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Understanding the crosstalk between immune cells and the tumor microenvironment in cancer and its implications for immunotherapy, 2nd Edition

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Table of contents

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Editorial: Understanding the crosstalk between immune cells and the tumor microenvironment in cancer and its implications for immunotherapy

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Editorial on the Research Topic
Understanding the crosstalk between immune cells and the tumor microenvironment in cancer and its implications for immunotherapy

This editorial features the articles published in this Research Topic in Frontiers in Medicine. This Research Topic aimed to uncover the complex interactions between tumor cells, immune cells, and their microenvironment, as well as their implications in cancer immunotherapy. Also, this topic aimed to provide insights into various crosstalk mechanisms that could be translated into the clinics. A case report by Liu et al. reported a 68-year-old male with chemotherapy-intolerable stage IV intrahepatic cholangiocarcinoma. This study revealed that the biomarkers predicting the response to immunotherapy failed to accurately capture the treatment response and clinical benefit of anti-PD-1 immunotherapy (Liu et al.). Moreover, lung metastasis occurred despite the shrinkage of the primary liver tumor and metastasis in the lymph nodes when anti-PD-1 immunotherapy was combined with radiotherapy. However, with the continued administration of radiotherapy and immunotherapy, a complete response was evident for the primary tumor and metastatic lesions with no treatment-related adverse effects.

Another study discussed another immunotherapeutic approach which is cytokine-based therapy (Razeghian et al.). The toxicity of cytokine-based therapeutics is attributed to the high doses required to reach the anticipated outcome, which limited their clinical utility and led to the employment of mesenchymal stem/stromal cells (MSCs) as potential vehicles for cytokine delivery in various tumors owing to their relatively low immunogenicity and tumor tropism (Razeghian et al.). Despite their unfavorable effects on drug resistance and metastasis, the use of MSC-based cytokine delivery systems can lead to effective immune cell-induced anti-tumor response and provide sustained cytokine release. Current research advances suggest that the combined use of engineered MSCs and small molecules could result in their notable safety and therapeutic efficacy.

The systemic review by Numprasit et al. highlighted the association between the expression of carbonic anhydrase IX (CAIX), a reliable endogenous marker of hypoxia, and BC patients' survival. It was reported that high expression of CAIX was associated with poor disease-free survival (DFS) in 9,157 BC patients. Furthermore, upon classifying BC patients according to their molecular subtypes, high CAIX expression was found to be associated with poor DFS and overall survival (OS) in the triple-negative subtype and a shorter DFS in the hormonal-positive subtype. This indicated that high CAIX expression is a poor prognostic indicator regardless of the subtypes and could be a potential therapeutic target in BC.

Hua et al., in this study, focused on the association between ovarian aging and BC risk. In this research article, the authors performed a multicohort genetic analysis, where clinicopathological data and gene expression data for 3366 BC patients were retrieved and analyzed. The results showed that the eight-validated Ovarian aging-related genes (OARG)-based signature established a prognostic model for BC using independent cohorts. Furthermore, a nomogram with good predictive performance was implemented by incorporating the OARG risk score with the clinicopathological factors. It is also worth noting that the OARG-based signature correlated with DNA damage repair, immune cell signaling pathways, and immunomodulatory functions. Collectively, this study postulated a comprehensive analytical method for BC assessment based on a unique eight OARG signature, which could accurately predict clinical outcomes and drug sensitivity of BC patients.

Decoding genomic and epigenetic changes in tumor cells has helped scientists comprehend the nature of cancer and find curative ways, including the contemporary notion of immunotherapies. The mini-review article by Talaat and Kim discussed the tumor microenvironment (TME) as a compartment guiding the dynamic interplay of different cell types. Also, they reviewed numerous initiatives, such as data-driven strategies, that will quickly advance our knowledge of the environment in which tumor cells thrive, leading to novel findings of prognostic indicators and eventually resulting in overcoming resistance to management.

The TME is known to consist of tumor-infiltrating lymphocytes (TILs), tumor-associated macrophages (TAMs), and tumor-associated neutrophils (TANs). The review by Talaat et al. highlights the several immune checkpoint molecules that are expressed on these immune cells and their interaction with colon cancer cells. Thus, novel approaches for therapy for solid tumors such as colorectal cancer (CRC) are targeting immune checkpoint markers; however, there are still obstacles to successful treatment. On the other hand, the article by Liu and Wang reviewed the use of TAMs in immunotherapy. Whilst macrophages are phagocytic cells that perform a variety of roles in the protection against external invaders, TAMs enhance tumor development and progression by supporting tumor cell division and invasion, immunosuppression, and angiogenesis, which is linked to the poor prognosis in the majority of solid tumors. As a result, an in-depth understanding of TAMs can lead to the discovery of more successful cancer treatment methods. Currently, a significant number of TAM-targeting medicinal drugs are in clinical studies.

The article by Banna et al. explored new techniques for quantitative image analysis, like radiomics or pathomics, which may provide a thorough method for analyzing spatial and temporal data from macroscopic imaging features that may be indicative of underlying molecular drivers and tumor-immune microenvironment in addition to the prognosis after immunotherapy. Additionally, merging data from other sources, such as blood levels, molecular characteristics, radiomics, and pathomics can boost the precision of their models. As a result, "digital biopsy", as a non-invasive digital method, may have the ability to enable a tailored strategy for cancer patients.

Due to the limitations of immunotherapy in CRC, the review by Mahgoub et al. explores the manipulation of autophagy as a possible adjuvant therapeutic method for patients with different molecular subtypes of CRC. The molecular regulation of autophagy in CRC and how it impacts numerous mechanisms and processes that regulate TME, as well as its role in the development of CRC, tumor immunity, hypoxia, and oxidative stress. Moreover, the clinical efforts and difficulties associated with combining autophagy modulators with other cancer-targeted drugs were discussed to improve CRC patients' survival and slow disease progression.

Rashid et al. reviewed the diagnosis, prognosis, and therapeutic approaches of CRC by shedding light on non-steroidal anti-inflammatory drugs (NSAIDs) that are commonly used as analgesics and anti-inflammatory agents. They have highlighted that NSAIDs possess a potent chemo-preventive effect on several gastrointestinal malignancies, including CRC, in several epidemiological and preclinical studies. The authors also described the molecular mechanisms postulated by which NSAIDs could act as chemo-preventive agents by preventing the synthesis of prostaglandins and resulting in NSAID-induced apoptosis and CRC growth inhibition.

Currently, an increasing number of studies examine the role of RNA modifications such as N7-methylguanosine (m7G) in tumors. A significant m7G-related signature, known as the m7G score, was elucidated based on four principal genes, namely *E2F7*, *FAM83A*, *PITX3*, and *HOXA13*, for predicting the immune infiltration and prognosis of lung adenocarcinoma (LUAD) (Li et al.). The m7G score could preferentially differentiate between two distinct molecular subtypes of LUAD. Moreover, the higher m7G score indicated poorer prognosis, higher immune infiltration, significant PD-1 and PD-L1 upregulation, higher tumor mutational burden, and lower tumor immune dysfunction and exclusion scores. Such an approach could aid in the advancement of novel therapeutic strategies for LUAD.

Zajac et al. focused in their review article on MAGE-A antigens, which are the first identified molecular human tumor-associated antigens. The authors shed light on their high tumor specificity and their potential usage as attractive targets for cancer immunotherapies. The review article was mainly focusing on structural features and functional aspects of MAGE-A antigens. Nonetheless, the authors reviewed all past and ongoing clinical studies targeting MAGE-A antigens, as well as the pros and cons of different therapeutic approaches.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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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MAGE-A Antigens and Cancer Immunotherapy

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MAGE-A antigens are expressed in a variety of cancers of diverse histological origin and germinal cells. Due to their relatively high tumor specificity, they represent attractive targets for active specific and adoptive cancer immunotherapies. Here, we (i) review past and ongoing clinical studies targeting these antigens, (ii) analyze advantages and disadvantages of different therapeutic approaches, and (iii) discuss possible improvements in MAGE-A-specific immunotherapies.

Keywords: MAGE-A, cancer–testis antigens, cancer immunotherapy, clinical trials, adoptive immunotherapy

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MAGE-A TUMOR-ASSOCIATED ANTIGENS

MAGE-A were the first human tumor-associated antigens identified at the molecular level (1). They belong to the larger family of cancer/testis antigens (CTA), whose expression is consistently detected in cancers of different histological origin and germinal cells (2). The MAGE-A sub-family includes 12 highly homologous genes located on chromosome Xq28 (3, 4). Specific gene products have been identified by immunohistochemistry in cancers of different histological origin, including high percentages of non-small cell lung cancers (NSCLC), bladder cancers, esophageal and head and neck cancers, and sarcomas (5). These antigens are also frequently expressed in triple negative breast cancers (6), myeloma (7), and Reed–Sternberg cells (8) in Hodgkin's disease, with the highest frequency being detected in synovial sarcoma (9). Among healthy tissues, the expression of specific members of the family has been observed in spermatogonia, placenta (10), and fetal ovary (11). However, recently, MAGE-A1 and -A12 genes have been shown to be expressed in CNS as well, as discussed below (12).

FUNCTIONAL ASPECTS OF MAGE-A ANTIGENS

Preferential intracellular location may be different for different antigens, e.g., mostly cytoplasmic for MAGE-A1, -A3, and -A4, but mostly nuclear for MAGE-A10 (13–16).

Functions are still unclear, although different studies have associated MAGE-A2, -A3/6, and -A9 expression with pro-tumorigenic activities such as p53 dysregulation (17–19), enhanced tumor cell proliferation potential, or maintenance of a cancer-stem cell-like functional profile (20).

In a variety of tumors of different histological origin, a clear correlation between expression of MAGE-A antigens and poor prognosis has been observed. In this context, data on bladder cancer (21, 22), NSCLC (23, 24), head and neck cancers (25–27), and ovarian cancer (28, 29) have consistently been reported. Indeed, MAGE-A antigen expression, at the gene and protein level, has repeatedly been shown to be associated with widespread DNA demethylation frequently

observed in advanced cancers. On the same line, it has been shown to be inducible by demethylating agents, including chemotherapeutic compounds widely used in cancer treatment such as 5-aza-2′-deoxycytidine (30, 31), thus realistically envisaging the possibility of treatments combining chemotherapy and specific vaccination (32).

IMMUNOGENICITY OF MAGE-A ANTIGENS

Although peptides restricted by both HLA classes I and II have been identified (33), naturally occurring adaptive immune responses to MAGE-A antigens are usually characterized by a very low frequency of specific precursors (34) in both healthy donors and patients bearing cancers expressing them (35). However, responses to MAGE-A10 have been more frequently detected (36, 37). Responses in tumor-associated lymphocytes (TIL) have seldom been explored, but we have observed that MAGE-A10-specific CTL could be expanded from TIL infiltrating NSCLC displaying a high expression of the target antigen (38). On the other hand, CTL recognizing peptide motifs shared by multiple MAGE-A proteins may be generated from peripheral blood from patients and healthy donors (39). Most recently, tumor reactive CD8+ T cells, isolated based on their expression of activation marker (PD-1) from peripheral blood of melanoma patients, have been shown to relatively frequently target MAGE-A antigens (40).

CLINICAL TRIALS TARGETING MAGE-A ANTIGENS

In the past 10 years (2006–2016), a total of 44 clinical trials could be identified in "https://clinicaltrials.gov" database using "MAGE-A" as keyword: a total of 16 phase 0 or I, 13 phase I/II, 13 phase II, and 2 phase III studies. Regarding immunogen formulations, 16 studies utilized entire proteins in the presence or absence of adjuvants (41, 42), 11 used peptides (43–45), 6 used mRNA-transfected DC (46, 47), 1 was based on tumor cell lysate-pulsed DC, 2 took advantage of recombinant viral vectors (48, 49), and more recently, 6 and 2 trials, respectively, have focused on adoptive treatments by using specific T cell receptor (TCR)-transduced T cells (12) or expanded CTL (50).

Efficacy clinical data published so far, from patients immunized in the context of the 15 larger studies (phase II or III, Table S1 in Supplementary Material) mainly using MAGE-A protein ($n = 11$), do not appear to support significant clinical effectiveness (51).

Of interest, a chronological analysis of these 44 studies clearly underlines a strategy shift in the most recent years. Indeed, in the past 4 years, among the (only) 10 clinical studies initiated and including MAGE-A as antigens, there are no phase II or III studies. Moreover, the majority of the phase I or I/II studies are based on adoptive cell transfer. This "shift" in MAGE-A translational research strategy clearly results from the combined effect of "protein/peptide" efficacy failure and from the confidence generated by new approaches focusing on personalized effector T-cell treatment. In addition, one should also mention the shift in target paradigm from classical TAA to neo-antigens also contributing to the decreased use of MAGE-A antigens.

MAGE-A3 PROTEIN AS IMMUNOGEN

One of the most important clinical trials ever performed in MAGE-A cancer immunotherapy, involving thousands of patients with NSCLC, was focusing on the administration of recombinant MAGE-A3 protein together with adjuvants (52, 53). Despite promising initial data and the proven ability of the immunization protocol to induce detectable humoral responses in vaccinated patients (54), disease-free interval in patients with completely resected stage IB, II, and IIIA NSCLC did not appear to be significantly prolonged, as compared to patients of control group, in phase III studies in the context of an adjuvant therapy setting (41).

Why did these trials fail to reach efficacy? First, similar to MAGE-A antigens, a large majority of classical TAA-specific cancer vaccines clinically tested so far have been shown to induce heterogeneous immune responses rarely resulting in significant clinical effects.

However, specific issues should be considered for CTA-specific immunization. For instance, MAGE-A CTA expression, a pre-requisite for the eligibility of patients for treatment in these studies, has usually been assessed at the gene level by quantitative RT-PCR (RT-qPCR) (41, 54), which cannot provide insights into the actual numbers of CTA-positive tumor cells. Immunohistochemical studies using available MAGE-A-specific mAbs consistently underline that expression of these antigens might be highly heterogeneous in cancerous tissues with high expression often only detectable in relatively low percentages of tumor cells (10, 55). Remarkably, due to the high homology of sequences from different components of the MAGE-A family, a majority of currently available reagents do recognize multiple antigens. Our own experience based on the use of a MAGE-A10 highly specific mAb (**Figure 1**) suggests that expression of these antigens may be highly heterogeneous in a variety of tumors of different histological origin, with percentages of "positive" cells ranging between 5 and >60% (16). One could speculate that criteria based on the expression of target antigen(s), at the protein level, in high percentages of tumor cells and in multiple areas of primary and metastatic cancers could be applied for a more stringent selection of patients potentially eligible for MAGE-A-targeted antitumor immunization. Additionally, it might be of interest to verify the expression of the target MAGE-A antigen in recurrent tumors following specific immunization protocols, to verify possible selective immune editing (56). It is worth noting, however, that successful antigen-specific vaccination has also been shown to be able to promote responsiveness against unrelated antigens, the so-called "antigen spreading" phenomenon (57), thus potentially overcoming the requirement for a uniform expression of target antigens in tumors to be treated.

Importantly, the recombinant protein used in most efficacy studies was shown to induce humoral response and HLA class II-restricted lymphoproliferation, as expectable (41, 53, 54). However, the ability of these antigen formulations to promote

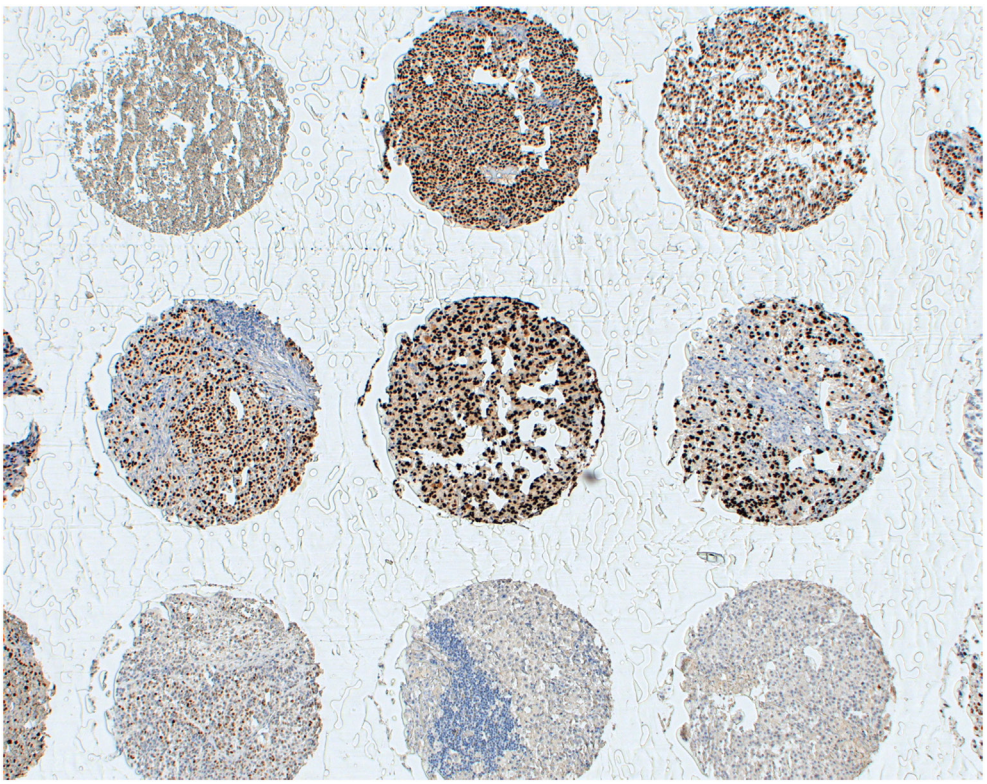

FIGURE 1 | Heterogeneity of MAGE-A10 expression at the protein level. Melanoma tissues from a multi-tumor tissue microarray were stained with a MAGE-A10-specific reagent by standard techniques, as previously detailed (16). Antigen expression displays a high heterogeneity, regarding both percentages of antigen-positive tumor cells and staining intensity.

class I-restricted responses appears to be more limited. One could speculate that libraries of overlapping "long" peptides (58), or highly immunogenic recombinant vectors (38, 59), could be more effective in this regard.

HETEROGENEOUS EXPRESSION OF MAGE-A GENES IN PRIMARY AND METASTATIC CANCERS

Studies from our group clearly document the heterogeneity of MAGE-A antigens expression at the gene expression level as well. We tested by RT-qPCR the expression of Mage-A1, -A2, -A3, -A4, -A10, and -A12 genes in primary NSCLC from 33 patients (**Table 1**). In keeping with published data (23, 24), a total of 22 tumors (66%) showed evidence of expression of at least one of the antigens under investigation. Similar to recently published data in oral cancer (60), out of these patients with MAGE-A+ NSCLC, 10 (45%) had lymph nodes (LN) showing evidence of tumor metastasis, as compared with only 2 (18%) from the 11 MAGE-A(−) primary tumors. Interestingly, among the 10 metastatic LN from MAGE-A+ primary cancers, only half showed evidence of MAGE-A gene expression. Furthermore, in four LN, classified as non-metastatic, based on pathological evidence, expression of MAGE-A genes could be observed by RT-qPCR. Intriguingly, among LN associated with MAGE-A− primary cancers, 1/2 and 1/8 metastatic and non-metastatic samples, respectively, showed evidence of MAGE-A gene expression.

TABLE 1 | MAGE-A gene expression, as detected by RT-qPCR in primary non-small cell lung cancers (NSCLC) and in corresponding lymph nodes (LN) showing evidence of metastatic outgrowth by standard clinical pathology techniques.

Total number of patient	1 MAGE-A + RT-qPCR	2 LN-met histo	3 LN-MAGE-A + RT-qPCR
33	22+	10+	5+
			5−
		12−	4+
			8−
	11−	2+	1+
			1−
		9−	1+
			8−

Tissues obtained from surgical resections from patients with NSCLC were tested by RT-qPCR for Mage-A1, -A2, -A3, -A4, -A10, and -A12 gene expression. Positivity (+) was defined by expression of at least one target gene above threshold (threshold = delta Ct to β-actin < 10). LN were similarly assessed by RT-qPCR and standard clinical pathology scoring.

Taken together, these data suggest a higher sensitivity of RT-qPCR as compared to standard techniques for the detection of cancer cells within LN draining primary tumor tissues.

Most importantly, however, they confirm the dynamic nature of MAGE-A antigens expression during cancer progression and may support the concept of combination therapies including treatments promoting MAGE-A antigen expression together with specific immunization procedures (61).

ADOPTIVE IMMUNOTHERAPIES

In recent clinical studies, effector T cells, transduced with vectors encoding for specific TCRs recognizing peptides from MAGE-A3 or MAGE-4, have been adoptively transferred into patients bearing tumors expressing these antigens. Unfortunately, upon anti-MAGE-A3, HLA-A0201-restricted TCR gene therapy, despite measurable clinical responses in some patients, treatment-related severe adverse events and deaths were also reported. These events may possibly be due to the high affinity of these TCRs (see below) and to the recognition ("on-target/off-tumor") of highly homologous peptide(s) from other MAGE-A proteins expressed in the CNS (12, 62). Similarly, myocardial toxicity, resulting in treatment related death, has also been observed following gene therapy with a MAGE-A3-specific HLA-A0101-restricted TCR (63, 64). In the latter case, the "off-target" effect was attributed to the high homology between the target peptide and a peptide from Titin muscle protein.

It is worth noting that the TCR transduced into T cells in the first study originally derived from "humanized" mice expressing HLA-A0201 and its affinity toward the target antigen was further improved by site-directed mutagenesis (65), thus increasing the chances of "on-target_off-tumor" adverse events affecting tissues characterized by low but detectable expression of defined MAGE-A antigens (12). The affinity of the TCR used in the second study, originally derived from a patient immunized with a recombinant viral vector (66), was also enhanced by site-directed mutagenesis.

By contrast, T cells expressing a MAGE-A4-specific TCR have been safely used in adoptive immunotherapy of patients with recurrent esophageal cancer (67).

Taken together, these data suggest that the clinical use of enhanced TCR effectors targeting MAGE-A antigens for cancer immunotherapy should be carefully evaluated in order to minimize potential "off-tumor" side effects.

However, natural MAGE-A-specific TCRs, from clones derived from tumor bearing patients or healthy donors, might also be of interest. Such CTLs would probably be characterized by a lower affinity for cognate HLA–class I peptide complex and possibly by a lower antitumor effector potential, but they would also likely have less toxic side effects. Considering the cumulative potency related to the high numbers of transduced cells usually infused into treated patients, and their ability to proliferate and generate "memory," the effectiveness of this type of treatment should reasonably be further tested.

CONCLUSIONS

Taken together, published data may suggest that therapeutic strategies targeting MAGE-A antigens have so far failed to fulfill the promise of representing effective tools for cancer treatment. However, the understanding of mechanisms controlling immune response as a whole and cancer-specific immune responses in the tumor microenvironment in particular has made enormous progress in the past decade, generating an unprecedented "momentum" for cancer immunotherapy.

Successful utilization of therapeutic mAbs recognizing "immunological checkpoints" is currently generating enormous interest in clinical oncology. Their mechanisms of actions (MoA) are not fully clarified (68, 69). However, one of the main MoA is arguably represented by the "release of brakes" hampering T cell responses specific for tumor-specific or associated antigens. This hypothesis is supported, for instance, by the higher effectiveness of treatment with anti CTLA-4 therapeutic mAbs in cancers characterized by a high mutational load, likely to result in a higher expression of mutated proteins potentially recognized as "non-self" by the adaptive immune system (70). It is therefore reasonable to postulate that adequately timed combinations of vaccination procedures and administrations of therapeutic "checkpoint inhibitor" specific mAbs could be of high clinical relevance. Within this framework, a critical point might be represented by the choice of antigens of potential clinical use. Neo-antigens, e.g., tumor-specific mutated proteins have been successfully identified by whole exome sequencing (71–73), and the expression of defined antigenic "non-self" peptides associated with restricting HLA class I determinants may be detected by mass spectrometry techniques (74). Although highly appealing, the "personal" nature of neo-antigens might possibly also represent their Achilles' heel, not only because of regulatory hurdles (75) but also because it would likely prevent the performance of conventional randomized trials, thereby complicating a reliable assessment of the effectiveness of innovative treatment procedures.

Based on these considerations, vaccination with tumor-associated or CTA could still realistically find an important place in cancer immunotherapy in the era of "immunological checkpoint" inhibitors (76). Considering that MAGE-A antigens are expressed in tumors with poor prognosis and a scarcity of therapeutic options, such as TNB, and lung and esophageal cancers, it is easy to predict that the interest of the scientific community in CTA might actually be revived in the light of the enormous advances in cancer immunotherapy of the last years.

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All authors participated in writing the manuscript and/or revising it critically for important intellectual content or providing the data mentioned in the manuscript.

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The Promise of Digital Biopsy for the Prediction of Tumor Molecular Features and Clinical Outcomes Associated With Immunotherapy

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Immunotherapy by immune checkpoint inhibitors has emerged as an effective treatment for a slight proportion of patients with aggressive tumors. Currently, some molecular determinants, such as the expression of the programmed cell death ligand-1 (PD-L1) or the tumor mutational burden (TMB) have been used in the clinical practice as predictive biomarkers, although they fail in consistency, applicability, or reliability to precisely identify the responding patients mainly because of their spatial intratumoral heterogeneity. Therefore, new biomarkers for early prediction of patient response to immunotherapy, that could integrate several approaches, are eagerly sought. Novel methods of quantitative image analysis (such as radiomics or pathomics) might offer a comprehensive approach providing spatial and temporal information from macroscopic imaging features potentially predictive of underlying molecular drivers, tumor-immune microenvironment, tumor-related prognosis, and clinical outcome (in terms of response or toxicity) following immunotherapy. Preliminary results from radiomics and pathomics analysis have demonstrated their ability to correlate image features with PD-L1 tumor expression, high CD3 cell infiltration or CD8 cell expression, or to produce an image signature concordant with gene expression. Furthermore, the predictive power of radiomics and pathomics can be improved by combining information from other modalities, such as blood values or molecular features, leading to increase the accuracy of these models. Thus, "digital biopsy," which could be defined by non-invasive and non-consuming digital techniques provided by radiomics and pathomics, may have the potential to allow for personalized approach for cancer patients treated with immunotherapy.

Keywords: radiomics, pathomics, omics, predictive, immunotherapy, cancer, digital biopsy, prognostic

INTRODUCTION

In the *data deluge* era, there is a unique opportunity to explore biological processes at multiple scales. Deriving useful information from data, often poorly structured, at large scales, led to the emergence of the so-called "*-omics*" disciplines (genomic, transcriptomic, proteomic, metabolomic, etc.) (1). Powerful bioinformatic tools allow for high-throughput extraction processes that convert images into data, from which biostatistical analysis, combined with clinical or other "*-omics*" data, may enhance diagnostic accuracy and find new predictive or prognostic factors (2). Applied to radiological images (most often computed tomography [CT], magnetic resonance [MR] imaging, and positron-emission tomography [PET]), it is called radiomics, which has been the pioneer in the field of images data analysis. Pathomics, that is a more recent discipline, ensues when the same processes are being applied to histopathological images.

In this review, we describe the basic background based on which these new disciplines have emerged and the important steps involved in imaging acquisition to clinical supporting correlations. Selected radiomics and pathomics reports will illustrate achievements in this field, with a focus on immunotherapy. Challenges and future development will be then considered.

BACKGROUND FOR RADIOMICS AND PATHOMICS

The founder hypothesis supporting the use of radiomics and pathomics in medical care is that data derived from images have a correlation with the underlying biological processes. More precisely, data derived from images would give additional information in relation with the underlying biological processes in comparison with the visual interpretation of the image as a picture, which is the traditional way of interpreting images (3).

Radiomics and, at a lesser extent, pathomics, fill the need to assess tumor heterogeneity. The presence, within the tumor, of distinct molecular cell clones, is a hallmark of cancer physiopathology (4). Natural history of cancer, as well as resistance mechanisms acquired through therapeutic selective pressure, manifest spatial and temporal heterogeneity of tumor cells (5, 6). Addressing tumor heterogeneity is one of the major goals of new therapeutic approaches and blood biomarkers may present limitations that could be overcome by radiomics and pathomics. In particular, radiomics represents a promising non-invasive and repeatable tool during the course of the disease.

Furthermore, traditional medical practice, based on human visual interpretation of images, is known to be inaccurate in up to 20% of cases in radiology and almost the same discrepancy rates are found in pathology reports (2). Despite many explanations accounting for these reporting errors, the result is the high prevalence of diagnosis unreliability, with clinical consequences for patients.

As far as cancer immunotherapy is concerned, immune checkpoint inhibitors (CPIs) have emerged as an effective therapeutic option for patients with aggressive tumors such as lung cancer (7, 8), although a few patients seem to benefit from the long-term benefit from this treatment (9). Aiming at identifying these patients, the expression of programmed cell death ligand-1 (PD-L1) has been widely explored as a predictive biomarker with contrasting results across different tumor subtypes and several methodological issues, mostly related to its variability and spatial intratumoral heterogeneity, that have been undermining its role and use (10). Other predictive biomarkers, such as the tumor mutational burden (TMB), are currently poorly applicable in the clinical practice and, noteworthy, identify a different sensitive population from the one selected by the PD-L1 (11). Thus, there is a need for new biomarkers to integrate into clinical practice in order to early identify patient response (or progression) to CPIs and avoid their potential sever toxicity (12–14).

PROCESS DESCRIPTION AND METHODS

Every "-omics" analysis requires a multistep process. Each stage has its own specificities. Radiomics process has been established as a model for other disciplines in image data analysis (such as pathomics) and essentially consists in the following five steps: image acquisition, identification of the target volumes, segmentation of the volumes, features extraction from the volumes and analysis [see **Figure 1**; (3, 15)].

After the first step, the identification of the volumes must identify tumor location and determine distinct parts within the tumor. These regions will be called *habitats,* and present specific biological properties (blood flow, cell density, edema, necrosis). Image data analysis can help to identify such *habitats* (16) before data extraction. This step is intentionally done before data extraction, thus giving additional data that would not be automatically detected by subsequent data analysis (17).

The next step, the most critical one, is the segmentation. It consists in contouring the volumes of interest. Its importance derives from the fact that all the data extraction process will be generated by each segmented volume, and any error at this point could mislead further interpretation. Given inter-operator variability and the time consuming of manual delineation, semi-automated tools seem to be the most reliable and cost-effective approaches to this step (18).

Next stages, highly technical, allow for high-throughput extraction of quantitative data and their analysis. Data extraction results in image-based "features." These features are mathematically and bioinformatically derived from images through first-, second-, or higher order statistical processes.

Radiomics features could be "texture" feature, "tumor heterogeneity" feature, etc. Quantitative features may be presented based on histograms for each volume of interest.

Analysis of radiomics features, along with clinical data or other "*-omics*" data try to find correlations with biological processes. The analysis aims to define and validate image-derived features as biomarkers that could have prognostic or predictive values helping thus to support medical decisions.

Different methods could apply to exploit this process, but we will exclusively describe, as an example to understand the

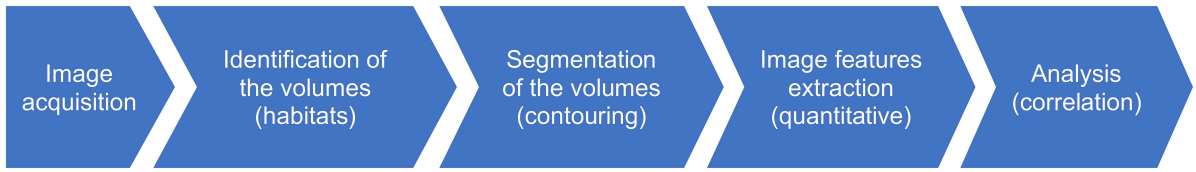

FIGURE 1 | Essential steps of the radiomics/pathomics process.

full operation, the bio-inspired system we have been currently investigating within a multi-disciplinary joint lab (engineers, mathematicians, and clinicians) for pathomics and radiomics. The mathematical core is based on recent Machine Learning (ML) approaches. The high capability of the ML systems in addressing complex problems and, in particular, those related to healthcare and medical applications, has already been confirmed (19, 20). As an additional validation, we have also implemented a joined mathematical-ML system for the early discrimination of skin lesions by dermoscopic images with high diagnostic accuracy (21). The bio-inspired system is based on the correlation between the tumor aggressiveness and fractal dimension of the related lesions (22).

Currently, we have been testing this approach within two specific subject areas. The first one in the field of pathomics for lung cancer (reported in **Figure 2A**), regards specifically the prediction of PD-L1 overexpression (a biomarker predictive of response to immunotherapy in this tumor subtype) by the analysis of histopathological hematoxylin stained images; this could represent a useful guide to pathologists (and physicians). The second one concerns radiomics for urothelial cancer and it is aimed to correlate tumor response to immunotherapy with CT-scans medical images and other blood data (i.e., radiomics).

Starting from these premises, for pathomics, we have implemented a hyper-filtering pre-processing of histopathological hematoxylin stained images (**Figure 2A**). Each of the analyzed images has been converted from RGB (red-green-blue) color spaces to luminance (Y) chrominance information (CbCr) spaces with the divided gray-level representation of the histopathologic image. The luminance Y gray-level images have been then pre-processed by the hyper-filtering layer inside the "Pre-processing Block" using an *ad-hoc* adaptive thresholds-based approach in order to obtain a 1D representation of the source gray-level Y images. From every pre-processed Y images, the system computes the corresponding fractal dimension according to the Hausdorf model allowing to obtain, through an additional computing analysis, a time-series collection of those fractal dimensions (23). These pathomics features, ensued along with histopathologic image-features extracted by the AutoEncoder system (that is designed with one hidden layer of 20 neurons) also included in the "Pre-processing Block" are fed into a regression neural network learned by a classical Scalable Conjugate Gradient (SCG) back-propagation algorithm, with the final classification layer based on the SoftMax approach (21).

For the learning process (training phase), the authors used 70 percent of the histopathologic images while the remaining 30 percent serves for testing and validation. The learning dynamic of the bio-inspired system and an example of the fractal dimension time-series extracted from images are represented in **Figure 2B**.

For our radiomics project, the system is basically the same as above described (**Figure 2A**) with the input being the sequence of segmented CT-scan slices in which the lesion is visible along with the possible association of normalized representation of laboratory data (i.e., blood values). Through an innovative patented approach, time-series mapped signals are extracted in the pre-processing layer, starting from an *ad-hoc* analysis of the morpho-geometric dynamic of the CT-scan lesion in each of the slices. The resulting output (time-series data) feed, as a new input, the regression neural layer and then the SoftMax classificatory, which finally provide the binary discrimination of the positive or negative response to the immunotherapy (**Figure 2C**).

RADIOMICS AND PATHOMICS APPLICATIONS

Diagnosis (Early) and Classification

Computer-aided diagnosis and detection system (CAD) help for better detection and diagnostic accuracy (24). Radiomics analysis, although sharing some principles with CAD, do not answer only a precise question (detection) but it is a complex process looking for a correlation with biological mechanisms. Magnetic resonance (MR) images from 147 patients with confirmed prostate cancer showed that several MR derived "texture" features were significantly different in benign and malignant prostate tissue and in samples with different Gleason scores (25). Another study confirmed that texture features extracted from MR prostatic images could define with accuracy not only the Gleason score but also score patterns: two patterns of Gleason score 7 ("4 + 3" vs. "3 + 4") were correctly discriminated with 92% accuracy (26).

Pathomics studies were preceded by computer-aided-system tools, with for instance a fractal analysis set, showing powerful discrimination in grading prostatic cancer (27). In another study, analysis from 39 patients with colorectal lesions finds that analysis of multiscale texture features, extracted through a "3D wavelet transform filter" from histopathological images, were able to correctly distinguish different colorectal cancer grades (28).

In the context of immunotherapy, Tang et al. associated radiomics features with PDL1 expression and CD3 count in two cohorts (training and validation cohort of $n = 114$ and $n = 176$, respectively) lung cancer patients (29). Sun et al. developed a radiomic signature for tumor-infiltrating CD8 cells

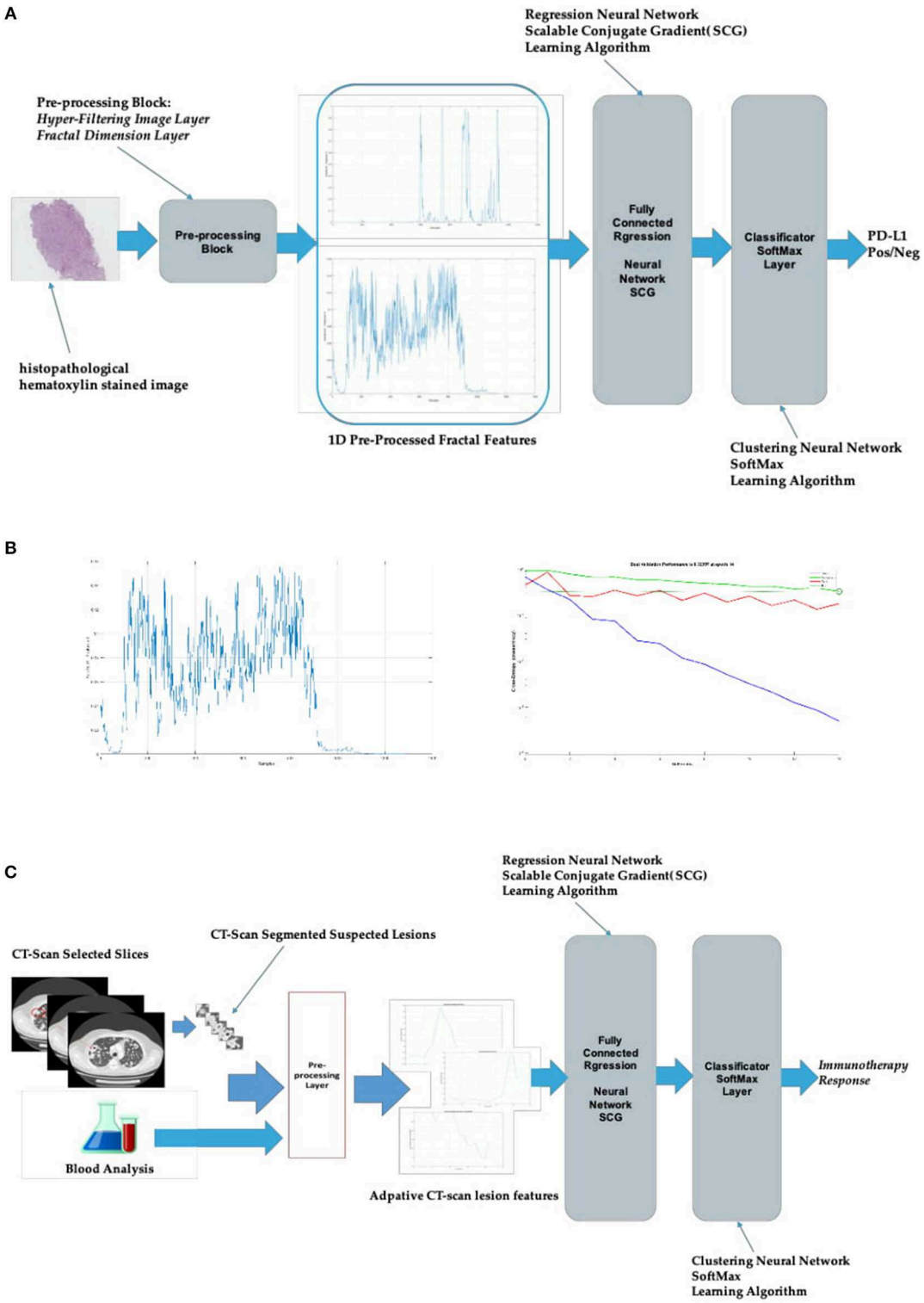

FIGURE 2 | Bio-inspired system for radiomics and pathomics. **(A)** Bio-inspired system for pathomics in lung cancer; **(B)** the diagram on the left shows an example of fractal dimension time–series extracted from a single histopathological hematoxylin stained image. The one on the right illustrates the learning dynamic of the system during the training session: the lower (blue) curve shows the training dynamic (i.e., the progressive error reduction) while the middle (red) and the upper (green) curves show the testing and validation, respectively; **(C)** bio-inspired system for radiomics in urothelial cancer. The pre-processing input data used arise from CT-scan images and blood analysis data.

in a retrospective multicohort study on overall 491 patients with advanced solid tumors (30).

Prognosis

The prognostic value of radiomics was reported in 108 patients with lung adenocarcinoma (separated in two independent cohorts), radiomics features (including tumor shape complexity and intratumor density variation) were strongly correlated with overall survival (31). Furthermore, Aerts et al. analyzed 440 image-related features extracted from CT images of 1019 patients with lung or head and neck cancer. They could find many radiomic features having a prognosis value and built a prognostic radiomic signature, which was found to be correlated with underlying gene-expression patterns (32).

Pathomics could also yield prognostic information. Pathomics features derived from the analysis of 2186 histopathological images were explored to distinguish short-term and long-term survivors in patients with non-small lung cancer. The survival prediction model was validated on 294 additional images (33).

Pathomics and radiomics studies in glioblastoma patients illustrated how correlations derived from different data scales (neuroimaging, pathologic and genomic) may give a deeper understanding of tumor biology and predict clinical outcomes (34–37).

Regarding immunotherapy, in the above-mentioned study of Tang et al. (29), a radiomic immune pathology-informed model was developed. The model defined four subsets of lung cancer patients significantly associated with overall survival. A group of patients with favorable prognosis was identified, harboring low CT intensity and high heterogeneity (as radiomic features) and low PDL1 with high CD3 infiltration, indicating a favorable immune activity.

Outcome Prediction

To date, fewer works have explored the predictive value of radiomics and pathomics features. MR images-derived texture features from 58 breast cancer patients showed that radiomic features before neoadjuvant chemotherapy could predict response (38).

As far as immunotherapy is concerned, in the study of Sun et al. (30), the radiomic-based biomarker of tumor-infiltrating CD8 cells was validated in 3 independent cohorts and showed predictive value for tumor response to the anti-PD-1 or anti-PD-L1 therapy. Moreover, Colen et al. elaborated a two-feature radiomic model in order to predict immunotherapy-induced pneumonitis characterized by strong internal accuracy (100%) (39).

FUTURE CHALLENGES OF IMAGE-DERIVED FEATURES

Some challenges regarding the multistep process of radiomics and pathomics still need to be adequately addressed. Methodologically, quantitative image-derived features biomarkers should undergo a multicenter prospective trial to be validated, as it is for other biomarkers. Technically, each step of image data analysis needs proper benchmarking and reproducibility. Furthermore, curation of big data, time processing and data sharing are other major challenges. In this sense, great efforts have been made by the scientific community to share tools (software, web-based platforms) allowing physicians to explore image data analysis (40–42). The Quantitative Imaging Network, for instance, initiated in 2008 and supported by the National Cancer Institute, is an example of the importance of these new disciplines. Along with the identification of biological biomarkers, assessed by longitudinal repeated tumor samples taken by tissue biopsy and/or liquid biopsy, we postulate that "digital biopsy," as previously defined, could allow to find potential correlation between biological biomarkers and "radiomics and pathomics biomarkers," and have the potential to better define prognosis and prediction of response. Interdisciplinarity and integration within "-omics" disciplines and clinicians will certainly be of key importance for greater precision in oncology diagnosis and treatment in the next future.

DATA AVAILABILITY

All datasets for this study are included in the manuscript and the supplementary files.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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CASE REPORT
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Anti-PD-1 Immunotherapy and Radiotherapy for Stage IV Intrahepatic Cholangiocarcinoma: A Case Report

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Due to the unsatisfactory robustness of current predictive biomarkers in many cases, application of immunotherapy in advanced cancers with limited treatment options, such as stage IV intrahepatic cholangiocarcinoma (ICC), was quite common. Hence, strategies to enhance the therapeutic effect of immunotherapy or to extend the scope of potential beneficial patients were urgently needed. Combination of radiotherapy and anti-programmed death receptor-1 (PD-1) immunotherapy was a promising one, since they were found to have a synergistic anti-tumor effect in animal models and a couple of patients. We here present a 68-years-old male with chemotherapy-intolerable stage IV ICC, whose primary tumor had low PD-L1 expression level, scarce CD8+ cells in tumor microenvironment, high microsatellite instability (MSI), and high tumor mutation burden (TMB). These biomarkers showed a conflicting prediction of the treatment response and clinical benefit of anti-PD-1 immunotherapy. Combination therapy of anti-PD-1 immunotherapy and radiotherapy was adopted as first-line treatment for the patient. After six cycles of immunotherapy, shrinkage of the primary liver tumor and metastatic lymph nodes happened, alongside with new lung metastasis, which indicated a mixed response. Radiotherapy was then administered to both the liver and lung lesions, accompanied with continued immunotherapy. The combined therapy eventually led to a complete response for both the primary tumor and all metastases without treatment-related adverse effects. The patient has survived for 26 months after the combined therapy and remains tumor-free currently. This case demonstrates the high inconsistency between immunotherapy response biomarkers and the synergetic anti-tumor effect of immunotherapy and radiotherapy in ICC.

Keywords: intrahepatic cholangiocarcinoma, immunotherapy, radiotherapy, combination therapy, biomarkers

INTRODUCTION

Stage IV intrahepatic cholangiocarcinoma (ICC) patients have very poor survival outcomes. Gemcitabine plus cisplatin chemotherapy is currently recommended as the only first-line treatment for these patients, with a median overall survival (OS) of only 11.7 months (1). The worst is that more than 70% of patients are intolerable to the chemotherapy regimen because of severe complications. Therefore, the use of current chemotherapy for most stage IV ICC patients is limited and the requirement for a novel treatment option is urgent (1).

Recently, immune checkpoint blockades showed promising therapeutic effects in a wide range of solid tumors, including a small number of ICC cases (2). However, robust biomarkers for predicting treatment response remains one of the most crucial issues. Although several biomarkers including PD-L1 expression level, microsatellite instability (MSI), tumor mutation burden (TMB), and immune cell infiltration have been applied for selecting target patients, their accuracies were all limited and diverse across different types of tumors. Only MSI was reported to be predictive in a few ICC cases (2). On the other hand, general outcomes of anti-PD-1 immunotherapy for ICC remain controversial. Thus, considering the lack of robust biomarkers and the limited treatment options for cholangiocarcinoma, it is more urgent to find out universal strategies for applying immunotherapy. Most evidence by far shows the inadequate efficacy of immunotherapy alone for the control of advanced cancer.

Radiotherapy is another treatment option for unresectable ICC, which showed a local control effect (3, 4). However, due to limited evidence, recommendations of anti-PD-1 immunotherapy and radiotherapy are both category 2A. It has been reported that local tumor destruction combined with immunotherapy may have a synergetic effect against solid tumors (5). Radiotherapy is a powerful local treatment that can only reduce tumor burden to the minimal but also trigger the anti-tumor immunity and reprogram the tumor microenvironment. Yet, present evidence of the synergistic anti-tumor effect of radiotherapy and immunotherapy for ICC is lacking.

Here we comprehensively investigated the current predictive markers and showed their inconsistency and complexity in a chemotherapy-intolerable stage IV ICC patient with metastases to lymph nodes and lungs, who had a complete response and survival benefit to the combination therapy of immunotherapy and radiotherapy as the first-line treatment.

CASE PRESENTATION

A 68-years-old male complained with xanthochromia, scleral icterus, and abdominal distension for over 20 days was admitted to our hospital in January 2018. He lost about 10 kg of body weight. Physical examination showed deep jaundice of the patient and the left supraclavicular lymph nodes were palpable. The performance status (PS) score was 3. Laboratory tests showed that total bilirubin (TB) was 707.9 umol/L, and CA19-9 level was over 12,000 U/mL, while AFP level was <20 ug/L (**Table 1**). Magnetic resonance imaging (MRI) found a 47 × 42 mm space-occupying lesion in Segment 4 (S4) and S5 of the liver and a mass in the common bile duct, suspicious for ICC. Subsequent positron emission tomography (PET) showed multiple distant metastases to lungs, abdominal lymph nodes, and left cervical lymph nodes. Histology of the liver lesion biopsy found numerous tubular structures of adenocarcinoma and a fibrous stoma (**Figures 1A,B**). Immunohistochemistry (IHC) analysis showed the following: CK(+), CK7(+), CK20(weak +), and Ki-67(3%+). The diagnosis was confirmed as stage IV ICC. The presumed survival time was only 3–5 months (6).

TABLE 1 | Clinical variables of the patient during treatment.

Variables	22 Jan 2018 (baseline)	8 Apr 2018	31 Jul 2018	10 Dec 2018	14 Feb 2019	31 May 2019
Size of the liver lesion (mm × mm)	47 × 42	38 × 33	35 × 29	32 × 23	29 × 21	10 × 7
Total bilirubin (umol/L)	707.9	114.0	21.2	9.7	23.0	12.9
CA199 (U/mL)	>12,000	>12,000	4620.49	109.31	36.41	14.70
CEA (ug/L)	19.69	8.90	2.97	1.39	1.62	1.82
CA125 (U/mL)	114.90	60.10	15.50	12.50	12.70	10.60
AFP (ug/L)	2.20	5.26	3.01	3.84	2.99	3.15

According to the opinion of the ICC multi-discipline team in our hospital, the patient was not a candidate for conventional treatments including surgery and chemotherapy, considering both tumor and PS status. Then, percutaneous transhepatic cholangial drainage (PTCD) was performed to relieve the jaundice and the patient's appetite recovered and the PS score was still 3. To comprehensively investigate the immune microenvironment, the tumor tissue of the liver lesion was submitted for subsequent tests. Additional IHC analysis found a low expression level of programmed cell death ligand 1 (PD-L1) and a low frequency of CD8+ T cells (**Figures 1C–F**). The whole-exome sequencing (WES) data showed high levels of both MSI and TMB (16.9 mutations/Mb), which indicated the potential benefit of immunotherapy. Additionally, there were 420 indels (insertions and deletions) and 660 single nucleotide variants (SNVs), with five mutations (including *MLH1*, *SMARCA4*, *BRCA2*, *POLE2*, and *ARID1A*) known to be associated with sensitivity to immunotherapy while one gene (*B2M*) conferred resistance to immunotherapy. We further included another 36 ICC cases in the Cancer Genome Atlas (TCGA) dataset to comparatively analyze the patient's tumor immune microenvironment based on the RNA-seq data. This case was found to have a moderate level of immune infiltration under a comprehensive immune signature (**Figures 2A,B**) (7–11). Analysis of immune cell components in the tumor microenvironment using the CIBERSORT algorithm revealed scarce CD8+ cells but a large number of M2 macrophages, which is consistent with the IHC result and indicates an immunodeficient state (**Figure 2C**) (10). After all, anti-PD-1

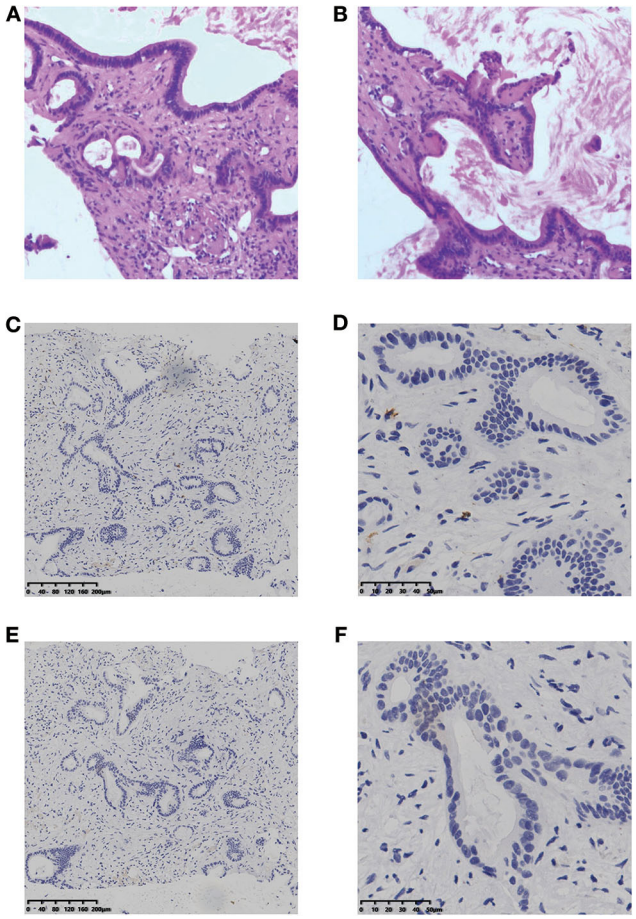

FIGURE 1 | H&E staining **(A, B)** and IHC result of CD8 **(C,D)** and PD-L1 **(E,F)** for the liver lesion. H&E staining, hematoxylin and eosin staining; IHC, immunohistochemistry; PD-L1, programmed cell death ligand 1.

immunotherapy (pembrolizumab, at a dose of 200 mg every month) combined with radiotherapy was considered as treatment for the patient, which was initiated in February 2018 (**Figure 3A**).

After two cycles of immunotherapy, the patient's symptoms relieved and his PS improved. The size of the liver lesion slightly reduced to 38 × 33 mm but CA-199 was still over 12,000 U/mL (**Figure 3B**; **Table 1**). After six cycles, PS score was 1 and CA-199 was decreased to 4620.49 U/mL (**Table 1**). Contrast-enhanced computed tomography (CT) scans showed that the liver lesion reduced to 32 × 23 mm (**Figure 3B**). However, the number of lung metastases increased, which indicated a mixed response to immunotherapy (**Figure 3C**). Anti-PD-1 immunotherapy continued while radiotherapy was introduced to control the liver and lung lesions, with doses of 50.0 and 48.0 Gy, respectively. All visible tumors reduced in size gradually in the follow-up and the PTCD was removed 3 months later (**Figure 3C**). Currently, after 26 months of treatment, the patient is alive with high life quality. There aren't any symptoms and PS score is 1. The patient regained 5 kg of body weight. All tumor biomarkers including CA19-9 level are normal. The latest imaging examinations show invisible signs of the liver lesion, the metastatic lymph nodes, and the lung metastases. CR is achieved in this stage IV ICC case (**Figure 3C**; **Table 1**).

DISCUSSION

Currently, emerging evidence shows the therapeutic effect of anti-PD-1 immunotherapy in various types of cancers, yet target patient selection remains one of the biggest problems. Although several biomarkers including PD-L1 expression level, TMB, MSI, or immune cell infiltration, have been used to select patients and predict treatment response in anti-PD-1 immunotherapy, they were still not reliable in many situations. As for cholangiocarcinoma, only weak evidence showed that MSI had the potential to be an appropriate predictive marker. Undoubtedly, the anti-tumor immune response is a very complicated biological process that involved cancer cells and cells in the microenvironment. Each biomarker only reflected some aspect of the whole process and it was no wonder that they would be inconsistent with others and fail to predict in some situations. In this case, we comprehensively analyzed the immune microenvironment of the patient and found that although both MSI and TMB were high, the PD-L1 expression level was low and the immunosuppressive tumor microenvironment of the liver lesion had scarce CD8+ cells but lots of M2 macrophages. High infiltration of M2 macrophages in the tumor stroma could suppress T cell infiltration and down-regulate antitumor immune responses. The contradiction between biomarkers resulted in difficulty in predicting response. Even though both MSI and TMB are currently the most valuable predictive biomarkers for anti-PD-1 immunotherapy, there are also lots of cancer patients with MSI-H or/and TMB-H that do not respond well. According to previous studies, only approximately half of solid tumors with MSI-H achieved object response to anti-PD-1 immunotherapy (2). Besides, low lymph cell infiltration in this case might also indicate immune escape, which allows tumor evolution and thus higher genomic diversity. The tumor with this situation was considered to be unresponsive to immunotherapy (12). On the other hand, tumor heterogeneity also influences the accuracy in determining the status of these markers (13). Intratumor genetic heterogeneity was found obvious in ICC and multi-point aspiration was needed to evaluate the markers accurately, which was impossible in patients that did not receive surgery or underwent tumor recurrence. In a word, there is currently no robust marker for predicting the response to anti-PD-1 immunotherapy. On one hand, further studies are needed to develop robust predictive markers for selecting those patients that might benefit from anti-PD-1 immunotherapy. On the other hand, strategies such as combination therapy of anti-PD-1 immunotherapy and radiotherapy in this case that make patients with limited treatment options benefit from immunotherapy might be applicated at present.

The possible mechanisms of the synergistic anti-tumor effect of combination therapy have been investigated by many researchers so far. We summarized them as follows, including tumor burden reduction, immunity activation, and tumor microenvironment modification. First, radiotherapy could

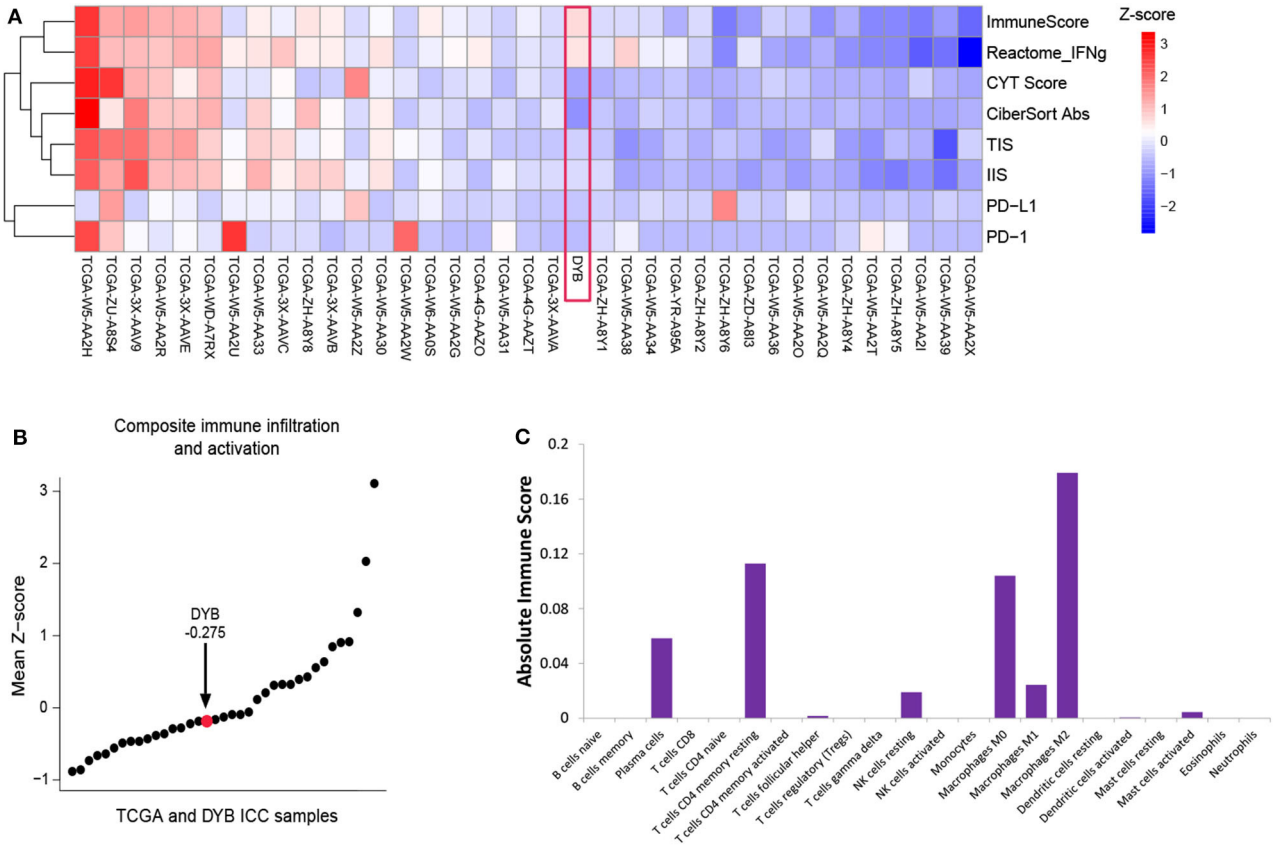

FIGURE 2 | Immune characterization of the primary tumor before treatment. **(A)** The heatmap of the ICC case and 36 ICC cases in the TCGA dataset, with the measurement of ImmunoScore, interferon-γ signaling (Reactome.org), CYT score, Cibersort Absolute Score, TIS, IIS, PD-L1, and PD-1. **(B)** Plot of the mean Z-scores across this ICC case and 36 ICC cases in TCGA dataset. **(C)** The absolute immune score shows the components of the immune cells in primary tumor of the ICC case. ICC, intrahepatic cholangiocarcinoma; TCGA, the Cancer Genome Atlas; TIS, T cell Infiltration Score; IIS, Immune Infiltration Score; PD-L1, programmed cell death ligand 1; PD-1, programmed death receptor-1.

reduce the tumor burden and create a background of minimum tumor burden for immunotherapy. Second, radiotherapy can fully trigger the recognition of tumor cells by antigen-presenting cells. Irradiation can directly destroy the DNA, allowing more neoantigens released by tumor cells to trigger immune responses (14). Some innate immune pathways can be activated during radiotherapy to regulate the anti-tumor immune responses. Irradiation-induced cGAS-STING pathways can lead to the recruitment of dendritic cells and trigger the type I IFN signaling, thus regulating the adaptive immune response and reinforcing the cytotoxic T cells (15). Third, radiotherapy can modify the tumor microenvironment, potentially affecting the immune compositions, and priming the adaptive immunity. Localized irradiation can induce chemokines involved in the recruitment of effector T cells, converting the tumors into tissues susceptible to immune attack (16). In our case, the primary tumor had significantly high infiltration of M2 macrophages, which contributed to the immunosuppressive tumor microenvironment. Klug et al. have recently shown that low doses of radiotherapy can reprogram tumor-associated macrophages to a M1 phenotype, which conversely enhanced the efficacy of adaptive immunity (17). Probably, the macrophages of the primary and metastatic tumors in this patient had experienced such a conversation from M2 to M1 under irradiation, initiating a significant change in the tumor immune microenvironment, which deserves further studies and clinical trials on the dynamic evolution of ICC under combined therapy.

Currently, chemotherapy such as gemcitabine plus cisplatin is considered as the only first-line treatment for metastatic ICC. However, the chemotherapy regimen results in a severe (grade 3 or 4) toxic effect rate of about 70% (1). Due to the toxicities of traditional chemotherapeutic drugs, good performance status is often required for chemotherapy. But in fact a large number of advanced-stage patients have bad performance status, so that they are intolerable to chemotherapy. On the contrary, immunotherapy combined with radiotherapy has relatively slighter short-term side effects and may be more suitable for these patients. Clinical trials that investigated the possibility of anti-PD-1 immunotherapy combined with radiotherapy as first-line treatment in ICC patients could be conducted.

During the treatment, new lung lesions occurred while the other lesions demonstrated controlled, which indicated different treatment responses across organs, namely a mixed response. This atypical response pattern has been noticed

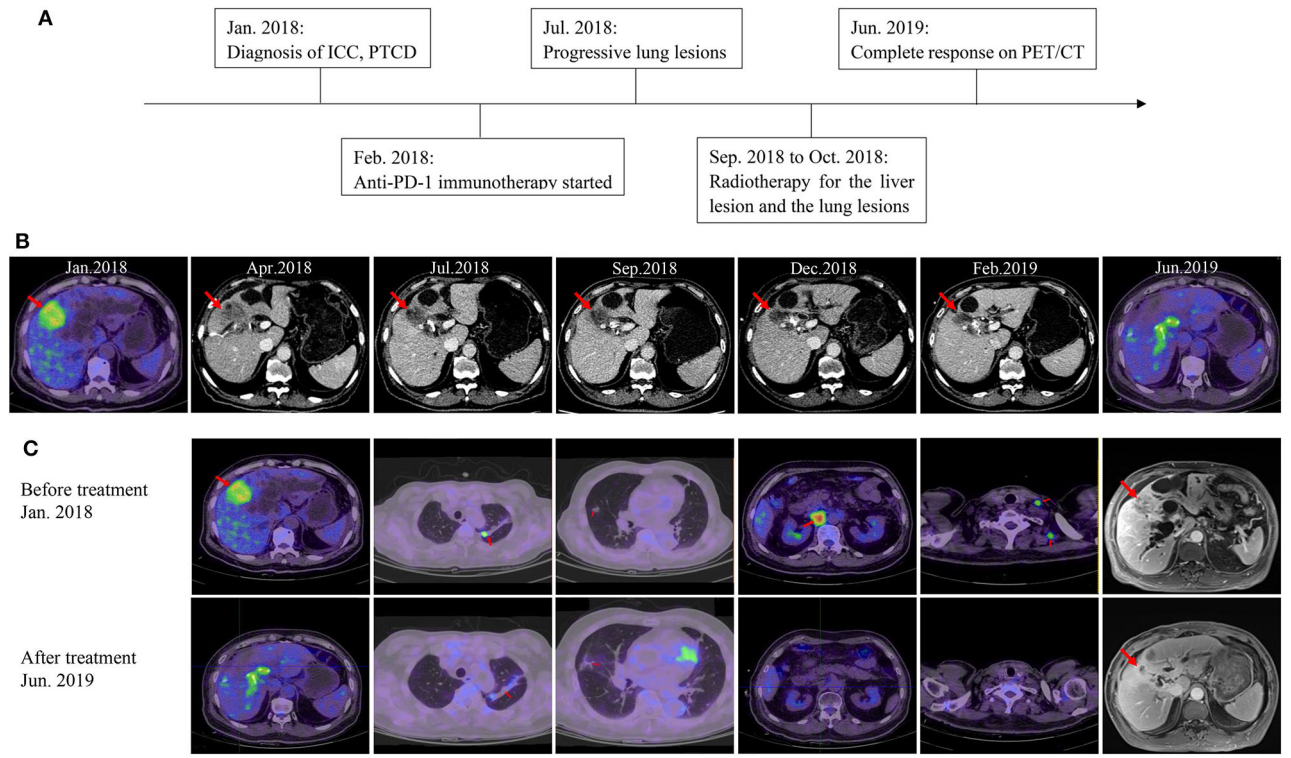

FIGURE 3 | Imaging of the patient during treatment. **(A)** The timeline of his clinical course. **(B)** Imaging shows the change of the liver lesion over time. **(C)** Imaging of the patient at baseline and the latest follow-up.

in previous studies (18, 19). According to the conventional radiological response criteria, the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1, this would be evaluated as PD. However, patients with the response pattern were found to have non-inferior OS compared with those who had controlled diseases, which means the RECIST underestimates the clinical benefit of immune checkpoint blockade. Thus, several novel response evaluation criteria have been proposed recently, including the iRECIST, the immune-related response criteria (irRC), and the immune-modified RECIST (imRECIST). According to these criteria, the patient in this case should not be characterized as PD in the situation, and the combination therapy could be continued, which was proven to be a sensible choice afterwards.

CONCLUSIONS

In conclusion, we analyzed the most valuable biomarkers for immunotherapy response and demonstrated their complexity and inconsistency in an ICC patient who had limited treatment options. The current dilemma made us adopt the combination therapy of anti-PD-1 immunotherapy and radiotherapy as his first-line treatment, which led to a complete response and prolonged survival time. This suggests their synergic anti-tumor effect and the bright prospect of combination therapy. Further efforts are required to investigate the combination therapy in ICC patients.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. 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

Z-LL, XL, and HP drafted the manuscript and performed data analysis. Z-WP, DT, and SP were involved in manuscript editing. J-TL, YB, and MK treated the patient and designed the study. All authors read and approved the final manuscript.

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MINI REVIEW
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Immunotherapy Targeting Tumor-Associated Macrophages

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Macrophages are phagocytic cells that play a broad role in maintaining body homeostasis and defense against foreign pathogens; whereas tumor-associated macrophages (TAMs) support tumor growth and metastasis by promoting cancer cell proliferation and invasion, immunosuppression, and angiogenesis, which is closely related to the poor prognosis in almost all solid tumors. Hence, deep-insight knowledge into TAMs can provide an opportunity to discover more effective strategies for cancer therapeutics. So far, a large number of therapeutic agents targeting TAMs are in clinical trials. In this review, we introduce an extensive overview about macrophages and macrophage-targeting agents.

Keywords: macrophage, tumor microenvironment, cancer, immunotherapy, polarization

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INTRODUCTION

Cancer, a global public health problem, is the first or second leading cause of death in most countries, and its incidence and mortality are rapidly growing (1). Clinically it is well-acknowledged that tumor sites contain not only cancer cells, but also immune cells, including macrophages, regulatory T (T_{reg}) cells (2), neutrophils (3), mast cells (4), natural killer (NK) cells (5), etc. Macrophages, the main component of the mononuclear phagocyte system (6), are phagocytic cells which play a broad role in maintaining body homeostasis and defense against foreign pathogens; whereas there are a large number of TAMs in tumor microenvironment (TME), which support tumor growth and metastasis by promoting cancer cells proliferation, immunosuppression, invasion, and angiogenesis. Therefore, scientists pay special attention to TAMs when looking for effective cancer treatment strategies. In recent decades, several types of immunotherapies targeting TAMs are playing more and more important roles in the treatment of cancer.

This comprehensive review first summarizes most recent updates regarding macrophage recruitments and functions in tumor, then focuses on the development and evaluation of cancer immunotherapy strategies targeting TAMs including drugs in pre-clinical and clinical stages. Finally, we would like to provide some views and visions of immunotherapy targeting TAMs.

ORIGINS AND POLARIZATION OF MACROPHAGES

Macrophages were first discovered and isolated by Ilya Metchnikoff in the nineteenth century (7). For decades, most people thought that blood-circulating monocytes derived from adult bone marrow (BM) continuously repopulate tissue-resident macrophages (TRMs). It is now well-accepted that a large number of TRMs derive from embryonic precursors, which are from both fetal yolk sac and fetal liver progenitors (8–12). All precursors seed different tissue and differentiate into specialized TRMs on the basis of tissue-specific context (10, 13). Moreover, most tissues also contain macrophages derived from monocytes after birth (13–15). However, some tissues are different, such that monocytes derived from hematopoietic stem cells (HSCs) fleetly take the place

of embryonic macrophages after birth in the colon, but microglia are rarely from monocytes derived from HSCs under homeostatic conditions (16, 17) (**Figure 1A**). In tumors, TAMs are usually thought to primarily derive from circulating monocytes, and most recent studies have shown that functions and phenotypes of embryonic-derived and monocyte-derived macrophages are different (13, 18, 19). For example, Pierre-Louis Loyher et al. showed that embryonic-derived TAMs largely correlated with tumor cell growth *in vivo*, while monocyte-derived TAMs accumulation was associated with enhanced tumor spreading (18). Furthermore, several studies have suggested that TRMs are up to 50% in some murine models such as lung and brain cancer (18, 20).

Macrophages are a type of remarkable plastic cells and can be easily induced by surrounding microenvironment (21, 22). According to different activation methods, macrophages are divided into two extremes (23), Classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages). M1 and M2 macrophages have significant differences in surface receptor expression, tissue distribution, metabolism, cytokine and chemokine production, function, and intracellular signal transduction. M1 macrophages are polarized by lipopolysaccharide (LPS), which binds to the Toll-like receptor 4 (TLR4). Then an inflammatory response is elicited (24), and pro-inflammatory cytokines are released, such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α). These downstream signals recruit more macrophages to resist pathogenic insult (25). M2 macrophages are polarized by cytokines such as IL-4 and IL-13, and release anti-inflammatory cytokines including transforming growth factor-β (TGF-β) and IL-10, inducing processes like membrane remodeling and angiogenesis to promote tissue repair (26, 27). Depending on specific inducing signals and their biological roles, M2 macrophages could be further divided into M2a, M2b, M2c, and M2d (28–32) (**Figure 1B**). Generally speaking, M1 macrophages mainly kill and clear cancer cells (33, 34), while M2 macrophages mainly support tumor development (35, 36). This M1/M2 concept can easily explain macrophage heterogeneity, but it is too simple to explain the complexity of macrophage activation. Actually, TAMs seem to consist of various populations with a wide range of polarization features or activation states, and their function is determined by microenvironment. Hence, additional studies are necessary to better classify macrophages, and there are several articles about other classifications (37–39).

Abbreviations: AMT, adoptive macrophages transfer; Arg-1, arginase-1; BM, bone marrow; BTK, Bruton's tyrosine kinase; CAR-M, chimeric antigen receptor macrophage; CAR-T, chimeric antigen receptor T cells; CCL, CC chemokine ligand; CCR, CC chemokine receptor; CSF-1R, CSF-1 receptor; CXCL8, CXC chemokine ligand 8; ECM, extracellular matrix; FLT1, FMS-like tyrosine kinase 1; HIF1α, hypoxia-inducible factor 1α; HSCs, hematopoietic stem cells; IFN-γ, interferon-γ; iNOS, inducible nitric oxide; Jak2, Janus kinase 2; IL-1β, interleukin-1β; LPS, lipopolysaccharide; MAMs, metastasis-associated macrophages; M-CSF/CSF-1, macrophage-colony stimulating factor; MHC II, major histocompatibility complex class II; MPS, mononuclear phagocyte system; MR, mannose receptor; NF-κB, nuclear factor-κB; NK, natural killer; PI3Kγ, PI-3 kinase γ; PMN, pre-metastatic niche; SIRPα, signal regulatory protein α; SR, scavenger receptor; STAT3, signal transducer and activator of transcription 3; TAMs, tumor-associated macrophages; TGF-β, transforming growth factor-β; T_H2, T helper 2; TIE-2, tumor endothelium releases angiopoietin-2; TLR4, Toll-like receptor 4; T_{reg}, regulatory T; TRMs, tissue-resident macrophages; TME, tumor microenvironment; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

FUNCTIONS OF MACROPHAGES IN TME

Promoting Tumorigenesis and Progression

TAMs are believed to be the bridge between cancer and inflammation. Some studies show that about 25% of all cancers are related to chronic infection and inflammation (40). The production of chemokines and cytokines are induced by key transcription factors [such as nuclear factor-κB (NF-κB)], hypoxia-inducible factor 1α (HIF1α), and signal transducer and activator of transcription 3 (STAT3) when chronic inflammation occurs, which activates the innate immune system and especially macrophages (41). There is a lot of evidence that the inflammatory microenvironment promotes genetic instability of tumor epithelial cells and tumor-infiltrating immune cells (42, 43). Recently, the inflammatory cytokines IL-23 and IL-17 secreted by TAMs have been shown to be closely related to human colorectal cancer progression (44). For instance, Kupffer cells can promote the progression of hepatocellular carcinoma by secreting mitogens, which relies on the NF-κB signaling pathway (45). Other results show that IL-6 produced by TAMs promotes the development of liver cancer through STAT3 signaling pathway (46), and IL-10 produced by TAMs promotes the development of non-small cell lung cancer through STAT1 signaling (47).

Formation of the Immunosuppressive Microenvironment

Macrophages cannot only kill tumor cells directly when they are activated by interferon-γ (IFN-γ), but also recruit and activate $CD8^+$ cytotoxic T lymphocytes and NK cells by presenting antigens and secreting cytokines to promote the adaptive immunity (48). In addition, T cells can activate monocytes through CD40-CD40L interplay to enhance their expression of major histocompatibility complex class II (MHC II), inducible nitric oxide (iNOS), and TNF (49). In fact, the T helper 2 (T_H2) cells, dominating in the TME, activate macrophages to be polarized toward M2 macrophages, which promotes the development of immune suppression (50). Numerous studies have shown that TAMs can directly or indirectly inhibit T cell immune response through different mechanisms. The direct mechanisms include TAMs expressing inhibitory receptors to negatively regulate the activation of T cells by interaction with CD94 (51), expressing T cell immune checkpoint ligands to inhibit T cell functions (52, 53), producing cytokines to maintain a immunosuppressive microenvironment through inducing T_{reg} cell expansion and inhibiting $CD4^+$ and $CD8^+$ T cells (54, 55), and depleting L-arginine and tryptophan to inhibit cytotoxic T cells (56, 57). The indirect mechanisms include TAMs regulating the release of chemokines to control the recruitment of T_{reg} cells (58, 59), and blunting T cell recruitment by regulating the extracellular matrix (ECM) (60).

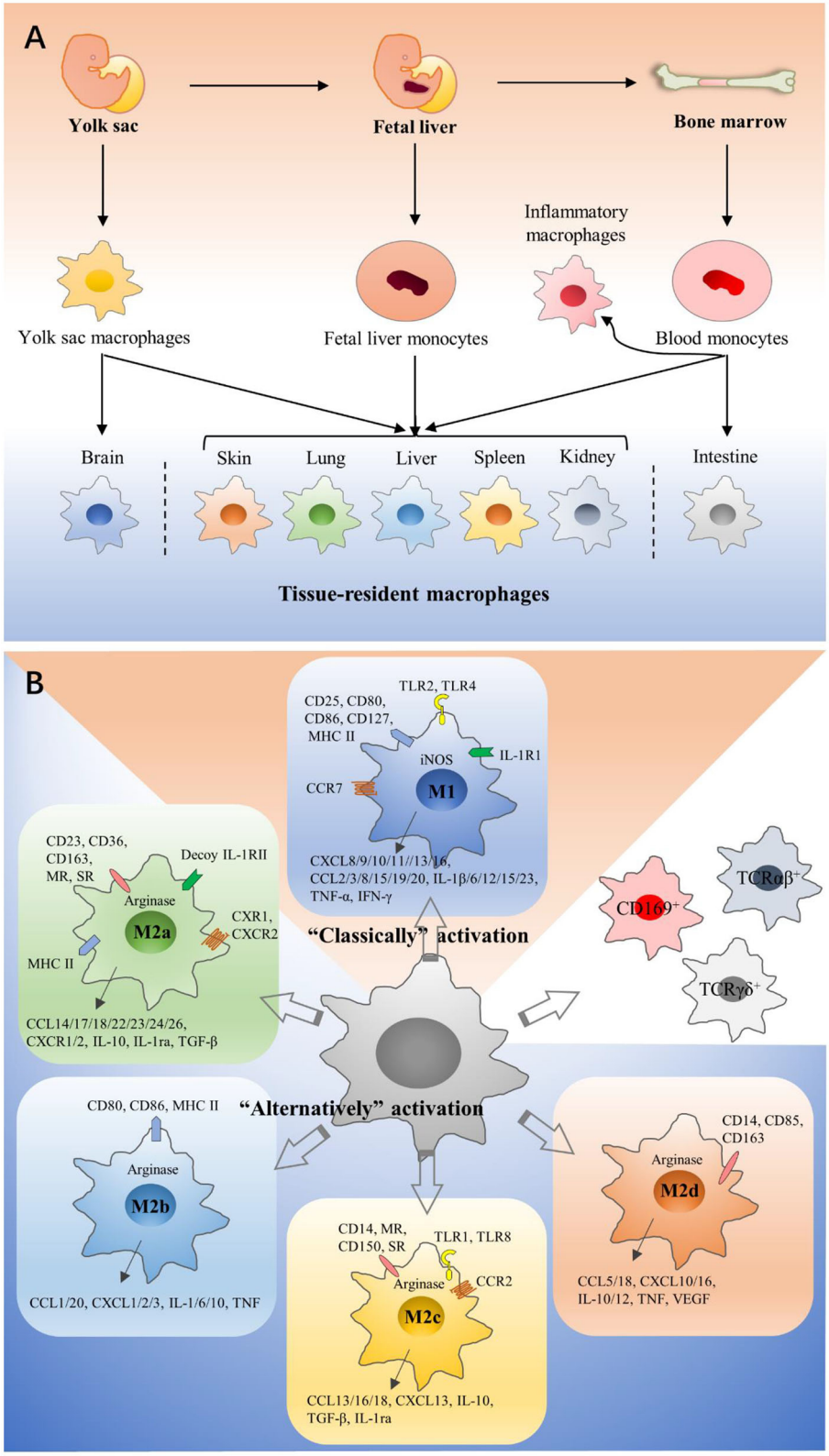

FIGURE 1 | Origins and polarization of macrophages. **(A)** Macrophages can have three different developmental pathways: fetal yolk sac, fetal liver, and bone marrow. Precursors seed different tissues and differentiate into specialized tissue-resident macrophages on the basis of tissue-specific context, and they have dramatical

(Continued)

FIGURE 1 | differences in their phenotypes and functions. In tumors, TAMs are usually thought to primarily derive from circulating monocytes. **(B)** According to activation methods, macrophages are divided into M1 and M2 macrophages. M1 macrophages are polarized by LPS, which binds to TLR4. M2a macrophages are induced by IL-4 and IL-13. M2b macrophages are polarized by immune complexes and some TLR ligands. M2c macrophages would increase in the presence of IL-10 or glucocorticoids. M2d macrophages are induced by TLR agonists and adenosine. They have significant differences in surface receptor expression, metabolism, cytokine, and chemokine production. $CD169^+$ macrophages, $TCR\alpha\beta^+$, and $TCR\gamma\delta^+$ macrophages are classified into neither M1 macrophages nor M2 macrophages.

Promoting Invasion and Metastasis

Cancer metastasis is a complicated event, which plays a crucial role in the cause of morbidity and mortality (61, 62). It is worth noting that macrophages play an important role in tumor cells invasion and metastasis. They facilitate the escape of tumor cells from the basement membrane through the dense stroma by producing proteases to promote ECM degradation (63). Furthermore, several factors, such as macrophage-colony stimulating factor (M-CSF/CSF-1), can stimulate macrophages to promote tumor invasion (64, 65). Metastasis-associated macrophages (MAMs), a unique population of macrophages, have been identified are found to be recruited by CC chemokine ligand (CCL) 2 (66, 67). MAMs promote cancer cell invasion and metastasis by FMS-like tyrosine kinase 1 (FLT1) receptor tyrosine kinase signaling in a mouse model of breast cancer (68). In addition, several studies show that the activation of the CCL2/CC chemokine receptor (CCR) 2 axis is very important in MAM-mediated metastasis (66, 67, 69). Recent studies have shown that pre-metastatic niche (PMN) is a pre-requisite in mediating tumor cell metastasis. Primary tumor cells are thought to initiate the formation of PMN by the secretion of proinflammatory cytokines, chemokines, and angiogenic factors that recruit BM-derived cells into future metastatic sites, and these cells induce PMN formation in reverse (70). For example, CXCL1 secreted by TAMs was reported to recruit $CXCR2^+$ myeloid suppressor cells to promote liver PMN formation (71, 72).

Promoting Angiogenesis

Angiogenesis is necessary for tumor growth and metastasis, which is regarded as a "hallmark" of cancer (73). Accumulating evidence emphasizes the crucial roles of macrophages in promoting tumor angiogenesis, and TAMs is closely related to the number of blood vessels in the tumor (74). Hypoxia is the primary driver of angiogenesis, and some studies show that anoxic areas of tumors, especially the necrotic tissue, have large numbers of macrophages due to the releasing of endothelins, vascular endothelial growth factor (VEGF), high mobility group 1, CCL2, CXC chemokine ligand 8 (CXCL8), CXCL12, and CSF-1 (75). The increased expression of hypoxia-inducible transcription factors on TAMs up-regulates the transcription of various genes in hypoxic tumor sites, which responds to hypoxia and promotes tumor cells proliferation, metabolism, and angiogenesis (75–77). In a CSF-1 knockout mice model, macrophage number was found to significantly reduce in the tumor site, accompanied by impaired vascular development (78). In addition, tumor endothelium-released angiopoietin-2 (TIE-2) was reported to play an significant role in tumor angiogenesis by recruiting monocytes that express the TIE-2 receptor (79). Furthermore, results of gene analysis indicated that TAMs could up-regulate the expression of various factors, which participate in tumor angiogenesis and provide nutrients for tumor growth (39).

IMMUNOTHERAPY-TARGETING TAMS IN CANCER

Restoration of Macrophage Phagocytosis

CD47 has been found expressed on many tumor cells, and it can bind with signal regulatory protein α (SIRPα) on the membrane surface of macrophages, which down-regulates macrophage phagocytosis of tumor cells (80, 81). In the past few years, a number of clinical trials have been conducted to determine various treatments that block CD47/SIRPα (**Figure 2**) (82). Anti-CD47 antibody treatment could inhibit tumor growth in a pediatric brain malignancies model (83). Anti-CD47 antibody in combination with TTI-621, a SIRPα-Fc fusion protein that could block the binding between SIRPα and CD47, promotes phagocytosis of tumor cells in s B-cell lymphoma mouse model (84). Hu5F9-G4, a human monoclonal antibody directing against CD47 has been tested in a tumor therapy as a single agent, as well as in combination with cetuximab. Nevertheless, anti-CD47 therapies may increase the occurrence of transient anemia, because HSCs and red blood cells extensively express CD47 (85, 86). Furthermore, there are other "don't eat me" signals including programmed cell death ligand 1 (PD-L1), MHC 1 component β2-microglobulin, and CD24, and antibodies which direct against the interaction of these signals with their macrophage surface receptors have demonstrated therapeutic potential in several cancers (87–89).

Inhibition of Macrophage Recruitment

Under tumor microenvironment, monocytes are rapidly recruited into tumor (90). Chemokines CCL2, CCL3, CCL4 and cytokines IL-1β, and CSF-1 have proven to contribute to the monocyte recruitment into tumors (91, 92). It is shown that CCL2 expression is up-regulated by macrophages and tumor cells in TME (93–95). Moreover, the high expression of CCL2 has a correlation with the poor prognosis in many human and murine tumors (96). CCL2 promotes monocytes recruitment by stimulating CCR2. In fact, blocking CCL2/CCR2 not only inhibits the monocyte infiltration but also prevents immunosuppressive polarization of macrophages (97, 98). Currently, a number of treatments targeting CCL2/CCR2 are in clinical trials (99) (**Figure 2**). A CCR2 inhibitor, PF-04136309, has been demonstrated to effectively inhibit tumor growth in pancreatic cancer patients (100, 101). CCL2 antibody treatment has proven to suppress tumor metastasis in a breast cancer model (96). Moreover, IL-1β has been identified as a chemoattractant target for cancer treatment. An IL-1 receptor antibody has been demonstrated to suppress inflammatory

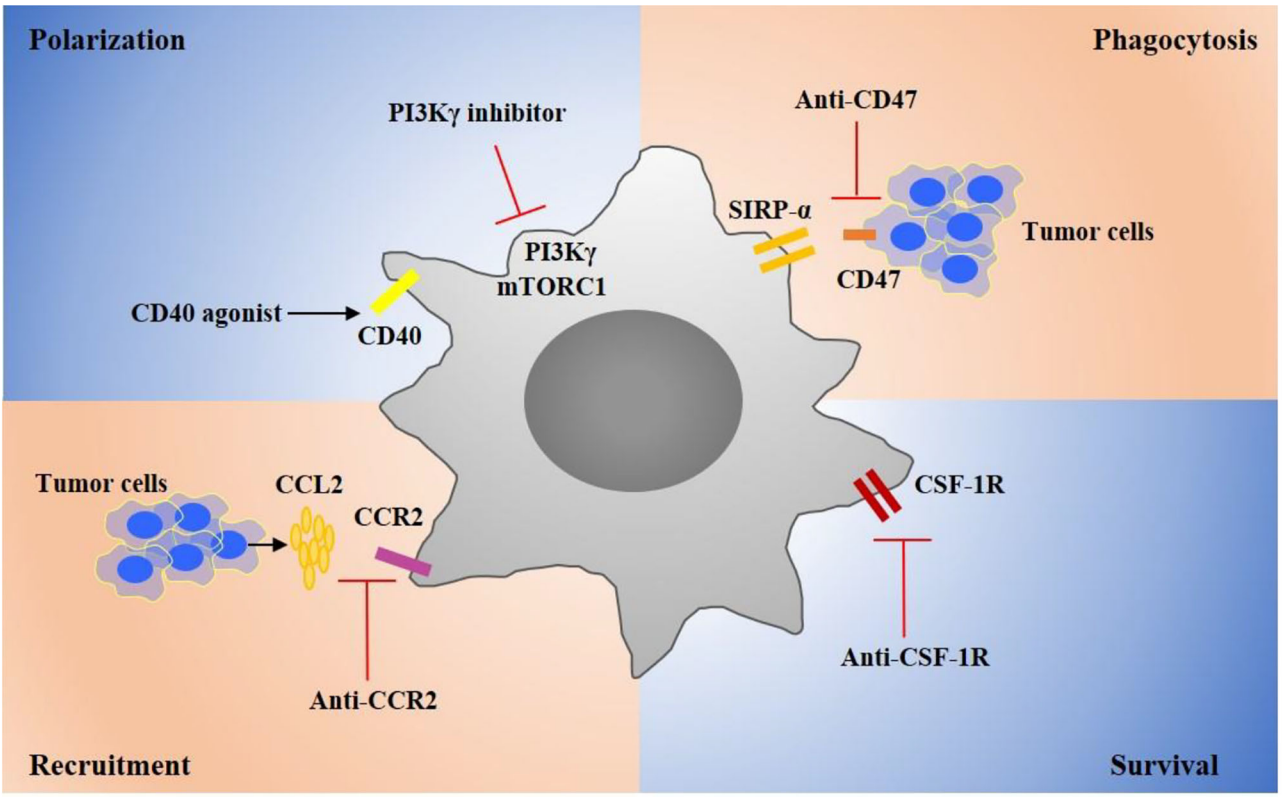

FIGURE 2 | Targeting TAM strategies in cancer treatments. Several critical targets have been identified that regulate TAMs recruitment, polarization, survival, and phagocytosis during tumor progression. Targeting key receptors or signaling molecules can modulate these macrophage properties and suppress tumor progression. For example, targeting CSF1R can suppress the survival of TAMs. Agonists of CD40 can promote TAMs toward a proinflammatory phenotype that can suppress tumor. Inhibitors of CCR2 or CXCL2 can inhibit monocyte recruitment. Targeting CD47 on tumor cells can promote macrophage phagocytosis of tumor cells. These therapeutic strategies are developed to promote effective antitumor immune responses and many drug candidates are currently investigated in clinical trials for cancer therapy.

macrophage accumulation and tumor growth in lung and breast cancer mouse models (100). Moreover, in combination with fluorouracil and bevacizumab, Anakinra, an IL-1 receptor antibody, has been shown to prolong patients' life in a colorectal carcinoma Phase II clinical trial (102) (**Table 1**).

Controlling Macrophage Proliferation and Survival

CSF-1 receptor (CSF-1R), a tyrosine kinase receptor, plays a key role in regulating macrophage proliferation and survival (103). Several studies show that blocking CSF-1/CSF-1R inhibited immunosuppressive macrophage polarization, reduced tumor cell proliferation, and promoted apoptosis, therefore suppressing tumor progression and prolonged life survival (104, 105) (**Figure 2**). M279, a CSF-1R antibody, blocking both CSF-1 and IL-34, has been shown to inhibit tumor growth and improve survival rate in a spontaneous breast tumor model (106, 107). BLZ945, a small-molecule CSF-1R inhibitor has been reported to be therapeutically effective in glioma and breast cancer mouse models (108). Moreover, a number of CSF-1R-specific inhibitors, including PLX3397, PLX7486, and BLZ945, have been tested in clinical trials (109, 110). Especially, PLX3397, exhibiting higher affinity to CSF-1R, has demonstrated a better effect for tenosynovial giant cell tumor therapy, and the drug has been advanced into clinical trial phase III (111). In addition, several FDA-approved tyrosine kinase inhibitors, such as targeting c-KIT and VEGFR, have also been shown to have a binding activity with the CSF-1R kinase (112).

Modulation of Macrophage Phenotype

PI-3 kinase γ (PI3Kγ) has been identified as a promising target for modulating macrophage phenotype and proinflammatory cytokine expression (113) (**Figure 2**). IPI-549, a PI3Kγ inhibitor, is currently tested in Phase 1b clinical trials for several solid tumors, in combination with nivolumab. Bruton's tyrosine kinase (BTK), a downstream of PI3Kγ, has been investigated as a target for cancer treatment. In line with studies, ibrutinib, a BTK inhibitor, has been advanced in Phase III clinical trials for pancreatic adenocarcinoma treatment and in Phase II clinical trials for relapsed or refractory solid tumor therapy in combination with durvalumab. Janus kinase 2 (JAK2) and STAT3 also have been regarded as potential targets for macrophage

TABLE 1 | Clinical trials of macrophage-targeting agents.

	Drug	Company	Clinical trial number	Tumor type	Phase
CD47	Hu5F9-G4	Forty Seven	NCT02953782	Advanced solid malignancies and colorectal carcinoma + cetuximab	I
			NCT02216409	Advanced solid malignancies	I
	TTI-621	Trillium	NCT02663518	Small cell lung cancer	I
			NCT02890368	Relapsed and refractory solid tumors	I
CD40	SEA-CD40	Seattle Genetics	NCT02376699	Solid tumors + pembrolizumab	I
	APX005M (Agonist antiCD40)	Apexigen	NCT03389802	Pediatric CNS	I
	CP-870,893 (agonist antiCD40)	VLST Corporation	NCT01103635	Metastatic melanoma + tremelimumab (antiCTLA-4)	I
	R07009879 (selicrelumab, agonist antiCD40	Roche	NCT02760797	Advanced solid tumors + anti-PDL1	I
			NCT02665416	Advanced solid tumors + bevacizumab or vanucizumab	I
			NCT02588443	PDAC + gemcitabine + nab-paclitaxel	II
CSF1R	BLZ945	Novartis	NCT02829723	Advanced solid tumors single agent	I
				Advanced solid tumors + PDR001	II
	Emactuzumab	Hoffman La Roche	NCT02323191	Advanced solid tumors + atezolizumab	I
			NCT03708224	Advanced HNSCC + atezolizumab	I
			NCT03193190	PDAC + additional therapies	I
	IMC-CS4 (antiCSF1R)	Lilly	NCT01346358	Advanced solid tumors	I
			NCT02265536	Advanced breast, prostate cancer	I
			NCT03153410	PDAC + cyclophosphamide pembrolizumab, GVAX	I
CCR2	BMS-813160	Bristol Meyers Squibb	NCT02471716	Tenosynovial giant cell tumor	II
			NCT03158272	Advanced malignancy + nivolumab	I
			NCT02526017	Advanced solid tumors + nivolumab	I
	CCX872-B	ChemoCentryx	NCT03778879	PDAC + SBRT	II
	MLN1202 (antiCCR2 antibody)	Millennium	NCT01015560	Bone metastases	II
IL1Ra	Anakinra	Swedish Orphan Biovitrum	NCT0255032	7 PDAC + abraxane, gemcitabine, cisplatin	I
TLR4	GSK1795091	GlaxoSmithKline	NCT03447314	Advanced solid tumors + GSK3174998 antiOX40) or (GSK3359609 anti-ICOS) or pembrolizumab	I
Stat3	TTI-101	Tvardi Therapeutics	NCT03195699	Advanced cancers	I
PI3Kγ	IPI-549	Infinity Pharmaceuticals	NCT02637531	Advanced solid tumors + nivolumab	Ib
BTK	Ibrutinib	Pharmacyclics/AbbVie	NCT02599324	Renal cell, urothelial, gastric, colon, pancreatic adenocarcinoma	III
			NCT02436668	PDAC, gemcitabine + nab-paclitaxel	Ib/II
			NCT02403271	Relapsed or refractory solid tumors + durvalumab	III

repolarization (114). The STAT3 inhibitor TTI-101 is currently investigated in a Phase I clinical trial for advanced cancers, and the JAK2 inhibitor has been applied for the treatment of psoriasis, myelofibrosis, and rheumatoid arthritis in clinic (115).

CD40 is mainly expressed on antigen presenting cells, monocytes, and some tumor cells. CD40 ligation in macrophages induces secretion of proinflammatory cytokines and promotes macrophage polarization toward a proinflammatory macrophage. Several anti-CD40 antibodies and CD40 ligands, such as RO7009789, APX005M, are currently under test and evaluation in clinical trials for solid tumors (**Figure 2**). Interestingly, unlikely other activatory Fc receptors, the antibody Fc domain with inhibitory FcγRIIb is required for anti-40 antibody because of its agonistic immunostimulatory activity. In particular, CP-870893, a Pfizer anti-CD40 antibody of IgG2 subclass, has been shown to be more competitive in immunostimulation compared to other drugs in clinical trials (116). Moreover, TLR agonist treatment has been studied and

developed for cancer therapy because TLRs stimulation can polarize macrophages toward a proinflammatory phenotype.

Metabolic Modulation of TAMs

To support specialized cellular activities, macrophages use diverse metabolic pathways for energy and metabolite at different states (117). Metabolic changes contribute to the regulation of macrophage polarization, and TAMs display an immunosuppressive phenotype that is defined by the production of ornithine and polyamines through the arginase pathway as well as by expression of T_H2 cytokines that include IL-10 (118–120). Several studies have shown that the tumor microenvironment, featured poor nutrient and acidic environment, directly induced macrophages to adopt immunosuppressive phenotypes (121–123). For example, lactate, a byproduct of tumor cells, can promote monocytes and macrophages toward to immunosuppressive macrophage polarization in B16 melanoma and lung carcinomas mouse model (121). Moreover, the tumor microenvironment in melanomas characterized by acid has been reported to promote immunosuppressive polarization of TAMs, including upregulating arginase and VEGF expression (124). Collectively, these studies have shown that altering the metabolic pathways of TAMs to repolarize macrophages might be an effective strategy for antitumor functions.

The PI3K/Akt/mTOR myeloid signaling pathway plays a key role in regulation of TAMs metabolism by promoting L-arginine metabolism, a curial section that could promote immunosuppression. The gene and protein expression of Arginase-1 (Arg-1) in TAMs up-regulates and inhibition of PI3Kγ can suppress Arg-1 expression and activity (90). Additionally, the deletion of PI3Kγ promotes the expression of the enzyme NOS, which promotes the production of the free radical and NO to function as anti-tumor. Kaneda et al. reported that IPI-549, a PI3kγ inhibitor, inhibited lung carcinoma and breast tumors by promoting TAM-immunostimulatory response (125). Moreover, mTORC1 and mTORC2 also play a key role in the metabolic programming of macrophages by sensing nutrients, oxygen, and metabolites. Rapamycin, an mTORC1 inhibitor, has been reported to promote macrophages toward the proinflammatory phenotype with an anti-tumor effect (126) (**Figure 2**).

Adoptive Macrophages Transfer

Adoptive cell transfer is an emerging method of immunotherapy, which kills and removes cancer cells by the infusion of immune cells (127). Macrophages have the capacity to penetrate tumors (128), which may kill tumor cells where CAR-T therapy has fallen (129). Therefore, adoptive macrophage transfer (AMT) has become a hot research field for tumor detection and treatment lately. Amin Aalipour et al. used engineered macrophages as diagnostic sensors to successfully detect tumors as small as 4 mm in diameter and show better sensitivity than traditional cancer biomarkers (130). Recently, Michael Klichinsky et al. described an anti-HER2 CAR-macrophage (CAR-M) that significantly reduced metastatic tumor burden (131). A cellular IFN-γ "backpack" for macrophages was reported to promote phagocytosis and polarize macrophages toward the M1 phenotype, which further slows down the tumor growth in a murine breast cancer model (132). Overall, the adoptive transfer therapy of macrophages is still in the research stage, and there are many problems to be solved, such as the establishment of pre-clinical models to evaluate the efficacy and safety of AMT. In addition, the way to efficiently transfer genes into human macrophages is still challenging and needs further study.

DISCUSSION

Various strategies targeting TAMs have been studied for cancer therapy, and some treatments have been advanced into clinical trials. However, because of complexity of tumors, a combination therapy is usually adopted to maximize the anti-tumor effect; whether currently targeted signaling pathways therapeutically overlap or synergize *in vivo* remains to be explored. More importantly, current researches do not have a thorough understanding of these targets, and their other functions are often overlooked in cancer treatment. Besides, with multiple targets being identified and drugs being tested for the modulation of TAMs, drug delivery technologies have been advanced to further enhance the efficacy of these drugs, through the way of improving stability, selectivity, and intracellular delivery efficiency, etc. CAR-M, as an emerging strategy for cancer therapeutic, is still in research stage. Currently, overcoming the challenge that genes transfer into human macrophages and finding effective solid tumor targets are the main tasks. Perhaps CAR-M in the future is to adopt multiple macrophages having different functions rather than a single population.

TAMs represent a heterogeneous population with different functions according to different origins and contexts. Consequently, it is necessary to understand this heterogeneity and how it evolves during the progression of cancer and also following therapy in human, not mouse, models. In this context, the extensive use of single-cell RNA sequencing, multiplex immunohistochemistry, and mass cytometry will considerably increase our knowledge about TAMs, which is essential for the adoption of precision medicine and good prediction of patient responses. Admittedly, many questions remain regarding to properties and functions of macrophages in TME. However, with the deeper understanding of macrophage diversity through single-cell sequencing and other technologies, we believe that TAM-targeted treatment will be an important addition for cancer immunotherapy.

AUTHOR CONTRIBUTIONS

YL conceived the concept and wrote the manuscript. RW edited and improved the manuscript. All authors contributed to the article and approved the submitted version.

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A brief glimpse of a tangled web in a small world: Tumor microenvironment

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A tumor is a result of stepwise accumulation of genetic and epigenetic alterations. This notion has deepened the understanding of cancer biology and has introduced the era of targeted therapies. On the other hand, there have been a series of attempts of using the immune system to treat tumors, dating back to ancient history, to sporadic reports of inflamed tumors undergoing spontaneous regression. This was succeeded by modern immunotherapies and immune checkpoint inhibitors. The recent breakthrough has broadened the sight to other players within tumor tissue. Tumor microenvironment is a niche or a system orchestrating reciprocal and dynamic interaction of various types of cells including tumor cells and non-cellular components. The output of this complex communication dictates the functions of the constituent elements present within it. More complicated factors are biochemical and biophysical settings unique to TME. This mini review provides a brief guide on a range of factors to consider in the TME research.

KEYWORDS

tumor microenvironment, Immune Checkpoint Inhibitors, immune system, network, immunotherapy

Introduction

The earliest form of cancer immunotherapy using infection started around 1550 BCE (1). In the modern era, an incidental observation of tumor regression after surgical wound infection was advanced into a more controlled approach using bacterial vaccines to treat sarcoma (2). This journey was then succeeded by application of Bacillus Calmette-Guerin (BCG), various types of oncolytic viruses and Immune Checkpoint Inhibitors (ICIs) (3). Substantial efficacy and superior safety profiles with tumor-agnostic features have immediately positioned ICIs in the main treatment arm in most advanced cancers. This has turned the focus from genetic and epigenetic alterations of tumor cells to immune cells. However, ICIs are no exception in primary or secondary resistance of drugs. This has led the investigators to place a heavier emphasis on other players and the surroundings of tumor cells. Long before the era of ICIs, histologic description of tumor tissues had already provided some insights in tumor surroundings. For instance, melanomas are characterized by fibrosis, melanophages (a type of macrophage), new blood vessels and infiltration of lymphocytes in and around the nests of dying tumor cells (4). Exuberant lymphoid reaction was the hallmark of colorectal cancer (CRC)

with high microsatellite instability (MSI-high) (5). The study of CRC with MSI-high, either in Lynch syndrome or sporadic cases has indicated the hypermutator phenotype and MSI is still the most relevant predictive biomarker of ICIs currently (6). It is quite logical to speculate that the tumor mutational burden (TMB) follows MSI. However, the TMB is not a one-marker-fit-for-all (7). An example that displays this fact to the furthest extent was from an animal study where fibroblasts having inactivated TGF-β type II receptor induced precancerous lesions and carcinomas from an otherwise normal epithelium (8). With all these factors to consider, the center of attention always has been revolving around tumor cells. Environment is defined as the circumstances, objects, or conditions by which one is surrounded (9). The circumstances surrounding tumor cells theoretically ranges from ions, humoral factors and matrikines to various types of cells and tissues and even to host itself. Like the stem cell niche, tumor cells reside in their own niche or TME, and also have a reciprocal non-static spatiotemporal coordination with each other to regulate functions and differentiation of tumor cells and non-tumor cells, under the influence of specific physicochemical conditions (10–16). The current mini-review aims to cover as many attributes in this complex system, ranging from ions to cell and extracellular matrix (ECM), to physico-chemical properties of TME in an attempt to assist future studies.

Definition of tumor microenvironment

The National Cancer Institute defines the TME as "The normal cells, molecules, and blood vessels that surround and feed a tumor cell. A tumor can change its microenvironment, and the microenvironment can affect how a tumor grows and spreads." (17). This definition may appear simple at first, but encompasses the idea of reciprocal interaction and regulation of a tumor cell behavior. The most common ones are based on a structural view (18). Regularly emphasized is the dynamic nature of the cell population, such as the resident players and non-resident cellular components (19, 20). However, these definitions do not specifically identify other elements, such as tumor interstitial fluid, and physicochemical properties. To better depict a dynamic symbiotic system, "Seed and Soil," an analogy of the stem cell niche, was introduced (14). "The TME comprises of a diverse cellular and acellular milieu, in which cancer stem cells (CSCs) develop and thrive, and various stromal and immune cells are recruited to form and maintain this self-sustained environment" (21). In that regard, the definition of "seed and soil" is comprehensive enough to cover all components in TME.

Cellular component

Histologic observation of tumors shows cancer cells intricately mixed with various inflammatory cells, fibroblasts, fibrotic stroma and blood vessels. One of the most studied examples is colorectal cancer (CRC) with high microsatellite instability (MSI). The tumor cells exhibit morphologic alterations such as mucinous change, signet ring cell feature and medullary histology (22). The presence of other cellular players is observed such as high number of tumor infiltrating lymphocytes (TILs) and peritumoral lymphoid follicles reminiscent of the inflammatory pattern of Crohn's disease (5). There are many cases providing morphologic evidence of multiple players in tumor tissues (6). On the other hand, data-driven approach was able to characterize complex alterations from genes to transcription, and has brought in molecular classifications agnostic about morphology (23). However, immune cells are still the major focus in the era of ICIs, and the classification systems based on proportion of these cells have been proposed (24–26). Two tier system such as a hot tumor vs. a cold tumor is widely accepted one. A three tier system, such as immune infiltrated/inflamed, immune excluded, and immune silent/desert is also a commonly used method of classification (25).

Back to the role of each population in TME, cells are generally classified as tumor-promoting vs. tumor-suppressing (27) (Table 1). In this scheme, players are not simply dysfunctional in TME, but also actively suppress other immune cells and promote tumor cells, ranging from growth, invasion, metastasis to immune evasion (27). Members found to promote tumors are regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), M2 tumor-associated macrophages (TAMs), resident or derived from bone marrow/spleen, N2 tumor-associated neutrophils (TANs), cancer-associated fibroblasts (CAFs), tolerogenic dendritic cells (DCs) and more details are summarized in Table 1 (76–78). Once cells migrate into the TME, they are polarized or differentiated under the local condition, and in return, these cells accelerate the immune-suppressive and tumor-promoting environment (37). Hence, the state is not static but can be dynamic depending on the context or milieu of cytokines and signaling molecules. For example, M1 macrophage can turn into the M2 type and vice versa, while an intermediate form between M1 and M2 has been discovered (37). Proportion-wise, cancer-associated fibroblasts (CAFs) are the most abundant component in the tumor tissue (13). CAFs have a critical position in all steps, from tumor initiation to metastasis, and even being related to therapeutic resistance (8, 79). CAFs are derived from resident fibroblasts and other cells such as smooth muscle cells, vascular pericytes and bone marrow-derived mesenchymal cells, adipocytes and this process is caused by various factors [stromal cell-derived factor 1 (SDF1), platelet-derived growth factor (PDGF), transforming

growth factor-β (TGF-β), fibroblast growth factor 2 (FGF2)] produced by tumor cells and immune cells (18, 80–83). CAFs then reciprocally promote tumor progression by production of growth factors (PDGF, TGF-β, epidermal growth factor (EGF), bone morphogenetic proteins (BMP) and C-X-C motif chemokine 12 (CXCL12), CXCL13) and these cells also stimulate angiogenesis by secreting vascular endothelial growth factor (VEGF), CXCL12 and FGF2 (72–75). Recently, focus was turned to rare cell populations in TME such as mast cells, basophils, eosinophils (84–86). The next-generation pathology, together with the single-cell analysis and systems pathology, will provide new insightful hints for developing effective therapeutic protocols targeting the TME (87, 88).

Extracellular matrix

Tumor stroma shows fibrosis or even desmoplasia in certain types of tumors, such as biliary cancer and gray-colored myxoid change, likely due to the ECM alteration (89, 90). ECM undergoes a remodeling process in physiologic and pathologic conditions, and it is an intricate phenomenon involving more than 700 proteins (91, 92). The characteristics of the remodeled ECM eventually affect the fate of cells (91, 92). The major alterations of tumor ECM are degradation, stiffening and physical remodeling (18, 93). In TME, acidic condition, excessive amount of proteases [i.e., matrix metalloproteases (MMPs), disintegrin and metalloproteinases (ADAMs), disintegrin and metalloproteinases with thrombospondin motifs (ADAMTS)] and production of reactive oxygen species (ROS) from tumor cells, CAF, TAN and TAM cause degradation of ECM (18). During this process, Extracellular Matrix-Derived Fragments are produced. These undertake active biological functions as matrikines leading to various effects such as acceleration of matrix production, promoting or suppressing tumor progression and angiogenesis (93, 94). Neoplastic tumors are stiffer than adjacent normal tissues and this is due to an excessive laydown of ECM and altered post-translational modification (PTM) (18). At first, CAFs secrete ECM in excess, including collagens, glycoproteins, proteoglycans, and polysaccharides (18). Then, the hypoxic condition enhances the cross-linking *via* production of lysyl oxidase (LOX) and transglutaminase from CAGs (95, 96). These modified rigid collagen fibrils are known to facilitate tumor cell migration and progression (97–100). In addition to the structural changes, PTM of ECM directly controls the tumor cell behavior by modulating the function of various growth factors embedded in the matrix (46, 101–103). For example, heparan sulfate proteoglycans (HSPGs) have different binding and releasing capacity of growth factors, depending on the sulphation pattern. This pattern is modified by the enzyme called endosulphatase (Sulf). In tumor tissue, the isotypes of Sulf are differentially expressed that the sulphation pattern made by Sulf1 inhibits the signaling pathways promoting tumors, while contrastingly, the other formed by Sulf2 enhances them (101, 102). Altered glycosylation patterns are reported in tumor tissues, and are currently under research (22, 104, 105). Lastly, mechanical force causes physical remodeling of the ECM, and makes fibers aligned to make routes for tumor cell migration (93). In TME, the ECM is continuously remodeled in terms of the amount, structure and chemical properties and this process shapes the interplay of the components modulating the fate of tumor cells in their progression (93). High-throughput proteomics approach is expected to acquire more insight from this process (91, 106).

Biochemical component

One of the approaches to understand the biochemical property of TME is to look into the fluid of tumor or tumor interstitial fluid (TIF) (107, 108). TIF is characterized by high P_{CO2}, low P_{O2} and low pH, and these parameters are linked with each other (11, 12). Hypoxia in tumor tissues is the major contributor to acidic environment. Rapid proliferation of tumor cells and insufficient oxygen supply cause hypoxia. This condition reprograms tumor cells favoring aerobic glycolysis with production of lactate (109). Major regulators in this process are hypoxia-inducible factor (HIF)-1α, c-Myc, and p53 (110–114). Hypoxia induces inhibition of prolyl-hydroxylases and this stabilizes the HIFs. HIF-1α switches metabolisms in tumor by upregulating the transcription of enzymes of glycolysis, such as hexokinase 1/2 (HK I/II) and pyruvate kinase isoenzyme M2 (PKM2), glucose transporters (Glut) such as Glut-1 and 3, alongside other genes inhibiting oxidative phosphorylation (115–118). As the dimer form of PKM2 prevails in the tumor, glucose metabolism is shifted to lactate production (118, 119). Abnormal vessels are unable to clear hydrogen ions effectively and hydration of CO_2 by carbonic anhydrase IX in hypoxic areas further increase acidity (120). This altered biochemical environment reconditions the cells under its influence forming a selective pressure which favors cancer cells over normal cells (120–128). This situation promotes tumorigenesis, tumor progression and immune evasion and is related with a poor clinical prognosis and resistance to therapy. Recently reported findings suggest that the lactic acid not only intensifies acidity but also directly impacts cellular signaling pathways preferentially polarizing TAM to M2 type (129).

What about the ions in TME? Previous studies have shown that the concentration of ions in TIF is similar to that in plasma (130). Recently, this notion has been revisited. More sophisticated analysis revealed that the potassium concentration is higher in TIF, while other ions such as sodium, chloride and magnesium remain within normal range (131). Higher potassium level was found to suppress activation and effector function of T cells (131). A starvation response is induced by local hyperkalemia, and this in turn reduces nutrient uptake, resulting in the imbalance of Acetyl Co-A (AcCoA) level in

TABLE 1 Tumor-suppressing and tumor-promoting roles of diverse cells in tumor microenvironment.

	Tumor-suppressing	Tumor-promoting	References
T lymphocyte	• Th1→ ↑CTL, M1, NK • *via* IFN- γ, IL-2 • CTL??direct killing • CTL→ ↓angiogenesis *via* IFN-γ • Th9 → ↑CTL *via* IL-9 and ↑NK *via* IL-21 • Th17 recruit CTL, PMN, DC *via* CCL2, CCL7, CCL20, CXCL9, CXCL10	• Treg suppress CTL • Treg→ ↓costimulatory molecules on DC • Treg modulate homeostasis of NK *via* IL-2 • Treg→ ↑tumor growth *via* GFs • Treg→ ↑angiogenesis • Th2→ ↓Th1 and ↑M2 • Th17→ ↑angiogenesis	(25, 28–36)
B lymphocyte	• B cell as APC to T cell • B cell→antibody & • IFN-γ → ↑CTL	• Breg → ↓CTL, macrophage, TAN *via* IL-10, TGF-β	(25)
Macrophage	• M1 cells as APC to Th1, NK • M1 produces inflammatory cytokine, ROS, RNS and ADCC→killing tumor cells	• M2 produce IL-10→induce PD-L1 on monocyte → ↑infiltration of Treg and ↓CTL • M2→ ↑PD-1→ ↓macrophage phagocytosis via tumor PD-L1 • M2→ ↑PD-L2→immune escape and tumor promotion *via* PD-1 • M2→ ↑tumor growth *via* EGF, FGF, PDGF, IL-4 • M2→ ↑angiogenesis *via* VEFG, IL-8, FGF, MMP-9	(25, 37–43)
Dendritic cell	• DC as APC and stimulate CTL *via* ICAM-1, CD86, CD40, CD80 • DC recruit naïve T cell *via* CCL17, CCL19, CCL22, IL-32 • DC stimulate Th1, CTL, NK *via* IL-12, IL-15 • DC→ ↑Ag expression by tumor *via* TNF-α, IL-6	• IL-10, TGF-β in TME→ ↑PD-1 on DC →immune-suppressive DC • DC→ ↑Treg but ↓CTL, Th, macrophage, PMNs *via* IL-10, PDL1, IDO, Arginase-1	(44–50)
NKT cell	• NKT as APC *via* CD1d • NKT activates NK, DC, CTL *via* IL-12, CD40	• NKT II→ ↑M2, MDSC and ↓CTL via IL-4, IL-13	(51, 52)
NK cell	• NK kill tumor cells *via* ADCC, Fas-FasL, perforin-granzyme and cytokines (TNF, IFN-γ, GM-CSF, IL-6, and CCL5) • NK stimulate DCs *via* FLT3L	• TGF-β in TME→ ↑dysfunctional NK • NK→ ↑autonomous inhibitory checkpoint molecules (PD-1, TIGIT, CD96, TIM-3, LAG-3, CTLA-4, KIR2DL-1/2/3 and NKG2A)	(53–56)
Neutrophil	• N1 TANs kill tumor cells *via* ADCC and pro-inflammatory factors (IFN-γ, MMP-8) & ROS • N1 TAN recruit DC *via* CCL19, CCL20 and T cells *via* CXCL9, CXCL10 and stimulate CTL, NK *via* TNF-α	• Tumor cells produce GM-CSF→ PD-L1 expression in TAN *via* JAK/STAT pathway→PD-L1+ TAN inhibit T-cell immunity (N2 TAN) • TAN suppress immune cells *via* Arginase-1, i-NOS • TAN recruit Treg *via* CCL17 • TAN→ ↑angiogenesis *via* MMP-9, VEGF	(25, 57–62)
Myeloid-Derived Suppressor Cell (MDSC)		• MDSC→ ↓immune cells *via* TGF-β, ROS, NO, Arginase-1, PGE-2 through PD-L1/PD-1 • MDSC→ ↓metabolites in TME • MDSC block lymphocyte homing *via* ↓e-selectin • MDSC→ ↑angiogenesis *via* VEGF	(56, 63–65)
Mast cell	• Mast cells regulate immune cells (T, B, APC) *via* cytokines	• Mast cells secrete angiogenic (VEGF-A, CXCL8, and MMP-9) and lymphangiogenic factors (VEGF-C and VEGF-F) • Mast cells secrete IL10→ ↑Treg in draining lymph nodes • Tumor cells secrete TNF-α → ↑PD-L1 in mast cells *via* NF-κB pathway	(66–69)

(Continued)

TABLE 1 Continued

Tumor-suppressing	Tumor-promoting	References
Endothelial cell	• Tumor-derived HIF→ ↑endothelial cell sprouting *via* PDGF, EGF, VEGF, FGF, Ang2, IL-8 → ↑endothelial cell migration→ support nutrient and metabolite to tumor cells • ↓ICAM-1, VCAM on endotheleial cells → ↓immune cell infiltration • ↑TGF-β, BMP in TME convert endothelial cells to CAF	(25, 70, 71)
Cancer Associated Fibroblast (CAF)	• Tumor cells secrete FGF, PDGF, SDF→ ↑CAF→ ↑PDGF, TGF-β → ↑tumor growth • CAF→immunosuppression *via* TGF-β • CAF→ ↑angiogenesis *via* VEGF, CXCL12 • CAF→ ↑MDSC recruitment *via* CCL7 • CAF→glucosaminoglycans and MMP-2→ ↑tumor migration	(72–75)

ADCC, antibody-dependent cellular cytotoxicity; Ag, antigen; Ang, angiopoietin; APC, antigen presenting cell; BMP, bone morphogenetic protein; Breg, B-regulatory lymphocyte; CAF, cancer-associated fibroblast; CAM, cell adhesion molecule; CAR, chimeric antigen receptor; CCL, CXCL, chemokines; CD, Cluster of differentiation; CTL, cytotoxic lymphocyte; DC, dendritic cell; ECM, extracellular matrix; EGF, epidermal growth factor; FasL, Fas-ligand; FGF, fibroblast growth factor; GF, growth factors; HIF-1, hypoxia-inducible factor-1; ICOS, inducible T-cell costimulator; IDO, Indoleamine 2, 3-dioxygenase; IL, interleukin; i-NOS, inducible nitric oxide synthase; M1, M1 macrophage; M2, M2 macrophage; MAB, monoclonal antibody; MDSC, myeloid-derived suppressor cell; MMP, matrix metalloproteinase; NK cell, natural killer cell; NKT cell, natural killer T cell; NKT II; type II NKT cells; NO, nitric oxide; PDL-1, programmed death-ligand-1; PGE2, prostaglandin E2; PMN, Polymorphonuclear neutrophil; RNS, reactive nitrogen species; ROS, reactive oxygen species; TAN, Tumor associated neutrophil; N2 TAN, N2 type tumor associated neutrophil; TGF-β, transforming growth factor-β; Th, T helper lymphocyte; Th1, type 1 T helper lymphocyte; Th17, T helper lymphocyte 17; Th2, type 1 T helper lymphocyte; Th9, T helper lymphocyte 9; TLR, Toll-like receptors; TME, tumor microenvironment; TNF-α, tumor necrosis factor-α; TRAIL, TNF-related apoptosis-inducing ligand; Treg, T regulatory lymphocyte; VEGF, vascular endothelial growth factor; →, influence; ↑, increase; ↓, decrease.

subcellular compartments (132). In this setting, mitochondrial AcCoA is relatively higher than nucleocytosolic AcCoA, and this disproportionate state causes reduction of histone acetylation promoting stemness of T cells, eventually impeding the activation of effector genes (132).

ROS are known as the byproduct of hypoxic environment produced by tumor cells in TME, and the up-to-date interpretation is that ROS are not only radicals having damaging effect, but also have diverse biologic effects such as stabilization of HIFs to promote angiogenesis, activation of cell proliferation, as well as survival pathways, metabolic reprogramming, differentiation of CAFs and deregulation of immune cells (133). Reactive Nitrogen Species (RNS) are also rich in TME, due to an increase in arginine metabolism within tumor cells and tumor-infiltrating myeloid cells (134). RNS causes nitration of chemokine (C-C motif) ligand 2 (CCL2), and this modification suppresses infiltration and effector function of lymphocytes (134, 135).

Altered metabolic condition is a common survival strategy by tumor cells (136–139). Clinically, cachexia represents increased catabolic status to feed cancer cells (140, 141). Abnormally increased anabolism is also seen in cancer patients. Non-Islet Cell Tumor Hypoglycemia (NICTH) is a paraneoplastic syndrome where non-endocrine tumors cause hypoglycemia, while promoting anabolism of tumor cells by aberrantly producing insulin-like growth factor II (IGF-II), insulin receptor antibodies and various cytokines (tumor necrosis factor-α, interleukin-1 and−6) (142–145). Metabolic condition comes into play at microscopic level as well. As immune cells enter into tumor tissue, those cells face hypoglycemia and a scant amount of essential amino acids including glutamine and lipids. This condition hinders all steps of immune cell functions such as infiltration, proliferation and effector because these tasks have great demand for energy, nutrition and metabolic reprogramming (136–139). This competitive condition places the immune system in an anergy and exhaustion state (146, 147).

Extracellular vesicles (EVs) are rich in TIF (148). EVs such as exosomes, microvesicles, and apoptotic bodies carry active signaling and regulatory molecules like mRMA, miRNA, signaling proteins, microRNAs (miRNAs), long non-coding RNAs (LncRNAs), and circular RNAs (circRNAs) (149–151). All types of cells including cancer stem cells are known to secrete them (152, 153). Isolated EVs enriched in TME have the capability of promoting angiogenesis, modulating immune cells, enhancing tumor migration and epithelial-mesenchymal transition (EMT), metastasis and increasing drug resistance (148, 154, 155). However, EVs in TIF are not always tumor-promoting. Some EVs were found to exhibit anti-tumor effects (156, 157). This concept can be applied to patient treatment *via* an EV engineering. EVs derived from proven fighters such as active TILs and chimeric antigen receptor (CAR)-T cells may potentially recondition dysfunctional or anergic immune cells in tumor tissue (158–162). There

are other humoral factors not mentioned here. Proteomic approach is expected to find unique signatures of TIF and further develop our understanding of the complex nature of TME.

Biophysical component

Highly cellular tumors like lymphoma, seminoma, and Ewing sarcoma frequently present characteristic bulging cut surfaces. These features are related to an increased pressure inside tumor tissue (163). High tissue pressure is due to an increase in the proliferation and migration of tumor cells, alteration of ECM and increased interstitial fluid pressure (IFP) (163). The increased IFP is caused by the abnormal vessels having higher permeability, lack of pericytes, vascular compression by tumor growth and abundant ECM (164–167). IFP is elevated by 10–40 mmHg in tumor tissues (168, 169). Increased IFP generates an outward tissue flow and cell velocity flow, which hinders an inward penetration of cells, antibodies and drugs (164, 165, 170, 171). Interestingly, high pressure itself has been shown to enhance tumor proliferation and is often related to a poor clinical outcome (172–174). Vascular endothelial growth factor inhibitors, pegylated human recombinant hyaluronidase-α, collagenase and angiotensin inhibitors are suggested for potential drugs which can reduce IFP and promote the delivery of various molecules into tumor tissues (165). Migration and homing of immune cells is an entrenched process involving various chemokines, gradients and APC interaction (175–179). However, movement of immune cells under high IFP and altered ECM are not well studied, requiring further research.

Conclusion

The main stream in cancer research has been about decoding genetic and epigenetic alterations in tumor cells. This scheme has been powerful to understand the nature of cancer diseases, and has led to the discovery of means to restore it. Meanwhile, a distinct course of ideas appeared long ago from the ancient time to the modern concept of immunotherapies and ICIs. This different perspective has widened sight to other attributes within tumor tissue. TME is a system consisting of a reciprocal communication network among components under unique physicochemical conditions. This process influences all components and the output influences TME in an iterative way. Various attempts such as data-driven approaches will rapidly improve understanding of surroundings of tumor cells and lead to several discoveries of predictive biomarkers and an eventual control of resistance. Another aspect not discussed in this mini review is about the host factors such as host genetic makeup. Certain single nucleotide polymorphisms (SNPs) in genes of the immune system were found to affect cancer susceptibility of an individual and these may also influence response to ICIs (180–182). There are case reports on renal cell carcinomas undergoing regression after transfusion of plasma from another patient of the same family (183, 184). This may indicate the presence of an inherited resistance to cancer. Even though these are still speculative and can be explained by other mechanisms, this macro-environment also needs to be considered in the dimension of future studies.

Author contributions

BK drafted the initial version of the manuscript. IT reviewed it and added comments. Both 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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Checkpoint molecules on infiltrating immune cells in colorectal tumor microenvironment

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Colorectal cancer (CRC) is one of the most prevalent cancer types worldwide, with a high mortality rate due to metastasis. The tumor microenvironment (TME) contains multiple interactions between the tumor and the host, thus determining CRC initiation and progression. Various immune cells exist within the TME, such as tumor-infiltrating lymphocytes (TILs), tumor-associated macrophages (TAMs), and tumor-associated neutrophils (TANs). The immunotherapy approach provides novel opportunities to treat solid tumors, especially toward immune checkpoints. Despite the advances in the immunotherapy of CRC, there are still obstacles to successful treatment. In this review, we highlighted the role of these immune cells in CRC, with a particular emphasis on immune checkpoint molecules involved in CRC pathogenesis.

KEYWORDS

tumor microenvironment, macrophages, neutrophils, lymphocytes, colorectal cancer, immune checkpoint

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in 2020, affecting 10% of the global population (1). The increasing mortality rate in patients with advanced CRC is of concern and reflects the limited range of treatment options. This could be attributed to the diagnosis of CRC at a late stage when the tumor has already metastasized. Furthermore, in most CRC patients, surgical resections are not the ultimate cure as there is a high possibility of recurrence of the disease in a more aggressive form; thus, using additional therapeutic modalities is mandatory (2). CRC is not a single disease and every patient has a unique illness due to distinctive genetic/epigenetic causes (3). The molecular classification of CRC is changing over

time. Global genomic status [microsatellite instability (MSI) status and chromosomal instability (CIN) status] and epigenomic status [CpG island methylator phenotype (CIMP) status] contribute significantly to the clinical, pathological and biological properties of CRC. CIN tumors are mostly microsatellite stable (MSS) and have been associated with an aggressive clinical picture (4–6). Such tumors usually have large genomic abnormalities that lead to higher average DNA copy number compared with MSI tumors (7). MSI is typically diagnosed by the variable lengths of DNA microsatellites (mononucleotide and dinucleotide repeats) (8), which are caused by epigenetic silencing (9, 10) or mutation of DNA mismatch repair (MMR) genes, leading to accumulated mutations at 10–100 times the normal rate promoting cancer progression (8). CRC tumourigenesis has been reported to be triggered by gene mutations associated with multiple signaling pathways such as KRAS, BRAF, and PIK3CA (11). Several studies have confirmed that association between BRAF and KRAS mutations, in addition to BRAF mutations being more linked to MSI status (3, 12–14).

The tumor microenvironment (TME) is a dynamic and ever-changing phenomenon that has a pivotal role in determining CRC initiation and progression. The TME is a unique environment that develops during tumor progression due to its interactions with the host. It comprises several components, such as immune cells, stromal cells, myofibroblasts, vessels, and extracellular matrix (ECM), which differ according to tumor type (15). The tumor growth occurs in a multi-step process, where the neoplastic cells recruit stromal and immune cells to establish the TME. Then, within the tumor site, the deranged production of inflammatory cytokines and growth factors by cellular components in the TME leads to further recruitment of various immune cells (16). Finally, angiogenesis and ECM degradation occur during the tumor growth, eventually leading to invasion and metastasis. Several multiplexed technologies, such as single-cell RNA sequencing and mass cytometry, explore the functional diversities of tumor-infiltrating immune cells and the recent progress in the cancer immunotherapy (17). Furthermore, multiplex immunohistochemistry/immunofluorescence (mIHC/IF) provides throughput staining and standardized quantitative analysis that could be a proficient approach to detect specific proteins or molecular aberrations as well as explore the immune evasion (18). Thus, it could have a great potential to discover novel prognostic and predictive biomarkers in cancer immunotherapy and contribute in translational research and clinical practice (19). During multiplex IHC, more than three markers can be analyzed simultaneously in a single cut of formalin fixed parrafin embedded tissue (FFPE) with good cell discrimination and spatial information due to recent developments in multiple immunolabeling and multispectral imaging (20–23). A valuable method for assessing the expression of numerous markers simultaneously in a single tissue section was a multiplex IHC with tyramide signal amplification (TSA) (20–24). This is a more sensitive method than standard chromogenic IHC and may be able to identify proteins that are expressed at lower quantities (20, 25). In this review, we aim to discuss the various cellular immune components, focusing on the impact of immune checkpoint molecules on the CRC TME.

Immune checkpoint molecules

The therapeutic use of antibodies that disrupt immune checkpoints was a critical turning point in the cancer immunotherapy (26). Blocking inhibitory coreceptors and pathways, which constrain immune cell activities in normal physiologic contexts, might "loosen the brakes" on immunological response, thus eliminating tumors. Immune cell activities are known to be exploited in malignancies (27). In addition, multiple immune checkpoint molecules have been identified in CRC pathogenesis and on various cell types, including lymphocytes, macrophages and neutrophils (28).

The co-inhibitory receptor programmed death-1 (PD-1), also known as CD279, is expressed inducibly on $CD4^+$ T cells, $CD8^+$ T cells, B cells, natural killer T cells, and macrophages (29). PD-L1 (B7-H1) and PD-L2 (B7-DC) are two known PD-1 ligands. PD-L1 is constitutively expressed on various immune and non-immune cells. However, PD-L2 expression can be induced in response to microenvironmental stimuli (30). The upregulation of PD-1 on tumor-infiltrating lymphocytes (TILs) and the increased expression of its ligands on tumor cells have been linked to tumor immune evasion, resulting in the suppression of tumor-specific $CD8^+$ T cells. This receptor upregulation has also been linked to T cell exhaustion in malignant tumors, defined as a reduction in the proliferation and cytokine production (31). Thus, blocking PD-1 and PD-L1 using monoclonal antibodies (mAbs) might be effective in stage IV solid tumors by overcoming this immune suppression (32, 33).

A well-known immune checkpoint molecule is cytotoxic T lymphocyte antigen-4 (CTLA-4), expressed on T lymphocytes' surfaces. CTLA-4 binds to B7-1 (CD80) and B7-2 (CD86) costimulatory receptors present on antigen-presenting cells (APCs), leading to inhibition of T cell activity by competitive blocking of CD28 (29). Therefore, CTLA-4 has been a hot target for mAbs cancer immunotherapy such as Ipilimumab (28). A remarkable target for immune checkpoint blockade (ICB) is lymphocyte activation gene-3 (LAG-3), a surface molecule of the immunoglobulin superfamily. LAG-3 interacts with MHC class II markers, thus leading to negative regulation of T cells, natural killer (NK) cells, B cells, and plasmacytoid dendritic cells (DCs) (34, 35). T cell immunoglobulin and mucin-containing protein-3 (TIM-3) is another immune checkpoint marker expressed on T helper 1 (Th1) and $CD8^+$ cytotoxic T cells (CTLs). TIM-3 plays a critical role in inhibiting Th1 responses by causing cell

death and is also known as hepatitis A virus cellular receptor 2 (HAVCR2) (36). Hence, blocking TIM-3 boosted the anti-tumor activity, with a greater efficiency upon combinatorial effect with PD-1 blockade (36). On the other hand, blockage of the inducible T-cell co-stimulator (ICOS), belonging to the B7-CD28 immunoglobulin superfamily, gained promising results in the treatment of different malignancies. Its expression is linked to a better prognosis in CRC patients, as the percentage of $ICOS^+$ $CD4^+$ cells operating as Th1 cells in either primary tumor tissue or peripheral blood could be a clinical predictive marker for a favorable prognosis (37).

CD40, a member of the tumor necrosis factor (TNF) family, was characterized on immune cells such as DCs, B cells and macrophages, as well as non-immune cells. The ligand of CD40 (CD40L) is expressed by activated B and T cells as well as platelets (38). CD40/CD40L interactions regulate T cell activity, cytokine production and antigen presentation (38, 39). In some cases, this interaction could inhibit tumor growth (40). On the other hand, tumors could utilize the CD40/CD40L to manipulate both T-cell and antigen-presenting compartments, thus contributing to the establishment of the immunosuppressive TME (38, 41). For instance, this immunosuppression could be achieved by inducing their proliferative capacity, growth, and survival (42).

Sialic acid-binding immunoglobulin-type lectins (Siglecs) are expressed on most white blood cells of the immune system, as well as TILs, DCs, and macrophages. Hypersialylation of neoplastic cells was identified as a hallmark of poor clinical outcomes and contributes to tumor escape from immune surveillance (43). Therefore, they are considered potential immune checkpoint targets for anticancer therapy (44, 45). Another promising target for cancer immunotherapy is the T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT). Its expression was known to be upregulated by various immune cells such as activated T cells, regulatory T (Treg) cells and NK cells. In addition, it can bind to two known ligands, CD155 and CD112, expressed by tumor and antigen-presenting cells in the TME (46).

Therapies targeting immune checkpoint molecules in colorectal cancer

Several immunotherapeutic strategies are under clinical trials, especially in metastatic CRC; however, the results in MSS-CRC are generally modest. The ongoing studies investigate the outcome and potential biomarkers of metastatic CRC using various immunotherapy-based modalities, including immune checkpoint blockers (ICB) such as PD-1 blockers (e.g., nivolumab, pembrolizumab, atezolizumab, avelumab, durvalumab) and CTLA-4 blockers (e.g., ipilimumab, tremelimumab). This is besides the use of other approaches such as cancer vaccines (autologous, peptide, viral vector, and dendritic cell-based) that aim to stimulate an immune response against tumor cells, as well as adoptive cell transfer using chimeric antigen receptor T-cell therapy to kill the tumor cells directly, and oncolytic virus therapy (e.g., herpes simplex virus and NV 1020) where the viruses selectively replicate in cancer cells to destroy them with no harm to normal cells. Also, among immunotherapies under clinical trials are indoleamine 2,3-dioxygenase 1 (IDO-1) inhibitors, OX40 antagonists (e.g., epacadostat, indoximod) that enhance the immune response, and biphasic antibody targeting carcinoembryonic antigen (e.g., RO6958688) on T cells (47, 48).

Multiple clinical trials in this research area are at different phases, and some of which have been completed and the results are expected to be published soon. To mention a few examples, a phase II clinical trial investigated a combination of pembrolizumab and azacytidine in metastatic CRC refractory to chemotherapy. The findings demonstrated the safety and tolerability of this regimen, however, the clinical effect was modest in the investigated cohort, likely due to DNA methylation and immunomodulation of the tumor as an effect of azacitidine therapy (NCT02260440) (49). Another remarkable study was IMblaze370, which did not meet its primary endpoint of improved overall survival with atezolizumab plus cobimetinib or monotherapy using atezolizumab vs. regorafenib in previously treated metastatic CRC (NCT02788279). The study findings highlighted the challenge of using immunotherapy in tumors with low baseline levels of immune inflammation, such as that observed in the MSS metastatic CRC (50). Results from ongoing comparative clinical trials, such as Morpheus-CRC, are likely to thoroughly evaluate the role of immunotherapy in CRC. Morpheus-CRC is an ongoing study to evaluate the efficacy and safety of multiple immunotherapy combinations in metastatic CRC (NCT03555149) (48).

There are several challenging factors in using immunotherapeutic agents in CRC. In contrast to melanoma, which represents a successful example of immunotherapy, patients with metastatic CRC responded modestly to immunotherapy treatment, with many trials with high failure rates. Several mechanisms may explain the discrepancy in immunotherapy outcomes in different types of cancer. The tumor mutational burden (TMB) has been early identified as a potential predictor for effective response to immunotherapy. For example, MSI in CRC, where there is deficient DNA repair, gives rise to high TMB. In addition, appropriate immune response in the intestine could be preserved by ameliorating the host immune system that must tolerate commensal bacteria while maintaining the ability to face infections, otherwise, severe chronic inflammatory reactions might occur (51). Another important aspect of the poor outcome of CRC to immunotherapy is the fact that most tumors are associated with activated WNT/β-catenin signaling which can promote dendritic cell and T-cell exhaustion (52). This is

similar to metastatic melanoma, where the activation of the WNT/β-catenin signaling pathway resulted in T-cell exclusion and resistance to anti-PD-L1/anti-CTLA-4 monoclonal antibody immunotherapy (53). Similarly, in a mouse model of hepatocellular carcinoma, the β-catenin pathway enhanced immune escape and suppressed the recruitment of DCs, and consequently led to impaired T-cell activity (54). Apart from the MSI status of the tumor, at the moment, no predictive biomarkers of immunotherapy response in CRC are available.

Immune components of the colon cancer microenvironment

The cellular landscape of the TME includes various immune cells, namely, TILs such as T, B, and NK cells, as well as tumor-associated macrophages (TAMs) and tumor-associated neutrophils (TANs). Various immune checkpoint molecules are expressed on these immune cells, thus modulating the colon cancer microenvironment and regulating the pathogenesis and response to therapy (**Figure 1**). The anti-tumor and pro-tumor roles of these immune cells on the TME have been previously discussed in CRC context [reviewed in (55)].

Tumor-infiltrating lymphocytes

TILs mainly include $CD8^+$ T cytotoxic and $CD4^+$ T helper lymphocytes, in addition to B and NK cells. They are usually considered the host protecting element against tumor formation, as they induce the recruitment, maturation, and stimulation of immune cells that repress tumor growth (56).

T cells

In conventional terms, TILs represent the heterogeneous population of αβ T cells, both $CD4^+$ and $CD8^+$ subsets, present within the TME (57). $CD8^+$ T cells (CTLs) recognize tumor-associated antigens (TAAs) along with proteins of HLA class I. These cells become differentiated into killer cells, release perforins, and express the apoptotic inducer FasL after expansion. Perforins disrupt the cell membrane, aiding the entry of granzymes inside the cells, causing cleavage of caspases' precursors, thus directing the neoplastic cells toward apoptosis. Additionally, $CD4^+$ T helper cells proved to have an essential role in the anti-tumor immunity by responding to antigens presented by antigen-presenting cells (APCs) such as macrophages (58).

Increased TILs is a favorable prognostic factor in many malignancies, including CRC (59). In addition, the quantification of lymphocyte infiltration has prognostic significance, suggesting that lymphocyte infiltration is not passive but may actively modulate tumor growth (60). This was supported by a large multicenter study spanning more than 10 years, which demonstrated levels of lymphocyte infiltration into primary tumors to be a strong independent predictor of relapse and overall survival (61). Using expression profiling of CRC, they further defined the relevance of specific immune signatures, demonstrating that Th1 type interferon-γ (IFN-γ) dominant immune profiles signified an improved prognosis. In contrast, Th17 type IL-17 dominant immune profiles signified a poor prognosis (61).

A recent study of most tumor-infiltrating immune cell subtypes revealed that $CD8^+$ T cells had the most significant impact on patients' survival (62). $CD8^+$ CTLs mediate tumor rejection by recognizing TAAs and directly killing transformed cells. Effector $CD8^+$ T cells in the TME generate IL-2, IL-12, and IFN-γ, which enhance the cytotoxic potential of $CD8^+$ CTLs, leading to a targeted tumor cell killing (63, 64). On the other hand, $CD4^+$ helper T cells present in the TME are involved in activating CTLs against tumor cells (65). Exhaustion of CTLs could be caused by long-term interaction between CTLs and antigens, leading to loss of their efficiency and function.

Similarly, tumor cells suppress the immune response by inducing the exhaustion of CTLs in the TME through the expression of inhibitory immune checkpoint receptors such as PD-1, CTLA-4, and LAG-3 (66, 67). In CRC pathogenesis, PD-1 was shown to be upregulated on $CD8^+$ T cells in the TME, and its ligand was associated with cytokines and perforin impairment (30). Furthermore, a study by Hua et al. reported an inverse relationship between T cell density in the TME and the expression of PD-L1 on CRC cells (68). This was accompanied by an expansion of Treg cells, further linking the presence of $PD\text{-}L1^+$ tumor cells and poor prognosis (68).

CTLA-4 was found to be expressed on TILs within the epithelial component of the tumor, the surrounding tumor stroma and the invasive front of the tumor. Further, CTLA-4 was identified on subsets of Treg cells, where high expression of CTLA-4 was revealed along with a significant increase of activated Tregs (CD45R $Foxp3^+$ T cells) in the blood and tissues of CRC patients (69). Also, a highly suppressive subset of the $CD4^+$ $Foxp3^-$ T cell population was described in CRC patients to express multiple immune checkpoints (such as LAG-3, PD-1, and CTLA-4) and produce immunosuppressive cytokines such as IL-10 and transforming growth factor (TGF)-β (70). Therefore, CTLA-4 expression on Treg cells highlighted its potential role as a therapeutic target in CRC, such as in the case of Tremelimumab, which has been investigated in a phase II study for CRC patients with refractory metastatic adenocarcinoma who failed standard chemotherapy (70). Additionally, LAG-3 was reported to regulate the function of Treg cells, and its expression on $CD4^+$ $CD25^+$ cells was associated with potent inhibitory activity (71). Exhausted $CD8^+$ T cells were observed to express LAG-3 along with other inhibitory receptors, such as PD-1, and thus inhibition of both PD-1

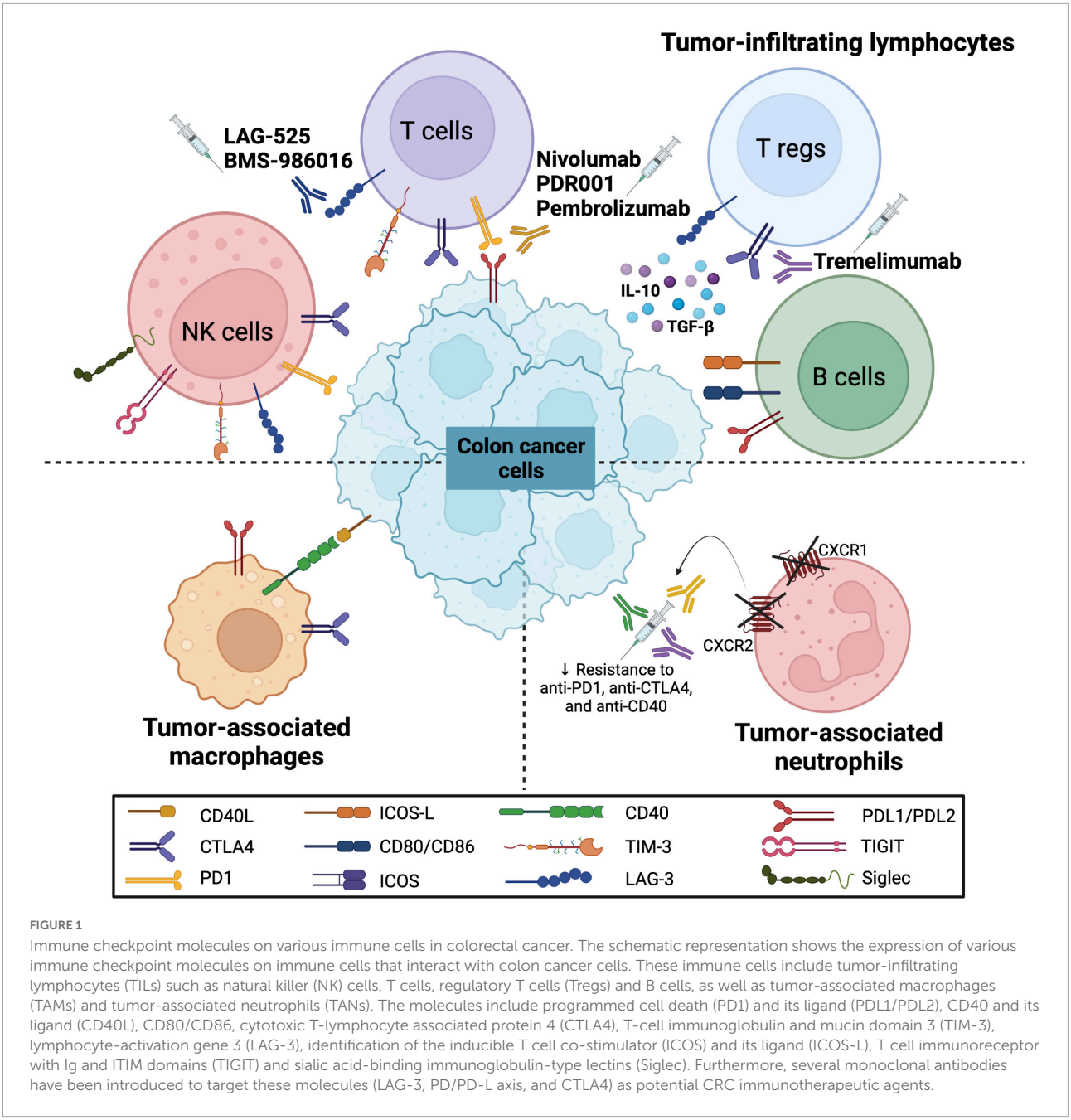

FIGURE 1
Immune checkpoint molecules on various immune cells in colorectal cancer. The schematic representation shows the expression of various immune checkpoint molecules on immune cells that interact with colon cancer cells. These immune cells include tumor-infiltrating lymphocytes (TILs) such as natural killer (NK) cells, T cells, regulatory T cells (Tregs) and B cells, as well as tumor-associated macrophages (TAMs) and tumor-associated neutrophils (TANs). The molecules include programmed cell death (PD1) and its ligand (PDL1/PDL2), CD40 and its ligand (CD40L), CD80/CD86, cytotoxic T-lymphocyte associated protein 4 (CTLA4), T-cell immunoglobulin and mucin domain 3 (TIM-3), lymphocyte-activation gene 3 (LAG-3), identification of the inducible T cell co-stimulator (ICOS) and its ligand (ICOS-L), T cell immunoreceptor with Ig and ITIM domains (TIGIT) and sialic acid-binding immunoglobulin-type lectins (Siglec). Furthermore, several monoclonal antibodies have been introduced to target these molecules (LAG-3, PD/PD-L axis, and CTLA4) as potential CRC immunotherapeutic agents.

and LAG-3 could boost T cell activity (72). There are several clinical trials with LAG-3 inhibitors (LAG-525 and BMS-986016) with or without the combination of PD-1 inhibitors (Nivolumab and PDR001) in patients with advanced solid malignancies (28).

Xu et al. found considerably greater levels of circulating TIM-3$^+$ PD-1$^+$ CD8$^+$ T cells in CRC patients' peripheral blood samples than in healthy subjects' blood (73). The expression of TIM-3 and PD-1 on CD8$^+$ and CD4$^+$ T cells was also revealed in peripheral blood collected after surgery. Furthermore, both TIM-3 and PD-1 expression appeared to be linked to decreased T cell activity (74). In comparison to adjacent colonic tissues, tumor tissue had a higher number of TIM-3$^+$ PD-1$^+$ CD8$^+$ T cells. Together with the lack of quantifiable responses to PD-1 blockage in a large group of CRC patients, these findings point to TIM-3 as a more prominent inhibitory receptor in CRC patients, thus limiting T cell responses. Furthermore, inhibiting this route may help to restore damaged cell-mediated immunity following surgical resection. These findings support the development of TIM-3 inhibitors and show considerable promise in CRC patients as single or combined treatments (34).

Immunoregulatory cells such as Treg cells, mesenchymal derived stem cells (MDSCs), and M2 macrophages possess the ability to control and modulate T cell function by releasing cytokines such as IL-10 and TGF-β that can activate specific inhibitory immune checkpoints (75–77). Likewise, tumor cells and other cells in the TME can express these inhibitory ligands and activate their receptors, thus impairing T cells' activity (78). This was reported to disrupt the proliferation of CTLs and reduce the immune response against CRC (79).

A known prognostic approach for immune checkpoint inhibitor therapy is MSI. Furthermore, MSI is linked with an MMR system that recognizes and repairs DNA damage. Several clinical trial data highlight that deficient MMR (dMMR) or MSI were able to predict treatment response across different solid tumor types, including CRC (80). In particular, MSI is known to be a good predictor of CRC prognosis, as it is closely associated with the abundance of tumor-infiltrating T cells. Several immunohistochemical studies have revealed high infiltration of intraepithelial activated $CD8^+$ T cells within MSI colorectal tumors (81–83). Furthermore, Dolcetti et al. found that cytotoxic infiltrating structures were highly abundant in tumor epithelial cells of MSI-high (MSI-H) patients. The exact pathophysiology of TILs accumulation in MSI-H CRC has not been elucidated. However, an early proposal was that MSI-H tumors produce many abnormal proteins that trigger a host immune response. This was supported in a study by Smyrk et al. which reported an active immune microenvironment in MSI/dMMR tumors that are characterized by a more favorable prognosis compared to MSS/MMR-proficient (pMMR) tumors (8). In the MSI/dMMR subset of CRC, the high accumulated mutation creates many tumor-specific neoantigens, typically 10–50 times that of MSS/MMR-proficient subset (84), which might be the reason for the high level of TILs and active Th1/CTL immune microenvironment in MSI/MMR-tumors observed in many previous studies (8).

Moreover, granase B expression and other cytotoxic effects were more active in MSI-H tumors (85). Additionally, pMMR-microsatellite instability-low (MSI-L)/MSS have low tumor mutational burden, poor infiltration by TILs and often have a worse prognosis than dMMR-MSI-H as well as a poor response to immune checkpoint inhibitors (86). In the TME, the PD/PD-L1 pathway leads to the escape of tumor cells from the immune response via the inhibition of CTLs (87, 88). Additionally, the expression of PD-L1 on tumor cells is related to the exhaustion of T cells, therefore blocking this pathway has been demonstrated to be a successful approach for the treatment of different types of cancers, including non-small cell lung cancer, melanoma, breast, renal cell carcinoma, and CRC (87–92). In particular, higher expression of PD-1 and PD-L1 has been associated with a better prognosis in CRC patients. Furthermore, PD-1 expression in TILs has been found to be an independent prognostic factor for overall survival and disease-free survival of CRC patients, especially for MMR-proficient tumors (93). Therefore, the upregulation of the PD-1/PD-L1 axis in CRC is correlated with a favorable clinical outcome. Such a pattern could be a compensatory upregulatory mechanism in the TME in order to identify the tumor and trigger an immune response. Furthermore, an association between PD-L1 on tumor cells and a high TILs density could further support this hypothesis, similar to that observed in breast cancer (94, 95). Moreover, there is a remarkable high expression of checkpoint molecules such as PD-1, PD-L1, CTLA-4, and LAG-3 in MSI CRC in comparison to MSS CRC, which could contribute to the immunosuppressive microenvironment that aids MSI tumors evade immune destruction by the infiltrating immune cells. Therefore, this explains why the MSI subset of CRC could be a potentially good candidate for the checkpoint immunotherapy (9). ICB was described as more effective in MSI CRC in a phase 2 trial of Pembrolizumab, a fully human mAb targeting PD-1. In addition, another PD-1 mAb, Nivolumab, showed efficacy in CRC, where a patient showed complete response with no disease recurrence and demonstrated MSI (27, 96). Therefore, MMR status is a critical key for response to therapy, as shown by different clinical trials with anti-PD-1 and anti-PD-L1 therapy. Moreover, it was also demonstrated that CTLA-4 expression is increased in MSI tumors compared to MSS cancers (84).

B cells

Tumor-infiltrating B cells constitute a significant proportion of the immune infiltrates in CRC. Until recently, B cells have not been considered an important population of TILs, despite that they compose around 40% of TILs (97, 98). They are considered positive regulators of immunity, often collaborating with T cells to generate potent, unrelenting immune responses (98).

B cells can exert anti-tumor effects by activating antibody-dependent cell cytotoxicity (ADCC) and the complement cascade (99). In tumor tissues, B cells can be found in lymphoid aggregates, known as tertiary lymphoid structures (TLSs) or could be sparsely distributed in the TME. B cells present in the immature TLSs were reported to possess immune-regulatory functions by the secretion of anti-inflammatory cytokines and thus leading to the inhibition of anti-tumor immunity (100). Also, B cells can act as APCs besides their main function as antibody producers. Furthermore, B cells possess the unique capability of concentrating antigens through membrane immunoglobulin mediated uptake, which might also facilitate T cell activation above certain thresholds for TAAs (98, 101). Autoantibodies were shown to react primarily with autologous tumor targets or allogeneic tumors of the same tissue type, suggesting recognition of TAAs (102). Antibodies were believed to play a negligible role in the TME, so their relevance in tumor biology has been overlooked. However, studies revealed that B cell markers such as CD20 and CD138 correlated significantly with a lower CRC stage (103).

A study by Maletzki et al. observed that tumor-infiltrating B cells in primary CRC were of a mature immunophenotype, suggesting activation and antigen-induced maturation (104). This was supported by other studies where most tumor-infiltrating B cells reside in follicular aggregates in CRC. Likewise, peritumoral follicular aggregates of lymphocytes have been previously reported as a "Crohn's-like reaction" and interpreted as an immune-mediated anti-tumor effect in CRC (105, 106). Similar to T cells, B cells express checkpoint ligands on their surface, such as PD-L1, CD80/CD86, and ICOS-L (107–109). Furthermore, a study by Helmink et al. observed significantly higher levels of B-cell-related gene expression, increased B cell receptor diversity, and clonal expansion in tumor samples from melanoma patients who responded to ICB treatment compared to other patients (110).

Natural killer cells

Being members of the innate immunity, NK cells can lyse tumor cells without prior sensitization or clonal expansion, unlike T cells. NK cells can be classified into two major groups, where the $CD56^{bright}$ $CD16^{-}$ subset represents 10-15% of circulating NK cells and are more immunoregulatory by releasing cytokines such as IFN-γ. They mainly reside in the secondary lymphoid organs, such as lymph nodes and tonsils (111). In contrast, $CD56^{dim}$ $CD16^{+}$ cells represent the significant population (90% of circulating NK cells) and predominantly mediate cytotoxicity (112, 113). NK cells play a fundamental role in cancer immunosurveillance through their anti-tumor activity (114). This has been supported by studies where the elimination of NK cells led to increased malignancy occurrence (115). NK cells perform their anti-tumor activity mainly when the expression of MHC class I molecules is downregulated. Moreover, upregulation of stress-induced molecules such as ligands of the activating receptor C type lectin receptor D (NKG2D) on cancerous cells makes them prone to NK-cell killing (116).

Most neoplastic cells and tumor-associated cells in the TME secrete factors that block the activation of NK cells, such as IL-6, IL-10, IDO, TGF-β, and prostaglandin E2 (PGE2), through downregulating NK cells activating receptors including NKG2D (117). Thus, NK cells, which infiltrate the tumor stroma, might proficiently lose their tumor-killing function due to these immunosuppressive mediators (118). For instance, IDO causes tryptophan depletion and kynurenine accumulation leading to immunosuppression of T and NK cell functions as well as the stimulation of Treg cells (119). Additionally, PGE2 suppresses IFN-γ production and responsiveness to IL-12 and IL-15 (120). Moreover, there is a reduction in the cytokine production of intra-tumoral NK cells (121). TGF-β affects the IL-15 signaling pathway, thus dampening NK cell proliferation and cytotoxicity (122). Furthermore, hypoxia and poor nutrient levels in the TME suppress NK cell activity (116). On another note, NK cell migration and penetration into the tumor growth site might be halted by ECM accumulation and increased interstitial fluid (123).

Furthermore, the recruitment of immunosuppressive cells such as MDSCs and the emergence of NK cell-resistant tumor variants result in primary tumor overgrowth. On the other hand, other tumor cells try to increase the expression of MHC class I molecules, such as human leukocyte antigen (HLA)-E, which engages the inhibitory receptor NKG2A on NK cells. This has been supported by studies where high expression of HLA-E and NKG2A led to a high inhibitory signal, potentially leading to poor outcomes and tumor growth (124–126).

NK cells have the potential to regulate the function of the adaptive immune system. For example, NK cells have been found to enhance T cell infiltration, thus triggering immune responses through their cytokine and chemokine secretion turning tumors immunologically "hot." In contrast, the absence of these immune cells leaves the tumors immunologically "cold" (127). Consequently, $CD8^{+}$ T cell recruitment in the TME and their interaction with NK cells elicit tumor regression. In addition, NK cells possess anti-metastatic activity by possible elimination of circulating tumor cells, "i.e., metastatic clones" (118, 127). However, tumors could escape NK cell activity through several mechanisms, including immune checkpoints expression by NK cells: PD-1, CTLA-4, LAG-3, and TIM-3. Upon binding to their receptors, NK cell activity is dampened (128), which can be surpassed by ICB, thus restoring NK and $CD8^{+}$ T cell anti-tumor immunity. Nevertheless, many tumors still develop resistance to ICB therapy, representing a potential therapeutic target (129).

Another major obstacle in solid tumors is the homing of immune cells such as NK cells to tumor growth sites. This could be attributed to a dysregulation in the chemokine gradient in the TME, thus preventing NK cells from reaching the tumor growth sites (130). This has been reported in several studies where aberrant signaling pathways led to alterations in chemokines, including CCL27, CCL2, and CXCL11, hence impairing leukocyte migration (131–133). In CRC, loss of MHC class I expression is quite common, allowing NK cell recognition and killing of tumor cells (134, 135). However, like other types of cancer, a decreased number of NK cells in CRC patients was reported, which was associated with an increased frequency of CRC tumor recurrence (136, 137). This has been further supported where a negative correlation between peripheral NK cells and the CRC staging was reported, especially at early (I) and late (IV) stages of the disease (138). Phenotypically, CRC patients exhibited a reduction in the expression of the natural cytotoxicity receptors, NKp44 and NKp46 (139).

Furthermore, other activating receptors such as NKG2D, NKp30, NKp46, and DNAX accessory molecule-1 (DNAM-1) were reduced in the peripheral blood of patients with CRC (140–142). Upon tumor progression, the percentages of $NKG2D^{+}$ NK

cells were decreased, indicating a role in the metastasis of CRC (143). It has been shown that reduced expression of NKG2D on NK cells was correlated with high soluble serum levels of its ligand MHC-class I related molecule A (MICA) (144). The pathway of NKG2D and its ligands has been reported to be affected by TGF-β, which is highly expressed by colorectal cells (145). Hence, ligands of the activating receptor NKG2D were detected in the early stages of CRC, but as an immune evasion strategy, their expression decreased upon disease progression (146). Additionally, dysregulated NK cells displayed impaired function in CRC, including IFN-γ secretion and degranulation (140). Moreover, phenotypic alteration has been observed in the circulating $CD56^{dim}$ population of NK cells in CRC patients (139). Interestingly, a subpopulation of NK cells that is positive for CD16 and CD56 was studied and correlated negatively with the occurrence of CRC and the staging of CRC (147). The inhibitory receptor, NKG2A, has been reported to be an interesting target as a checkpoint molecule in cancer (148). Thus, blocking the inhibitory NKG2A receptor enhances tumor immunity by promoting both NK and $CD8^+$ T cell effector functions. Monalizumab, a humanized anti-NKG2A antibody, was reported to induce NK cell activity against various tumor cells, especially in combination with PD axis blockade (149). This is under investigation in multiple clinical trials in solid tumors such as CRC (149).

Differentiated CRC cells were found to be more resistant to NK cells compared to cancer-initiating cells that were more susceptible to NK cell killing (150). It has been established by both *in vitro* and *in vivo* studies, where NK cells were shown to mediate the direct killing of human tumor cells in colon cancer (151–153). This has been implemented in clinical settings, where autologous NK cells were utilized in patients with advanced gastric or colorectal cancers combined with trastuzumab or cetuximab chemotherapy (154, 155). Colon adenocarcinomas exhibited low NK cell infiltration rates, thus causing the NK cell population to remain in the outer stroma and halting them from performing their anti-tumor activity (60, 134, 156, 157). Additionally, infiltration of NK cells was proposed to be a potential predictive marker of therapy. The homing and migration of NK cells are dependent on selectins, adhesion molecules and chemokines. Hence, future clinical trials should target the trafficking of NK cells into tumor sites rather than focusing on the simple administration of a single cytokine/chemokine as a therapeutic approach (157).

Another interesting aspect that is critical for immunotherapy for CRC is the expression of immune checkpoint molecules on NK cells (158). These include CTLA-4 and PD-1 receptors as well as TIGIT, CD96, LAG-3, and TIM-3. In CRC animal models and human patients, NK cell exhaustion was reported to be associated with the expression of TIGIT. Furthermore, the presence of NK cells was critical for the efficacy of TIGIT and PD-L1 checkpoint inhibitors, as they regulate the frequency of effector $CD8^+$ T cells secreting IFN-γ and TNF-α (159). The combination of these checkpoint inhibitors showed a synergistic effect in their anti-tumor potential that was accompanied by prevention of NK cell exhaustion in both animal models and CRC patients (159, 160). In addition, PD-1 was found to be upregulated on tumor-infiltrating and peripheral NK cells in digestive cancers such as esophageal, gastric, biliary, and CRCs (161).

Other recently reported immune checkpoints are the Siglec family receptors, such as Siglec-7 and -9, CD47, and CD200. On another note, NK cells express Siglec-7 and Siglec-9 receptors, with a further upregulation on the cytotoxic $CD56^{dim}$ NK cell subset (162, 163). In addition, Siglec-9 was found to be upregulated on tumor-infiltrated $CD8^+$ cytotoxic T cells in various solid tumors, including CRC (164, 165). An interesting fact about the Siglec immune checkpoint molecules is that they are expressed on various immune cells and are usually expressed on T cells that concomitantly express PD-1, further enhancing the co-inhibitory signal (165). Furthermore, they were known to play an inhibitory effect on NK cell function against tumor cells, particularly cytotoxicity.

On the other hand, blocking these immune checkpoint molecules such as Siglec-9 antibodies improved the anti-tumor cytotoxic potential of NK cells. This was due to the blockage of Siglec markers on tumor cells as well as the NKG2A receptor on NK cells (164). Also, sialidase treatment was found to enhance NK cell killing against various cell lines, including the colon cell lines. Therefore, anti-Siglec-7 and anti-Siglec-9 blocking antibodies could be developed to be used for cancer immunotherapy, along with other immune checkpoint inhibitors.

Tumor-associated macrophages

TAMs are the dominant inflammatory constituent in the TME and are ample in all stages of carcinogenesis. Activated infiltrating TAMs secrete a plethora of proteolytic enzymes as well as growth and inflammatory mediators, known to modulate different molecular pathways involved in tumor progression and metastasis (166).

Macrophages can be classified into two well-defined subtypes: M1 macrophages "classically activated" and M2 macrophages "alternatively activated." M1 macrophages have a pivotal role in eradicating different organisms and cancerous cells, as they have an inflammatory function by secreting pro-inflammatory cytokines like TNF-α, IL-6, and IL-1β. On the contrary, M2 macrophages release anti-inflammatory cytokines, such as TGF-β, IL-10, and IL-13, and have been implicated in tissue healing and tumor progression. M1 and M2 are distinguished with certain markers in the tumor samples, where M1 macrophages are characterized by the expression of HLA-DR, CD11c, CD86, inducible nitric oxide synthetase (iNOS), and phosphorylated signal transducer and

activator of transcription 1 (pSTAT1), while M2 macrophages express CD163, CD204, and CD206 (167). In the TME, TAMs are mostly pro-tumorigenic/anti-inflammatory "M2 phenotype form." Their significance in tumor evolution and progression is accentuated by the fact that they may comprise up to 80% of the tumor mass (168). The suppression of an immune response, activation of angiogenesis, and remodeling of ECM are important functional characteristics of TAMs. Furthermore, TAMs produce proteolytic enzymes such as matrix metalloproteinases (MMPs) and cathepsins that cause ECM breakdown, leading to the intravasation of tumor cells into the bloodstream, thus enhancing metastases (169). Additionally, TAMs release angiogenic factors, allowing tumor cells to spread beyond the primary tumor site and contributing to metastasis (170). They also provide a favorable environment for metastatic tumor cells by releasing inflammatory mediators like IL-1β. Furthermore, reactive oxygen species (ROS) produced by TAMs are implicated in malignant cell instability, a hallmark of cancer (168). On another note, TAMs could promote cancer cell proliferation by releasing growth factors such as epidermal growth factor (EGF) (170).

Recently, the effect of colon cancer ECM on macrophage polarization was investigated, where it was discovered that tumor ECM-educated macrophages could develop into M2 macrophages. The anti-inflammatory markers (IL-10, CCL18, and TGF-β) were upregulated, and the pro-inflammatory markers (TNF-α and IL-6) were downregulated by the macrophages that are differentiated within the tumor matrices. It was also found that MMP1, the MMP responsible for M2 polarization, was upregulated in tumor matrices. These results indicated that tumor-derived matrices caused an anti-inflammatory M2-like macrophage polarization significantly (171). Additionally, clinical staging and lymph node metastases were found to be associated with macrophage infiltration and vascular density in CRC (172). Moreover, blocking the colony-stimulating factor 1 receptor (CSF1R), required for TAMs' recruitment, differentiation, and survival, is one of the most effective ways to target TAMs (173). Small molecule inhibitors or mAbs against CSF1R diminish the number and/or affect the behavior of TAMs in mice models of solid tumors such as CRC, breast cancer, and glioblastoma, thus impairing tumor formation and progression (174–176).

TAMs were reported to express molecular triggers of checkpoint proteins that regulate T-cell activation. Such proteins are the site of action of checkpoint-blockade immunotherapies (177). On another note, TAMs are key players in immunological resistance and their manipulation could improve the efficiency of immunotherapies, possibly through the NF-κB pathway. Such a pathway could be inhibited to increase the efficacy of immunotherapies by repolarizing M2 TAMs and to decrease the expression of PD-L1 on them (178). A recent study in CRC by Fiegle et al. showed that the combined blockade of CTLA-4 and PD-L1 increased the levels of the pro-inflammatory Th1/M1-related cytokines, increased NOS^+ macrophages in the tumor tissue and reduced PD-$L1^+$ macrophages (179). The role of TAMs as therapeutic targets was reviewed by Malfitano et al. (177). Also, $CD40^+$ TAMs and plasma sCD40 in CRC tissues have been identified as favorable prognostic markers (180). Apoptotic susceptibility is dependent on the "quality" of the signal, as death occurs when the CD40 signal is delivered in membrane-bound form (mCD40L), whereas the soluble CD40 agonists are non-apoptotic (181). Blocking of CD40 using membrane-bound CD40L showed pro-apoptotic signal and pro-inflammatory cytokine production in CRC cells, thus suggesting CD40 as a promising therapeutic in CRC (182).

Tumor-associated neutrophils

Neutrophils play an intricate and complex role in cancer (183). Many reports support the dual function of neutrophils, including anti-tumoral and pro-tumoral roles, and thus TANs are segregated into anti-tumor (N1) and pro-tumor (N2) phenotypes (184). However, these cells do not have specific cell surface markers to discriminate N1 and N2 neutrophils. Some studies indicate that N1 neutrophils have a higher expression of CD54, CD95, TNF-α, CXCL10, and low production of IL-8, while N2 neutrophils have high expression of CD182 and IL-8 production (185). In addition, neutrophils play a role in the immunosuppression of tumors (186), through the release of different mediators, including IL-4, TGF-β, immune checkpoint ligands, ROS, and reactive nitrogen intermediates (187). On the other hand, releasing nitric oxide by neutrophils could enhance cancer cell killing and suppress CRC growth and metastasis (188).

Under the effect of TGF-β present in the TME, neutrophils polarize into pro-tumor N2 neutrophils, which produce proangiogenic factors and exert immunosuppressive activity through the secretion of arginase-1 (Arg1) (184, 189, 190). TANs mediate direct suppression of Th1 and CTL in tumors (191). On the other hand, upon blockade of TGF-β or administration of type 1 IFN, neutrophils could polarize into anti-tumor N1 neutrophils, which activate $CD8^+$ T cells, thus exerting anticancer cytotoxic activity, by reducing the expression of the proangiogenic factors (e.g., VEGF and MMP-9), and increasing the expression of T cell-attracting chemokines (e.g., CCL3, CXCL9, and CXCL10) (184, 189, 192).

Neutrophils are recruited to the tumor site through inflammatory molecules such as granulocyte-colony stimulating factor (G-CSF), tumor-derived cholesterol derivatives (oxysterols) (193) and anaphylatoxin C5a (complement component) (99, 194). In CRC, neutrophils play an anti-tumoral role through the secretion of IFN-β, IFN-γ and

Granulocyte macrophage-colony stimulating factor (GM-CSF), and are known to express CD66b, CD11b, CD101, and CD177 (187). Neutrophils may promote tumor metastasis by accumulating in the metastatic niche. Tumor and stromal cells expressing G-CSF, CXCL1, and CXCL2 enhance neutrophil recruitment in the metastatic sites (195).

In solid tumors, neutrophils' accumulation is a poor prognostic marker associated with tumor progression and metastases (196–198). However, in CRC, high infiltration of TANs was reported to be associated with a better response to 5-FU-based chemotherapy (199). In this regard, CRC represents an exception from other solid tumors in which a high number of TANs is associated with poor response to chemotherapy and radiotherapy (200). Different key players in tumor immunobiology among different cancers may explain the discrepancy of TANs function in CRC compared to other tumors (e.g., ovarian and gastric).

Noteworthy, neutrophils interact with TILs. Using an inducible colon tumor mouse model, Germann et al. reported that the most potent inhibitor of T-cell activity in the TME was the TANs. The suppression is exerted through matrix metalloproteinase-mediated activation of TGF-β (201). Interestingly, MMP-9 secreted by TANs, converts TGF-β precursor into an active form. Thus, inhibiting the MMP-9/TGF-β axis eliminates the immunosuppressive effect of neutrophils and suppresses their tumor-promoting functions (201). On the other hand, a recent study reported that the pre-operative and post-operative neutrophil to lymphocyte ratio was associated with histological markers of CRC progression. Also, there was a trend of association between post-operative neutrophil count and disease-free survival (202). Different factors affect neutrophil polarization and may, at least in part, explain the apparent paradoxical impact of TME neutrophil count.

The link between TANs infiltration and tumor angiogenesis determines to a great extent the response to ICBs. It has been reported that neutrophil infiltration in the TME is associated with significant resistance elements to ICBs and their adjuvant anti-angiogenic agents. More than 100 clinical trials investigate the combination of bevacizumab (Avastin; anti-VEGF-A antibody) with ICBs (203). In addition, inhibition of CXCL1 or CXCL5/CXCR2 signaling in tumors with low TILs causes a reduction in TANs infiltration, with an increase in the number of PD-1^{+} CD8^{+} T cells. Furthermore, this enhances the sensitization of cancer cells to the anti-CD40, anti-CTLA-4, and anti-PD-1 combination immunotherapy (204). Moreover, the use of CXCR2 inhibitors might overcome the resistance to anti-PD-1 immunotherapy in KRAS^{G12D}-expressing CRC (205). Such findings, together with similar ones in other cancers, promoted the development of phase I and II clinical trials, using CXCR1 and CXCR2 inhibitors in combination with anti-PD-1 in patients with metastatic CRC with MSI-L and Ras-mutation (195). Furthermore, the "neutrophil extracellular trap" or "NET" is considered an important element of the TME that leads to resistance to ICB therapy (206, 207). Accordingly, DNase I, an inhibitor of NETs, was reported to significantly enhance the therapeutic effects of anti-PD-1 in an MC38-bearing mouse model of CRC (208).

Conclusion

Blocking immune checkpoints has ushered in a new era of cancer treatment. Targeting immunological checkpoints in CRC TME is an intriguing novel cancer therapeutic approach via altering the immune cells' function. Increasing evidence suggests that patients' responses are linked to different pro-tumor and anti-tumor immune cells in the TME, such as TILs, TAMs, and TANs. Anti-PD-1, anti-PD-L1 and anti-CTLA-4 are well-known ICBs showing promising results in CRC patients. In addition, other intriguing immunological checkpoints that can suppress T or NK cell activity have emerged in recent years, such as TIM-3 and LAG-3. As a result, combining ICBs with other therapeutic modalities has shown encouraging results and could be a successful step forward in CRC treatment.

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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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Identification of N7-methylguanosine related signature for prognosis and immunotherapy efficacy prediction in lung adenocarcinoma

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Background: Lung adenocarcinoma (LUAD) is one of the most frequent causes of tumor-related mortality worldwide. Recently, the role of N7-methylguanosine (m^7G) in tumors has begun to receive attention, but no investigation on the impact of m^7G on LUAD. This study aims to elucidate the significance of m^7G on the prognosis and immunotherapy in LUAD.

Methods: Consensus clustering was employed to determine the molecular subtype according to m^7G-related regulators extracted from The Cancer Genome Atlas (TCGA) database. Survival, clinicopathological features and tumor mutational burden (TMB) analysis were applied to research molecular characteristics of each subtype. Subsequently, "limma" package was used to screen differentially expressed genes (DEGs) between subtypes. In the TCGA train cohort (n = 245), a prognostic signature was established by univariate Cox regression, lasso regression and multivariate Cox regression analysis according to DEGs and survival analysis was employed to assess the prognosis. Then the prognostic value of the signature was verified by TCGA test cohort (n = 245), TCGA entire cohort (n = 490) and GSE31210 cohort (n = 226). Moreover, the association among immune infiltration, clinical features and the signature was investigated. The immune checkpoints, TMB and tumor immune dysfunction and exclusion (TIDE) were applied to predict the immunotherapy response.

Results: Two novel molecular subtypes (C1 and C2) of LUAD were identified. Compared to C2 subtype, C1 subtype had poorer prognosis and higher TMB. Subsequently, the signature (called the "m^7G score") was constructed according to four key genes (*E2F7*, *FAM83A*, *PITX3*, and *HOXA13*). The distribution of m^7G score were significantly different between two molecular subtypes. The patients with lower m^7G score had better prognosis in TCGA train cohort and three verification cohort. The m^7G score was intensively

related to immune infiltration. Compared with the lower score, the higher m^7G score was related to remarkable upregulation of the PD-1 and PD-L1, the higher TMB and the lower TIDE score.

Conclusion: This study established a m^7G-related signature for predicting prognosis and immunotherapy in LUAD, which may contribute to the development of new therapeutic strategies for LUAD.

KEYWORDS

N7-methylguanosine, lung adenocarcinoma, molecular subtype, prognosis, immunotherapy efficacy

Introduction

Lung adenocarcinoma (LUAD) accounts for the largest proportion in non-small cell lung cancer (NSCLC) (1). Since patients with LUAD suffer from advanced disease or have distant metastasis when first diagnosed, they have a poor prognosis, and the overall 5-year survival rate is still below 20% (2, 3). Impressively, immune checkpoint blockade (ICB) has become a promising therapy strategy for NSCLC (4). However, some patients have a low response rate to ICB treatment, or even drug resistance, thus resulting in disease relapse or dead cases (5, 6). Therefore, it is essential to identify a novel biomarker in LUAD, in order to improve the outcomes of patients and formulate personalized treatment strategies.

Increasing evidence indicates that the initiation and progression of lung cancer depends not only on genetic variation, but also on epigenetic dysregulation (7, 8). As an important part of epigenetic modification, RNA modification is involved in regulating many physiological processes and disease occurrence (9). Besides, dynamic regulation and disruption of these RNA modifications are also related to the tumorigenesis, maintenance and progression of lung cancer (10, 11). Among numerous RNA dynamic modifications, N6-Methyladenosine (m^6A), 5-Methylcytosine (m^5C), and N7-methylguanosine (m^7G) are extremely common (12). Importantly, m^7G is the most prevalent modifications of RNA caps (13), which occurs in various RNAs of eukaryotes (14). m^7G modification has a significant impact on RNA metabolism, processing and function (15). Nevertheless, the exploration of m^7G-related regulators on tumors have only recently begun to receive attention owing to technological limitations. Mis-regulated m^7G modification could disturb the translation of many oncogenic transcripts involved in RPTOR/ULK1/autophagy pathway, which contributed to esophageal squamous cell carcinoma oncogenesis (16). *EIF4E* is regarded as one of m7G-related regulators, whose phosphorylation could increase the translations of oncogene mRNAs to promote prostate cancer tumorigenesis (17). Moreover, one study demonstrated that *METTL1* and *WDR4* were upregulated in lung cancer samples and vital for the progression (18). Besides, RNA dynamic modification could influence the response function and maturation of tumor immune cells (19). So far, the overall impact of m^7G-related regulators on the immunotherapeutic response in LUAD and its relationship with patient prognosis and treatment are still unclear.

With the advances in high-throughput sequencing technique, research on tumor genes is more in-depth, which can help to classify tumors to some content. There are many signatures that assess the prognosis of LUAD according to various subtypes (20–22). However, these signatures are still far from guiding precise treatment, which urgently requires a reliable signature. Here, two molecular subtypes of LUAD were constructed according to the gene expression of m^7G-related regulators. We further evaluated the relation between survival, clinical characteristics, immune infiltration and molecular subtype. Then, a novel m^7G score was established to quantify the m^7G modification patterns, which was proven to be an independent predictor of LUAD prognosis. Moreover, the prognostic signature effectiveness was validated by the internal and external cohort (GSE31210). Furthermore, we elucidated whether this signature could provide reference for clinical immunotherapy and chemotherapy. In conclusion, this study not only provides a novel understanding of molecular subtype by m^7G regulators, but also built a robust signature to estimate prognosis and guide individualized treatments in LUAD.

Abbreviations: LUAD, lung adenocarcinoma; m^7G, N7-methylguanosine; TCGA, The Cancer Genome Atlas; TMB, tumor mutational burden; DEGs, differentially expressed genes; NSCLC, non-small cell lung cancer; ICB, immune checkpoint blockade; GEO, Gene Expression Omnibus; PCA, principal component analysis; GSEA, Gene Set Enrichment Analysis; FDR, false discovery rate; LASSO, least absolute shrinkage and selection operator; ROC, receiver operating characteristics; ssGSEA, single-sample gene set enrichment analysis; TIDE, tumor immune dysfunction and exclusion; IC50, half-maximal inhibitory concentration; CDF, cumulative distribution function; AUC, areas under the curves; C-index, concordance index.

Materials and methods

Lung adenocarcinoma datasets

We obtained gene expression profile, clinical and somatic mutation data of LUAD from The Cancer Genome Atlas (TCGA) database.[1] Four hundred ninety LUAD cases were included in the follow-up study after removing patients with survival time less than 30 days. We acquired the external verification cohort (GSE31210) from Gene Expression Omnibus (GEO) database.[2] 226 cases were finally included after processing as TCGA data. 29 m^7G regulators were extracted from previous report (23) and three m^7G-related gene sets in MSigDB database[3] (**Supplementary Table 1**).

Landscape of genetic variation and identification molecular subtype

The expression of m^7G regulators was extracted from TCGA-LUAD dataset. Then various methods were applied to depict the genetic variation of m^7G regulators. The expression of m^7G regulators was compared between tumor and normal groups. The mutation map was presented by using "maftools" package. Next, Cox analysis was used to filter genes correlated with LUAD prognosis ($P < 0.05$).

The "ConsensusClusterPlus" R package was employed to identify molecular subtype by consensus clustering of prognostic gene (parameters: reps = 50, pItem = 0.8, pFeature = 1, clusterAlg = "pam," distance = "Pearson") (24). Pam and Pearson distances were used as the clustering algorithm and distance measure, respectively. Furthermore, the sample's distribution was characterized by principal component analysis (PCA). Moreover, we employed "survival" package to investigate the survival differences among subtypes. Besides, "heatmap" package was applied to explore the relation among molecular subtypes, expression of prognostic gene and clinicopathological features.

Biological function analysis and immune infiltration profile estimation

We investigated the biological process of distinct molecular subtype by Gene Set Enrichment Analysis (GSEA). The "h.all.v7.5.1.symbols.gmt" gene set was obtained from MSigDB database. We applied the CIBERSORT algorithm (25) to assess the immune status among different molecular subtypes. In recent years, tumor mutational burden (TMB) was widely applied to measure the effectiveness of ICB therapy (26). TMB score was calculated by using the somatic mutation data of each patient and then we compared TMB score in different subtypes. In addition, patients were further separated into low and high TMB groups on the basis of the threshold value (27) (10 mutations/megabase) of TMB and then we compared the frequency of high TMB in different subtypes. GSE135222 immunotherapy cohort including 27 cases was obtained from GEO database, which used to verify immunotherapy efficacy of different subtypes.

Screening of differentially expressed genes

We calculated the differentially expressed genes (DEGs) between molecular subtypes by using the Bioconductor "limma" package. The significance criteria were $|\log2FC| > 1$ and false discovery rate (FDR) < 0.01. The upregulated and downregulated of DEGs were visualized by volcano map. The heatmap was also applied to show the distribution of DEGs in different subtypes.

Construction and validation of the m^7G related signature

The all patients ($n = 490$) were randomly separated into train and test cohort according to the ratio of 1:1 by using "caret" package. In train cohort ($n = 245$), univariate Cox analysis was applied to screen genes related to the survival ($P < 0.01$). Then, least absolute shrinkage and selection operator (LASSO) regression was employed to further reduce the overfitting genes. Finally, a m^7G related signature was established by multivariate Cox analysis, and we also called it m^7G score. The previously reported formula (28) was used to calculate m^7G score: Σ(gene expression level $\times$ corresponding coefficient). Patients was separated into high and low m^7G score groups according to median m^7G score. Then the sample's distribution was characterized by PCA. We applied "survival" package to investigate the survival differences between two groups. We also plotted the receiver operating characteristics (ROC) curve to estimate the accuracy of the m^7G Related signature by using "timeROC" package. The test cohort ($n = 245$) and the entire cohort ($n = 490$) were employed to validate the signature power by using the same analyses. We further used GSE31210 dataset ($n = 226$) to verify the robustness of the signature.

m^7G related signature analysis and nomogram construction

Univariate and multivariate analysis were applied to demonstrate the independent prognosis of the m^7G score. Then

1 https://portal.gdc.cancer.gov/

2 https://www.ncbi.nlm.nih.gov/geo/

3 http://www.gsea-msigdb.org/gsea/index.jsp

these results were visualized with the forest plots. We also constructed a nomogram by combining the age, gender, stage and m^7G score for clinical practice. Additionally, calibration curve was applied to evaluate the predictive accuracy of the nomogram by using "rms" package.

Analysis of immune infiltration and anti-cancer treatment

We applied different bioinformatics methods including XCELL, TIMER, QUANTISEQ, MCPCOUNTER, EPIC, CIBERSORT-ABS, CIBERSORT, and single-sample gene set enrichment analysis (ssGSEA) (29, 30) to study the relation between m^7G score and immune score. Subsequently, we also compared the expression of immune checkpoints between two groups. Mutation maps were manifested by using "maftools" package in two groups. In recent years, in addition to immune checkpoints, tumor immune dysfunction and exclusion (TIDE) was also widely employed to assess the effectiveness of ICB therapy (31). The TMB score was calculated by using the somatic mutation data of each patient and the TIDE score was calculated in the TIDE website[4] ($P < 0.05$). Moreover, the drug sensitivity of each group was estimated by "pRRophetic" package (32). The half-maximal inhibitory concentration (IC50) of drugs was compared through Wilcoxon rank test between different m^7G score groups ($P < 0.05$).

Statistical analysis

R software (version 4.1.0) was employed for all data analysis. Wilcoxon rank test was applied to compare m^7G regulators expression between normal and LUAD groups. All above survival distribution was evaluated through survival analysis. The relation among molecular subtype, clinicopathological features and high TMB distribution was estimated by the chi-squared test. Immune infiltration, TMB and TIDE were also compared through Wilcoxon rank test. $P < 0.05$ was regarded as statistically significant.

Results

Genetic variation profile and m^7G modification pattern

The overall research procedure is shown in **Figure 1**. Twenty-four regulators were manifested significant downregulation or overexpression in different groups according to *P*-value less than 0.05 (**Figure 2A**). The result of mutation map showed that *EIF4G3* had the highest mutation frequency followed by *LARP1* (**Figure 2B**). The correlation and prognostic significance of m^7G regulators were presented in **Figure 2C**. Four genes including *EIF4E3*, *LARP1*, *WDR4*, and *NCBP1* were significantly associated with prognosis. m^7G regulators were also showed a remarkable interaction, which was critical for the development of the different m^7G modification patterns. The above-mentioned results suggested m^7G regulators may relate to tumorigenesis and progression in LUAD.

The LUAD patients were classified into two molecular subtypes (C1 and C2) by using "ConsensusClusterPlus" package according to prognostic genes. The intergroup correlation was lowest and intragroup correlation was highest when $k = 2$ (**Figure 3A**). Cumulative distribution function (CDF) curve performed the highest partition efficiency when $k = 2$ (**Figures 3B,C**). Taken together, two molecular subtypes were established according to the m^7G modification pattern, including 245 patients of C1 and 245 patients of C2. The PCA analysis also demonstrated that the two subtypes could be completely distinguished (**Figure 3D**). The result of Kaplan–Meier analysis showed distinct survival outcome between two subtypes ($P < 0.001$) (**Figure 3E**), suggesting that C1 subtype had worse prognosis than C2. Subsequently, the clinical features and gene expression were compared, then we found patients in C1 subtype had poorer tumor stage than C2. *EIF4E3* was upregulated in C2 subtype, while *LARP1*, *WDR4*, and *NCBP1* were upregulated in C1 subtype (**Figure 3F**).

Analysis of biological functional and immune infiltration

The results of GSEA presented diverse functional pathways between two subtypes. Functional analysis showed E2F_targets, G2M_checkpoint, glycolysis, MITOTIC spindle, MTORC1_signaling, MYC_targets_V1, MYC_targets_V2 were significantly enriched in C1 subtype (**Figure 4A**). While, there were significantly different pathways were enriched in C2 subtype, such as allograft rejection, complement, inflammatory response, interferon gamma response, IL2_STAT5_signaling, IL6_JAK_STAT3_signaling (**Figure 4B**).

Subsequently, we found that two subtypes had markedly different immune infiltration patterns (**Figure 4C**). The result of CIBERSORT algorithm showed the expression level of T cells follicular helper, resting NK cells, M0 macrophages, activated mast cells were high in C1 subtype, while resting CD4 memory T cells, T cells gamma delta, monocytes, resting dendritic cells, resting mast cells are high in C2 subtype. These suggested that two subtypes may have different immunotherapeutic response, so we further compared TMB score in two subtypes. Then we observed that C1 had

4 http://tide.dfci.harvard.edu/

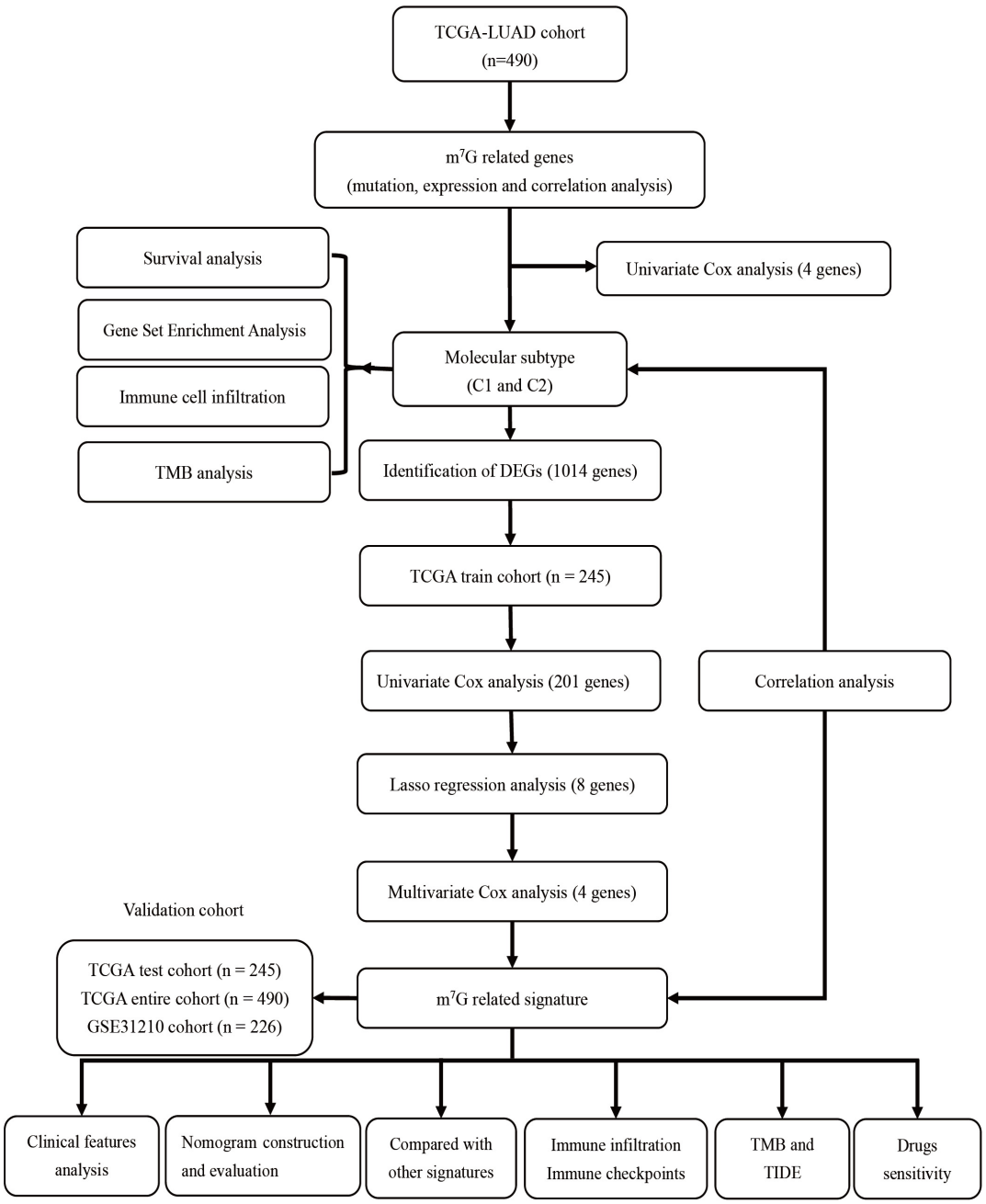

FIGURE 1
The flowchart of the overall study.

higher TMB score as well as the proportion of high-TMB compared to C2 subtype (22 vs. 10%) (Figures 4D,E). GSE135222 immunotherapy cohort was divided into two subtypes by using the same method mentioned above, including 15 cases in C1 subtype and 12 cases in C2 subtype. The results presented that the proportion of response to immunotherapy was higher in C1 subtype than C2 subtype (40 vs. 17%) (Figure 4F).

Screening differentially expressed genes between m^7G subtypes and construction of m^7G related signature

Based on "limma" package, we identified 1,014 DEGs between m^7G Subtypes, including 534 upregulated genes and 480 downregulated genes. Then the significant DEGs were visualized with volcano map (Figure 5A). The expression

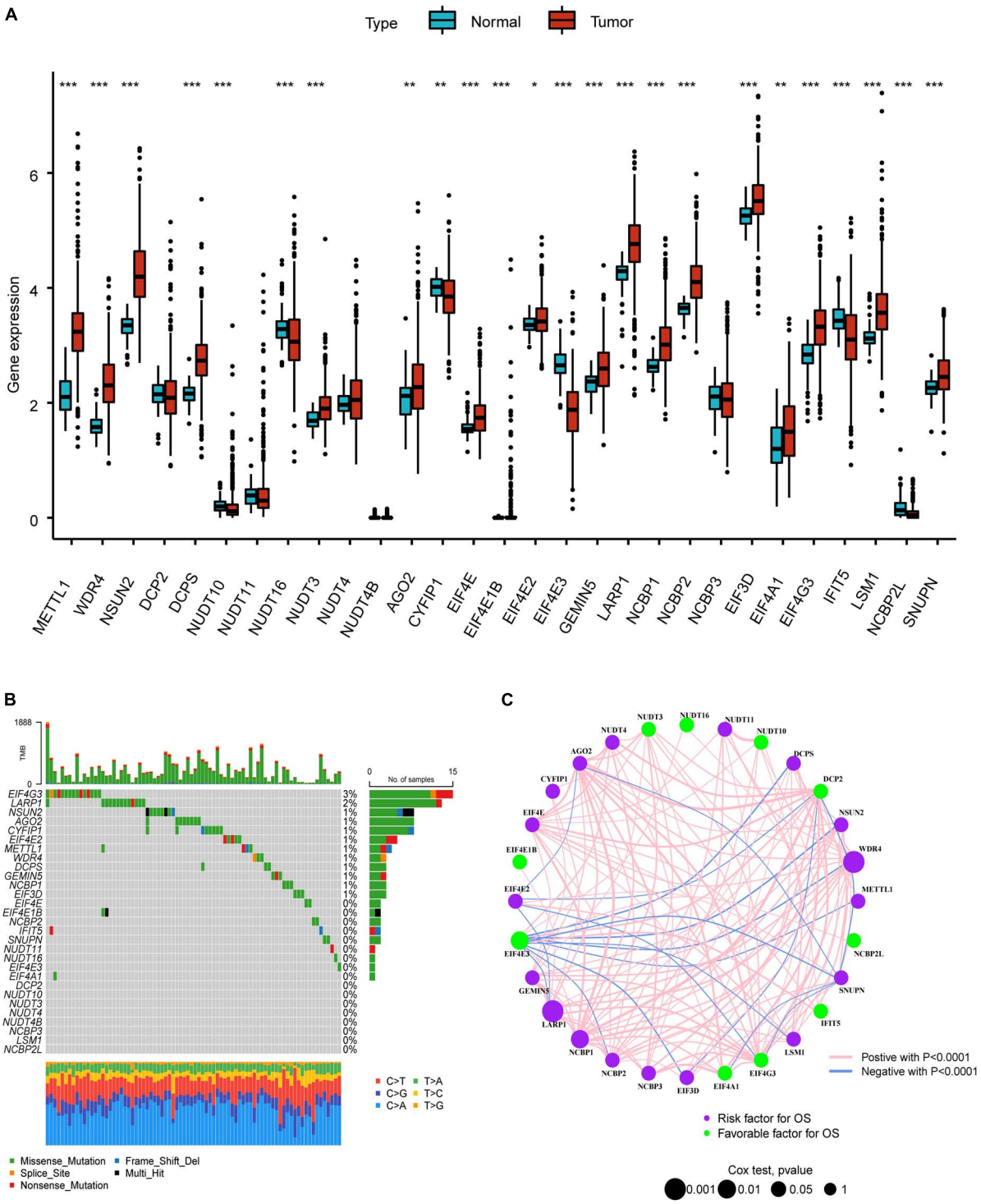

FIGURE 2
Landscape of genetic variation in LUAD. **(A)** The difference of m^7G regulators expression level in normal and tumor group. **(B)** The mutation map of m^7G regulators in LUAD. **(C)** The interplay and prognostic significance of m^7G regulators. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

profiles of DEGs between C1 and C2 subtype were visualized with heatmap (Figure 5B). We utilized the train cohort to establish m^7G related prognostic signature (n = 245). First, 201 genes correlated with patient prognosis were found by univariate analysis (Supplementary Table 2). Then we further applied LASSO regression to filter eight genes for the

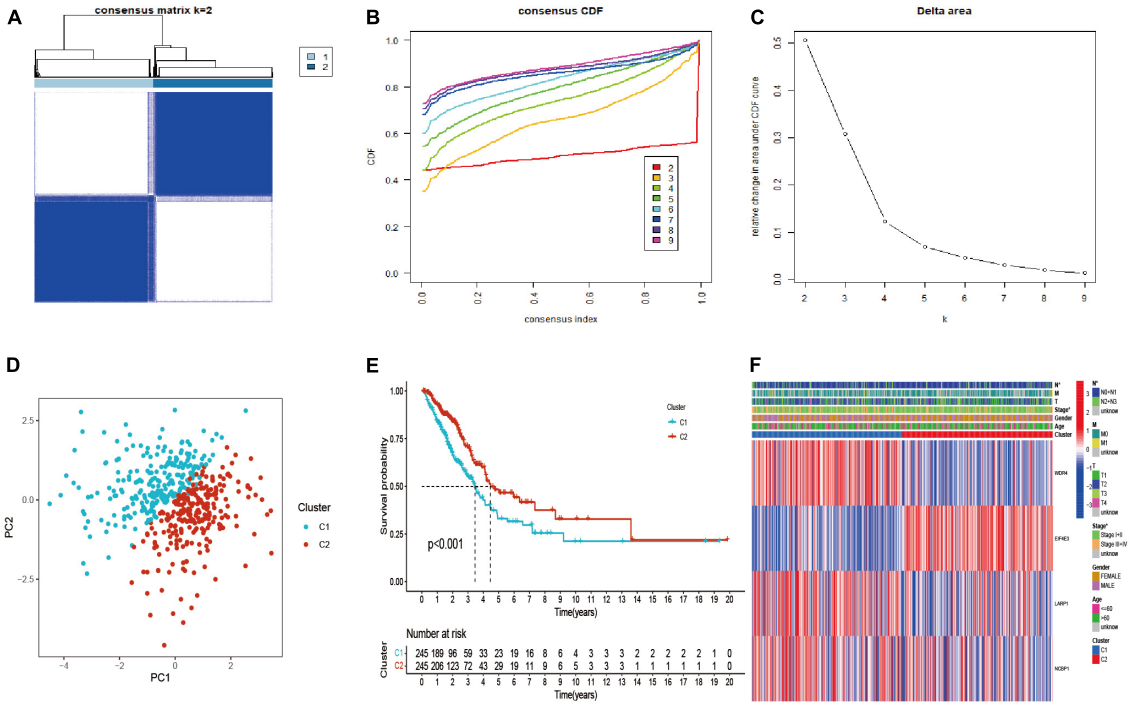

FIGURE 3
Identification, survival, and clinical characteristics analysis of molecular subtype. **(A–C)** The optimal value of consensus clustering. **(D)** Distribution of all patients. **(E)** Survival analysis in C1 and C2 subtypes. **(F)** Heatmap of prognostic gene and clinicopathological features between two subtypes. *$P < 0.05$.

subsequent multivariate analysis (Figure 5C). Finally, four key genes including *E2F7*, *FAM83A*, *PITX3*, and *HOXA13* were identified by using multivariate Cox regression (Figure 5D). The m^7G score was calculated with the following formula: 0.5171 × *E2F7* (mRNA level) + 0.1888 × *FAM83A* (mRNA level) + 1.5576 × *PITX3* (mRNA level) + 0.5210 × *HOXA13* (mRNA level). Then we split patients into high and low m^7G score groups according to approach mentioned above (Figures 5E–G). The relative expressions of *E2F7*, *FAM83A*, *PITX3*, and *HOXA13* in two groups were presented in Figure 5H. Patients had significant poor survival in high m^7G score group (Figure 5I). The areas under the curves (AUC) for predicting survival rates at 1-, 3-, and 5-year were 0.736, 0.732, and 0.672, respectively (Figure 5J).

Verification of m^7G related signature and analysis of survival in different clinical subgroups

Patients in the internal verification cohort (test and entire cohort) and the external verification cohort (GSE31210) were categorized into two groups on the basis of the same risk formula in train cohort. Patients of test cohort were classified into two groups (Figures 6A–C), which were consistent with the result of the train cohort. The heatmap showed the expression profile of four key gene were apparently different between two groups (Figure 6D). The Kaplan–Meier curve also indicated that two groups had distinct survival (Figure 6E). The area of AUC verified that the signature was a great indicator for assessing prognosis in LUAD (Figure 6F). The similar results were acquired in the entire cohort and GSE31210 cohort (Figures 6G–L, 7A–F). On the basis of Kaplan–Meier analysis of entire TCGA cohort, we also found that patients presented lower survival rate in high m^7G score group among different clinical subgroups compared to low m^7G score group (Supplementary Figure 1).

Evaluation of association between m^7G score, clinicopathological features, and molecular subtype

The results showed strikingly distinct of m^7G score in age, gender, N-stage, M-stage, clinical stage and T-stage (Figures 8A–F) ($P < 0.05$). We also investigated the relation between m^7G score, m^7G subtype and survival state by using Sankey diagram (Figure 8G). We found that C2 subtype has a strong correlation with low m^7G score, while C1 subtype has a strong correlation with high m^7G score. Moreover, the majority

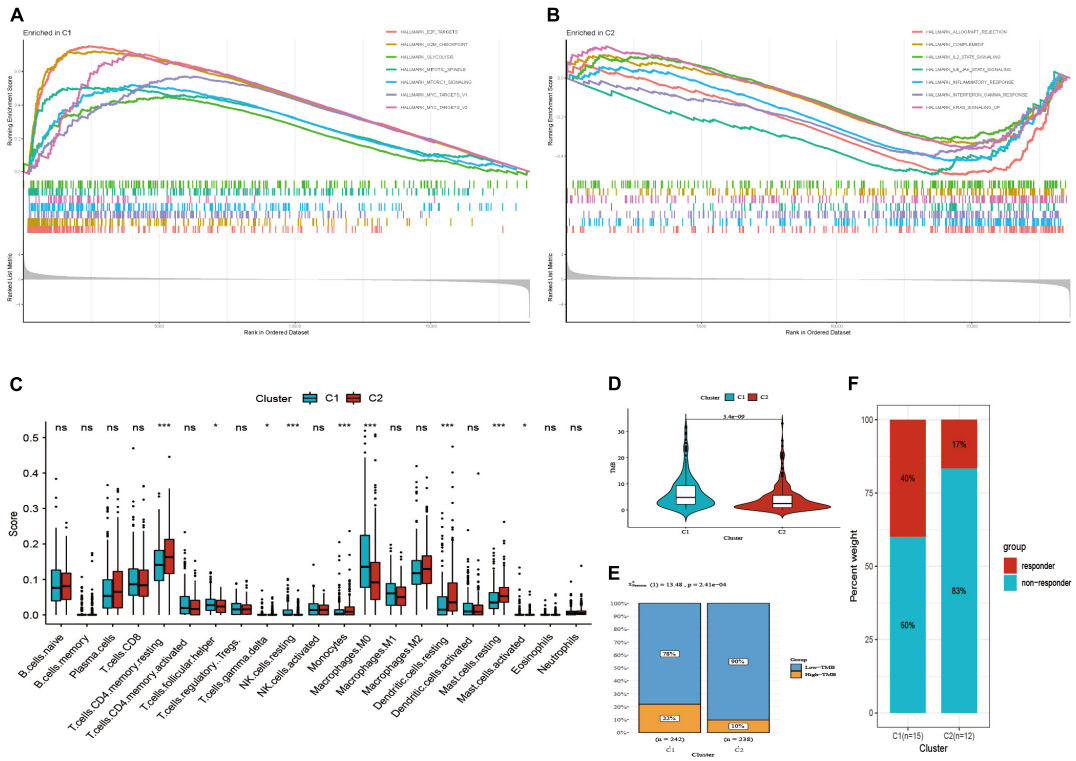

FIGURE 4
Functional pathways, immune infiltration and TMB analysis. **(A)** Functional pathways in C1 subtype. **(B)** Functional pathways in C2 subtype. **(C)** Comparison of immune score. **(D)** Comparison of TMB score. **(E)** Distribution of low and high TMB between two subtypes. **(F)** Distribution of response to immunotherapy between two subtypes in GSE135222. *$P < 0.05$; ***$P < 0.001$; ns: not significant.

of patients with low m^7G score were alive, which was consistent with preceding survival analysis. Furthermore, stacked bar chart also presented C1 subtype has a strong correlation with high m^7G score (**Figure 8H**). Similarly, the m^7G score was higher in C1 subtype than that in C2 subtype (**Figure 8I**).

Construction of nomogram and comparison of prognostic signatures

Univariate analysis identified that m^7G score was related to poor prognosis in LUAD (**Figure 9A**). Moreover, m^7Gs core was still an independent prognostic indicator after using multivariate analysis (**Figure 9B**) ($P < 0.001$). Subsequently, we used m^7G score and other clinical factors to establish a nomogram (**Figure 9C**). Calibration curve demonstrated that 1, 3, 5-year predicted survival rates matched the veritable condition (**Figure 9D**). These evidences revealed that the m^7G score could potentially assist clinical practice to evaluate the prognosis of LUAD patients.

After reviewing previous researches, we further compared the m^7G related signature with other prognostic models, including 5-gene signature (Wang) (33), 4-gene signature (Wu) (34), 3-gene signature (Yue) (35), and 5-gene signature (Zhai) (36). In order to ensure comparability among models, the risk score of each LUAD sample in entire TCGA cohort was calculated with the same formula according to corresponding genes in four signatures, and then patients were categorized into two groups based on same cut-off value (37). The results of survival analysis showed significant difference in four models (**Figures 10A–C,G**) ($P < 0.05$). However, all AUC at 1-, 3-, and 5-years in four models were lower than that corresponding AUC of our prognostic signature (**Figures 10D–F,H**). Furthermore, we conducted "survcomp" package to calculate the concordance index (C-index) of each signature. The C-index was highest in our prognostic signature (**Figure 10I**). Therefore, our signature was more efficient to estimate prognosis in LUAD.

Investigation of immune microenvironment and anti-cancer therapy

Firstly, the results of bubble plot exhibited that CD8+ T cell, common lymphoid progenitor, plasmacytoid dendritic cell, macrophage M1/M0, CD4+ Th1/Th2 cell, neutrophil,

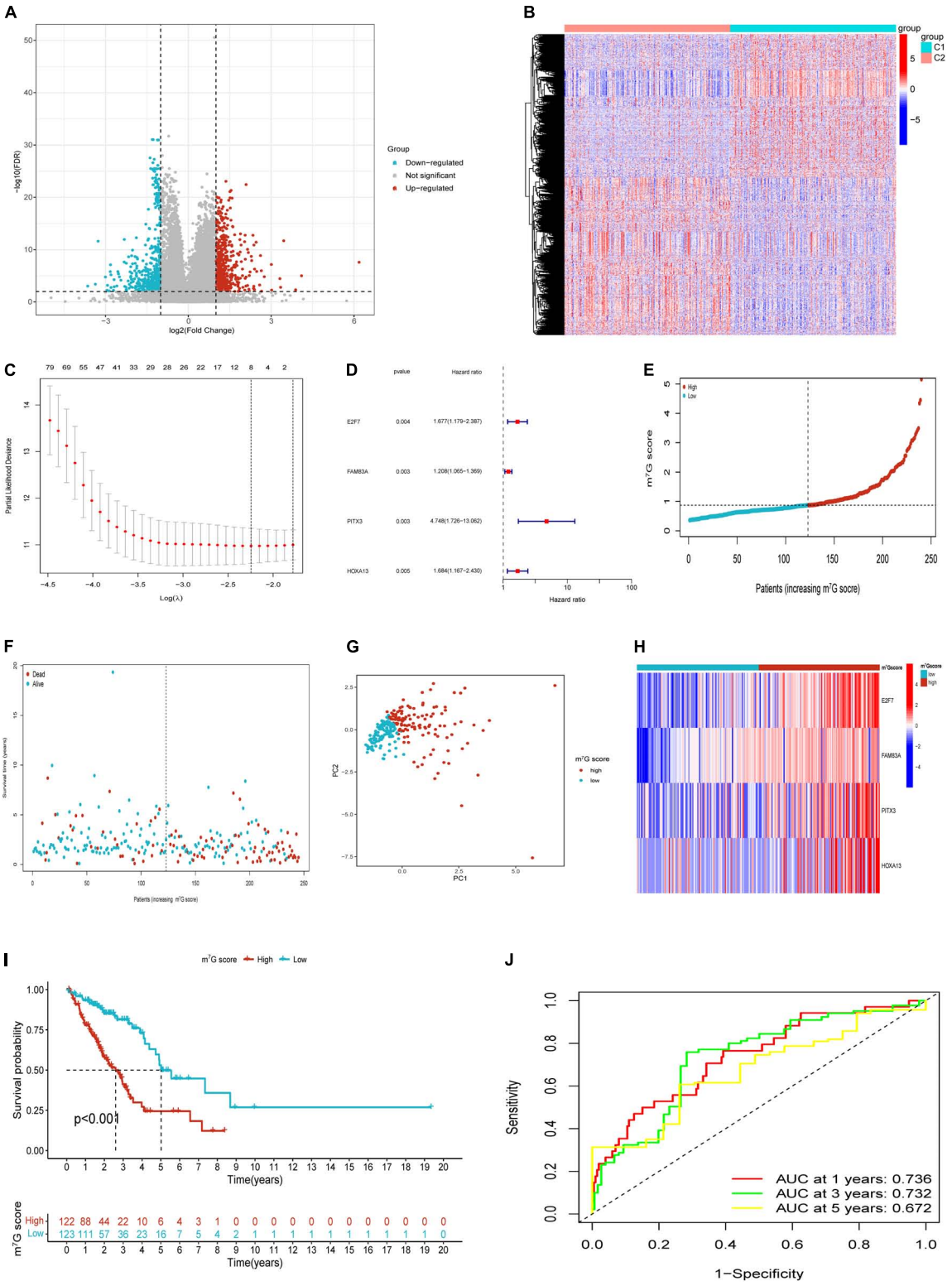

FIGURE 5
Construction of m^{7}G related signature based on TCGA train cohort. **(A)** Volcano plot of DEGs. **(B)** Heatmap of DEGs. **(C)** Eight genes through Lasso regression analysis. **(D)** Four key genes through multivariate Cox regression. **(E,F)** Distribution of m^{7}G score and survival state. **(G)** Distribution of patients according to m^{7}G-related signature. **(H)** Heatmap of four genes expression between high and low m^{7}G score groups. **(I)** Survival analysis in two groups. **(J)** AUC for predicting 1-, 3-, 5-years survival rates.

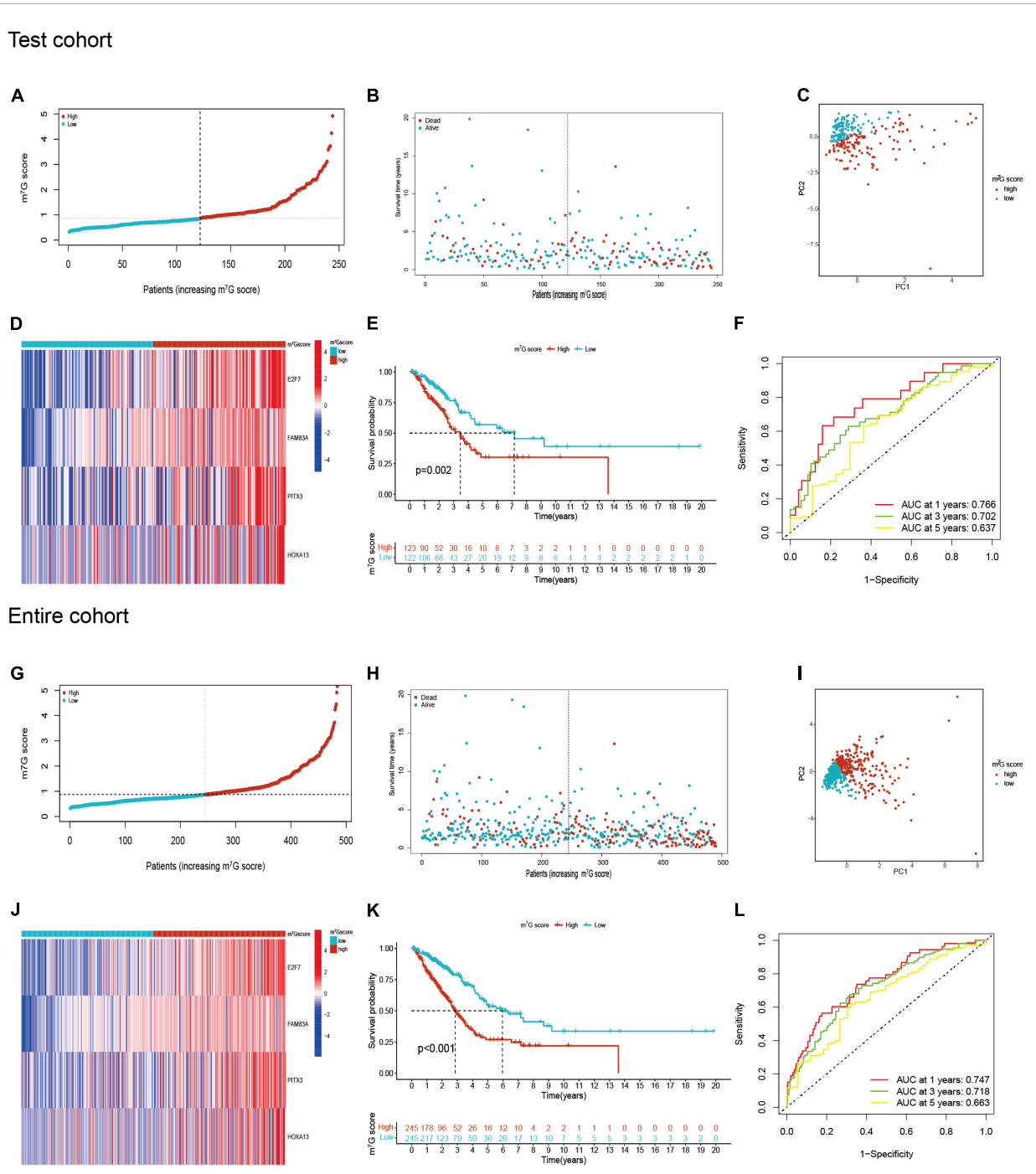

FIGURE 6
m^7G related signature validation in TCGA test cohort and TCGA entire cohort. **(A,B)** Distribution of m^7G score and survival state in TCGA test cohort. **(C)** Distribution of patients in TCGA test cohort. **(D)** Four genes expression between two groups in TCGA test cohort. **(E)** Survival analysis between two groups in TCGA test cohort. **(F)** AUC for predicting 1-, 3-, 5-years survival rates in TCGA test cohort. **(G–L)** The results of validation in TCGA entire cohort.

cytotoxicity score, NK cell, cancer associated fibroblast, monocyte, Myeloid dendritic cell, and mast cell resting were positively correlated with m^7G score (Figure 11A). The ssGSEA displayed that activated CD4 T cell, CD56dim natural killer cell, natural killer T cell, neutrophil, Type-2 T helper cell were more active in high m^7G score group (Figure 11B) ($P < 0.05$). Subsequently, the level of CD274 (PD-L1) and PDCD1 (PD-1) were presented upregulated in high m^7G score

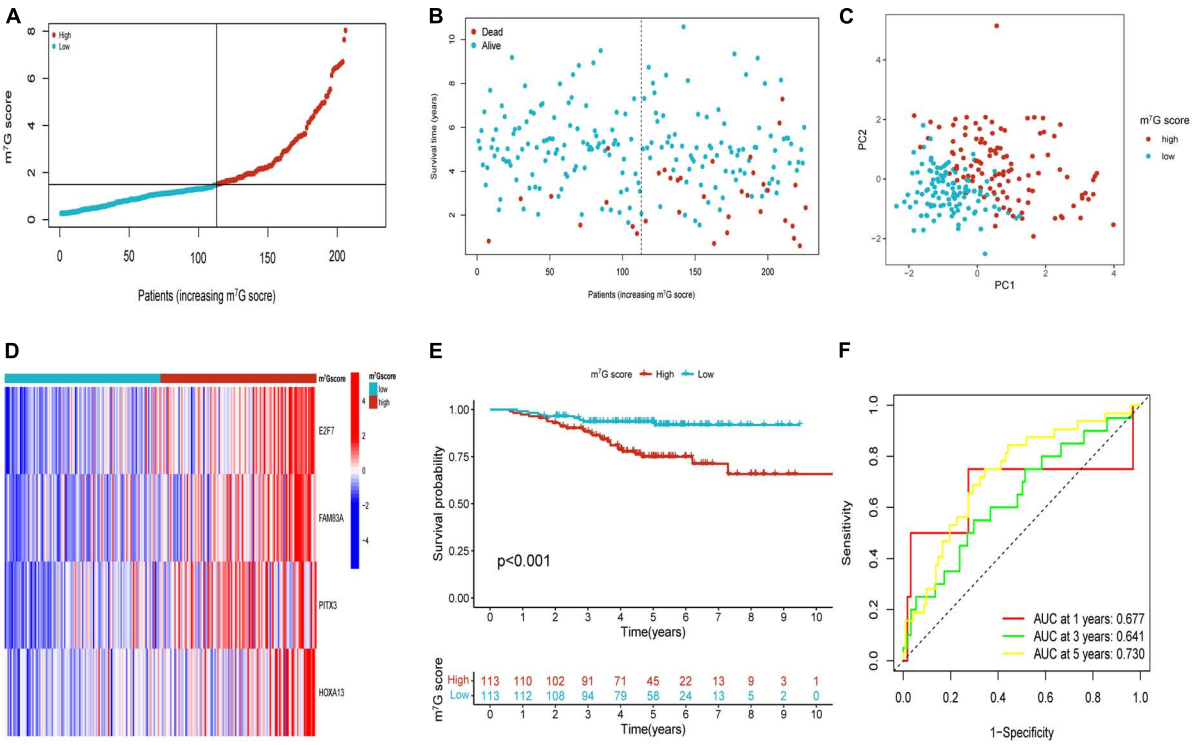

FIGURE 7
Validation of m^7G related signature in GSE31210. **(A,B)** The distribution of m^7G score and survival state. **(C)** Distribution of patients according to m^7G-related signature. **(D)** Heatmap of four genes expression between two groups. **(E)** Survival analysis between two groups. **(F)** AUC for predicting 1-, 3-, 5-years survival rates.

group (Figures 11C,D) ($P < 0.05$). Moreover, we compared somatic mutations in two risk groups. The results of mutation map showed remarkably high mutational rate in high m^7G score group. *TP53* was the highest mutational gene in both groups (Figures 12A,B). We also found that high m^7G score group was related to higher TMB score (Figures 12C,D) ($P < 0.05$). Compared to low m^7G score group, high m^7G score group had strikingly lower TIDE score (Figure 12E) ($P < 0.05$).

We further investigated common drug sensitivity in two groups (Figure 13). Patients presented lower IC50 of Cisplatin, Docetaxel, Doxorubicin, Etoposide, Gemcitabine, Paclitaxel, and Rapamycin in high m^7G score group, representing these drugs were more effective for high m^7G score group. Meanwhile, IC50 of Bicalutamide, Erlotinib, Axitinib, Imatinib, Metformin, Methotrexate, Bexarotene, Sorafenib, and Temsirolimus were lower in low m^7G score group, representing these drugs were more effective for low m^7G score group.

Discussion

Recently, the role of m^7G in tumors has begun to receive increasing attention. However, there are no reports on studying the molecular subtype correlated with m^7G and the implications of m^7G related signature on the prognosis and immunotherapy in LUAD. Therefore, we expect to discover more tumor phenotypes through this classification, which could be used to evaluate the prognosis of LUAD patients. Here, we first extracted twenty-nine m^7G related regulators expression profiles from TCGA, four of which were demonstrated to have prognostic value. Then, two novel molecular subtypes were identified according to theses prognostic genes. Results showed that patients in C1 subtype had more poor survival outcomes and advanced tumor stages compared to C2 subtype through survival analysis and clinicopathological features comparison, indicating m^7G regulators correlated with prognosis and progression of LUAD. And the two subtypes presented markedly different molecular features. Compared with C2 subtype, m^7G regulators were more activated in C1 subtype, including *LARP1*, *WDR4*, and *NCBP1*, while only *EIF4E3* was activated in C2 subtype. Xu et al. (38) demonstrated that the expression level of *LARP1* was upregulated in NSCLC, which positively related to poor prognosis and progression of cancer. A study of *WDR4* uncovered that knockdown of *WDR4* could restrain the aggressiveness of NSCLC cells, demonstrating that *WDR4* may have tumorigenic function in lung cancer (18). *NCBP1* was significantly overexpressed in LUAD, combined with CUL4B, which promoted the proliferation, migration and invasion of

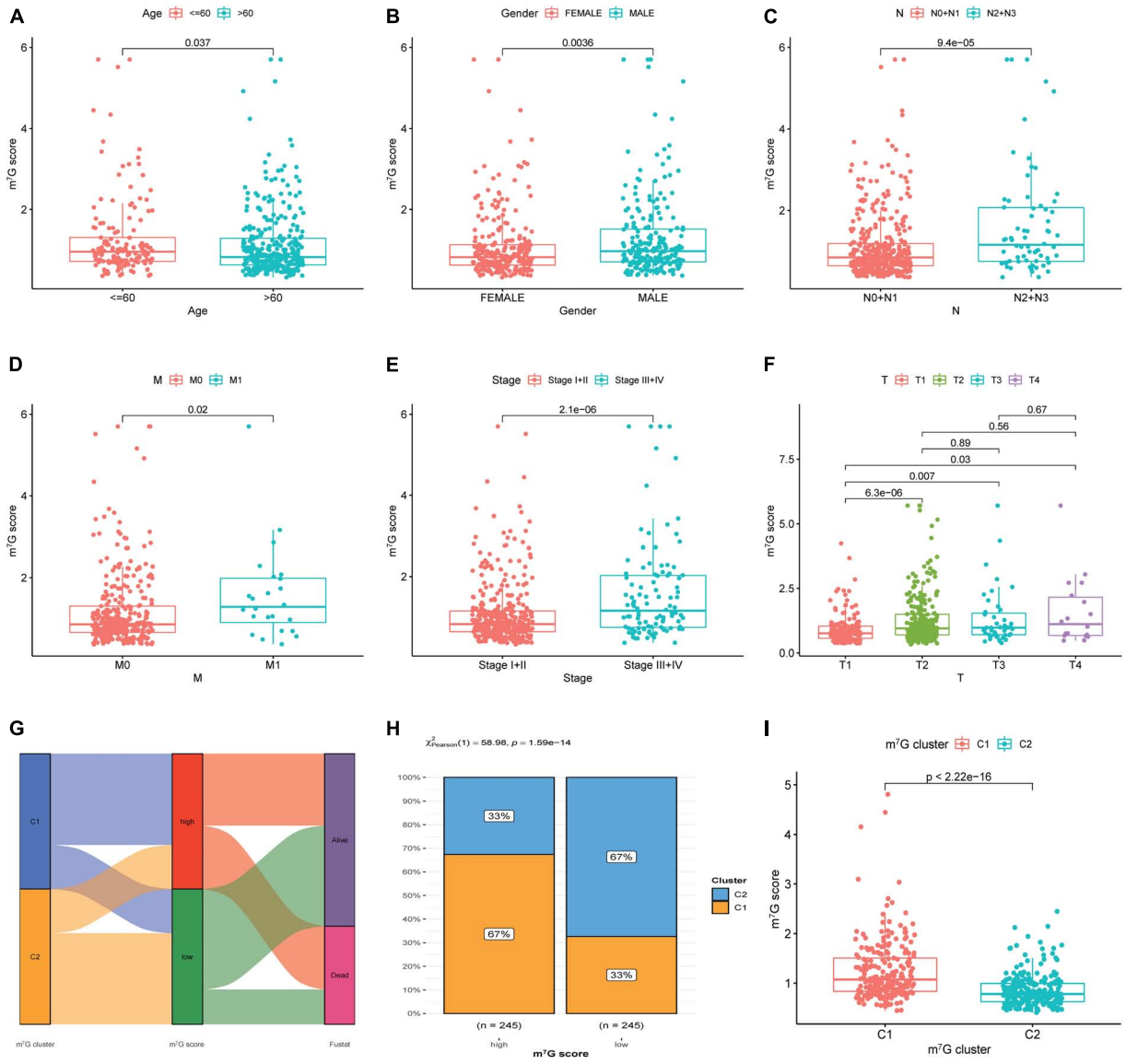

FIGURE 8
Correlation among clinical features, molecular subtype and m^{7}G score. **(A–F)** Correlation between clinical features and m^{7}G score. **(G)** Relation between m^{7}G score, m^{7}G subtype and survival state by using Sankey diagram. **(H)** Distribution of m^{7}G subtype between high and low m^{7}G score groups. **(I)** Comparison of m^{7}G score between C1 and C2 subtypes.

tumor cells (39). It was reported that compared with patients with lower expression of EIF4E3, patients with high expression of *EIF4E3* had markedly better survival rates in various cancers, including LUAD (40). These results are consistent with our study, indicating LUAD patients with C1 subtype have poor prognosis compared to C2 subtype. Furthermore, we investigated the possible functional mechanisms in both two subtypes by using GSEA analysis. Interestingly, the functional pathways enriched in C1 subtype were mainly cell proliferation-related pathways, which may indicate advanced clinicopathological staging, adverse survival outcomes and aggressive tumor subtypes. This evidence also suggested the worse prognosis of C1 subtype. Immune cells as an important part of the tumor microenvironment intensively relate to the response to immunotherapy (41). Recently studies showed that RNA modifications were correlated with the differentiation of immune cells in the tumor microenvironment (42). As one of the RNA modifications, m^{7}G also influenced immune cells in the tumor microenvironment. Chen et al. (43) quantified the tumor-infiltrating lymphocytes and found that CD4+ T exhaustion and Tregs decline after knockout of m^{7}G regulators. Besides, Devarkar et al. (44) presented that m^{7}G was involved in innate immunity mediated by RIG-I. Therefore, the immune score was applied to characterize immune microenvironmental

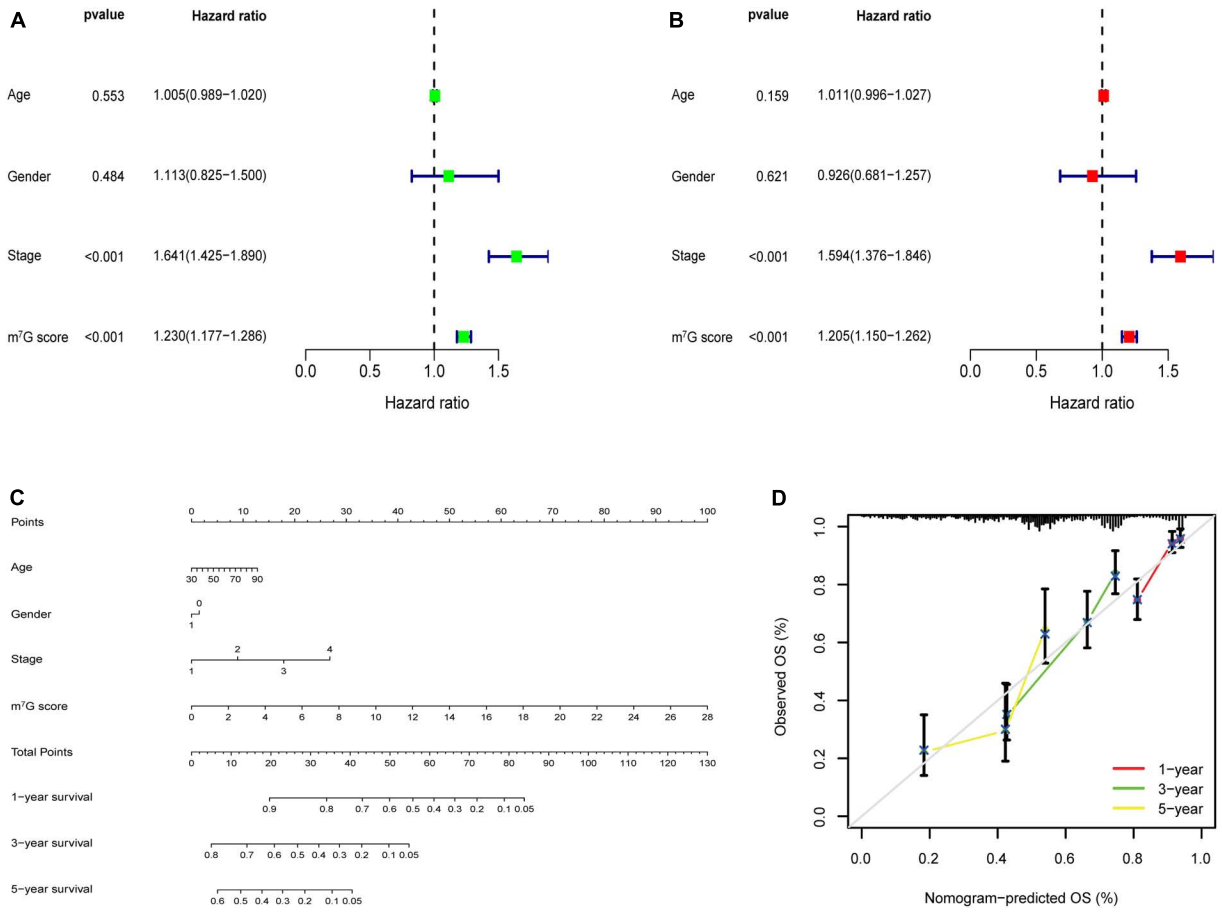

FIGURE 9
m^7G related signature analysis and nomogram construction. **(A)** Univariate analysis of m^7G score and clinical factors. **(B)** Multivariate analysis of m^7G score and clinical factors. **(C)** Establishment of nomogram. **(D)** Calibration curve of the nomogram.

landscapes in two subtypes. Then we found that follicular helper T cells, resting NK cells and activated mast cells were increased in C1 subtype. It was reported that follicular helper T cells could recruit CD8+ T cells to enhance antitumor immune response (45). The resting NK cells could secrete various cytokines to kill target cells, for example tumor cells (46). The TLR4 activation by mast cells resulted in the secretion of CXCL10, which could recruit effector T cells to influence antitumor immune response (47). These evidences indicated that C1 subtype with activated m^7G regulators may more sensitive to immunotherapy compared to C2 subtype. Increasing investigations suggested that TMB is a biomarker of response to immunotherapy and is positively relate to the effectiveness of ICB in various cancers, including NSCLC (48). In this study, the TMB score in patients with C1 subtype was distinctly higher compared with C2 subtype. After dividing TMB into high and low groups, more percentage of high TMB was observed in patients with C1 subtype compared with C2 subtype. Consequently, patients with C1 subtype may present better immunotherapeutic response than C2 subtype. Besides, by using an immunotherapy cohort of lung cancer, we also demonstrated that patients with C1 subtype had better immunotherapy efficacy than C2 subtype.

Considering the individual heterogeneity of m^7G modification, we utilized a novel m^7G score to quantify the m^7G modification patterns in LUAD. In the TCGA train cohort, we identified four key genes (*E2F7*, *FAM83A*, *HOXA13*, and *PITX3*), and then calculated the m^7G score through the previously mentioned algorithm. After separating patients into high and low m^7G score groups, we found that the four genes were overexpressed in the high m^7G score group. It was reported that overexpression of *E2F7* correlated with poor prognosis and microRNA-935 could inhibit tumor metastasis and invasion by targeted suppression the level of *E2F7* in NSCLC (49). Wang et al. (50) found that activating the expression of *E2F7* expression by targeting microRNA-140-3p could promote the progression of LUAD. Studies presented that *FAM83A* was significantly related to TMB and DNA damage response pathways in NCSLC (51, 52), indicating that it may play an important part in tumor progression and immunotherapy.

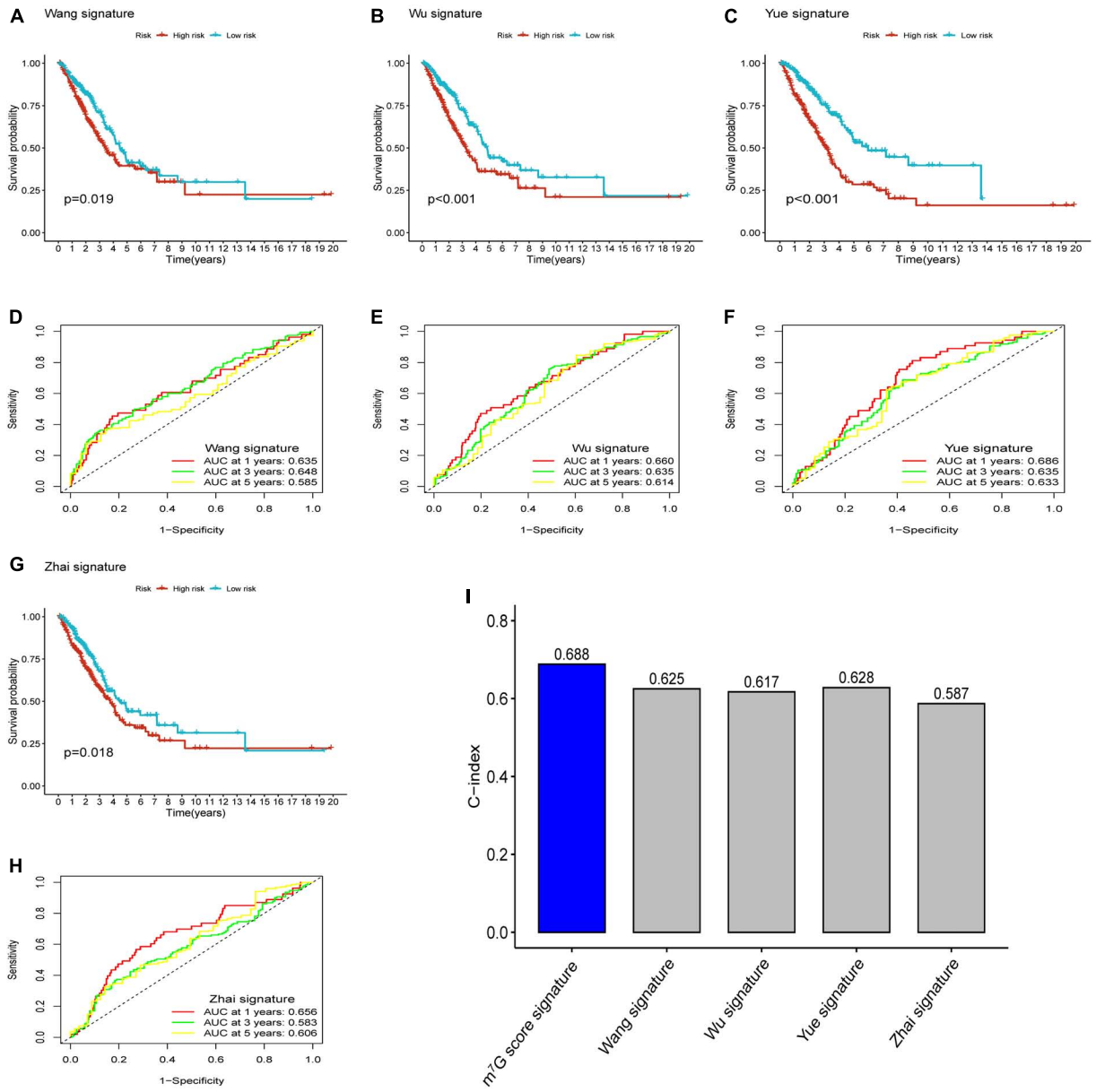

FIGURE 10
Comparison of prognostic signatures. **(A–C,G)** Survival analysis of 5-gene signature (Wang), 4-gene signature (Wu), 3-gene signature (Yue) and 5-gene signature (Zhai). **(D–F,H)** AUC of 5-gene signature (Wang), 4-gene signature (Wu), 3-gene signature (Yue), and 5-gene signature (Zhai) for predicting 1-, 3-, 5-years survival rates. **(I)** C-index of each signature.

Hu et al. (53) demonstrated that the expression of *FAM83A* regulated the proliferation and invasiveness of NSCLC through PI3K/Akt/mTOR pathway. Investigations showed *HOXA13*, as a nuclear transcription factor, was related to tumor cells proliferation and differentiation, which could accelerate tumor aggressive characteristics through disturbing P53 and Wnt/β-catenin signaling pathways in NSCLC (54). One research reported that *HOXA13* was markedly upregulated and strongly correlated with tumorigenesis and progression in LUAD (55). Some studies demonstrated that *PITX3* as a transcription factor was involved in many tumors (56, 57). Zhang et al. (58) presented that high expression of *PITX3* was strongly associated with the poor prognosis in LUAD. According to these evidences, we indicated that patients with high m^7G score in which these four genes were activated, had poor survival. Also, survival analysis demonstrated that the high m^7G score group presented worse survival outcomes. ROC curves further showed the great efficacy of m^7G score to predict survival rate. And, the TCGA test cohort, entire cohort and GSE31210 cohort were applied to validate the accuracy and reliability of the m^7G related

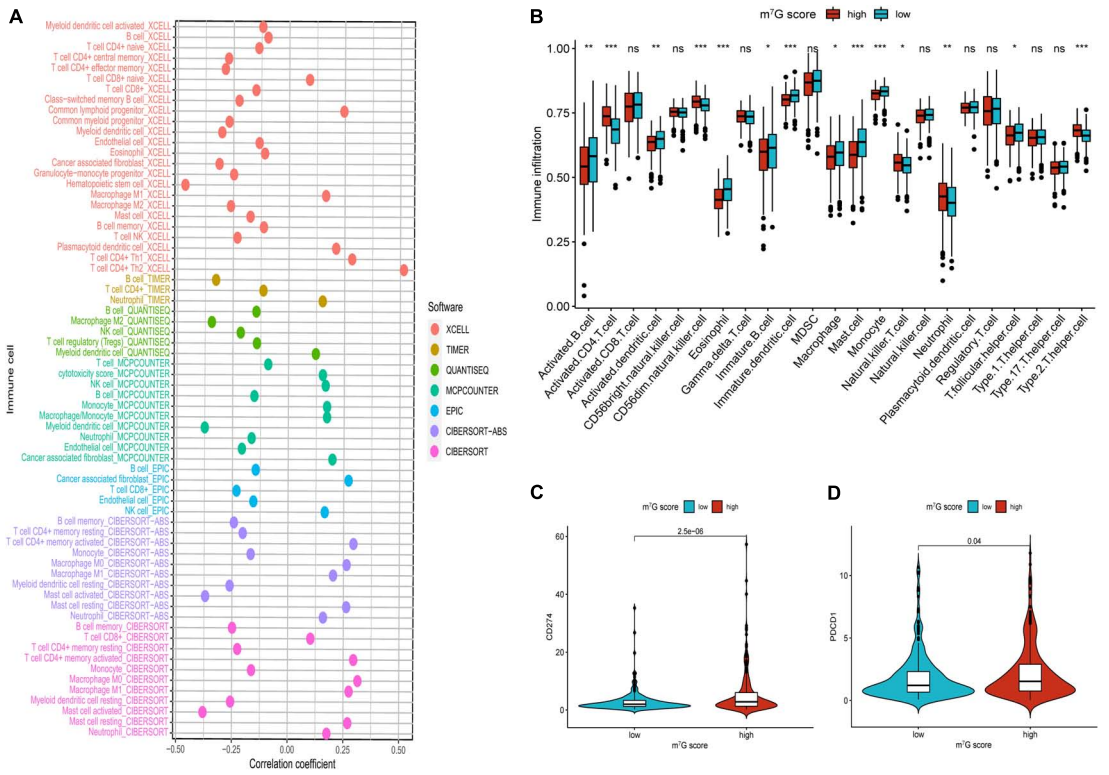

FIGURE 11
Comparison of immune infiltration and immune checkpoints between high and low m^{7}G score groups. **(A)** Correlation between immune cell and m^{7}G score. On the right side of the correlation coefficient = 0 indicates a positive correlation with m^{7}G score. **(B)** Immune infiltration analysis by ssGSEA. **(C)** Comparison of CD274 (PD-L1). **(D)** Comparison of PDCD1 (PD-1). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

signature. Similar results were acquired from all validation cohorts, demonstrating that the prognostic signature may be a robust biomarker to evaluate the prognosis in LUAD. Patients with high m^{7}G score also presented remarkably poor survival condition among different clinical subgroups. After analyzing the association between m^{7}G score and clinicopathological parameters, we observed that m^{7}G score was significantly high in N2 + N3, M1, Stage III + IV and T2-T4, suggesting high m^{7}G score is associate with cancer progression. The characterization of m^{7}G modification patterns showed C2 subtype had lower m^{7}G score compared with C1 subtype. And, the high m^{7}G score correlated with poor survival and cancer progression was consistent with characteristics of C1 subtype. Univariate and multivariate analysis presented that the m^{7}G score was an independent prognosis predictor of LUAD patients. Subsequently, the nomogram also presented high accuracy in predicting survival rate of 1, 3, 5-years. In recent years, many signatures were built to predict the prognosis of LUAD patients. Furthermore, we presented that the AUC area and C-index of our signature were higher than the other four public prognostic signatures, suggesting our signature have better performance in predicting clinical prognosis in LUAD patients.

Currently, although the immunotherapy of lung cancer has got great progress, how to choose the appropriate therapeutic regime for patients is still a clinical challenge. Besides, a part of patients did not obtain effective benefits from immunotherapy (59), and even some patients will undergo obvious side effects during therapy (60). Therefore, it is critical to explore a novel method to guide individualized and precise treatment in LUAD patients. The results of various evaluation methods of immune cell infiltration showed distinct activation of immune cells in both groups, which were similar to molecular subtypes. We speculated there was different immunotherapeutic response in two groups, so we further investigated the association between m^{7}G score groups, immune checkpoint, TMB and TIDE. The immune checkpoints are also an integral part of the immune system and participate in regulating immune escape (61). In recent years, immunotherapy targeting immune checkpoints has obtained huge clinical therapeutic results, especially anti-PD-1/PD-L1 antibody (62). In the study, we observed that patients with high m^{7}G score had upregulated PD-1/PD-L1, indicating that these patients may be more sensitive to ICB than low m^{7}G score. Subsequently, compared with the low m^{7}G score group, the high m^{7}G score group had markedly higher TMB which was consistent with the

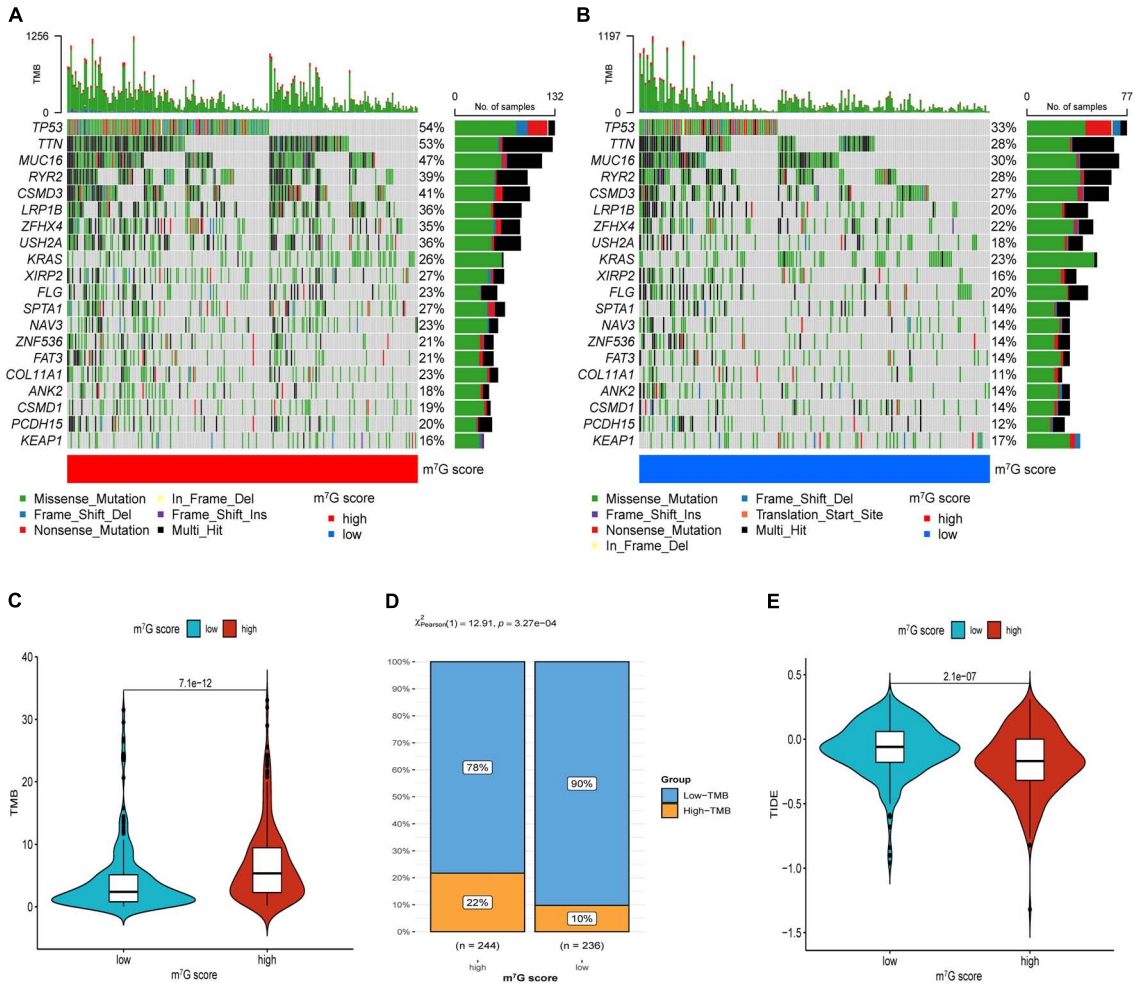

FIGURE 12
Immunotherapeutic response evaluation in high and low m^{7}G score groups. **(A)** Mutation plot in high m^{7}G score group. **(B)** Mutation plot in low m^{7}G score group. **(C)** Results of TMB between two groups. **(D)** Distribution of low and high TMB between two subtypes. **(E)** Results of TIDE between two groups.

C1 subtype. In addition, we found that compared with the patients with low m^{7}G score, patients with high m^{7}G score had more percentage of *TP53* mutation. Studies showed that *TP53* mutation was remarkably related to high PD-L1 expression and patients with *TP53* mutation could acquire benefits from ICB therapy in LUAD (63, 64). Investigations presented TIDE was an accurate biomarker used to predict the immunotherapeutic effects of NSCLC, which was negatively associated with the efficacy of ICB (31). Meanwhile, recent studies have reported the clinical application of TIDE in predicting and evaluating immunotherapeutic response (65, 66). In our study, compared with patients with low m^{7}G score patients with high m^{7}G score had lower TIDE score, suggesting patients in the high m^{7}G score group may obtain clinical benefits from immunotherapy. Integration analysis of the m^{7}G score, immune cell infiltration, immune checkpoint, TMB, and TIDE indicated that the signature is a potential biomarker to assess immunotherapeutic response and tailor individualized treatment for LUAD patients.

Chemotherapy is a classic treatment for lung cancer, but patients have different response rates to chemotherapy drugs. Selecting an appropriate chemotherapy regimen is helpful to improve the prognosis and reduce the economic burden of patients. Our study revealed that common drugs including Cisplatin, Docetaxel, Doxorubicin, Etoposide, Gemcitabine, Paclitaxel, and Rapamycin were suitable for patients with high m^{7}G score, while Axitinib, Bexarotene, Bicalutamide, Erlotinib, Imatinib, Metformin, Methotrexate, Sorafenib, and Temsirolimus were more appropriate for patients with low m^{7}G score.

Our research may assist in judging the prognosis in LUAD, but there are also some limitations. First, the research is a

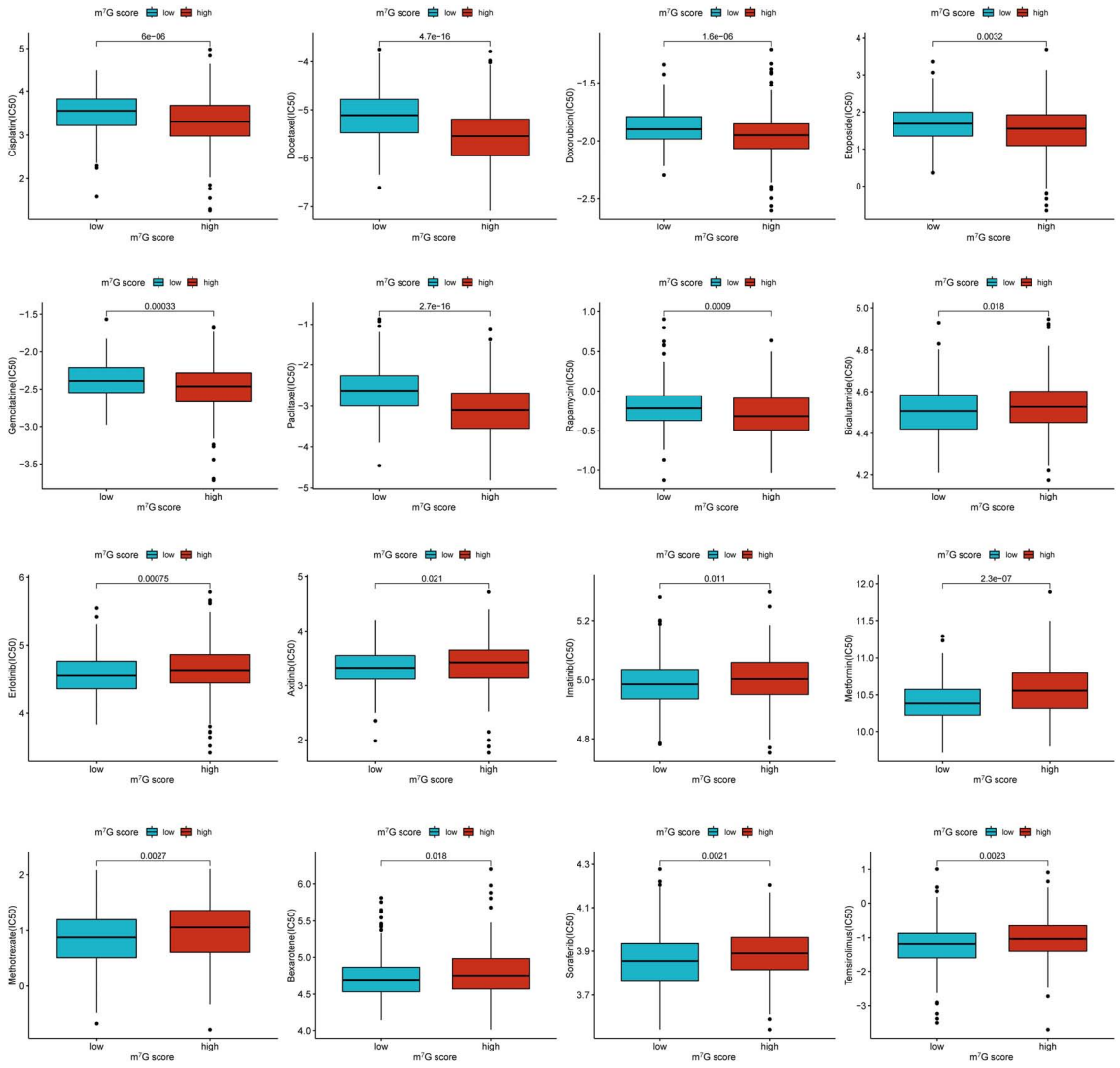

FIGURE 13
Comparison of drug sensitivity between low and high m^{7}G score groups.

retrospective study according to the data from TCGA and GEO datasets, so it's crucial to collect prospective clinical data to further verify the signature. Second, the potential functional mechanisms of m^{7}G score are not fully verified, so these need to be further verified by experiments at the molecular level *in vivo* and *in vitro*. Finally, the drug response in patients is based on methodological prediction, so clinical trials need to be implemented in the future.

Conclusion

In summary, we identified two novel molecular subtypes of LUAD according to m^{7}G regulators. The survival, immune infiltration, and TMB are significantly different in two subtypes. The m^{7}G related signature to quantify the heterogeneity of the two subtypes was constructed. The signature can be employed to predict prognosis in LUAD, then the internal and external cohort were applied to verify the prognostic value. And the signature was elucidated be helpful to guide immunotherapy and chemotherapy. Therefore, this research provides a new direction for improving prognosis and current anti-cancer strategies in LUAD.

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 in the article/ **Supplementary material**.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

ZL and XY conceived and designed the study. ZL and WW performed the data analysis. ZL wrote the manuscript. WW and JW participated in collecting the data and helped draft the manuscript. XY and WW prepared and edited the manuscript. All authors reviewed and approved the 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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.962972/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Survival analysis among different clinical subgroups.

SUPPLEMENTARY TABLE 1
Three m^7G-related gene sets.

SUPPLEMENTARY TABLE 2
201 prognostic genes through univariate Cox analysis.

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The role of autophagy in colorectal cancer: Impact on pathogenesis and implications in therapy

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Colorectal cancer (CRC) is considered as a global major cause of cancer death. Surgical resection is the main line of treatment; however, chemo-, radiotherapy and other adjuvant agents are crucial to achieve good outcomes. The tumor microenvironment (TME) is a well-recognized key player in CRC progression, yet the processes linking the cancer cells to its TME are not fully delineated. Autophagy is one of such processes, with a controversial role in the pathogenesis of CRC, with its intricate links to many pathological factors and processes. Autophagy may apparently play conflicting roles in carcinogenesis, but the precise mechanisms determining the overall direction of the process seem to depend on the context. Additionally, it has been established that autophagy has a remarkable effect on the endothelial cells in the TME, the key substrate for angiogenesis that supports tumor metastasis. Favorable response to immunotherapy occurs only in a specific subpopulation of CRC patients, namely the microsatellite instability-high (MSI-H). In view of such limitations of immunotherapy in CRC, modulation of autophagy represents a potential adjuvant strategy to enhance the effect of those relatively safe agents on wider CRC molecular subtypes. In this review, we discussed the molecular control of autophagy in CRC and how autophagy affects different processes and mechanisms that shape the TME. We explored how autophagy contributes to CRC initiation and progression, and how it interacts with tumor immunity, hypoxia, and oxidative stress. The crosstalk between autophagy and the TME in CRC was extensively dissected. Finally, we reported the clinical efforts and challenges in combining autophagy modulators with various cancer-targeted agents to improve CRC patients' survival and restrain cancer growth.

KEYWORDS

colorectal cancer, autophagy, tumor microenvironment, endothelial cells, hypoxia, oxidative stress, targeted therapy, MSI-H

Introduction

Colorectal Cancer (CRC) is counted as one of the most predominant cancers in both genders with high death rates. CRC is third in terms of prevalence which accounted for 6.1% of new cases and second in terms of the cause of death which accounted for 9.2% of deaths by cancer worldwide (1). There is a high incidence of colorectal cancer at young age (15–39 years) which was estimated by 70.2–82.9 thousand cases in 2019 with a mortality rate of 26.2–30.5 thousand in the same year (2). By the year 2035, it is estimated that the total number of deaths will increase by 71.5 and 60% from colon and rectal cancers, respectively (3). CRC is a heterogeneous disease with numerous variations in its molecular profiles, clinical manifestations and prognosis. CRC prognosis depends on the tumor staging at the time of diagnosis. Currently, the best therapeutic option for stage I and most of the stage II CRC patients is the aggressive surgical resection of the primary tumors which showed high success rates, with/without adjuvant radio-chemotherapy for high risks patients in stage II and stage III of CRC. Notably, stage III CRC patients usually suffer from recurrent disease, which may be associated with micro-metastasis. Stage IV CRC represents a metastatic state with a high risk of relapse and with less/no benefit from surgery. Instead, chemotherapy combinations are usually used at this stage, such as oxaliplatin/irinotecan and folinic acid, 5-fluorouracil (5-FU)-based regimens (4, 5). However, adjuvant treatment is highly accompanied by drug resistance, and ultimately disease progression in metastatic CRC. Recent advances in cancer-targeted therapy as second-line treatment of CRC in combination with chemotherapy, to disrupt signaling pathways or cellular mechanisms, have led to enhanced overall survival (OS) and progression-free survival (PFS). Currently, anti-angiogenic drugs including bevacizumab, regorafenib and aflibercept, are approved as a treatment of metastatic stage of CRC, whereas immunotherapy for CRC is still limited to the MSI-H tumors (6).

Classification system of CRC, based on molecular structure, was established to categorize both the tumor and the surrounding tumor microenvironment (TME) through variations in CRC gene expression (7). TME is a dynamic ecosystem that plays a crucial role in the support and progression of tumors. The composition of TME may significantly affect the tumor response to immunotherapy. TME includes different types of cells, e.g., tumor-infiltrating lymphocytes (TILs), tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), cancer-associated fibroblasts, natural killer (NK) cells, regulatory T cells and dendritic cells (DCs). There are four main consensus molecular subtypes: CMS1, CMS2, CMS3, and CMS4 (8). Both CMS1 and CMS4 subtypes are characterized by increased immune cells infiltration, while CMS1 tumors, in particular, is identified by enhanced Th1-cell response as well as inflamed and stimulated TME. Whereas CMS4 TME is characterized by being inflamed and highly angiogenic, hence a good target for combination therapy. CMS2 tumors are caused by β-catenin pathway activation, with subsequent dendritic and T-cell exhaustion. Therefore, this subtype of tumors does not elicit anti-tumor immune response. CMS3 tumors are characterized by several metabolic pathways dysregulation such as nitrogen, glucose pentose, fatty acids, etc. (7).

Tumor microenvironment of colorectal cancer

Tumors are cellular networks characterized as being different and complex with de-differentiated malignant cell types, tumor stem cells, fibroblasts and endothelial and immune cells. TME is a dynamic ecosystem that plays a crucial role in supporting the progression of tumors. Cytotoxic CD8+ T-lymphocytes (CTL) are considered the major defense mechanism against tumor cells, hence T-cell abundance is a decisive and crucial prognostic factor for immunotherapy and chemotherapy response, particularly at the early tumor initiation stage, where an increased activity of T cells has been reported (9). The PD-L1/PD1 axis is identified as an inhibitor of CTL activity in several CRC phenotypes including Mismatch repair deficiency (MMRd)/Microsatellite instability-high (MSI-H) phenotype in which anti-PD1 monoclonal antibodies are highly beneficial in fighting the tumor (10, 11). Another essential type of T-cells highly associated with colorectal tumors is the Regulatory T-cells (Tregs) (12).

Other cell types in the TME include TAMs involved in regulating metastatic phenotype of cancer and modulating growth and invasion of cancer cells (13, 14). Two sub-populations of TAMs have been identified, the pro-tumorigenic (M2) and the anti-tumorigenic (M1) phenotypes, which are characterized by high plasticity (15). TAMs and myeloid-derived suppressor cells (MDSCs) are the most abundant cells in solid tumors including CRC. Moreover, other immune cell types have been identified in the CRC microenvironment, such as NK cells, TANs, eosinophils and mast cells, with variable roles in CRC progression (16, 17). CRC stroma is well-known for its ability to promote tumor-associated blood vessels. Immune cells and fibroblasts supply tumor cells with VEGF (18). Moreover, matrix metalloproteinase and associated proteases, expressed by CAFs, are abundant in TME.

Autophagy and colorectal cancer

Autophagy signaling in cancer

Autophagy has a diverse and dynamic impact on cancer cells that can affect both tumor initiation, progression and cancer

response to therapy. Recently, vast published data indicate a crosstalk between autophagy-related genes (ATG's) associated pathways with oncogenes and/or tumor suppressor genes. Indeed, the precise role of autophagy in modulating cancer tumorigenicity is highly complicated and is dependent on the context (19). Several autophagy genes might be involved in switching normal cells to CRC under particular conditions. The first autophagy marker indicated to be involved in colorectal carcinogenesis is LC3 (20). One of the LC3 isoforms, named LC3-II, is overexpressed in CRC cells particularly in advanced stages, compared to normal colon cells (21). Notably, low LC3 level has been interrelated to good CRC prognosis, particularly in advanced stages (22). Moreover, ATG5 and ATG10 showed a major role in CRC progression and chemotherapy resistance in several studies. ATG5 was found to be down-regulated in 95% of CRC cases, and its high expression level indicates lympho-vascular invasion (23). In contrast, ATG10 was upregulated in CRC tissues and increased protein expression of ATG10 was accompanied by tumor lymph node metastasis and invasion (24). Another essential protein implicated in autophagy is the activating molecule in Beclin-1-regulated autophagy (Ambra1) protein encoded by the *AMBRA1* gene. Mutated *AMBRA1* gene was found in a subset of colorectal neoplasms (25). Additionally, Beclin-1 gene, UVRAG gene and *Bif-1* gene were highly correlated with CRC carcinogenesis which is explained in the following sections.

Role of autophagy in colorectal cancer initiation

Autophagy is an equilibrating mechanism that promotes anti-malignant mechanism by clearance of unhealthy damaged proteins, DNA abnormalities and reactive oxygen species (ROS). A proper autophagic mechanism is crucial for the mutagen's elimination and appropriate genomic stability as it avoids the genetic defects accumulation that proceeds to malignant transformation. Thereby, autophagy might act as a tumor-suppressor in the early stages of the tumor. Evidence demonstrates that the tumor-suppressive effect is derived from some ATG-proteins such as Beclin-1, which shows anti-oncogenic properties. Tumor suppressor role of Beclin-1 is validated genetically in breast, ovarian and prostate tumors, as mono-allele deletion of Beclin-1 occurs (26, 27). However, Beclin-1 has a debatable role in CRC in that it promotes tumorigenesis, but may paradoxically inhibit CRC cell growth. Increased Beclin-1 expression was associated with better OS in patients with locally advanced colon carcinomas who received postoperative 5-FU chemotherapy for 6 months (28). Beclin-1 Overexpression in cases with resected stage II and stage III colon carcinomas, who received 5-FU-based therapy was associated with worse OS, denoting a potential effect of autophagy in drug resistance (29).

Moreover, allelic loss of UVRAG, an autophagy component, and attenuation of *Bif-1* expression that both interact with Beclin-1 directly, might be correlated to CRC initiation and development (30). UVRAG protein is needed to form a complex with Beclin-1 to induce autophagy; therefore, the loss of this protein results in impaired autophagy machinery. Similarly, Bif-1 serves to induce autophagy *via* interacting with Beclin-1 and UVRAG.

Autophagy displays an important defense mechanism against pathogens and therefore plays an anticarcinogenic role in combatting viral and bacterial infections. For example, autophagic machinery was shown to effectively eliminate digestive cancer-associated pathogens such as *Streptococcus bovis* (*S. bovis*) that may cause CRC (31). In the same study, using autophagy-deficient *ATG5*-/- cells showed *S. bovis* pathogen survival and enhanced multiplication within the cells (31). The presence of infectious endocarditis of *S. bovis* may be followed by colonic neoplasia in an estimated incidence of 18–62% of cases, even after years of its presentation in the host (32, 33). Similarly, 25 to 80% of *S. bovis* bacteremia cases induce colorectal tumors (34). Despite this, the relationship between CRC and *S. bovis* bacteremia has been underestimated for a long time and is under the controversy of whether this association is a result of gastro-intestinal tumor or the *S. bovis* itself could be the etiology of CRC (35).

Role of autophagy in colorectal cancer cell survival and metastasis

In previous studies, autophagy seems to support tumor progression. Autophagy helps tumor cells overcome induced metabolic stress resulting from high proliferative rate, hypoxia and nutrient deprivation due to insufficient blood supply needed by these tumors for proliferation and progression (36, 37). Cancer cells consume more energy and metabolites than normal cells due to their rapid proliferative rate. Both energy and metabolites can be provided to cancer cells by increasing autophagy (38). Autophagy is considered a survival mechanism for cancer cells under hypoxic and metabolic stress conditions to provide them with the energy required for their survival and proliferation (39). In this regard, down-regulation of crucial autophagy proteins level led to restraining cancer growth and reduced oxygen consumption along with the accumulation of abnormal mitochondria, and specifically, autophagy was demonstrated to be essential to promote the growth of *Ras*-driven tumors, including CRC (40). Several *in vitro* studies indicated that gaining autophagy activity in *Ras*-driven cancer cells shows a significant increase in the survival and progression of those cancer cells in several settings of metabolic stress (41).

Besides its critical role in regulating protein turnover and cancer immunogenicity, autophagy has been involved in epithelial-to-mesenchymal transition (EMT), a crucial multistep mechanism needed by tumor cells to metastasize (42, 43). The commonly identified EMT inducer TGFβ is known to induce EMT through the stimulation of SMAD, MAPK, Rho-GTPases and PI3K/AKT (44). During tumor progression, cells that

undergo EMT need to stimulate autophagy machinery for their survival and metastases. In this regard, it has been demonstrated that autophagy is essential for EMT activation and cancer cell metastasis in hepatoblastoma cells (45). Similarly, autophagy is needed in TGFβ1-mediated EMT in non-small-cell lung cancer cells (46). In CRC cells and upon using rapamycin, a specific mTOR inhibitor and an autophagy inducer, starvation-mediated autophagy was demonstrated to induce invasion and migration and increase EMT marker expression; and interestingly, this was reverted by *Beclin-1* knockdown (47).

Effect of autophagy on cancer stem cells

Cancer stem cells (CSCs) are recognized to promote tumor initiation, progression and contribute to therapy resistance. CSCs drive tumor heterogeneity *via* EMT and inflammatory signaling activation (48). Autophagy is identified to promote the survival and control the pluripotency of CSCs in the TME. IL-17B/IL-17RB signaling induces autophagy, and subsequently, autophagy controls and maintains CSCs homeostasis. Interestingly, TRAF6 is recruited in the cytoplasm by IL-17B, which would induce autophagosome formation through Beclin-1 ubiquitination, thus promoting self-renewal and sphere-forming potential in gastric carcinoma (49). Likewise, IGF-2/insulin receptor signaling controls CSCs stemness and pluripotency through autophagy regulation. In CRC, loss of imprinted gene expression of IGF-2 indicated increased autophagy, leading to higher sphere-forming potential, and increased *CD133* expression, which is a marker of stemness (50).

Increased autophagic flux is highly maintained and required by CSCs to promote therapy resistance. In CRC, SOX2 transcriptional factor increases the expression of EMT and *ABCC2* genes and promotes chemotherapy resistance through translocation and activation of β-catenin. Interestingly, SOX2 tends to increase *Beclin-1* expression to induce autophagy and promote chemoresistance. Thus, SOX2-β-catenin/Beclin-1/autophagy pathway is involved in tumor progression and chemotherapy resistance (51). A graphical illustration of the autophagy signaling pathway and its dual role in CRC initiation and progression is displayed in **Figure 1**.

Autophagy signaling modulates tumor microenvironment

Autophagy is actively involved in remodeling TME *via* unconventional secretion of several peptides, proteins and hormones that are typically operated and secreted through the conventional secretory system controlled by the endoplasmic reticulum–Golgi pathway (53). Knockdown of autophagy in both stromal cells and cancer cells is associated with a reduction of several cytokines and chemokines release including IL-1β, IL-2, IL-6, IL-8, IL-18, CCL2, CCL20, TNFα, and LIF. Herein, autophagy is capable of modulating tumor growth, metastasis and angiogenesis as well as immune evasion and stemness maintenance, through autophagy-dependent secretion of pro-inflammatory and pro-invasive factors (54–57). Another tumor secretome released in an autophagy-dependent manner includes growth factors (TGF-β1, b-FGF), extracellular matrix proteins (MMP2, MMP9) and the angiogenesis stimulant (VEGFA) (**Table 1**) (55, 58, 59). Additionally, autophagy deficiency impedes the release and secretion of crucial cytokines and chemokines involved in T cells and DC recruitment, including IFN-γ, CXCL9, CXCL10, and CXCL11, thus immune surveillance escape occurred (**Table 1**) (60).

In contrast, autophagy stimulates the release of specific proteins known as DAMPs (damage-associated molecular patterns) that enhance an immunomodulatory effect by triggering immune cells. Therefore, it enhances the anti-tumor immunity and restricts tumor progression (61, 62).

Cross-talk of autophagy and anti-tumor immunity

In the age of immunotherapy success to fight cancer, there is an increasing demand to know how autophagy modulation affects the response to anti-cancer medications. Evidence suggested a decline in autophagy levels in aging T lymphocytes, indicating that autophagy inhibition might contribute to hematopoiesis and/or systemic immunity impairment (64). Furthermore, the survival of hematopoietic stem cells and memory T cells are dependent on autophagy (65, 66). In the myeloid compartment, autophagy supports B1 cell self-renewal and provides free fatty acids needed by the differentiating cells (67, 68). Additionally, autophagy has a major influence on the tumor-specific CD8+ T cells (69) and memory T-cells (70). Autophagy has been shown to dictate the degradation of cytolytic granules secreted by cytotoxic CD8+ T cells and NK cells (71, 72). Intriguingly, autophagy has a crucial role in protein degradation, thus allowing antigen-presenting cells (APCs), like DCs, to utilize such proteins as antigens on major histocompatibility complex (MHC)-I and II. The process occurs through three main pathways; namely, exogenous, cross-presentation, and endogenous pathways (**Figure 2**). Such role was previously reviewed by Koustas et al. (73).

Furthermore, immune suppressor cells have variable responses to autophagy inhibition. For instance, the immunosuppressive effect of Tregs is highly autophagy-dependent (12). Interestingly, it has been indicated that *ATG5 or ATG7* deletion in T cells produces severe tumor implant rejection in the syngeneic mouse tumor model (74). Another published work demonstrated that inhibition of *Beclin-1* gene expression enhances T cells infiltration into the TME (75).

In the developed TME, TAMs, M2 phenotype, are vital in the growth and metastasis of cancer cells, as well as

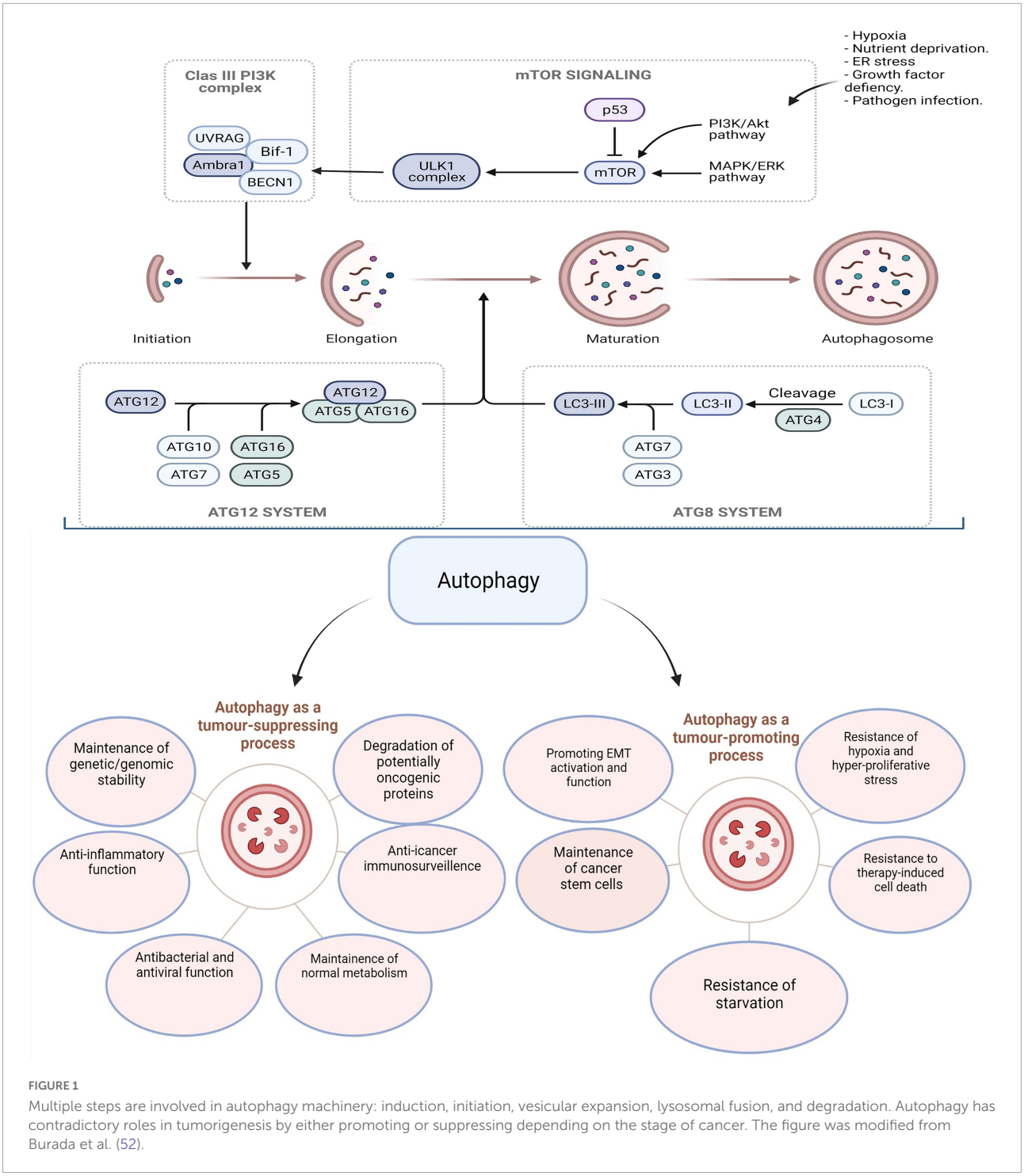

FIGURE 1
Multiple steps are involved in autophagy machinery: induction, initiation, vesicular expansion, lysosomal fusion, and degradation. Autophagy has contradictory roles in tumorigenesis by either promoting or suppressing depending on the stage of cancer. The figure was modified from Burada et al. (52).

angiogenesis (76). On the other hand, several studies proposed that M1 macrophages inhibit tumor progression (77). Autophagy has been shown to participate in the production and polarization of macrophages. Toll-like receptor-2 (TLR2) deficiency is associated with autophagy inhibition and subsequently results in the biosynthesis of M2-type macrophages, which in turn supports tumor progression (78). In addition, autophagy initiation in TAMs promotes apoptotic cell death, restrains proliferation, and enhances radiosensitivity of CRC (79). Altogether indicated that autophagy in TAM plays an essential role in suppressing cancer (**Figure 2**).

Furthermore, other native immune cells critically participate in CRC tumorigeneses, such as tumor-associated neutrophils

TABLE 1 Summarized list of the crucial autophagy-dependent secretome and inflammatory mediator in TME.

Substances/ Secretome	Definition and function
TGF-β1	Transforming growth factor β-1 (TGF-β1) is an important pleiotropic cytokine in wound healing, immunoregulation, angiogenesis and cancer. TGF-β1 isoform is produced by immune cells that exert powerful anti-inflammatory functions.
β-FGF	Beta- Fibroblast Growth Factors (β-FGF) are involved in cell proliferation, differentiation, normal development, wound repair, and angiogenesis. β-FGF is mostly produced by stromal cells in bone marrow, leukemic cells, and T cells. β-FGF is an important regulator in the self-renewal and differentiation of multipotent hematopoietic progenitor cells.
MMP2	Matrix metalloproteinase-2 (gelatinase a); is a type IV collagenase that plays a role in vasculature remodeling, angiogenesis, tissue repair, tumor invasion, inflammation, and atherosclerotic plaque rupture. Also, MMP2 functions as degrading extracellular matrix proteins.
MMP9	Matrix metalloproteinase-9; potentially involved in local proteolysis of the extracellular matrix, leukocyte migration and bone osteoclastic resorption. Also, it cleaves type IV and type V collagen and fibronectin degradation.
VEGFA	Vascular endothelial growth factor-A is involved in angiogenesis, vasculogenesis and endothelial cell growth. As well as it Induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and induces permeabilization of blood vessels.
IFN-γ	Interferon γ; Produced mostly by lymphocytes, has antiviral activity, and an important immunoregulatory functions. It acts as an activator of macrophages and has anti-proliferative effects on transformed cells. IFN-γ can potentiate the antitumor effects of the type I interferons.
CXCL9	C-X-C motif chemokine 9; is a cytokine that impacts the growth, movement, or involved in the immune and inflammatory response. It acts as a chemotactic for activated T-cells.
CXCL10	C-X-C motif chemokine 10; Chemotactic for monocytes and T-lymphocytes. Binds to CXCR3; Belongs to the intercrine alpha (chemokine CxC) family.
CXCL11	C-X-C motif chemokine 11 is an important chemotactic for interleukin-activated T-cells, neutrophils, or monocytes. CXCL11 induces calcium release in activated T-cells. Also, it is participating in CNS diseases that involve T-cell recruitment.

(TANs) and NK cells (**Figure 2**). For instance, promoting autophagy in TANs enhances the migration and metastasis of cancer cells (80). Analogous outcomes have been reported in other cancer types such as melanoma and renal cell carcinoma (81).

Autophagy as a regulator of immune-checkpoints

Additionally, autophagy has an impact on immune tolerance in response to immunotherapy, since immunologic molecules such as indoleamine 2,3-dioxygenase (IDO), Programmed cell death protein 1 (PD-1), and T-lymphocyte-associated protein 4 (CTLA-4) are regulated by autophagy pathways. IDO can inhibit tumor immunity through its inhibitory effects on cytotoxic T-cell responses, DC maturation, and Treg proliferation, thus promoting immune tolerance and tumor development. However, autophagy can inhibit the production of IDO in tumor sites (82, 83). Tumor cell PD-1 interacts with T-cells PD-L1 and serves as an inhibitory checkpoint molecule, preventing tumor cells from being recognized, thus suppressing the antitumor immunity. It has been reported that PD1 inhibits the availability of nutrients to nearby T-cells by interacting with its ligand, inducing autophagy (84). Results from experiments with murine melanoma cells and human ovarian cancer cells suggest that PD-L1-overexpressing cells are more responsive to autophagy inhibitors than cells with weak PD-L1 expression. This finding suggests that autophagy inhibitors may become an important therapeutic tool in PD-L1-overexpressing cancer cells (85). However, further experiments are warranted to explore how PD-L1 signaling and autophagy operate in different cell types, including CRC. This will assist in determining whether anti-PD-L1 therapy combined with autophagy inhibitors will enhance antitumor responses. The CTLA-4 protein is another immune tolerance checkpoint that can be targeted to treat tumors. A cancer-antigen called MAGE-A is associated with CTLA-4 inhibitor resistance and is known to suppress autophagy, suggesting that autophagy induction may be used therapeutically as a way to improve the efficacy of CTLA-4 inhibitors in human melanomas (86). Further experiments are needed to explore cross-talk of autophagy and immune checkpoints in CRC as well. Immune checkpoint therapy for CRC, as a whole, remains unsatisfactory at present. However, there has been renewed interest in examining additional immune checkpoint molecules. New immune checkpoint targets have been identified like the T cell immunoglobulin and mucin domain containing-3 (TIM-3), the V-domain Ig suppressor of T cell activation (VISTA), the T cell immunoglobulin and ITIM domain (TIGIT), and the lymphocyte activation gene-3 (LAG-3) (87–89). Despite an exponential growth in clinical trials for emerging immune modulators, such as anti-LAG-3 antibodies and anti-TIM-3 antibodies, registered on ClinicalTrials.gov, no drugs have yet been approved for clinical use. Despite promising monotherapy results, more effort needs to be integrated toward developing rational combinations of immune-therapy to inhibit cancer growth through non-redundant pathways that work synergistically.

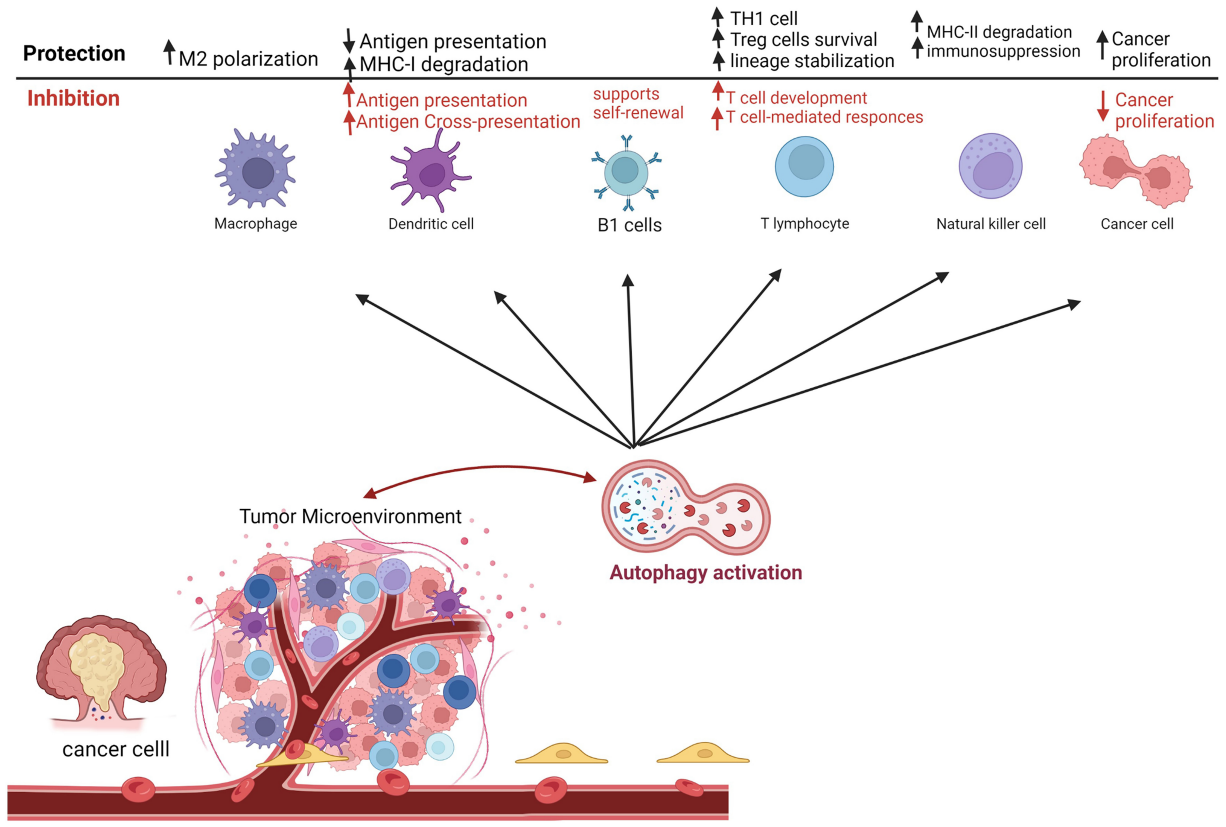

FIGURE 2
Autophagy roles in immune responses as a tumor-suppressive and tumor protective mechanism in the tumor microenvironment. This figure was modified from Zada et al. (63).

Cross-talk of autophagy and endothelial cells

The innermost layer of blood vessels is lined by endothelial cells. In addition to being essential for normal tissue function, new blood vessels also play an important role in cancer pathology. For tumor cells to grow and spread, neovascularization is necessary. Tumor endothelial cells have a multifaceted functional role since they are not only responsible for enhancing angiogenesis, but are also important in immune regulation in the TME (90). Regulatory mechanisms profoundly influence peripheral immune cell recruitment into the TME by acting as significant gatekeepers during cellular transmigration (91–93). Furthermore, tumor endothelial cells act as antigen-presenting cells (APCs), which are associated with T cell activation, proliferation, and priming (92). Furthermore, tumor endothelial cells are required for the development of "tertiary lymphoid structures," which are associated with the response to checkpoint antibody therapy (94). Other qualities that distinguish tumor endothelial cells from normal endothelial cells are their high proliferation potential and markedly changed gene expression profile (i.e., an increase in pro-angiogenic, extracellular matrix remodeling, and stemness genes), leading to increased secretion of immunomodulatory cytokines and altered cell-surface receptors, e.g., MHC and immune checkpoints (90, 95). It is possible that the tumor endothelial cells phenotype is rooted in an aggressive tumor micro-milieu driven by hypoxia and ROS (96, 97). In clinical practice, chemotherapy combined with angiogenesis inhibitor results in marked enhancement of anti-cancer effects in patients with metastatic CRC (98).

Increasing evidence suggests that autophagy impacts endothelial cell survival, proliferation, migration and angiogenesis. However, whether autophagy regulates angiogenesis positively or negatively is still debated. For instance, according to Du et al., overexpressing *ATG5* induced autophagy in bovine endothelial cells resulting in enhanced formation and migration in those endothelial cells while 3-methyladenine (3-MA) or siRNA targeting *ATG5* reduced angiogenesis (99). A study by Goyal et al. discovered that decorin-induced autophagy provided protection against tumor neovascularization and epithelial death (100). Autocrine VEGF released from endothelial cells and gastrin-releasing peptide (GRP) secreted by tumors promote angiogenesis, endothelial survival, and proliferation of endothelial cells by inhibiting autophagy (101). Moreover, a study carried out by Seon-Jin Lee

et al. established that genetically disrupting *Beclin1* can increase tumor growth and angiogenesis in hypoxic environments (102). A broader view suggests that autophagy can influence the angiogenesis process, which is important to tumor growth, by affecting the function and survival of endothelial cells, which has a pro- or anti-tumor effect on CRC.

Autophagy and colorectal cancer metabolism

Autophagy is a conserved catabolic process by which various proteins, cytoplasmic constituents and organelles can re-enter the different metabolic processes. Cancer cells altered their metabolism, thus promoting their proliferation, progression, and long-term survival. Cancer cells enhance glucose uptake and metabolize glucose to lactate even when completely functioning mitochondria support the oxidative phosphorylation mechanism, altogether is known as Warburg effect (103). In the normal process, pyruvate kinase (PKM2), the enzyme catalyzing the last step in the glycolytic process, takes control of the glycolytic flux, preventing the excessive accumulation of glycolytic metabolites (104, 105). However, pyruvate kinase (PKM2) enzyme breakdown is enhanced in cancer cells *via* chaperon-mediated autophagy, thus associated with increased accumulation of glycolytic metabolites (106). Also, hexokinase 2 (HK2), rate-limiting enzyme of the glycolytic pathway, is selectively damaged by autophagy in liver carcinoma (107, 108). Therefore, autophagy plays a vital role in cancer metabolism *via* controlling glycolysis at different stages and levels. Warburg effect elevates lactate level in the TME that disturbs the extracellular environmental pH, resulting in autophagy activation (109). For instance, acute acidification of breast cancer cells results in increased expression of *LC3, ATG5, and BNIP3* (110). Therefore, autophagy destructive effect on vital metabolic enzymes may critically influence many features of central metabolism in cancer. Hence, autophagy contributes to malignancy progression and transformation by providing cancer cells with the efficient ability to re-distribute metabolites allowing metabolic rewiring.

Moreover, as a result of starvation, infections, and cancer, glutamine homeostasis is disturbed and the need for exogenous glutamine to promote cell survival and growth is increasing (111). Due to the Warburg effect, glutamine is excessively required to sustain oxidative phosphorylation through its role as a key intermediate in the tricarboxylic acid (TCA) cycle. Furthermore, it is the main nitrogen source for many aminotransferase enzymes involved in the synthesis of nucleotides and non-essential amino acids (112). Glutamine participates in redox homeostasis by contributing to NADH/NADPH synthesis and glutamate synthesis, which is critical for glutathione synthesis (112). Therefore, with such a wide range of glutamine functions, it is critical for some cancers including CRC to ensure an adequate glutamine supply (113). Targeting glutamine transport and metabolism has therefore been a promising approach for treating CRC (113). As soon as glutamine is deficient or lacking, the cells show differential manifestations, including a pronounced decline in ATP and NADH, as well as a significant accumulation of ROS (114, 115). Herein, Autophagy plays an important role in this adaptive response by suppressing glutamine-consuming processes and elevating glutamine content in the body. Macro-pinocytosis is one of the mechanisms by which activated autophagy restores glutamine levels *via* recycling intracellular proteins and extracellular compartments (116). Meanwhile, some reports claim that autophagy plays a crucial role in cancers that escape death with high success rates (117). Upon limitation of exogenous glutamine, inhibition of autophagy in SW620 and SW480 colorectal cell lines resulted in increased apoptotic activity (118). In the same way, chronic activation of mTORC1 may result in severe mTORC1-dependent cell death (later termed glutamoptosis), ultimately inhibiting autophagy (119). In nutrient starvation, autophagy activation is often associated with cell survival. However, over-activating autophagy in specific contexts has shown anti-tumor potential.

Role of autophagy in the regulation of hypoxia and oxidative stress in tumor microenvironment

Autophagy plays a pivotal role in helping cancer cells adapt and survive under hypoxic TME. Intriguingly, autophagy promotes the survival of cancer cells through its main effector, hypoxia-inducible factor-1α (HIF-1α), which is mostly the case in solid tumors, specially CRC (120). Tumor cells can endure hypoxia through Beclin1-mediated cytoprotective autophagy by upregulating the transcription of *BNIP3 and BNIP3L* (121). Moreover, BNIP3L/NIX functions as a selective receptor for autophagy that is highly expressed in tumor cells, which is crucial to promote mitophagy under hypoxic TME through NFE2L2/NRF2 transactivation. In addition, cells overexpressing NIX, are more susceptible to acquire glioma stem cell-like properties *via* mTOR/AKT/HIF pathway (122). Under hypoxic conditions, a crucial adaptor protein, FUNDC1, is triggered to eliminate dysfunctional mitochondria. FUNDC1 protein functions critically in autophagy *via* engaging with LC3 protein through LC3 interacting region (LIR) of FUNDC1 (123). Additional form of autophagy regulation under hypoxia occurs *via* HMGB1 signaling through upregulating *YAP* expression in tumor cells. Similarly, ATG5 and ATG12 are stimulated by PAK1 acetylation and PTBP3, respectively, resulting in promoting pro-survival autophagy. Furthermore, an important kinase, PRKCA/PKCα, that regulates hypoxia-mediated autophagy *via* ATG5 and Beclin1, stimulates tumor-initiating cell renewal in CRC (124). Likewise, *YTHDF1* gene is activated by HIF-1α to promote autophagy protective effect through ATG2A and ATG14. Of note, protein phosphatase 2 (PP2A) along with mTOR downstream kinase signaling pathways control the prolyl hydroxylase domain-containing protein 2 (PHD2) phosphorylation to govern and promote HIF-1α mediated

autophagy in CRC cells survival (125). Also, *ANKRD37* gene is demonstrated to induce HIF-1α mediated autophagy in hypoxic colon cancer once it translocates to the nucleus (126).

Hypoxia-mediated HIF-1α induction is reported to promote autophagy, thus controlling glycolytic processes to maintain energy supply and cell progression. In this regard and under hypoxic conditions, proline gets metabolized into pyrroline-5-carboxylate (P5C) with the help of proline oxidase (POX) enzyme, which elicits ROS production that promotes protective autophagy mechanism, which is necessary for the survival of HT29 cells (127). Proline oxidase (POX) enzyme role is AMPK-dependent; however, it is controlled in HIF-1α and HIF-2α independent manner (127).

Interestingly, autophagy was demonstrated to restrain oxidative stress-dependent inflammation and promote tumor-suppressor mechanisms. For instance, the transcription activator "BRG1" stimulates autophagosome biogenesis by regulating the transcription of *ATG7, AMBRA1, and Wipi2*, thus attenuating colonic inflammation and CRC development in an oxidative stress-mediated autophagy manner (128).

Autophagy targeted therapy in colorectal cancer

Recent and ongoing clinical trials

Despite the controversial and contextual relationship between cancer and autophagy, it is still considered a promising target for treatment, as many shared regulatory pathways of carcinogenesis and autophagy are involved. Some studies demonstrated that autophagy induction is highly correlated to the resistance of cancer cells to chemotherapy, immunotherapy, and radiotherapy *via* directly modulating cancer cell metabolism or diminishing cell death pathway (72, 129–131). Thus, various preclinical and clinical studies have been conducted to develop pharmacological autophagy inhibitors (132). The most recent development of autophagy inhibitors can be known by tracing the clinical trials (**Table 2**). The most effective targeted therapies recognized in CRC treatment, so far, are anti-angiogenesis such as cabozantinib, apatinib and bevacizumab, and the inhibitors of epidermal growth factor receptor (anti-EGFR) such as cetuximab (133).

For decades, chloroquine has been approved in malaria and arthritis treatment and is currently an inhibitor of autophagy *via* inhibiting the fusion of autophagosomes with lysosomes in the last step of autophagy machinery. Hence, many clinical trials are investigating chloroquine or chloroquine derivatives either alone or in chemotherapy or radiotherapy combinations in patients suffering from different forms of cancers. One trial named CHOICES (Chloroquine and Imatinib Combination to Eliminate Stem cells), a phase II trial, is investigating and comparing the effect of imatinib and hydroxychloroquine combination versus imatinib alone in patients with chronic myeloid leukemia, establishing evidence of autophagy inhibitors concept (134). Apatinib, a tyrosine kinase inhibitor of VEGFR2, has been indicated to stimulate autophagy *via* AKT- mTOR signaling pathway in colon cancer cells (135). Additionally, Cabozantinib is an inhibitor of various kinases responsible for angiogenesis, cell growth and metabolism that showed a major autophagy induction in HCT116 and HT29 CRC cell lines. Notably, cabozantinib in combination with autophagy inhibitors promotes apoptosis in HT29 and HCT116 cells (136). In a study using CRC cell lines, bevacizumab stimulates autophagy as evidenced by punctate patterns of LC3, autophagic vacuoles presence and Beclin-1 accumulation. Autophagy inhibition by targeting *ATG5 and Beclin-1*, *via* RNA interference or chloroquine, enhances the ability of bevacizumab to induce apoptosis and prevent proliferation, verifying the protective role of autophagy. Similarly, *in vivo* studies using small interfering RNA or chloroquine and bevacizumab combination showed significant inhibition in tumor growth when compared to bevacizumab monotherapy (137).

Of note, a combination of temozolomide and hydroxychloroquine is indicated to be safe and tolerable as well as exerted beneficial anti-tumor effect in phase I trial in patients with solid tumors, including CRC, and in advanced melanoma (138). Similarly, another phase I trial documented the significant efficacy of hydroxychloroquine in combination with mTOR inhibitor temsirolimus in tumor suppression (139). On the other hand, a recent phase I study showed that hydroxychloroquine treatment with AKT inhibitor MK-2206 is tolerable but with minimal anti-tumor activity in solid tumors including CRC (140). As evidenced by multiple instances previously reported, autophagy inhibitors as monotherapy might not be a good treatment choice for cancer therapy (141). Treatment combination of hydroxychloroquine with HDAC inhibitor vorinostat in an ongoing phase I study for patients with advanced renal and colorectal cancers shows no significant clinical improvement in the safety profile and in the patient PFS, indicating a limited benefit of adding hydroxychloroquine (**Table 2**) (142).

In a study on CRC cell lines, autophagy inhibition by 3-MA showed significant 5-FU-induced apoptosis, thus autophagy might have a crucial role in enhancing response of colon cancer cells treated with 5-FU (143). Likewise, another study using chloroquine, an autophagy inhibitor, in combination with 5-FU showed an enhanced anti-proliferative effect of 5-FU in CRC cells (144). More, inhibiting late-stage autophagy has been demonstrated to enhance the apoptotic cell death activity of the pyrrolo-1,5-benzoxazepines (PBOXs) in human CRC cells (145). Moreover, UAMC-2526 displays inhibitory effects on ATG4. This compound abolishes autophagy in mice bearing colorectal tumors and promotes chemotherapy-induced cell death (146). Recent *in vitro* assays and *in silico* screening has

TABLE 2 Previous and current clinical trials involving hydroxychloroquine (HCQ) in combination with a variety of anti-cancer targeted agents in CRC.

Treatment	Target of the treatment	Phase	Patients number	Status	Outcome	Trial reference number at ClinicalTrials.gov/References
Vorinostat + HCQ	Histone deacetylase (HDAC) inhibitor.	I	72	Active not recruiting	No significant clinical improvement in the safety profile and the progression-free survival.	NCT01023737 (142)
Temsirolimus + HCQ	mTOR inhibitor.	I	40	completed	Safe and tolerable, Significant tumor suppression effect.	NCT00909831 (139)
Temozolomide + HCQ	DNA alkylating agent/induce cell cycle arrest at G2/M.	I	38	completed	Safe and tolerable, beneficial anti-tumor effect.	NCT00714181 (138)
Protein kinase B Akt inhibitor (MK-2206) + HCQ	Akt inhibitor.	I	62	Active not recruiting	Tolerable, minimal anti-tumor activity.	NCT01480154 (140)
HCQ, FOLFOX and bevacizumab.	FOLFOX: chemotherapy that inhibits DNA synthesis. Bevacizumab: VEGF/VEGF receptor inhibitor.	II	38	completed	Increases in autophagy marker LC3 with a complete response rate of 11% but without improved OS in the 28 evaluable patients.	NCT01006369 (98)

identified a new, important ATG4B inhibitor (S130) that has the ability to interfere with ATG4 proteolytic activity but not with other proteases. Also, S130 is well distributed in tissues *in vivo*, enhances cell death in CRC and reduces the tumor size (147). These findings identify ATG4B as a potential anti-cancer target.

Challenges and potential solutions of the autophagy targeted treatment

Based on studies and clinical trials described above, it seems that autophagy inhibitors have a different clinical response in cancer therapy. Identification of good biomarkers with suitable pharmaco-dynamic properties that can estimate any change in autophagy, is of the major difficulties facing scientists (148). It remains to be explored whether the limited clinical efficacy of chloroquine is correlated with its lack of specificity in inhibiting autophagy. In fact, both chloroquine and hydroxychloroquine are non-selective autophagy inhibitors which are evident by their role in the reduction of nutrient scavenging (149). This diminished targeted delivery results in plummeting the bioavailability of the drugs. However, hydroxychloroquine is characterized by higher bioavailability compared to chloroquine. Moreover, both drugs have been identified to modify the pH of tumors, hence resulting in bioavailability modulation of different cytotoxic drugs when used in combination (150). Furthermore, frequent use of chloroquine has been identified for a long time to elicit renal failure (151). Noteworthy, both hydroxychloroquine and chloroquine could affect pacemaker channels and voltage-gated Na^+, Ca^{2+}, and K^+ ion channels in the heart, leading to serious dysrhythmias.

In this regard, there is an urgent need for novel safe autophagy inhibitors with selective targets and a good bioavailability; properties that many proposed drugs failed to reach. One of the major advancements in the field is the discovery of Lys05, a dimeric form of chloroquine, which shows higher accumulation capabilities in the lysosome. Also, Lys05 has been identified to exert potent monotherapy anti-tumor activity in both *in vitro* and preclinical mouse models with limited toxicity in the treated mice. Of note, Lys05 potent characteristic in autophagy inhibition is dependent on C7-Chlorine, bivalent aminoquinoline rings and a short tri-amine linker (152).

Recently, new druggable autophagy target proteins have been established, including Vps34 (or class III PI3K) and Beclin-1. Notably, both proteins are involved in the early autophagy initiation process. A kinase inhibitor, SAR405, inhibits both Vps34 and Vps18, thus diminishing the lysosomal function *via* disturbing the vesicle trafficking between the lysosome and the late endosome. Further, SAR405 has been found to prevent mTOR- and starvation-dependent stimulation of autophagy (153).

Another druggable protein for autophagy modulation which has been recently proposed is the serine/threonine kinase ULK1/ATG1. Identification of small-molecule SBI-0206965, a potent ULK1 inhibitor, was happened through cell-based screen. This inhibitor was found to be high *in vitro* selective for ULK1 kinase as well as suppressed phosphorylation events mediated

by ULK1 kinases. Markedly, SBI-0206965 anti-tumor effect has been evidenced *in vivo* as it showed potent tumor inhibition when combined with mTOR inhibitors, hence allowing it for use in the clinic (154). However, a major limitation of this molecule is that it could affect the activity of other kinases including JAK3, FLT3, FAK, and Src.

Conclusion and perspectives

A large number of proteins involved in the complex process of autophagy, which appears to play a significant role in all stages of carcinogenesis as it impacts tumor progression, initiation and metastatic capacity. Although the role of autophagy is not fully understood in cancer, it is thought to play both a promoting and inhibiting role depending on the context. Thus, it is imperative to identify how these apparently paradoxical roles of autophagy are regulated in CRC, and to constitute an overall view of the mechanisms that enable autophagy to play one role, not the other.

Autophagy modulates the effect of hypoxia and oxidative stress, regulates metabolism, promotes cancer stem cells and constrains the surveillance of immune cells to support cancer progression. The development of several therapeutic agents that modulate autophagy in CRC has led to promising results, supporting their use to enhance the action of other medications. Currently, autophagy inhibitors used in cancer therapy are limited to hydroxychloroquine and chloroquine that require close monitoring, when used for a prolonged period, for hepatic and renal adverse effects. Therefore, there is an urgent need for more translational and basic research to clarify the intricate role of autophagy, and to resolve unanswered questions about the enhanced efficacy of autophagy-targeted cancer therapy. Notably, there is an increased interest in personalized cancer treatment by joining the TME modulation status with advanced technology to explore the alteration in cancer progression. This will hopefully propose a major success in cancer therapy.

Author contributions

EM and MS-A: conceptualization. EM: writing—original draft preparation and visualization. JT, NS, and MS-A: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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Non-steroidal anti-inflammatory drugs and biomarkers: A new paradigm in colorectal cancer

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Colorectal cancer is a sporadic, hereditary, or familial based disease in its origin, caused due to diverse set of mutations in large intestinal epithelial cells. Colorectal cancer (CRC) is a common and deadly disease that accounts for the 4th worldwide highly variable malignancy. For the early detection of CRC, the most common predictive biomarker found endogenously are KRAS and ctDNA/cfDNA along with *SEPT9* methylated DNA. Early detection and screening for CRC are necessary and multiple methods can be employed to screen and perform early diagnosis of CRC. Colonoscopy, an invasive method is most prevalent for diagnosing CRC or confirming the positive result as compared to other screening methods whereas several non-invasive techniques such as molecular analysis of breath, urine, blood, and stool can also be performed for early detection. Interestingly, widely used medicines known as non-steroidal anti-inflammatory drugs (NSAIDs) to reduce pain and inflammation have reported chemopreventive impact on gastrointestinal malignancies, especially CRC in several epidemiological and preclinical types of research. NSAID acts by inhibiting two cyclooxygenase enzymes, thereby preventing the synthesis of prostaglandins (PGs) and causing NSAID-induced apoptosis and growth inhibition in CRC cells. This review paper majorly focuses on the diversity of natural and synthetic biomarkers and various techniques for the early detection of CRC. An approach toward current advancement in CRC detection techniques and the role of NSAIDs in CRC chemoprevention has been explored systematically. Several prominent governing mechanisms of the anti-cancer effects of NSAIDs and their synergistic effect with statins for an effective chemopreventive measure have also been discussed in this review paper.

KEYWORDS

colorectal cancer, biomarkers, NSAIDs, colonoscopy, KRAS, chemoprevention, COX-pathways, statins

1. Introduction

Colorectal cancer remains one of the fourth most common malignancies worldwide after lung, liver, and stomach cancer. It majorly develops after the age of 50 whereas, a dramatic increase in the younger generation has been observed with an expected increase rate of 140% by the year 2030. A significant disparity in the incidence and survival rates of CRC between developed and developing countries depicts a difference in socioeconomic development (1).Genetic inheritance has been proved to play an important role in the development of CRC, with men being the major targets. Apart from genetic predisposition, lifestyle factors such as inactivity, type-2 diabetes mellitus (TDM), alcohol consumption, smoking, and obesity also influence the risk of CRC (2). Familial adenomatous polyposis and lynch syndrome are the two most prominent inherited syndromes that account for approximately 5% of all CRC. The accumulation of genetic mutations results in the transformation of normal colonic epithelium to a precancerous lesion and ultimately to invasive carcinoma over 10-15 years. Whereas people having adenomatous polyps or polyps with villous or tubulovillous dysplasia are at higher risk of developing synchronous and metachronous CRC primary cancer. Unfortunately, people who survived cancer at a childhood age and received abdominal radiation are at higher risk of developing CRC thus, it is recommended to adopt a screening session after 10 years or at the age of 35 (3). Hence, the early detection and removal of preformed or developing polyps will eliminate the chances of CRC. Polyps which are hamartomatous and serrated have also proven to be responsible for leading to CRC. The molecular pathways such as chromosomal instability, mismatch repair and hypermethylation has been attributed to the major pathways linked to CRC (4). Adenocarcinomas accounts for more than 90% of CRC whereas adenosquamous, spindle, squamous and undifferentiated are frequently not seen. Among the treatments for CRC, surgical resection is commonly adopted for localized non-metastatic stage CRC. Additionally, palliative systemic chemotherapy and the use of NSAIDs as chemoprevention are offered to non-surgical candidates and may prove to be a curative option (5).

Surgical removal of polyps and increasing death of CRC requires the demand of risk assessment, screening, differential diagnosis, prognosis determination, treatment response prediction, and disease progression monitoring. These potentialities are determined with the help of biomarkers in oncology. Biomarkers help in biological observation, which ideally predicts the endpoint or intermediate outcome of a disease at an early stage where it is difficult to be observed (6). Biomarkers must undergo a thorough evaluation, including analytical validation, clinical validation, and assessment of clinical utility, before being incorporated into routine clinical care because of the crucial role they play at all stages of the disease (7). In CRC treatment biomarkers, molecular pattern act as a tool for the early detection of colorectal cancer. These biomarkers play an important role in the early detection and well-individualized treatment of people suffering from cancer. The various categories of biomarkers are predictive, prognosis, and diagnostic which help to determine the progression and recurrence of cancer whereas, it also proves to be an effective therapeutic target (8). The detail view of various biomarkers along with their potentiality in CRC has been mentioned systematically in the next section.

After an early detection of CRC, the intervention of therapeutics to curb the progression of colorectal cancer becomes an important task. Hence, NSAIDs are believed to have a chemopreventive impact on gastrointestinal malignancies, and more especially, on colorectal cancer, according to a significant body of data from epidemiological and preclinical research (9). Non-steroidal anti-inflammatory drugs (NSAIDs) are a group of chemical compounds that are typically unrelated yet have some therapeutic qualities and side effects. They are among the most widely used medicines in the world and have potent analgesic, antipyretic, and anti-inflammatory properties (10). NSAIDs are among the most widely used medications, supporting their inclusion on the WHO's Model List of Essential Medicines due to their effectiveness in lowering pain and inflammation (11). They primarily work by inhibiting two cyclooxygenase enzymes, which stop the production of prostaglandins (PGs). Numerous cellular activities, including gastrointestinal cytoprotection, hemostasis and thrombosis, inflammation, renal hemodynamics, cartilage turnover, and angiogenesis, depend heavily on PGs. A lot of different illnesses' pathophysiologies are heavily influenced by inflammation. PGs, coagulation cascade-derived peptides, interleukin IL-2, IL-6, and tumor necrosis factor (TNF) are among the inflammatory mediators whose production and activity are affected by NSAIDs (12). Long-term use of NSAIDs has also been linked to renal illness, which can cause both acute and chronic abnormalities in kidney function (13). The US Food and Drug Administration (FDA) was led by these consequences to issue a scientific statement in 2005 that emphasized, "the necessity of utilizing the lowest effective dose for the shortest time feasible if therapy with an NSAID is necessary for an individual patient" (14).

2. Biomarkers

According to the National Cancer Institute, a biomarker is a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal activity, as well as of a condition or disease, such as cancer (NCI). A patient with the condition can frequently be distinguished from a healthy person using biomarkers. The adjustments could be brought on by post-translational modifications, somatic or germline mutations, transcriptional changes, or other factors. Proteins (such as an enzyme or receptor), nucleic acids (such as a microRNA or other non-coding RNA), antibodies, and peptides are only a few examples of the wide variety of biomarkers. A few examples of the kinds of alterations that can be regarded biomarkers are changes in gene expression, proteomic signatures, and metabolomic signatures. In order to be analyzed non-invasively and serially, biomarkers can be detected in the circulation (whole blood, serum, or plasma), excretions or secretions (stool, urine, sputum, or nipple discharge), or they may be formed from tissues, necessitating a biopsy or specialized imaging. Sequence variations in germ-line DNA recovered from whole blood, sputum, or buccal cells are examples of inherited genetic biomarkers. Mutations in DNA extracted from tumor tissue are examples of somatic genetic biomarkers (8). Briefly tabulated certain biomarkers and their significance in various type of cancer in Table 1. Prostate-specific antigen (PSA) is a frequently employed but contentious biomarker for screening (22). Biomarkers can be used to assess a patient's prognosis, or the likelihood of the disease returning without regard to treatment,

TABLE 1 Biomarkers and their significance in various type of cancers.

S. no.	Type of cancer	Biomarkers	Significance	Drawbacks	References
1.	Lung cancer	- Plasma CD4 levels	- Identification of benign lung nodules-89% Specificity	- No validation for high-risk individuals-Mild CT screening trial	(15)
		- miRNA	- 81 % specificity - 87% sensitivity	-	
		- ctDNA - CTCs	- Tumor shed Product	- Advanced tumor stages - Sensitivity 57%	
		- Blood antigens: CYFRA 21-1, CEA, NSE, SCC-Ag	- 88–95% specificity	- Multi-antigen approach is required	
2.	Liver cancer	- GP73 - CA19-9 - GPC3 - Hep Par 1 - Gs - Arg 1	- Helps in Diagnosis - Prominent Indicators - Average 95–100% specificity	- Combined identification is required	(16)
3.	Stomach cancer	- CEA - CA19-9 - CA72-4 - CA125 - HER2	- Helps in early detection - Involved in diagnosis and prognosis	- HER2-Prognosis is not established	(17)
4.	Colorectal cancer	- KRAS - BRAF	- 94–98% specificity - Prognostic and predictive factor	-	(18)
		- PTEN	-Predictive factor		
		- TP53	- 58% sensitivity - 88% specificity		
		- CEA	- Screening - Prognostic factor		
5.	Ovarian cancer	- CA125	- Predicts prognosis EOC	- Low sensitivity 67.39% - No clinical value	(19)
		- HE4	- Detection of Endometrioid - 91.4% specificity	-	
		- OPN	- Early detection	-	
6.	Prostate cancer	- PCA3	- Significant biomarker - Approved biomarker - Specificity 88%	-	(20)
		- PSA glycoforms - MPRSS2-ERG	- Detection, potential new biomarkers	- Not approved yet	
7.	Breast cancer	- BRCA 1/2	- 98-100% specificity - Early diagnostic and prognosis of cancer	-	(21)

after a cancer diagnosis. More lately, the prognosis for specific malignancies is being determined using modern methods. Additionally, biomarkers can be used to modify the response to a particular therapy, or as "predictive factors," or to determine which therapy is most likely to be successful. Because somatic mutations in KRAS are linked to poor response to anti-epidermal growth factor receptor (EGFR) focused therapy, KRAS is a predictive biomarker for colorectal cancer (23).

Overexpression of the estrogen receptor in breast cancer predicts sensitivity to anti-endocrine therapy like tamoxifen (24) whereas overexpression of the HER2 gene or gene amplification in gastric and breast cancers predicts response to anti-Her2 drugs like trastuzumab. Chemotherapy sensitivity and resistance assays, which have been researched in a variety of tumor types, are potential somatic biomarkers for predicting response to therapy. These assays are offered commercially and have been the subject of numerous published clinical investigations (25).

Biomarkers can be utilized to identify early disease recurrence in patients who have finished adjuvant therapy before they experience symptoms. For instance, serial monitoring of CEA after adjuvant treatment for colon cancer is done to look for liver metastases while they are still treatable and resectable (26). Similar to this, beta HCG, lactate dehydrogenase, and alpha-fetoprotein are serially examined in non-seminomatous germ cell tumors to look for early disease recurrence. Additionally, biomarkers can be used to monitor the efficacy of treatment in the context of metastatic disease. Circulating soluble protein tumor indicators such CEA, PSA, CA125, the MUC1 antigens CA15, CA27.29, and CA19, as well as the efficiency of palliative care in metastatic colorectal, prostate, ovarian, breast, and pancreatic cancers, are suggested (27).

2.1. Synthetic biomarkers

Some researchers are adopting a different strategy rather than depending on endogenous signals, which come from the body. They are tricking a tumor into secreting synthetic biomarkers that can be detected in biofluids while the tumor is still undetectable by any existing technique by using cunning engineering technologies and tumor-specific biological knowledge. When ingested, the exogenously supplied bioengineered sensors can send out a signal indicating the presence of cancer cells. These techniques have successfully detected significantly lower tumor sizes in animal models (28). The biological, physiological, and statistical constraints of endogenous biomarkers serve as a justification for the development of synthetic biomarkers. Endogenous biomarkers such as proteins in the pool of blood and having varying secretion rates are difficult to detect due to short periods of retention and frequent clearance from circulation (29). These represent a new class of diagnostics that use bioengineered sensors such as molecular probes or genetically encoded vectors that take the advantage of the potentially dysregulated characteristics of early stage tumors or their precursors which could become lethal, inside the body to scan for early stage tumors and amplify illness signals to levels that may be greater than those of shed biomarkers detectable from body fluids such as blood and urine. Several imaging techniques also employ synthetic biomarker approach including reporter gene imaging, in which an exogenous molecular tracer (such as a positron emitting probe) is systematically infused (29). Synthetic biomarkers on the basis of their activities are called protease-activated synthetic biomarkers that are particularly effective molecular amplifiers. Apart from it vector-based, mammalian cell-based, and bacterial cell-based synthetic biomarkers are also employed on the basis of their advantages (Figure 1). Moreover, some preclinical studies have reported the potential use of activity-based sensor composed IONPs synthetic biomarkers for early detection of LS174T colorectal cancer (30).

3. Biomarkers for early detection of colorectal cancer

Colorectal cancer is treatable if caught early enough. As a result, early identification of colorectal cancer can minimize mortality. The categories of colorectal biomarkers that are now studied include proteins, mutated and methylated DNA, RNAs that are mostly microRNAs, volatile organic chemicals, alterations, and variations in gut microbiota makeup. It is generally known that early-onset CRC is becoming more common and is more deadly among those under the age of 50. These patterns have prompted thorough research aimed at clarifying the epidemiology and characteristics of early-onset CRC as well as formulating tactics for early identification and prevention. It is generally known that during the past 30 years, early-onset CRC incidence has grown globally (31). The identification of blood-based biomarkers may be a useful screening method for CRC due to how simple it is to donate or collect blood. A significant percentage of sporadic, non-hereditary malignancies have genetic abnormalities in the initial phases of carcinogenesis. Large numbers of these aberrant cells are shed from the expanding tumor, and their cell-free nucleic acids can be found in biological effluents, especially in urine, serum, and faeces. To promptly detect genetic disorders, molecular biomarkers with higher sensitivity and specificity than the faecal occult blood test (FOBT) or faecal immunochemical test (FIT) can be utilized (32). The biomarkers can be grouped into broader categories: Blood, Tissue, Stool, and Others.

3.1. Blood biomarkers

1. **Tumor cells in circulation**: According to a recent study, a limited fraction of circulating tumor cells (CTCs) with the ability to cause metastasis include those that express the molecules; EpCAM, CD44, CD47, and MET. It has been found that individual CTCs from the same patient have different KRAS, BRAF, and PIK3CA mutations. CTC are detected using flow cytometry and immunocytochemical analysis are also highly sensitive methods to detect biomarkers for CRC (33).
2. **Tumor DNA in circulation**: There is circulating tumor DNA or ctDNA called cell-free DNA (cfDNA) in cancer patients that are a diagnostic biomarker for CRC (34). cfDNA contains mutations, methylation, microsatellite instability, and loss of heterozygosity that contribute to tumor-specific alterations (35). There is a high concentration of cfDNA in neoplastic disease. The ctDNA/cfDNA is considered a novel biomarker for the early detection of colorectal malignancies.

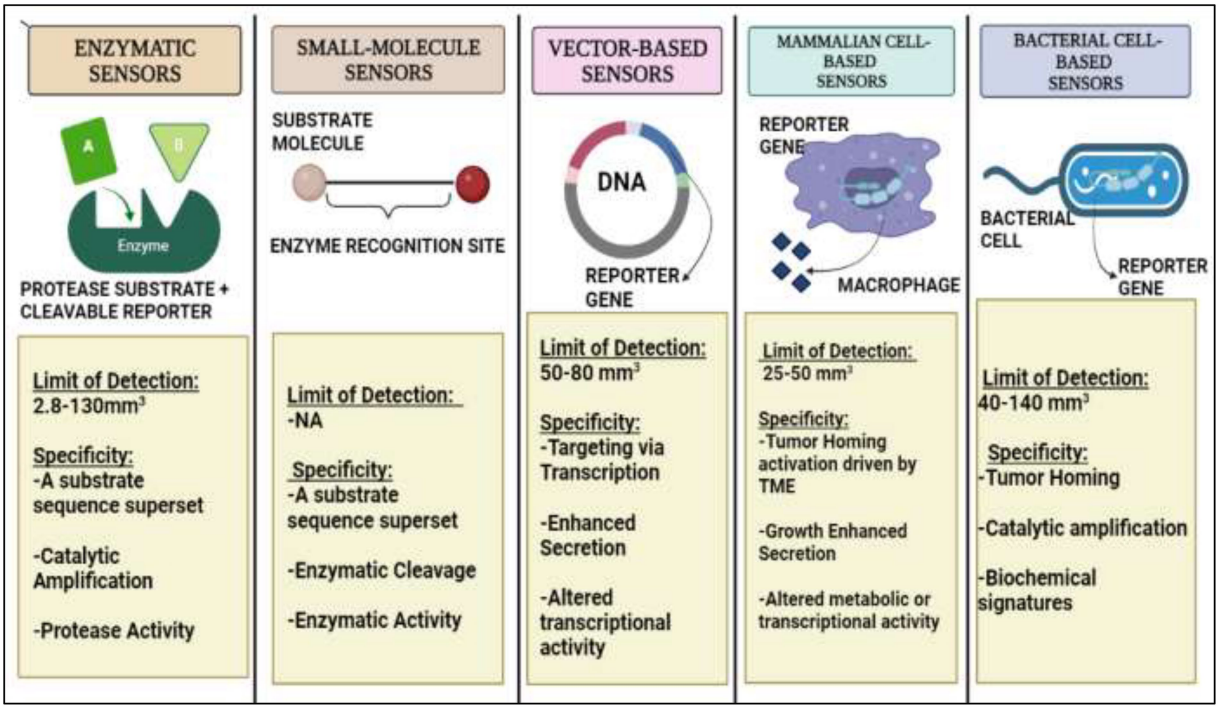


FIGURE 1
A detailed diagrammatic overview of Synthetic Biomarkers on the basis of their activity and various methodologies for endogenous administration.

3. **Micro RNAs in circulation**: Non-coding RNAs subclass contains more than 38,500 microRNAs in human beings discovered so far. Circulating upregulated or downregulated miRNAs like miR-18a, miR-31, miR-145, miRNA-486, miRNA-320, miRNA-451, etc., are sensitive biomarkers for CRC (36). Other Biomarkers like ctDNA, mSEPT9 DNA, miR-31, miR-141, miR-224-3p, miR-576-5p, miR-4669, miR-21, exosomal miR-548c-5p, lncRNA CRNDE-h, etc. were identified as biomarkers of CRC. Dysregulation of miRNAs is frequent in CRC and hence are potential biomarkers. RT-qPCR and next-generation sequencing (NGS) are a few methods to detect these miRNAs.
4. **DNA methylation-based biomarkers**: In CRC the most frequent process to occur as compared to genetic mutation is the methylation of the CpG island of the promoter. More than 600 hypermethylated genes have been identified so far. Among these, the best-known biomarker for CRC is the *SEPT9* methylated DNA. There are 13 genes in the *SEPT* gene family, which is located on chromosome 17q25 in the human genome (37).
5. **Long non-coding RNA-based biomarkers**: Long noncoding RNAs (lncRNAs) have been shown to be stable in blood and to have diagnostic potential during the past 10 years (38). Through processes, such as chromatin remodeling, chromatin interaction, competing endogenous RNAs, and natural antisense transcripts, they can affect cancer cells (39). Because lncRNAs may pass across cell membranes, they can be discovered in a variety of bodily fluids, including blood, plasma/serum, and urine. Various biomarkers used for CRC detection based on these lncRNAs are CCAT1, HOTAIR, LOC285194, RP11-462C24.1, BCAR4, BLACAT1, UCA1, 91H, PVT-1, MEG3, ATB, CCAT1, NEAT1, etc.
6. **Others**: Pyruvate kinase M2 (PKM2), an isoform of pyruvate kinase enzyme is reported as overexpressed in CRC. PKM2's great sensitivity makes it appear like a viable blood and fecal biomarker for CRC screening (40).

3.2. Tissue biomarkers

1. **Transcription factors**: The caudal type homeobox transcription factor (CDX2) is one of many transcription factors that contribute to the development and differentiation of the intestine (41). It is a widely used immunomarker for CRC as it is a tumor suppressor gene in CRC and its expression is lacking in CRC cases. Another transcription factor special AT-rich sequence-binding protein 2 (SATB2) regulates skeletogenesis and is a CRC biomarker with a positivity rate of 83.7% of stage III/IV colorectal adenocarcinomas, 91.4% of stage II and 92.4% of stage I of this malignancy (42).
2. **Transmembrane glycoproteins**: The A33 antigen, a type I transmembrane glycoprotein of the immunoglobulin superfamily, is expressed in the basolateral membranes of both proliferating cells in the lower regions of the crypts and differentiating cells in the upper regions of the crypts, as well as in 95% of colon tumors in the colon and small intestine (10). Another glycoprotein, a member of the cadherin superfamily, cadherin-17 (CDH17) is a calcium-dependent transmembrane

glycoprotein (43). In normal, metaplastic, and neoplastic tissues of the gastrointestinal tract, CDX2 binds to elements in the 50 flanking regions of the gene to regulate this cadherin's transcription. With a specificity of 50–83.8% and a sensitivity of 96–100%, CDH17 is a helpful immunohistochemical marker for the identification of primary and metastatic colorectal adenocarcinomas (44).

3. **Telomerase**: Telomeres, which guard the ends of chromosomes, have certain hexameric repeats (TTAGGG)n in them. They control the longevity of cells and chromosomal integrity. A telomere-specific reverse transcriptase (hTERT) found in telomerase is similar in structure and function to viral transcriptase. The replicative capacity of CRCs and the risk of recurrence are increased by the overexpression of hTERT (45). hTERT appears to be a recurrent biomarker that may be utilized to track systemic treatment responses.
4. **Cytokeratins**: The intermediate filament-forming protein known as cytokeratins is found in the cytoplasmic cytoskeleton. The only cells that exhibit them are epithelial cells. Numerous cellular processes, including cell size determination, apical-basal polarization, protein translation regulation, organelle location, and membrane protein targeting, are regulated by cytokeratins (46). In CRC diagnoses, cytokeratins are frequently utilized as immunohistochemistry markers. Various cytokeratins involved in the prognosis expressed in CRC patients are cytokeratin 7, cytokeratin 20, cytokeratin 20+/cytokeratin 7-, cytokeratin 15, and cytokeratin 18 (47).

3.3. Stool biomarkers

As the exfoliating tumor cells first occur in the large intestine or rectal lumen during colorectal carcinogenesis, stool specimens are more suitable for the early identification of CRC than blood samples (48). The presence of stool biomarkers has resulted in the early detection of CRC. The guaiac-based fecal occult blood testing (gFOBT) and fecal immunochemical test are mostly used for the screening of rectal blood loss, which is the biomarkers in the stool. The fecal microRNA-106a test is used to detect mRNAs in stool (37). Tumor suppressor genes are rendered inactive by hypermethylation at every stage of carcinogenesis, from polyps to colorectal adenocarcinomas. Many genes, particularly those in the promoter region, are methylated in CRC, including APC, MLH1, MGMT, SFRP1, SFRP2, CDK2A, TIMP3, VIM, SEPT, CDH1, and HLTF (49). There are numerous methylated DNA stool biomarkers used in CRC like SFRP methylation, CDKN2A, MGMT methylation, Vimentin methylation, NDRG4 methylation, BMP3 methylation, K-*ras* mutation, hypermethylated SCNA, etc.

4. Techniques and current advancements in biomarkers for early detection of CRC

4.1. Techniques for early detection of CRC

Early detection and screening for CRC is necessary and could potentially be lifesaving in many cases, as the symptoms of CRC often tend to develop late in the natural course of the disease (50). Multiple methods can be used to screen and perform early diagnosis of CRC, each with its associated advantages and disadvantages. The most important feature of these tests is the test's sensitivity, and to a certain degree, its specificity (51).

4.1.1. Colonoscopy

Endoscopic procedures involve passing a camera attached to a long flexible tube into the gut of the patient. These procedures can be used to visualize and non-surgically remove adenomas and early cancers (52). Currently, colonoscopy is the most prevalent method for diagnosing CRC or confirming the positive result from other screening methods, with most doctors suggesting regular colonoscopy at a gap of 10 years for patients over the age of 45. A colonoscopy can be performed to spot and remove pre-cancerous lesions and tumors across the entire large bowel (53). Its sensitivity for CRC detection is around 95%, and for advanced adenomas (about 10 mm in diameter) its sensitivity is around 88–98%. It has been seen in case–control studies that with the use of colonoscopy there was a decline of about 53–72% in the incidence of CRC and a 31% decline in CRC-associated mortalities (54). But colonoscopy has its associated disadvantages, like high dependency on the operator, significant burden to the patient, expensive nature, post-colonoscopy CRC risk, etc. (55).

4.1.2. Sigmoidoscopy

Flexible sigmoidoscopy (FSIG) enables the endoscopic examination of the rectum and, distal colon (56). FSIG is most performed without sedation, unlike colonoscopy (57). Concerning colonoscopy, has its advantages it requires less intestinal preparation, takes less time, causes less discomfort for the patient without anesthesia, has fewer complication rates, and is cheaper (58). The common risk associated includes bleeding and perforations (59). Within this test's reach, the sensitivity and specificity for large adenomas and CRC have been found to be 95 and 87%, respectively.

4.1.3. Colon capsule endoscopy

Colon capsule endoscopy or CCE is a recent development in the field of CRC screening and involves swallowing a wireless camera, which has the size of a pill, which moves along the GI tract taking images of its surroundings (60). For advanced neoplasia, 10 mm or larger, the CCE-2 has a sensitivity of 76.7% and a specificity of 90.7% (61). g-FOBT and fecal immunochemical tests Guaiac Faecal Occult Blood Tests or g-FOBT involve the testing of stool for the presence of blood in it. The stool sample is tested using peroxidase enzyme for the presence of the heme group using a guaiac card. A positive g-FOBT necessitates a follow-up colonoscopy test (12). Fecal Immunochemical Tests or FITs incorporate antibodies that specifically bind to the globin protein of hemoglobin. Thus, like g-FOBT, they also search for the presence of blood in the stool of the patient (62). The biggest advantage of such stool-based tests is the ease of use. An issue with these techniques is that most polyps do not bleed. Thus, their presence goes undetected with these tests.

4.1.4. Stool DNA testing

This non-invasive method tests for the presence of molecular debris and occult blood in the stool samples (63). This debris might include mutant DNA seen in tumor cells, like mutant KRA, p53, aberrantly methylated BMP3, NDRG4 promoters, etc. (64). Cologourd®, an FDC-approved multi-target stool test, has been

shown to have higher sensitivity than FIT (92 and 72% respectively) but lower specificity (92 and 74% respectively), in a study that tested both on nearly 10,000 patients, using colonoscopy as reference. It also had a low detection rate for large advanced melanomas of only 42%, therefore limiting its preventive role (64).

4.1.5. Computed tomography colonography

Computed tomography colonography, or CTC, provides images of the entire abdomen and pelvis, not just the colon. It uses a radiographic agent to non-invasively tag stool for digital imaging. CTC's per-person sensitivity for adenomas below 10 mm varied between 66.7 to 93.5% in a meta-analysis evaluating its effects with colonoscopy, while its specificity values ranged from 96.0 to 97.9% (65).

4.1.6. Double-contrast barium enema

Double-contrast barium enema or DCBE is performed without any use of sedative and involves the injecting of air and rectal contrast and is therefore an unpleasant experience for the patient. But it can evaluate the entire colon for any abnormality (51). A study between DCBE and colonoscopy showed that DCBE detected only 32% of polyps less than 5 mm, 53% of polyps 6 to 10 mm, and only 48% of those greater than 1 cm (66).

4.1.7. Serological tests

A blood-based detection test, or liquid biopsies, checks for DNA markers floating in blood. The presence of methylated septin 9 in plasma has been assessed in many studies (67). According to a meta-analysis study that was based on 25 research articles, the SEPT9 assay is only better than the FIT in the symptomatic group (68). The test's current commercially available iteration has a sensitivity for advanced neoplasia and CRC of 25 and 68%, respectively, with a specificity of 79% (69, 70) (Table 2).

4.2. Current advancements in biomarkers for early detection of colorectal cancer

The need for more specific and sensitive biomarkers to detect CRC arises from the fact that CRC is one of the top four most prevalent cancers worldwide (71) with a high mortality rate. The current non-invasive techniques used for screening, for example, are not very sensitive to the earlier stages of cancer and may miss any pre-cancerous lesions and polyps. According to Imperiale et al. (72) "In asymptomatic persons at average risk for colorectal cancer, multitarget stool DNA testing detected significantly more cancers than did FIT but had more false positive results" (73). The finding further establishes the need for more sensitive biomarkers along with the already used screening techniques. The emergence of gene expression analysis along with transcriptome studies has allowed scientists to categorize CRC into subtypes for developing a better understanding of the disease and for devising better treatment strategies based on the subtype of CRC a patient may have. (74)Maida et al. (Maida et al., 2017) performed Molecular sub-typing of colorectal cancer, dividing it into 4 major subtypes: CMS1, CMS2, CMS3, and CMS4. This analysis has also elucidated new biomarkers. Similarly, Multi-Omics studies analyzing large amounts of data on the structure and function of several biological molecules in their totality have led to a better understanding of multifaceted and complex diseases like cancer. The omics studies including genomics, transcriptomics, proteomics, metabolomics, glycomics, etc. have revealed many new promising biomarkers for CRC. These biomarkers include several different kinds of molecules which may be DNA–RNA-based, protein-based, metabolite-based, or even volatile substances found in a patient's breath. They can be detected utilizing techniques like genomic analysis, mutation analysis using hybridization arrays, micro-arrays, bioinformatics analysis, mass spectroscopy, Gas- Chromatography MS, Gel electrophoresis, etc. (9).

5. Effect of NSAIDS on the gastrointestinal system

PGs increase mucus production and PGI2 and PGE2 have a vasodilator effect on the vasculature of the gastric mucosa and reduce gastric acid output. On the other side, NSAIDs could prevent the effects of PG on the gastrointestinal tract. Mucosal proliferation, HCO_3 secretion, and mucin synthesis are all inhibited by this action. NSAIDs can damage the gastrointestinal tract by impairing this function, which can lead to stomach problems (75). Gastric hypermotility results from NSAID usage that inhibits COX-1. Although the exact process is unknown, it is possible that tissue hypoxia and microvascular damage arise from high-amplitude, limited blood flow. There is some evidence that NSAID usage may lower the chances of GI malignancies, including gastric, pancreatic, and colorectal cancers, in contrast to the acute effects of NSAIDs on the GI tract (76). For instance, multiple studies have discovered that NSAIDs without aspirin are linked to a lower risk of gastric cancer (77) and, in the case of celecoxib, a higher rate of per-cancerous gastric lesions regressing when compared to placebo. To identify these possibly beneficial effects more fully, more research is nonetheless required (78).

5.1. Effect of NSAIDS and relation between cancer and inflammation

Acute inflammation, also known as resolved inflammation, is a self-limiting adaptive host defense mechanism that brings the body back to a state of homeostasis. However, persistent, unchecked, or unresolved inflammation can result in a number of diseases, including cancer. Nonsteroidal anti-inflammatory drugs (NSAIDs), like aspirin, lower the risk and death from several malignancies, which is significant evidence that connects inflammation and cancer. Clinical studies using COX-2 inhibitors for cancer prevention or therapy were justified by the overexpression of COX-2 in the colon and many other malignancies. NSAIDs, on the other hand, do not need COX-2 to prevent cancer (79).

Since ancient times, it has been understood that the primary reaction to damage is "Inflammation." Hippocrates, a Greek physician, may have been the first to view inflammation as the start of a healing process and used terms like *erysipelas* and *edema* to characterize its symptoms (80). The body's reaction to an exposure, such as an infection or an injury, is inflammation. NSAIDs have been identified as the prototype chemopreventive drugs against several types of cancer by more than 30 epidemiological investigations that combined

TABLE 2 Summary of detection techniques used for CRC detection based on cost-effectiveness.

Techniques used	Sensitivity	Specificity	Cost effectiveness	References
Colonoscopy	95%	88–98%	Higher cost when compared to other methods.	(54)
Sigmoidoscopy	95%	87%	More affordable than a colonoscopy	(59)
Colon Capsule Endoscopy	76.7%	90.7%	More expensive than a colonoscopy	(12)
g-FOBT	96–98%	50–75%	More affordable than a colonoscopy	(12)
FITs	94%	74%	More affordable than a colonoscopy	(70)
stool DNA testing	85%	93%	More expensive than a FITs	(64)
Computed tomography colonography	66.7–93.5%	96.0–97.9%	More affordable than a colonoscopy.	(65)
DCBE	80%	95%	Almost same as colonoscopy	(66)
Serological tests	68%	79%	More affordable than a colonoscopy	(69, 70)

reported findings on more than one million participants. NSAIDs can affect the microenvironment of tumors by slowing cell migration, boosting apoptosis, and decreasing chemosensitivity. Targeting the molecules (COX-2 cyclooxygenase 2, NF-kB, VEGF) involved in the inflammatory process might offer a useful technique for cancer prevention and therapy since they can predispose to tumors (81). Several NSAIDs like aspirin, celecoxib, piroxicam have shown preventive effects on inflammation in colorectal cancer. Colorectal cancer-related prevention by NSAIDs mostly works by acting on the pathway of the eicosanoids (82). NSAIDs have been shown in the past to have anti-tumor effectiveness, less toxicity, and non-specific side effects than those caused by conventional chemotherapy. They were also able to limit tumor growth by causing changes in the inflammatory environment of the tumor (83). NSAIDs have demonstrated chemoprotective and anti-inflammatory effects on inflammations associated with tumors. The fact that COXIB has more notable protective advantages than non-selective NSAIDs against a variety of malignancies is associated with a larger reduction in the risk of cancer (84, 85).

6. Role of NSAIDs in colorectal cancer chemoprevention

Cyclooxygenase (COX)-dependent and independent pathways participate in anti-tumorigenesis, albeit their mechanisms are not completely known (86). The primary anticancer action of NSAIDs is assumed to be a COX-2 inhibition-mediated suppression of prostaglandin E2 production, which reduces tumor cell proliferation, angiogenesis, and enhances apoptosis. Various signal transduction pathways like nuclear factor-kappa B, NF-κB, have been proven as COX-independent NSAID-induced effects, despite the fact that many of the anticancer mechanisms of NSAIDs are described as COX-dependent. Numerous studies have been conducted on the relationship between expression of COX and colorectal cancer, including prognostic variables and potential chemo-preventive drugs (87).

6.1. Mechanism of anti-cancer activity of NSAIDs

6.1.1. COX dependent pathway

COXs are regulators that have critical roles in carcinogenesis, angiogenesis, and inflammation. COXs found on luminal side of the ER (endoplasmic reticulum) are connected with the nuclear envelope and have three isoforms: COX 1, COX 2, and COX 3 (58, 88). The pharmacological basis for anti-inflammatory activity of NSAIDs is believed to be the inhibition of COX 1 and COX 2 enzymes, which catalyse conversion of arachidonic acid into prostaglandin H2, a precursor for the formation of prostacyclins, thromboxanes and prostaglandins. These eicosanoids have been associated to pain, fever, and inflammation. Moreover, they protect stomach and gut lining from harmful impact of the acid, stimulate blood clotting by activating blood platelets, and control kidney function. COX 2 is triggered by inflammatory stimuli, whereas on the other hand COX 1 is constitutively expressed in several tissues and has a significant role in the tissue homeostasis (89).

Several molecules linked to inflammatory and malignant processes have their gene transcription and protein synthesis regulated by aspirin and NSAIDs (90). The ability of NSAIDs and aspirin to decrease COX expression and downstream signals, that are essential for CRC cell diffusion, survival proliferation, allows for differentiation of these actions. Arachidonic acid is transformed into prostaglandin G2 by cox enzymes which is an unstable intermediate that is quickly degraded into PGH2. After that, PGH2 is transformed in a number of PGs with comparable structural properties, such as Thromboxane (TX) A2, PGD2, PGF2, PGI2, and PGE2 (91). Despite the fact that research in experimental CRC models has shown that COX 1 may promote cancer growth, in mammalian tissues, COX 1 is expressed

constitutively, and PGs synthesized from COX 1 are required for physiological functions (56).

On the other hand, COX 2 is activated in various cell types by tumor promoters, growth factors, and inflammatory cytokines (92). In 80-90% of carcinomas and 40–50% of human colorectal adenoma cancers, COX-2 expression is elevated, which increases PG synthesis (93, 94). Platelet-derived growth factor, matrix metalloproteinases, and vascular endothelial growth factor are all vital for the genesis, development, and advancement of tumors. COX 2 stimulates the synthesis of these molecules. Additionally, COX-2 restricts the development of immune cells with antineoplastic activity and controls the production of proteins that are both pro- and anti-apoptotic (61, 95).

Aspirin is the NSAID which has the ability to permanently suppress COX 1 and COX 2 action. On antiplatelet therapeutic levels of 75-100 mg daily, aspirin is 100-fold more effective than monocyte COX-2 in suppressing platelet COX-1 (96). Platelet activation in CRC patient stimulates the generation of proteolytic enzymes and chemokines which promote metastasis, angiogenesis and cancer cell proliferation (97). Activated platelets may potentially contribute to COX 2 overexpression in CRC by producing TGF, IL-1 and platelet-derived growth factor (97). Aspirin's anti-platelet activity may thus be responsible for some of its anti-tumorigenic actions. Aspirin and other NSAID suppression of PGE2 and COX 2 synthesis may depend on modulating a variety of signals which also includes sphingosine-1-phosphate (S1-P) synthesis suppression and activation of NAG-1, a gene induced by NSAID.

6.1.2. COX independent pathway

COX inhibition does not account for all of the NSAID-mediated anticancer effects. In fact, not all NSAIDs which are COX inhibiting possess anticancer effects and reactivating COX does not release the CRC cells from the arrested cell growth induced by NSAIDs. Additionally, CRC cells deficient in COX experience NSAID-induced apoptosis and growth inhibition (98).

6.1.2.1. NF-κB activation

Different subunits of the NF-κB family are regulated by NSAIDs in Colorectal cancer and can combine to produce homodimers and heterodimers. Among these, the binding of the RelA (p65) and p50 heterodimer occurs in an inactive state in cytoplasm with the help of I-kappaB (IκB) inhibitor protein. The translocation of this heterodimer to nucleus occurs in response to the activating stimuli which leads to phosphorylation of IκB with its subsequent degradation by proteasome. Translocated p50/RelA heterodimer controls the transcription of various genes (99). Reduced NF-κB transcriptional activity is resulted from the nucleolar sequestration of RelA induced by a dose of 5–10 mM of aspirin in cultured CRC cells (100). A dose of 50 μM of Sulindac sulfide limits the HCT-116 invasion of cells by inhibiting transcription (mediated by NF-κB) of some particular microRNAs like miR 9, miR 17, miR 21 which regulates gene expression implicated in metastasis and tumor cell invasion (101).

6.1.2.2. Wnt/β-catenin pathway

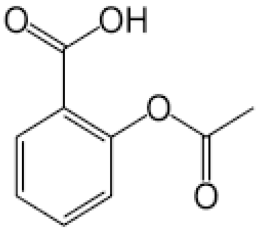
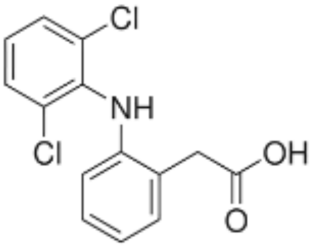
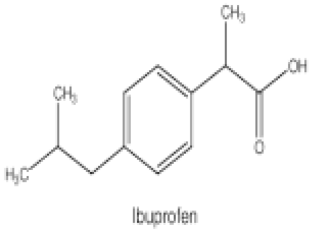
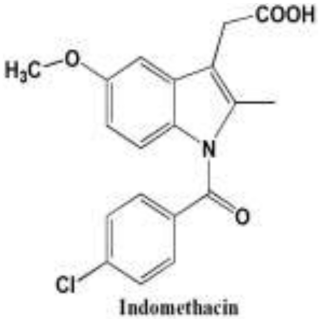
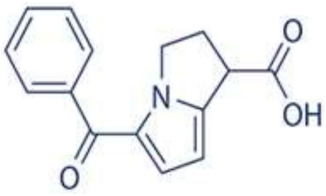
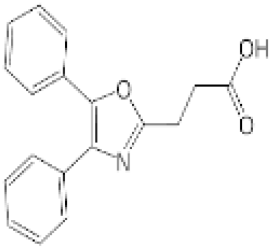
Wnt/β-catenin pathway (Wingless and integration site growth factor (Wnt)/-catenin) is a pathway that NSAIDs can target easily, since it is active in most of the CRC cells. A protein called cytoplasmic disheveled (Dsh) protein is activated by binding of Wnt with TFR (transmembrane frizzled receptor). The glycogen synthase kinase 3 (GSK3), casein kinase 1 (CK1), protein phosphatase 2A (PP2A), axin, and Apc, make up the catenin destruction complex, which Dsh protein binds with. The ubiquitination and degradation of the destruction complex is facilitated by the phosphorylation of the cytoplasmic β-catenin while Wnt signaling being absent (102). However, a decrease in the β-catenin degredation in response to the Wnt signals is seen with the aggregation of cytoplasmic β-catenin and gradual translocation to nucleus. Therefore, gene expression that promote tumor, for example, c-jun, peroxisome proliferator-activated receptor delta, c-myc, cyclin D1, and matrilysin is stimulated by the binding of β-catenin with the family components of LEF (lymphoid enhancer factor) and TCF (T-cell factor) (103).The β-catenin phosphorylation is enhanced by 5 mM and 100 mM doses of aspirin and celecoxib which reduces its nuclear aggregation and, as a result, transcription of Wnt/-catenin target genes in colorectal cancer cells (104). A study reported more data supporting the Wnt/-catenin pathway as a target of NSAIDs in CRC chemoprevention. According to this study, a 50 μM dosage of sulindac sulphide suppresses TCF transcriptional action of Wnt/β-catenin without enhancing phosphorylation of β-catenin, hence downregulating cyclin D1, and specifically inhibiting CRC cell proliferation (105). Several types of NSAIDs and their chemical structures have been discussed under Table 3 (Figures 2, 3).

6.2. Combined use of statins and NSAIDS for synergistic effect in CRC-chemoprevention

The drugs which lower cholesterol, also known as statins are made of tiny molecules called 3-hydroxy-3-methyl glutaryl coenzyme-A (HMG-coA) reductase inhibitors. Since statins show anti-carcinogenic characteristics in several *in vitro* and *in vivo* preclinical tests, there is a great interest in finding out how they might be used in cancer chemoprevention. Statin use may offer some preventive benefits against total cancer risk, according to some observational human research, but not others (113).

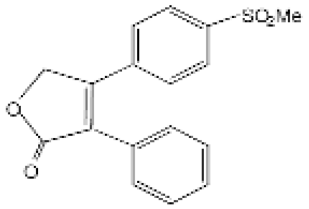
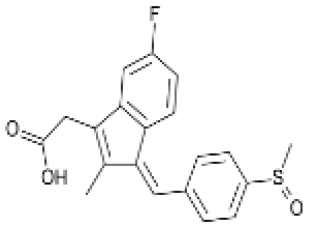
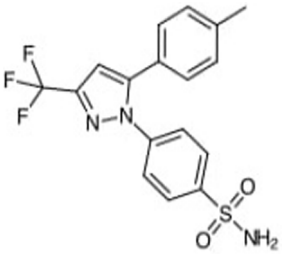
Statins are routinely used to reduce cholesterol and NSAIDs are mainly used to treat inflammation. Recent studies have focused on their potential function as cancer chemo-preventive drugs. Human studies have not shown solid data on the protective benefits of statins against various malignancies, although NSAIDs have yielded more compelling results for cancer prevention, particularly in CRC. Combining statins with NSAIDs may induce synergy and result in a reduction in the doses needed for each agent, which is a potential technique for improving cancer prevention effectiveness. This method is of particular importance for the prospective long-term utilization of low dosages of NSAIDs and statins for cancer chemoprevention. Significantly, colorectal cancer chemo-preventive studies have shown elevated possibility for gastrointestinal and cardiovascular adverse effects linked to NSAID usage. A growing body of research has conclusively shown that NSAIDs help prevent cancer, particularly colorectal cancer. Because of the potential elevated risk of severe cardiovascular and gastrointestinal side effects, relatively high dose needed to produce the observed chemo-preventive benefit in human studies can dissuade the long-term usage of NSAIDs alone for cancer prevention (114). Emerging research suggests that combining cancer chemo-preventive drugs, NSAIDs with distinct mechanisms of action

TABLE 3 A systematic representation of mechanism of action of NSAIDs along with their advantages and drawbacks.

Name of the NSAID	Chemical structure	Mechanism of action	Advantages in CRC	Drawbacks	References
Aspirin		- Inhibit COX1 and COX2 in CRC tissues. - PIK3CA pathway inhibition (COX independent)	Reduces colorectal polyps and inflammation	GI bleeding	(106, 107)
Diclofenac		- Inhibit Wntβ catenin signaling *via* NF-kβ	Reduce inflammation	Abdominal discomfort, nausea, diarrhea	(108, 109)
Ibuprofen		- Inhibition of MAPK, NFkβ (COX independent) - COX dependent inhibition	Reduce inflammation	GI bleeding	(110, 111)
Indomethacin		- NF-kβ, PPARδ inhibition - COX dependent inhibition	Anti-proliferative and apoptotic effects	Gastric ulceration and renal toxicity	(108, 109)
Ketorolac		- COX1 and COX2 dependent inhibition	Anti-metastatic effects	Post surgical anastomotic leak	(112)
Oxaprozin		- COX1 and COX2 dependent inhibition	Anti-metastatic effects	Cardiovascular risk, GI ulceration	

(Continued)

TABLE 3 (Continued)

Name of the NSAID	Chemical structure	Mechanism of action	Advantages in CRC	Drawbacks	References
Rofecoxib		- COX dependent inhibition	Anti-inflammatory and analgesic properties	Cardiovascular risk, strokes	(110, 111)
Sulindac		- COX dependent inhibition - Inhibition of Wnt/ βcatenin pathway	Reduced colorectal polyps, anti-inflammatory roles	GI ulceration and bleeding	(106, 107)
Celecoxib		- COX2 inhibition - MAPK pathway inhibition	Decreased recurrence of colorectal adenoma	GI bleeding, ulceration and cardiovascular risk	(106, 107)

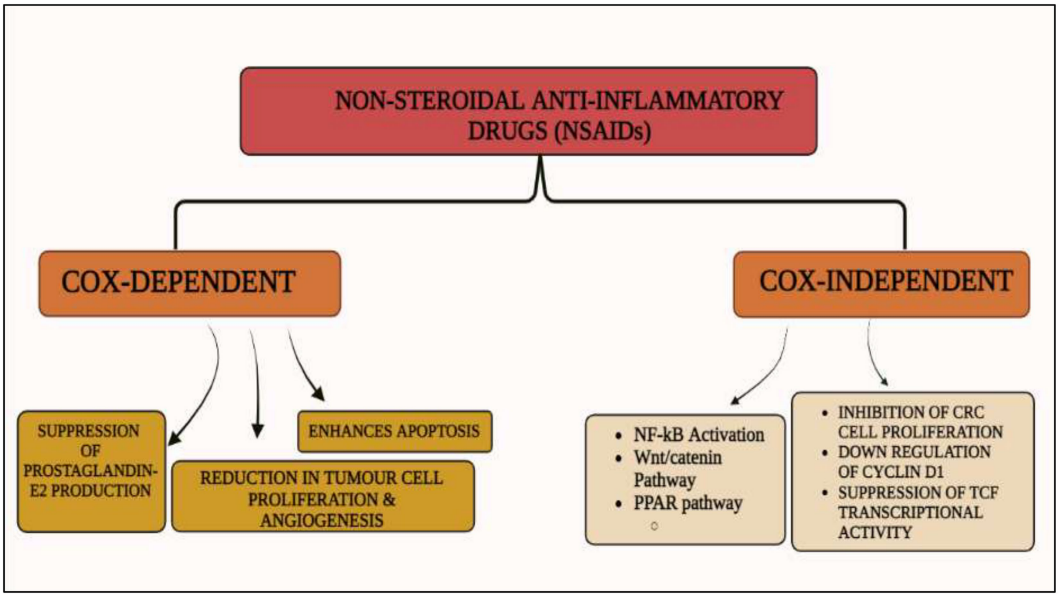


FIGURE 2
Cox-dependent and independent mechanism overview associated with NSAIDs. Cyclooxygenase dependent and independent pathways play a significant role in anti-tumorigenesis. The major anticancer action of NSAIDs is thought to be COX 2 suppression mediated decrease of prostaglandin E2 synthesis, which inhibits tumor cell proliferation and angiogenesis while increasing apoptosis.

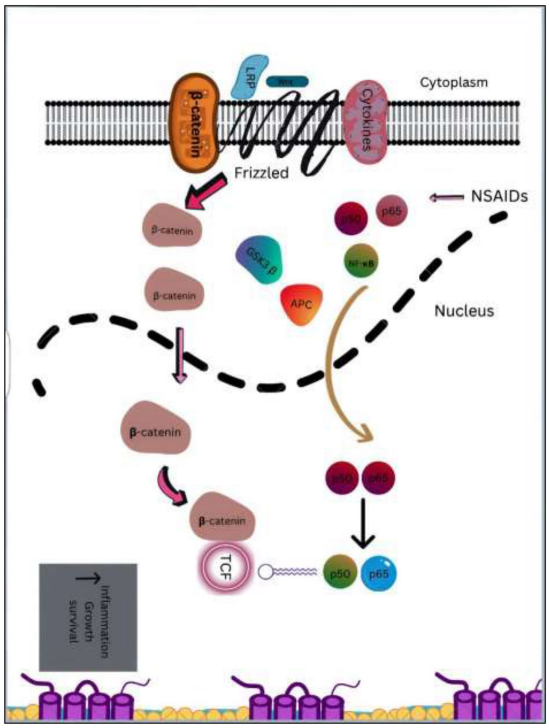


FIGURE 3
An overview of downstream targets in colorectal cancer & NF-κB and β-catenin/Wnt pathways. Catenin accumulates as a result of APC gene or activating mutations in the β-catenin, which leads to the formation of complex with the TCF/LEF transcription factors. TCF can interact with extra to stimulate the transcription of genes which are proliferative in the colon, including c-Myc and cyclinD1. With the release of p65, that is subsequently translocated to nucleus, inflammatory cytokines activate NF- κB, which leads to an increase in target gene transcription. NSAIDs in combination with other drugs like naproxen or sulindac targets β-catenin /Wnt and NF-κB pathways and suppresses downstream signaling.

may result in synergistic interactions, which might result in far higher anti-carcinogenesis benefits than each chemo-preventive agent could independently. NSAIDs have demonstrated synergistic effect in various other *in vitro* studies when treated with other therapeutic agents for example EGFR family inhibitors, statins, TRAIL receptor ligands, and PPARg ligands (115).

The combined use of statins and NSAIDs is particularly intriguing for cancer prevention. Atorvastatin is an example of the drug that was prescribed most in the year 2006 in US. In a significant experiment, to examine the results in individuals with coronary artery disease, pravastatin usage was found to be linked to a 43% decrease in several newly detected instances of colon cancer. Notably, 83% of individuals in both placebo and pravastatin groups received aspirin every day, implying that interaction between aspirin and pravastatin may have an improved protective impact (116).

The effects of statins and aspirin on risk of CRC were studied in a population-based case control research (117). This study comprised 612 controls and 537 patients with CRC cases that had been histologically proven. Frequent use of aspirin at a low dosage level was linked to a moderate reduced risk for CRC, whereas frequent use of statins, primarily simvastatin and atorvastatin, was linked to a stronger risk reduction. The most intriguing finding was that taking statins and lower dose of aspirin together for 5 years or more was linked to 62% risk of risk in CRC.

Utilizing the AOM rat model, effectiveness of celecoxib, aspirin, and atorvastatin against colon carcinogenesis when given separately on high dosage levels and when combined at low dosage levels (118). In comparison to single high doses of atorvastatin given at 150 ppm or celecoxib given at 600 ppm, the combination of 100 ppm atorvastatin and 300 ppm celecoxib reduced the prevalence and multiplicity of adenocarcinomas. Accordingly, low-dose combination of atorvastatin and aspirin significantly inhibited the prevalance and multiplicity of adenocarcinoma when compared to higher doses of each treatment alone. The effects of celecoxib and atorvastatin was examined on growth of adenomatous polyps in intestines in a different experiment utilizing the ApcMin/+ mouse model. Combining atorvastatin and celecoxib at 100 ppm and 300 ppm, respectively, was found to completely suppress colonic adenomatous polyps and reduce adenomatous polyps in small intestines by 86%. However, these effects were more potent than those brought on by either celecoxib or atorvastatin treatment administered separately (119). Together, these findings certainly showed that statin/NSAID combination regimens significantly increased the effectiveness of either type of agent administered alone in preventing cancer. This strongly supports the use of the statin/NSAID combination as a promising method for cancer chemoprevention.

6.2.1. Pathway involved

The pathways through which statins and nonsteroidal anti-inflammatory drugs (NSAIDs) limit cancer cell proliferation, induce apoptosis, and block other procarcinogenic processes are not completely known. Examples of celecoxib and atorvastatin were selected to briefly describe the potential mechanism of statins and NSAIDS as cancer chemo-preventive medications. By inhibiting HMG-CoA reductase, the rate-limiting enzyme in the mevalonate pathway, statins reduce the formation of isoprenoids such geranylgeranylpyrophosphate (GGPP) and farnesylpyrophosphate (FPP; FPP). These isoprenoids are necessary for the isoprenylation, membrane localization, and subsequent activation of a number of signaling proteins, such as Ras, Rho, and Rac. In contrast to GGPP, which can stop the apoptosis that statins cause in cancer cells, add-back assays showed that FPP had little to no protective benefits (120). These results demonstrated that GGPP had a more significant contribution to statin-induced effects than FPP. Studies have shown that geranylgeranylated Rho proteins play a part in the effects that statins induce, whereas the findings on farnesylated Ras have been contentious (121).

The specific mechanism by which statins and NSAIDs operate synergistically to create improved anti-carcinogenic effects remains largely unknown. A study was carried out on colon cancer HCT 29 and HCT116 cells. The mode of action was studied, and a strong synergistic effect was observed (122). Cell cycle arrest in the G0/G1 phase was brought on by the atorvastatin/celecoxib combination therapy for 24 h, and this effect was substantially stronger than those brought on by atorvastatin or celecoxib alone. These results are in line with those from animal studies, which showed that atorvastatin and celecoxib combination therapies reduced proliferative index and elevated apoptotic index in tumor tissues. Other studies in cancer cells demonstrated increased apoptosis with statin and NSAID co-treatments (123).

According to research, atorvastatin lowers the level of membrane bound RhoA, probably by isoprenylation inhibition and its impact is greatly boosted when combined with celecoxib (124). This may inhibit RhoA's carcinogenic actions, which have been linked to cell cycle progression, enhanced tumor invasiveness, and metastasis (125). The suppression of RhoA's membrane attachment is one potential method by which the combination of atorvastatin and celecoxib might cause cell cycle arrest. This can cause disruption of RhoA's negative control on both p21Cip1/Waf1 and p27Kip1 and that may raise the levels of these two CDK inhibitors (126). Unlike RhoA, the combination of atorvastatin and celecoxib raised the membrane-bound RhoB by an unknown mechanism. Due to the potential tumor-suppressing action of RhoB, the enhanced membrane association of RhoB may contribute to the inhibitory effects of atorvastatin/celecoxib combo on cancer cell proliferation (127). Celecoxib was discovered to strongly synergize with atorvastatin to abolish phosphorylation of Akt in colon cancer cells, even at low doses when little or no inhibition on Akt was shown on its own (128). The same treatments decreased Akt's upstream kinases, PDK1 and PI3K, phosphorylation levels. Furthermore, by reducing PTEN's phosphorylation at Ser380, the combination therapy may have elevated PTEN activity. In colon cancer cells treated with celecoxib or atorvastatin alone, all of these effects were either completely absent or markedly diminished. The apoptosis brought on by the combination of atorvastatin and celecoxib therapy may be significantly influenced by the suppression of the Akt pathway (128). It is crucial to note that neither of the two human colon cancer cell lines used had enough COX-2 expression. HCT11 cells lack the enzymatically inactive COX-2 protein that HT29 cells express (129). As a result, the effects of celecoxib and its combination with atorvastatin reported in this study were COX-2 activity independent. Findings on the combined treatment of licofelone (a dual inhibitor of COX-1 and 2) as well as 5-lipoxygenase, and atorvastatin did not show a significant synergy in inhibiting HCT116 cellular proliferation. More research is required to validate the involvement of COX-2 in the statin/NSAID combined treatment (Figures 4, 5).

6.2.2. The Nanoformulation of NSAIDs for CRC chemoprevention

Nanotechnology encompasses a wide range of novel and extraordinary nanomaterials with diagnostic and therapeutic potential. Carbon nanotubes, liposomes, dendrimers, gold nanoparticles, silica nanoparticles, and other nanomaterials are employed in colorectal cancer diagnosis and therapeutic delivery. Various drugs loaded on gold and silica nanoparticles are engaged in the death of CRC cells by targeted delivery of anticancer medications to cancer cells. With technological innovation, new approaches incorporating the utilization of nanotechnology have paved the way for the manufacture of nanomaterials capable of treating CRC cancer as well as other tumor types. These approaches have also aided in the identification and screening of CRC. The use of nanotechnology in CRC is crucial for the development of tailored drug delivery systems, the early detection of malignant tumors (which are nanomaterial-based), and several other improved therapeutic approaches. Regarding the present progress of nanotechnologies in the treatment of CRC, it has gained global attention due to its capacity to enhance screening techniques as well as diagnosis and therapy. Nanoparticles have been shown to increase current information on biochemical and physiological principles underlying a few diseases and their therapies. Nanoparticles have shown improved performance in few techniques like PET (positron emission tomography) and MRI (magnetic resonance imaging) with the respective use of radioisotope chelator-free nanoparticles in PET and iron-oxide based nanoparticles in MRI.

Due to their small size, remarkable sensitivity, and unique chemical constitution, nanoparticles are ideal contrast agents and are frequently employed in the treatment of cancer. When used therapeutically, it enhances the aggregation and discharge of pharmacologically active substances at the diseased site, increasing therapeutic efficacy and minimizing adverse toxic side effects. Additionally, NPs which have been recently developed have the capacity to combine diagnostic and therapeutic compounds into a single nanoparticle that is simple to employ for theranostic applications. Theranostic nanoparticles (NPs) may also be used in individualized nanomedicine-based therapeutics, according to studies. To develop an efficient treatment for colorectal cancer, new technologies for detecting proteins, genes, and other components in an individual's cancer should be devised. Anti-angiogenesis therapy is an alternative for CRC treatment in addition to EGFR inhibitor therapy. The most prevalent negative effects of targeted treatment are appearance of upper body and facial rashes. Poor drug responsiveness to chemotherapy while treating CRC is commonly observed, and this may be largely because of the development of multidrug resistance in tumor cells. Nanomedicine is believed to be a current method to improve the prognosis and treatment for CRC patients in order to combat multidrug resistance.

Numerous significant nanotechnological applications in cancer biology have been established, including early cancer screening and diagnosis as well as the development of novel therapy modalities that cannot be achieved with the currently available conventional technologies. In fact, particles bearing nano sizes of various forms and constitution have evolved as crucial and promising innovative tools for colorectal cancer screening, diagnostics, and treatments.

Different nano-formulations have been developed throughout the years to enhance curcumin delivery to cancer cells or tissues. Nano-formulations are generally utilized to improve solubility of curcumin in water and provide more constant curcumin administration (107, 130). Also, Curcumin nano-formulations treating tumors should ideally have increased anticancer efficacy when compared to curcumin alone and be harmless to normal cells.

Various studies have reported the documentation of curcumin nano-formulation for colorectal cancer treatment. The studies involve the use of polymeric nanoparticles, nano gels, liposomes, gold nano particles, cyclodextrins, solid lipid nanoparticles etc. Even though several nano formulations are through clinical testing, the number of nano formulations employed in CRC clinical trials is restricted. With the improvement in the designing of nano devices, nanomedicine has demonstrated its effectiveness in transforming the treatment and diagnosis of cancer. The drug-encapsulation methods that are on the nanoscale are particularly effective in passively retaining additional drug-loaded NPs close to cancer cells. These tactics have aided in the establishment of the subsequent generation of anticancer nanomedicine.

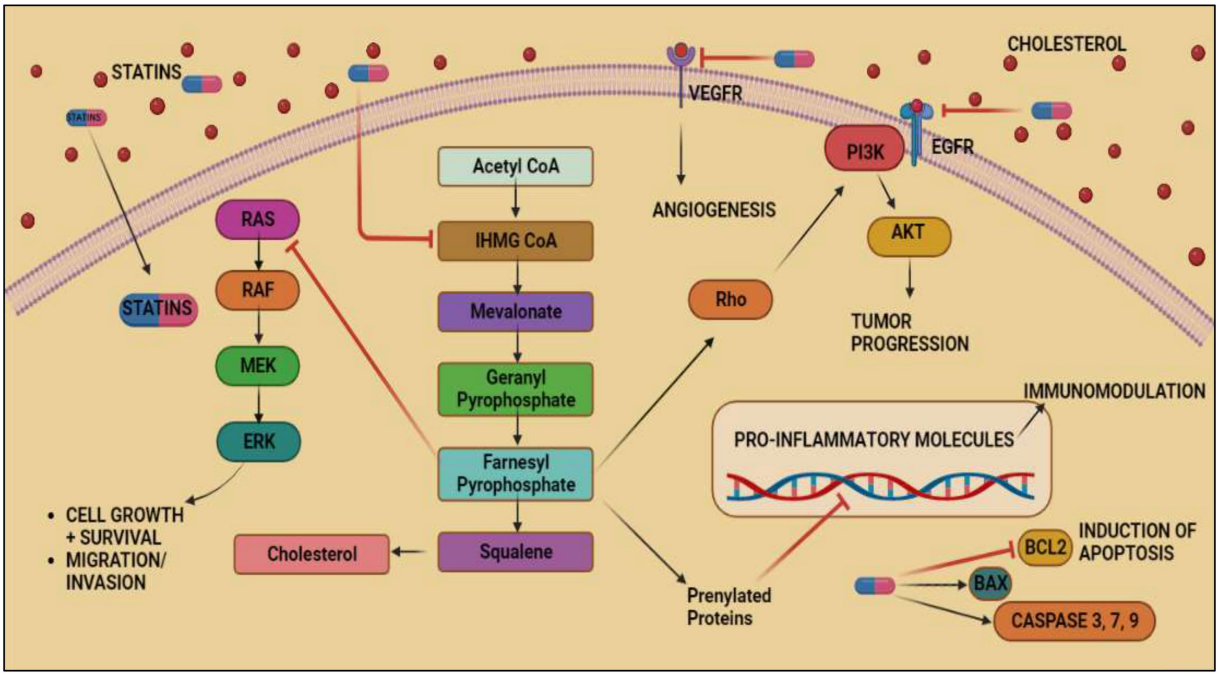


FIGURE 4
Anticancer effects exerted by Statins by inhibiting mevalonate pathway. Acetyl-CoA, the byproduct of glycolysis, is converted into mevalonate, IPP, GPP, FPP, GGPP, and cholesterol through a series of enzymatic processes that make up the mevalonate pathway. FPP and GGPP may both be supplemented to proteins post-translationally, particularly minor monomeric GTPases such as Ras predominantly part of MAPK/ERK pathway responsible for inducing VEGF expression in colorectal cancer. The inhibitory effect of FPP on MAPK/ERK pathway and inhibition of mevalonate pathway by statins causes tumor cell death and prevents migration of tumor cells. Statins shows its inhibitory effect on VEGFR and EGFR thus, inhibiting angiogenesis and tumor progression in cancer. It also inhibits BCL2 and induces aoptosis of cancerous cells.

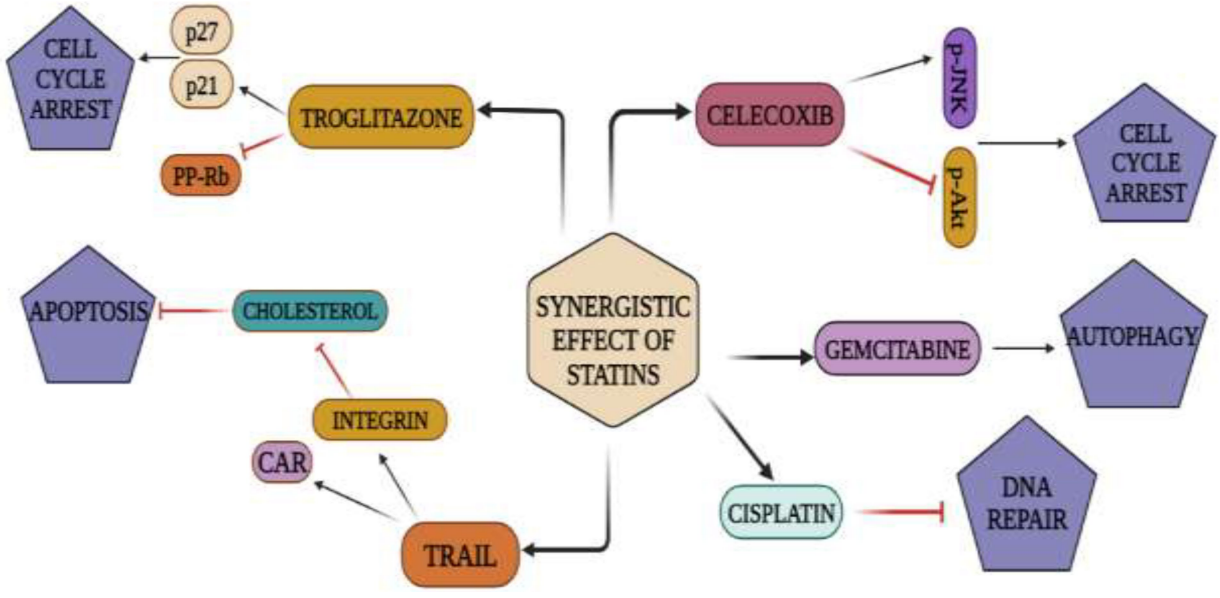


FIGURE 5
Synergistic action of statins and NSAIDs: Statins repress and activate signaling cascades that result in cell-cycle arrest, cell death, apoptosis, and autophagy when used with anti-cancer medications such TRAIL, troglitazone, celecoxib, gemcitabine, cisplatin.

The main purpose of NSAIDs is to prevent colon cancer. The epidemiological studies show that aspirin is the most promising NSAID of all the reported ones. Whereas the prevention of colon cancer by aspirin either alone or in combination has been demonstrated, nano encapsulation of aspirin can increase its effectiveness at a lower dose. A study conducted on seven-week-old

male Sprague Dawley rats which were treated with azoxymethane revealed the chemo protective impact of calcium, folic acid, and aspirin. It was discovered that this combination was 1.7-fold more effective than their unmodified complement routine (111, 131). The clinical uses of another NSAID, known as celecoxib in context of chemo preventive activity has been widely explored. The preparation of celecoxib polymeric Nanoparticles with ethyl cellulose, lipid hybrid nanoparticles, sodium casein ate bile salt, and micro emulsions improved the drug's bioavailability and permitted a reduction in dosage, crystallization, and associated toxicity. Phytochemicals are naturally derived plant-based compounds that are widely explored as possible chemo preventive agents and they are non-toxic and have pleiotropic properties. Curcumin has demonstrated effective chemoprotective effects in colon and intestine cancer, although it exhibits limited absorption, minimal solubility in water, and poor bioavailability. To address the issue, nano capsules of curcumin whey protein were produced, which not only demonstrated >70% discharge after 48 h but also increased bioavailability and cell internalization. In subsequent studies, it was discovered that encapsulating curcumin using polymeric nano carriers improved its solubility and the treatment group receiving curcumin nanoparticles demonstrated fewer structural abnormalities, a significant decrease in tumors, and beta-catenin levels than the group receiving curcumin alone.

In the future, this knowledge might be utilized to generate new approaches for the continued development of nanotechnology to upgrade existing medications and produce newer therapeutics.

6.3. NSAIDs – Dosage and duration and their therapeutic effects

Studies on the detection of colorectal cancer and its prevention are currently an expanding area of clinical oncology because it is one of the most prevalent tumors in the world. An analysis of randomized controlled, double blinded clinical studies including a few NSAIDs such as aspirin, sulindac, and celecoxib and colorectal cancer chemoprevention was done for this study. People taking NSAIDs had a decreased incidence of CRC, which points to the medications sustained chemo-preventive effects in both per-clinical and clinical studies. This advanced method of treating colorectal cancer could make it less fatal and more manageable. Clinical trials have examined and analyzed different NSAIDs for their proper dosage, duration, and therapeutic effects on CRC chemoprevention (110, 132, 133). Evidence from these clinical trials determined the extent of their chemopreventiveness in CRC. Seven trials on the use of aspirin in monotherapy, polytherapy with folic acid or eicosapentanoic acid for the prevention of CRC has been completed to date. For aspirin one such study involved the people with a history of CRC and not the ones with FAP or HNPCC. Patients had to wait for at least 5 years following tumor removal before experiencing a relapse to be eligible for carrying out colorectal adenoma prevention study (CAPS). It was found that the groups receiving aspirin 325 mg per day for 3 years had reduced average number of adenomas recurrence by 35% (134). Similar encouraging results were reached in the Asian population in the clinical trials with ASA 100 mg/day for 2 years, which involved participants with adenoma and a history of colon cancer (135). The Rothwell team also looked at if there was any weight or height dependence and how aspirin affected the risk of colon cancer over the next 20 years. In people weighing 70 kg or more, they found that 75-100 mg of aspirin used once day was ineffective at avoiding cardiovascular events, sudden cardiac death, or cancer, especially in those who smoked or took enteric-coated forms, suggesting that its dosage is too low for treatment (136). Sulindac was the subject of a further double-blind, placebo-controlled investigation in FAP patients. It was discovered that standard sulindac doses did not prevent adenomas from developing in younger patients with FAP (137) despite the fact that the number of scientific experiments with sulindac was significantly lower and was too small to be trusted. They were either given 75 or 150 mg orally twice a day of sulindac or identically looking placebo tablets for 48 months. Contrarily, celecoxib has a proven track record of protecting patients who have previously experienced sporadic colorectal adenomas from developing the condition again. Over 1,500 patients participated in the PreSAP (prevention of sporadic adenomatous polyps) and APC (adenoma prevention with celecoxib) trails. Both studies findings-one evaluating celecoxib at a daily dose of 400 mg for 3 years and the other evaluating daily doses of 400 and 800 mg are in an agreement with each other. Celecoxib's effectiveness in preventing adenoma recurrence improves with dosage (138, 139). Celecoxib's effectiveness in treating various tumor types when administered in conjunction with cystostatic medicines or monoclonal antibodies such as gemcitabine, cisplatin, fluorouracil, or cyclophosphamide has been studied. Moreover, in studies involving certain patient population, rofecoxib has shown to have a lower incidence of adenoma recurrence (58). Usually non aspirin NSAIDs use is associated with increased risk of cardiovascular risk and gastrointestinal bleeding which limit their use in CRC chemoprevention (140). However certain case control studies such as the one based on Danish population analyzing non aspirin NSAID use (average daily dose & gt; or = 0.3) was associated with a substantial reduction in CRC risk. Aspirin and non-selective NSAIDs (SIR 0.74 [0.71-0.77]), but not COX-2i, were linked to lower risk of GI malignancies including CRC, according to a Swedish population-based analysis of persons taking frequent NSAIDs (cumulative exposure of 6 months) (141). Another prospective cohort study analysis found that using non-aspirin NSAIDs was linked to a decreased risk of CRC in postmenopausal women (142).

Chemoprevention necessitates the continuous administration of NSAIDs. The case for prescribing a chemopreventive medication is more convincing when the patient's CRC risk is higher, and the drug's cumulative side effects are less severe. Traditional NSAIDs have adverse effects that worsen over time, particularly in older patients who take other drugs due to comorbidities that interact with the chemopreventive agent (107, 143). As a result, the CRC risk must be significantly more than the 5% likelihood that a person at average risk will develop CRC in order to sustain the lifetime treatment of a typical NSAID. The use of NSAIDs for cancer chemoprevention is not advised despite the substantial evidence of activity because of the risk of serious renal, gastrointestinal, and cardiovascular adverse effects that arise from COX inhibition and the suppression of physiologically significant prostaglandin (111, 133). The chemopreventive efficacy of NSAIDs is also insufficient, albeit it is unclear whether this deficiency is brought on by dosage restrictions or resistance mechanisms. Hence preventing NSAIDS from getting into more clinical trials and FDA approval in CRC chemoprevention.

7. Advantages, challenges and future perspective of NSAIDs

7.1. Advantages of NSAIDs

Patients who smoked heavily and had a high BMI had decreased ability to benefit from the chemo-preventive effects of NSAIDs, especially aspirin. Ibuprofen use was linked to a lower incidence of CRC in a different cohort study of patients with germline mismatch repair gene mutations (144, 145). The use of both aspirin and non-aspirin NSAIDs was associated with a reduced risk of cancer, including CRC. The FDA has authorized the use of NSAIDs as analgesics, antipyretics, and anti-inflammatory drugs. These qualities allow NSAIDs to be used to treat a wide range of illnesses, such as migraines, pyrexia, gout, arthritic disorders, muscle pain, and dysmenorrhea, and as an opioid alternative in some cases of severe trauma (145). Colorectal cancers with PIK3CA mutations or COX2 overexpression appear to have a stronger correlation between NSAID and aspirin usage and decreased mortality. Thus lending credence to the idea that NSAIDs might be used as adjuvant therapy for CRC. Optimizing the timing of NSAID use as an adjuvant treatment is clinically important. The synergistic anticancer effect of aspirin, biologically, might be explained by the stimulation of apoptosis through a COX-dependent or COX-independent mechanism (107, 146), the decrease of metastatic risk by preventing the contact between platelets and circulating cancer cells (109, 111, 147, 148), or the modification of the antitumor immune response (112, 149). In the end, NSAIDs may have more than one target and most likely has several adjunctive effects.

7.2. Challenges In The Use of NSAIDs As CRC chemopreventive

Despite the immense potential of NSAIDs as chemo-preventive agents, their use in CRC chemoprevention encounters many challenges. The poor acceptability and cost of screening colonoscopies are the two factors that make chemoprevention of colorectal cancer (CRC) a viable approach. The most promising treatment agents are those NSAIDs, which are presently not advised for the prevention of CRC (150). NSAIDs' limited chemo-preventive effectiveness is exacerbated by their considerable toxicity, which can be cumulative. These limitations can be curbed by the use of drug combinations, and the development of certain classes of NSAIDs that are chemically modified (for example – phospho-NSAIDs, nitro-NSAIDs, sulindac) and thus have prolonged safety than any other type of NSAIDs like those of conventional ones (150). One of the major challenges for using NSAIDs as a chemo-preventive drug is identifying the subjects who will gain the most from the chemo-preventive medication and those who are at potentially higher risk (151). The development of biomarkers that are predictive and techniques to reliably evaluate risk would be immensely beneficial in this case. Another challenge is optimizing chemo-preventive drug delivery time, dosage, and duration. According to research, very brief durations of agent administration may be necessary and can prevent colon carcinogenesis at an extremely early stage (152). The dosage and duration of NSAID administration might therefore be adjusted to ensure that the least amount of NSAID is utilized for the shortest duration of time. Furthermore, for individuals at risk, starting such an intervention at an early age may be beneficial. Also, the use of other new or combined agents or those agents that prevent other diseases in addition to colorectal cancer has its own merits (153). A meta-analysis of aspirin's role in preventing CRC and other malignancies in recent years published in May 2009 showed frequent aspirin use is linked to a lower risk of cancer. However, this theory raises various issues, such as the best aspirin dose and the prevention of gastrointestinal bleeding brought on by prolonged aspirin usage. Thus, there is still much debate about the use of aspirin in the prevention and treatment of cancer (154).

NSAIDs are associated with other serious non-cancerous conditions also. As it was recently revealed that using NSAIDs increases the risk of myocardial infarction (155). Among the medications examined were Celecoxib, ibuprofen, diclofenac, naproxen, and rofecoxib (156). According to a study, many NSAID users reported gastrointestinal side effects ranging from nausea, slight pain, and dyspeptic symptoms to serious problems like bleeding, peptic ulcer rupture, and intestinal blockage (157). Peptic ulcer illness in the past, age, and concurrent aspirin usage are all significant risk factors for developing GI side effects in NSAID users (158, 159). NSAIDs are known for having substantial renal side effects, which in extreme situations might result in renal failure, in addition to cardiovascular and gastrointestinal problems (160). A higher risk has been noted in previous research for acute renal failure. Thus, the use of NSAIDs in the treatment and prevention of cancer must be carefully evaluated and there must also be a balance between the risks and the benefits (5).

7.3. Future perspectives

The number of studies on CRC chemoprevention has grown. Although NSAIDs have shown the most promise, only those with a greater risk of CRC predisposition syndromes, such as Lynch syndrome or FAP, have been advised to take them as chemopreventive medicines (161). The ideal CRC chemoprevention drug is elusive for the majority of patients. Finding new colonic neoplastic pathways that can be targeted as well as developing drug combinations that maximize efficacy and reduce toxicity are obstacles to CRC chemoprevention. It's crucial to establish if more typical intermediate endpoints, like ACF or adenomas, may be employed given the generally low incidence of CRC in populations at average risk. Identifying subgroups based on genetic characteristics that influence treatment response, a history of polyps, and the subtype of a polyp is vital to determine which subgroups are most likely to benefit from chemoprevention drugs with the lowest degree of risk. CRC chemoprevention research must overcome obstacles including the necessity for funds to finance lengthy trials that enlist lots of participants and the requirement to validate results in various ethnic groups and geographical regions. Since many possible chemoprevention medicines are sold as over-the-counter drugs or dietary supplements, it is crucial to get reliable data on risk since their widespread usage might skew study results. It seems doubtful that CRC screening will ever be replaced as the main form of prevention by chemoprevention. The ability to prove the effectiveness of chemoprevention techniques in clinical trials will become more challenging as screening rates rise and CRC incidence and death decline (162, 163). Therefore, studies in groups who

regularly receive CRC screening will need to show a stronger protective impact to significantly support chemoprevention in addition to screening. In conclusion, a chemopreventive drug that is generally effective, safe, affordable, accessible, and simple to use is appropriate for CRC. The promise of lowering CRC risk and lowering its morbidity and mortality makes CRC chemoprevention an activity worth continuing to pursue, even if it is difficult to discover a chemoprevention medication that complies with these requirements.

8. Conclusion

CRC being the second leading cause of cancer death globally is a major concern among the WHO (World Health Organization). Its preventive measures and treatments have become one of the challenging issues in the public health sector. CRC has been regarded as a sporadic and hereditary disease caused due to accumulation of genetic and epigenetic abnormalities in epithelial cells of the large intestine. It comprises of several modifiable (diet, alcohol, obesity, exercise) and non-modifiable risk factors such as age, genes, family history, etc. Several biomarkers such as KRAS, a major CRC biomarker, help in the early detection of colorectal cancer in patients. With the advent of technology and biological, physiological, and statistical constraints of endogenous biomarkers unavoidable need for the development of synthetic biomarkers in cancer sectors became a priority. Hence, several vector-based, mammalian cell-based, and bacterial cell-based synthetic biomarkers have been employed for the early detection of CRC on the basis of their advantages. Furthermore, among the various invasive techniques, colonoscopy is the most preferred method for early detection of CRC due to its better sensitivity-95% and specificity- 98% whereas sigmoidoscopy is more cost-efficient as compared to the expensive colonoscopic procedure. However, due to better specificity, colonoscopy is the most preferred procedure followed by sigmoidoscopy. Apart from this several non-invasive analytical methods based on DNA–RNA, protein, and metabolites found in a patient's breath, blood, urine, and stool can be detected by utilizing genomic and mutation analytical techniques.

Chemoprevention techniques may help to further lower the incidence and mortality of CRC. Chemoprevention medications can be used for both low- and high-risk populations, as well as to stop colorectal cancer from returning following treatment. Aspirin, non-aspirin non-steroidal anti-inflammatory medications, statins, medicines that target metabolic pathways, vitamins, and minerals are examples of CRC chemoprevention treatments that have been explored (164).

NSAIDs are powerful anti-inflammatory, antipyretic and analgesic drugs having a chemopreventive impact on gastrointestinal malignancies, especially CRC, whereas long-term use of NSAIDs has also been linked to renal illness, myocardial infarction, gastrointestinal illness etc. Several NSAIDs, especially aspirin lower the risk and death from several malignancies, which is significant evidence that connects inflammation and cancer. The primary anticancer action of NSAIDs is assumed to be a COX-2 inhibition-mediated suppression of prostaglandin E2 production, which reduces tumor cell proliferation, and angiogenesis, and enhances apoptosis. Numerous studies have been conducted on the relationship between the expression of COX and colorectal cancer potential impact of NSAIDs-chemo-preventive drugs. It has been noted that statins and NSAIDs together show the synergistic effect as anticarcinogenic drugs in several *in vitro* and *in vivo* preclinical investigations, and this has drawn significant interest in examining their potential collaborative impact in cancer chemoprevention and combating the problems associated with the use of NSAIDs. This synergistic effect of combinational use of drugs proves to be beneficial in terms of reduced dosage and duration which is a potential technique for improving cancer prevention effectiveness.

Author contributions

GR and NAK played a role in designing the study as well as drafted the review paper. NAK, DE, AR, Tanzeelah, HM, HA, AR, and MSU did the writing part. AB, MAK, and WH helped in the revision. All authors contributed to the article and approved the submitted version.

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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The prognostic significance of human ovarian aging-related signature in breast cancer after surgery: A multicohort study

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Background: Recent studies have shown that ovarian aging is strongly associated with the risk of breast cancer, however, its prognostic impact on breast cancer is not yet fully understood. In this study, we performed a multicohort genetic analysis to explore its prognostic value and biological features in breast cancer.

Methods: The gene expression and clinicopathological data of 3366 patients from the The Cancer Genome Atlas (TCGA) cohort, the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) cohort and the GSE86166 cohort were analyzed. A total of 290 ovarian aging-related genes (OARGs) were included in the establishment of the prognostic model. Furthermore, functional mechanisms analysis, drug sensitivity, and immune cell infiltration were investigated using bioinformatic methods.

Results: An eight OARG-based signature was established and validated using independent cohorts. Two risk subgroups of patients with distinct survival outcomes were identified by the OARG-based signature. A nomogram with good predictive performance was developed by integrating the OARG risk score with clinicopathological factors. Moreover, the OARG-based signature was correlated with DNA damage repair, immune cell signaling pathways, and immunomodulatory functions. The patients in the low-risk subgroup were found to be sensitive to traditional chemotherapeutic, endocrine, and targeted agents (doxorubicin, tamoxifen, lapatinib, etc.) and some novel targeted drugs (sunitinib, pazopanib, etc.). Moreover, patients in the low-risk subgroup may be more susceptible to immune escape and therefore respond less effectively to immunotherapy.

Conclusions: In this study, we proposed a comprehensive analytical method for breast cancer assessment based on OARG expression patterns, which could precisely predict clinical outcomes and drug sensitivity of breast cancer patients.

KEYWORDS

ovarian ageing, breast cancer, prognosis, drug sensitivity, immune infiltration

Introduction

Breast cancer is a hormone-sensitive tumor and its development and progression are closely related to the host's hormone levels (1, 2). The decline in ovarian function, known as ovarian aging, results from a decrease in the quantity and quality of oocytes and is one of the key intrinsic determinants of hormonal changes (3). Numerous studies have shown that ovarian aging is strongly associated with the risk of breast cancer, but its prognostic impact on breast cancer is not yet fully understood. Therefore, it is of great significance to explore the prognostic implications of ovarian aging and its potential as an alternative individual therapeutic target for breast cancer.

Menarche and menopause mark the origin and end points in the process of ovarian ageing, as well as affect breast cancer risk. It has been well-documented that women who experienced menarche at an early age have an exponentially increased risk of developing breast cancer (4–7). Large cohort studies have also demonstrated that breast cancer incidence decreases with an earlier onset of menopause (8–10). Ovarian aging is a complex process with multi-linked genetic, etiological, or influencing factors and its molecular mechanisms remains largely unelucidated (3, 11). Fortunately, a new study in *Nature* conducted a large-scale genome-wide association study of ovarian ageing and identifies 290 genetic determinants of ovarian aging (12). Therefore, a comprehensive understanding of the relationship between the expression of the 290 ovarian aging-related genes (OARGs) and survival outcomes in breast cancer, would be important in determining the effects of ovarian aging in breast cancer.

Herein, this study was conducted to evaluate the prognostic profiles of OARGs in breast cancer. A novel ovarian aging-based signature for evaluating breast cancer prognosis was developed and validated in multiple cohorts. Furthermore, the present study aimed to present the prognostic landscape of OARGs in breast cancer, and screen for survival-related OARGs as biomarker candidates and potential therapeutic targets.

Methods

Data collection

RNA-sequencing (HTSeq-fragments per kilobase per million [FPKM]), clinicopathological, and survival data were obtained from three individual large breast cancer cohorts, namely The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/repository, accessed in July 2022), The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) (https://www.cbioportal.org/, accessed in July 2022) and the GSE86166 dataset from Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/, accessed in July 2022). Subjects who met the following criteria were included in the study: (a) had a histologically confirmed breast cancer without metastatic disease; (b) from post-surgery; (c) with available follow-up data of overall survival (OS), and an OS of not less than 30 days. The OS was defined as the time from the date of diagnosis to the date of death due to any cause or to the date of the last follow-up. A total of 290 OARGs were identified from the study of Ruth et al. (Table S1) (12). The overall workflow followed in this study was presented in Figure 1.

Screening for prognostic genes

The Kaplan-Meier and univariate Cox regression analyses, using OS as an outcome, were employed to estimate the predictive values of the 290 OARGs and screen for prognostic genes (with both $P < 0.05$) in the TCGA cohort.

The prognostic pattern of ovarian aging in breast cancer

Consensus cluster analysis was carried out based on the identified prognostic genes to classify patients into different groups by a non-negative matrix factorization (NMF) algorithm

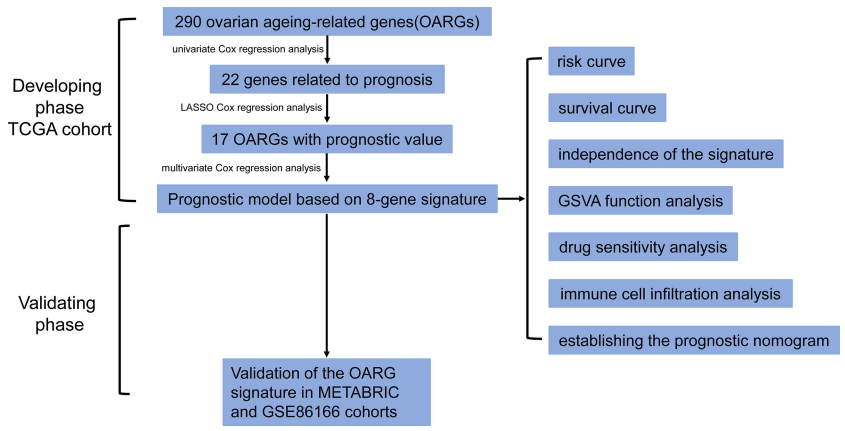

FIGURE 1
The flow chart detailing the comprehensive analysis of ovarian aging patterns in postoperative breast cancer patients.

using the NMF package (13). This was done to ensure maximum differences between the groups and minimum differences within the groups. The samples were clustered using the Brunet criterion. The K's range was set at 2 to 10. According to cophenetic, dispersion, and silhouette, the ideal K was found. The prognostic pattern of ovarian aging in breast cancer

Development and validation of the prognostic OARG signature

To further screen candidate genes for the prognostic model, the identified prognostic genes were subjected to LASSO Cox regression analysis to avoid potential co-linearity and simplify the number of independent variables (14). Then, multivariate Cox regression analysis was performed to evaluate the prognostic contributions of the selected candidate genes from the LASSO Cox regression analysis (hazard ratio, HR, 95% confidence interval, CI should not cross HR 1; $P < 0.05$), and establish the OARG risk score using the following formula: risk score = sum (each OARG normalized expression level × corresponding coefficients). Based on this, we calculated the OARG risk score for each patient and determined the optimal cut-off value for the OARG risk score according to maximally selected rank statistics method with OS for an outcome (15). Thus, according to the cutoff value, we divided each patient into different risk-stratified groups: the patient would be assigned into high-risk group if the patient's calculated OARG risk score was larger than the cutoff value; otherwise assigned into low-risk group. The survival differences between the two risk groups were compared using Kaplan-Meier analyses with a log-rank test. Furthermore, in the TCGA cohort, a nomogram was constructed, which incorporated the OARG risk score and additional prognostic clinicopathological characteristics identified from the multivariate Cox regression analysis. Calibration curves for the survival probability at one, three, and five years were also plotted to assess the prognostic precision of this nomogram. The same procedures and calculations were performed in the METABRIC and GSE86166 cohorts for validation.

Functional enrichment analysis of the OARG signature

Gene Set Variation Analysis (GSVA) using the "GSVA" package and Gene Set Enrichment Analysis (GSEA, https://www.gsea-msigdb.org/gsea/index.jsp) were conducted to determine the pathway and biological function differences between the two risk groups (16, 17). We used the c2.cp.kegg.v7.4.symbols.gmt in the Molecular Signatures Database (MSigDB) for board hallmarkers (17). Gene sets with normal $P < 0.05$ and false discovery rate < 0.10 were considered to be significantly enriched. Gene ontology (GO) enrichment analysis was performed using Metascape (https://metascape.org/gp/index.html#/main/step1) and plotted using the "ClusterProfiler" and "Cytoscape" package.

Identification of potential target drugs for high-risk group patients

The "pRRophetic" package, which was developed upon statistical models calculated from huge collections of cancer cell lines gene expression and drug sensitivity data (18), was used to predict the drug sensitivity of the two risk groups. The half maximal inhibitory concentrations (IC50) of potential target drugs were compared between the two risk groups.

Estimation of the immune cell infiltration landscape

The "GSVA" package with single-sample GSEA (ssGSEA) was used to evaluate the infiltration scores of immune cell types and immune-related pathways between the two risk groups. In addition, the variations in the compositions of immune cell types between the two risk groups were evaluated using the CIBERSORT method (19). Then, the differences in the reported famous six immune subtypes of wound healing (Immune C1), IFN-γ dominant (Immune C2), inflammatory (Immune C3), lymphocyte depleted (Immune C4), immunologically quiet (Immune C5), and TGF-β dominant (Immune C6) subtypes (20) were compared between the two groups. We also estimated the immunogenicity and immunome infiltration characteristics of breast cancer using the Estimation of STromal and Immune cells in MAlignant Tumours using Expression data (ESTIMATE) and Tumor Immune Dysfunction and Exclusion (TIDE) approaches (21, 22), and further investigated how well the risk signature performed in predicting the effects of immunotherapy. More specifically, a higher TIDE score means a higher likelihood of immune escape and a lower likelihood that the patient will benefit from immunotherapy.

Statistical analysis

Continuous data were reported as medians with interquartile ranges (IQR), while categorical data were reported as frequencies with percentages, and compared using the Mann-Whitney U test, chi-square test, continuity corrected chi-square test, or Fisher's exact test, whichever is appropriate. Disease-free survival (DFS) was defined as the time from the date of diagnosis to the date of recurrence/metastasis or to the date of death due to any cause or to the last follow-up. Meanwhile, recurrence-free survival (RFS) was defined as the time from the date of diagnosis to the date of recurrence or to the date of death due to any cause or to the last follow-up. The survival outcomes were estimated using the Kaplan-Meier method and compared by the log-rank test. The Cox proportional hazards model was performed to calculate the adjusted HRs and corresponding 95% confidence intervals (CIs). All statistical analyses were conducted with R version 4.1.2 (http://www.r-project.org). Statistical significance was set at two-sided $P < 0.05$.

Results

Screening for prognostic OARGs

A total of 1096 subjects from the TCGA cohort, 1904 subjects from the METABRIC cohort, and 366 subjects from the GSE86166 cohort were included in this study. After filtering out subjects who did not meet our selection criteria, a total of 3267 subjects were enrolled in the final analysis, including 1017 subjects in the TCGA cohort for training, as well as1888 subjects in the METABRIC cohort and 362 subjects in the GSE86166 cohort for validation.

The Kaplan-Meier and univariate Cox regression analyses, using OS as an outcome, were conducted to screen for prognostic genes among the 290 OARGs. In total, the expression of 22 genes was found to be significantly related to OS, with 11 genes having a negative association and 11 genes with a positive association (Figure S1).

The prognostic pattern of ovarian aging in breast cancer

The selected 22 prognostic OARGs were subjected to cluster analyses using the Brunet selection criterion for 50 iterations. The classification of clusters (K) was limited to 2-10. Three were chosen as the optimal cluster number based on the homogeneity, discreteness, and silhouette (Figures S2A, B). The results show that the OS ($P < 0.001$; Figure S2C) and DFS (P < 0.001; Figure S2E) of C2 were worse than those of C1 and C3.

Development and validation of the prognostic OARG signature

The selected 22 prognostic OARGs were also subjected to LASSO Cox regression analysis to avoid potential co-linearity and simplify the number of independent variables in the prognostic signature (Figures 2A, B). Subsequently, the LASSO Cox analysis yielded a total of 17 genes and therefore multivariate Cox regression analysis was performed to establish the prognostic OARG signature (Figure 2C). Finally, an 8-OARG risk signature was established in the TCGA cohort. The corresponding risk score of each patient was calculated using the following formula: risk score = *HLA-B* × (-0.24351) + *RBBP8* × (-0.34470) + *SPRY4* × 0.31174 + *WT1* × 0.29836 + *WWOX* × 0.39556 + *UPRT* × 0.40719+ *PELO* × 0.43603+ *ZNF208* × (-0.23972). The patients in the TCGA cohort were grouped into risk-stratified groups (high-risk group, n = 337; low-risk group, n = 680) based on the cut-off value of 4.49 which was determined using maximally selected rank statistics (Figure S2). The distributions of patient risk score and survival status, as well as each patient's 8-OARGs expression levels, are summarized in Figures 3A, B, respectively. The Kaplan-Meier survival curves demonstrated that the high-risk group patients had significantly worse survival OS ($P < 0.001$; Figure 3C) and DFS ($P < 0.001$; Figure 3D) than the low-risk group patients. Moreover, the OARG risk signature remained significantly associated with OS (HR = 3.79, 95% CI = 2.42-5.95, $P < 0.001$; Figure 3E) and DFS (HR = 2.20, 95% CI = 1.28-3.76, $P = 0.004$; Figure 3F) after adjusting for other clinicopathological variables.

Using the same formula and the cut-off value from the TCGA cohort, the risk scores and risk-stratified groupings weredetermined for patients in the METABRIC and GSE86166 cohorts for validation (Figures S3, S4). Consistently, the Kaplan-Meier survival curves also showed that the high-risk group patients had significantly worse OS ($P < 0.001$; Figure S3C) and RFS ($P < 0.001$; Figure S3D) in the METABRIC cohort, and worse OS ($P = 0.016$; Figure S4C) and RFS ($P = 0.022$; Figure S4D) in the GSE86166 cohort, respectively. Furthermore, after adjusting for other clinicopathological variables, the OARG risk signature remained associated with OS (HR = 1.35, 95% CI = 1.14-1.60, $P < 0.001$; Figure S3E) and RFS (HR = 1.22, 95% CI = 1.00-1.49, P = 0.050; Figure S3F) in the METABRIC cohort and OS (HR = 1.94, 95% CI = 1.05-3.60, P = 0.035; Figure S4E) and RFS (HR = 1.86, 95% CI = 0.91-3.82, P = 0.090; Figure S4F) in the GSE86166 cohort, respectively.

Establishment of a prognostic nomogram based on the OARG signature

A risk score-based visualized nomogram, which integrates the risk signature and three important clinicopathological factors (age, stage and subtype) selected from the multivariate Cox regression analysis, was established to individually quantify and assess the OS probability at 1-, 3- and 5-years of breast cancer patients in TCGA cohort (Figure 4A). We conducted a bootstrap validation and calculated the nomogram's C-index to be 0.812 (95% CI: 0.768-0.856) in the TCGA cohort and 0.757 (95% CI: 0.734-0.779) in the METABRIC cohort, respectively. To evaluate the predictive efficacy and clinical application of the nomogram, calibration curves were plotted for both the TCGA cohort (Figure 4B) and the METABRIC cohort (Figure 4C). The calibration curves demonstrated satisfactory consistency among the actual and anticipated OS probabilities at 1-, 3- and 5-years.

Gene set variation analysis of OARG signature

We performed GSVA to determine the potential biological functions of the OARG signature in breast cancer. In the training cohort of TCGA, the pathway sets DNA sensing, primary

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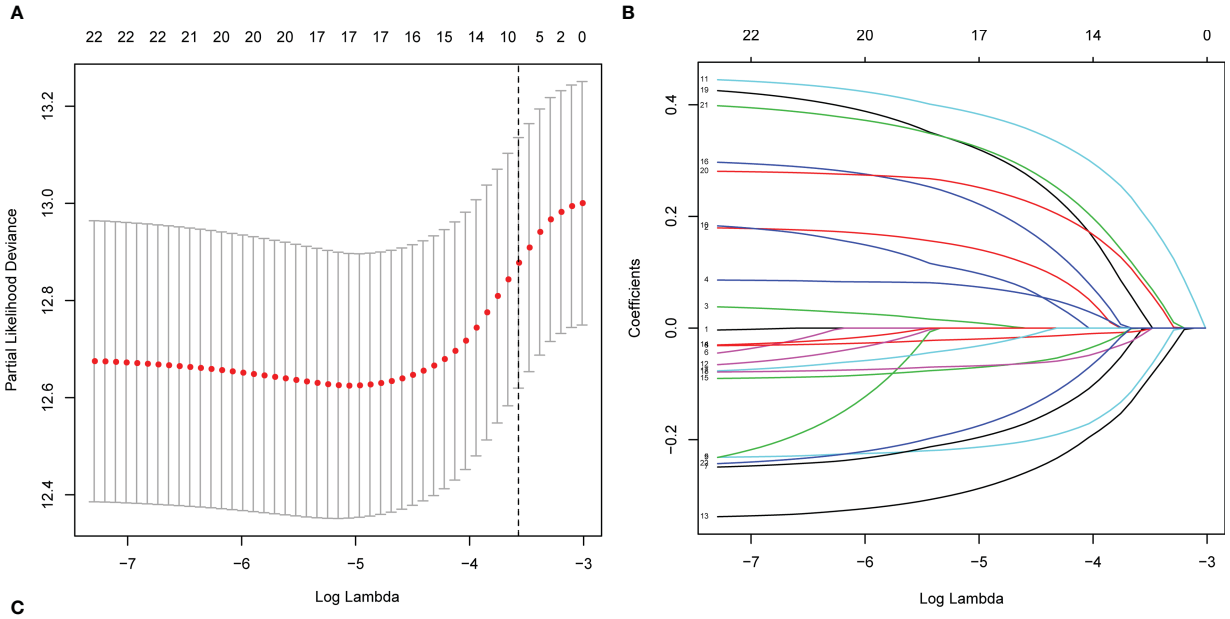

C

Variable	N	Hazard Ratio (95% CI)	Hazard Ratio (95% CI)	P value
CPEB1	1035		1.20 (0.90, 1.60)	0.222
EIF4E	1035		1.03 (0.85, 1.25)	0.778
EIF4EBP1	1035		1.10 (0.96, 1.26)	0.164
GSTM4	1035		0.79 (0.60, 1.05)	0.102
HLA-B	1035		0.78 (0.64, 0.96)	0.021
HLA-DQA1	1035		0.97 (0.87, 1.09)	0.643
OPA1	1035		1.16 (0.78, 1.71)	0.467
PELO	1035		1.55 (1.21, 1.98)	<0.001
RBBP8	1035		0.71 (0.58, 0.87)	<0.001
RUNX1	1035		0.92 (0.69, 1.22)	0.547
SPRY4	1035		1.37 (1.05, 1.77)	0.018
TIPARP	1035		0.94 (0.70, 1.25)	0.651
TP63	1035		0.93 (0.76, 1.14)	0.492
UPRT	1035		1.50 (1.14, 1.98)	0.004
WT1	1035		1.35 (1.15, 1.57)	<0.001
WWOX	1035		1.49 (1.21, 1.83)	<0.001
ZNF208	1035		0.79 (0.62, 1.00)	0.047

FIGURE 2
Screening and identification of prognostic ovarian ageing-related genes (OARGs) in the TCGA cohort. **(A)** Selection of the optimal candidate genes in the LASSO model. **(B)** LASSO coefficients of prognosis-associated OARGs, each curve represents a gene. **(C)** Forest plots showing results of univariate Cox regression analysis between the candidate OARGs expression and overall survival.

immunodeficiency, and nutrients metabolism were found to be activated in the high-risk group (Figure S5A). Meanwhile, the pathway sets with the immune network, autoimmune system, and immune disease were activated in the low-risk group (Figure S6D). GO enrichment analysis confirmed that the immune-related biological processes were enriched in the low-risk group (Figure S6A). These results were further validated in the METABRIC (Figures S5B, S6B, E) and GSE86166 (Figures S5C, S6C, F) cohorts and similar functional results were found. These results support the comprehensive DNA repair and immunomodulatory function effects of the OARG signature in the development and progression of breast cancer.

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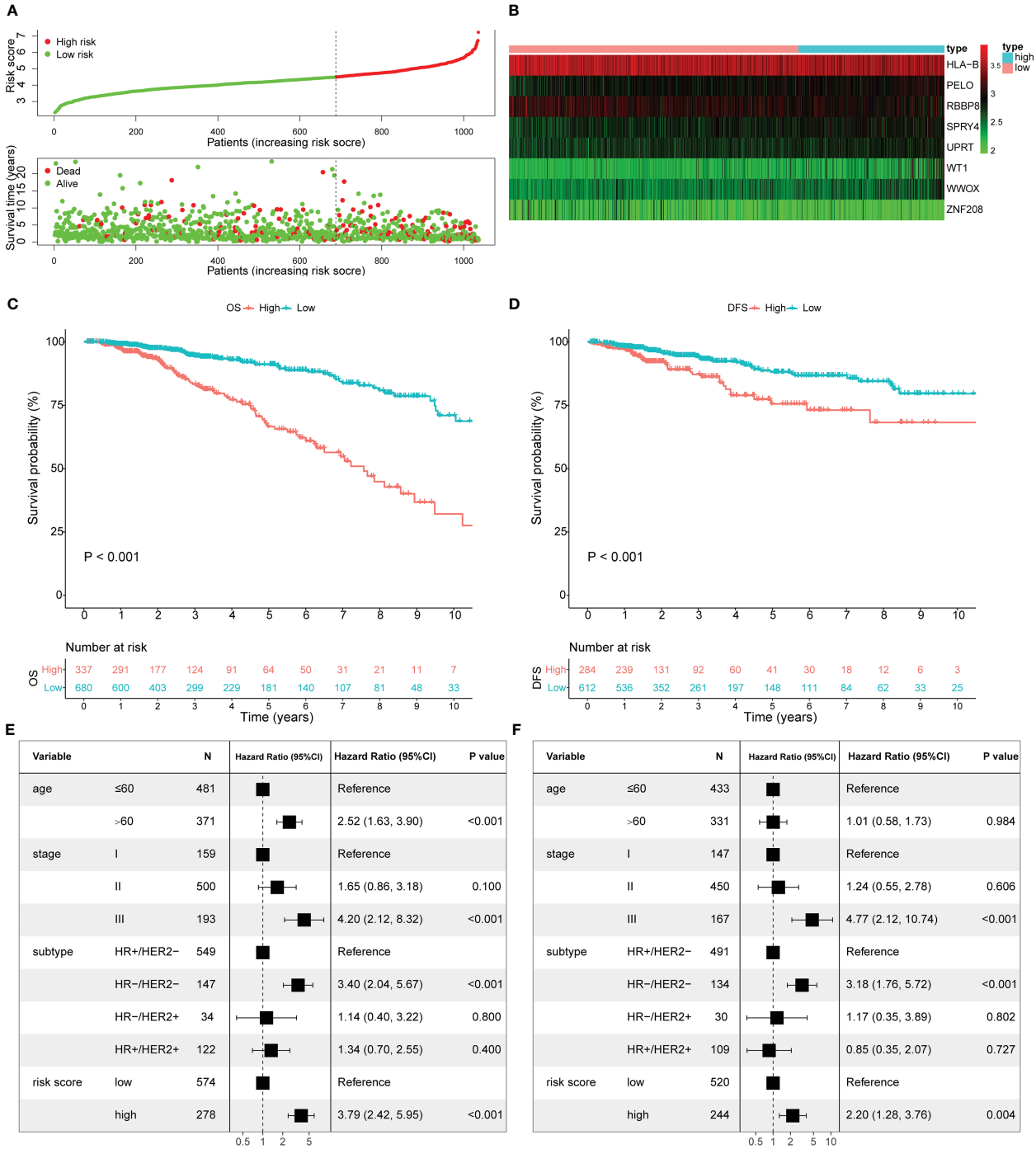

E

Variable		N	Hazard Ratio (95%CI)	P value
age	≤60	481	Reference	
	>60	371	2.52 (1.63, 3.90)	<0.001
stage	I	159	Reference	
	II	500	1.65 (0.86, 3.18)	0.100
	III	193	4.20 (2.12, 8.32)	<0.001
subtype	HR+/HER2−	549	Reference	
	HR−/HER2−	147	3.40 (2.04, 5.67)	<0.001
	HR−/HER2+	34	1.14 (0.40, 3.22)	0.800
	HR+/HER2+	122	1.34 (0.70, 2.55)	0.400
risk score	low	574	Reference	
	high	278	3.79 (2.42, 5.95)	<0.001

F

Variable		N	Hazard Ratio (95%CI)	P value
age	≤60	433	Reference	
	>60	331	1.01 (0.58, 1.73)	0.984
stage	I	147	Reference	
	II	450	1.24 (0.55, 2.78)	0.606
	III	167	4.77 (2.12, 10.74)	<0.001
subtype	HR+/HER2−	491	Reference	
	HR−/HER2−	134	3.18 (1.76, 5.72)	<0.001
	HR−/HER2+	30	1.17 (0.35, 3.89)	0.802
	HR+/HER2+	109	0.85 (0.35, 2.07)	0.727
risk score	low	520	Reference	
	high	244	2.20 (1.28, 3.76)	0.004

FIGURE 3
Estimate the prognostic value of ovarian ageing-related gene (OARG) signature model in TCGA cohort. **(A)** The distribution of risk scores in the TCGA and patient distribution in the high- and low-risk group according to overall survival (OS) status. **(B)** The heatmap showing expression profiles of the 8 OARGs. **(C)** Kaplan-Meier curves for the OS of patients in the high- and low-risk groups. **(D)** Kaplan-Meier curves for the diseases-free survival (DFS) of patients in the high- and low-risk groups. **(E)** Multivariate Cox regression analysis of OS. **(F)** Multivariate Cox regression analysis of DFS.

Clinical implications of the OARG signature in predicting therapeutic effects

The potential intrinsic connections between the OARG signature and therapeutic effects of chemotherapeutic, endocrine, and targeted agents were further explored. In the training cohort of TCGA, the low-risk group had a lower IC50 for chemotherapeutics such as doxorubicin, etoposide, gemcitabine, paclitaxel, vinorelbine and 5-fluorouracil, indicating the predictive potential of the model for chemosensitivity (Figures 5A–F). For the endocrine and targeted drugs, the low-risk patients had a lower IC50 for tamoxifen and

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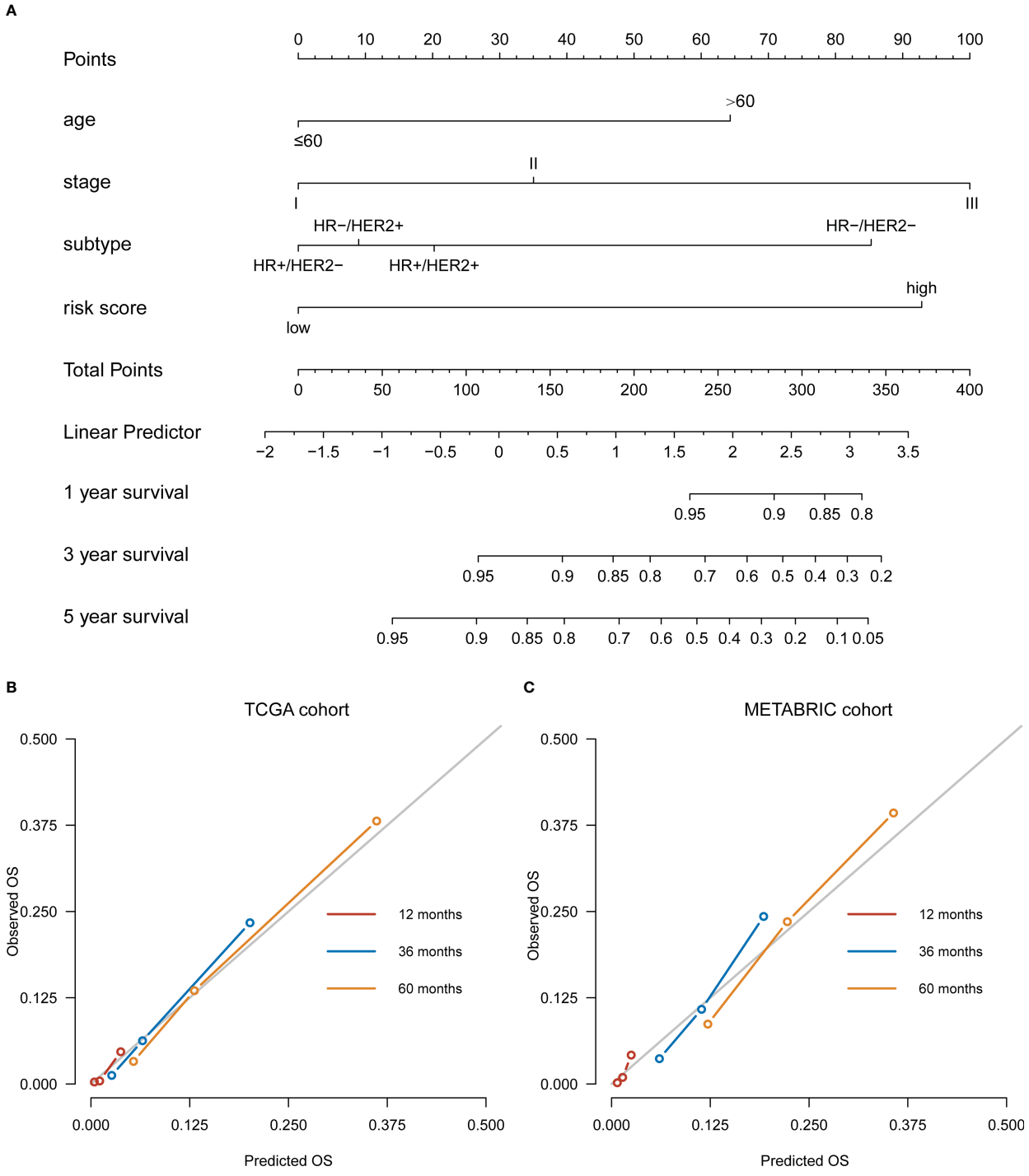

FIGURE 4
Development of a nomogram based on ovarian ageing-related genes (OARGs) signature for predicting overall survival (OS) of patients with breast cancer. **(A)** The nomogram plot integrating OARG risk score, age, stage and subtype in the TCGA training cohort. **(B)** The calibration plot for the probability of 1-, 3-, and 5-year OS in the TCGA training cohort. **(C)** The calibration plot for the probability of 1-, 3-, and 5-year OS in the METABRIC validation cohort.

fulvestrant (Figures 5G, H), as well as for lapatinib, sunitinib, dasatinib, crizotinib, pazopanib, and ruxolitinib (Figures 5I–N). Most of the results were validated in the METABRIC (except for crizotinib; Figure S7) and the GSE86166 (except for vinorelbine, crizotinib, and ruxolitinib; Figure S8) cohorts. The better prognosis for the low-risk group could be partially explained by these findings. These findings also imply that the low-risk group would benefit more from therapy with traditional and novel drugs.

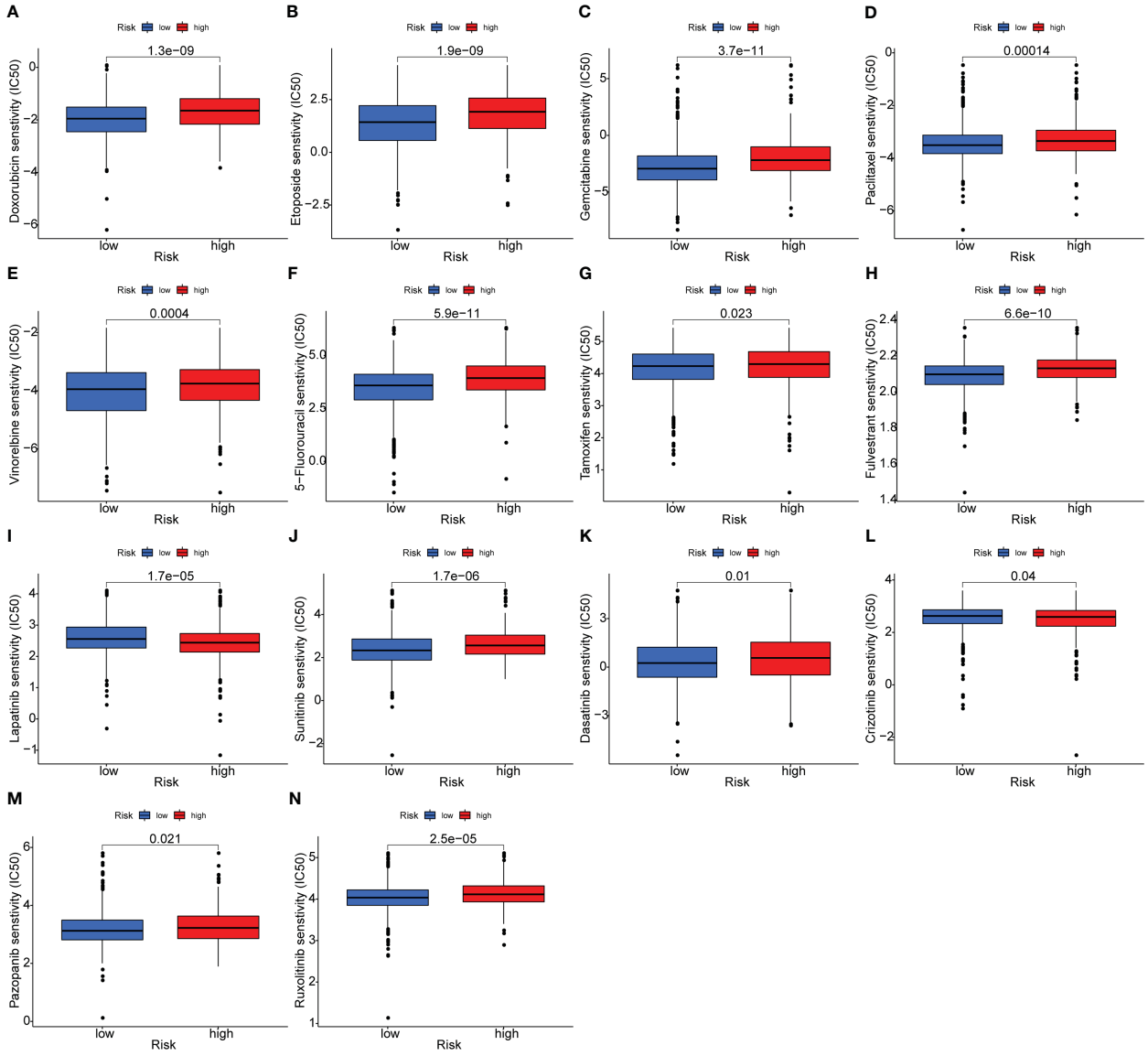

FIGURE 5
Analysis of the association between the risk model and chemotherapeutics, endocrine therapy, and targeted therapy. **(A–F)** The model predicting the sensitivity to chemosensitivity. It was estimated that low-risk patients had lower IC50 for chemotherapeutics of doxorubicin, etoposide, gemcitabine, paclitaxel, vinorelbine and 5-fluorouracil. **(G, H)** The model predicting the sensitivity to endocrine therapy. It was estimated that low-risk patients had lower IC50 of tamoxifen and fulvestrant. **(I–N)** The model predicting the sensitivity to targeted therapy. It was estimated that low-risk patients had lower IC50 of lapatinib, sunitinib, dasatinib, crizotinib, pazopanib and ruxolitinib.

Immunocyte infiltration profiling of the OARG signature in breast cancer

The profiling of immune infiltration was performed using the ssGSEA and CIBERSORT methods, and the outcomes showed noticeably different immune infiltration landscapes between the two risk categories. Specifically, functions such as APC_co_inhibition, APC_co_stimulation, CCR, Check-point, Cytolytic_activity, HLA, Inflammation-promoting, MHC_class_I, Parainflammation, T_cell_co-inhibition, T_cell_co-stimulation and Type_I_IFN_Reponse were elevated in the low-risk group patients (Figure 6A). Moreover, the patients in the low-risk group exhibited a higher percentage of B cells naive, Macrophages M0 and Macrophages M2. In contrast, the percentages of B cells memory, T cells CD8, T cells CD4 memory activated, T cells follicular helper, NK cells activated, Monocytes, Macrophages M1, Dendritic cells resting and Dendritic cells activated were all higher in high-risk group individuals (Figure 6B). In addition, the high-risk group had significantly lower immune and ESTIMATE scores than the low-risk group (Figure 6C). There was no immune C5 subtype in our cohort and the risk scores between the immune subtypes significantly differed. The immune C4 subtype had the highest risk score and the immune C2 subtype had the lowest risk score (Figure 6D). In contrast, the low-risk group presented with higher TIDE scores indicating that the low-risk group patients may be more susceptible to immune escape (Figure 6E). The patients

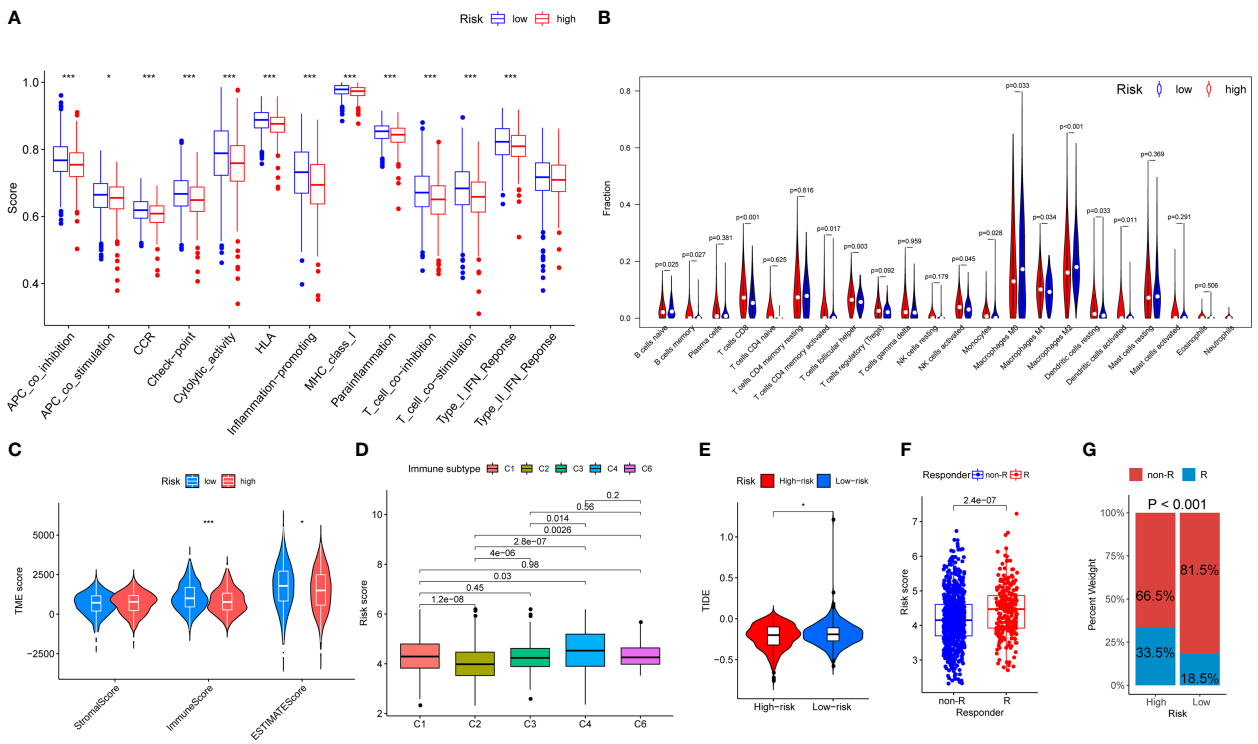

FIGURE 6
The landscape of immune function and immune cell infiltration between the high- and low-risk group in the TCGA cohort. Red represents high-risk samples; blue represents low-risk samples. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. **(A)** Barplot showing differences of immune functions between the low- and the high-risk group. **(B)** Violin plot showing differences of infiltrating immune cell types between the low- and the high-risk group. **(C)** Comparison of tumor microenvironment scores calculated by ESTIMATE between the low- and the high-risk group. **(D)** Comparison of risk scores between different immune subgroups. **(E)** Comparison of tumor microenvironment scores calculated by TIDE between the low- and the high-risk group. **(F)** Comparison of risk scores between different responder subgroups. **(G)** Comparison of the immunotherapy responding proportion between the low- and the high-risk group.

responding to immunotherapy also had higher risk scores than those non-responding to immunotherapy (Figure 6F). We also discovered that the proportion of patients responding to immunotherapy in the high-risk group was higher than that in the low-risk group (33.5% vs 18.5%, $P < 0.001$, Figure 6G). Overall, these findings showed that the immune infiltration profiles in breast cancer are linked with the risk stratification based on the OARG signature, and the immunotherapy effects could be also predicted.

Discussion

The current multicohort genetic association research provided a bioinformatics-based analysis model, which incorporated clinical information collection, transcriptome profiling, survival analysis, functional evaluation, and immune infiltration estimation to interpret the possible molecular mechanisms of ovarian aging and its implication in breast cancer. Moreover, this analysis model proposes a comprehensive perspective to explore the ovarian aging microenvironment in breast cancer and could reveal the potential outcomes and mechanisms related to the prognostic OARG signature.

Ovarian aging, involves complex genetic variants regulation and elaborate biological mechanisms. It is linked to several unfavorable consequences of hormone-sensitive cancers (23, 24). In recent years, increasing evidence suggests that ovarian aging is crucial in the female reproductive longevity biological processes, which have been demonstrated to be associated with the tumorigenesis and development of endocrine tumors (25–29). This study developed a signature featuring 8 OARGs (HLA-B, RBBP8, SPRY4, WT1, WWOX, UPRT, PELO, ZNF208) and determined its prognostic and functional implications in breast cancer patients. HLA-B has been previously demonstrated to have significant immunogenic involvement in breast cancer by supporting multiple downstream immunogenic pathways (30, 31). Our research showed that a better prognosis was related to a relatively higher expression of HLA-B. On the other hand, RBBP8 functions as a tumor suppressor protein in breast cancer by interacting with some distinct tumor-suppressing factors, including BRCA1 and retinoblastoma (32, 33). Our findings also suggest that RBBP8 served as a protective factor for breast cancer. An *in vivo* research revealed that SPRY4 may influence the characteristics of cancer stem cells, as well as tumor cell migration and proliferation (34). Numerous studies have demonstrated that WT1 plays an oncogenic role in various solid cancers including breast cancer, by promoting epithelial-to-mesenchymal transition and lowering chemotherapy efficacy (35, 36). Although previous studies found that WWOX expression was reduced in various cancers, our study has shown that it may be a

risk factor affecting the prognosis of breast cancer (37). Moreover, the current study found that the overexpression of UPRT was associated with a worse prognosis in breast cancer and is closely related to cancer gene-therapy efficacy (38). PELO is a new HER-signaling regulator and was suggested to play a role in inhibiting tumor cell proliferation and metastasis (39, 40). ZNF208 is a member of the zinc finger family of proteins and its mutations were found in many cancers, such as pancreatic cancer, gastric cancer, esophageal cancer and laryngeal cancer (41–43). We discovered its prognostic significance for breast cancer in our investigation.

The functional analysis results support the comprehensive DNA damage repair and immunomodulatory functions of the OARG signature in the development and progression of breast cancer. DNA damage repair mechanisms can trigger an innate immune response, resulting in a reduction in cell proliferation and the production of interferon, which is a crucial mechanism for promoting immune regulation (44–46). The tumor microenvironment enables tumor cells to avoid immune monitoring and medication interference, which permits them to survive (47). Previous studies have found that numerous pathways and genes associated with DNA damage repair networks play a role in genetic instability and immune activity (46, 48–50). Our results revealed that patients in the low-risk group exhibited a higher percentage of B cells naive, Macrophages M0 and Macrophages M2. Macrophages M0 have been polarized into M1-like and M2-like subtypes, both of these two macrophages are strongly linked to inflammatory reactions. Specifically, M1-like macrophages are primarily involved in pro-inflammatory reactions, while M2-like macrophages primarily participate in anti-inflammatory reactions (51). Ovarian aging activity is typically connected to the trigger of the anti-inflammatory signal, which is consistent with our results. Many studies have revealed that a better outcome is associated with the abundance of M1-like macrophages, while a worse outcome is suggested by the predominance of M2-like macrophages in breast cancer (52, 53). Therefore, the increased enrichment of M2-like macrophages that occurs with ovarian aging may be a possible explanation for the poor prognosis and may serve as a novel prognostic biomarker for breast cancer. Additionally, patients in the low-risk group had lower IC50 values for chemotherapeutic agents (doxorubicin, etoposide, gemcitabine, paclitaxel, vinorelbine, and 5-fluorouracil), endocrine agents (tamoxifen and fulvestrant), and targeted agent (lapatinib), which may have contributed to their better prognosis, since they were more responsive to systemic therapeutic drugs. Moreover, patients in the low-risk group have a higher sensitivity to sunitinib, pazopanib, ruxolitinib and crizotinib, which are currently being tested in ongoing clinical trials and may be potential targets for breast cancer therapy.

Although the present study shows that the OARG signature has an excellent performance in multicohort of breast cancer datasets, the study also has some limitations. Firstly, the participants were retrospectively enrolled, which may inevitably introduce bias to some extent. Secondly, the functional results of OARG genes from our bioinformatics analyses have not yet been confirmed in *in vitro* and *in vivo* experimental studies. Thirdly, we recognize that it is essential for well-designed clinical trials to investigate the prognostic significance of this model and its therapeutic implications in selecting novel drugs for breast cancer.

In conclusion, the current multicohort genetic association research comprehensively explored the OARGs in breast cancer based on their biological functions, linked pathways, regulatory immune infiltration, efficacy levels, and clinical implications. The survival-related OARG signature proposed in the current study has the potential to distinguish prognosis and may be clinically applied as useful biomarker and candidate targets in breast cancer.

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.

Author contributions

XH did the literature search. XH designed the study. XH, Q-WZ, Y-NZ, LC, M-DW, Y-SG, and J-YC participated in the analysis and interpretation of data. XH and J-YC developed an early draft. 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.2023.1139797/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Screening of ovarian ageing related prognostic genes by univariate Cox regression analysis.

SUPPLEMENTARY FIGURE 2
The prognostic pattern of ovarian aging in breast cancer and determination of the optimal cutoff value of the vitamin C index according to maximally selected rank statistics.

SUPPLEMENTARY FIGURE 3
Estimate the prognostic value of ovarian ageing-related gene (OARG) signature model in METABRIC cohort. **(A)** The distribution of risk scores in the TCGA and patient distribution in the high- and low-risk group according to overall survival (OS) status. **(B)** The heatmap showing expression profiles of the 8 OARGs. **(C)** Kaplan-Meier curves for the OS of patients in the high- and low-risk groups. **(D)** Kaplan-Meier curves for the recurrence-free survival (RFS) of patients in the high- and low-risk groups. **(E)** Multivariate Cox regression analysis of OS. **(F)** Multivariate Cox regression analysis of RFS.

SUPPLEMENTARY FIGURE 4
Estimate the prognostic value of ovarian ageing-related gene (OARG) signature model in GSE86166 cohort. **(A)** The distribution of risk scores in the TCGA and patient distribution in the high- and low-risk group according to overall survival (OS) status. **(B)** The heatmap showing expression profiles of the 8 OARGs. **(C)** Kaplan-Meier curves for the OS of patients in the high- and low-risk groups. **(D)** Kaplan-Meier curves for the recurrence-free survival (RFS) of patients in the high- and low-risk groups. **(E)** Multivariate Cox regression analysis of OS. **(F)** Multivariate Cox regression analysis of RFS.

SUPPLEMENTARY FIGURE 5
Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analysis of ovarian ageing-related gene (OARG) signature. **(A)** TCGA cohort. **(B)** METABRIC cohort. **(B)** GSE86166 cohort.

SUPPLEMENTARY FIGURE 6
Gene ontology (GO) and Gene set enrichment analysis (GSEA) functional enrichment analysis functional enrichment analysis of ovarian ageing-related gene (OARG) signature. GO functional enrichment analysis for **(A)** TCGA cohort. **(B)** METABRIC cohort. **(B)** GSE86166 cohort; GSEA functional enrichment analysis for **(D)** TCGA cohort. **(E)** METABRIC cohort. **(F)** GSE86166 cohort.

SUPPLEMENTARY FIGURE 7
Analysis of the association between the risk model and chemotherapeutics, endocrine therapy, and targeted therapy in the METABRIC cohort. **(A–F)** The model predicting the sensitivity to chemosensitivity. It was estimated that low-risk patients had lower IC50 for chemotherapeutics of doxorubicin, etoposide, gemcitabine, paclitaxel, vinorelbine and 5-fluorouracil. (GH) The model predicting the sensitivity to endocrine therapy. It was estimated that low-risk patients had lower IC50 of tamoxifen and fulvestrant. **(I–M)** The model predicting the sensitivity to targeted therapy. It was estimated that low-risk patients had lower IC50 of lapatinib, sunitinib, dasatinib, pazopanib and ruxolitinib.

SUPPLEMENTARY FIGURE 8
Analysis of the association between the risk model and chemotherapeutics, endocrine therapy, and targeted therapy in the GSE86166 cohort. **(A–E)** The model predicting the sensitivity to chemosensitivity. It was estimated that low-risk patients had lower IC50 for chemotherapeutics of doxorubicin, etoposide, gemcitabine, paclitaxel and 5-fluorouracil. (FG) The model predicting the sensitivity to endocrine therapy. It was estimated that low-risk patients had lower IC50 of tamoxifen and fulvestrant. **(H–K)** The model predicting the sensitivity to targeted therapy. It was estimated that low-risk patients had lower IC50 of lapatinib, sunitinib, dasatinib and pazopanib.

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Carbonic anhydrase IX-related tumoral hypoxia predicts worse prognosis in breast cancer: A systematic review and meta-analysis

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Background: Tumoral hypoxia is associated with aggressiveness in many cancers including breast cancer. However, measuring hypoxia is complicated. Carbonic anhydrase IX (CAIX) is a reliable endogenous marker of hypoxia under the control of the master regulator hypoxia-inducible factor-1α (HIF-1α). The expression of CAIX is associated with poor prognosis in many solid malignancies; however, its role in breast cancer remains controversial.

Methods: The present study performed a meta-analysis to evaluate the correlation between CAIX expression and disease-free survival (DFS) and overall survival (OS) in breast cancer.

Results: A total of 2,120 publications from EMBASE, PubMed, Cochrane, and Scopus were screened. Of these 2,120 publications, 272 full texts were reviewed, and 27 articles were included in the meta-analysis. High CAIX was significantly associated with poor DFS (HR=1.70, 95% CI=1.39–2.07, $p<0.00001$) and OS (HR=2.02, 95% CI 1.40–2.91, $p=0.0002$) in patients with breast cancer. When stratified by subtype, the high CAIX group was clearly associated with shorter DFS (HR=2.09, 95% CI =1.11–3.92, $p=0.02$) and OS (HR=2.50, 95% CI =1.53–4.07, $p=0.0002$) in TNBC and shorter DFS in ER$^+$ breast cancer (HR=1.81 95% CI =1.38–2.36, $p<0.0001$).

Conclusion: High CAIX expression is a negative prognostic marker of breast cancer regardless of the subtypes.

KEYWORDS

breast cancer, carbonic anhydrase IX, meta-analysis, prognosis, survival

Introduction

The incidence of breast cancer has increased in recent decades, with an estimated 13% of women developing breast cancer in their lifetime and over 40,000 deaths per year (1, 2). The survival depends on clinicopathological factors, such as tumor size, nodal status, evidence of distant metastasis as well as biological markers, including estrogen receptor (ER), progesterone

receptor (PR), and human epidermal growth factor receptor 2 (HER2) status (3–5). The intrinsic breast cancer subtypes are currently significant prognostic and predictive markers. Five-year overall survival (OS) was the highest in the ER/PR-positive subtype (94%) as compared to the HER2-positive subtype (85%) and the triple-negative (TNBC) subtype (77%) (1). Breast cancer has distinct phenotypes as evidenced by patients who have a similar staging and molecular classification but have a different treatment response and prognosis (6–8). Thus, additional predictive and prognostic markers are warranted to improve the treatment and prognostic outcomes.

Tumoral hypoxia is a common characteristic of many solid tumors (9, 10). In breast cancer, median oxygen partial pressure is approximately 10 mmHg, which is less than that of the normal breast tissue (52–65 mmHg) (11, 12). Cancer cells adapt to survive under hypoxic conditions *via* hypoxia-inducible factor-1α (HIF-1α), leading to the transcription of targeted genes resulting in tumor progression and invasion (13). Subsequently, HIF-1α can trigger the transcription of targeted genes, leading to tumor progression and invasion (14).

The expression of carbonic anhydrase IX (CAIX) is targeted by the HIF-1α transcriptional activity and controls the pH between intracellular and extracellular compartments (15). It is mainly dependent on HIF-1α regulation; therefore, it can also be a marker of tumor hypoxia (16, 17). However, hypoxia is not an obligated factor, and the inactivation of the von Hippel–Lindau (VHL) gene can stabilize HIF-1α under a non-hypoxic condition and subsequently activated the CAIX overexpression (15, 18). CAIX catalyzes extracellular hydrating CO_2 into HCO^-_3 and H^+ and cooperates with other acid/base transporters to maintain extracellular acidosis and intracellular neutral/slight alkalosis (19). In contrast, CAIX-bound Cl^-/HCO^-_3 exchangers (AEs) can import or provide or export HCO^-_3 from intracellular compartment during cell migration (20). CAIX expression mediates cancer cell growth, migration, and invasion (18) by directly binding to β-catenin, resulting in the disruption of the E-cadherin/cytoskeleton/β-catenin complex; and an acidic extracellular pH also suppresses the function of cytotoxic T-cells (21).

Many studies have shown that high CAIX expression was associated with adverse survival outcomes. In breast cancer, some studies evaluated the importance of CAIX expression in relation to survival; however, those results were controversial and mostly included a small number of patients. Ong et al. reported that CAIX expression was the independent prognostic factor for disease-free survival (DFS) and OS in TNBC. Similarly, Brennan et al. reported that high CAIX was associated with shorter OS, breast cancer-specific survival (BCSS), and relapse-free survival (RFS) (22, 23). In contrast, Currie et al. found no association between the level of CAIX and DFS and OS (24), while Pinheiro et al. reported that only a high CAIX expression was related to DFS but not to OS (25).

To address this issue, a meta-analysis was conducted to evaluate the prognostic value of CAIX in breast cancer and to determine the correlation between CAIX and breast cancer subtypes. To date, this is the first meta-analysis to focus on the prognostic role of CAIX in breast cancer. The meta-analysis revealed that a high CAIX protein expression was associated with unfavorable survival outcomes and could discriminate the prognosis in the ER-positive and TNBC subtypes.

Materials and methods

Search strategy

This study used EMBASE, PubMed, Cochrane, and Scopus electronic databases to search for articles. The keywords including [(Prognos*) OR (surviv*) OR (hazard) OR (disease-free) OR ("disease free") OR (progression-free) OR ("progression-free") OR (Kaplan–Meier) OR ("Kaplan Meier") OR (predict*) OR (outcome) OR (efficacy) OR (effective*)] AND [(CAIX) OR (ca9) OR ("carbonic anhydrase IX") OR ("carbonic anhydrase 9") OR ("carbonic anhydrase-IX") OR ("carbonic anhydrase-9") OR (CA-IX) OR (ca-9) OR (G250)] AND [(breast cancer) OR (breast tumors*) OR (breast carcinoma)] were used.

Selection criteria

The inclusion criteria of the present study were as follows: (a) the patients in the study cohorts who were confirmed to have invasive breast cancer, regardless of the subtype, (b) CAIX expression which was detected by immunohistochemistry (IHC), (c) the studies that reported DFS or OS with hazards ratios (HRs) and 95% confidence intervals (CIs) or the Kaplan–Meier survival curves from which HRs and 95% CIs could be extracted, and (d) the studies that were published in English. The exclusion criteria for the present study were studies that failed to meet any of the inclusion criteria, were related to non-human studies, or contained duplicated and unavailable full texts.

Data extraction and quality assessment

The search with regard to data extraction and quality assessment was reviewed by three independent reviewers (WN, JP, and SY). The following information was extracted from each study: the first author's name, year of publication, the total number of patients, the scoring method and cut-off level for high or low CAIX expression, breast cancer subtypes, HRs, 95% CIs of DFS and OS, and whether univariate or multivariate analysis was performed.

Statistical methods

Pooled HRs and their 95% CIs were used to determine the association between CAIX expression and survival. Heterogeneity among studies was assessed using the chi-squared test and I^2. A *p*-values of <0.1 or an I^2 statistic of >50% was indicative of significant heterogeneity between studies; in these cases, a random-effects model was used. The meta-analysis was performed with Review Manager 5.4 (RevMan the Cochrane Collaboration; Oxford, England). The *p*-values of <0.05 were considered statistically significant.

Results

Study selection and characteristics

A PRISMA flow diagram for the process of study selection is summarized in Figure 1. Initially, 275 articles from EMBASE, 242

from PubMed, 19 from Cochrane, and 1,897 from Scopus were identified, and subsequently, 313 duplicated records were removed. A total of 2,120 papers were screened. A total of 1,848 studies were excluded based on the titles and abstracts resulting in 272 full texts being reviewed. Of these, 245 articles were excluded. Finally, 27 papers met the eligibility criteria (Figure 1; Table 1).

Study characteristics

The 27 included studies were published between 2001 and 2022. DFS was reported in 22 articles, 10 of which provided HRs and 95% CIs, while the OS analysis was included in 16 articles, 7 of which provided HRs and 95% CIs (Table 1). Most of the articles (20 out of 27, 74%) were reported on mixed breast cancer subtypes and provided data on ER, PR, and/or HER2 staining, with survival analysis on all cases, regardless of the subtype. Three studies focused on TNBC, two articles on ER-positive (ER^+), one on ER-negative (ER^-), and one on male breast cancer. The mean age of patients was between 46 and 62 years. Fifty percent of the studies used the primary antibody clone M75 to detect the CAIX expression. In most studies (80%), the level of CAIX expression was determined by estimating both staining intensity and the percentage of tumor cells stained. The remaining studies (20%) used only intensity or percentage. The low-–high cutoff value varied across all studies. Overall, high CAIX expression in patients with breast cancer varied in each study, ranging from 8 to 91.1%. Most studies (45.5%, 12 out of 27 studies) did not report on the cellular location of CAIX expression. In 36% of studies, expression was reported in the cell membrane, in 9% of studies, CAIX expression was reported in the membrane and cytoplasm/nucleus, and in two studies, CAIX expression was reported in the exclusive cytoplasm or nuclear staining (9%).

High CAIX was associated with poor DFS in breast cancer

Twenty-two studies totaling 9,157 patients were analyzed for the effect of CAIX expression on DFS. Shamis et al. studied CAIX expression in two independent cohorts with specific HRs and 95% CIs and DFS in each cohort, and both cohorts were included in this meta-analysis (26). The study by Jubb et al. did not define the low/high cutoff for CAIX expression, but it provided the HR and 95% CI for each CAIX score of 1, 2, and 3 and compared each with that of the negative CAIX group (41). Hence, the HR and 95% CI for each CAIX score were included in the meta-analysis. High CAIX was significantly associated with poor DFS in patients with breast cancer (HR = 1.70, 95% CI = 1.39–2.07, $p < 0.00001$) with heterogeneity $I^2 = 83\%$ (Figure 2).

High CAIX was associated with poor OS in breast cancer

A total of 3,591 patients from the selected 17 studies were investigated for the association between CAIX expression and

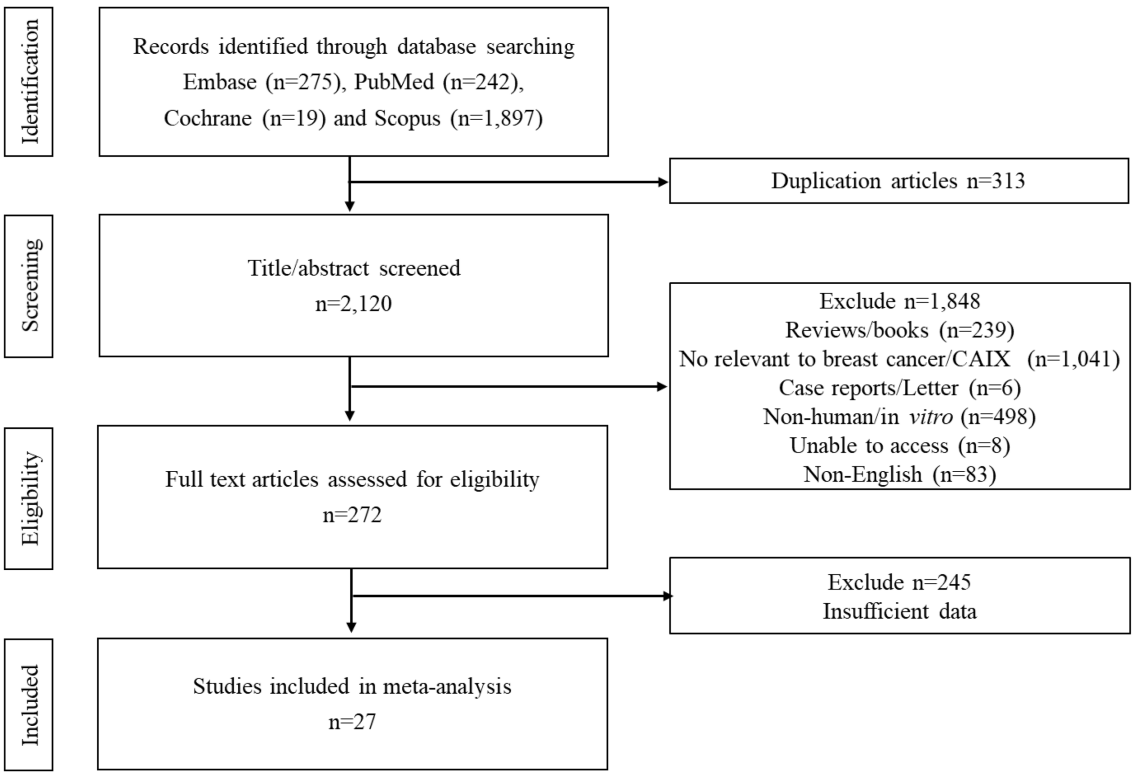

FIGURE 1
The PRISMA flow diagram for the process of study selection.

TABLE 1 Characteristics of the eligible studies for meta-analysis in this study.

References	Country	Mean age	BC subtypes (*n*)	Stage	Treatment (*n*)	IHC score method	CAIX cut-off level	CAIX high (%)	Ab clones	HR (95% CI) for DFS	*p*-value	HR (95% CI) for OS	*p*-value
Shamis et al. (26)	United Kingdom	NA	ER+ (373)	I–III	CMT (110)	Weight H score	Log-rank statistics by R Studio	9	M75	UV = 1.81 (1.12–2.92)	0.018	NA	NA
										MV = 1.04 (0.46–2.35)	0.926		
		NA	ER+ (285)	I–III	CMT (71)			28		UV = 1.64 (1.14–2.37)	0.008		
										MV = 1.74 (1.08–2.82)	0.023		
Ong et al. (22)	Singapore	55	TNBC (306)	NA	NA	I and P	≥1	39.3	NA	MV 2.77 (1.78–4.31)	<0.001	MV 2.48 (1.50–4.09)	<0.001
Li et al. (27)	China	49	ER+ (55)	Recurrence	NA	I and P	NA	34.5	ab108351	UV* 2.64 (1.28–5.44)	0.0086	NA	NA
Alves et al. (28)	Brazil	49.6	Mixed BC (196)	IIb or III	CMT (196)	I and P	≥3	7.4	ab15086	UV* 0.32 (0.19–0.55)	<0.00001	UV* 0.33 (0.15–0.66)	<0.00001
Ozretic et al. (29)	Croatia	60	TNBC (64)	NA	NA	I and P	>60	77	ab15086	NA	NA	UV 2.85 (0.36–22.25)	0.32
Jin et al. (30)	South Korea	NA	TNBC (270)	I–II	NA	NA	≥10%	21.9	NA	UV* 1.45 (0.77–2.67)	0.25	NA	NA
Chu et al. (31)	China	55.34	Mixed (149)	I–IV	CMT	I and P	Strong intensity in ≥10% cells	15	NA	MV 5.758 (2.28–14.50)	<0.001	NA	NA
Samaka et al. (32)	Egypt	48	Mixed (56)	I–IV	NA	I and P	>1%	91.1	ab107257	NA	NA	UV* 2.09 (1.05–4.19)	0.0358
Aomatsu et al. (33)	Japan	NA	Mixed (102)	IIA–IIIA	CMT (102)	I and P	Moderate to strong staining in >10% cells	46	M75	UV* 4.52 (2.05–9.97)	0.0002	UV* 3.31 (1.56–7.05)	0.0018
Deb et al. (34)	Australia	NA	Male (276)	I–IV	NA	I and P	Strong intensity in ≥10% cells	8	NA	UV 2.2 (0.8–5.7)	0.11	NA	NA

(Continued)

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TABLE 1 (Continued)

References	Country	Mean age	BC subtypes (*n*)	Stage	Treatment (*n*)	IHC score method	CAIX cut-off level	CAIX high (%)	Ab clones	HR (95% CI) for DFS	*p*-value	HR (95% CI) for OS	*p*-value
Kim et al. (35)	South Korea	52	Mixed metastasis (162)	IV	NA	I and P	≥2	19.8	NA	NA	NA	MV 1.69 (0.77–3.69)	0.189
Noh et al. (36)	South Korea	NA	ER-AR+ (127)	I–III	NA	I and P	≥2	28.7	NA	MV 2.231 (0.670–7.426)	0.191	MV 15.89 (1.82–131.6)	0.01
Betof et al. (37)	United States	48	Mixed (209)	I–III	CMT (209)	I and P	≥50	88	M75	UV* 1.75 (0.92–3.31)	0.088	UV* 2.73 (1.2–6.21)	0.0166
Kaya et al. (38)	Turkey	46	Mixed (111)	I–III	NA	I	Any staining	55.8	H-120	UV* 0.86 (0.54–1.36)	0.5253	UV* 2.77 (1.58–4.85)	0.0004
Beketic-Oreskovic et al. (39)	Croatia	61.5	Mixed (40)	I–III	NA	I and P	52.5	60	NA	UV 6.74 (2.27–20.03)	<0.001	UV 5.68 (2.11–15.31)	<0.001
										MV 4.14 (1.28–13.35)	0.018	MV 3.99 (1.38–11.59)	0.011
Lou et al. (40)	Canada	NA	Mixed (3,630)	I–III	NA	I and P	Any staining	15.6	M75	UV* 2.30 (1.91–277)	<0.00001	NA	NA
Pinheiro et al. (25)	Portugal	NA	Mixed (122)	T1-3anyN	NA	I and P	≥3	18	ab15086	UV* 2.24 (0.79–6.35)	0.1294	NA	NA
Jubb et al. (41)	United Kingdom	57 (27–80)	Mixed (151)	I–III	CMT (63)	I and P	>10%	32	M75	CAIX score 1; UV 0.63 (0.29–1.41)	0.26	NA	NA
										CAIX score 2; UV 1.24 (0.49–3.13)	0.65		
										CAIX score 3; UV 1.83 (0.86–3.89)	0.12		

(Continued)

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TABLE 1 (Continued)

References	Country	Mean age	BC subtypes (*n*)	Stage	Treatment (*n*)	IHC score method	CAIX cut-off level	CAIX high (%)	Ab clones	HR (95% CI) for DFS	*p*-value	HR (95% CI) for OS	*p*-value
Tan et al. (42)	United Kingdom	55	Mixed (407)	I–III	NA	I and P	≥10%	14	M75	UV* 1.81 (1.14–2.86)	0.0119	UV* 4.29 (2.61–7.04)	<0.00001
Crabb et al. (43)	Canada	NA	Mixed (602)	II–III	NA	NA	NA	16.7	M75	MV 1.58 (1.12–2.22)	0.008	NA	NA
Kyndi et al. (44)	Denmark	NA	Mixed (945)	II–III	NA	I and P	≥10%	16	M75	UV 1.29 (1.02–1.62)	<0.05	UV 1.3 (1.06–1.60)	<0.05
Hussain et al. (45)	United Kingdom	62	Mixed (144)	I–II	NA	I and P	Weak or strong staining and focal or diffuse distribution	26	M75	NA	NA	UV 2.63 (1.21–5.70)	0.01
												MV 2.43 (1.07–5.53)	0.035
Trastour et al. (46)	France	62	Mixed (132)	I–III	CMT/ET	I and P	>1%	29	M75	MV 2.0 (1.0–4.2)	0.05	NA	0.2
Brennan et al. (23)	Ireland	NA	Mixed (400)	II	ET (199)	I	Any staining	11	M75	UV* 1.62 (1.02–2.72)	0.041	UV* 1.92 (1.09–3.38)	0.0239
Generali et al. (47)	United Kingdom	NA	Mixed (166)	T2-4N0-1	CMT/ET (187)	I and P	Any staining	24.7	M75	UV* 1.79 (0.84–3.89)	0.1315	UV* 1.99 (0.79–5.02)	0.1443
Tomes et al. (48)	Canada	NA	Mixed (53)	any T,N	NA	P	NA	NA	M75	NA	NA	UV* 0.50 (0.30–0.85)	<0.0001
Chia et al. (49)	Canada	59	Mixed (103)	I–III	CMT (27)/ET (80)	I and P	≥1	48	M75	UV* 2.38 (1.34–4.22)	0.0031	UV 2.61 (1.01–6.75)	0.05

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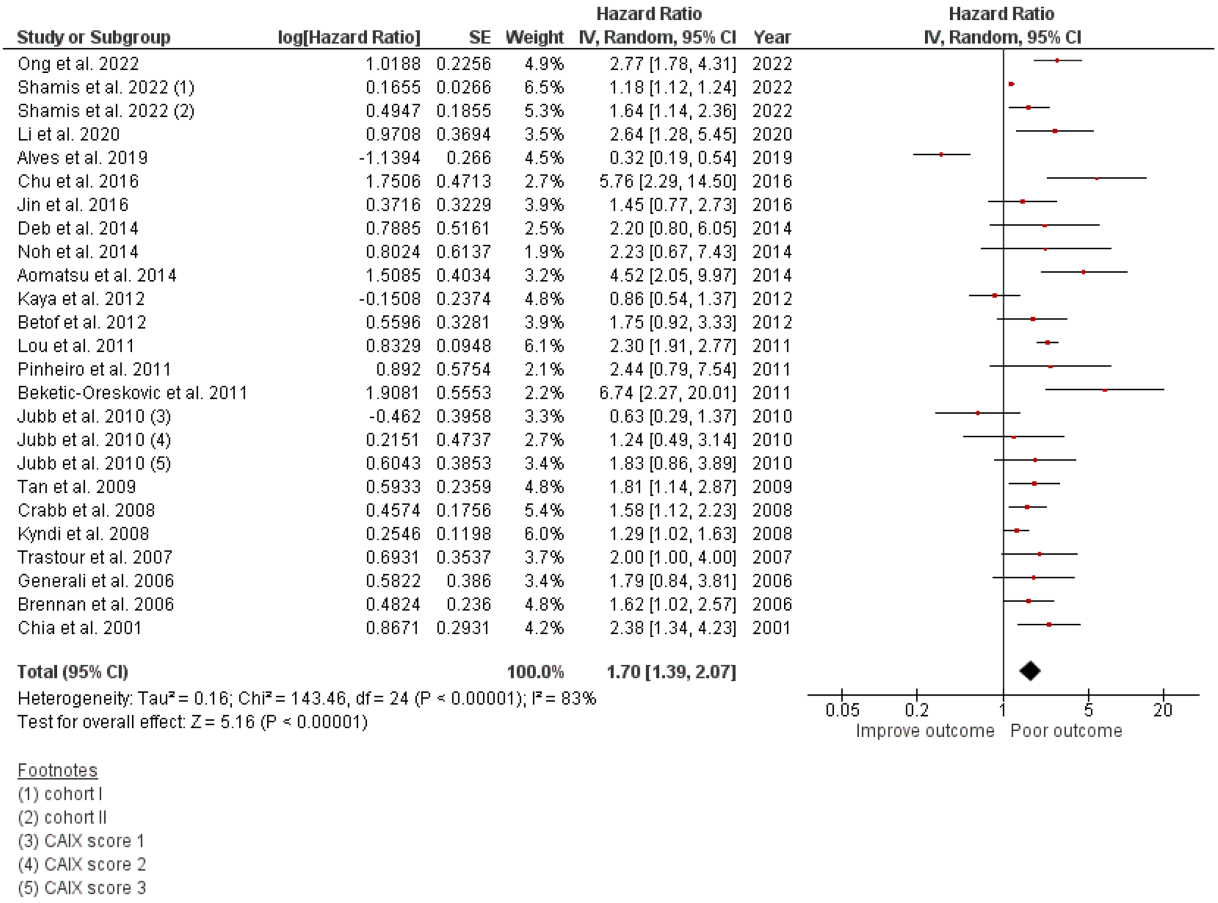

FIGURE 2
A Forest plot of HR and 95% CI for the association of CAIX with DFS of all patients with BC.

OS. High CAIX expression was statistically significantly associated with shorter OS (HR = 2.05, 95% CI 1.44–2.91, $p < 0.0001$) with heterogeneity $I^2 = 80\%$ (Figure 3).

High CAIX was associated with poor OS and DFS in ER$^+$ and TNBC subtypes

Three articles focused on the CAIX expression in 640 TNBC cases. One study reported both DFS and OS, while the other two reported either DFS or OS, resulting in 576 TNBC cases included in the DFS analysis and 370 TNBC cases included in the OS analysis. Two articles focused on CAIX expression and DFS in ER$^+$ breast cancer from 731 ER$^+$ breast cancer cases. The results revealed that, when compared to patients with a low CAIX expression, patients with a high CAIX expression were clearly associated with shorter DFS in TNBC (HR = 2.09, 95% CI =1.11–3.92, $p = 0.02$) with heterogeneity $I^2 = 63\%$ and OS (HR = 2.50, 95% CI =1.53–4.07, $p = 0.0002$) without heterogeneity $I^2 = 0\%$; and shorter DFS in ER$^+$ breast cancer (HR = 1.81 95% CI =1.38–2.36, $p < 0.0001$) without heterogeneity $I^2 = 0\%$ (Figure 4).

The antibody does not affect CAIX survival

The studies used a variety of CAIX antibodies for IHC. Twelve studies used an M75 antibody clone: 1 from BioScience, 1 from Novus Biologicals, and 1 from Bayer, but the other 9 could not be identified. The HR for DFS was 1.66 (95% CI: 1.35–2.0, $p < 0.00001$). Clones used in other studies were as follows: 6 studies used Abcam, 1 from Cell Marque, 1 from Novus Biologicals, and 2 from Santa Cruz Biotechnology (Table 1), which also demonstrated the effect of CAIX with HR for DFS 1.94 (95% CI: 1.06–3.57; $p < 0.0001$; Figure 5). There was no significant difference between the M75 antibody and other antibodies ($p = 0.63$; Figure 5). The HR for OS in the group stained with the M75 antibody was 2.01 (95% CI: 1.19–3.38; $p = 0.009$), and it was 2.10 (95% CI: 1.26–3.52; $p = 0.002$) for the other antibody group (Figure 6). There was no significant difference between the M75 antibody and the other antibodies in terms of OS ($p = 0.90$; Figure 6).

Discussion

This meta-analysis focused on the prognostic role of CAIX expression in breast cancer. Hypoxia, as determined by the CAIX

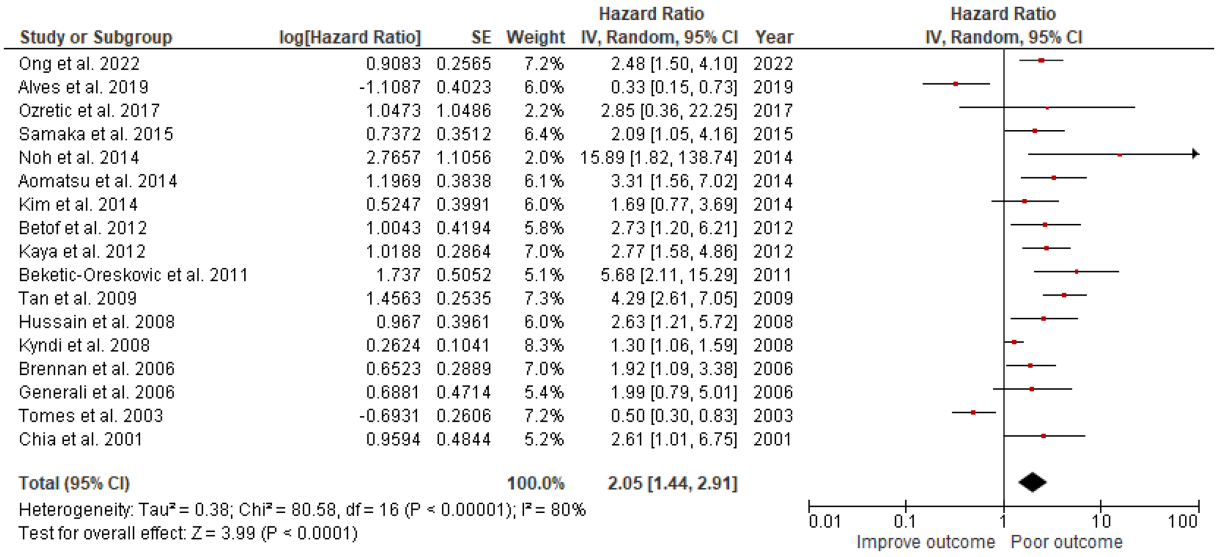

FIGURE 3
A Forest plot of HR and 95% CI for the association of CAIX with OS of all patients with BC.

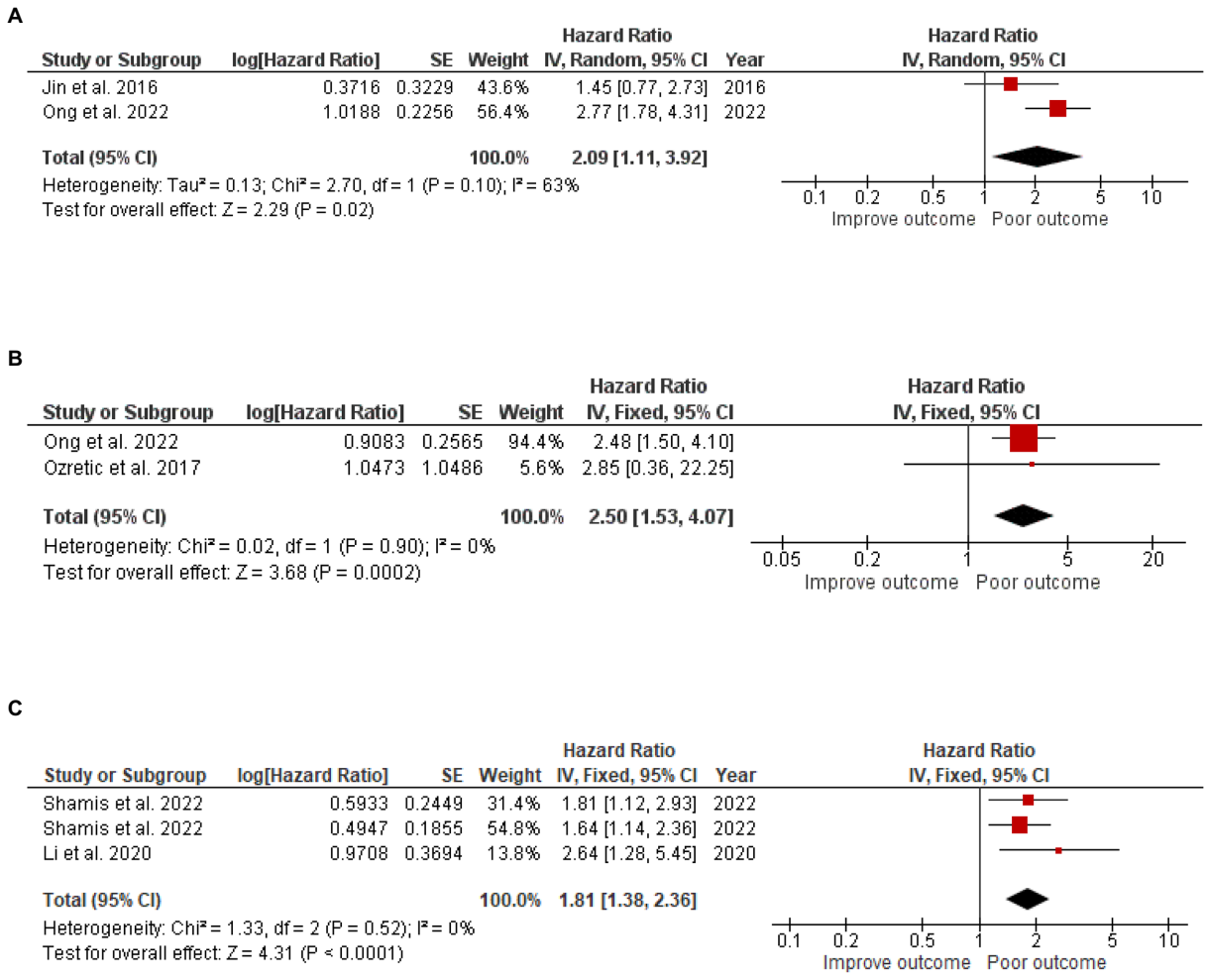

FIGURE 4
A Forest plot of HR and 95% CI for the association of CAIX with **(A)** DFS, **(B)** OS of patients with TNBC, and **(C)** DFS of patients with ER⁺ BC.

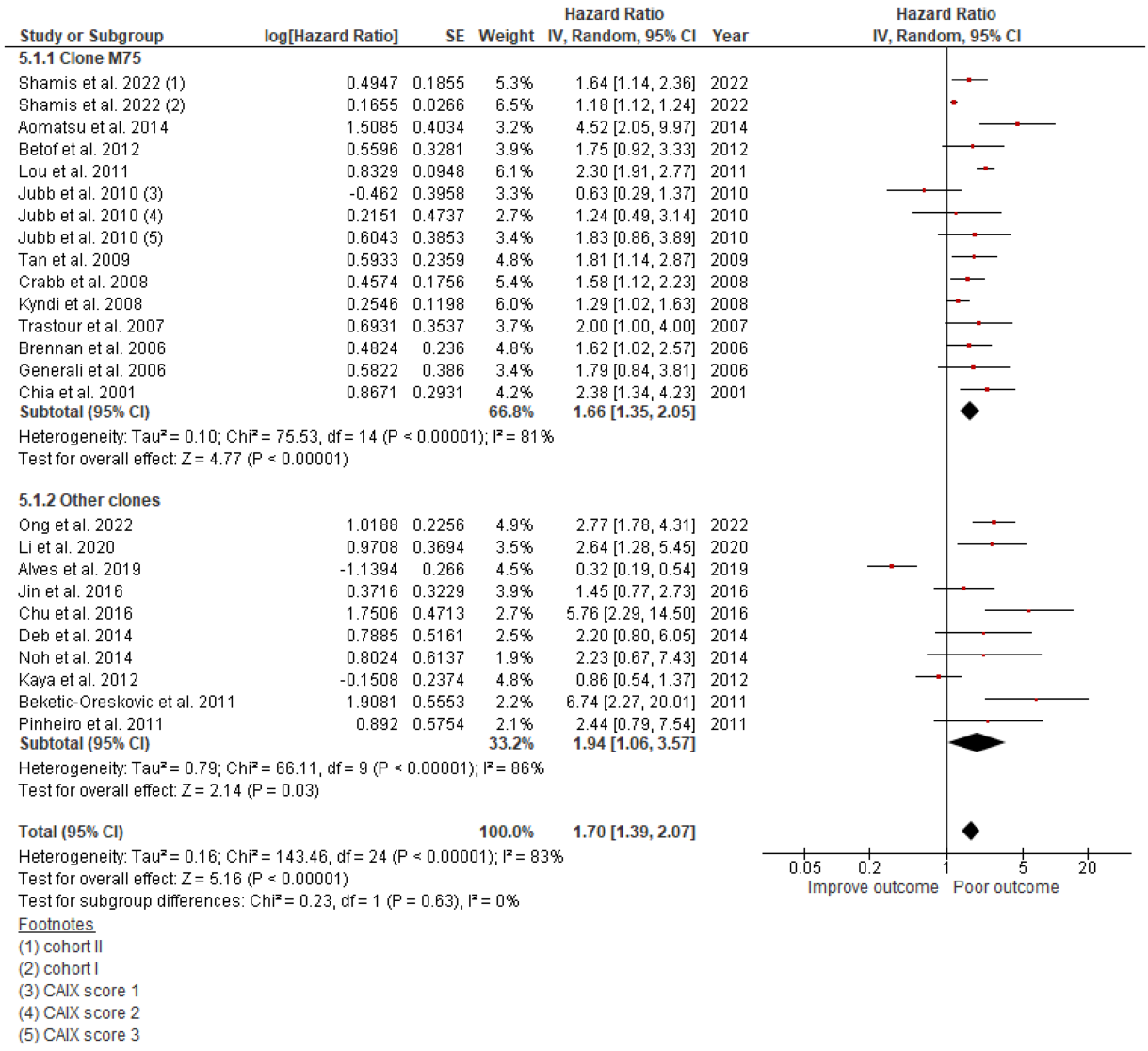

FIGURE 5
A Forest plot of HR and 95% CI for the association of CAIX expression with DFS in patients with BC stratified by the antibody clone.

expression, has been associated with poor survival outcomes, independent of other clinicopathological factors in many solid malignancies, including breast cancer (50). The current meta-analysis included a greater number of studies and confirmed a negative survival outcome in patients with breast cancer who had a high CAIX expression. To our knowledge, this is the first meta-analysis that has examined the CAIX expression exclusively in breast cancer. The results of this meta-analysis may lead to the use of CAIX expression as a prognostic marker, resulting in better treatment options for patients with breast cancer.

High CAIX was significantly associated with poor DFS (HR = 1.70, 95% CI = 1.39–2.07, $p < 0.00001$) and OS (HR = 2.02, 95% CI 1.40–2.91, $p = 0.0002$), despite the high heterogeneity of DFS, $I^2 = 83\%$, and OS, $I^2 = 81\%$. This heterogeneity could be explained by the bias in the scoring method and cutoff level as most of the studies determined the CAIX protein expression by the intensity and percentage of tumor cell staining and with individual cutoff levels. However, this meta-analysis did support the use of CAIX as a prognostic marker; therefore, the evaluation of CAIX expression should be considered in breast cancer.

Tumoral hypoxia has long been established as a factor in the progression and metastasis of cancer cells (51). CAIX protein expression is a reliable endogenous hypoxic marker as its expression is dependent on the HIF-1α activity (16). CAIX is a zinc metalloproteinase that is located at the transmembrane and acts to convert CO_2 to HCO^-_3 and H^+ (52). This process occurs extracellularly and results in an extracellular acidic pH. The cancer cells exploit the extracellular acidity to invade the stroma by promoting epithelial–mesenchymal transition (EMT) and cell motility as well as suppressing anti-tumor immunity by, for example, dysregulating cytotoxic T-cell functions while enhancing the function of M2 macrophages and myeloid-derived suppressor cells (MDSCs) (53, 54). These effects may explain the correlation between the increased expression of CAIX and poor survival outcomes.

Carbonic anhydrase IX is highly induced in a HIF-1-dependent manner and is constitutively expressed in VHL-defective cells. While

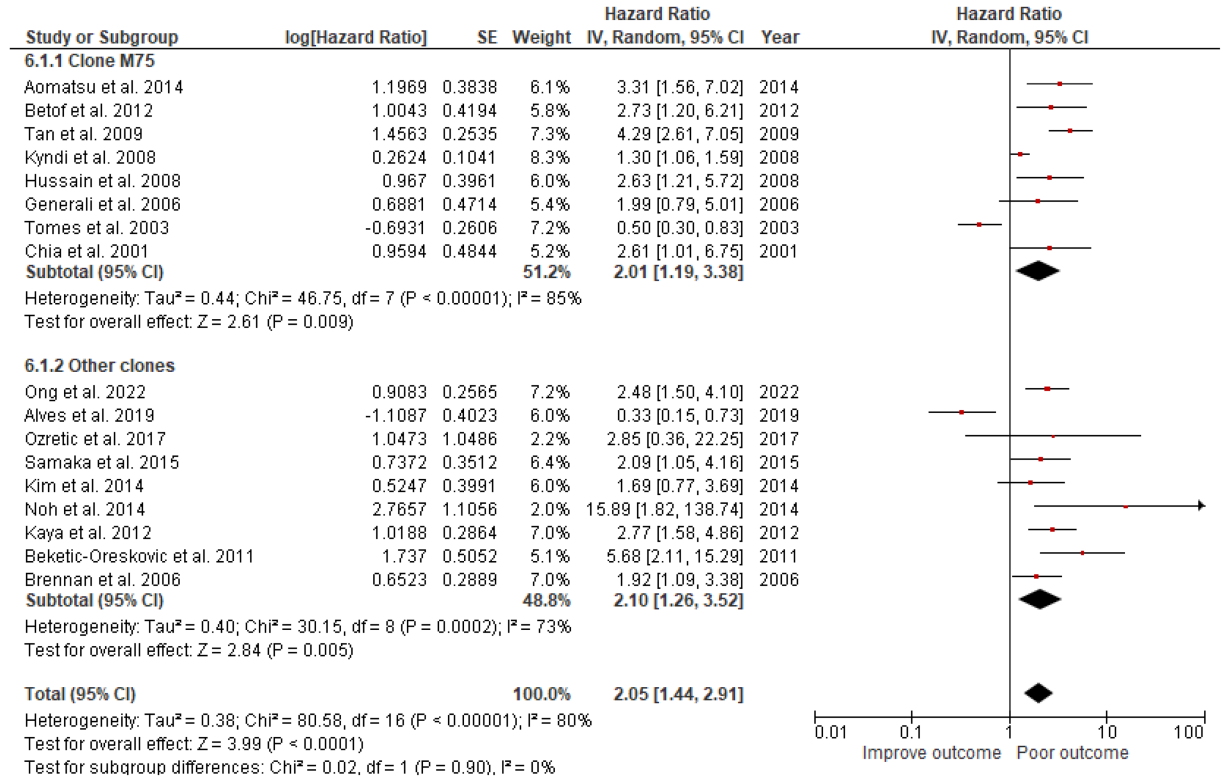

FIGURE 6
A Forest plot of HR and 95% CI for the association of CAIX expression with OS in patients with BC stratified by the antibody clone.

CAXII is upregulated in VHL-defective renal tumors and induced hypoxia in tumor cells, its dependence on HIF is not well established (15). Additionally, it is well known that the tumor expression of HIF-1α and CAIX was correlated with poor patient survival, CAXII, which lacks the extracellular proteoglycan domain of CAIX implicated in cell adhesion, had a less obvious survival effect (17). CAXII expression is related to better survival statistics for patients (55–57). In breast cancer, there is a strong association between luminal cancers and CAXII expression. Moreover, CAXII is also a biomarker of favorable prognosis in lung (58) and brain (59) tumors but is associated with a poor prognosis in colorectal cancer (60).

Additionally, this meta-analysis clarified the importance of CAIX expression associated with survival outcomes in both ER^+ and TNBC. Li et al. reported increased tamoxifen resistance in ER^+ breast cancer with a high CAIX expression (27). Similarly, a study by Tan et al. demonstrated the adverse effect of CAIX expression on basal-like breast cancer subtypes by escalating the chemotherapy resistance (42). This may imply that CAIX overexpression is a hostile factor mediating treatment resistance. Thus, a combination of chemotherapy and CAIX inhibitors may be helpful in the prevention of chemoresistance. This meta-analysis had several limitations. The high degree of heterogeneity of the study indicated that we were unable to accurately define a CAIX expression scoring method and optimal threshold values. Further studies to standardize the IHC protocol for CAIX are needed. The publication bias might overestimate the survival outcome as articles reporting positive findings were selected.

Conclusion

Our results highlight the importance of a high CAIX expression being associated with poor DFS and OS in patients with breast cancer. This information may be useful for future studies, leading to the incorporation of CAIX inhibitors in treatment regimens for patients with breast cancer. High-quality studies with larger homogeneous samples are required to determine the prognostic role of CAIX in different breast cancer subtypes.

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.

Author contributions

WN and CT contributed to the framework and the overall perspective of the study design. The literature search was carried out by WN, SY, and JP. SY and JP extracted the data and assisted with quality control. JP carried out the statistical analysis. WN

wrote the manuscript and created the tables and figures. The statistical analysis was supervised and verified by JE and CT. WN, JE, and CT contributed to the study's quality assessment and manuscript revision. JQ checked and edited the English grammar. 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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