD16^+$NK cells was higher in the Epigenetic_Mut group (Figures 6A, B).

Discussion

Although ICI-based combination therapies have shown certain effectiveness in pMMR/MSS CRC, especially in combination with antiangiogenic agents (Lenvatinib or Regorafenib) that resulted in an ORR of 20-30% (6, 33), most patients still cannot benefit from the combination therapy because of the high heterogeneity of pMMR/MSS CRC. Recently, the MAYA phase II trial (NCT03832621) showed that MSS-CRC patients with silenced MGMT could benefit from ICIs combined with temozolomide treatment (34). This trial showed 36% for 8-month PFS, 42% for ORR, and 18.4 months for the median OS. Therefore, screening the MSS CRC patients with active anti-tumor immune response may be the key to improving the efficacy of immunotherapy. However, the predictive biomarkers for ICI therapy in MSS-CRC patients are limited.

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TABLE 1 Patient and disease characteristics of the validation set of MSS-CRC patients receiving ICI therapy.

Characteristics	Epigenetic_Mut (n = 24)	Epigenetic_Wt (n = 65)	P-value*
Age			0.651
<60	12	36	
≥60	12	29	
Sex			0.423
Male	13	36	
Female	11	29	
ECOG PS			0.857
0	16	42	
≥1	6	23	
ICI line			0.967
1	0	2	
2	6	16	
≥3	18	47	
Primary tumor sidedness			0.334
Right	10	20	
Left	14	45	
Liver metastases			0.683
With Liver	10	24	
Without Liver	14	41	
Regimen			0.951
ICIs + TKIs	10	26	
ICIs + Chemotherapy	11	32	
ICIs + Chemoradiotherapy	3	7	
Best overall response**			0.026
CR/PR	9	10	
SD	7	14	
PD	8	41	

* Fisher's exact test or Wilcoxon-Mann-Whitney test, as appropriate.
** CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

pMMR/MSS CRC is a cold tumor that contains few neoantigens and either no or inactive TILs (35). Meanwhile, CRC is a multilayered heterogeneous disease with specific treatment challenges and opportunities (36). Additionally, Previous studies have reported that epigenetic-related gene mutations affect both the tumor microenvironment and efficacy of ICIs (24–26). Mechanistically, epigenetic modification can reshape the tumor microenvironment by affecting genomic instability and enhancing the immunogenicity of tumor cells. First, epigenetic modification can affect the DNA damage repair response by regulating the accessibility of chromatin. Studies have shown that epigenetic-related gene mutations can lead to increased TMB in tumor cells, such as ARID1A and KMT2D. ARID1A specifically has a 6.7% mutation rate in MSS-CRC (22) and may increase the instability of the genome by adjusting the MMR pathway (21, 37). Mutations in the KMT2D gene are common in cancer patients, and their deficiency can increase the levels of genomic DNA damage and TMB, as well as increase transcription instability. Clinical studies have shown that individuals with mutations in genes from the KMT family are more likely to benefit from ICI therapy (24, 25). Furthermore, epigenetic-related gene mutation enhances the immunogenicity of tumor cells. Accounting for 5%-10% of genomic DNA sequences, human endogenous retroviruses (ERVs) are remnants of the evolution of germline integrations of exogenous infectious retroviruses (38, 39). These exogenous genes are not expressed in healthy tissues other than germ cells but are often abnormally expressed in tumors with epigenetic regulation defects. Here, neoantigen expression increases immunogenicity and triggers an innate immune response against tumors (40, 41). Recently, genome-wide technologies have revealed frequent mutations in epigenetic modifier genes, particularly in cancers (42). It is therefore necessary to analyze systematically the immune activity and the effect of immunotherapy in MSS-CRC patients with epigenetic regulation impairment.

In our study, we systematically analyzed 68 epigenetic-related genes from 13 pathways involved in chromatin regulatory processes in MSS-CRC samples. The mutation rate of epigenetic-related genes in the TCGA cohort was 18.35%. This mutation frequency was higher than that of any previous marker in the population, such as *POLE* or DDR mutations, and was closer to the potential benefit ratio in MSS-CRC clinical trials. *ARID1A*, *KMT2C*, *RSF1*, *CHD9, PBRM1*, and *ATRX* were the most mutated genes in the TCGA cohort, accounting for approximately 75% of the epigenetic-related gene-mutated MSS-CRC patients. This is consistent with previous reports, and *ARID1A* is thus a marker gene that should be investigated in clinical practice.

Using bioinformatics algorithms, we also assessed whether the MSS-CRC samples with epigenetic-related gene mutations from TCGA had better immune activity, including immune signatures, tumor-infiltrating lymphocytes, and expression of immune checkpoints and key genes. Furthermore, we validated our bioinformatic findings using immunohistochemical analyses of $CD8^+$ and $FOXP3^+$ cells from a cohort of MSS-CRC patients, and similar results were obtained at the histopathological level. In the Epigenetic _Mut group, $CD8^+$ cells were higher and $FOXP3^+$ cells were lower. The Epigenetic _Mut group also had a higher proportion of CD8/FOXP3 cells than the Epigenetic_Wt group. The VOLTAGE trial demonstrated that among MSS-CRC patients receiving ICIs as neoadjuvant treatment, patients with an elevated CD8/FOXP3 cell ratio were more likely to achieve pathologic complete response (pCR), suggesting that the CD8/FOXP3 cell ratio may be a predictor for ICI therapy efficacy (43).

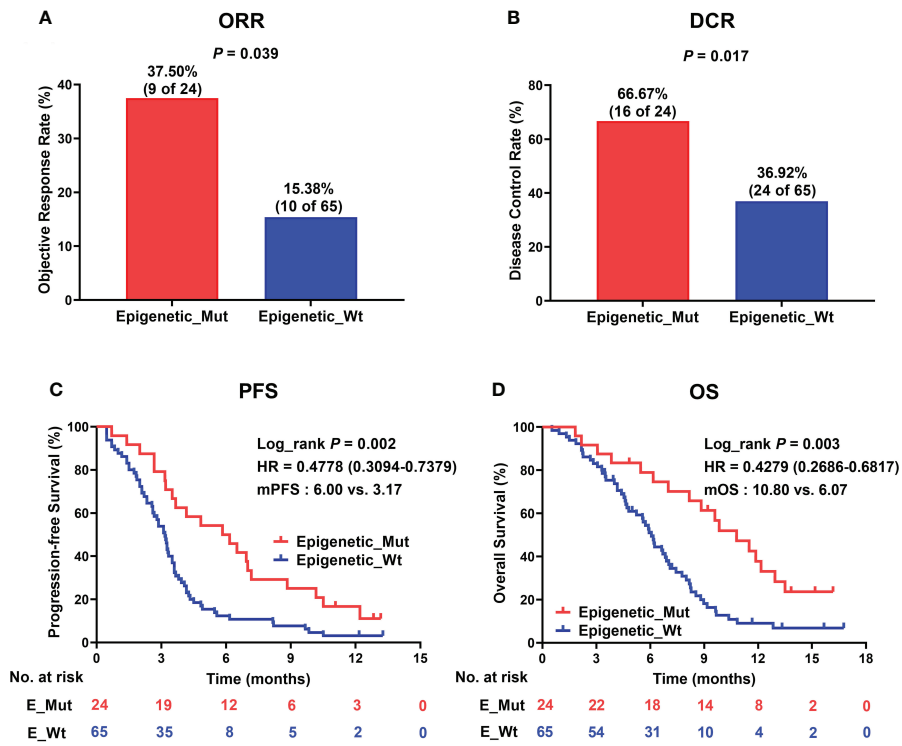

FIGURE 4
Epigenetic_Mut predicts favorable clinical outcomes following ICI therapy. **(A)** Histogram presenting the proportion of patients that acquired ORR in the Epigenetic_Mut and Epigenetic_Wt groups. **(B)** Histogram presenting the proportion of patients that acquired DCR in the Epigenetic_Mut and Epigenetic_Wt groups. **(C)** Kaplan–Meier estimates of PFS between Epigenetic_Mut or Epigenetic_Wt group patients in the discovery cohort. **(D)** Kaplan–Meier estimates of OS between Epigenetic_Mut or Epigenetic_Wt group patients in the discovery cohort.

Finally, we validated the predictive power of epigenetic-related gene mutations in the HMUCH cohort of 89 MSS CRC patients who received immunotherapy and discovered that patients with epigenetic mutations were more likely to benefit from ICI-based combination therapy and had better clinical outcomes. These preliminary results demonstrate that epigenetic-related gene mutations can predict the response to ICIs in MSS-CRC patients.

This study has several limitations, including the validation cohort coming from a single-center, the small size of the cohort, and the lack of validation in other populations. This is because ICI-based regimens have not been recommended by any clinical guidelines for MSS-CRC. Numerous patients included in this study experienced the failure of standard treatment, and the treatment compliance and completeness of the clinical information in many of these patients, were not ideal. Additionally, since the genetic information in the HMUCH cohort was obtained from clinical testing, transcriptomic data were lacking. Thus, our TCGA cohort findings could not be validated. Instead, we performed immunohistochemical staining analysis of pathological sections to validate the immune activation status of the Epigenetic _Mut group, but a larger-scale validation remains necessary. Furthermore, the application of ICIs in MSS-CRC has not been standardized, and most patients enrolled in our study were patients who had experienced multiple failed lines of treatment, bringing considerable heterogeneity to the population of this study. Therefore, future prospective studies with larger cohort studies are needed.

Conclusion

In conclusion, our data suggest that identifying epigenetic-related gene mutations might help select the right immunotherapy for MSS-CRC patients and can be used as a biomarker to predict ICI therapy effectiveness. Importantly, the status of epigenetic-related gene mutations is highly accessible from clinical genetic testing, although it is often overlooked by clinicians. Further exploration of the molecular mechanisms underlying the increased effectiveness in specific MSS-CRC patients and prospective clinical trials are therefore warranted.

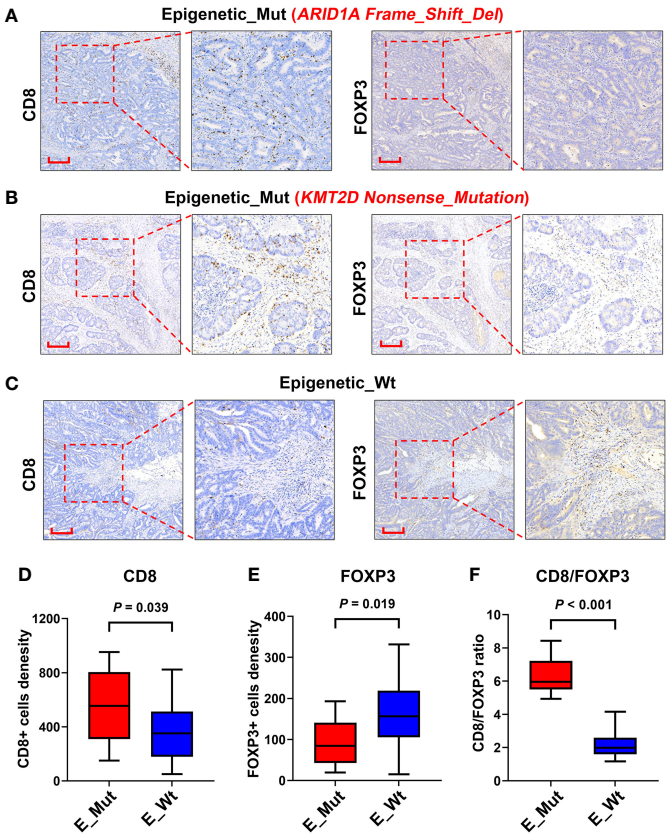

FIGURE 5
Infiltration of CD8$^+$ and FOXP3$^+$ lymphocytes in the tumors of patients with or without epigenetic-related gene mutations. **(A)** A Representative image of CD8$^+$ and FOXP3$^+$ lymphocytes infiltrating the MSS-CRC with ARID1A Frame_Shift_Del. **(B)** A Representative image of CD8$^+$ and FOXP3$^+$ lymphocytes infiltrating the MSS-CRC with KMT2D Nonsense_Mutation. **(C)** A Representative image of CD8$^+$ and FOXP3$^+$ lymphocytes infiltrating the MSS-CRC without epigenetic-related gene mutations. **(D)** Tumors with epigenetic-related genes mutation had significantly higher levels of intra-tumoral CD8$^+$ lymphocytes than tumors with wild-type epigenetic-related genes. **(E)** Tumors with epigenetic-related genes mutation had significantly lower levels of intra-tumoral FOXP3$^+$ lymphocytes than tumors with wild-type epigenetic-related genes. **(F)** The Epigenetic_Mut group had a higher CD8/FOXP3 cell ratio than the Epigenetic_Wt group.

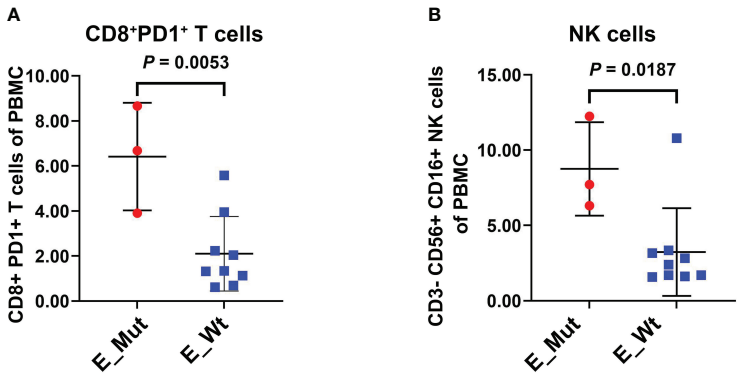

FIGURE 6
Proportion of CD8$^+$PD1+T cells and NK cells in the peripheral blood of patients with or without epigenetic-related gene mutations. **(A)** The Epigenetic_Mut group had a higher proportion of CD8$^+$PD1$^+$T cells compared to the Epigenetic_Wt group in peripheral blood. **(B)** The Epigenetic_Mut group had a higher proportion of NK cells compared to Epigenetic_Wt group in peripheral blood.

Data availability statement

The data presented in the study are deposited in the Genome Sequence Archive in National Genomics Data Center repository (https://ngdc.cncb.ac.cn/gsa-human), accession number GSA-Human: HRA003408.

Ethics statement

The study was approved by the Ethics Committee of Harbin Medical University Cancer Hospital (Harbin, China). Informed consents were obtained from all patients before surgery and during the experimental procedures.

Author contributions

CL: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. HX: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. LC: Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing. LF: Investigation, Methodology, Writing - review & editing. SH: Investigation, Writing - review & editing. YR: Investigation, Writing - review & editing. WZ: Conceptualization, Project administration, Resources, Supervision, Writing - review & editing. YZ: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing - review & editing. The work reported in the paper has been performed by the authors, unless clearly specified in the text. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1039631/full#supplementary-material

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Ensemble deep learning enhanced with self-attention for predicting immunotherapeutic responses to cancers

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Introduction: Despite the many benefits immunotherapy has brought to patients with different cancers, its clinical applications and improvements are still hindered by drug resistance. Fostering a reliable approach to identifying sufferers who are sensitive to certain immunotherapeutic agents is of great clinical relevance.

Methods: We propose an ELISE (Ensemble Learning for Immunotherapeutic Response Evaluation) pipeline to generate a robust and highly accurate approach to predicting individual responses to immunotherapies. ELISE employed iterative univariable logistic regression to select genetic features of patients, using Monte Carlo Tree Search (MCTS) to tune hyperparameters. In each trial, ELISE selected multiple models for integration based on add or concatenate stacking strategies, including deep neural network, automatic feature interaction learning via self-attentive neural networks, deep factorization machine, compressed interaction network, and linear neural network, then adopted the best trial to generate a final approach. SHapley Additive exPlanations (SHAP) algorithm was applied to interpret ELISE, which was then validated in an independent test set.

Result: Regarding prediction of responses to atezolizumab within esophageal adenocarcinoma (EAC) patients, ELISE demonstrated a superior accuracy (Area Under Curve [AUC] = 100.00%). AC005786.3 (Mean [|SHAP value|] = 0.0097) was distinguished as the most valuable contributor to ELISE output, followed by SNORD3D (0.0092), RN7SKP72 (0.0081), EREG (0.0069), IGHV4-80 (0.0063), and MIR4526 (0.0063). Mechanistically, immunoglobulin complex, immunoglobulin production, adaptive immune response, antigen binding and others, were downregulated in ELISE-neg EAC subtypes and resulted in

unfavorable responses. More encouragingly, ELISE could be extended to accurately estimate the responsiveness of various immunotherapeutic agents against other cancers, including PD1/PD-L1 suppressor against metastatic urothelial cancer (AUC = 88.86%), and MAGE–A3 immunotherapy against metastatic melanoma (AUC = 100.00%).

Discussion: This study presented deep insights into integrating ensemble deep learning with self-attention as a mechanism for predicting immunotherapy responses to human cancers, highlighting ELISE as a potential tool to generate reliable approaches to individualized treatment.

KEYWORDS

deep learning, immunotherapy, cancer, PD1/PD-L1, ELISE

Introduction

Avoiding immune surveillance by reconstructing the tumor microenvironment and compromising antigen presentation machinery to seize growth advantages has been widely recognized as a hallmark of human cancers (1), which makes adoptive cell transfer and therapies targeted to immune checkpoints the new therapeutic pillars within oncology (2). Many immunotherapies have received durable clinical responses, including pancreatic (3), gastric (4), bladder (5), and lung cancer (6); however, limited response rates and unclear underlying mechanisms hinder further immunotherapy development, so only subsets of cancer patients can benefit from them (7). For instance, although nivolumab renewed melanoma clinical treatment, about 39% of patients had progressed at the 5-year follow-up (8). Failure of immunotherapies to reach tumor remission is ascribed to many molecular and cellular mechanisms, such as altered tumor microenvironment (9, 10) and defects in antigen presentation machinery (11), which makes the key points of clinical success of future immunotherapeutics likely to lie in the pre-evaluation of individual responses in order to tailor strategies (9).

The emerging deep learning technologies have the potential to drive away the shadows hanging over immunotherapy and offer a glimmer of hope, since it has already powered recent disease diagnosis and prognosis prediction (12). For example, prognostication of clear cell renal cell carcinoma significantly benefits from deep learning, even in a previous study where a very simple neural network was deployed (13). The immunotherapeutic responses prediction is a classification issue that can be greatly improved with many state-of-the-art (SOTA) neural network architectures that have demonstrated their outstanding performances in computational science fields but have yet to be applied in medical areas. For example, Autoint (Automatic feature interaction learning *via* self-attentive neural networks), a deep neural network with residual connections and a multi-head self-attention, can map both numerical and categorical features into the same low-dimensional space to explicitly model the feature interactions, and has demonstrated its SOTA performance in the benchmark comparison (14). RNA-seq data are typically ultra-high-dimensional data, which are difficult to be fitted accurately by a single algorithm. Secondly, the data distribution of gene expression profiles approximates a Poisson distribution. However, considering the different sequencing platforms, the actual distribution may be a mixture of multiple distributions. Therefore, a combination of different algorithms is needed

In the present study, we proposed ELISE (Ensemble Learning for Immunotherapeutic Response Evaluation) by combining Linear Neural Network (LNN), Deep Neural Network (DNN), Deep Factorization Machine (DeepFM), Compressed Interaction Networks (CIN), and Autoint. ELISE inputted a pre-selection phase to erase irrelevant features and employed MCTS algorithm to output the best model. ELISE was validated to be a general pipeline for predicting immunotherapeutic responses to many human cancers and featured high potential for predicting any immunotherapeutic response against any tumor.

Materials and methods

Patients

Responses data of atezolizumab on resectable EACs were obtained *via* Gene Expression Omnibus (GEO) (GEO Access ID: GSE165252), which presented RNA expression data in the form of normalized counts. In the present study, GSE165252 were converted to TPM (Transcripts Per Million, or Transcripts Per kilobase of exon model per Million mapped reads) using R

software (version 4.1.0), as normalized counts are not acceptable for any prediction models.

Responses data and RNA-seq data of PD-1/PD-L1 suppressor on metastatic urothelial cancers and MAGE–A3 on metastatic melanoma, were respectively obtained *via* GEO Access IDs GSE176307 and GSE35640.

ELISE architecture

The feature selection phase was conducted with R software, implementing logistic regression as per our previous study (15), for selecting features that impact outcomes significantly. Features met p-value < 0.001 in their corresponding logistic regression model were retained and considered as the important features for clinical outcomes.

The remaining phases of ELISE were conducted with Python software (version 3.8). LNN and DNN are the base neural network architecture, differing in the number of hidden layers according to our previous study. LNN and DNN performed well in some cases, so both were included in ELISE (12). DeepFM combines the power of factorization machines for recommendation and deep learning for feature learning in a new neural network architecture (16). CIN aims to generate feature interactions in an explicit fashion at the vector-wise level (17). AutoInt can be applied to both numerical and categorical input features, and maps these into the same low-dimensional space. Then, a multi-head self-attentive neural network with residual connections was used to explicitly model the feature interactions in the low-dimensional space (14). All these neural networks were applied using package DeepTable in python, and MCTS used for hyperparameters tuning (github.com/DataCanvasIO/DeepTables).

For each trail in the model training, ELISE used MCTS to decide what models should be trained, and then optimized their hyperparameters based on the observation of hyperparameters optimization history. After all models were trained, they were considered as "weak learners". ELISE stacked all predictions of "weak learners" to output final prediction.

Area Under Curve (AUC) of receiver operating characteristic curve (ROC) and calibration were employed to evaluate performance of ELISE in the test and train cohorts. These analyses were conducted in R with pROC and rms packages.

Interpretability

SHAP provides a game theory-based approach to interpret any deep learning models' output, connecting optimal credit allocation with local explanations using the classic Shapley values from game theory and their related extensions (18). We employed SHAP to interpret ELISE using the shap package in python.

Dissecting molecular mechanisms

Gene set enrichment analysis (GSEA) was employed to elucidate the dysregulated biological processes, molecular functions, cellular components, and signaling pathways of ELISE subtypes. The differential expressed genes (FDR < 0.05, $\log_2$ Fold-Change >1) were involved in GSEA analyses. GSEA relied on Gene Ontology dataset and KEGG dataset curated in GSEA official database (19).

Estimation of stromal and immune cells in malignant tumor tissues using expression data (ESTIMATE) algorithm is a sophisticated algorithm which is designed for measuring the degree of infiltration of cancer cells and different normal cells by exploiting the unique properties of tumor cell transcriptional profiles (20), with its robustness having been validated in various cancers. The present study employed ESTIMATE algorithm which was provided by ESTIMATE package in R. This was used to quantify the global tumor microenvironment into four characterized indictors, including stromal score, immune score, ESTIMATE score, and tumor purity, representing infiltration abundance of stromal cells, immune cells, overall normal cells, and tumor cells, respectively. Since the resultant data had a skewed distribution, a grouped comparison was performed with a Wilcoxon test, and Spearman coefficients evaluated their correlation. All p-values were corrected using Benjamini–Hochberg method to avoid false positive results.

According to our previous study, we used single sample GSEA (ssGSEA) to dissect immune cell infiltration between ELISE subtypes (12).

Statistical analysis

Raw data were collated by R software. The statistical analyses were based on R and Python software. The statistical results and interactive network data analysis were visualized with Cytoscape version 3.7.1 (Cytoscape Consortium, San Diego, California, USA). According to the previous study (15), Pearson's and Spearman's correlation coefficients were utilized to calculate continuous and categorical variables, respectively.

Results

ELISE methodology

We proposed ELISE as a computerized approach for individualized prediction of immunotherapeutic response to human cancers based on their transcriptomic data (Figure 1). ELISE consists

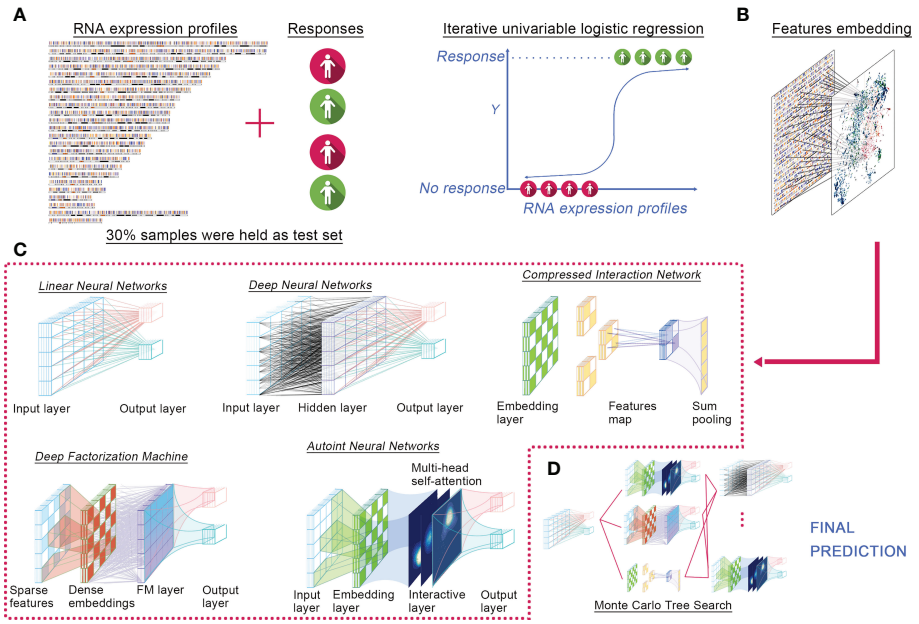

FIGURE 1
ELISE pipeline. **(A)** Inputted data. **(B)** Feature embedding. **(C)** Neural networks. **(D)** Hyperparameters tuning.

of four core components: Feature Selection, Feature Embedding, Deep Learning Models, and Hyperparameter Optimization modules. The collated transcriptome data and clinical immunotherapy response data were first loaded into the Feature Selection module, which employed iterative univariable logistic regression to parallelize and evaluate the impact of all input features on the outcomes, where features with *p*-values less than the pre-defined screening threshold were subsetted as input data of the next module, Feature Embedding (Figure 1A). Subsetted features were either directly loaded into a dense layer of the next training model, LNN, or those features were discretized or categorized to the embedding layer (Figure 1B). The subsequent module is Deep Learning Models (Figure 1C), which incorporated five of the most prevalent neural network architectures available recently, including DNN, Autoint, DeepFM, CIN, and LNN. After pre-defining the hyperparameter search space or directly adopting the default settings, the Hyperparameter Optimization module was initiated for hyperparameter optimization *via* MCTS algorithm (Figure 1D). In each trial, the module trained a different number of neural networks, performed individual hyperparameter tuning for each network, and subsequently stacked all networks using the concatenate or add strategy and offered the final prediction. Notably, ELISE employed a sigmoid function as the activation function, a binary cross-entropy as the loss function, an AUC as the evaluation metric, and an Adam optimizer in all trials.

ELISE was designed to process different normalized data, no matter TPM or RSEM. The potential user just needs to ensure their data in a standalone task is homogeneous, i.e., normalized *via* the same method. For evidence these hypotheses, we implemented ELISE for three different tasks. Data normalization methods among these tasks were different, but each task's data was normalized *via* the same method to ensure their homogeneous.

ELISE performed with outstanding accuracy in predicting atezolizumab responses to EAC

A total of 76 EAC suffers were randomly split into initial train and test cohorts at a proportion of 8:2, and 10% of those in the initial train cohort were randomly shuffled out as the validation cohort with the remaining 90% defined as the final train cohort. Then, ELISE trained the prediction model only with the train cohort, which was validated using the validation cohort and then independently tested within the test cohort. Feature Selection module identified 442 RNAs as the most important contributors to atezolizumab responses (all $p < 0.001$, Figure 2A). The retained features were loaded into Deep Learning Module for launching the training process, in which the drifting features were corrected with Adversarial Validation algorithm. After ten trials, MCTS identified the ninth trial as the best trial with the smallest validation loss and relatively small train loss; the train history and the best hyperparameters are

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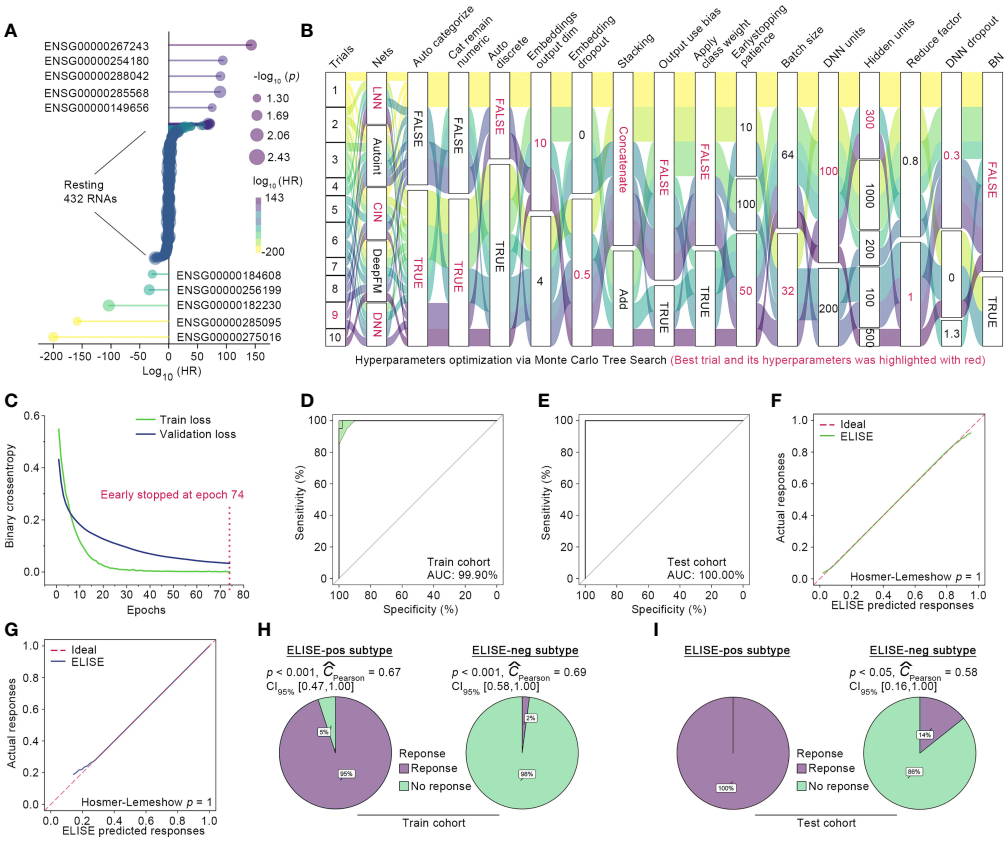

FIGURE 2
ELISE applied to EACs. **(A)** Resultant data of feature selection. **(B)** Hyperparameter optimization. **(C)** Loss curves of the best trial. **(D, E)** presented AUCs of ELISE in the train and test cohort. **(F, G)** are the calibration plot of ELISE in the train and test cohort, respectively. **(H, I)** displayed the actual outcomes distribution in the ELISE-neg and ELISE-pos subtypes.

presented in Figure 2B. Specifically, the ninth trial was stopped early at the epoch 74 with a train loss of 0.0011 and a validation loss of 0.0329 (Figure 2C).

The independent test cohort was then employed for testing ELISE performance. The existing expert consensus is that a prediction model is considered to feature high discrimination when its AUC is higher than 75% (21). As expected, ELISE presented outstanding discrimination in terms of atezolizumab responses prediction, which could be evidenced by the AUC of 100.00% in the test cohort and AUC of 99.90% in the training cohort (Figures 2D, E). Calibration plots also demonstrated that ELISE performed a good calibration (Figures 2F, G), which means ELISE could correctly estimate the absolute risk (21). ELISE ultimately distinguished EAC patients into two subtypes, the ELISE-pos subtype (ELISE-identified subtype with positive response to immunotherapies) and the ELISE-neg subtype (ELISE-identified subtype with negative response to immunotherapies), in which the ELISE-pos subtypes displayed a predominant proportion of patients with immunotherapeutic response and the ELISE-neg subtype held the opposite, with most patients without an immune response (Figures 2H, I).

Interpreting ELISE

Deep learning models are deemed "black boxes," despite the good predictions made; however, it is difficult to understand the logic behind the predictions (22). The correct interpretation of these "black boxes" is of great importance, as they engender appropriate user trust and support the understanding of the process being modeled (23). However, the prevailing method to interpret deep learning or machine learning model in the medical field remains Variable Importance algorithm (12, 24), which is a biased method that fails to explain how the features affect the specific or overall predictive ability of the models (23, 24). A novel algorithm, SHAP, has been proposed to overcome these limitations (23). SHAP is a game theoretic approach to interpret the output of any deep learning model. It computes the

global interpretation by calculating and combining the SHAP values for a whole dataset and measures the impact direction of each feature (23). In the present study, SHAP was used to interpret ELISE and improve the user trust of it.

Figure 3A summarizes the top 20 SHAP-identified important features, ranked according to Mean (|SHAP value|) to quantify the impact of all features on ELISE prediction (unfavorable immunotherapeutic response). *AC005786.3* (Mean [|SHAP value|] = 0.0097) was distinguished as the most valuable contributor to ELISE output, followed by *SNORD3D* (0.0092), *RN7SKP72* (0.0081), *EREG* (0.0069), *IGHV4-80* (0.0063), *MIR4526* (0.0063), etc. SHAP values includes an essential property that always sum up the difference between the players-present and players-absent game outcomes. For ELISE, a deep learning model, SHAP values of all the input features will always sum up to the difference between baseline (expected) and real-time ELISE outcomes for the prediction being explained (25). Thus, the SHAP algorithm interpreted how ELISE summed up each features' contribution and made the final predication accordingly. The stacked force plot presented in Figure 3B displayed features contributing to pushing the ELISE individual prediction from the base value (the mean ELISE prediction over the train set) to the final prediction (features pushing the prediction higher are marked in red and those pushing the prediction lower are in blue). The decision plot in Figure 3C further highlights the contributions of the top 20 features' observed values to ELISE outputs and how they push the model prediction in each sample. The impacts of the top six features on the output of ELISE were further quantified with dependent plots (Figure 3D); *AC005786.3* and *EREG* demonstrated negative contributions to ELISE, predicting poor responses to immunotherapies, with Spearman's ρ to their SHAP values of 0.73 and 0.80, respectively. On the contrary, *SNORD3D*, *RN7SKP72*, *IGHV4-80*, and *MIR4526* raised risk to unfavorable responses, which were evidenced by their Spearman's ρ of -0.90, -0.89, -0.84, and -0.84, respectively. *SNORD3D*, *RN7SKP72*, *IGHV4-80*, and *MIR4526* held much higher expression profiles within ELISE-neg subtypes than other

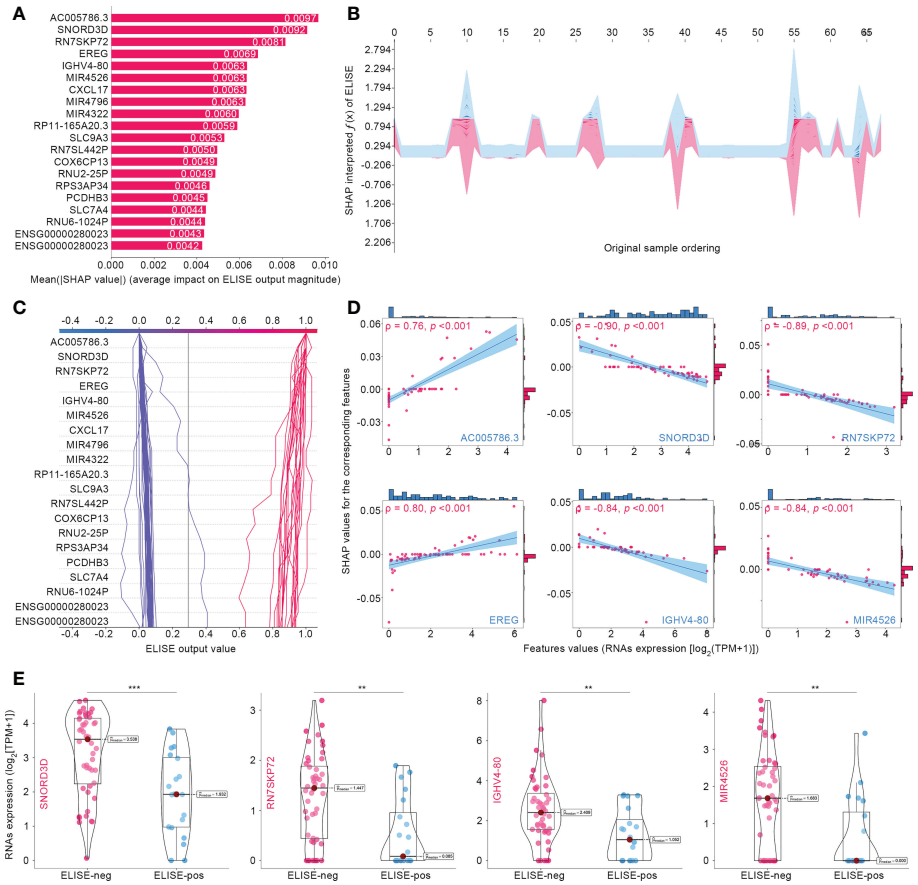

FIGURE 3
Interpreting ELISE in EACs. **(A)** SHAP summary plot ranked and presented the top 20 important features. **(B, C)** exhibited how ELISE makes the global and individual prediction. **(D)** Dependent plot indicated the affection directions of top 6 features. **(E)** Many top important features identified by SHAP presented differential expression profiles between ELISE-neg and ELISE-pos subtypes. The symbols ** represents $p < 0.01$, and *** represents $p < 0.001$.

ELISE-pos counterparts (Figure 3E), which was in the line with resultant data of dependent plots and reaffirmed that they served as risk factors to poor responses to immunotherapies given to EAC patients.

Molecular mechanisms leading to poor responses to atezolizumab in ELISE-neg EAC subtype

It is of great clinical relevance to provide deep insight and elucidate the molecular mechanisms underlying the failure to immunotherapies in the ELISE-neg EAC subtype. GSEA was employed to offer an atlas of dysregulated biological processes, molecular functions, cellular components, and signaling pathways of ELISE subtypes. As resultant data shown in Figure 4A, certain critical biological processes, molecular functions, and cellular components involved in immunosurveillance and the cytotoxic effect mediated by immune cells towards human EAC, including immunoglobulin complex (NES: 0.656, adjusted < 0.001), immunoglobulin production (NES: 0.607, adjusted < 0.001), production of molecular mediator of immune response (NES: 0.532, adjusted < 0.001), adaptive immune response (NES: 0.462, adjusted < 0.001), antigen binding (NES: 0.563, adjusted < 0.001), and T cell receptor complex (NES: 0.548, adjusted < 0.001), were upregulated in ELISE-pos EAC and downregulated in ELISE-neg EAC subtypes. Further GSEA to analyze dysregulated signal pathways also revealed that key immune pathways were enriched in ELISE-pos EAC subtypes, which were downregulated in ELISE-neg subtypes, such as antigen processing and presentation (Figure 4B), and the proteins encoded by key RNAs involved in the ELISE model had significant interactions (Figure 4C). These

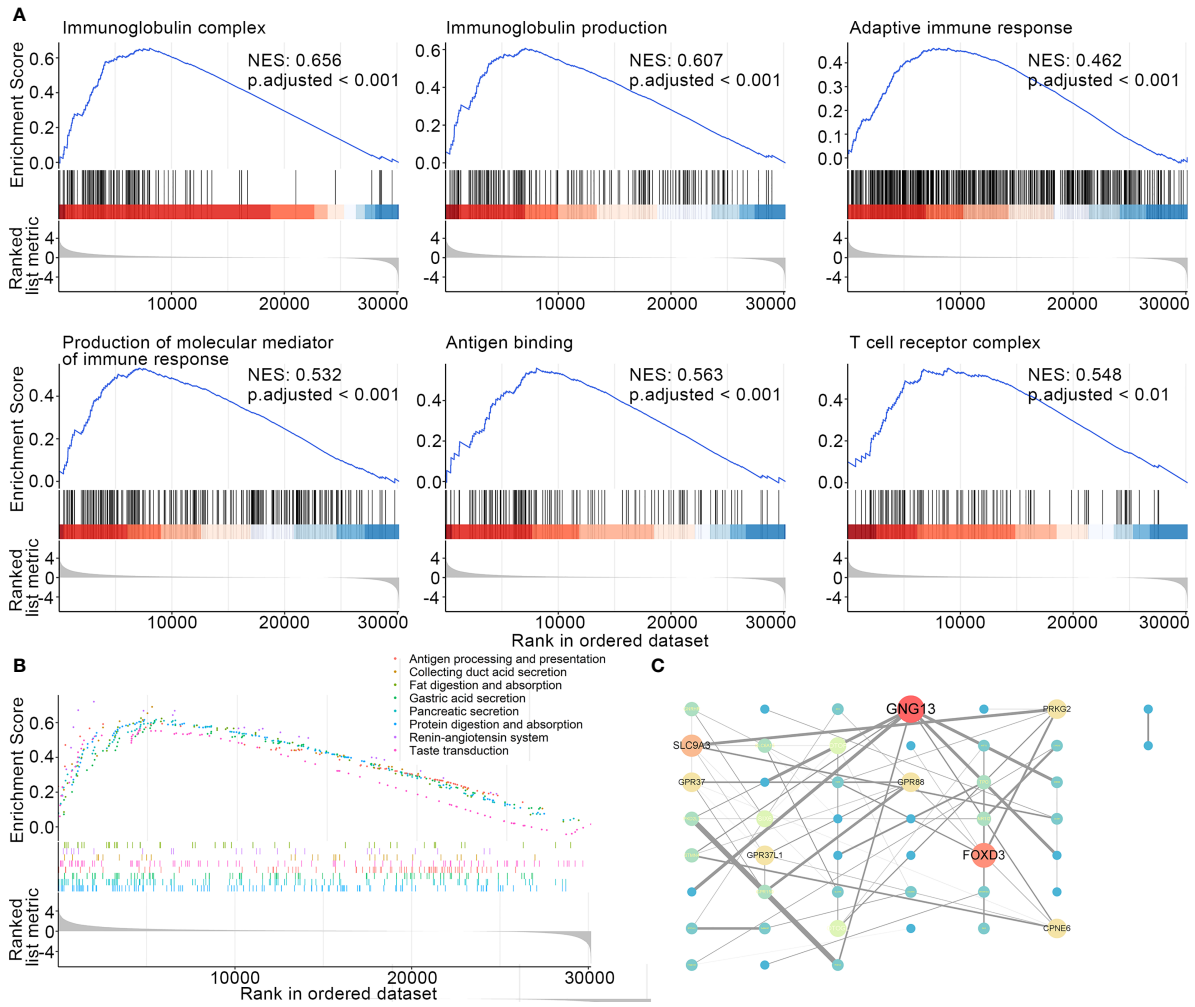

FIGURE 4
Dissecting underlying mechanisms leading to different outcomes. **(A)** Resultant data of GSEA (BP, CC, MF). **(B)** GSEA results (signaling pathways). **(C)** Protein-protein interaction network.

positive findings strongly indicate that critical molecules synergistically mediate the downregulation of immune signaling and result in failure of atezolizumab treatment on EACs.

Since the immune microenvironment in EACs plays a vital role in their tumorigenesis, malignant progression, and remote migration, we further investigated the tumor microenvironment of ELISE-subtyped EACs. However, as displayed in Figure 5, tumor purity, immune microenvironment, 29 types of immune cellular component infiltrations, and stromal cells infiltration, did not show any significant differences between ELISE-pos and ELISE-neg subtypes. These results indicate that atezolizumab may affect the immune microenvironments less, but still affects immune cell functions and their downstream pathways in EACs.

ELISE is a general pipeline to predict immunotherapeutic responses to human multi-cancers

To evidence the general applicability and robustness of ELISE in the prediction of pan-cancer responses to immunotherapies, it was tested in two different human cancers, metastatic urothelial cancer (UC) and metastatic melanoma.

For prediction of responses to PD1/PD-L1 suppressor against metastatic UCs, ELISE included 89 subjects' RNA expression profile data, in which 70% were assigned to a train cohort, 10% to a validation cohort, and the remaining 20% to an independent test cohort. During the feature selection phase, ELISE distinguished 624 RNAs as the most critical factors that caused high responses to immunotherapies of metastatic UCs (Table S1) that were then fed into the model training phase. ELISE employed 10 trials to select and ensemble a final prediction model and chose Trial 6 as the best trial (Figure 6A). The best hyperparameters included: models ensembled (DNN, Autoint, and DeepFM), parameters activated (Auto Categorization, Cat Remaining Numeric, Output Use Bias, Class Weight), parameters disabled (Auto Discrete, Batch Normalization), Stacking by Add, Embedding Output Dim of 4, Embedding Dropout of 0, Early Stopping Patience of 50, Batch Size of 64, DNN Units of 200, Hidden Units of 300, Reduce Factor of 1, DNN Dropout of 0.3, and Space Vectors of [21, 1, 1, 0, 0, 0, 0, 1, 1, 1, 1, 1, 2, 0, 1, 0]. Loss curves of Trial 6 are presented in Figure 6B, and early stopping was activated at Epoch 64. Finally, ELISE performed well in pre-evaluating responses to PD1/PD-L1 suppressor against metastatic UCs, which could be evidenced by high AUCs in the train cohort (Figure 6C) and the test cohort (Figure 6D). ELISE prediction, also as expected, was highly in line with the actual responses to PD1/PD-L1 suppressor upon metastatic UCs in the train cohort (Figure 6E) and the test cohort (Figure 6F).

When applied to foresee MAGE−A3 responses to metastatic melanoma, ELISE also performed outstandingly. ELISE trained a prediction model with RNA expression data of 56 melanoma suffers, in which 713 RNAs were identified as the most significant impactors for raising unfavorable responses to MAGE−A3 treatment (Table S2). After all trials were completed, Trial 2 was triumphed as the best trial (Figure 6G), offering the best hyperparameters of models ensembled (DNN, LNN, and DeepFM), parameters activated (Auto Categorization, Auto Discrete, Class Weight, Batch Normalization), parameters disabled (Cat Remaining Numeric, Output Use Bias), Stacking by Concatenation, Embedding Output Dim of 4, Embedding Dropout of 0.5, Early Stopping Patience of 100, Batch Size of 32, DNN Units of 100, Hidden Units of 100, Reduce Factor of 0.8,

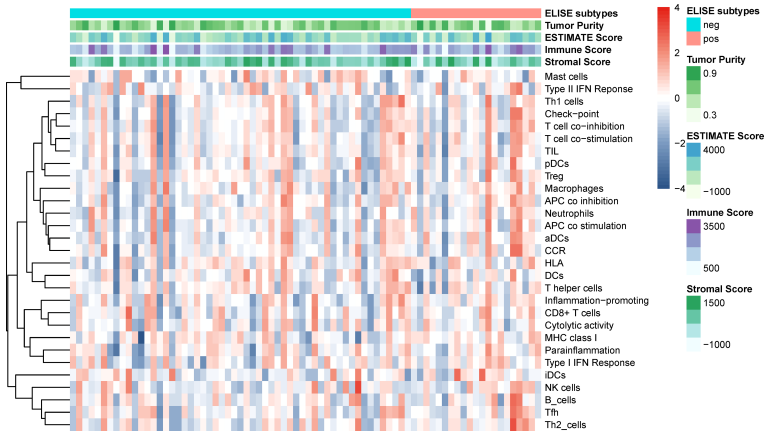

FIGURE 5
Tumor microenvironments and immune cell infiltration.

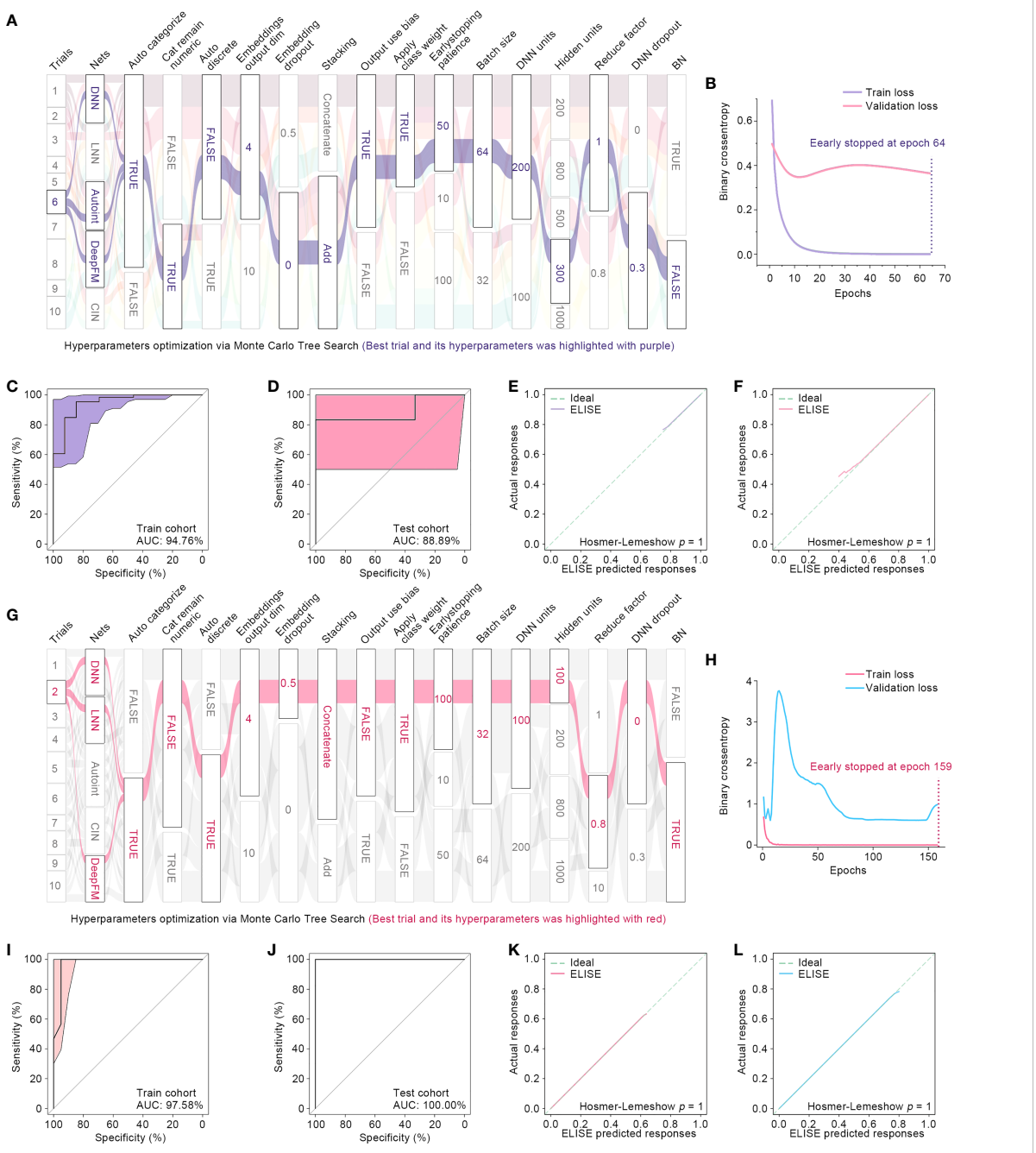

FIGURE 6
ELISE applied in UCs and melanoma. **(A)** Hyperparameter optimization in UCs. **(B)** Loss function curves in UCs. **(C, D)** AUCs of ELISE applied in UCs in the train and test cohort. **(E, F)** are the calibration plots of ELISE in the train and test cohort, respectively. **(G)** Hyperparameter optimization in MELANOMAs. **(H)** Loss function curves in MELANOMAs. **(I, J)** AMELANOMAs of ELISE applied in MELANOMAs in the train and test cohort. **(K, L)** are the calibration plots of ELISE in the train and test cohort, respectively.

DNN Dropout of 0, and Space Vectors of [25, 1, 0, 1, 0, 1, 1, 0, 1, 2, 0, 0, 0, 1, 0, 1]. ELISE reached high AUCs in the train and test cohorts of 97.58% and 100%, respectively, which demonstrated that ELISE presented high discrimination in predicting MAGE−A3 responses against metastatic melanoma.

Subsequently, ELISE was tailored to predict responses to PD1/PD-L1 suppressor against metastatic UCs, picked for interpretation owing to the large sample size of the metastatic UCs cohort, to demonstrate the general interpretability of ELISE in the human pan-cancers. As demonstrated in Figure 7A, SHAP

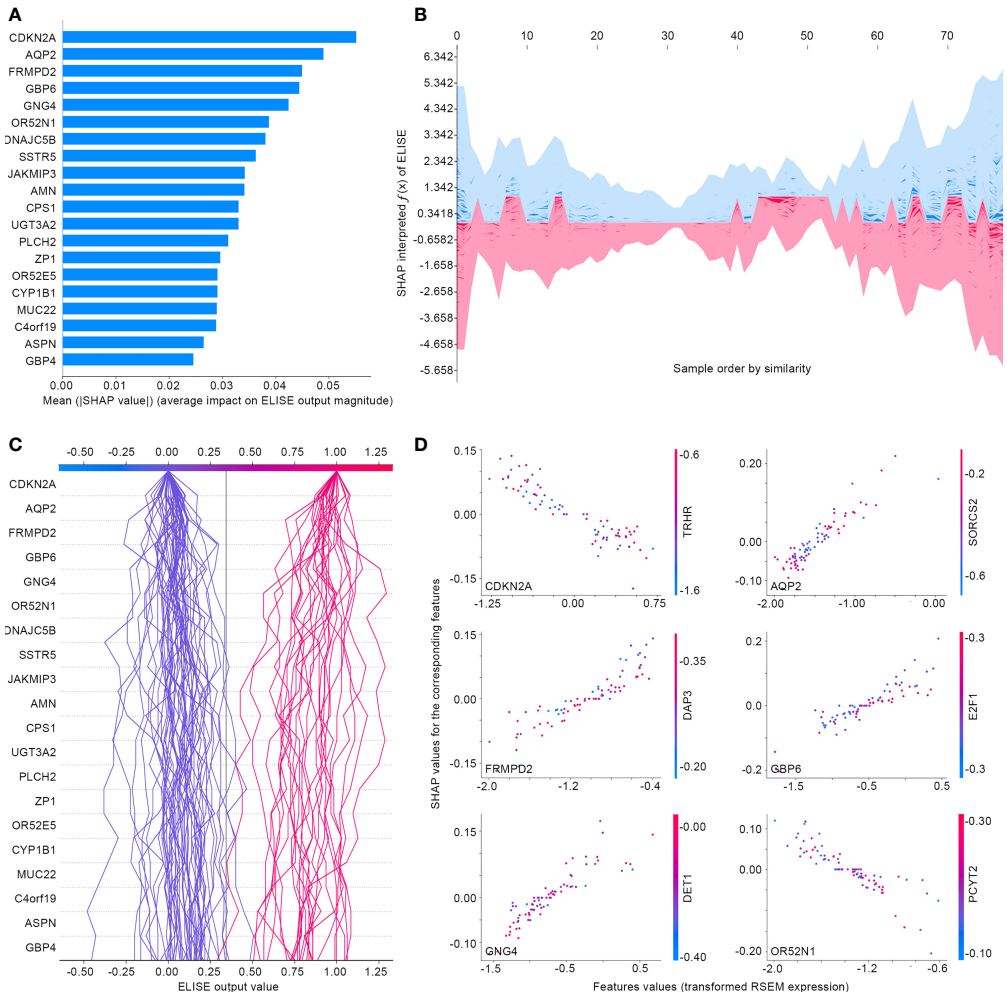

FIGURE 7
Interpreting ELISE in UCs. **(A)** SHAP summary plot ranked and presented the top 20 important features. **(B, C)** exhibited how ELISE makes the global and individual prediction. **(D)** Dependent plot indicated the affection directions of top 6 features.

algorithm ranked all inputted features according to their mean |SHAP values| to discover important features that made the decisive contributions to unfavorable immunotherapeutic responses. *CDKN2A* was outstanding as the most pivotal contributor with the highest mean |SHAP values| of 0.0551, followed by *AQP2*, *FRMPD2*, *GBP6*, *GNG4*, *OR52N1* etc. Furthermore, SHAP algorithm stacked contributions of all participants to directly visualize how ELISE made the individualized prediction according to the original inputted features values, shown in Figures 7B, C. As learned from Figure 7D, *AQP2*, *FRMPD2*, *GBP6*, and *GNG4* served as catalysts to increase resistance to PD1/PD-L1 suppressor within metastatic UCs. Conversely, *CDKN2A* and *OR52N1* declined the PD1/PD-L1 resistance, which demonstrates that suffers with metastatic UCs will be more sensitive to PD1/PD-L1 suppressor with the increased expression of *CDKN2A* and *OR52N1*.

Discussion

The present study conducted based on real-world patient data represents, to the best of our knowledge, the first attempt to develop a general pipeline for predicting responses of various immunotherapies against human pan-cancers. The contribution of our findings to the related scientific fields is not only the proposed ELISE pipeline that has already been attested for its generalization and robustness, but also offers an interpretable tool that could highly foster user trust and has the prevailing advantages for clinical application. With the assistance of the

present ELISE, oncologists and clinicians will be able to pre-evaluate individual responses to specific immune treatment more rapidly and decide on tailored therapeutic approaches with a high confidence level provided by ELISE.

As a state-of-the-art bioinformatic tool, deep learning has achieved an overwhelming advantage in disease diagnosis and treatment response prediction (12, 15, 26). Traditionally, diagnosing cancers relies highly on histopathology or cytopathology, which mainly involves assessment under microscopy to detect aberrant cells within a clinical sample, evaluate biomarkers of certain cancers and determine cancers' subtype, stage, and grade (27, 28). However, the high-throughput feasibility and reliability of such approaches has been compromised by their nature of labor-intensive and human subjectivity (29). Benefiting from deep learning, clinicians are now able to automatically or semi-automatically stage many malignant tumors, including prostate (30), colon (31), and skin cancer (32), with comparable accuracy to pathologists. Notably, deep learning plays a critical role in cancer treatment decisions that cannot be ignored, as one of the promises of precision oncology is individualizing treatment to achieve tumor remission and prolong the overall survival of patients (29, 33, 34). A large-scale study investigated over 650 drug sensitivity data on thousands of cell lines and raised a deep learning tool called "DrugCell", which is designed as an interpretable model to predict response to therapies and is successfully validated with *in vitro* and *in vivo* data (35). More encouragingly, deep learning techniques have raised many opportunities to discover and identify drugs sensitive to human cancers, such as cimetidine sensitive to lung adenocarcinoma (36), emetine to atypical meningiomas (37), and vinorelbine to *TTN*-mutated tumors (38). These enlightening shreds of evidence prove that deep learning could be greatly beneficial in predicting immunotherapeutic responses.

With these exciting techniques, we propose ELISE, one of the present study's most important findings, for offering highly accurate pre-evaluation of immunotherapeutic responses. ELISE powers many immune treatments for human cancers, and theoretically could be employed for predicting any immunotherapeutic response against any tumor. Taking EACs as an example, ELISE demonstrated high discrimination when employed to predict atezolizumab responses (AUC = 100.00% in the test cohort). When applied to predict other immunotherapies on different tumors, including PD1/PD-L1 suppressor against metastatic UCs and MAGE−A3 responses to metastatic melanoma, ELISE also performed outstandingly, which could be evidenced by our findings in Figure 6. Compared to other studies, ELISE exhibited its overwhelming advantages in terms of therapeutic outcomes prediction. For predicting atezolizumab responses of EAC patients, ELISE reached AUC value of 100.00%, yet previous study only achieved AUC value less than 80.00% (39). These positive results are attributed to the design of the ELISE pipeline. ELISE employed feature selection and feature embedding modules to pre-erase "noise" i.e., features with less or no influences upon outcomes, ensembled many state-of-the-art deep learning networks architecture including LNN, DNN, Autoint, DeepFM, and CIN, and implemented a state-of-the-art hyperparameters optimization algorithm, MCTS, for tuning hyperparameters and stacking networks to generate the best model. Moreover, ELISE does not require specific data normalization processes if batch effects are pre-removed; in fact, it can process any RNA expression data, regardless of TPM data (Figure 3) or RSEM data (Figure 6).

Autoint is a deep neural network with residual connections and multi-head self-attention; it works with the same low-dimensional space, which is mapped from both numerical and categorical features, to explicitly model the feature interactions (14). With the assistance of multi-head self-attentive neural networks, Autoint can further refine interactions of high-order features and satisfactorily fit large-scale RNA expression data in an end-to-end fashion (14). DeepFM, which was designed as an end-to-end wide & deep learning framework for CTR prediction, offers a novel, state-of-the-art neural network architecture that integrates factorization machines and deep learning for recommendation and feature learning (16). CIN aims to generate feature interactions in an explicit fashion at the vector-wise level (17). Besides, DNN and LNN have widely been employed for modeling medical data and reached remarkable performances in many cases (12, 38). Furthermore, for hyperparameters tuning, MCTS is a notoriously advanced algorithm that has led to remarkable successes of many landmark artificial intelligences, including AlphaGo. In the ELISE pipeline, all these state-of-the-art network architectures and hyperparameters optimization method were included, which endowed ELISE with outstanding performance when predicting immunotherapeutic responses to cancers.

ELISE allows model interpretation *via* SHAP algorithm to transparentize the decision process of the "black box" model and increase clinician trust. Taking metastatic UCs as an example, SHAP algorithm elucidated each inputted features' contribution to ELISE output, determined their affecting direction, and offered the global and individual interpretation for ELISE decision processes. *CDKN2A*, in the present study, was distinguished as the most important contributor with a negative correlation to unfavorable responses to PD1/PD-L1 suppressor, consistent with previous publications. *CDKN2A* encodes p16, an endogenous inhibitor of the cyclin-dependent kinases CDK4 and CDK6, which restrict the G1/S phase transition and induce cell senescence (40). A large-scale clinical study attested that *CDKN2A* is identified as a significant transcriptional correlate of response, highlighting the association of non-immune pathways to the outcome of checkpoint blockade (41). These data emphasize the high consistency that ELISE provides to prior experiences of routine clinical practices and lab works.

The present study is limited due to the inherent disadvantages of retrospective cohort studies, and ELISE

warrants further validation and improvement in large and well-designed prospective clinical trials. Moreover, the potential of ELISE is limited by the samples size, despite we searched the related dataset as much as possible. A well-designed study will be conducted if more samples are obtained in the future. Besides, we could not access survival difference between different ELISE group due to their survival data was not available. The survival analyses will be preformed as planed if more survival data is available.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

Participated in study conception design: WJ, ZX. Data analysis: WJ, QY, HC, PZ, KW and GZ. Wrote or contributed to the writing of the manuscript: WJ, QY, HC, SC, ZX and XL. Obtained the funding: XL. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1025330/full#supplementary-material

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PD-1 inhibitor combined with radiotherapy and GM-CSF in MSS/pMMR metastatic colon cancer: a case report

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Patients with chemo-refractory metastatic colorectal cancer (mCRC) have poor prognoses. The application of programmed cell death protein 1 (PD-1)/programmed cell death ligand 1 (PD-L1) inhibitors encouragingly improved the survival of mCRC patients with microsatellite instability-high (MSI-H)/mismatch repair-deficient (dMMR). Unfortunately, it was ineffective for mCRC with microsatellite-stable (MSS)/proficient mismatch repair (pMMR), which accounted for 95% of mCRC. Radiotherapy can promote local control by directly killing tumor cells and inducing positive immune activities, which might help synergistically with immunotherapy. We present the report of an advanced MSS/pMMR mCRC patient who had progressive disease (PD) after first-line chemotherapy, palliative surgery and second-line chemotherapy combined with targeted therapy. Then the patient received the therapy of PD-1 inhibitor combined with radiotherapy and granulocyte-macrophage colony-stimulating factor (GM-CSF). According to Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST1.1), the patient showed a complete response (CR) after triple-combined therapy with progression-free survival (PFS) for more than 2 years so far. The patient had no other significant adverse reactions except for fatigue (Grade 1). The triple-combination therapy provided a promising strategy for metastatic chemo-refractory MSS/pMMR mCRC patients.

KEYWORDS

colorectal cancer, immunotherapy, radiotherapy, GM-CSF, MSS/pMMR, case report

Abbreviations: mCRC, metastatic colorectal cancer; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; MSI-H, microsatellite instability-high; dMMR, mismatch repair-deficient; MSS, microsatellite-stable; pMMR, proficient mismatch repair; GM-CSF, granulocyte-macrophage colony-stimulating factor; RECIST, Response Evaluation Criteria in Solid Tumors; CR, complete response; PR, partial remission; PD, progressive disease; PFS, progression-free survival; PET/CT, Positron Emission Tomography/Computed Tomography; IHC, Immunohistochemical; NGS, Next Generation Sequencing; CEA, Carcinoembryonic antigen; CA242, Carbohydrate antigen 242; AEs, adverse events; NSCLC, non-small cell lung cancer; ORR, Objective response rate; APCs, antigen presenting cells; TDLNs, Tumor-draining lymph nodes; FDA, Food and Drug Administration; CMS, consensus molecular subtypes.

Introduction

By 2021, colorectal cancer (CRC) was the third most common cause of cancer mortality worldwide. Meanwhile, metastasis was found at the first diagnosis in 20% of CRC patients (1). Although the United States Food and Drug Administration (FDA) has approved PD-1 inhibitor pembrolizumab for the treatment of microsatellite instability-high (MSI-H) metastatic CRC (mCRC), about 95% of mCRC patients are MSS/pMMR and cannot benefit from PD-1 inhibitor monotherapy (2). The clinical trials of KEYNOTE-016 and KEYNOTE-028 showed no response in MSS mCRC patients treated with pembrolizumab (3, 4). The preclinical studies of MSS colorectal cancer mice models have shown synergy between radiotherapy and anti-PD-1 in modulating anti-tumor immune responses (5, 6). It provides a theoretical basis for the combination of radiotherapy and immunotherapy. Recently, several I/II clinical studies have shown that the combination of radiotherapy and immunotherapy could improve clinical outcomes in mCRC patients with MSS/pMMR with acceptable toxicity (7–9). Granulocyte-macrophage colony-stimulating factor (GM-CSF), known as an immunomodulatory cytokine, might improve the efficacy of immunotherapy in advanced biliary cancers (10). It is necessary to explore novel strategies for treating MSS/pMMR mCRC, and combining anti-PD-1 immunotherapy with radiotherapy and GM-CSF therapy might be a potential one.

We present the report of a refractory mCRC patient with MSS/pMMR who received PD-1 inhibitor combined with Radiotherapy and GM-CSF. The patient demonstrated a sustained tumor response and prolonged progression-free survival (PFS) for over 2 years so far.

Case presentations

A patient in his mid 40s was diagnosed with ascending colon adenocarcinoma through colonoscopy biopsy and pathological examination on 21 January 2020. Further Positron Emission Tomography/Computed Tomography (PET/CT) imaging showed metastasis of retroperitoneal and celiac lymph nodes (Figure 1A). According to the AJCC 8th TNM staging system, the patient was staged T3N2bM1a (c-Stage IVA). Immunohistochemical (IHC) staining was AE1/AE3(+), MSH2 (+), MSH6 (+), MLH1 (+), PMS2 (+), HER-2 (0), PD-L1 (+,CPS=10), CD8 (+,15%), CD68 (+,80%) (Supplementary Figure 1). Genetic testing of tumor tissue revealed that missense mutation A146T was found in exon 4 of the KRAS gene. No mutation was found in the BRAF/NRAS. The microsatellite state detection showed a microsatellite-stable (MSS) phenotype by Next Generation Sequencing (NGS).

Considering that the patient was young and was willing to receive surgery, two cycles of conversion therapy of mFOLFOX4 were administered from January to February 2020. Bevacizumab was not added to the conversion therapy, which may cause surgical complications such as bleeding and gastrointestinal perforation. After the conversion chemotherapy, the patient underwent palliative surgery (R2 resection) in February 2020. All of the primary lesion and the part of the mesenteric lymph nodes were resected. The mesenteric and retroperitoneal lymph nodes were not

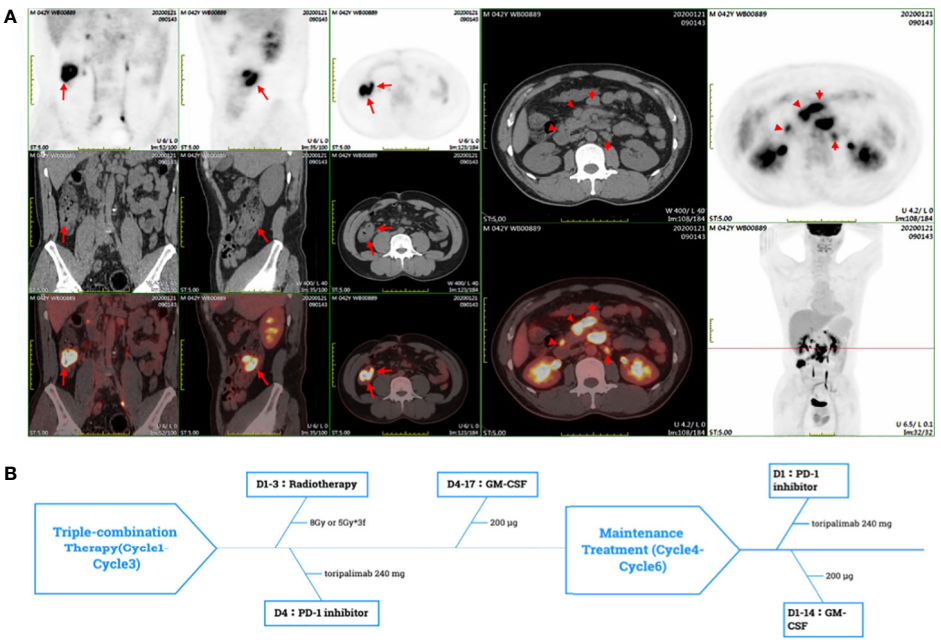

FIGURE 1
(A) The PET-CT showed the high SUV_{mean} of ascending colon and enlarged lymph nodes of retroperitoneal and peritoneal. **(B)** In a PRaG cycle, radiotherapy was delivered for metastases, followed by GM-CSF subcutaneous (sc) injection once daily for two weeks, and toripalimab was intravenous(iv) once within one week after radiotherapy. PRaG Therapy was repeated every three weeks, and three cycles of triple-therapy were administered. Subsequently, the patient underwent three cycles of PD-1 inhibotor and GM-CSF maintenance treatment.

resected. Then the patient received six cycles of chemotherapy of capecitabine plus irinotecan (mXELIRI) combined with Bevacizumab from March to July 2020. CT scan observed new lymph node metastases at mesenteric and retroperitoneal in August 2020, which indicated progressive disease (PD). It suggested the patient was insensitive to chemotherapy combined with vascular-targeted Therapy. In addition, neutropenia and gastrointestinal reaction (Grade 2) were observed during chemotherapy.

Considering the side effects of chemotherapy, the patient refused to continue the chemotherapy. Then the patient was enrolled in a prospective phase II clinical trial which was conducted to assess the clinical efficacy and safety of PD-1 (toripalimab) inhibitor combined with Radiotherapy and GM-CSF (Recombinant Human Interleukin-2(I) for Injection) in patients with advanced metastatic solid tumors on 10 August 2020 (ChiCTR1900026175, http://www.chictr.org.cn/index.aspx). We defined the triple-combined therapy of PD-1 inhibitor combined with Radiotherapy and GM-CSF as PRaG therapy. The patient underwent three cycles of PRaG therapy in August 2020 and September 2020. In the PRaG cycle, radiotherapy (8Gy or 5Gy/d, d1-3) was delivered for lymph node metastases, followed by subcutaneous injection of GM-CSF (200μg once daily, d4-17) and intravenous injection of toripalimab (240mg, d4). PRaG regimen was repeated every three weeks (Figure 1B). After the 3 cycles of PRaG therapy, the patient achieved partial response (PR) according to RECIST1.1 by CT scan. CT showed a significant decrease of irradiated lymph node metastases in retroperitoneal and celiac (Figures 2A, B), and a reduction of nonirradiated lymph node metastases in celiac was also observed (Figure 2C). Moreover, tumor markers of carcinoembryonic antigen (CEA) and carbohydrate antigen 242 (CA242) decreased to normal range after two cycles of PRaG therapy (Figure 3A).

Three different metastatic sites were chosen for irradiation in three cycles. In the first two cycles, radiotherapy (8Gy/d, d1-3) was delivered for celiac metastatic lymph node and retroperitoneal metastatic lymph node. Considering the tolerance dose constraints of normal tissues, the radiation dose was reduced to 5Gy/d (d1-3) for celiac lymph node in the third cycle.

After the PRaG therapy, the CT scan showed that lymph node metastases in retroperitoneal and celiac almost disappeared compared with before (Figure 2). The clinical response of PRaG therapy was complete remission (CR) based on RECIST1.1. Due to the significant decrease of the lesions, no lesions can be irradiated in the follow-up treatment. Subsequent maintenance therapy was implemented with toripalimab and GM-CSF for three cycles from October 2020 to January 2021. The patient exhibited no tumor progression or recurrence in the next two re-examinations by CT scan. The patient had no other significant adverse reactions except for fatigue (Grade 1). The PFS has more than 2 years so far (Figure 3B). Due to the influence of COVID-19, the re-examination interval was longer than expected. The recent follow-up in August 2022 showed that the patient maintained a good physical condition and exhibited sustained CR.

Discussion

Backline treatment of MSS mCRC is a hard nut to crack. Regorafenib or trifluridine and tipiracil (TAS-102) were recommended, but the survival benefit was still limited. A phase II trial of TAS-102 combined with nivolumab showed no tumor response in MSS/pMMR mCRC (11). The mPFS was 2.8 months (95% CI,1.8 to 5.1). 72% of the patients experienced grade grade ≥3 adverse events (AEs). The REGONIVO study from Japan showed that the combination of regorafenib and nivolumab achieved encouraging results in treating MSS mCRC (12). The mPFS was 7.9months (95%CI,2.9 to not reached [NR]), but outcomes of this trial were not reproduced in subsequent clinical studies. These clinical trials showed that the existing combined therapies were limited in overcoming the immune resistance of MSS/pMMR mCRC. A recent study revealed that MSS mCRC patients might benefit from the combination of radiotherapy, anti-PD-1 and anti-CTLA-4 immunotherapy (8).

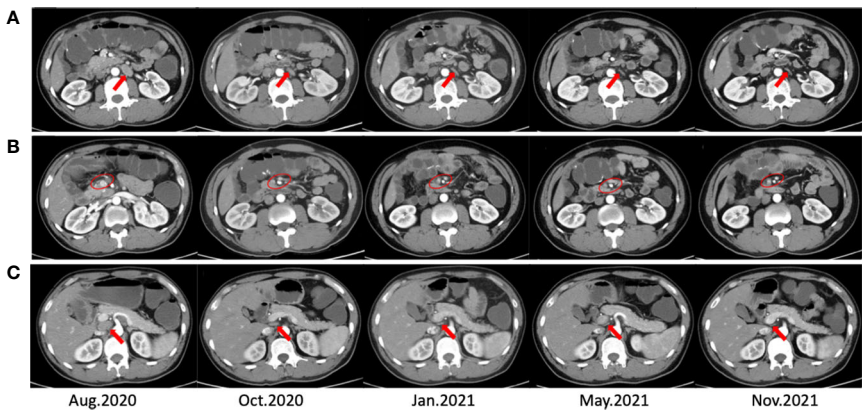

FIGURE 2
CT scans before, during, and after the PRaG therapy. The CT scans **(A, B)** showed shrunk and disappeared of irradiated lymph node metastases. The CT scans **(C)** showed shrunk and disappeared of nonirradiated lymph node metastases. The arrows point to individual lymph nodes, and the circles include fusion lymph nodes.

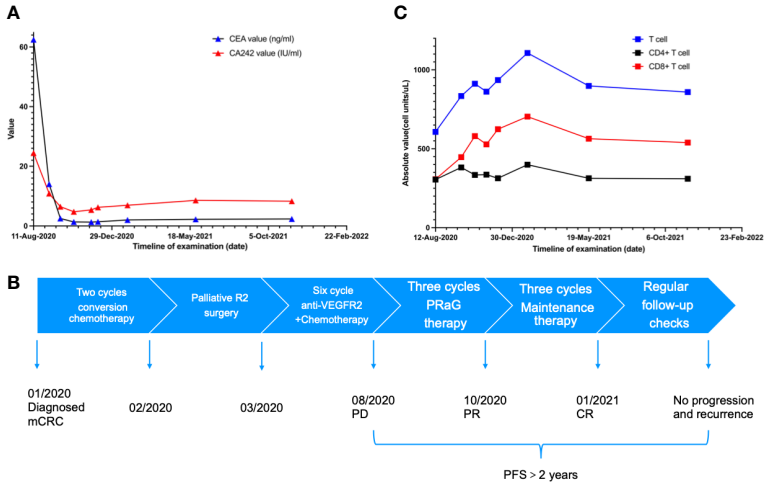

FIGURE 3
(A) The carcinoembryonic antigen (CEA) dropped to the normal range after two cycles of PRaG Therapy. In the whole course of treatment, the patients had no obvious adverse reactions. Due to the influence of COVID-19, the re-examination interval was longer than expected. **(B)** The scheme shows the complete treatment process of the patient. **(C)** The number and activation of lymphocytes are related to the efficacy of immunotherapy.

It is necessary to develop suitable combination strategies to improve the efficacy of immunotherapy for the MSS/pMMR mCRC.

Radiotherapy is an essential local tumor control treatment method. In recent years, studies have found that adding radiotherapy can enhance the anti-tumor effect of immunotherapy. Radiation can transform tumor cells into *in-situ* vaccines that can promote tumor cells to release tumor-associated antigens (TAAs) and induce immunogenic cell death (ICD) (13). The PEMBRO-RT study has reported the synergistic effect between immunotherapy and radiotherapy in advanced metastatic NSCLC. The ORR of combined therapy was 36% vs. 18% of the control group (pembrolizumab alone). The combined therapy's mPFS and mOS were better than the control group (mPFS: 6.6 vs. 1.9 months, mOS: 15.9 vs. 7.6 months). The adverse reactions between the combined and control groups have no significant difference (14). The same results were obtained in PEMBRO-RT and MDACC clinical trials pooled analysis. The combined therapy prolonged the mPFS and mOS than the pembrolizumab alone in patients with metastatic NSCLC (PFS: 9.0 vs. 4.4 months, median OS: 19.2 vs. 8.7 months) (15).

GM-CSF is a cytokine used to promote the growth of leukopenia or neutropenia and is widely used to promote the production of granulocytes or APCs. Preclinical studies supported that GM-CSF combined with immune checkpoint inhibitors (ICI) can improve the activity of innate immune cells, and indirectly recruit T cells by promoting the antigen cross-presentation (16, 17). Ipilimumab combined with GM-CSF can prolong the OS of advanced melanoma more than ipilimumab alone (mOS: 17.5 vs. 12.7 months) (18). In addition, a prospective clinical study has shown that local radiotherapy combined with GM-CSF can improve the prognosis of patients with advanced metastatic solid tumors (19).

The doses and frequency of radiation have not been standardized when radiotherapy is combined with PD-1/PD-L1 inhibitors. Preclinical studies have shown that hypo-fractionated radiotherapy (5Gy × 3f) boosted more proliferation and activation of antigen-presenting cells compared with conventionally fractionated radiotherapy (2Gy × 5f) (20). The conventional fraction also caused more lymphocyte death than hypo-fraction regimens, which affects the response to immunotherapy (21). When the fraction dose of radiation exceeds 5Gy, radiotherapy can indirectly promote the ICD of the tumor (22). However, a higher fraction dose did not represent a better response for treatment. Studies have shown that the increase of Tregs will offset the local control effect when therapy with a single high dose (15Gy × 1f), while the medium fraction regimen (7.5-10Gy × 2-3f) can maintain the low level of Treg and activate the immune response effectively (23). In the PEMBRO-RT study, the fraction regimen (8Gy × 3f) combined with PD-1 inhibitors had excellent clinical efficacy in advanced metastatic NSCLC (14). No additional adverse reactions of immunotherapy were added at this fraction dose. The fraction regimen (3×8Gy or 3×5Gy) we used in PRaG Therapy could be a reasonable choice.

Considering the heterogeneity of the tumor, irradiation of a single site may not induce sufficient exposure to TAAs. Chang et al. suggested multisite radiotherapy of metastases to enhance the synergistic effect (24). However, multisite irradiation may increase the volume of irradiation and lead to a higher incidence of adverse reactions. The number and activation of lymphocytes are related to the efficacy of immunotherapy (25). The decrease in lymphocyte number caused by lymph node irradiation directly reduces the efficacy of immunotherapy (26). Considering side effects and lymphocyte depletion caused by irradiation, it is difficult to irradiate all sites in one cycle for patients with large masses and multiple metastases. Therefore, we chose one lesion to irradiate each cycle. The treatment consists of multiple cycles. Compared to conventional radiotherapy, the range of irradiated lesions was smaller and the total radiation dose was lower (Supplementary Figures 2–4). We suggested multiple cycles of radiotherapy, with

each cycle targeting a small volume that might protect the lymphocytes and produce sustained immune activation (27). Moreover, irradiation of the Tumor-draining lymph nodes (TDLNs) can benefit patients with lymph node metastases. In this case, there was no significant decrease in lymphocytes, which may be one reason for the excellent efficacy (Figure 3C).

After three irradiation cycles achieved an excellent local control effect with a significant decrease in the irradiated lymph node metastases. At the same time, the regression of the nonirradiated lesion was also observed. Regression of the nonirradiated tumor was called the abscopal effect, which was not frequently in patients with radiotherapy alone (28, 29). However, we cannot be sure that the regression of the nonirradiated lesion was caused by abscopal effect in this case. The regression of nonirradiated lesions might be due to the sensitization of radiotherapy or GM-CSF to immunotherapy. Moreover, MSS CRCs were divided into multiple subtypes by consensus molecular subtypes (CMS) consortium. Most of MSS CRCs are immune desert and has no immune cell infiltration, a small portion of them do have CD8+ cell infiltration but suppressed by TME (30). According to the results of IHC and NGS, the immune phenotype of this patient was low CD8+ cell infiltrated. We think that only a subgroup of MSS mCRCs should be considered for this combinational therapy of anti-PD1, radiotherapy and GM-CSF. The specific mechanisms remain to be further studied.

Conclusions

The MSS mCRC patient achieved terrific results through PRaG triple-combination therapy with well-tolerated. The efficacy and safety of PRaG therapy for MSS mCRC patients need to be confirmed in future prospective studies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

JY composed the manuscript as the first authorship. LZ designed and conducted the study as corresponding authors. PX and YK helped with data collection and interpretation. MX collected and sorted out part of the image data. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1078915/full#supplementary-material

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