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Original Research

Discovery of core gene families associated with liver metastasis in colorectal cancer and regulatory roles in tumor cell immune infiltration

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ABSTRACT

In this study, we aimed to uncover genes that drive the pathogenesis of liver metastasis in colorectal cancer (CRC), and identify effective genes that could serve as potential therapeutic targets for treating with colorectal liver metastasis patients based on two GEO datasets. Several bioinformatics approaches were implemented. First, differential expression analysis screened out key differentially expressed genes (DEGs) across the two GEO datasets. Based on gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, we identified the enrichment functions and pathways of the DEGs that were associated with liver metastasis in CRC. Second, immune infiltration analysis identified key immune signature gene sets associated with CRC liver metastasis, among which two key immune gene families (CD and CCL) identified as key DEGs were filtered by protein-protein interaction (PPI) network. Some of the members in these gene families were associated with disease free survival (DFS) or overall survival (OS) in two subtypes of CRC, namely COAD and READ. Finally, functional enrichment analysis of the two gene families and their neighboring genes revealed that they were closely associated with cytokine, leukocyte proliferation and chemotaxis. These results are valuable in comprehending the pathogenesis of liver metastasis in CRC, and are of seminal importance in understanding the role of immune tumor infiltration in CRC. Our study also identified potentially effective therapeutic targets for liver metastasis in CRC including *CCL20, CCL24* and *CD70*.

Introduction

Colorectal cancer (CRC) is one of the most malignant tumors with a mortality rate of 9% among all cancer-related deaths [1]. It is also viewed as a refractory malignancy because a considerable proportion (>20%) of CRC was metastatic upon diagnosis, and nearly one-third of CRC relapsed after the surgical resection [2]. Such neoplasm usually originates from the epithelial cells lining the colon or rectum in the gastrointestinal tract [3]. Early CRC has almost no obvious symptoms, with progression of the disease, symptoms may include changes in defecation habits with hematochezia, diarrhea, constipation, and abdominal pain among others [4]. Later stages of the disease are characterized by severe anemia, weight loss, and other systemic symptoms [5]. Due to its higher morbidity and refractory nature, CRC has become a common malignant tumor worldwide, second only to gastric, esophageal, and primary liver cancers of the digestive system [6]. One of the major CRC risk factors is age [7]; however, over the last 25 years, the morbidity of CRC in young adults has increased in the European countries. Between 2004 and 2016, CRC incidence increased 7.9% per year in the 20–29 years age group, 4.9% per year in the 30–39 years age group, and 1.6% per year in the 40–49 years age groups [8]. The percent increase in CRC among all new cancer cases and all cancer-related deaths were 10% and 8.5%, respectively [9]. Therefore, CRC represents a serious risk and challenge to human health globally.

CRC cells can migrate to other organs and viscera through the lymphatic and blood circulation or by direct diffusion [10]. Liver receives dual blood supply from both the hepatic artery and portal vein, and the competence of the abnormal blood flow provides a convenient site for the spread of malignant tumors such as CRC, gastric cancer, and esophageal cancer among others [11–13]. Development of liver metastasis is quite frequent among CRC patients, and approximately one-half of the liver metastasis can be ascribed to CRC [14]. The mortality risk remains high in patients with liver metastasis who are not treated with any form of therapy including drugs, chemotherapy, or surgery, with survival rarely exceeding 6–9 months. Furthermore, patients with unresectable tumors who are treated with even the best chemotherapeutic

Abbreviations: CRC, Colorectal cancer; DEG, differentially expressed gene; TIP, tumor immunophenotype; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; COAD, colon adenocarcinoma; READ, rectal adenocarcinoma.

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drugs rarely survive beyond 13–18 months [14]. Thus, liver metastasis in CRC remains a critical challenge for the medical community and identification of improved therapeutic targets is vital to improve the prognosis and treatment outcomes. Given that liver metastasis in CRC is associated with several complex physiological processes and stages that drive multiple signaling pathways as well as interactive genes alterations [15], the paramount goal of the present study was to uncover genes with clinical value that drive the pathogenesis of CRC liver metastasis.

The resistance of metastatic cancer could be attributed to its partial dormancy, which confers resistance to the conventional therapy aimed at suppressing proliferation [16]. Encouragingly, cancer immunotherapy has emerged as a promising anti-cancer strategy, whereby aberrant cells can be obliterated, irrespective of their activation status [16]. This novel therapy has brought significant benefits in liver metastasis [17]. In previous report, transfer of adaptive T cells into several types of cancer patients could effectively increase the chance of survival [18,19]. Specifically, seven CRC patients with lung metastasis participated in the project of Rosenberg's team, and received reinfusion of *in vitro*-proliferated CD8+ T cells, the clinical outcomes were satisfactory with high eradication rate of metastasis [20]. A crucial step of immune therapy, namely intratumoral immune cell infiltration, has been proposed to stimulate the host immune response through the release of cytokines, thereby shaping the progression of tumor cells in direct or indirect manner [21]. Inoue et al. demonstrated that treatment with cetuximab could significantly increase the infiltration of CD3+, CD8+, and CD56+ cells in the chemotherapy group, which may potentiate the immune-enhancing effect in CRC patients with liver metastasis [22]. The foregoing immune cells responding to cetuximab are quite heterogeneous; for instance, the presence of CD3 is typical among T-lymphocytes, since it can be observed in an overwhelming majority of T cells [23], whereas CD8 marker is only expressed by approximately one thirds of mature T cells, conferring them with cytotoxic effects [23], in addition, CD56+ cells is alias to NK (natural killer) cells that are distinguished by CD56 expression, and exerting analogous functions to that of cytotoxic T cells [24]. Despite the rapid progress in the research of immune infiltration, the implication of lymphocytes in this field requires further investigation [21] due to the diversity of immune cell characteristics as noted above. Aside from immune cells that play important roles in regulating metastasis, immune driving genes are of equivalent importance. In liver metastasis and non-liver metastasis CRC patients, IL-17, CD8 and CD45RO are relevant genes that show significant differential expression in the local immune microenvironment, similar to FAS and tryptase [25]. Collectively, in-depth investigations of the involvement of lymphocytes in tumor micro-environment and associated immune biomarker are imperative.

Nowadays, with the development of 'big data' and advancements in bioinformatics, analyzing large datasets has become faster, more thorough, and convenient. Large datasets such as screening of genomic markers of colorectal liver metastasis using next-generation sequencing (NGS) platforms are available publicly [26]. Differential expression analysis is effective in detecting the differentially expressed genes (DEGs) in various pathological groups. Miao et al. reported the detection of 66,772 sequenced PIWI-interacting RNAs (piRNAs) and 241 piRNAs differentially expressed in cancer and paracancerous tissues and 1634 piRNAs differentially expressed in metastatic and non-metastatic tumors in hepatocellular carcinoma patients [27]. With the deepened understanding of tumor micro environment, several methods have been developed to efficiently analyze tumor cell immune infiltration, these methods include the 'ssGSEA' and 'CIBERSORT', both of which were integrated in TIP (tracking tumor immunophenotype), a meta-server for tracking, analyzing, and visualizing the status of anticancer immunity and the proportion of tumor-infiltrating immune cells across seven-step cancer-immunity cycle using RNA-seq or microarray data [28].

Previous big data studies concerning CRC liver metastasis had achieved accomplishment in identifying altered transcriptome [29], lncRNAs [30], or fusion genes [31] that might be responsible for CRC liver metastasis. In all these studies, evidence that associate immune activities with CRC liver metastasis was scarce. In this study, we performed differential expression analysis to screen for the key DEGs that drive liver metastasis in CRC and utilized TIP website to uncover immune hub genes that drive tumor immune cell infiltration in liver metastasis in CRC. The overall design of the current study was depicted in (Supplementary Figure 1), which revealed potential genes that could serve as new anti-metastasis targets or provide effective clinical immunotherapy approaches in CRC with liver metastasis.

Materials and methods

Data collection

To uncover genes that drive the pathogenesis of liver metastasis in CRC, we downloaded two relevant data sets from the National Center of Biotechnology Information (NCBI) database (GEO Accession viewer: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi). The GSE75050 dataset contained mRNA/lncRNA data of 6 CRC liver metastasis samples and equivalent primary CRC counterparts [30]; The GSE131418 dataset was focused on deciphering transcriptomic differences between primary CRC and lung/liver CRC metastasis based on 517 CRC samples [29], among which 141 liver metastasis and 333 primary CRC samples with complete gene expression matrix were incorporated into our study.

Differential gene expression analysis

For differential gene expression analysis, samples in the two GEO datasets were divided into two groups depending on their metastatic status (liver metastasis or primary CRC, respectively). Then the Limma R package was used for intra-dataset differential gene expression analysis in both GSE75050 and GSE131418 datasets [32]. By comparing metastatic groups against their corresponding primary CRC controls, differentially expressed genes (DEGs) were screened under the threshold of $|\log2FC| > 1$ and q-value < 0.05 (FC: fold change, q-value: p-value adjusted by Benjamini-Hochberg procedure). The volcano plot and heatmap were generated using the R packages ggplot2 and complexheatmap, respectively.

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway analyses

To identify the biological functions and associated signaling pathways of the screened genes, we performed the GO and KEGG signaling pathway analyses. These methods included the gene functional annotation, information visualization, and integrated discovery for the terms of biological processes (BP), cellular components (CC), molecular functions (MF), as well as functional signaling pathway. The analysis was performed using the ClusterProfiler package in R. The significance threshold was $p < 0.05$

Tumor cell infiltration phenotyping

Tumor cell infiltration phenotyping was performed online using the TIP domain (http://biocc.hrbmu.edu.cn/TIP) based on data obtained from the GSE75050 or GSE131418 dataset. The samples were divided into liver metastasis and no metastasis (primary CRC) groups. CIBERSORT de-convolution algorithm was used to make inferences about 22 immune cells composition within each sample [33], the number of permutations was set to 100 to ensure the precision of deconvolution and the results were visualized using stacking barplots and dot plots; while the 7-step cancer immunity cycle was based on manually curated 178 signature genes that involved in "Step1: release of cancer cell antigens"; "Step2: cancer antigen presentation", "Step3: priming and activation",

"Step4: trafficking of immune cells to tumors", "Step5: infiltration of immune cells into tumors", "Step6: recognition of cancer cells by T cells" and finally, "Step7: killing of cancer cells", aside from Step1 and Step4, each signature gene set contained subsets of genes that facilitate (positive) or suppress (negative) the corresponding immune process. The enrichment of anti-cancer immune signature gene sets were quantified by ssGSEA algorithm [34] using gene expression profile regarding individual samples. In order to make anti-cancer properties comparable between different datasets, the final immune activity score of each signature step was calculated by subtracting Z-score-normalized ssGSEA (negative gene subset) score from corresponding Z-score-normalized ssGSEA (positive gene subset) score [28]. Immune signature gene families that strongly correlated with previously defined key DEGs (associate with CRC liver metastasis) were defined as immune hub genes (unearthed by MCODE, a Cytoscape Plugin).

Gene expression profiling in GEPIA

Colon adenocarcinoma (COAD) and rectal adenocarcinoma (READ) are two common subtypes of CRC defined by different anatomical sites, particularly, molecular mechanisms underlying READ resembled that in COAD [35,36]. Accordingly, these subtypes were used to interpret the biological significance of immune hub genes. As an interactive online platform for mining RNA sequencing data from the Genotype Tissue Expression (GTEx) and TCGA projects, GEPIA was employed for analyzing the expression profiles and pathological stages of immune hub genes in COAD and READ. The website was also used for survival analysis based on the immune hub genes, whereby samples were divided into high and low expression groups by using median expression as a threshold. The Kaplan-Meier plotter generated the survival plot containing the log rank p value and the hazard ratio (HR) with 95% confidence intervals (CIs). All analyses were carried out in GEPIA were grounded on TCGA and GTEx gene expression data preprocessed in the website [37].

Genetic variations and co-expression analysis in cBioPortal

The TCGA database covers genomic and clinical data on more than 30 types of cancers. A study called "Colorectal Adenocarcinoma (TCGA, PanCancer Atlas)" comprised both COAD and READ subtypes, was chosen for analyzing the immune hub genes in the cBioPortal (http://www.cbioportal.org). Genetic variations were analyzed by selecting copy number alterations (CNAs) and mutations as selected molecular profiles. Spearman's correlation (gene co-expression) analysis was also performed in cBioPortal based on the colorectal adenocarcinoma study as mentioned above, the results regarding immune hub genes were retrieved from the website and visualized locally.

Protein-protein interaction analysis

To establish PPI networks based on different sets of proteins, we queried them against the STRING online database (version 11.0) (http://string-db.org/), following the default parameters (medium confidence of interaction score). After retrieving interaction data from the website, we used Cytoscape (version 3.8.1) software (http://www.cytoscape.org/) to locally visualize the PPI network. A Cytoscape plug-in called "MCODE" was used to extract biologically significant subnetworks with higher intra-connectivity.

Results

Identification of genes that drive liver metastasis in CRC

To uncover genes that are differentially expressed between CRC patients with liver metastasis and those without liver metastasis, we performed differential expression analysis using the Limma package. The volcano plots showing the expression of the significantly upregulated and downregulated genes and those without differential expression in GSE75050 and GSE131418 datasets are shown in Fig. 1A and B. DEGs that were common to GSE75050 and GSE131418 were named intersecting DEGs (227 genes) (Fig. 1G), and underwent subsequent enrichment analysis. GO functional enrichment analysis indicated that these intersecting DEGs were predominantly aggregated in the biological processes (GO-BP) of "cytokine secretion", "regulation of cytokine secretion", "positive regulation of cytokine secretion" and "leukocyte chemotaxis" (Fig. 1C). In the cellular component (GO-CC), "apical part of cell", "ruffle membrane", "leading edge membrane" were the most enriched terms (Fig. 1D) while in the category of molecular function (GO-MF), "chemokine activity", "chemokine receptor binding" and "peptidase inhibitor activity" were the most representative (Fig. 1E). KEGG pathway analysis revealed that "complement and coagulation cascades", "nitrogen metabolism" and "viral protein interaction with cytokine and cytokine receptor" were the most enriched signaling pathways (Fig. 1F).

Among the 227 intersecting DEGs, we defined those which were consistently up- or down-regulated in both datasets as key DEGs. The expression pattern of key DEGs was depicted in a color gradient heatmap (Fig. 2A), the top 5 up-/down-regulated key DEGs were *TRIM48, OR5H6, TAS2R42, OR10A6, OR52A5/SERPINC1, PELI1, GSTA5, GPR171, GPR68* in GSE75050 dataset; and *INSL5, MUC12, PNLIPRP2, PHGDH, TRIM48/TMEM229A, CALML5, MMP12, MSH4* and *TREM1* as shown in GSE131418. TRIM48 was among the top 5 upregulated key DEGs in both datasets. In addition, the relationships among key DEGs were further visualized using PPI network (Fig. 2F). Enrichment analysis was also performed to uncover the biological roles of 118 key DEGs. Consistent with intersecting DEGs, key DEGs were further aggregated into GO-BP terms associated with cytokine, especially in "positive regulation of cytokine secretion", "regulation of cytokine secretion" and "positive regulation of cytokine production" (Fig. 2B). Likewise, key DEGs participated in CC pathways included "ruffle membrane", "leading edge membrane", and "apical part of cell" (Fig. 2C). In addition, notable GO-MF enrichment of key DEGs was observed in "peptidase inhibitor activity" and "carbonate dehydratase" activity (Fig. 2D). Finally, the KEGG analysis results of key DEGs were slightly different from that of intersecting DEGs, as the 3rd and the 4th top enriched pathways of the former were concerned with cytochrome P450 drug metabolism instead of "viral protein interaction with cytokine" and "cytokine receptor" in the latter. Nevertheless, the top 2 enriched KEGG pathway, "complement and coagulation cascades" as well as "nitrogen metabolism" remained the same by comparing with the intersecting DEGs (Figs. 1F and 2E).

Immune infiltration profiling based on CIBERSORT inference and ssGSEA algorithm

Immune response in carcinogenic process involved delicately modulated events that are best interpreted as a whole instead of being addressed solely [38,39]. These events can be summarized as step-wise processes forming a cancer immunity cycle that eradicate cancer cells by iterative initiation, proceeding and expansion [40]. To uncover the immune infiltration profile of CRC with liver metastasis, the tracking tumor immunophenotype (TIP) website was used for tracking, analyzing, visualizing the status of anticancer immunity across seven-step cancer-immunity cycle, as well as inferring the proportion of tumor-infiltrating immune cells using samples from CRC patients with or without liver metastasis. We first calculated step-wise immune activity score based on ssGSEA score of 7 signatures (corresponding to 7 steps) as indicated previously (Stepwise immune activity score = $ssGSEA_{positive\ score}$ - $ssGSEA_{negative\ score}$). In the following immune activity analysis, we used Wilcoxon rank-sum test to find immune signatures with conspicuous changes of immune activity score when comparing metastatic samples to primary CRC controls, the results were shown in dot plots (Fig. 3A, B), whereby we found that in both datasets, the immune activity score of 2 signatures (Eosinophil recruiting and Step 6, marked with red ellipses)

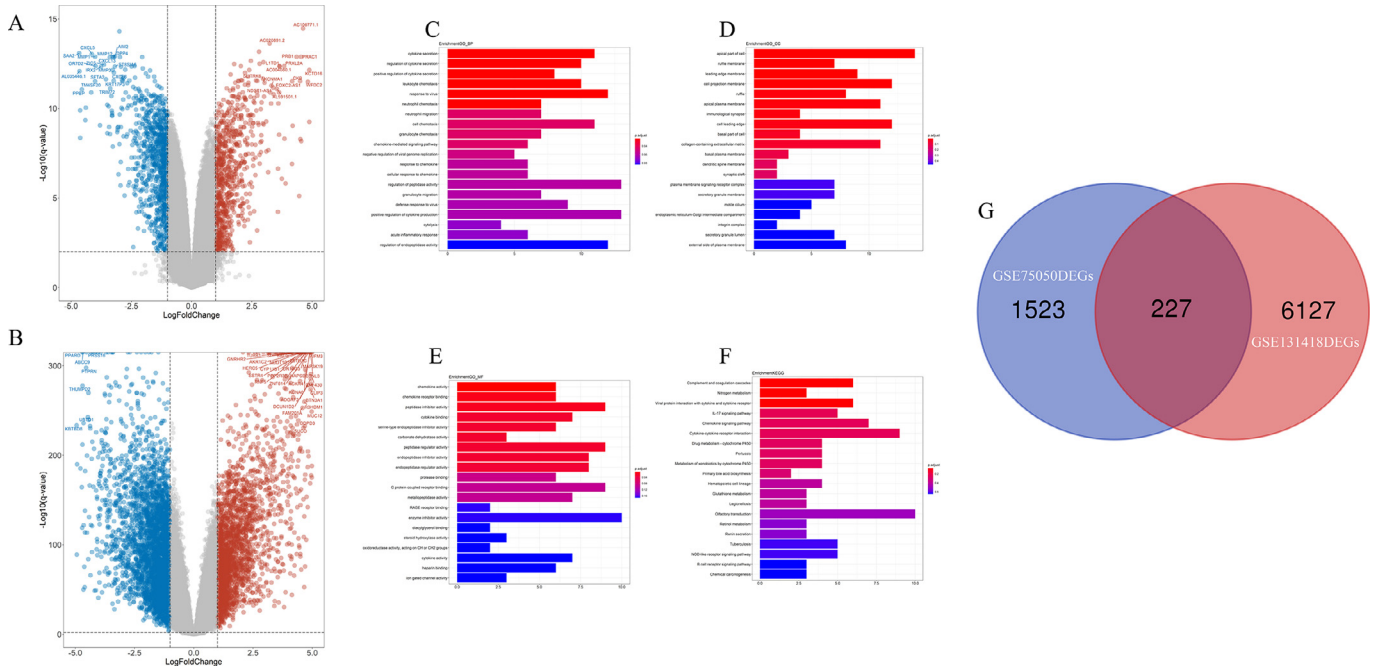

Fig. 1. Differentially expressed genes (DEGs) analysis between the liver metastasis and primary CRC samples in two datasets. TheDEGs between the liver metastasis and no liver metastasis groups in GSE75050 (A) and GSE131418 (B) were shown in volcano plots. Results of the intersecting DEGs functional enrichment by GO and KEGG pathway analyses were shown in (C, D, E, F). Intersection between DEGs in two datasets (227 intersecting DEGs) were shown in venn diagram (G).

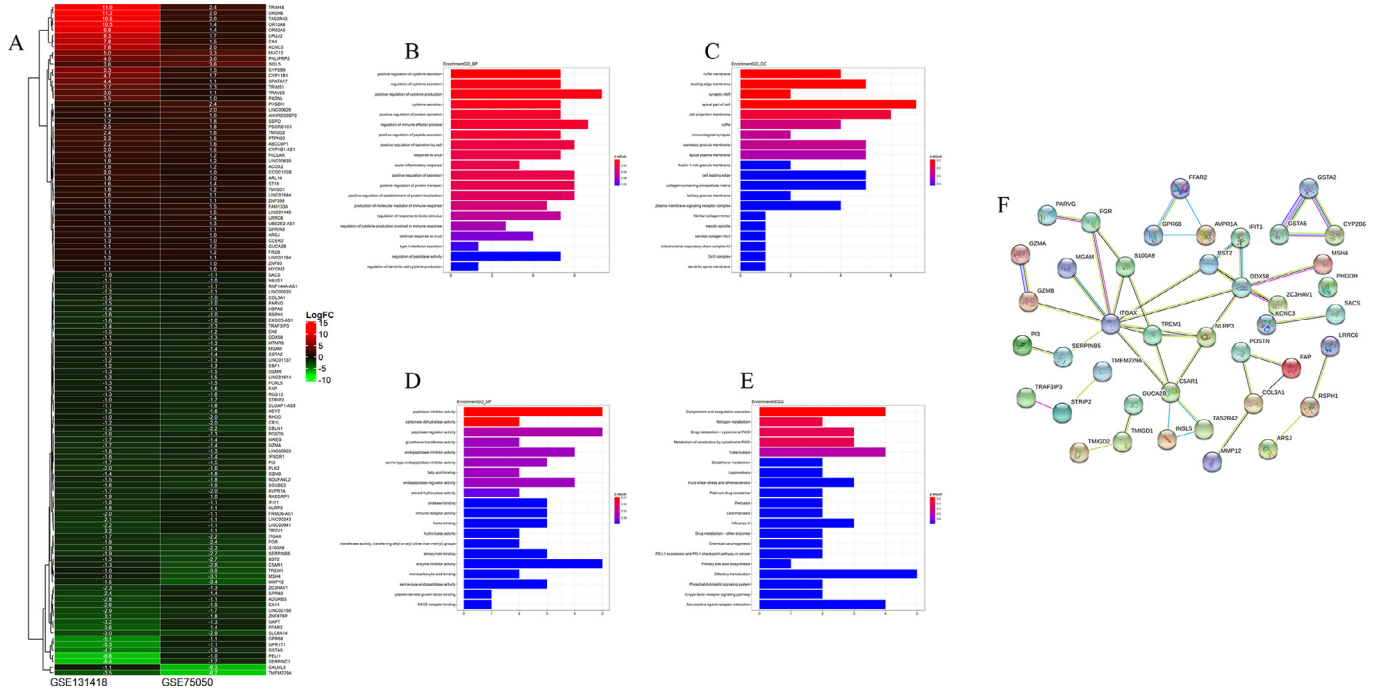

Fig. 2. Analyses of key DEGs. The key DEGs were consistently up/down-regulated in both datasets, whose expression patterns were shown in (A), the results of corresponding functional enrichment analysis were shown in (B, C, D, E). The PPI network of key genes was shown in (F).

were increased, while that of 4 signatures (CD4+ T cells recruiting, CD8 + T cells recruiting, Th1 cell recruiting and B cell recruiting, marked with blue ellipses) were distinctly decreased in metastatic groups by comparing primary CRC groups (Fig. 3A, B). These alterations were consistent across the two datasets, the corresponding gene sets were defined as key immune signature gene sets. The immune signature gene sets were further visualized in the form of ssGSEA score profile in the two datasets (Supplementary Fig. 2). The heatmaps of immune signature gene profiles (Fig. 4A and B) also helped distinguishing genes that belonged to key immune signature gene sets (the corresponding row names were marked in red/blue), among which the most representative gene families (with the greatest/second greatest number of member genes) were the CCL (6 genes) and CD gene families (5 genes).

The inference of immune cell infiltration in multiple samples by CIBERSORT indicated that, monocytes and resting dendritic cells infiltrated CRC liver metastasis samples in both GSE75050 and GSE131418

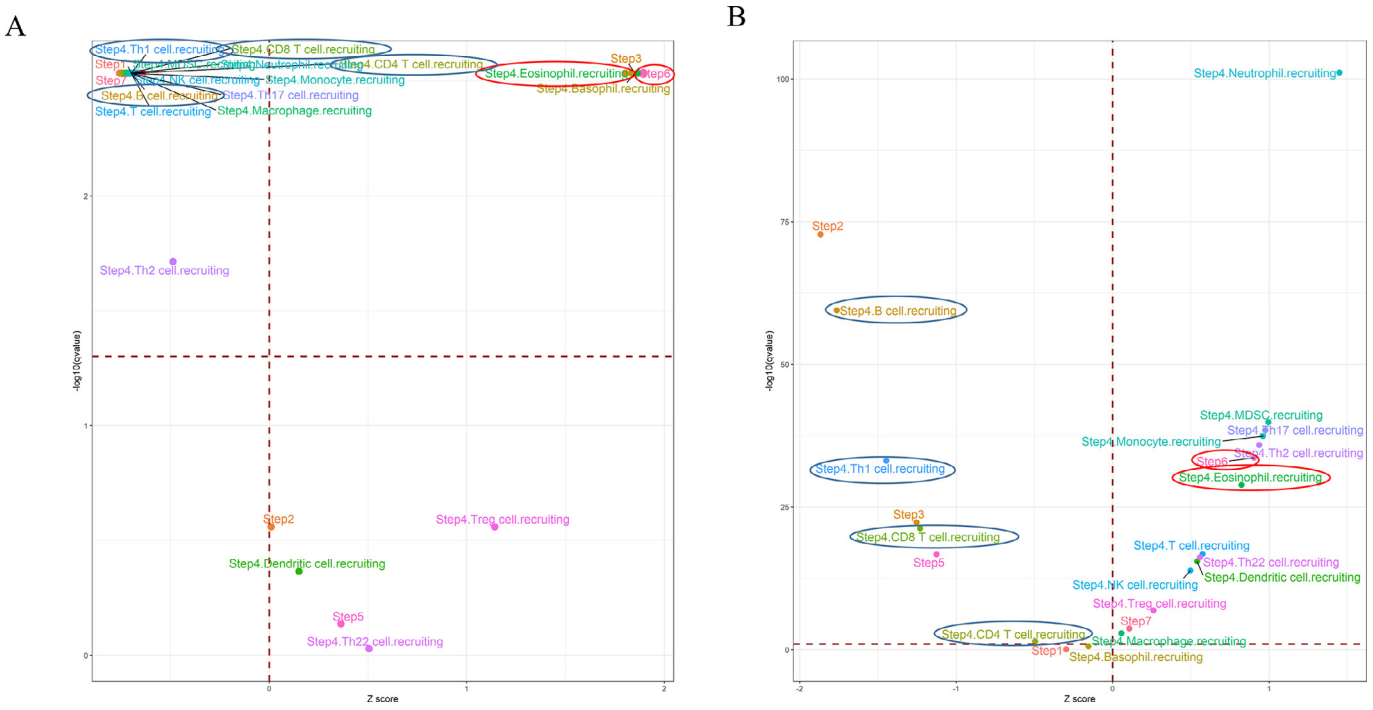

Fig. 3. Consistently altered immune signatures in two datasets. Wilcoxon rank-sum test and dot plot visualized 23 immune signatures, among which 6 immune signatures with consistent trend for immune activity score alteration (metastasis versus primary CRC) across GSE75050 (A) and GSE131418 (B) datasets were marked by ellipse, 2 signatures with elevated immune activity score in metastatic groups (Eosinophil recruiting and Step 6) were marked with red ellipses; while 4 signatures with decreased immune activity score in metastatic groups (CD4+ T cells recruiting, CD8 + T cells recruiting, Th1 cell recruiting and B cell recruiting) were marked with blue ellipses.

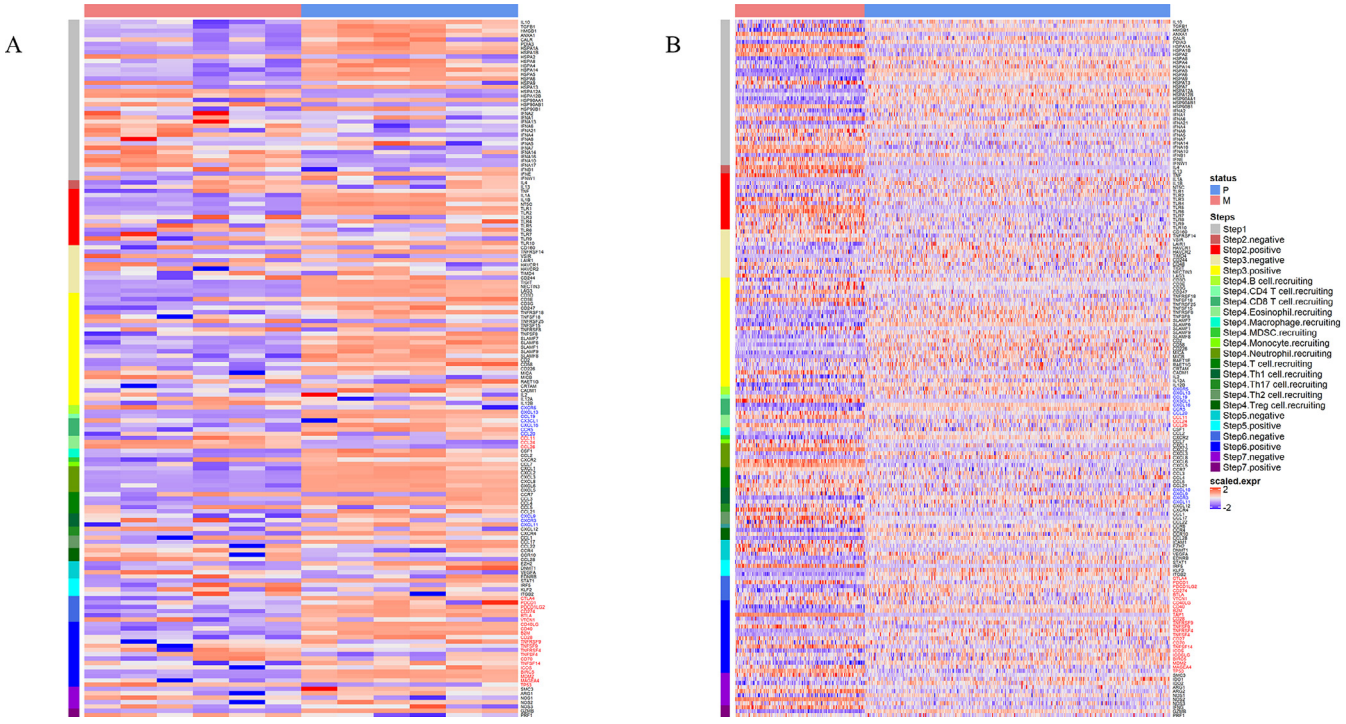

Fig. 4. Visualization of expression patterns concerning immune signature gene sets. Heatmaps showing expression profiles of signature gene sets of GSE75050 and GSE131418 were shown in (A) and (B), key immune genes in key immune signature gene sets were marked with red/blue depending on the alteration of activity score as defined by foregoing dot plots.

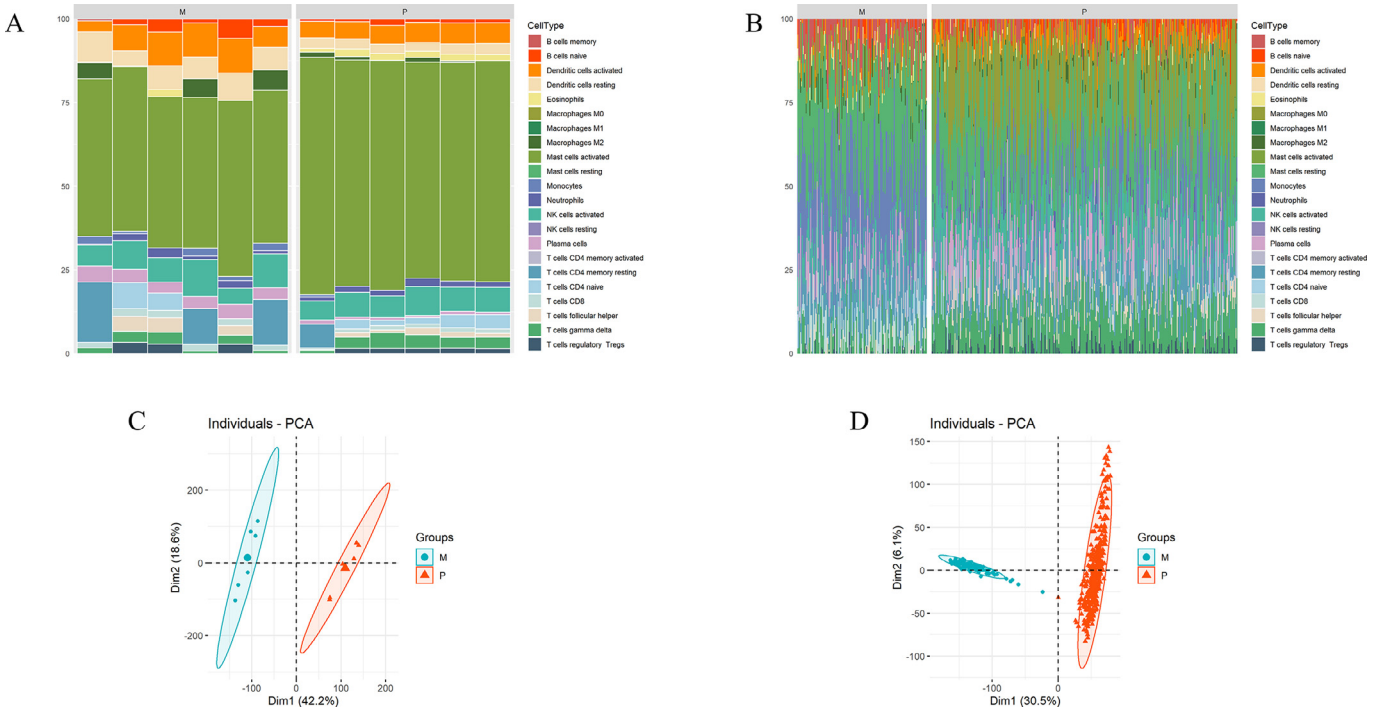

Fig. 5. CIBERSORT inference on immune cell composition. Stacking plot showing composition of 23 immune cells across two datasets (A, B), indicated that monocytes and resting dendritic cells infiltrated CRC liver metastases samples. Principal component analysis (PCA) showed distinct separation between metastatic and primary CRC samples in two datasets. (C, D)

datasets (Fig. 5A and C).The PCA plot showed a significant separation between the samples with liver metastasis and those without liver metastasis (Fig. 5B and D).

Identification of immune gene families driving immune infiltration and metastasis

A total of 118 key DEGs involved in liver metastasis were identified, together with previously defined key immune signature gene sets, we further investigated the way through which they work in concert to regulate CRC liver metastasis in the context of immune activity. The 118 key DEGs and key immune signature gene sets were merged and queried against STRING database, the PPI network was retrieved from the website and visualized locally using Cytoscape. As shown in Fig. 6, although CCL and CD families (diamonds, colored in red) were more closely interconnected by comparison to the 118 key DEGs (ellipse, colored in blue), a portion of key DEGs exhibited strong interaction with immune gene families. Therefore, we used a Cytoscape plugin MCODE to excavate clusters (subnetworks) whose nodes displayed higher interconnectivity. The largest subnetwork comprised 6 key DEGs and 33 immune genes was highlighted by yellow (Fig. 6), and we identified 11 immune hub genes (*CCL11, CCL19, CCL20, CCL24, CCL26, CD27, CD274, CD28, CD40, CD40LG* and *CD70*) belonging to CD/CCL family and strongly interacting with key DEGs. These immune hub genes might bridge immune regulation and CRC liver metastasis, and were chosen for subsequent analysis.

Expression patterns of immune hub genes in COAD and READ

Next, we explored the expression profiles of 11 immune hub genes (two hub gene families) in two common classification of CRC, namely COAD and READ. The GEPIA website was used to analyze the gene expression profiles of the immune hub genes and their correlation with clinicopathological traits. The expression profiles of immune hub genes are depicted in Fig. 7 and the corresponding boxplots of the expression between the tumor and normal samples are shown in (Supplementary Fig. 3). The results indicated that two genes in the CCL family (*CCL20* and *CCL24*) were markedly upregulated in COAD and READ compared to the normal samples. In contrast, the expression of *CCL11* was markedly decreased in patients as compared to normal controls, whereas no statistical significance concerning the expression patterns of CD families (*CD27, CD274, CD28, CD40, CD40LG* and *CD70*) were found between COAD/READ patients and controls.

To understand the implications of immune hub genes in different tumor stages, pathological stage analyses were performed; gene expression in 4 pathological stages (Stage I to Stage IV) defined by TCGA clinical annotation were visualized by violin plots (Supplementary Fig. 4), whereas the statistical significance of immune hub genes expression across these stages was determine by *F*-test. However, no significant alterations (Pr (>F) < 0.05) were found concerning immune hub genes expression across 4 pathological stages.

Prognostic value of two immune hub gene families in COAD and READ

To explore the prognostic value of immune hub genes that were found to be significantly associated with CRC liver metastasis and tumor infiltration phenotype, we performed OS and DFS analyses using the GEPIA website (Figs. 8 and 9). For the CCL gene family, patients with high *CCL24* (HR = 1.2) and *CCL26* (HR = 1.3) expression were predisposed to lower DFS as compared to their low-expression counterparts, whereas elevated levels of *CCL11* (HR = 0.89), *CCL19* (HR = 0.87) and *CCL20* (HR = 0.88) were associated with slightly improved DFS. Aside from *CCL24* (HR = 0.71), the DFS results of other CCL family members were corroborated with OS analysis results, where high *CCL26* (HR = 1.1) expression was related to increased risk, while high-expression *CCL11* (HR = 0.78), *CCL19* (HR = 0.83) and *CCL20* (HR = 0.71) contributed to improved OS. The DFS analysis of CD family showed that high *CD274* (HR = 0.8), *CD40* (HR = 0.8) and *CD40LG* (HR = 0.85) expression were associated with increased percentage of DFS, yet they contributed less to prolonged OS (HR = 0.93, 1.1, 1, re-

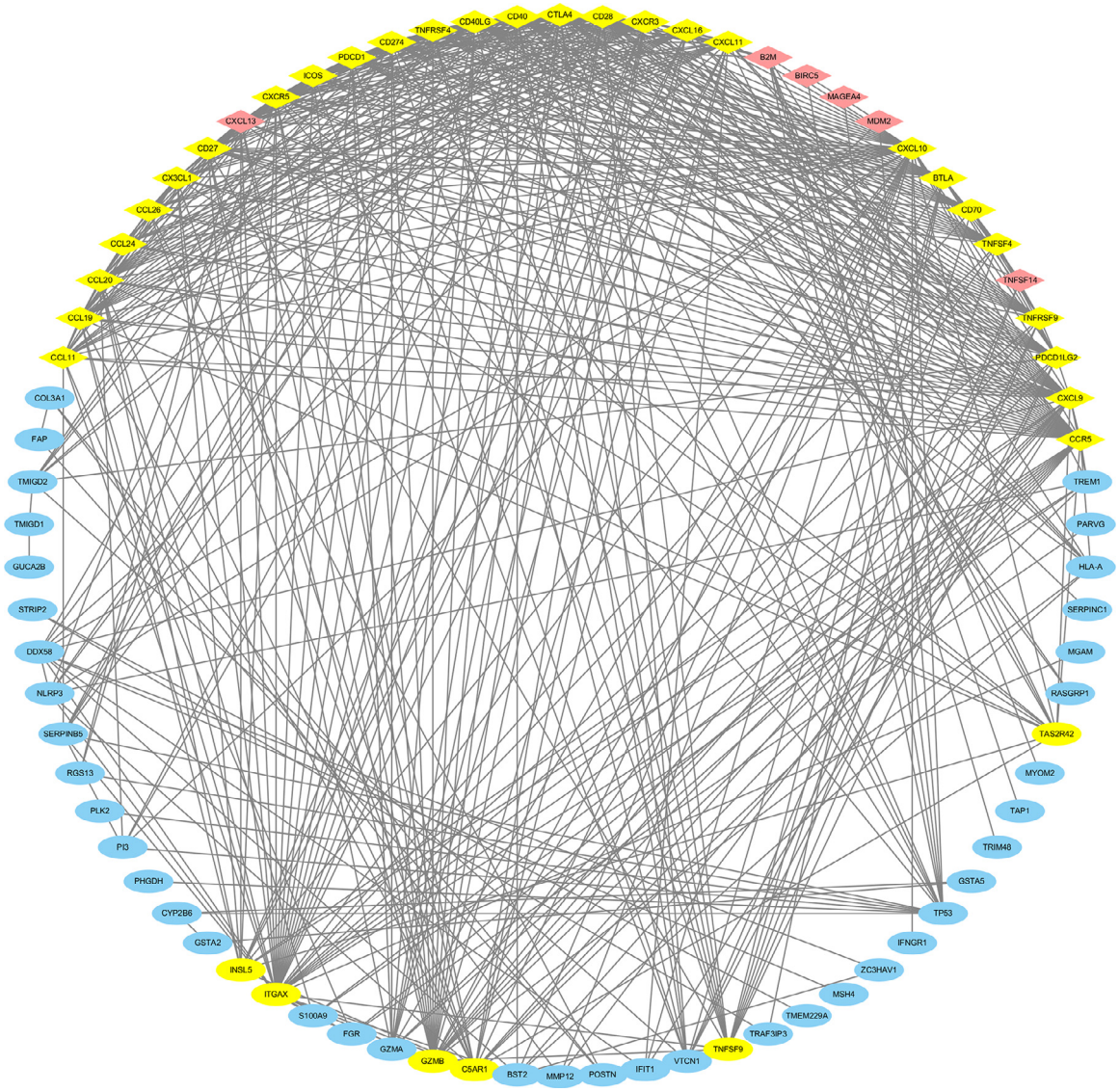

Fig. 6. PPI network involving key DEGs and key immune genes. As depicted in the PPI network, CCL and CD families were shaped as diamonds and colored in red, whereas 118 key DEGs were shaped as ellipse and colored in blue. Nodes marked in yellow represent the largest subnetworks with higher interconnectivity that was filtered by Cytoscape plugin MCODE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

spectively). High *CD27* and *CD28* expression displayed no influence on DFS (HR = 1), and acted differently on OS (HR:*CD27* < 1, HR:*CD28* >1). Of note, patients with high *CD70* expression exhibited the worst DFS (HR = 1.7) among immune hub genes, and this gene was consistently responsible for worse OS (HR = 1.2).

Genetic variation analysis of two immune hub gene families in COAD and READ

To assess the genetic variation of the CCL and CD families in COAD and READ, we performed oncoprint analysis in the cBioPortal platform. The results indicated that *CCL24* (7%) and *CD40* (13%) were the most frequently altered immune hub genes in CCL/CD families (Fig. 10B). *CCL24* was affected by amplification, missense mutation, and mRNA high alterations while *CD40* was affected by amplification (preferentially occurred in *CD40* among other CD family members), missense mutation, mRNA high alterations and truncating mutation. Genetic variation patterns in the two immune hub gene families were demonstrated in Fig. 10A, where we found that mRNA high alterations were the predominant genetic variation in both families, while mutation, amplification and multiple alterations were more frequent in CD family.

Functional enrichment of the regulatory network of immune hub gene families in COAD and READ

The co-regulation of the immune hub gene families (CD and CCL) involved in CRC liver metastasis and immune infiltration were analyzed based on COAD and READ samples. Genes that correlated with immune hub genes were analyzed in cBioPortal. The concordance between different genes was determined by Spearman's correlation analysis and downloaded before being visualized locally. As shown in Fig. 11A, B, genes in CD family were more closely correlated as compared to that in CCL family, indicating a higher intra-family coordination. Intriguingly, the expression of *CCL20* varied inversely with other members in CCL family (Fig. 11A). In contrast, high coordination of gene expression was demonstrated by CD family (Fig. 11B). We next selected the top 10 genes (from cBioPortal correlation results) with the highest absolute value of correlation to each member gene in CCL/CD families for in-depth analysis. A total of 50 genes (strongly correlated with CCL fam-

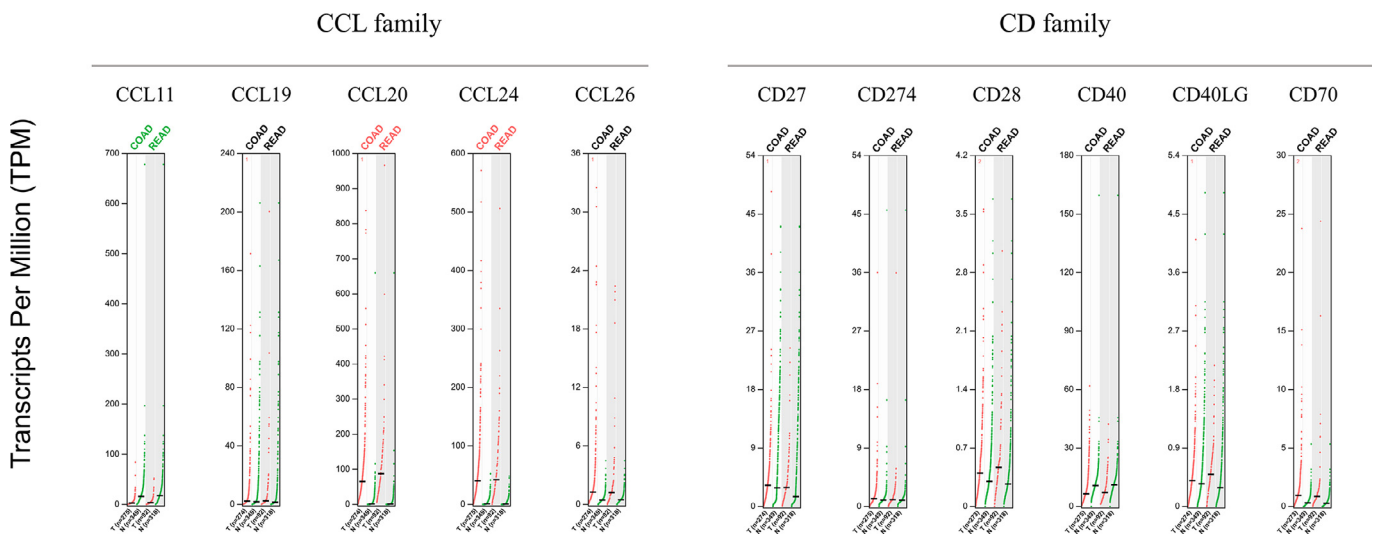

Fig. 7. Expression patterns of key immune gene families in COAD and READ. Scatter plots show the expression profiles of CCL and CD gene families among two CRC subtypes, expression of CCL11 were depleted among CRC patients, whereas the expression patterns of CCL20 and CCL24 were quite the opposite.

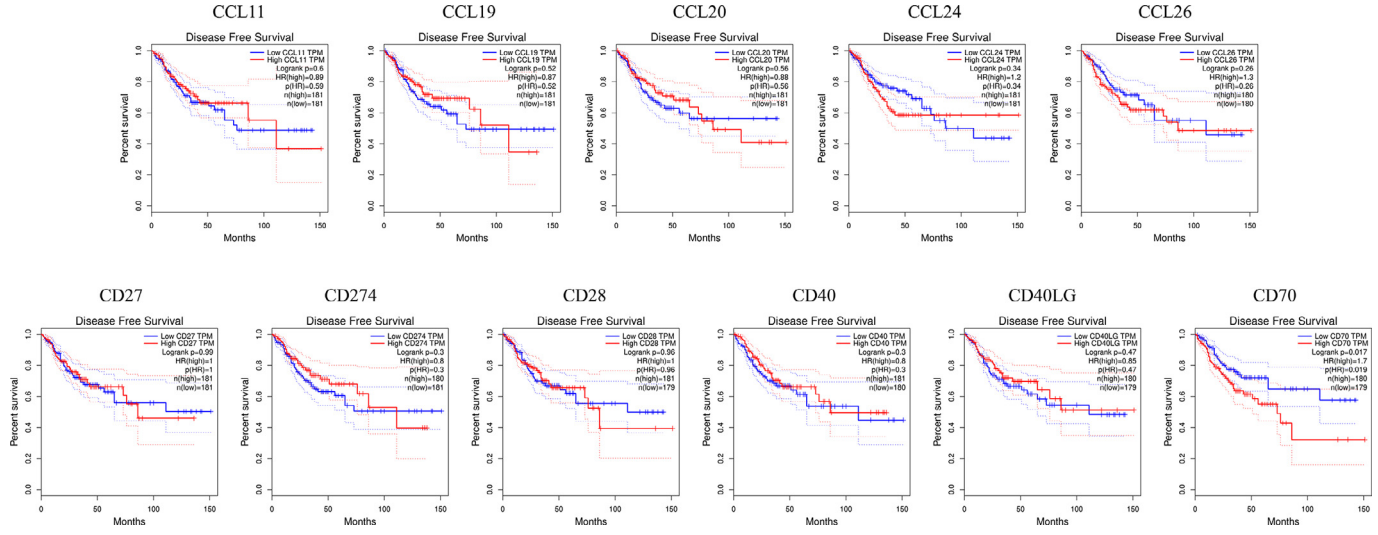

Fig. 8. Prognostic value of key immune gene families in COAD and READ (Disease free survival). Kaplan-Meier plotter showed that high CD70 expression was associated with DFS among CRC patients.

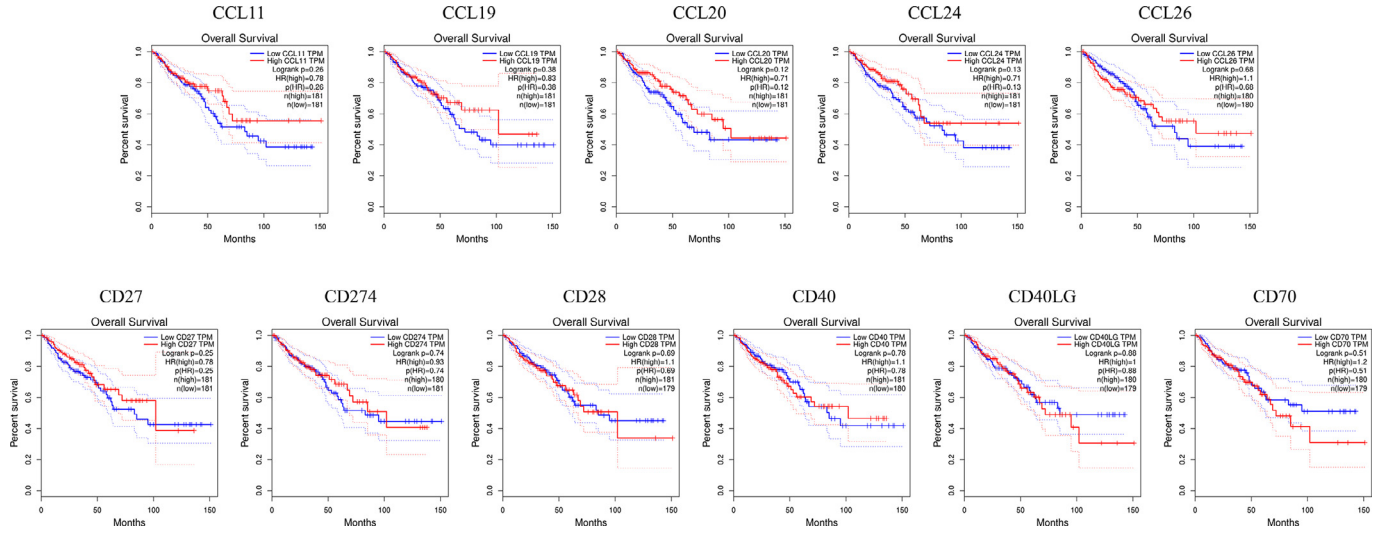

Fig. 9. Prognostic value of key immune gene families in COAD and READ (Overall survival). Key immune genes did not show significant influence on the OS of CRC patients, as demonstrated by Kaplan-Meier plotter. High CCL20 and CCL24 expression with p(HR) approximating 0.1 might be associated with improved OS.

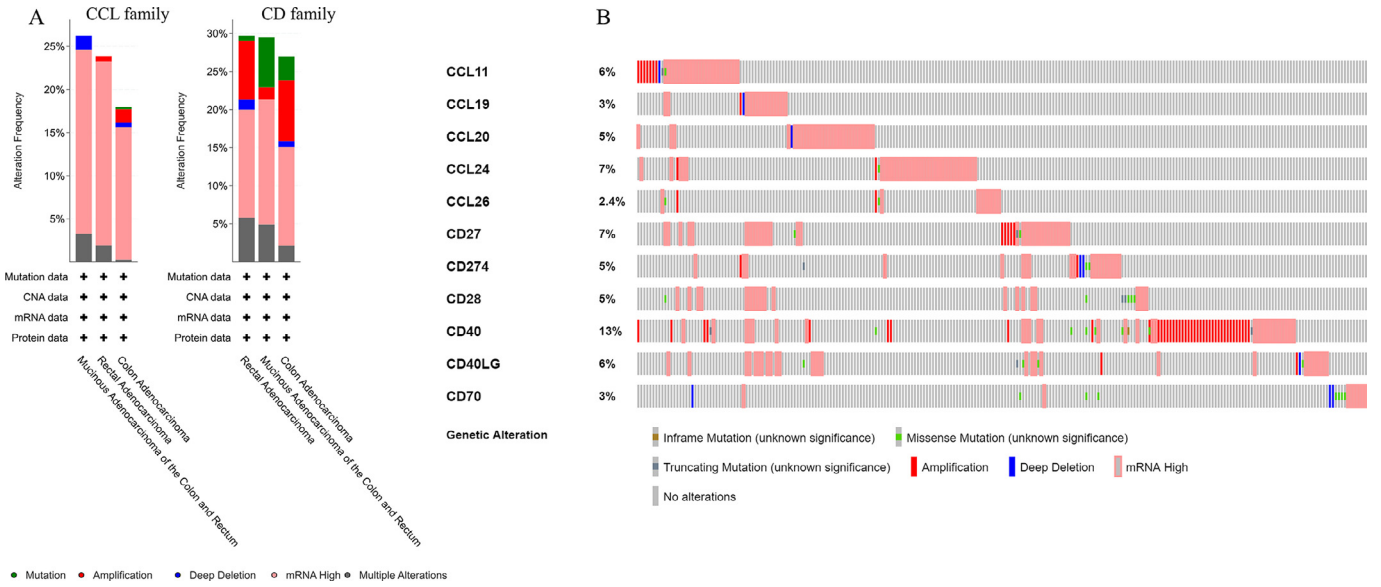

Fig. 10. Genetic variation analysis of key immune gene families in COAD and READ. Alteration frequency of genetic variations in two key immune gene families were shown in stacking plots (A), visualized summary of variations regarding individual key immune gene was shown in (B).

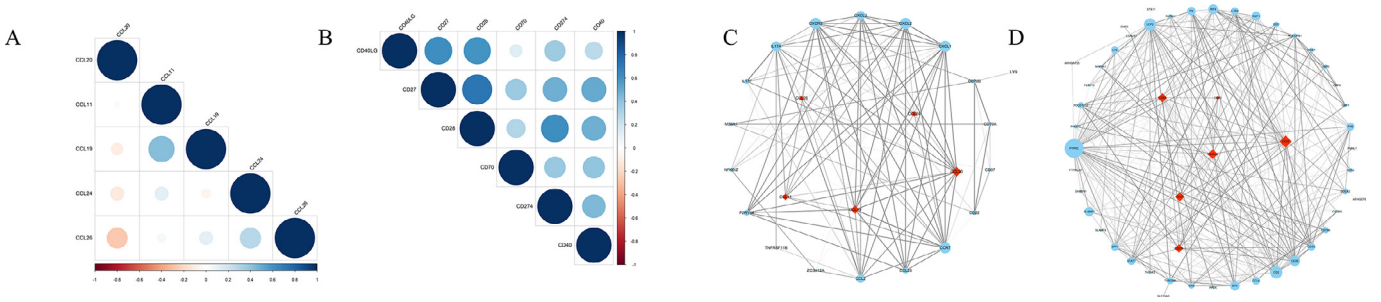

Fig. 11. Correlation analysis of key immune gene families. Correlations among CCL and CD families were calculated in cBioPortal and visualized locally (A, B), the size of colored circles was proportional to the absolute value of correlation between two family members. CD and CCL families, along with their respective neighboring genes, were respectively merged as CCL and CD clusters, prior to the construction of PPI networks (C, D). The node size corresponded to the number of edges, large nodes interact with more genes within the PPI network, and thus play a central role.

ily) and 60 genes (strongly correlated with CD family) were merged with corresponding family members (55 and 66 genes, respectively), through which two cluster, namely CCL cluster and CD cluster, were finalized. The two clusters were queried against STRING database following default setting. PPI network in (Fig. 11C and D) showed that CCL family interacted with less targets as compared to CD family. *CCL20* and *CCL19* displayed higher connectivity than *CCL11, CCL24* and *CCL26*, whereas *CD70* showed the minimum number of connections among other CD family members. Genes in the two PPI networks were finally subjected to functional enrichment analysis. CCL cluster was predominantly involved in the BPs of "response to chemokine", "cellular response to chemokine" and "chemokine-mediated signaling pathway". In the category of CC, CCL cluster was mainly involved in "external side of plasma membrane" and "cell" and "membrane raft". The representative MF terms affected by these genes included "chemokine activity", "chemokine receptor binding" and "cytokine activity". In the KEGG pathway enrichment analysis, significant involvement of CCL cluster in "Viral protein interaction with cytokine and cytokine receptor", "Cytokine-cytokine receptor interaction" and "Chemokine signaling pathway" was observed (Fig. 12A). In contrast, CD cluster displayed different biological roles, they were significantly enriched in "T cell activation", "lymphocyte differentiation", "regulation of lymphocyte activation" as well as "lymphocyte proliferation" under the category of BP. "external side of plasma membrane", "cell-cell junction", plasma membrane signaling receptor complex" and "membrane raft" were the most representative GO-CC terms for CD cluster. Regarding MF, CD cluster predominately participated in "tumor necrosis factor receptor binding", "signaling receptor complex adaptor activity" and "tumor necrosis factor receptor superfamily binding". In addition, significant enrichment of CD cluster in several KEGG pathways was revealed, including "Primary immunodeficiency", "Cell adhesion molecules", "T cell receptor signaling pathway", "Th17 cells differentiation" and "Chemokine signaling pathway" (Fig. 12B).

Discussion

Bioinformatic analysis was performed on the data from the dataset GSE75050 and GSE131418 that are available publicly. Through differential gene expression analysis between liver metastasis and primary CRC groups, we identified 227 common DEGs shared by both datasets that might drive CRC liver metastasis. These intersecting DEGs were mainly enriched in GO-BP terms of cytokine secretion associated pathways, as well as "leukocyte chemotaxis". Leukocyte chemotaxis referred to a process through which immune cells migrate to the target site at the presence of external stimulus, for instance, infiltration of neutrophil into stimulated target sites was essential for innate immune response [41], yet relatively limited reports described such process in metastasis, nevertheless, we hypothesize that this process might regulate leukocyte infiltration in tumor micro environment, as the migration of immune

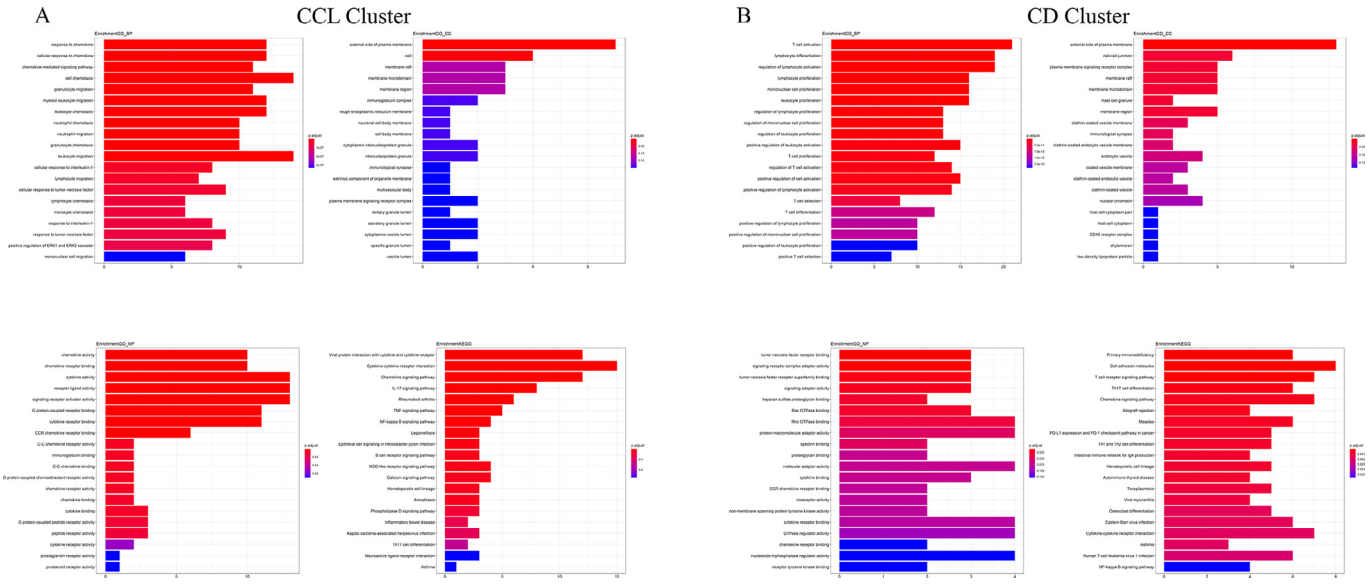

Fig. 12. Functional enrichment analyses of CCL and CD clusters. The results of function enrichment analysis regarding CCL and CD clusters were summarized in (A) and (B), respectively.

cells was coordinated with infiltration [42]. Cytokines can facilitate metastatic colonization in some tumors [43]; for instance, IL-6 and G-CSF were responsible for metastasis [44], specifically, in CRC patients, circulating galectin-3 could promote secretion of several cytokines in metastatic CRC [45]. Therefore, we assume that a portion of 227 intersecting DEGs in our current study might play similar roles. As for CC ontology, "apical part of cell", "ruffle membrane", "leading edge membrane" were the most enriched terms, among which membrane ruffle is essential for epithelial migration [46], and also contributed to the mobility and invasion of cancer cells [47]. Regarding MF, "chemokine activity", "chemokine receptor binding" and "peptidase inhibitor activity" were the most represented terms. Normally, chemokines coordinate intercellular positioning and migration; however, cancer cells also take advantages of these attributes, and disordered chemokine regulation was deemed culprit in carcinogenesis [48]. KEGG pathway analysis revealed that "complement and coagulation cascades", "nitrogen metabolism" and "viral protein interaction with cytokine" and "cytokine receptor" were the most enriched signaling pathways, the involvement of intersecting genes in cytokine-associated pathways agreed with the results of GO-BP category [43–45], while nitrogen shift (metabolism) that control the exploitation of nitrogen originated from glutamine was also reported to modulate progressive malignancy [49].

Given that 227 intersecting DEGs displayed biological significance that might regulate CRC liver metastasis, we further investigated their expression (logFC, liver metastasis versus primary CRC) in their respective datasets, and narrowed down our targets to 118 key DEGs with consensus expression patterns in both datasets. The biological functions of these genes were also obtained using GO/KEGG analysis. In general, most results of enrichment analysis concerning key DEGs were consistent with intersecting DEGs as indicated previously, specifically, consistency could be observed in significantly enriched pathways including: "positive regulation of cytokine secretion", "regulation of cytokine secretion" and "positive regulation of cytokine production" under GO-BP; "ruffle membrane", "leading edge membrane", and "apical part of cell" under GO-CC; "peptidase inhibitor activity" under GO-MF; "complement and coagulation cascades", as well as "nitrogen metabolism" under KEGG. These results indicated that 118 key DEGs were highly representative of 227 intersecting DEGs, and that these consistent pathways might play crucial roles in the pathogenesis of CRC liver metastasis. Notably, the KEGG analysis results of key DEGs were slightly different from that of intersecting DEGs, aside from the top two consistently enriched pathway—"complement and coagulation cascades" and "nitrogen metabolism", key DEGs were additionally concerned with cytochrome P450 drug metabolism, including "Drug metabolism – cytochrome P450" and "Metabolism of xenobiotics by cytochrome P450". Cytochrome P450 (CYPs) played a central role in metabolizing drug [50], whose dysregulation was observed in CRC, for instance, cytochrome P450 2W1(CYP2W1) could be detected in nearly one third of colon cancer [51]; overexpression of CYP family member *CYP24A1* was responsible for invasion and metastasis and had been proposed in various types of cancer, including CRC [52], these CYP family members were potential candidates as drug target, and we speculated that they might be associated with key DEGs.

Among 118 key DEGs, the top 5 up-regulated key DEGs in GSE75050 dataset were *TRIM48* (Tripartite Motif-Containing Protein 48), *OR5H6* (Olfactory receptors 5H6), *TAS2R42* (Taste receptor), *OR10A6* (Olfactory receptor 10A6), *OR52A5* (Olfactory receptor 52A5) and *INSL5* (Insulin-like peptide), *MUC12* (Mucin 12), *PNLIPRP2* (Pancreatic lipase), *PHGDH* (Phosphoglycerate dehydrogenase), *TRIM48* as shown in GSE131418, and we probed into the genes that might be associated with CRC liver metastasis. Olfactory receptor family were implicated in tumorigenesis, among which *OR51E2* was highly expressed in prostate cancer [53], overexpression of *OR2T6* was observed in breast cancer, serving as an initiator in epithelial-mesenchymal transition [54], notably, *OR7C1* was indispensable for maintaining colon cancer-initiating cells (CICs) [55]. Although several olfactory receptor family members in our current study did not overlap with forgoing results, they might exert analogous effects. *INSL5* was found in enteroendocrine cells, the expression increased from colorectum to rectum in a gradient manner, additionally, this marker was detected in rectal neuroendocrine tumors [56]. *MUC12* is a transmembrane mucin, whose homologue *MUC1* was increased in colon cancers, accompanied by worse prognostic outcome [57]. *PNLIPRP2* was a lypolytic enzyme attenuated in pancreatic ductal adenocarcinoma [58]. Elevated *PHGDH* expression as well as its functions as proliferation/migration promoter was documented in pancreatic cancer [59]. *TRIM48* (tripartite motif-containing 48) was among the top 5 up-regulated key DEGs in both two datasets, limited *in vitro* evidence indicated that elevated expression of this gene facilitate lung cancer cell death in xenograft model presumably by activating *ASK1* [60], as well as inhibiting growth of human glioblastoma cells through ERK1/2 pathway [61]; such tumor inhibitory effects might be used to counter metastasis in CRC due to the consistent upregulation

of *TRIM48* in metastatic samples across two dataset. Collectively, aside from *TAS2R42* that was not reported in cancer, and downregulated *PNLIPRP2* in pancreatic ductal adenocarcinoma, other top up-regulated genes were elevated in various types of cancers, their overexpression in current study might account for the increased malignancy of metastatic CRC.

As for the top 5 down-regulated key DEGs, *SERPINC1* (Serpin Family C Member 1), *PELI1* (Pellino E3 Ubiquitin Protein Ligase 1), *GSTA5* (glutathione S-transferase alpha 5), *GPR171* (G Protein-Coupled Receptor 171), *GPR68* (G Protein-Coupled Receptor 68) were observed in GSE75050 and *TMEM229A* (Transmembrane Protein 229A), *CALML5* (Calmodulin Like 5), *MMP12* (Matrix Metallopeptidase 12), *MSH4* (mutS homolog 4) *TREM1* (Triggering Receptor Expressed On Myeloid Cells 1) were detected in GSE131418. *SERPINC1* exert anti-coagulatory and anti-inflammatory effects, it also facilitates proliferative and apoptotic processes in nasopharyngeal carcinoma [62]. *PELI1* modulates biological activities through modifying protein at post-translational level or ubiquitination process, its high expression coincided with advanced B-cell lymphoma cases [63]. Up-regulation of *GSTA5* was reported to be responsible for irinotecan resistance in CRC. *In vitro* and *in vivo* studies indicated that *GPR171* was critical for lung cancer progression, this gene was up-regulated in nearly half of all lung cancer tissues incorporated into the study [64]. *GPR68* was overexpressed in numerous types of cancers, including colon cancer, and is responsible for tumor metastasis [65]. A previous study involving cell lines and tissue samples declared increased ubiquitination level of *CALML5* in breast cancer [66]. In a study of serum samples, the expression of *MMP12* was proportional to the progression of colon cancer, which was higher in patients suffering from vascular invasion than their V0-stages counterparts [67]. Mutated *MSH4* was detected in CRC patients [68], another case report described a metastatic bladder cancer patient with *MSH4* mutation who demonstrated complete response to PD-L1 blockade [69]. *TERM-1* could boost immune responses during pro-inflammatory processes, neutrophils who expressed high level of *TREM-1* was proposed as a promoting factor in CRC development [70]. However, the tumor-promoting nature of these genes were not consistent with their down-regulation in metastatic samples as depicted in our current study, which might be attributed to the sophisticated regulatory network underlying carcinogenesis and, in CRC progression, the key DEGs, as well as other DEGs with totally different biological functions, might orchestrate the regulation of CRC liver metastasis.

We next parsed immune activity in the two selected datasets using ssGSEA-based seven-steps immune cycle analysis to evaluate the activity of each step (signature), during which the combined action of positive and negative regulatory gene sets were put into consideration. The result unraveled that the immune activity score of 2 signatures (Eosinophil recruiting and Step 6, marked with red ellipses) and 4 signatures (CD4+ T cells recruiting, CD8 + T cells recruiting, Th1 cell recruiting and B cell recruiting, marked with blue ellipses) were distinctly altered in CRC liver metastatic samples by comparing primary CRC samples in both datasets. The consistency across these immune signatures suggests their importance in regulating CRC liver metastasis, gene sets representing these signatures were defined as key immune signature gene sets. Infiltration of eosinophils into solid tumor was frequent, and usually accompanied by improved prognosis [71], though recently there have been controversies in this regard [72]. The sixth step of cancer immune cycle, namely recognition of cancer cells by T cells played an essential part in eliminating cancer, as tumors cells can avoid being recognized by T cells through restraining MHC (Major Histocompatibility Complex) class I or by suppressing other participants underlying antigen processing [73]. Relatively lower immune activity score of these two signature in liver metastasis CRC samples indicated that recruitment of eosinophils and recognition of cancer cells might be hindered. The T cells have a protective role in immunity due to the ability of cellular differentiation and subsequent migration to target tumor tissues [74], among which CD4+ T cells could eliminate cancer through different ways including direct cytolytic effect or indirect regulation upon tumor micro environment or magnify the immune response of other lymphocytes including CD8+ T cells and B cells [75]. Th1 cells play a critical role in antitumor immunity by inducing CTL-mediated potency and lengthening the immune response to prolong the survival of cancer patients [76]. B cells are representative tumor-associated immune cells that enhance autoimmunity and anticancer ability [77]. Increased immune activity score regarding the recruitment of these cells in liver metastasis CRC samples might be associated with enhanced lymphocytes infiltration that counter the progression of metastasis. Recruitment of preceding lymphocytes might be crucial for CRC liver metastasis, however, the overall immune effect also depended on other immune processes in a dynamic fashion, and differences resided in other immune signatures score across two data sets, which could be ascribed to the varying sites of sample collection, different sample size, as well as the heterogeneity of CRC. Nevertheless, our results from immune phenotypic study offer different perspectives into the mechanism of CRC liver metastasis in the context of immune activity, and we focused our subsequent analyses on these consistent immune signature gene sets.

Based on the 118 key DEGs associated with metastasis, as well as key immune signature gene sets, we further explored their coordination in regulating CRC liver metastasis. STRING database was used to construct a PPI, after which the largest subnetwork was unraveled and extracted. The subnetwork comprised 11 genes (*CCL11, CCL19, CCL20, CCL24, CCL26, CD27, CD274, CD28, CD40, CD40LG* and *CD70*) that belonged to CD/CCL family and might bridge immune regulation and CRC liver metastasis. These genes (CCL and CD families) were defined as immune hub genes and chosen for subsequent analysis.

The analysis in GEPIA website showed that *CCL11* was significantly down-regulated in both COAD and READ samples, whereas the expression patterns of *CCL20* and *CCL24* were quite the opposite. In contrast, no significant alterations were found among genes in CD family. *CCL11* (C-C motif chemokine 11) originally served as a recruiter of eosinophils [78], and was found markedly elevated in prostate cancer patients and might be used to tell the difference between prostate enlargement and cancer [79]. In promoting chemotaxis, *CCL24* was more versatile, aside from eosinophils, this cytokine also promotes chemotaxis of T lymphocytes and neutrophils [80,81], as *CCL24* and *CCL11* were both responsible for eosinophils recruitment, their down-regulation in glandular cells of COAD might explain the lowered level of neoplastic infiltration of eosinophils [82]. *CCL20* is a potent chemotactic factor of lymphocytes [83]. However, a recent study of breast cancer showed that *CCL20* noticeably facilitated cell invasion and the secretion of *MMP-2/9 in vitro* cell model, which might account for why its overexpression in breast cancer was inversely correlated with metastatic-free/overall survival [84]. In general, the expression profile of *CCL11, CCL20* and *CCL24* were similar in two CRC subtypes, these results suggested that COAD and READ might share similar CCL family-associated molecular mechanisms. The results of survival analyses indicated that the expression of the majority of CD/CCL family members were related to survival, yet the association was still away from being statistical significant, except for *CCL20, CCL24* (high expression associated with improved OS, with p(HR) approximating 0.1) in OS and *CD70* (high expression associated with hindered DFS, with p(HR) of 0.019) in DFS. Combining with results of *CCL20* and *CCL24* expression profile where they were found elevated in two CRC subtypes, as well as increased immune activity score of “eosinophil recruiting” in CRC liver metastatic groups, we propose that these genes might impede CRC liver metastasis and improve prognosis through the recruitment of eosinophil, although further experiments were warranted for validation. The overexpression of *CD70* was documented in various malignancies [85,86], partially because of its interaction with *CD27* (a member molecule from tumor necrosis family), thereby controlling tumor proliferation [86]; the disease-prone nature of *CD70* was consistent in our current study. Unexpectedly, in the pathological stage analyses involving COAD and READ subtypes, no significant alterations were found concerning immune hub gene expres-

sion across 4 pathological stages, despite their influences on OS and DFS of CRC. Therefore, the stable expression of these genes implied that they might exert similar biological functions across the entire span of CRC carcinogenesis, specifically, stable *CD70* expression across different tumor stages was supported by a previous study on paired primary/metastatic NSCLC samples [87]. To sum up, in this part of the study, we identified *CCL20/CCL24* that might regulate CRC liver metastasis through the recruitment of eosinophils, and *CD70* that might promote CRC liver metastasis through facilitating tumor proliferation.

To further assess the genetic variation in two immune hub gene families, we performed oncoprint analysis and constructed an interaction network. The genetic variation analysis revealed that mRNA high alterations were the predominant genetic variation in both families, indicating that their elevated expression was responsible for carcinogenesis in a proportion of CRC patients. The total percentage of genetic variation for *CD40* has reached up to 13%, with the highest rate of amplification. Such variation was normally found in tumor and is restricted to tumor cells as long as the amplified gene is oncogenic [88]. Here, we pursued the genetic variation status of *CCL20, CCL24* and *CD70: CCL20* and *CCL24* displayed similar patterns of genetic variation, among which mRNA high alteration was still dominating, corroborating the results of gene profile analysis; *CD70* underwent less genetic variation (3%), indicating its stability in the tumorigenesis of CRC, which was consistent with pathological stage analyses. cBioPortal was also used to calculate the correlation within two immune hub gene families, we found close correlation among CD family members by comparing CCL family, specifically, *CCL20* was inversely correlated with other CCL family members. Such observation was not consistent with concurrent elevation of *CCL20* and *CCL26* in primary bronchial epithelial cells [89], indicating that the delicate balance within CCL family might be perturbed during malignant transformation.

Finally, the top neighboring genes with high correlation to CD/CCL family members, along with CD/CCL families themselves were defined as CD cluster or CCL cluster, respectively, both of which were used to construct PPI network. According to the number of edges (connectivity), *CCL19, CCL20* played more pivotal roles in CCL cluster, whereas *CD70* exhibited less connectivity by comparing other family members. Based on the functional enrichment analysis, we found that CCL cluster was mainly enriched in GO-BP pathways associated with migration/chemotaxis of immune cells such as leucocyte, neutrophil, and monocyte. The leucocyte acts as a moving vehicle carrying the anticancer drugs or agents to the targeted tissues and thereby exerts its anticancer activities [90], whereas monocytes has been described as a double-edged sword in confronting cancer [91]. These results were corroborated in marked enrichment of CCL cluster in pathways associated with immunoglobulin and chemokine/cytokine activity under GO-MF or KEGG category, as these pathways were responsible for the recruitment of the preceding cells. Results of CD cluster enrichment analyses were similar to that of CCL cluster, except for KEGG pathway, where CCL cluster was aggregated in pathway such as “Primary immunodeficiency”, “Cell adhesion molecules”, in the former case, primary immunodeficiency disorder (PID) hampered the development of immune system or disturbed the immune function [92], thereby prompting the carcinogenesis; in the latter scenario, weakened adhesion improved cancer cells’ motility, and contributed to metastasis, a good example was epithelial-mesenchymal transition [93]. Several other studies also reported the functions of aforementioned relevant signaling pathways in regulating tumor proliferation and metastasis [94–96]. The last part of our results suggested that CD and CCL might regulate CRC liver metastasis through regulating numerous down-stream target protein, through which they conspire to the pathogenesis of CRC liver metastasis.

In summary, this study screened out some immune genes that may serve as potential therapeutic targets for liver metastasis in CRC, among which *CCL19, CCL20* and *CD70* deserve further experimental validation.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Data for reference

All data generated or analyzed during this study are included in this published article.

CRediT authorship contribution statement

Wei-Qing Liu: Data curation, Writing - original draft, Visualization, Investigation, Writing - review & editing. **Wen-Liang Li:** Formal analysis, Data curation, Software, Validation. **Shu-Min Ma:** Formal analysis, Data curation, Methodology, Software, Validation. **Lei Liang:** Formal analysis, Data curation, Methodology, Software, Validation. **Zhi-Yong Kou:** Formal analysis, Data curation, Visualization, Investigation, Software, Validation. **Jun Yang:** Formal analysis, Conceptualization, Supervision, Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101011.

References

[1] M. Wiseman, The second World Cancer Research Fund/American Institute for Cancer Research expert report. Food, nutrition, physical activity, and the prevention of cancer: a global perspective, Proc. Nutr. Soc. 67 (3) (2008) 253–256.
[2] N. Takeuchi, et al., Treatment of metastatic refractory colorectal cancer following regorafenib failure, Mol. Clin. Oncol. 7 (2) (2017) 308–312.
[3] G. Xu, et al., Enhancing the anti-colon cancer activity of quercetin by self-assembled micelles, Int. J. Nanomed. 10 (2015) 2051–2063.
[4] M.S. Cappell, Pathophysiology, clinical presentation, and management of colon cancer, Gastroenterol. Clin. N. Am. 37 (1) (2008) 1–24 v.
[5] M.E. Del Giudice, et al., Systematic review of clinical features of suspected colorectal cancer in primary care, Can. Fam. Phys. 60 (8) (2014) e405–e415.
[6] E.J. Kuipers, et al., Colorectal cancer. Nature reviews, Dis. Primers 1 (2015) 15065 -15065.
[7] D. Cunningham, et al., Colorectal cancer, Lancet 375 (9719) (2010) 1030–1047.
[8] F.E.R. Vuik, et al., Increasing incidence of colorectal cancer in young adults in Europe over the last 25 years, Gut 68 (10) (2019) 1820–1826.
[9] L.A. Torre, et al., Global cancer statistics, 2012, CA: Cancer J. Clin. 65 (2) (2015).
[10] J. Robert, Biology of cancer metastasis, Bull. Cancer 100 (4) (2013) 333–342.
[11] D.V.F. Tauriello, et al., Determinants of metastatic competency in colorectal cancer, Mol. Oncol. 11 (1) (2017) 97–119.
[12] Y.J. Liu, et al., Surgical resection for gastric cancer patients with liver metastasis, Zhonghua Zhong Liu Za Zhi 39 (7) (2017) 532–535.
[13] Y. Tokairin, Y. Kumagai, S. Yamazaki, A case of postoperative liver metastasis of esophageal cancer remains in progression free after successfully resected, Gan To Kagaku Ryoho 36 (12) (2009) 2462–2464.
[14] C.L. Stewart, et al., Cytoreduction for colorectal metastases: liver, lung, peritoneum, lymph nodes, bone, brain. When does it palliate, prolong survival, and potentially cure? Curr. Problems Surg. 55 (9) (2018) 330–379.
[15] Y.J. Fang, D.S. Wan, Molecular mechanism of liver metastasis from colorectal cancer, Ai Zheng 27 (5) (2008) 549–554.
[16] P. Zhang, et al., Nanomedicine-based immunotherapy for the treatment of cancer metastasis, Adv. Mater. 31 (49) (2019) e1904156.
[17] W.H. Fridman, et al., Immune infiltration in human cancer: prognostic significance and disease control, Curr. Top. Microbiol. Immunol. 344 (2011) 1–24.
[18] Ledford, Heidi, Immune cells boost cancer survival from months to years, Nat. News 516 (7530) (2014) 156.
[19] M.P. Avanzi, R.J. Brentjens, Emerging role of CAR T cells in Non-Hodgkin’s lymphoma, J. Natl. Compr. Cancer Netw. 15 (11) (2017) 1429–1437.

[20] J. Fan, et al., Adoptive cell transfer: is it a promising immunotherapy for colorectal cancer? Theranostics 8 (20) (2018) 5784–5800.
[21] P. Ge, et al., Profiles of immune cell infiltration and immune-related genes in the tumor microenvironment of colorectal cancer, Biomed. Pharmacother. 118 (2019) 109228.
[22] Y. Inoue, et al., Cetuximab strongly enhances immune cell infiltration into liver metastatic sites in colorectal cancer, Cancer Sci. 108 (3) (2017) 455–460.
[23] C.S. Roxburgh, D.C. McMillan, The role of the in situ local inflammatory response in predicting recurrence and survival in patients with primary operable colorectal cancer, Cancer Treat. Rev. 38 (5) (2012) 451–466.
[24] R. Marechal, et al., Putative contribution of CD56 positive cells in cetuximab treatment efficacy in first-line metastatic colorectal cancer patients, BMC Cancer 10 (2010) 340.
[25] Y.F. Zou, et al., Comparison of local immune microenvironment between liver-metastasis colorectal cancer and non-liver-metastasis colorectal cancer, Zhonghua Wei Chang Wai Ke Za Zhi 16 (6) (2013) 547–551.
[26] K. Søreide, et al., Assessment of clinically related outcomes and biomarker analysis for translational integration in colorectal cancer (ACROBATICC): study protocol for a population-based, consecutive cohort of surgically treated colorectal cancers and resected colorectal liver metastasis, J. Transl. Med. 14 (1) (2016) 192 -192.
[27] P.Z. Miao, et al., Differential expressions analysis of piwi-interacting RNAs in hepatocellular carcinoma, Zhonghua Gan Zang Bing Za Zhi 26 (11) (2018) 842–846.
[28] L. Xu, et al., TIP: a web server for resolving tumor immunophenotype profiling, Cancer Res. 78 (23) (2018) 6575–6580.
[29] Y. Kamal, et al., Transcriptomic differences between primary colorectal adenocarcinomas and distant metastases reveal metastatic colorectal cancer subtypes, Cancer Res. 79 (16) (2019) 4227–4241.
[30] D. Chen, et al., Genome-wide analysis of long noncoding RNA (lncRNA) expression in colorectal cancer tissues from patients with liver metastasis, Cancer Med. 5 (7) (2016) 1629–1639.
[31] T. Oga, et al., Genomic profiles of colorectal carcinoma with liver metastases and newly identified fusion genes, Cancer Sci. 110 (9) (2019) 2973–2981.
[32] M.E. Ritchie, et al., limma powers differential expression analyses for RNA-sequencing and microarray studies, Nucl. Acids Res. 43 (7) (2015) e47 -e47.
[33] A.M. Newman, et al., Robust enumeration of cell subsets from tissue expression profiles, Nat. Methods 12 (5) (2015) 453–457.
[34] D.A. Barbie, et al., Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1, Nature 462 (7269) (2009) 108–112.
[35] V. Heinemann, et al., FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer (FIRE-3): a randomised, open-label, phase 3 trial, Lancet Oncol. 15 (10) (2014) 1065–1075.
[36] H. Brenner, C. Stock, M. Hoffmeister, Colorectal cancer screening: the time to act is now, BMC Med. 13 (2015) 262.
[37] Z. Tang, et al., GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses, Nucleic Acids Res. 45 (W1) (2017) W98–W102.
[38] S.L. Topalian, C.G. Drake, D.M. Pardoll, Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity, Curr. Opin. Immunol. 24 (2) (2012) 207–212.
[39] D.S. Chen, B.A. Irving, F.S. Hodi, Molecular pathways: next-generation immunotherapy-inhibiting programmed death-ligand 1 and programmed death-1, Clin. Cancer Res. 18 (24) (2012) 6580–6587.
[40] D.S. Chen, I. Mellman, Oncology meets immunology: the cancer-immunity cycle, Immunity 39 (1) (2013) 1–10.
[41] K. Kienle, T. Lammermann, Neutrophil swarming: an essential process of the neutrophil tissue response, Immunol. Rev. 273 (1) (2016) 76–93.
[42] Y. Wang, et al., Immunoreactive cells after cerebral ischemia, Front. Immunol. 10 (2019) 2781.
[43] M. Yao, et al., Cytokine regulation of metastasis and tumorigenicity, Adv. Cancer Res. 132 (2016) 265–367.
[44] Y. Yoshida, et al., Granulocyte colony-stimulating factor- and interleukin-6-producing large-cell carcinoma of the lung with sarcomatoid changes suggestive of epithelial-mesenchymal transition: an autopsy case report, Intern. Med. 58 (22) (2019) 3305–3311.
[45] C. Chen, et al., Increased circulation of galectin-3 in cancer induces secretion of metastasis-promoting cytokines from blood vascular endothelium, Clin. Cancer Res. 19 (7) (2013) 1693–1704.
[46] B. Borm, et al., Membrane ruffles in cell migration: indicators of inefficient lamellipodia adhesion and compartments of actin filament reorganization, Exp. Cell Res. 302 (1) (2005) 83–95.
[47] W.G. Jiang, Focus on science—membrane ruffling of cancer cells: A parameter of tumour cell motility and invasion, Eur. J. Surg. Oncol. 21 (3) (1995) 307–309.
[48] M.T. Chow, A.D. Luster, Chemokines in cancer, Cancer Immunol. Res. 2 (12) (2014) 1125–1131.
[49] M. Kodama, et al., A shift in glutamine nitrogen metabolism contributes to the malignant progression of cancer, Nat. Commun. 11 (1) (2020) 1320.
[50] F.P. Guengerich, Cytochrome p450 and chemical toxicology, Chem. Res. Toxicol. 21 (1) (2008) 70–83.
[51] S. Travica, et al., Colon cancer-specific cytochrome P450 2W1 converts duocarmycin analogues into potent tumor cytotoxins, Clin. Cancer Res. 19 (11) (2013) 2952–2961.
[52] H. Sadeghi, et al., miR-30a promoter variation contributes to the increased risk of colorectal cancer in an Iranian population, J. Cell Biochem. (2018).
[53] T. Abaffy, et al., A testosterone metabolite 19-hydroxyandrostenedione induces neuroendocrine trans-differentiation of prostate cancer cells via an ectopic olfactory receptor, Front. Oncol. 8 (2018) 162.
[54] M. Li, et al., The olfactory receptor family 2, subfamily T, Member 6 (OR2T6) is involved in breast cancer progression via initiating epithelial-mesenchymal transition and MAPK/ERK pathway, Front. Oncol. 9 (2019) 1210.
[55] R. Morita, et al., Olfactory receptor family 7 subfamily C member 1 is a novel marker of colon cancer-initiating cells and is a potent target of immunotherapy, Clin. Cancer Res. 22 (13) (2016) 3298–3309.
[56] H. Mashima, et al., INSL5 may be a unique marker of colorectal endocrine cells and neuroendocrine tumors, Biochem. Biophys. Res. Commun. 432 (4) (2013) 586–592.
[57] J.C. Byrd, R.S. Bresalier, Mucins and mucin binding proteins in colorectal cancer, Cancer Metastasis Rev. 23 (1-2) (2004) 77–99.
[58] G. Zhang, et al., Integration of metabolomics and transcriptomics revealed a fatty acid network exerting growth inhibitory effects in human pancreatic cancer, Clin. Cancer Res. 19 (18) (2013) 4983–4993.
[59] Z. Song, et al., PHGDH is an independent prognosis marker and contributes cell proliferation, migration and invasion in human pancreatic cancer, Gene 642 (2018) 43–50.
[60] Y. Hirata, et al., TRIM48 promotes ASK1 activation and cell death through ubiquitination-dependent degradation of the ASK1-negative regulator PRMT1, Cell Rep. 21 (9) (2017) 2447–2457.
[61] L.P. Xue, et al., Overexpression of tripartite motif-containing 48 (TRIM48) inhibits growth of human glioblastoma cells by suppressing extracellular signal regulated kinase 1/2 (ERK1/2) pathway, Med. Sci. Monit. 25 (2019) 8422–8429.
[62] J. Xu, et al., Knockdown of serpin peptidase inhibitor clade C member 1 inhibits the growth of nasopharyngeal carcinoma cells, Mol. Med. Rep. 19 (5) (2019) 3658–3666.
[63] J.Y. Choe, et al., PELI1 expression is correlated with MYC and BCL6 expression and associated with poor prognosis in diffuse large B-cell lymphoma, Mod. Pathol. 29 (11) (2016) 1313–1323.
[64] S.H. Dho, et al., GPR171 expression enhances proliferation and metastasis of lung cancer cells, Oncotarget 7 (7) (2016) 7856–7865.
[65] S.Z. Wiley, et al., GPR68: an emerging drug target in cancer, Int. J. Mol. Sci. 20 (3) (2019).
[66] M. Debald, et al., Specific expression of k63-linked ubiquitination of calmodulin-like protein 5 in breast cancer of premenopausal patients, J. Cancer Res. Clin. Oncol. 139 (12) (2013) 2125–2132.
[67] F. Klupp, et al., Serum MMP7, MMP10 and MMP12 level as negative prognostic markers in colon cancer patients, BMC Cancer 16 (2016) 494.
[68] Y.S. Chang, et al., Molecular characterization of colorectal cancer using whole-exome sequencing in a Taiwanese population, Cancer Med. 8 (8) (2019) 3738–3747.
[69] Y. Yang, et al., Complete response to anti-PD-L1 antibody in a metastatic bladder cancer associated with novel MSH4 mutation and microsatellite instability, J. Immunother. Cancer 8 (1) (2020).
[70] L. Saurer, et al., TREM-1 promotes intestinal tumorigenesis, Sci. Rep. 7 (1) (2017) 14870.
[71] S. Gatault, et al., Involvement of eosinophils in the anti-tumor response, Cancer Immunol. Immunother. 61 (9) (2012) 1527–1534.
[72] S. Sakkal, et al., Eosinophils in cancer: favourable or unfavourable? Curr. Med. Chem. 23 (7) (2016) 650–666.
[73] I. Mellman, G. Coukos, G. Dranoff, Cancer immunotherapy comes of age, Nature 480 (7378) (2011) 480–489.
[74] T. Yoshimura, et al., PCP4/PEP19 promotes migration, invasion and adhesion in human breast cancer MCF-7 and T47D cells, Oncotarget 7 (31) (2016) 49065–49074.
[75] J. Borst, et al., CD4(+) T cell help in cancer immunology and immunotherapy, Nat. Rev. Immunol. 18 (10) (2018) 635–647.
[76] M.A. Sayem, et al., Identification of glypican-3-derived long peptides activating both CD8(+) and CD4(+) T cells; prolonged overall survival in cancer patients with Th cell response, Oncoimmunology 5 (1) (2015) e1062209.
[77] L.V. Pham, E. Pogue, R.J. Ford, The role of macrophage/B-cell interactions in the pathophysiology of B-cell lymphomas, Front. Oncol. 8 (2018) 147 -147.
[78] A.L. Teixeira, et al., Revisiting the role of eotaxin-1/CCL11 in psychiatric disorders, Front. Psychiatry 9 (2018) 241.
[79] M. Agarwal, et al., CCL11 (eotaxin-1): a new diagnostic serum marker for prostate cancer, Prostate 73 (6) (2013) 573–581.
[80] V.P. Patel, et al., Molecular and functional characterization of two novel human C-C chemokines as inhibitors of two distinct classes of myeloid progenitors, J. Exp. Med. 185 (7) (1997) 1163–1172.
[81] J.R. White, et al., Cloning and functional characterization of a novel human CC chemokine that binds to the CCR3 receptor and activates human eosinophils, J. Leukoc. Biol. 62 (5) (1997) 667–675.
[82] H. Cho, et al., Eosinophils in colorectal neoplasms associated with expression of CCL11 and CCL24, J. Pathol. Transl. Med. 50 (1) (2016) 45–51.
[83] K. Hieshima, et al., Molecular cloning of a novel human CC chemokine liver and activation-regulated chemokine (LARC) expressed in liver. Chemotactic activity for lymphocytes and gene localization on chromosome 2, J. Biol. Chem. 272 (9) (1997) 5846–5853.
[84] S.K. Lee, et al., Human antigen R-regulated CCL20 contributes to osteolytic breast cancer bone metastasis, Sci. Rep. 7 (1) (2017) 9610.
[85] C.L. Law, et al., Lymphocyte activation antigen CD70 expressed by renal cell carcinoma is a potential therapeutic target for anti-CD70 antibody-drug conjugates, Cancer Res. 66 (4) (2006) 2328–2337.
[86] A. Nilsson, et al., Expression of CD27-CD70 on early B cell progenitors in the bone marrow: implication for diagnosis and therapy of childhood ALL, Exp. Hematol. 33 (12) (2005) 1500–1507.
[87] J. Jacobs, et al., Unlocking the potential of CD70 as a novel immunotherapeutic target for non-small cell lung cancer, Oncotarget 6 (15) (2015) 13462–13475.
[88] M. Debatisse, B. Malfoy, Gene amplification mechanisms, Adv. Exp. Med. Biol. 570 (2005) 343–361.

[89] S. Prakash, et al., Dendritic cells from aged subjects contribute to chronic airway inflammation by activating bronchial epithelial cells under steady state, Mucosal Immunol. 7 (6) (2014) 1386–1394.
[90] M.J. Mitchell, M.R. King, Leukocytes as carriers for targeted cancer drug delivery, Expert Opin. Drug Deliv. 12 (3) (2015) 375–392.
[91] C.E. Olingy, H.Q. Dinh, C.C. Hedrick, Monocyte heterogeneity and functions in cancer, J. Leukoc. Biol. 106 (2) (2019) 309–322.
[92] C. McCusker, R. Warrington, Primary immunodeficiency, Allergy Asthma Clin. Immunol. 7 (Suppl 1) (2011) S11.
[93] Y. Tsubakihara, A. Moustakas, Epithelial-Mesenchymal transition and metastasis under the control of transforming growth factor beta, Int. J. Mol. Sci. 19 (11) (2018).
[94] T. Ren, L. Zhu, M. Cheng, CXCL10 accelerates EMT and metastasis by MMP-2 in hepatocellular carcinoma, Am. J. Transl. Res. 9 (6) (2017) 2824–2837.
[95] H. Hu, et al., MEGF6 promotes the epithelial-to-Mesenchymal transition via the TGFbeta/SMAD signaling pathway in colorectal cancer metastasis, Cell Physiol. Biochem. 46 (5) (2018) 1895–1906.
[96] S.Q. Liu, et al., Sphingosine kinase 1 promotes tumor progression and confers malignancy phenotypes of colon cancer by regulating the focal adhesion kinase pathway and adhesion molecules, Int. J. Oncol. 42 (2) (2013) 617–626.