Novel biotargets of colorectal cancer peritoneal metastasis

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Abstract

Introduction: Peritoneal metastasis often predicts advanced progression and a poor prognosis in colorectal cancer (CRC). However, peritoneal metastases are extremely difficult to predict or diagnose by routine diagnostic methods.
Material and methods: In this study, a microarray containing 30 samples from peritoneal metastasis and their matched CRC primaries obtained during cytoreductive surgery were compared to take a long hard look at all the options on the significant differentially expressed genes. The potential interactions and mechanisms of these expressed genes in promoting peritoneal metastasis were analyzed and studied by multiple bioinformatics analysis.
Results: The results suggested that the functions of these genes are closely related to immune response and cytokine activity. Additionally, the top 10 core genes' correction with the leukocyte infiltration and serum cytokine profiles were identified and may be expected to become diagnostic and therapeutic targets of peritoneal metastasis in CRC.
Conclusions: The expression of IL-6, IL-10 and IL-17 in plasma and their correlation with leukocyte infiltration are proven potential diagnostic, prognostic, and therapeutic biomarkers for peritoneal management of CRC.

Key words: colorectal cancer, peritoneal metastasis, bioinformatic analyses, biomarker.

Introduction

Colorectal cancer (CRC) is the most common malignant tumor of the digestive tract with high incidence and mortality rates, and approximately 151,030 new individuals will be diagnosed with CRC in 2022 [1, 2]. Peritoneal metastases are accompanied by high incidence, representing the second most common metastatic site, and associated with a poor quality of life, significant morbidity and dismal disease outcome in advanced CRC patients [3]. Once peritoneal metastasis presents in CRC patients, the

stage classification is stage IV. Patients with peritoneal metastatic CRC have shorter overall survival and worse prognosis compared with those with other isolated sites of metastases [4, 5].

Appropriate screening and surveillance can further mitigate peritoneal metastatic CRC morbidity and mortality [1]. However, peritoneal metastases are extremely difficult to predict or diagnose by normal diagnostic methods [6]. Novel therapeutic and targeted biologic agents are urgently needed to enhance the prognosis of patients with peritoneal metastatic CRC [5, 7].

Notably, bioinformatic analyses has been effective offering multiple diagnostic and therapeutic biomarkers in prolonging survival and enriching treatment strategies in pan-cancer [8–10]. In this study, we analyzed in depth the difference between peritoneal metastases and their matched CRC tissues excised simultaneously at the time of surgery to improving the overall diagnosis and treatment of colorectal peritoneal metastasis.

Material and methods

Differentially expressed gene analysis

The original data of GSE161097 microarray was downloaded from GEO datasets (https://www.ncbi.nlm.nih.gov/geo/) [11]. This microarray contained 30 samples from peritoneal metastasis with their matched colorectal cancer primaries obtained during cytoreductive surgery [12]. The gene expression profiles of peritoneal metastasis and their matched CRC primaries were compared. Genes with adjusted $|\log2FC| \geq 1$ and $p < 0.05$ were identified as differentially expressed genes.

Functional enrichment analysis

Functional characteristics of the differentially expressed genes in CRC peritoneal metastases were performed by DAVID 6.8 (https://david.ncifcrf.gov/home.jsp) [13] and Metascape (http://metascape.org) [14]. In this research, the Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the differentially expressed genes were provided. Biological processes (BP), cellular components (CC), and molecular function (MF) were included in the GO enrichment analysis.

Gene network, interaction, mCODE and core genes analyses

GeneMANIA (http://www.genemania.org) [15] provided information for genetic interactions, pathways, co-expression, co-localization, and protein domain similarity of submitted differentially expressed genes in CRC peritoneal metastases. Cytoscape is an open-source software platform [16]. In this research, the list of differentially expressed genes was uploaded to Plugin STRING to analyze the protein–protein interaction network [17]. Plugin mCODE was used to identify the most highly interconnected region and hubs [18]. Plugin cytoHubba was used for exploring the top 10 core changed genes [19].

Leukocyte infiltration of the core changed genes in patients with CRC

In our study, correlative analysis between expression of the top 10 core genes and the infiltration of leukocytes was performed using "Gene module" on TIMER (https://cistrome.shinyapps.io/timer/) [20]. The *p*-value cutoff was 0.05.

CRC patients' serum collection and cytokine profile analyses

Peripheral blood samples were collected from 25 patients aged 18–70 years old with metastatic CRC (including 14 peritoneal metastases and 11 cases of metastases to other sites) at the time of the first diagnosis at Affiliated Hangzhou First People's Hospital, Westlake University School of Medicine, Hangzhou, China, from April 2021 to July 2021; these patients did not receive any pre-operative chemo-radiotherapy. This study was approved by the Research Ethics Committee of the Affiliated Hangzhou First People's Hospital, Westlake University School of Medicine and followed the Declaration of Helsinki. All participants signed written informed consent forms before the research. Then the cytokine profiles in serum were quantified using ELISA kits according to the manufacturer's protocol. ELISA sets were purchased from BioLegend and analyzed by LEGENDplex 8.0. Analyses were carried out using GraphPad Prism 8 and SPSS 22. Student's *t* test was used to generate a *p*-value ($*p < 0.05$, $***p < 0.001$).

Results

Differentially expressed genes in CRC peritoneal metastases

To reveal the super-specialized genes in promoting CRC peritoneal metastasis we compared the differences between 30 samples from peritoneal metastasis and their matched CRC primaries obtained during cytoreductive surgery (Figure 1 A). Genes with adjusted $|\log2FC| \geq 1$ and a $p < 0.05$ were obtained. We identified 95 genes dysregulated in CRC peritoneal metastasis, which included 84 up-regulated and 11 down-regulated genes (Figure 1 B).

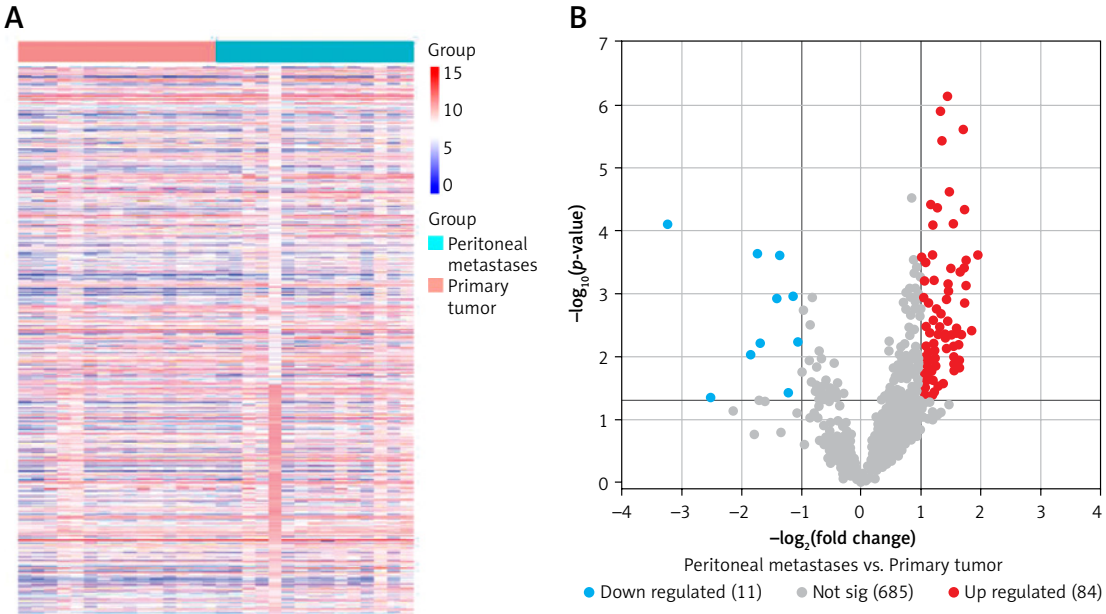


Figure 1. Differentially expressed genes in CRC peritoneal metastases. **A** – Gene heat map of 30 samples from peritoneal metastasis and their matched CRC primaries. **B** – 84 up-regulated (red) and 11 down-regulated (blue) differentially expressed genes were identified

Functional enrichment analysis of differentially expressed genes in CRC peritoneal metastases

Subsequently, overall consideration of the functional characteristics of the differentially expressed genes in CRC peritoneal metastases were performed. DAVID 6.8 and Metascape were utilized to analyze the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway items. Among the most highly enriched functions in the BP category, chemokine-mediated signaling pathway, leukocyte chemotaxis, response to lipopolysaccharide, and response to chemokine were associated with the peritoneal metastases of CRC (Figure 2 A). The external side of the plasma membrane and the secretory granule membrane were the most highly enriched items in the CC category (Figure 2 B). In the molecular function MF category, the differentially expressed genes in CRC peritoneal metastasis genes were mainly enriched in cytokine activity, pattern recognition receptor activity, G protein-coupled receptor binding, and chemokine activity (Figure 2 C). As expected, KEGG pathway analyses were also performed. Cytokine-cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptor, and chemokine signaling pathway were significantly associated with CRC peritoneal metastases (Figure 2 D).

Gene network, interaction, mCODE and core gene analyses of differentially expressed genes in CRC patients with peritoneal metastases

The results of GeneMANIA also revealed that the functions of differentially expressed genes in CRC patients with peritoneal metastases were primarily related to leukocyte migration, cell chemotaxis, and leukocyte chemotaxis (Figure 3 A). Moreover, we conducted a PPI network analysis of these differentially expressed genes with STRING to explore the potential interactions among them (Figure 3 B). The mCODE and the top 10 core genes associated with differentially expressed genes in CRC patients with peritoneal metastases were isolated with Cytoscape (Figures 3 C, D). The core genes included *itgam*, *il10*, *il6*, *cd86*, *gzmb*, *tlr7*, *ifna1*, *sell*, *cd28* and *tbx21*, all of which were up-regulated genes.

Leukocyte infiltration of the core changed genes in CRC patients

Functional enrichment analysis of differentially expressed genes showed that especially the core changed genes are involved in leukocytes response and cytokine interaction, which may affect peritoneal metastases in CRC. Therefore, we embarked on an exploration of the correlation between the core changed genes and leukocytes infiltration using the TIMER database. There was a positive correlation between itgam expression

and the infiltration of B cells, CD4+ T cells, CD8+ T cells, macrophages, neutrophils and dendritic cells. *il10* expression was positively associated with the infiltration of B cells, CD4+ T cells, CD8+ T cells, macrophages, neutrophils and dendritic cells. *il6* expression was positively associated with the infiltration of CD4+ T cells, CD8+ T cells, macrophages, neutrophils and dendritic cells. *cd86* was positively associated with the infiltration of B cells, CD4+ T cells, CD8+ T cells, macrophages, neutrophils and dendritic cells. *gzmb* was positively associated with the infiltration of CD8+ T cells, neutrophils and dendritic cells. *tlr7* was positively associated with the infiltration of B cells, CD4+ T

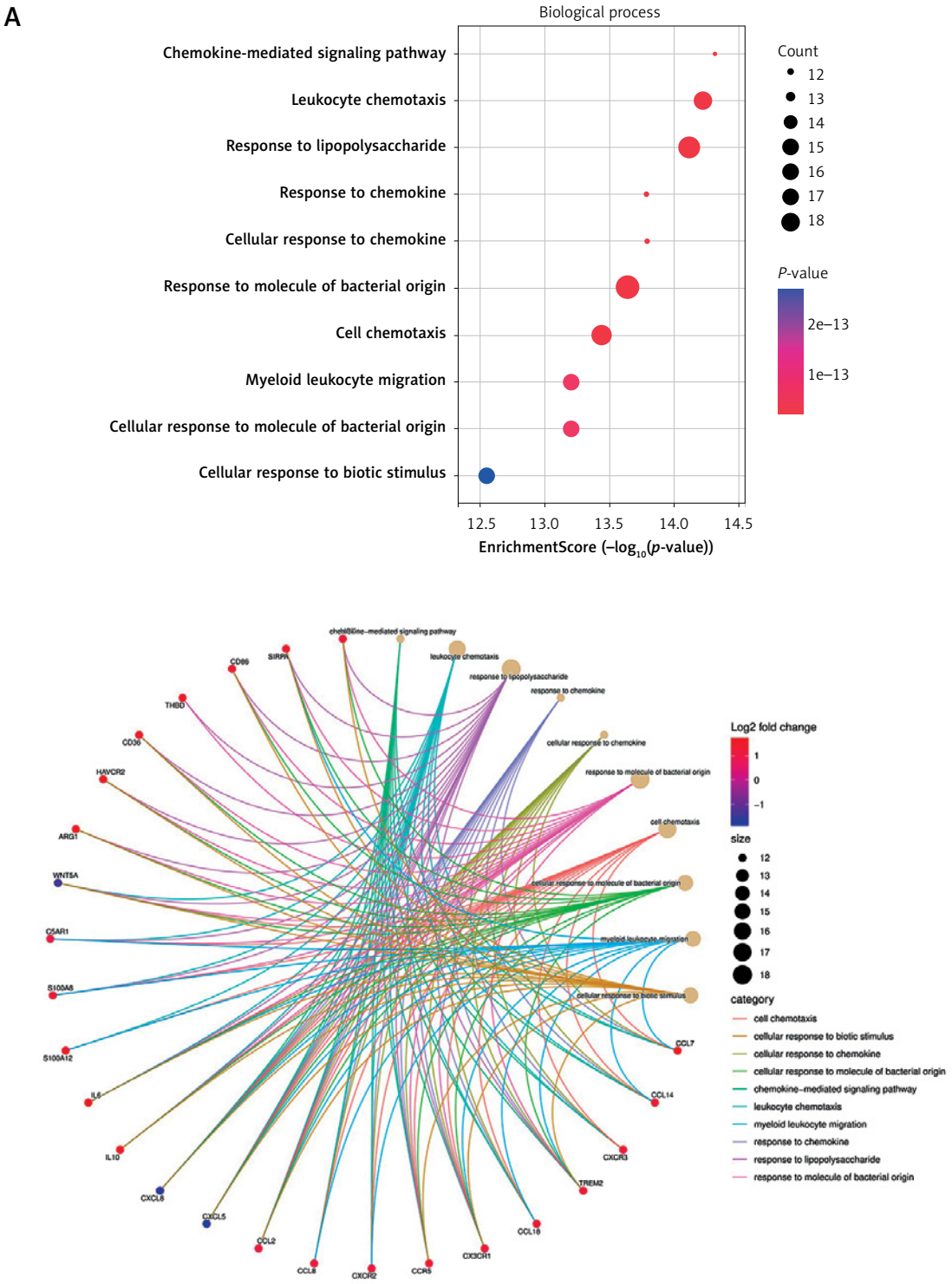


Figure 2. Enrichment analysis of differentially expressed genes in CRC peritoneal metastases. **A** – Bar plot of GO enrichment in biological process terms

B

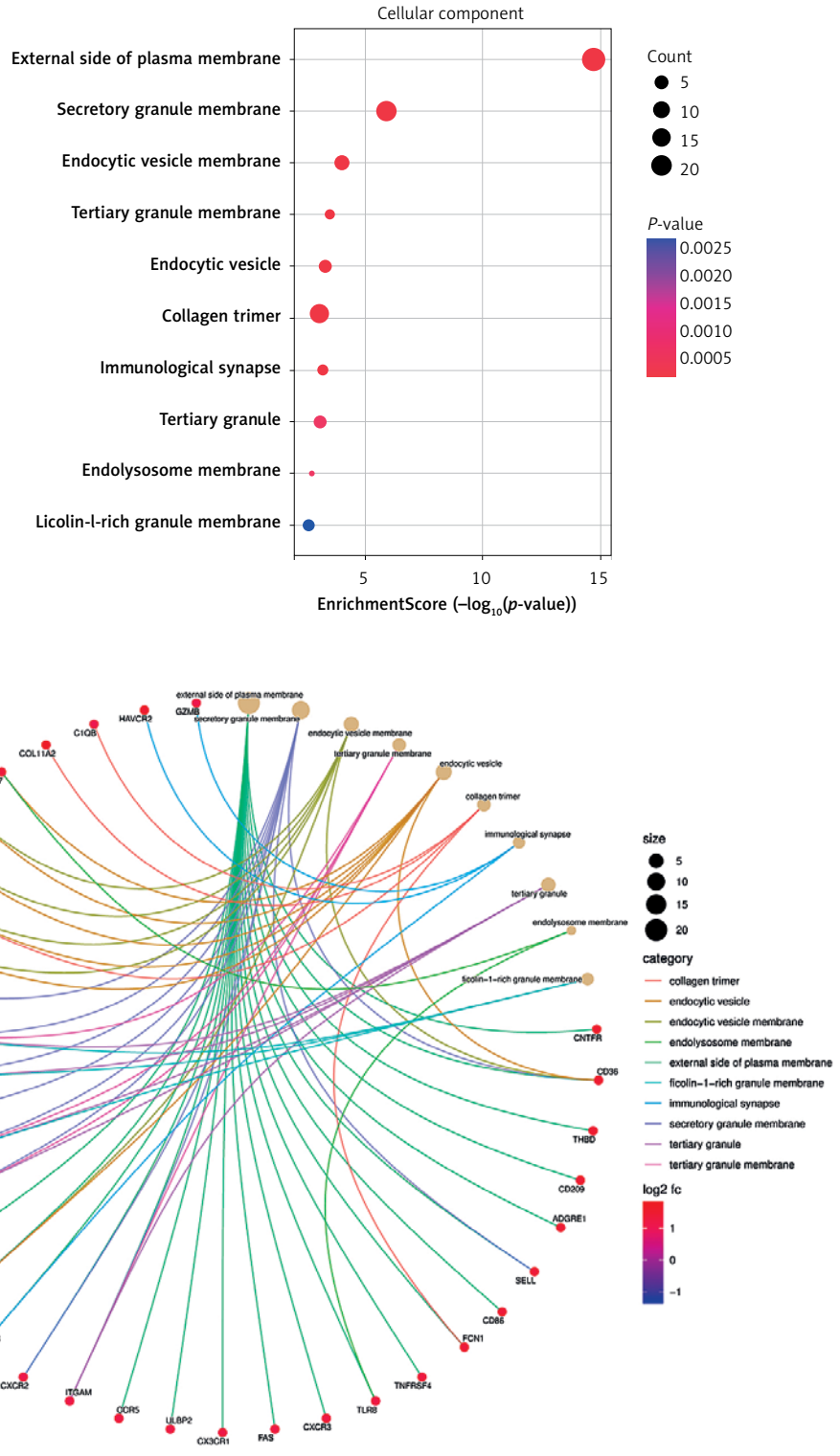


Figure 2. Cont. **B** – Bar plot of cellular component terms

C

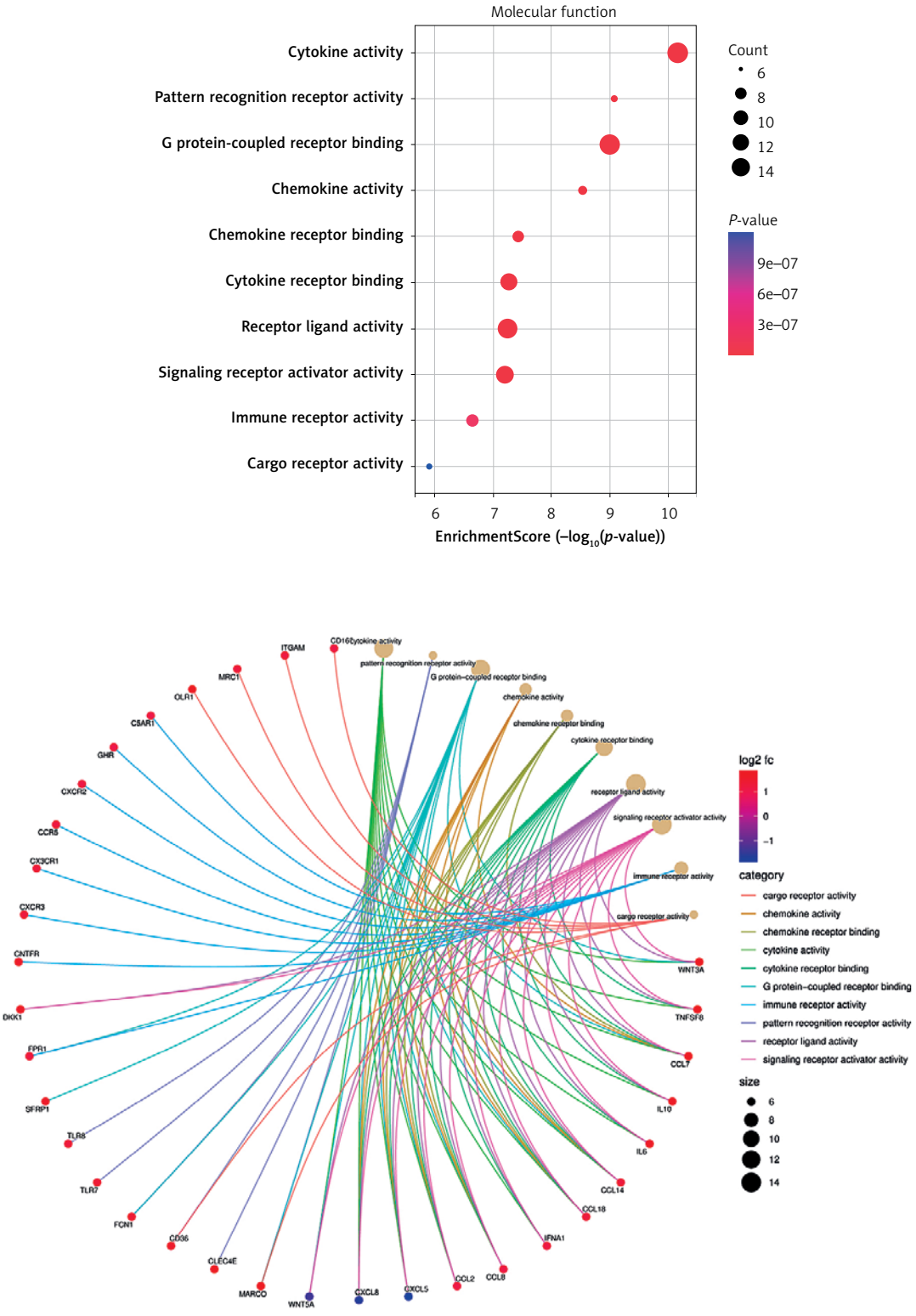


Figure 2. Cont. **C** – Bar plot of molecular function terms

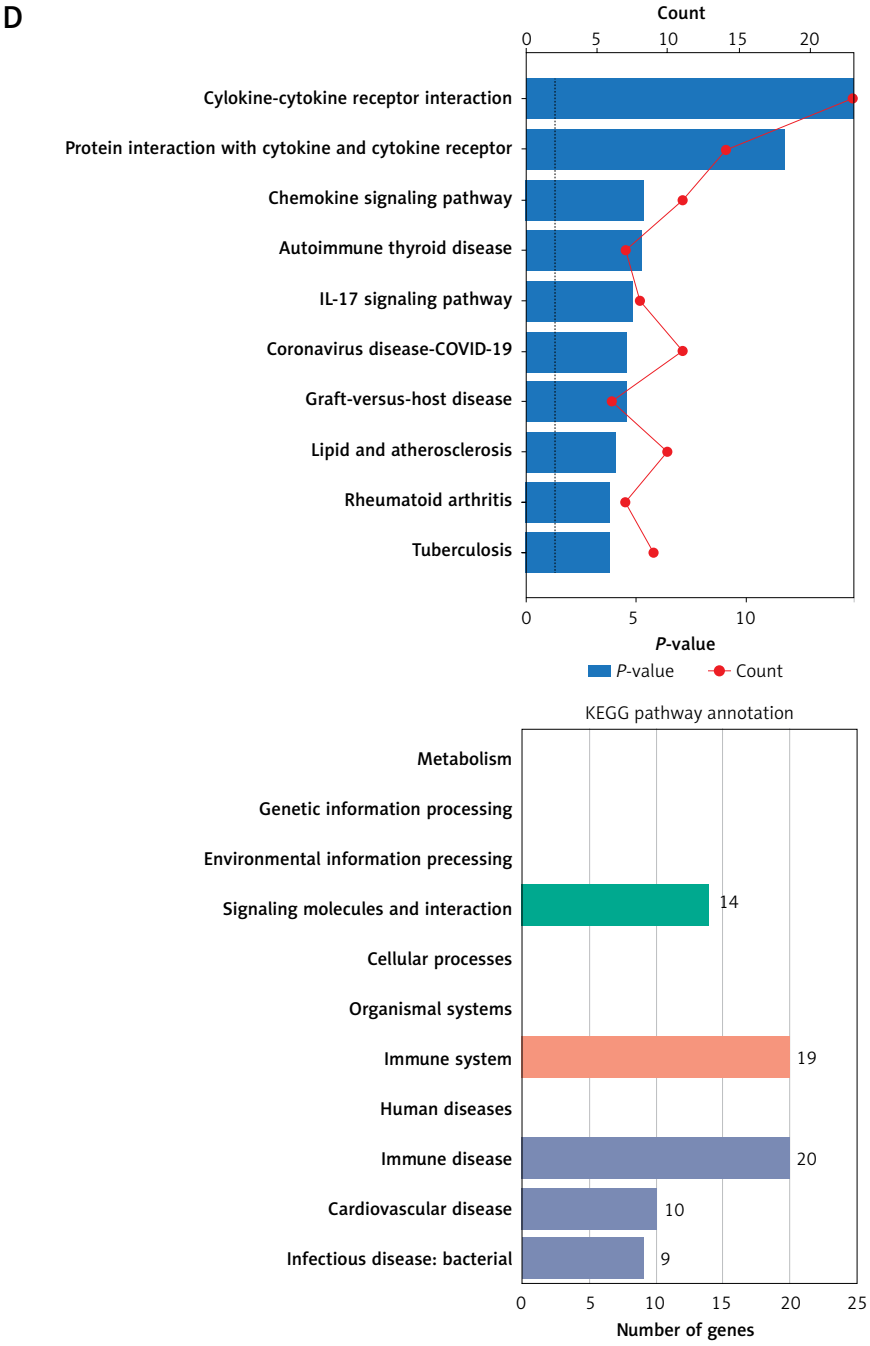


Figure 2. Cont. **D** – Bar plot of KEGG enriched terms

cells, CD8⁺ T cells, macrophages, neutrophils and dendritic cells. *ifna1* was positively associated with the infiltration of CD8⁺ T cells and dendritic cells. *sell* was positively associated with the infiltration of B cells, CD4⁺ T cells, CD8⁺ T cells, macrophages, neutrophils and dendritic cells. *cd28* was positively associated with the infiltration of B cells, CD4⁺ T cells, CD8⁺ T cells, macrophages, neutrophils and dendritic cells. *tbx21* was positively associated with the infiltration of B cells, CD4⁺ T cells, CD8⁺ T cells, macrophages, neutrophils and dendritic cells (Figure 4).

CRC patients' serum cytokine profiles

Subsequently, the levels of CRC patients' serum cytokine profiles were detected. Samples of peripheral blood were collected from 25 CRC patients at stage IV, including 14 peritoneal metastases and 11 other metastatic sites, to verify the correlation between cytokine and peritoneal metastases. There were clearly elevated levels of secretion of interleukin-6 (IL-6, $*p < 0.05$), IL-10 ($***p < 0.001$) and IL-17 ($*p < 0.05$) in CRC peritoneal metastases (Figure 5). These results validated the correlations between the core cytokine

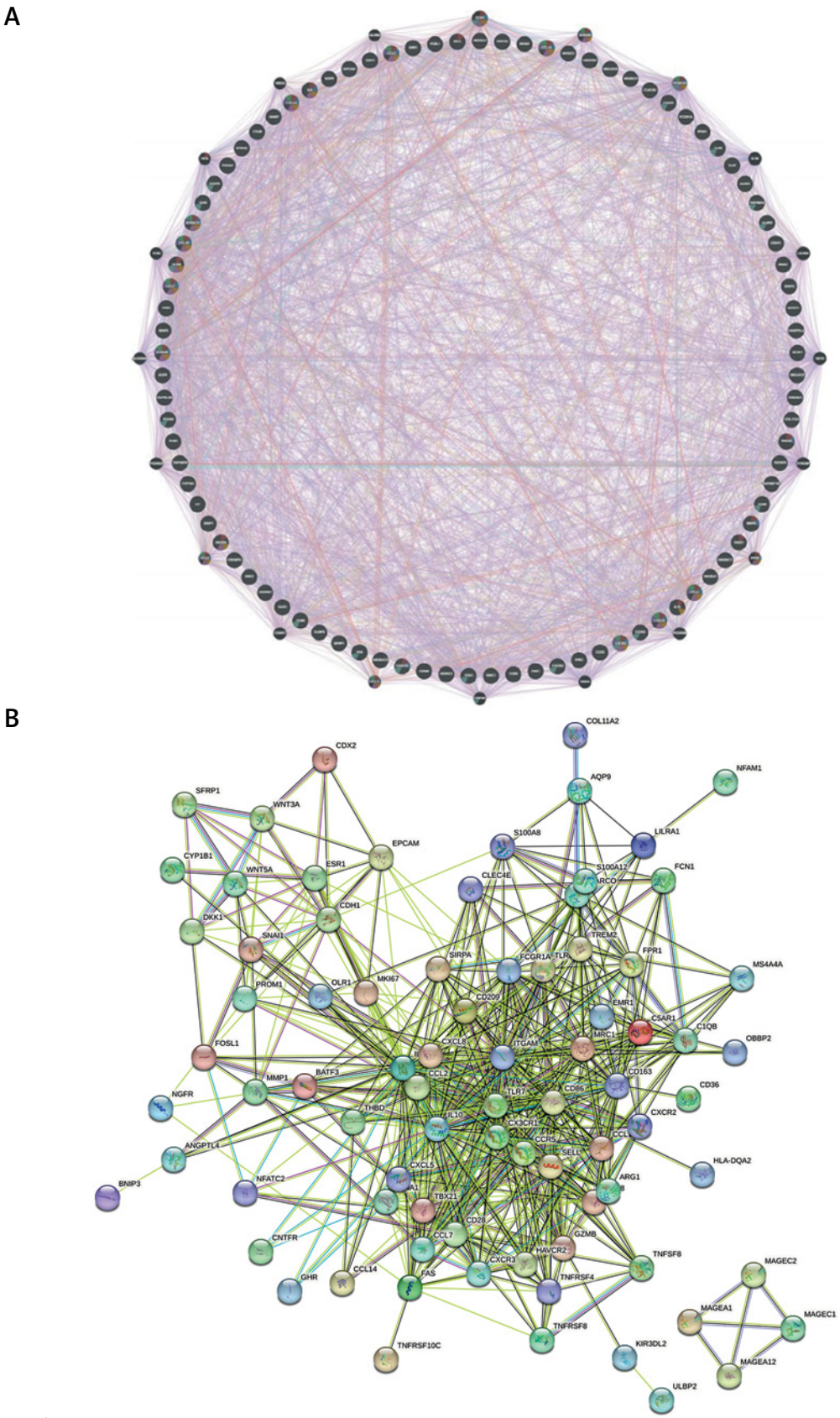
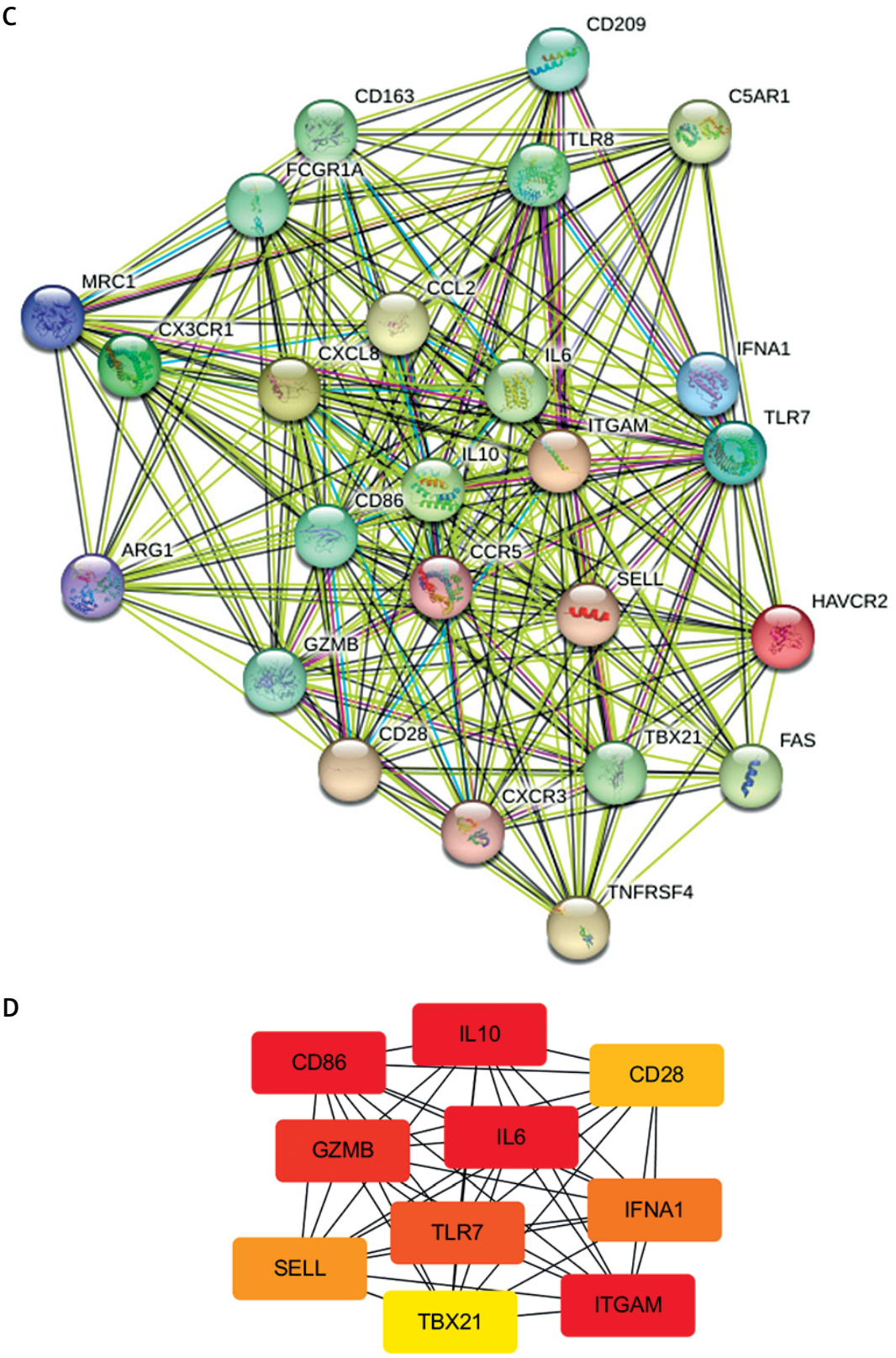


Figure 3. Gene network, interaction, mCODE and core gene analyses of differentially expressed genes in CRC patients with peritoneal metastases. Gene–gene interaction network (**A**) and protein–protein interaction network (**B**) of differentially expressed genes in CRC patients with peritoneal metastases. The most significant mCODE (**C**) and top 10 core changed genes (**D**) in CRC patients with peritoneal metastases



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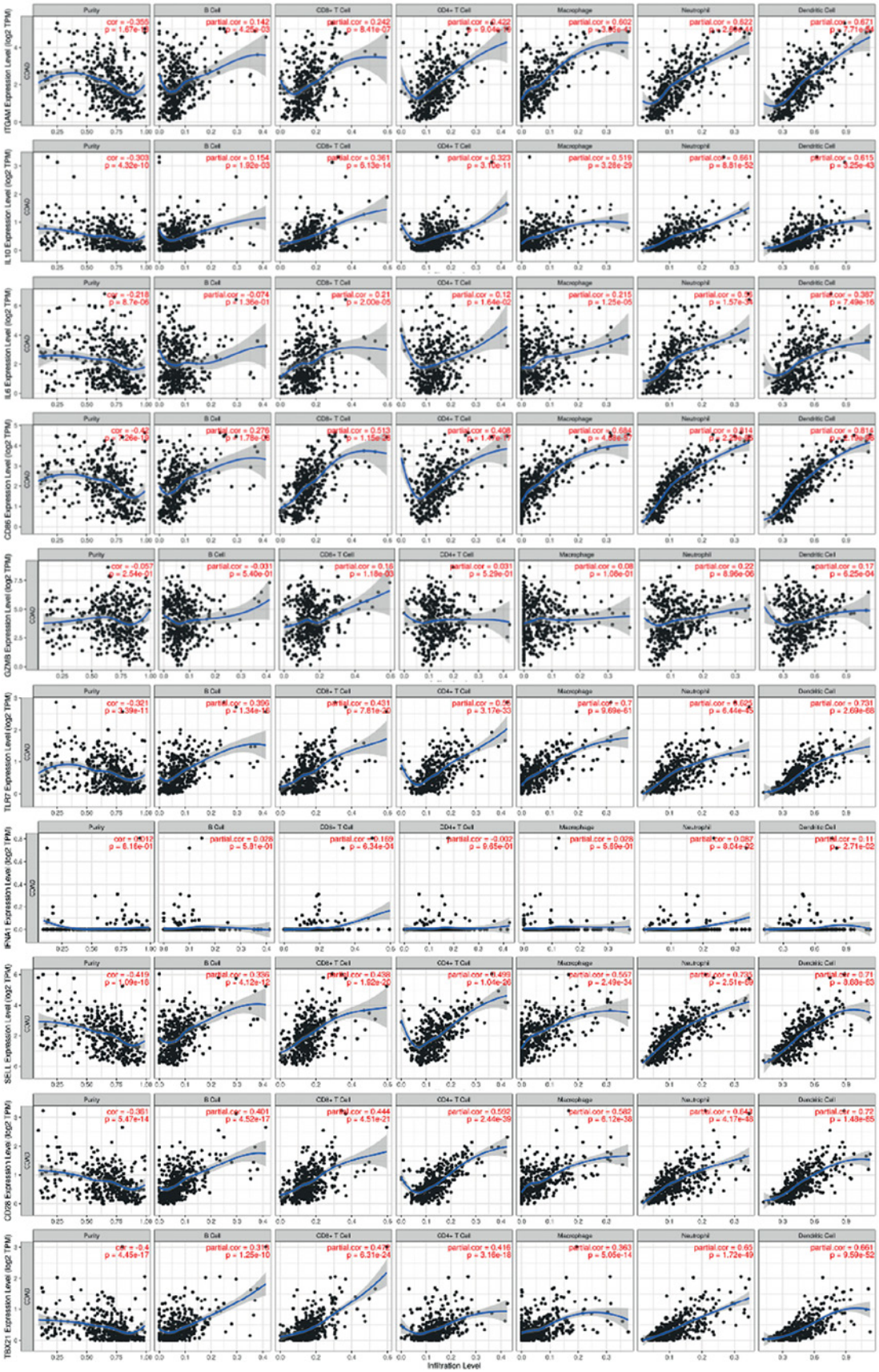

Figure 4. Correlation between the core changed genes and leukocyte infiltration (TIMER)

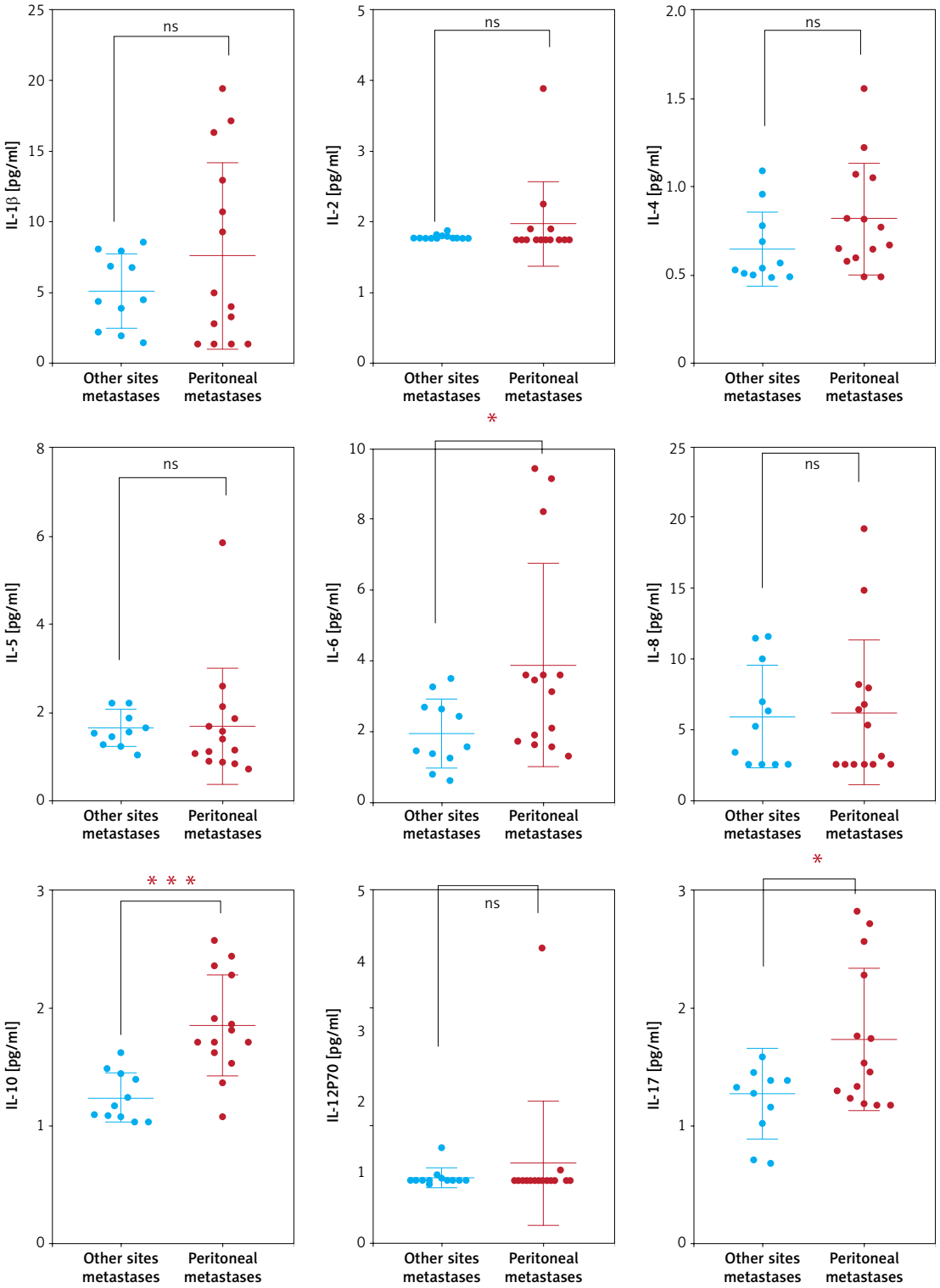

Figure 5. Cytokine profiles in plasma of CRC patients with peritoneal metastases and metastases to other sites

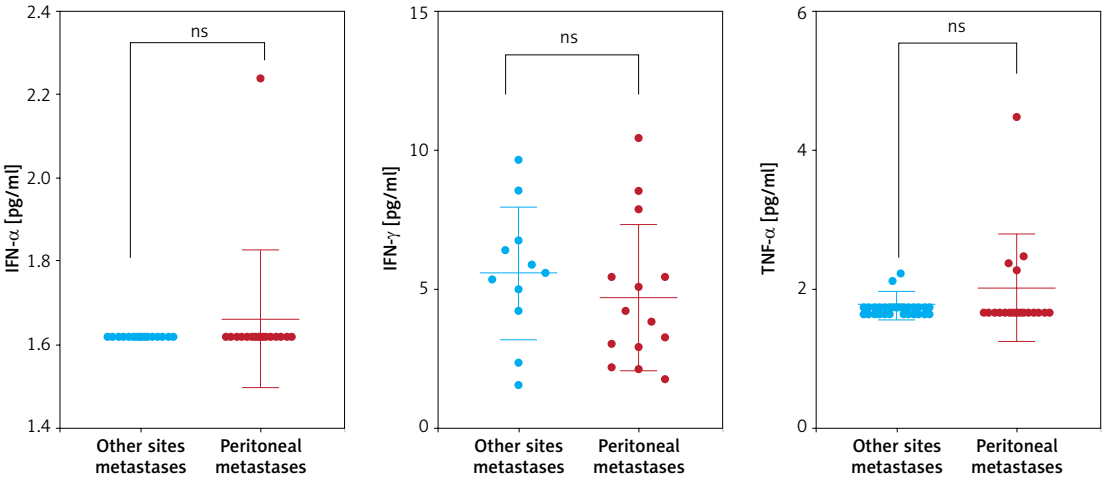

Figure 5. Cont.

accumulation and peritoneal metastases in clinical CRC patients.

Discussion

Compared with other sites of metastases, CRC peritoneal metastasis was regarded as a terminal disease stage only treated in a palliative setting and achieved survival rates of a few months [7]. The occurrence of peritoneal metastasis is a critical prognostic factor of CRC outcome. However, no predictors of peritoneal metastasis have been described in previous studies [21, 22]. This research aimed to discover the core diagnostic and treatment biomarkers of colorectal peritoneal metastasis to extend CRC patients' survival time and improve the quality of life. Firstly, we compared the differences between tissues from peritoneal metastasis and their matched CRC primaries obtained during cytoreductive surgery, and identified 95 genes dysregulated in CRC peritoneal metastasis. Afterward, bioinformatic analyses were utilized to deeply analyze the GO and KEGG pathway items of these differentially expressed genes. Functional enrichment analysis showed that these genes mainly participated in the immune response and cytokine activity in CRC peritoneal metastases. Furthermore, the neighbor gene network, PPI network and mCODE of these aberrant expressed genes were analyzed. Finally, 10 most potential diagnostic and therapeutic biomarkers of peritoneal metastases in CRC were selected: *itgam*, *il10*, *il6*, *cd86*, *gzmb*, *tlr7*, *ifna1*, *sell*, *cd28* and *tbx21*.

We found that these genes were significantly connected with the immune response through consulting the latest research on these top 10 core genes. *Itgam* encodes the integrin alpha M chain. The alpha M beta 2 integrin is important in the adherence of neutrophils and monocytes to stimulated endothelium and in the phagocytosis of complement coated particles [23]. *il10*, *il6 and ifna1* encode cytokines produced primarily by peripheral blood mononuclear cells. IL-10 is produced primarily by monocytes and to a lesser extent by lymphocytes; it has pleiotropic effects in immunoregulation and inflammation [24, 25]. *il6* functions in inflammation and the maturation of B cells. The functioning of this gene is implicated in a wide variety of inflammation-associated disease states [26]. Recently research reported that targeting the IL-6/JAK/STAT3 pathway might be a potential therapeutic strategy in treating CRC [27]. The protein encoded by *ifna1* is produced by macrophages and has antiviral activity. *ifna1* has been reported be associated with CRC susceptibility and survival [28]. *cd28* is essential for T cell proliferation and survival, cytokine production, and T helper type 2 development [29, 30]. *cd86* encodes a type I membrane protein, which is expressed by antigen presenting cells, and it is the ligand for two proteins at the cell surface of T cells, CD28 antigen and cytotoxic T cell associated protein 4. Binding of this protein with CD28 antigen is a costimulatory signal for activation of the T cell [31]. *gzmb* is crucial for cytolytic T lymphocytes' (CTL) and natural killer (NK) cells' rapid induction of the remarkable ability to recognize, bind, and lyse specific target cells in a cell-mediated immune response [32]. TLR7 encoded by *tlr7* is a member of the Toll-like receptor (TLR) family, which has roles in pathogen-associated molecular pattern (PAMP) recognition and mediates the production of cytokines necessary for activation of innate immunity [33, 34]. *sell* encodes a cell surface adhesion molecule, which contains a C-type lectin-like domain. The gene production is required for leucocyte binding on endothelial cells and facilitates their migration into secondary lymphoid organs and inflammation sites [35]. Finally, Tbx21 protein is a specific transcription factor that controls the expression of IFN-γ expression in Th1 and natural killer cells [36, 37].

Hence, we set about exploration of the investigation between the core changed genes and leukocyte infiltration, and detected the levels of CRC patients' serum cytokine profiles. IL-6, IL-10 and IL-17 were remarkably upregulated in CRC patients' serum with peritoneal metastases compared with other-site metastases. Underlying mechanisms of the concrete interaction of these genes is worth in-depth research.

Altogether, the evidence suggests that expression levels of IL-6, IL-10 and IL-17 in plasma, and their correlation with leukocyte infiltration, are potential diagnostic, prognostic, and therapeutic biomarkers for peritoneal management of CRC.

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Ye Gu, Qiang Liu, Lele Li contributed equally to this work.

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Ethical approval

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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