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Transcriptomic Analysis of Metastatic Colorectal Tumor with Low Mutational Burden

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Abstract: *Objective:* To identify potential drug targets for metastasis colorectal cancer (CRC) patients with low mutational burden by examining differences in immune-related gene expression. *Methods:* For this study, 623 samples were collected from The Cancer Genome Atlas (TCGA) database, comprising tumor mutational burden (TMB), RNA sequencing (RNA-Seq), and clinical data. Differential gene expression analysis, Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the identified genes were conducted using the R package. Additionally, a comparative analysis of immune infiltrating cell composition in metastatic and non-metastatic groups was performed. Hub genes, exhibiting high levels of interaction, were selected using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database. The Drug Gene Interaction Database (DGIdb) was then utilized to estimate drugs targeting the identified hub genes. *Results:* The transcriptome data of 326 colorectal cancer patients with low TMB were analyzed, comprising 58 patients with metastasis and 268 patients without metastasis. Among the differential expression in 1,111 genes for patients with metastasis compared to those without metastasis, 733 genes were upregulated, and 378 genes were downregulated. KEGG and GO enrichment analysis indicated significant differences in gene expression in CRC metastatic patients with low TMB compared to non-metastasis patients with low TMB. Enriched pathways included humoral immune response, immunoglobulin production, and regulation of AMPA receptor activity. Two genes related to interleukin-12 were identified through secondary enrichment for immune-related genes. Analysis of tumor-infiltrating immune cell data revealed significant differences in memory-activated T cell CD4 and T cell CD8. *Conclusions:* This analysis of RNA sequencing data and immune-filtrating cell data revealed significant differences between metastatic colorectal cancer patients with low TMB and their non-metastatic counterparts. These distinctions suggest the possibility of identifying more effective drugs or therapies for metastatic colorectal cancer patients with low TMB.

Keywords: Metastatic colorectal cancer (mCRC); RNA-seq; Differentially expressed genes; Functional enrichment; Protein-protein interaction; Immunity

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1. Introduction

Colorectal cancer (CRC) stands as the third most common malignant tumor globally, ranking second in cancer-

related mortality. With over 1.8 million new cases diagnosed annually worldwide, CRC patients face a daunting 50% mortality rate [1]. Notably, approximately 25% of CRC patients present with distant metastases at initial diagnosis, and 50% of those with stages I–III disease eventually develop metastases, primarily affecting the liver which cannot be resected [2]. CRC can be categorized into two groups: high microsatellite instability (MSI-H) and microsatellite stabilization (MSS), with the former caused by DNA mismatch pair (dMMR) and accounts for approximately 15% of cases, and the latter showed chromosomal instability and accounts for approximately 85% of cases.

Anti-programmed cell death 1 (PD-1)/programmed death ligand 1 (PD-L1) monoclonal antibody (MAb) immunotherapy has emerged as a standard strategy for lung, gastric, and renal cancers. Although initial trials of CRC immune checkpoint inhibitors (ICIs) showed less promising results, dMMR/MSI-H CRCs are now recognized as candidates for anti-PD-1/PD-L1 immunotherapy [3]. Despite significant progress in using ICIs for metastatic MSI-H tumors, 85% of CRC cases are MSS tumors with low TMB, and ICIs fail to induce a response [4].

Immune evasion, a hallmark of cancer, underscores the effectiveness of therapies that restore immune surveillance, particularly in cancers with a high tumor mutational burden (TMB), such as those with microsatellite instability. However, the concept of TMB has only recently been estimated from sequencing data [5]. Approximately 12% of CRC exhibit defects in dMMR, resulting in microsatellite instability. These microsatellite unstable samples are prone to base insertions/deletions, resulting in frameshift mutations that produce neoantigenic polypeptide fragments shared by some pantomytic species [6]. Consequently, TMB has become a clinical diagnostic index for solid tumors, including CRC [7]. However, TMB's predictive ability is tumor species-specific, exhibiting a clear positive correlation in colorectal cancer and endometrial cancer but lacking sensitivity in thyroid cancer samples [8]. This limitation stems from the unknown immunogenic neoantigens associated with TMB.

The majority of CRCs are MSS with lower TMB, but the TMB of MSS CRC is still higher than that of some cancers responding favorably to ICB. A small study of MSS CRC revealed the presentation of novel antigen-derived epitopes of human leukocyte antigen class I (HLA-I) and immune cell infiltration, suggesting that intrinsic tumor microenvironment factors contribute to the poor immunogenicity of these cancers [3]. Metastatic tumor samples exhibit unique mutational characteristics [9]. However, approximately 95% of metastatic CRCs are classified as proficient mismatch repair (pMMR), MSS, and lack an immunogenic phenotype.

Various strategies have been explored to make these cancers responsive to immunotherapy, combining ICIs with drugs designed to enhance the immune response against MSS pMMR CRC. These strategies, such as the administration of fluoropyrimidine alone or in combination with bevacizumab as maintenance therapy in metastatic CRC, atezolizumab and cobimetinib, or durvalumab and tremelimumab in previously treated advanced CRC, have disappointingly failed to show convincing results [10].

Recent studies highlight low TMB as a responsive factor to immunotherapy in recurrent gliomas [11], suggesting that the characteristics of low TMB tumor samples remain unexplored. Additionally, a correlation between TMB calculated at the DNA level and the RNA transcriptome level offers an opportunity to assess low TMB sample characteristics [12]. Nevertheless, detailed studies on changes in expression profiles and immune cell infiltration in low TMB metastatic samples are still lacking.

2. Materials and methods

2.1. General information

Data from 623 low TMB CRC patients, including TMB, RNA sequencing (RNA-Seq), and clinical data, were collected from The Cancer Genome Atlas (TCGA) database. Among these, 58 patients were associated with metastasis. Low TMB is defined as tumors with fewer than 10 mutations per megabase (mut/Mb) of tumor DNA.

2.2. Analysis of the differential gene expression and immune cell infiltration

For the analysis of differential gene expression and immune cell infiltration, R software (version 4.1.1) was utilized and the DESeq2 (version 1.34.0) and ClusterProfiler (version 4.2.2) packages were employed. Differential gene expression analysis involved grouping patients into metastasis vs. non-metastasis, with a significance cutoff of adjusted *P*-value < 0.05. Expression data was represented as fragments per kilobase million (FPKM), and logFC greater than 1 indicated upregulation, while logFC less than -1 indicated downregulation. Additionally, immune cell infiltration data from TCGA were obtained and differences between the two patient groups were analyzed.

2.3. Enrichment analysis

Enrichment analysis on differentially expressed genes was conducted using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. In the initial enrichment result, pathways related to the nervous system and nervous system development were excluded. Subsequently, the first set of results were re-enriched and presented using bubble and bar plots. Simultaneously, upon observing downregulation in genes associated with immunity in the GO results, in comparison to the non-metastatic group, these genes were further enriched using the Reactome database.

2.4. Protein-drug interaction and protein-protein interaction networks analysis

To build interaction networks for each set of differentially expressed genes, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (version 1.34.0) was utilized. The "score_threshold" parameter was set to 700, and for the algorithm parameter in "get_clusters," "fastgreedy" was opted. Finally, specific genes were selected for import into The Drug Gene Interaction Database (DGIdb) to obtain protein-drug interaction results.

3. Results

3.1. Differences in gene expression and tumor-infiltrating immune cells

RNA expression data from 326 samples obtained from TCGA revealed 733 upregulated genes and 378 downregulated genes after differential expression analysis. Heat maps (**Figure 1A**) and volcano plots (**Figure 1B**) generated using the R package illustrated significant differences in expression levels, exceeding 5-fold for genes such as PPP1R17 and CLDN18. Analysis of tumor-infiltrating immune cell data indicated higher proportions of B cell plasma, T cell CD4 memory resting, macrophage MO, and macrophage M2 in the metastatic group, while T cell CD8, NK cell resting, and macrophage M1 exhibited lower proportions (**Figure 1C**).

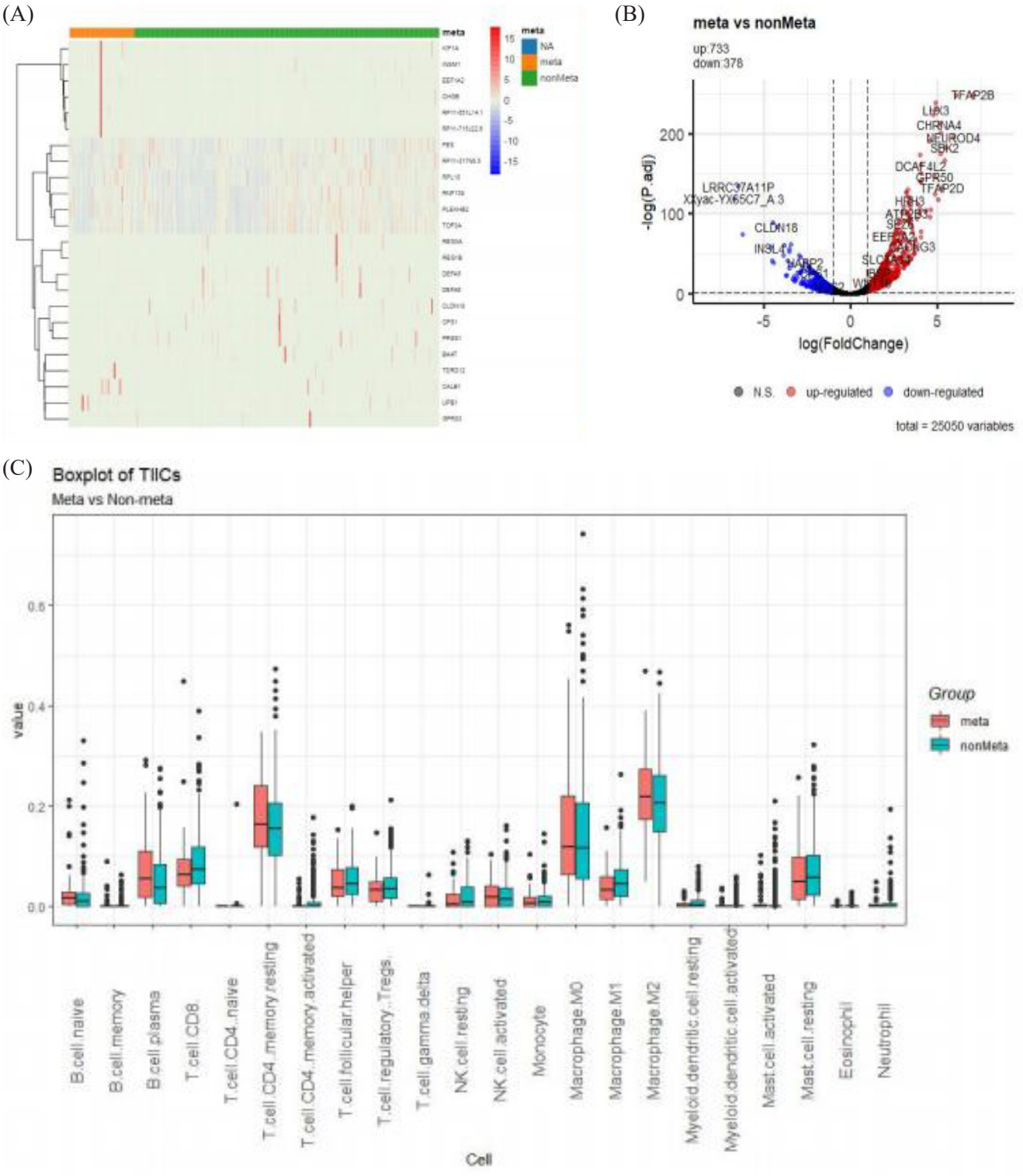


Figure 1. Differences in gene expression and tumor-infiltrating immune cells (TIICs) in low TMB CRC. **(A)** Heat map of metastatic versus non-metastatic CRC. **(B)** Volcano plot of metastatic versus non-metastatic CRC, pCutoff = 0.05. **(C)** Box plot of TIICs in the metastatic versus non-metastatic groups.

3.2. GO and KEGG analysis results of gene downregulation

A total of 378 genes were downregulated. KEGG enrichment analysis revealed that the differentially expressed genes were mainly associated with pancreatic development and retinol metabolism (**Figure 2A**). GO results showed the differential genes were predominantly distributed in antimicrobial humoral response, immunoglobulin production, and antimicrobial humoral immune response mediated by antimicrobial peptide (**Figure 2B**).

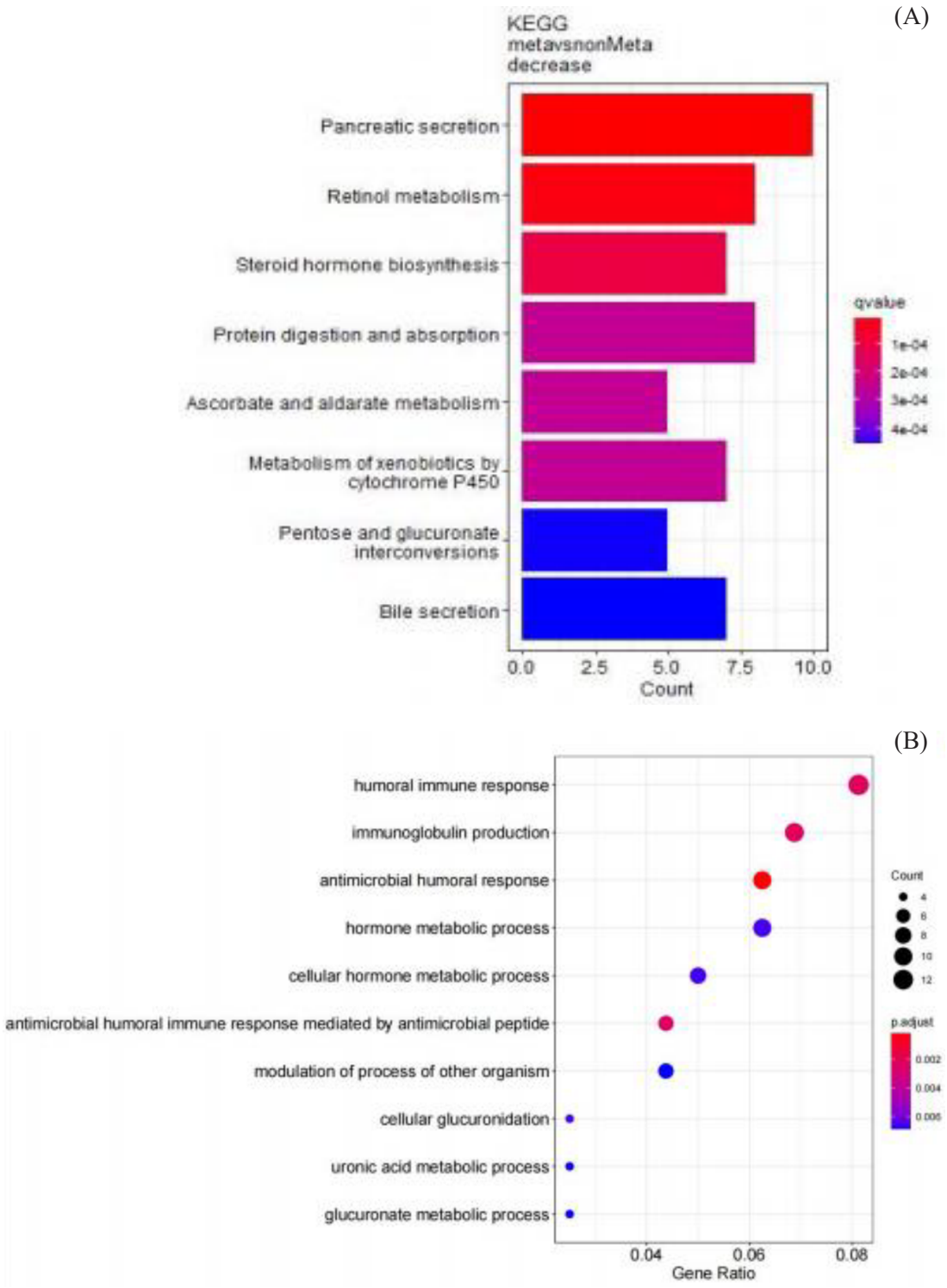


Figure 2. GO and KEGG analysis results of gene downregulation. **(A)** KEGG enrichment analysis results of downregulated genes. **(B)** GO enrichment analysis results of downregulated genes in biological processes.

3.3. GO and KEGG analysis results of gene upregulation

A total of 733 genes were upregulated. KEGG enrichment analysis showed that differentially expressed genes were primarily associated with neuroactive ligand-receptor interaction (**Figure 3A**). GO results indicated that the differential genes were mainly related to organic acid transport, regulation of AMPA receptor activity, and negative regulation of gliogenesis (**Figure 3B**).

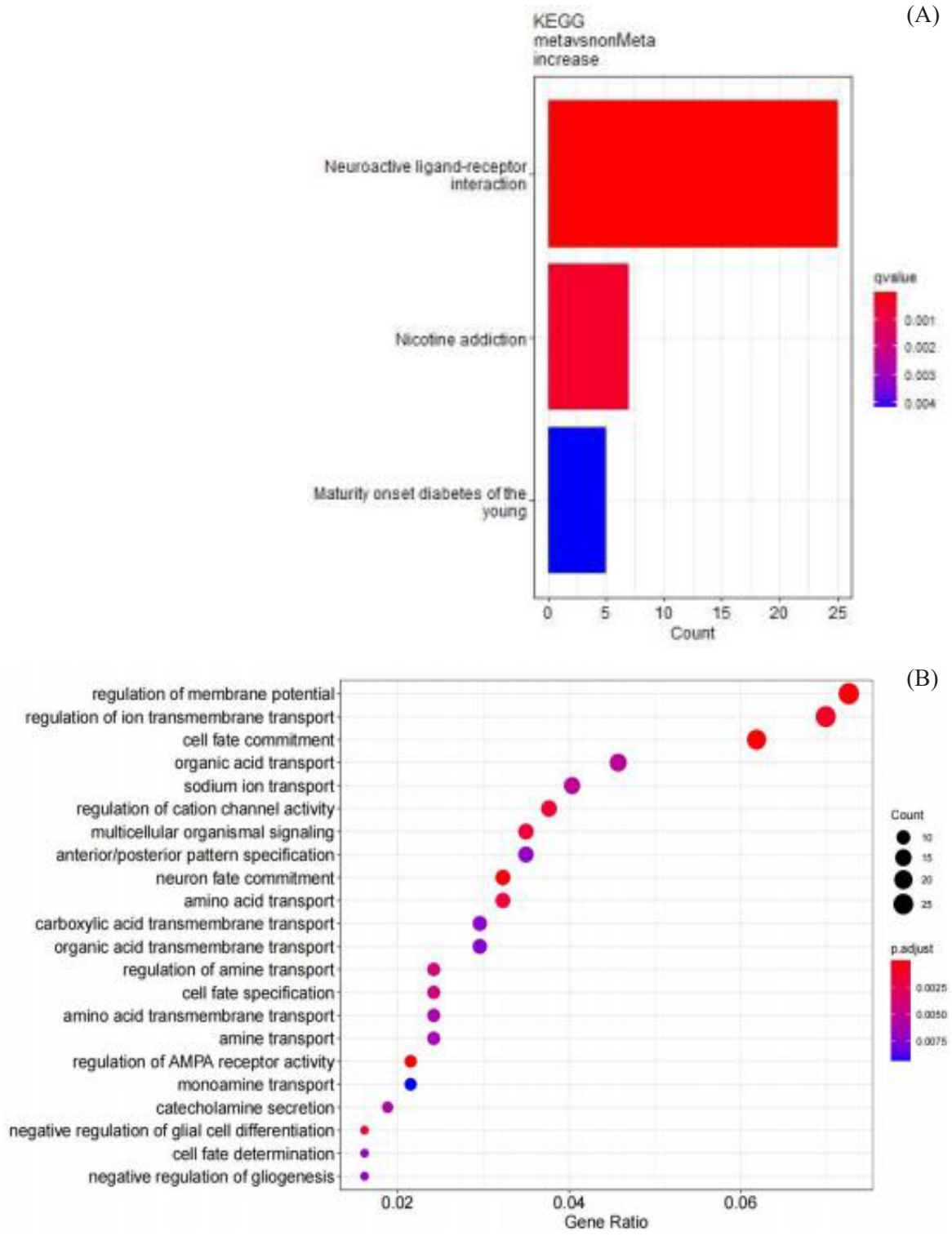


Figure 3. Go and KEGG analysis results of gene upregulation. **(A)** KEGG enrichment analysis results of upregulated genes. **(B)** GO enrichment analysis results of upregulated genes in biological processes.

3.4. Results of immune-related pathways

In the GO enrichment of downregulated genes, enrichment of genes associated with immunity was observed. These genes were re-enriched using the Reactome database, revealing associations with antimicrobial peptides, gene and protein expression of JAK-STAT signaling after interleukin-12 (IL-12) stimulation, and expression of IL-12 family signaling (**Table 1**).

Table 1. Re-enrichment analysis of immune-related genes using the Reactome database

Pathway name	Entities				Reactions	
	Found	Ratio	*P* value	FDR	Found	Ratio
Antimicrobial peptides	4/123	0.008	4.04×10^{-6}	1.54×10^{-4}	12/58	0.004
Alpha-defensins	2/11	7.24×10^{-4}	4.75×10^{-5}	9.02×10^{-4}	6/12	8.52×10^{-4}
RUNX1 and FOXP3 control the development of regulatory T lymphocytes (Tregs)	2/17	0.001	1.13×10^{-4}	0.001	3/20	0.001
Defensins	2/51	0.003	1.00×10^{-3}	0.009	7/19	0.001
Gene and protein expression by JAK-STAT signaling after IL-12 stimulation	2/73	0.005	0.002	0.014	1/36	0.003
IL-12 signaling	2/84	0.006	0.003	0.016	1/56	0.004

3.5. Results of protein-drug interactions

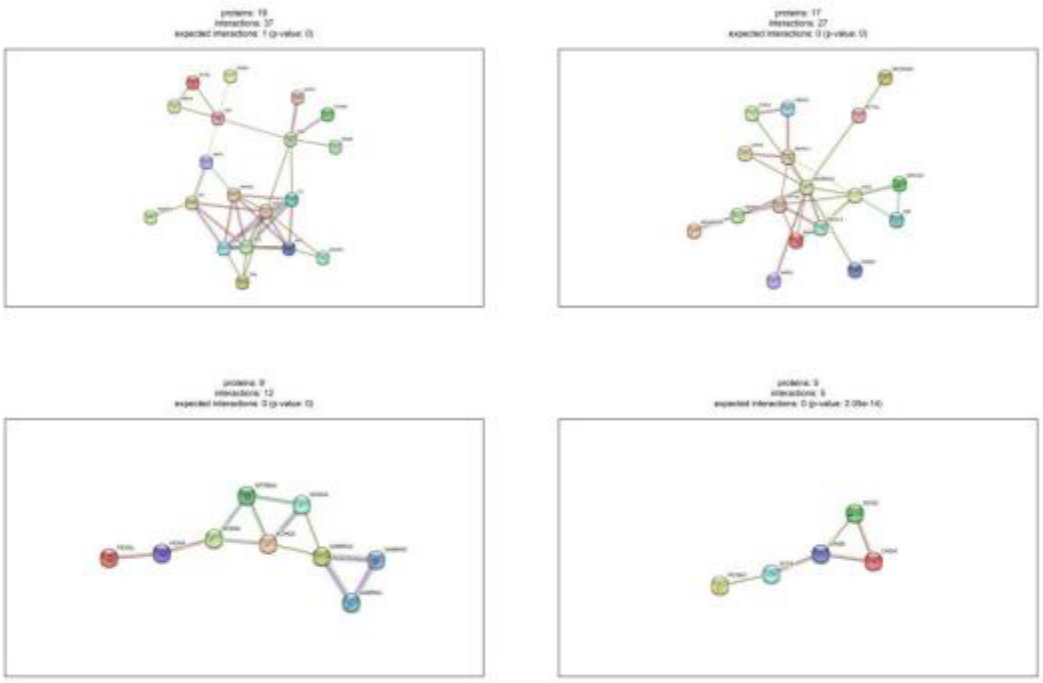
Differentially expressed genes were used to construct protein-protein interaction networks with the STRING database, resulting in 13 clusters (**Figure 5**). Of these, nine clusters were upregulated genes (**Figure 5A**) and four clusters were downregulated genes (**Figure 5B**). A total of 87 genes formed these clusters, and when introduced into the DGIdb, 331 drugs were identified. Among them, 14 drugs were categorized as anti-tumor and immunotherapy drugs, making them potential candidates for subsequent drug screening. Within the upregulated genes, associations with the Wnt signaling pathway were found, and among the downregulated genes, gamma-aminobutyric acid type A receptor alpha 5 (GABRA5), involved in GABA metabolism, was identified.

4. Discussion

CRC ranks as one of the most prevalent cancers globally, holding the third-highest incidence worldwide. With over 1.8 million new cases diagnosed annually [1], more than 50% of CRC patients exhibit a TMB of less than 10. Unfortunately, less than 25% of these patients can be treated with FDA-approved drugs [13]. In the pursuit to identify drug targets for metastatic CRC patients with low TMB, analyses on 326 gene expression data from CRC patients obtained from TCGA were conducted. GO and KEGG enrichment analysis was performed, and data on tumor-infiltrating immune cells (TIICs) were obtained concurrently. The analyses revealed significant differences in gene expression and immune cell infiltration between the metastatic and non-metastatic groups.

In the upregulated genes of the GO enrichment analysis, the observations highlighted genes associated with the AMPA receptor. Past studies have suggested that AMPA antagonists, such as GYKI 52466 and CFM-2, can inhibit the extracellular signal-regulated kinase (ERK1/2) pathway, potentially serving as useful compounds in cancer treatment [14]. Additionally, recent findings indicated that CFM-2 inhibits surviving and reduces cancer cell viability at both the mRNA and protein levels, offering a novel mechanism for promoting the anticancer effects of AMPA antagonists [15]. This suggests that AMPA inhibitors may exert anti-tumor effects in the metastatic group of CRC patients with low TMB.

(A)



(B)

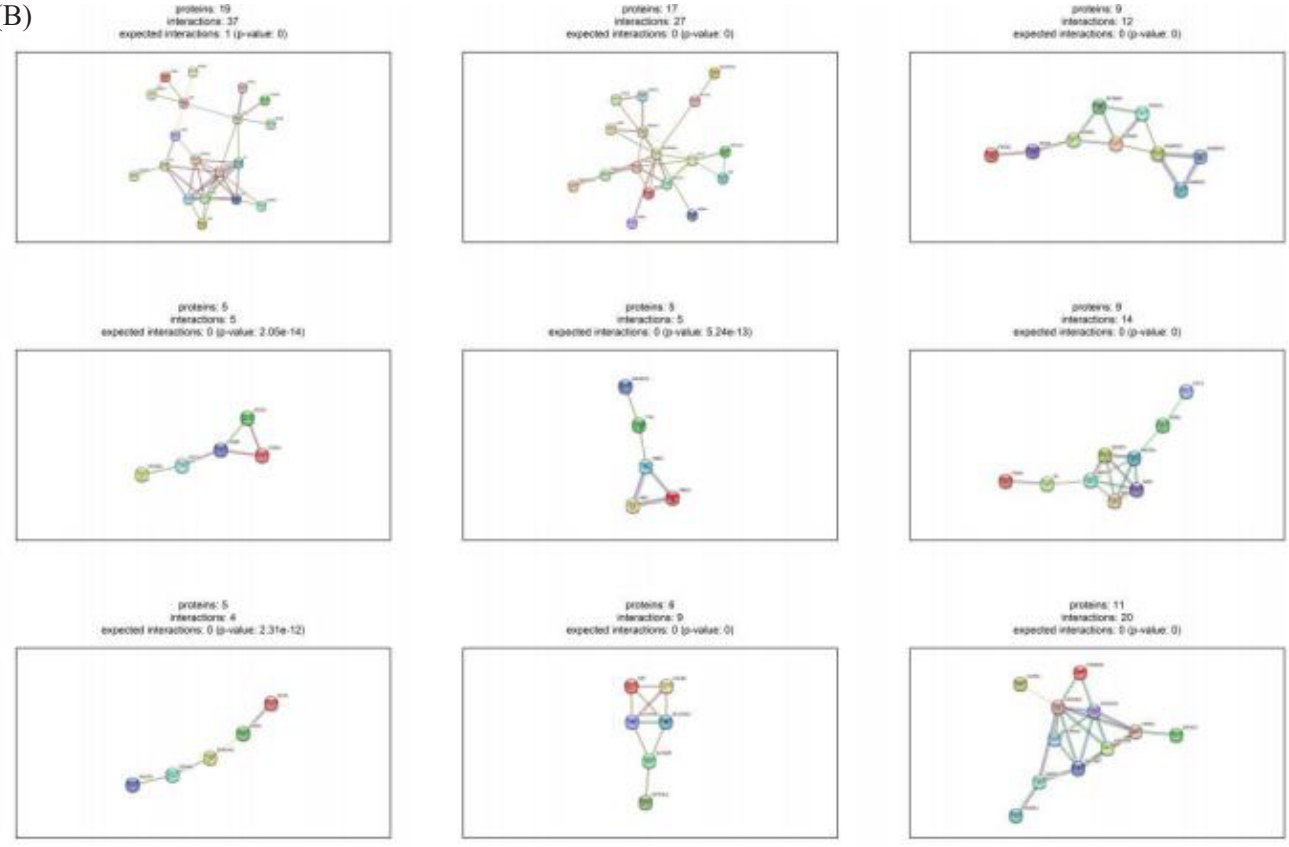


Figure 5. Results of protein-drug interactions. **(A)** Protein-drug interactions of downregulated genes. **(B)** Protein-drug interactions of upregulated genes.

Wnt, a crucial player in various cellular functions such as cell survival, maintenance, proliferation, differentiation, migration, and apoptosis, has been implicated in several cancers, including gastric, colorectal, liver, and breast cancer [16]. Activation of the Wnt/β-catenin signaling pathway is frequently observed in CRC patients and is a significant determinant of CRC pathogenesis. Some evidence suggests that IWR-1 may inhibit tumor metastasis by suppressing the Wnt/β-catenin pathway and survivin expression [17]. Another study found that SM08502, a novel small molecule for the treatment of solid tumors, reduces Wnt pathway signaling and gene expression by effectively inhibiting cdc-like kinase (CLK) activity [18]. Combination therapy with 36-077 and 5-fluorouracil (5-FU) significantly improves treatment efficacy and inhibits CRC tumor cell metastasis by impeding the GSK-3β/Wnt/β-catenin signaling pathway [19]. The protein-protein interaction network analysis in this study revealed upregulated gene clusters containing Wnt signaling genes such as WNT3a, WNT16, and CER1, suggesting that drugs targeting the Wnt pathway may be more effective in patients with low TMB metastatic CRC.

Similarly, in the protein-protein interaction network analysis, upregulated gene clusters were identified, revealing genes associated with gamma-aminobutyric acid (GABA), including GABA type A receptor subunit gamma 2 (GABRG2), GABRA5, and GABRG1. GABA, a neurotransmitter, and its receptors' subunits have been implicated in various diseases, including cancer [20]. Recent studies have even suggested the potential involvement of GABA and its receptor in the development and progression of pancreatic ductal adenocarcinoma [21]. Furthermore, B-cell-derived GABA has been shown to differentiate monocytes into anti-inflammatory macrophages, secrete IL-10, and inhibit the killing function of CD8+ T cells, indicating a potential immunomodulatory role in tumor immunotherapy [22].

IL-12 family cytokines, crucial in shaping innate and adaptive immune responses to tumors, have demonstrated their efficacy in activating anti-tumor immune responses and inhibiting immunosuppression. Research findings underscore the potential of IL-12 as an immunotherapy to control tumor growth [23], as supported by recent developments in cryo-electron microscopic imagery guiding the design of IL-12 agonists that activate T cells without toxicity [24].

The statistical analysis of immune cell infiltration revealed higher levels of B cell plasma and T cell CD4 memory resting in metastatic CRC patients compared to the non-metastatic group. Conversely, T cell CD8 and T cell CD4 memory activated were lower in the metastatic group. Immune cell infiltration in the tumor microenvironment (TME) has been recognized as a crucial contributor to tumor development and has a significant impact on the clinical prognosis of cancer patients [25]. These observations suggest a complex interplay of immune cell populations that may influence the progression and response to therapy in metastatic CRC patients with low TMB.

5. Conclusion

A comparative analysis of RNA expression data and tumor immune infiltrating cell data between CRC patients with low TMB in metastatic and non-metastatic groups was conducted in this study. The findings revealed numerous signaling pathways with differential expression and significant variations in immune cell profiles between the two groups. Previous studies have identified drugs that could potentially have therapeutic effects on these observed differences. This implies the existence of more promising therapeutic approaches for patients with low TMB CRC metastases. Nevertheless, it is important to note that, due to time and cost constraints, the clinical effects of the identified drugs were not validated, representing a shortcoming of this study.

Disclosure statement

The authors declare no conflict of interest.

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