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Brief Communication

Integrated multiplex network based approach reveled CC and CXC chemokines associated key biomarkers in colon adenocarcinoma patients

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Abstract: Colon adenocarcinoma (COAD) is a prevalent and aggressive form of cancer that necessitates the identification of robust biomarkers for early diagnosis and treatment. Therefore, this project was launched to identify a few key biomarkers from CC and CXC chemokine families for the accurate detection of COAD. Hub gene identification was performed using CytoHubba analysis. Clinical samples from COAD patients and normal individuals were collected and subjected to appropriate methods for DNA and RNA extraction. The expression levels of hub genes were analyzed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), while promoter methylation analysis was conducted using targeted bisulfite sequencing (bisulfite-seq). Additionally, The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) datasets were utilized to validate the findings based on clinical samples. CXCL10 (C-X-C motif chemokine ligand 10), CXCL12 (C-X-C motif chemokine ligand 12), CXCL16 (C-X-C motif chemokine ligand 16), and CCL25 (CC motif chemokine ligand 25) were denoted as the key hubs among CC and CXC chemokine families. Through RT-qPCR analysis, it was found that CXCL10, CXCL12, and CXCL16 were significantly up-regulated, while CCL25 was down-regulated in COAD patients compared to healthy controls. Later on, these findings were also validated using TCGA and GEO datasets consisting of COAD and normal control samples. Furthermore, we investigated the promoter methylation status of these chemokine genes in COAD patients. Our results revealed significant dysregulation of promoter methylation, suggesting an epigenetic mechanism underlying the altered expression of CXCL10, CXCL12, CXCL16, and CCL25 in COAD. In addition to this, various additional aspects of the CCL25, CXCL10, CXCL12, and CXCL16 have also been uncovered in COAD during the present study. This study highlights the dysregulation of CXCL10, CXCL12, CXCL16, and CCL25 chemokine members in COAD patients, emphasizing their significance as potential biomarkers and therapeutic targets in the management of this deadly disease. However, further investigations are warranted to elucidate the underlying molecular mechanisms and evaluate the clinical utility of these findings.

Keywords: Colon adenocarcinoma, biomarkers, CC and CXC chemokine

Introduction

Cancer poses a significant global public health challenge, standing as the second most common cause of mortality in the United States (U.S.) [1-3]. Based on the latest statistics from the American Cancer Society for 2022, colorectal cancer (CRC) holds the third position in terms of incidence and stands as the third primary contributor to cancer-related fatalities on a global scale [4]. Colorectal cancer (CRC) continues to be prevalent as one of the most fre-

quently occurring malignant tumors within the digestive system. Specifically, colon adenocarcinomas (COAD) represent the predominant subtype, constituting approximately 95% of all reported cases of colon cancer [5].

Multiple risk factors are associated with COAD [3, 6-8]. A variety of therapeutic approaches, such as surgery, radiation therapy, chemotherapy, targeted therapy, immunotherapy, and combination therapy, are employed in the treatment of colon cancer. Nevertheless, despite the utilization of these methods, the incidence and mortality rates of the disease remain persistently high [6, 9-12]. The inadequate early detection and diagnosis of colon cancer continue to contribute to its unfavorable prognosis. Consequently, it is of utmost importance to explore potential diagnostic biomarkers and effective therapeutic targets in order to enhance monitoring and overcome the challenges associated with COAD.

The complex interaction between the immune system and the progression of cancer has become a captivating field of investigation, attracting significant attention and extensive research endeavors over the years [13, 14]. Chemokines, which fall under the category of cytokines, are generated by diverse cell types, including tumor cells, leukocytes, and immune cells, among others. These molecules have been acknowledged for their pivotal role in regulating inflammation and immune responses [15-17]. Based on the number and position of the first two conserved cysteine residues found at the N terminus, chemokines can be classified into four main subgroups: CXC, CC, C, and CX3C [18]. Chemokines are further categorized into distinct subsets based on their functions and expression patterns, specifically as homeostatic and inflammatory chemokines [19]. Inflammatory chemokines are commonly induced during inflammatory processes and are expressed by a range of cell types, including leukocytes [19]. The presence of these chemokines is essential for promoting the recruitment of inflammatory leukocytes to the precise location of tissue damage or inflammation, aiding in the immune response and restoration of affected tissues [20, 21]. On the other hand, homeostatic chemokines demonstrate consistent expression in particular tissues, even in the absence of apparent activating stimuli [19]. These chemokines have a critical function in regulating the movement of cells and ensuring the effective operation of immune surveillance systems [20-22]. The roles of CC and CXC chemokines are pivotal in various aspects of tumor angiogenesis, growth, invasion, and metastasis, underscoring their significance in these intricate processes [18, 23].

In recent research, there has been a focus on investigating the expression patterns and exploring the diagnostic and prognostic implications of CXC and CC chemokine members in a range of human cancers, including gastric cancer, hepatocellular carcinoma, and non-small-cell lung cancer [24-29]. Hence, the aim of this study was to conduct a comprehensive analysis using both in silico and molecular experimental approaches, with the goal of revealing the diagnostic and prognostic relevance of the entire CXC and CC chemokine families in COAD.

Methodology

COAD and normal control tissue sample collection

After obtaining the necessary approval from the ethics committee, we conducted a prospective collection of 25 pairs of COAD tissues and their corresponding normal tissues. The patients included in the study were individuals who visited the Institute of Nuclear Medicine, Oncology and Radiotherapy Hospital and Ayub Medical Complex between August 2022 and May 2023. Prior to their participation, informed consent was obtained from all participants through the signing of consent forms. All patients included in the study were diagnosed with COAD and had not undergone any adjuvant or neoadjuvant therapy prior to their surgical procedures.

Construction of the CC and CXC families member PPI and the selection of hub genes

To investigate the protein-protein interaction (PPI) networks of the CC and CXC chemokine families, we utilized the STRING database [30]. Subsequently, we employed the Cytohubba function [31] within the Cytoscape tool to identify the critical module and detect the hub genes. The selection of hub genes was based on four different scoring algorithms, namely the maximum neighborhood component (MNC), the density of the maximum neighborhood compo-

nent (DMNC), the maximal clique centrality (MCC), and the Degree of the Cytohubba [32]. The top four genes shared by these four algorithms were chosen as the hub genes.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) validation analysis

The specific protocols were performed as follows: Initially, the PrimeScript™ RT reagent kit (Takara, Japan) was utilized to perform reverse transcription of the extracted RNA from tissue samples, resulting in the generation of complementary DNA. Subsequently, RT-qPCR was conducted using an ABI ViiA 7 Real-Time PCR System (Thermo Fisher, USA) with SuperReal SYBR Green Premix Plus (Tiangen Biotech, China) as the fluorescent dye. In this study, GAPDH was selected as the internal reference. All experiments were independently conducted in triplicate. The 2^(-ΔΔCt) method was employed to assess the relative expression of each hub gen [33]. The primer sequences for each hub gene are provided below.

GAPDH-F 5'-ACCCACTCCTCCACCTTTGAC-3', GAPDH-R 5'-CTGTTGCTGTAGCCAAATTCG-3' [34]. CXCL10-F 5'-GCTCAGGCTCGTCAGTTCTAAGT-3', CXCL10-R 5'-GGAAGATGGTGGTTAAGTTCGTC-3' [35]. CXCL12-F 5'-TCAGCCTGAGCTACAGATGC-3', CXCL12-R 5'-CTTTAGCTTCGGGTCAATGC-3' [36]. CXCL16-F 5'-CTGACTCAGCCAGGCAATGG-3', CXCL16-R 5'-TGAGTGGACTGCAAGGTGGA-3' [37]. CCL25-F 5'-A AGGCCCAGAGTTACTATCGC-3', CCL25-R 5'-TCTTCATCCCAGCCTGAACC-3' [38].

Targeted bisulfite sequencing (bisulfite-seq) analysis

The DNA samples were submitted to the Beijing Genomics Institute (BGI) company for bisulfite-seq analysis. After conducting targeted bisulfite-seq analysis, the methylation values were normalized as beta values. The beta values obtained from the COAD and normal control sample groups were compared to identify variations in the methylation levels of the hub genes.

UALCAN GEPIA, OncoDB, gene expression, KM plotter and GEO databases

The UALCAN database (http://ualcan.path.uab.edu/) provides a comprehensive analysis of cancer-related omics data derived from The Cancer Genome Atlas (TCGA) and MET500 databases [39]. To assess the mRNA expression levels of the identified hub genes, the "TCGA gene analysis" module of the UALCAN database was utilized. This analysis encompassed COAD samples from different clinical variables as well as normal tissues.

To further validate the expression of hub genes in COAD tissues and normal controls, we employed several online databases, including GEPIA [40], OncoDb [41], and Gene Expression Omnibus (GEO) [42]. These widely recognized platforms for cancer microarray-based expression analysis provided comprehensive results in the form of box plots, ensuring robust validation of our findings.

The UALCAN database was also utilized in this study to explore promoter methylation level of the hub genes in COAD patients and normal controls. Then, KM plotter [43] platform, which is a cutting-edge tool for conducting survival analysis was used in this study to perform survival analysis of the hub genes.

Examining hub gene expression in the Human Protein Atlas and across various immune and molecular subtypes of COAD

The Human Protein Atlas (HPA) database is a comprehensive resource for studying the human proteome [44]. The database employs immunohistochemistry-based profiling and offers high-quality images of protein expression patterns. This database was used in this study to explore hub gene proteomic expression in COAD tissue samples paired with controls. Furthermore, in order to assess the expression of hub genes in various immune and molecular subtypes of COAD, we utilized the TISIDB database (http://cis.hku.hk/TISIDB/index.php) [45].

Development of hub genes-based prognostic model

To construct the prediction model, we utilized the Lasso and multivariate Cox proportional hazard regression analysis in the "survival" package of the R language [46]. The CRC_TCGA dataset served as the training dataset, while the GSE72970, GSE71187, GSE39582, GSE39084, GSE29621, GSE17537, GSE17536, GSE106584, and GSE103479 dataset was used for validation purposes. The prognostic model for predicting the prognosis of COAD

patients was formulated as follows: the risk score was determined by summing the variations of the multivariate Cox regression coefficients for each mRNA.

Genomic alteration, functional enrichment, immune cell infiltration, miRNA, and drug prediction analyses of hub genes

For genetic alteration and mutual exclusivity analyses of hub genes among COAD patients, we leveraged the cBioPortal with a default setting [47]. With its extensive capabilities, the cBioPortal allowed us to query specific gene(s) of interest and explore relevant alterations across a vast collection of over 5,000 cancer samples derived from 20 different cancer studies.

We conducted a functional enrichment analysis of the hub genes utilizing the GSEA program [48]. This comprehensive analysis involved Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. By taking into account the biological attributes of the protein or gene list under investigation, the GSEA program identified pertinent GO terms and KEGG pathways [48].

To evaluate the infiltration of immune cells within tumors, the web-based TIMER database [49] was employed. This database employs various algorithms to estimate the abundance of immune cells across different types of cancer. In this research, the levels of immune cell infiltration in COAD were plotted against the expression levels of the identified hub genes.

The ENCORI database, known for its exploration of miRNA-ncRNA and mRNA-miRNA interactions using CLIP-seq and degradome-seq interactome data [50], was utilized in this study. Specifically, the ENCORI database was employed to construct the miRNA network associated with the identified hub genes.

Finally, We performed the DrugBank [51] research to find the drugs related to the hub genes because we believe that the identified hub genes can be interesting therapeutic targets.

Statistics analysis

For GO and KEGG enrichment analysis, we used the Fisher's Exact test for computing difference [52]. Correlational analyses were carried out using the Pearson method. For comparisons, a student t-test was adopted in the current study. All the analyses were carried out in R version 3.6.3 software.

Results

Construction of the CC and CXC families' member PPI and the selection of hub genes

To determine the interaction scores threshold, a minimum value of > 0.4 was selected. After that, we analyzed proteins belonging to the CC and CXC chemokine Families using the STRING database to construct the protein-protein interaction (PPI) network (**Figure 1A**, **1B**). The PPI network comprised 267 edges and 38 nodes. To identify the hub genes within this network, we applied a combination of scoring algorithms, including MNC, DMNC, MCC, and Degree, using the CytoHubba application. The top four shared differentially expressed genes (DEGs) identified by these 4 algorithms were considered the hub genes. Overall, we identified four genes as hub genes: CXCL10 (C-X-C motif chemokine ligand 10), CXCL12 (C-X-C motif chemokine ligand 12), CXCL16 (C-X-C motif chemokine ligand 16), and CCL25 (CC motif chemokine ligand 25) (**Figure 1C**).

RT-qPCR analysis of CXCL10, CXCL12, CXCL16, and CCL25 in normal and COAD clinical tissue samples

To quantify the mRNA expression levels of the hub genes (CXCL10, CXCL12, CXCL16, and CCL25), we conducted an RT-qPCR experiment. This analysis involved the use of 25 paired COAD tissue samples and their respective controls. The results, as depicted in **Figure 2A**, revealed notable differences in the expression levels of the four hub genes (CXCL10, CXCL12, CXCL16, and CCL25) between the COAD tissue samples and their paired controls. Interestingly, we observed a significant up-regulation of CXCL10 and CXCL16, as well as CCL25, whereas CXCL12 exhibited a significant down-regulation in the COAD tissue samples compared to their corresponding controls (**Figure 2A**). These findings shed light on the altered expression patterns of the hub genes in COAD, indicating their potential involvement in the disease occurrence.

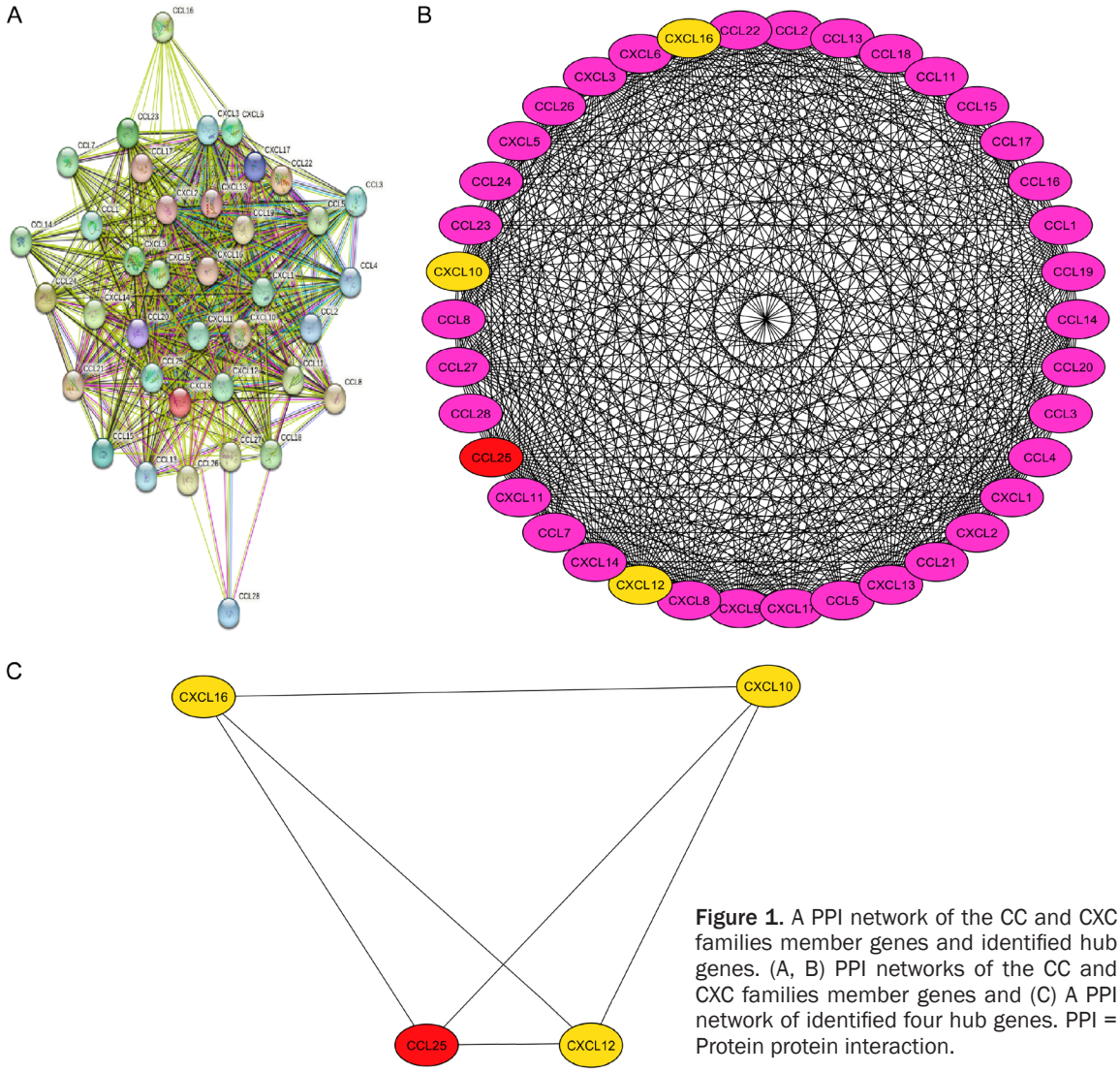

Figure 1. A PPI network of the CC and CXC families member genes and identified hub genes. (A, B) PPI networks of the CC and CXC families member genes and (C) A PPI network of identified four hub genes. PPI = Protein protein interaction.

Promoter methylation and survival analysis of CXCL10, CXCL12, CXCL16, and CCL25 in normal and COAD clinical tissue samples

To analyze the promoter methylation of the hub gene, we employed a targeted bisulfite-seq technique using 25 paired COAD samples and normal controls. In this analysis, beta values were utilized to validate the methylation levels. The results of the analysis, presented in **Figure 2B**, exhibited noticeable discrepancies in the beta values of the hub genes CXCL10, CXCL12, CXCL16, and CCL25 between the COAD samples and normal controls. Specifically, the beta values of CXCL10, CXCL16, and CCL25 were lower in the COAD samples, indicating decreased methylation, while the beta value of CXCL12 was higher, indicating increased methylation, in comparison to the control samples (**Figure 2B**).

Hub genes expression verification via UALCAN

After identifying CXCL10, CXCL12, CXCL16, and CCL25 as hub genes with differential expression, we proceeded to validate their expression levels in TCGA COAD samples and normal controls using the UALCAN database. Through this analysis, we gained insights into the distinct expression patterns of these hub genes in COAD compared to normal tissues. The results revealed a significant up-regulation of CXCL10, CXCL16, and CCL25 in COAD samples when compared to controls (**Figure 3A**, **3B**; Supplementary Tables 1, 3 and 4). Conversely, the hub gene CXCL12 exhibited a notable

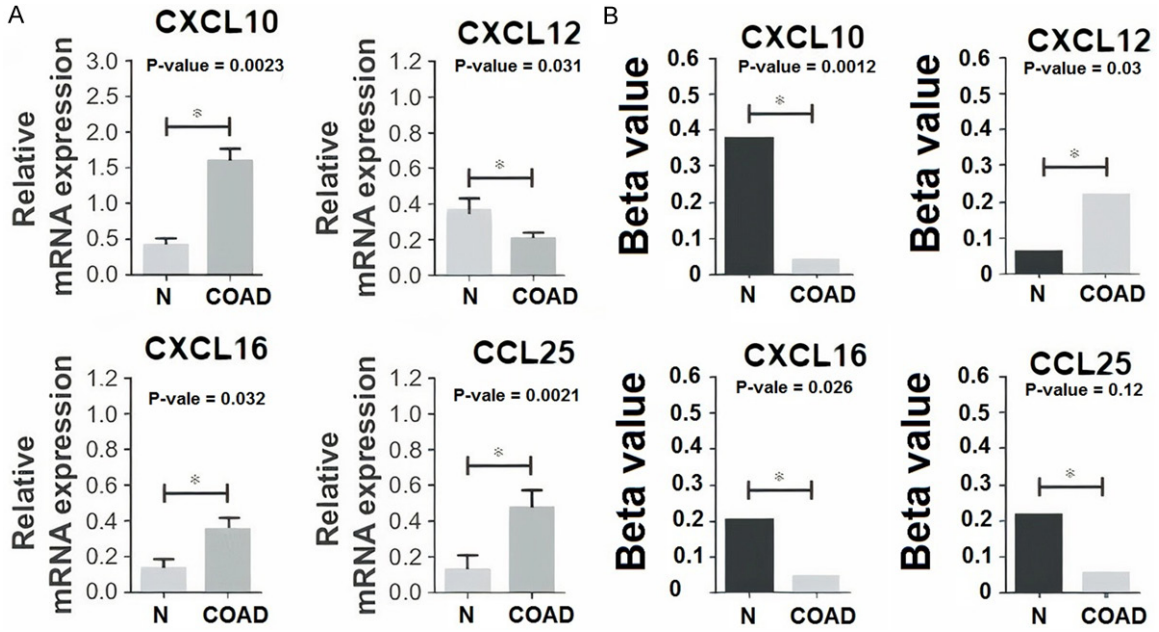

Figure 2. Expression and promoter methylation profiling of CXCL10, CXCL12, CXCL16, and CCL25 levels using COAD tissue samples paired with controls via RT-qPCR and targeted bisulfite-seq analyses. (A) Relative expression profile of CXCL10, CXCL12, CXCL16, and CCL25 across COAD tissue samples paired with controls via RT-qPCR, (B) Beta values based promoter methylation based validation of CXCL10, CXCL12, CXCL16, and CCL25 across COAD tissue samples paired with controls. COAD = Colon adenocarcinoma, RT-qPCR = Reverse transcription-quantitative polymerase chain reaction, Bisulfite-seq = Bisulfite sequencing.

down-regulation in COAD samples (**Figure 3A**, **3B**; Supplementary Table 2).

Furthermore, by considering various clinical variables such as cancer stage, race, gender, and age, we observed consistent trends in the expression levels of these hub genes in COAD patients relative to the control samples (**Figure 4**; Supplementary Tables 1, 2, 3, 4). Specifically, CXCL10, CXCL16, and CCL25 consistently exhibited higher expression levels, while CXCL12 consistently displayed lower expression levels in COAD patients across different clinical factors.

Additional validation of hub gene expression

To enhance the credibility of our hub gene expression findings, we performed expression validation analysis using additional TCGA and GEO datasets accessible through the GEPIA, OncoDB, and GEO databases. The results, illustrated in **Figure 5A-C** and Supplementary Tables 5, 6, 7, unequivocally showcased significant up-regulation of CXCL10, CXCL16, and CCL25 mRNA expression in COAD samples compared to normal individuals. On the other hand, the mRNA expression of CXCL12 was distinctly lower in COAD samples. This robust evidence further corroborates the dysregulation of these hub genes in COAD across multiple datasets, solidifying their potential role in the pathogenesis of COAD.

Correlation between hub gene expression and different immune and immune molecule subtypes of COAD

Using the TISIDB database, we conducted an investigation into the correlation between hub gene expression in COAD and various immune subtypes and immune molecule subtypes. The immune subtypes, designated as C1 to C6 (wound healing, IFN-γ dominant, inflammatory, lymphocyte depleted, immune quiet, and TGF-β dominant), were included in the analysis. The results revealed a significant association between hub gene expression and all immune molecule subtypes in COAD (Supplementary Figure 1A). Furthermore, there was a clear correlation observed between hub gene expression and different molecular subtypes of COAD tumors (Supplementary Figure 1B). Collectively, these findings highlight the differential expres-

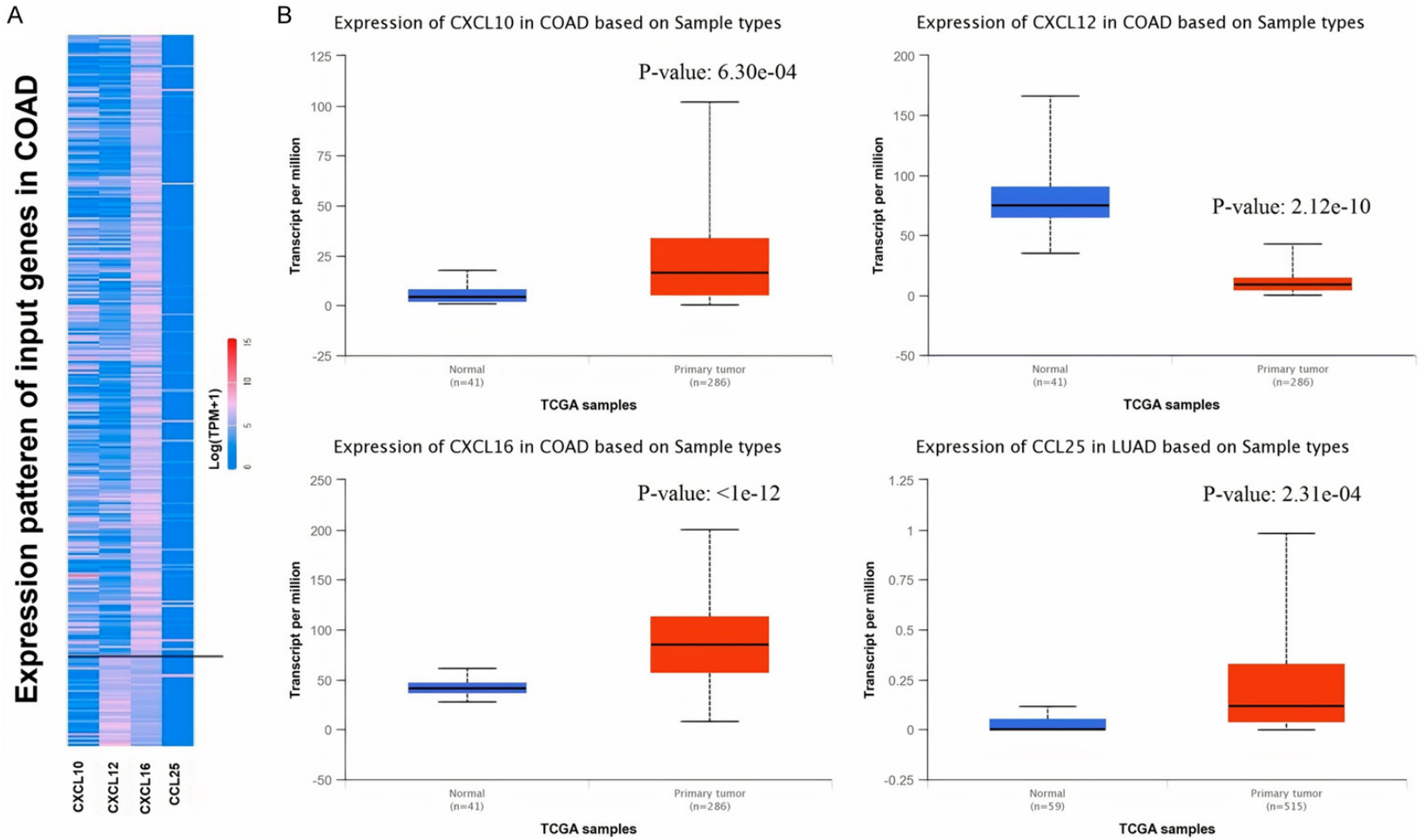

Figure 3. Expression profiling of the CXCL10, CXCL12, CXCL16, and CCL25 in COAD samples paired with controls via UALCAN. (A) A heatmap of CXCL10, CXCL12, CXCL16, and CCL25 hub genes in COAD sample group and normal control group and (B) Box plot presentation of CXCL10, CXCL12, CXCL16, and CCL25 hub genes expression in COAD sample group and normal control group. COAD = Colon adenocarcinoma.

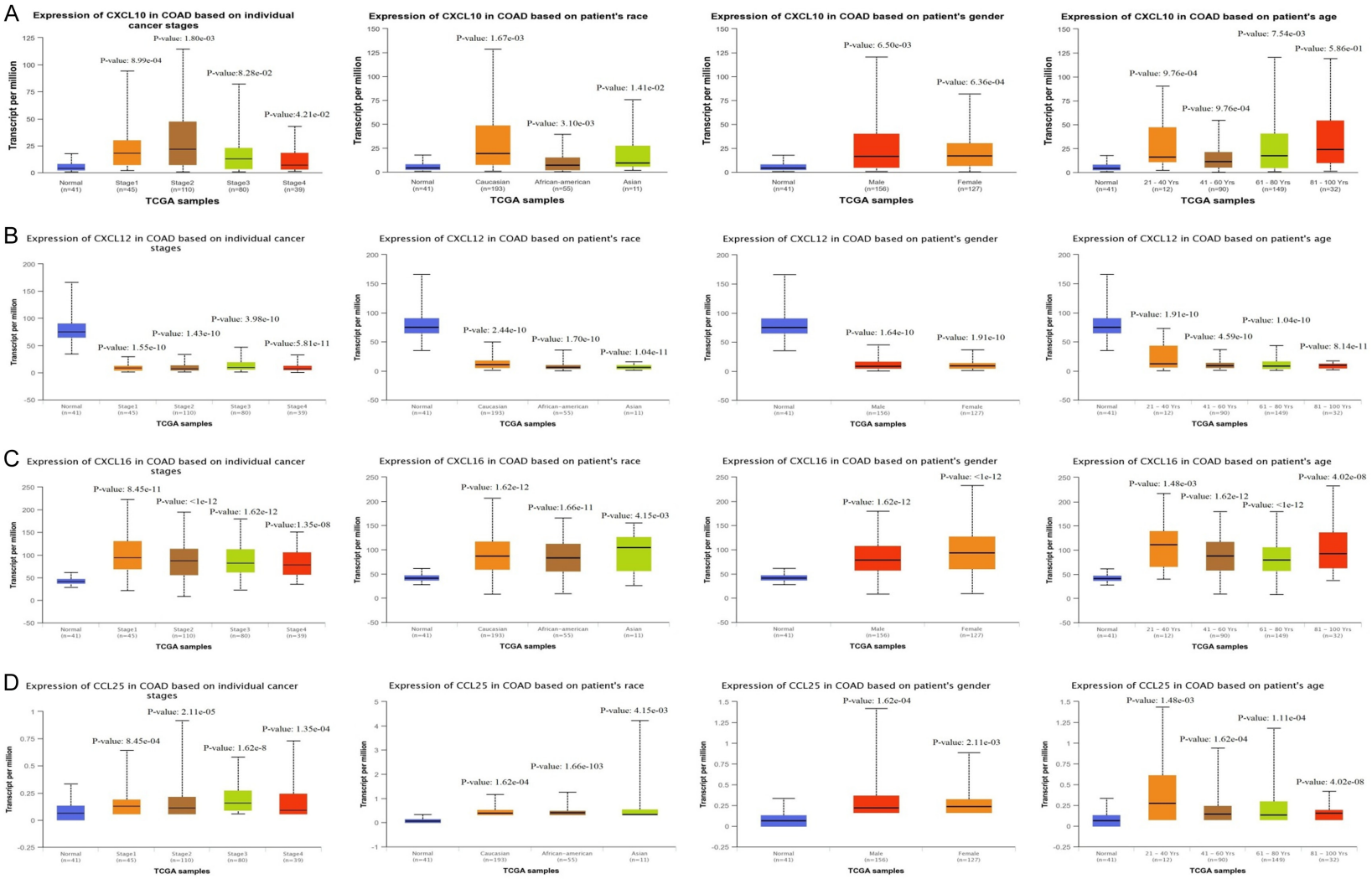

Figure 4. Expression profiling of CXCL10, CXCL12, CXCL16, and CCL25 in COAD samples of different clinical variables relative to controls via UALCAN. (A) Expression profiling of CCL25 in COAD samples of different clinical variables, (B) Expression profiling of CXCL10 in COAD samples of different clinical variables, (C) Expression profiling of CXCL12 in COAD samples of different clinical variables, and (D) Expression profiling of CXCL16 in COAD samples of different clinical variables. COAD = Colon adenocarcinoma.

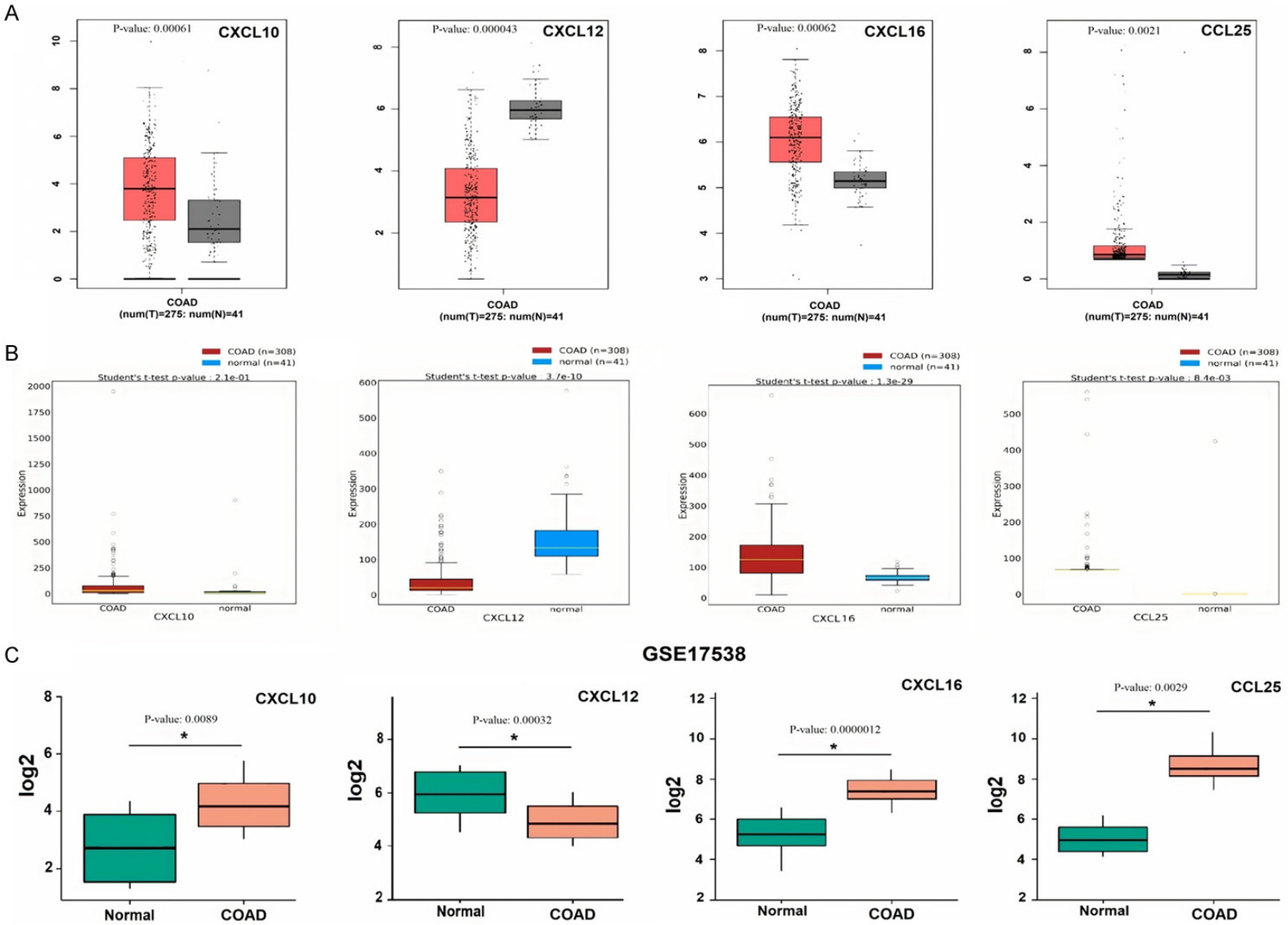

Figure 5. Expression validation of CXCL10, CXCL12, CXCL16, and CCL25 using additional TCGA and GEO datasets. (A) Expression validation of CXCL10, CXCL12, CXCL16, and CCL25 in COAD and normal samples via GEPIA database, (B) Expression validation of CXCL10, CXCL12, CXCL16, and CCL25 in COAD and normal samples via OncoDB database, and (C) Expression validation of CXCL10, CXCL12, CXCL16, and CCL25 in COAD and normal samples via GEO database using GSE17538 dataset. TCGA = The cancer Genome Atlas, GEO = Gene Expression Omnibus, COAD = Colon adenocarcinoma.

sion of CXCL10, CXCL12, CXCL16, and CCL25 in COAD tumors across diverse immune and molecular subtypes.

Promoter methylation validation and survival analysis of CXCL10, CXCL12, CXCL16, and CCL25

To validate the potential impact of promoter methylation on CXCL10, CXCL12, CXCL16, and CCL25 in COAD, we examined promoter methylation levels using both UALCAN and OncoDB. Remarkably, our analysis revealed intriguing findings regarding the promoter methylation patterns of these hub genes. Specifically, we observed significant hypomethylation in the promoters of CXCL10, CXCL16, and CCL25 genes, while the promoter of CXCL12 exhibited hypermethylation in COAD specimens when compared to controls (Supplementary Figure 2A, 2B). These results strongly suggest that the altered promoter methylation levels play a crucial role in driving the higher expression of CXCL10, CXCL16, and CCL25, as well as the reduced expression of CXCL12 in COAD.

Furthermore, using the KM plotter tool, we performed survival analysis of CXCL10, CXCL12, CXCL16, and CCL25 in COAD patients. The analysis demonstrated a significant correlation between the dysregulation of these genes and poor overall survival (OS) in COAD patients (Supplementary Figure 2C). This finding strongly suggests that the altered expression levels of CXCL10, CXCL12, CXCL16, and CCL25 could serve as valuable prognostic indicators for COAD. These genes hold the potential to be important prognostic biomarkers, aiding in the assessment of patient outcomes and providing valuable insights for guiding clinical decision-making in the management of COAD.

Protein expression analysis and development of prognostic model based on the annotated DEGS

In order to validate the protein expression levels of CXCL10, CXCL12, CXCL16, and CCL25 genes in COAD, we utilized the HPA database. Our analysis revealed intriguing findings regarding the protein expression profiles of these hub genes in COAD. Specifically, we observed significant up-regulation (Staining: High) of CXCL10, CXCL16, and CCL25 proteins in COAD tissues compared to normal controls (Supplementary Figure 3A). However, in contrast, we noticed a notable down-regulation (Staining: Low) of CXCL12 protein in COAD samples relative to controls (Supplementary Figure 3A). These results provide strong evidence supporting the dysregulation of these proteins in COAD and further underscore their potential role in the development and progression of COAD.

To develop a prognostic model based on the expression of CXCL10, CXCL12, CXCL16, and CCL25 genes, we utilized the CRC_TCGA dataset as the training dataset, while the GSE72970, GSE71187, GSE39582, GSE39084, GSE29621, GSE17537, GSE17536, GSE106584, and GSE103479 as the validation datasets. Our approach involved the implementation of a stepwise Cox regression model, which considered hazard ratio, c-index, and risk score as key parameters. By assessing the performance of our predictive prognostic model using the c-index, we demonstrated its effectiveness and reliability in accurately predicting the prognosis of patients with COAD (Supplementary Figure 3B).

Genetic alteration, mutual exclusivity, and immune infiltrate analyses of CXCL10, CXCL12, CXCL16, and CCL25

We utilized the cBioPortal database to investigate the genetic alteration profile of CXCL10, CXCL12, CXCL16, and CCL25 in COAD patients. Our analysis revealed that genetic alterations in these hub genes were infrequent among the COAD samples examined. Specifically, CXCL10 and CXCL16 exhibited genetic alterations in only 0.9% of the COAD samples (Supplementary Figure 4A), while CXCL12 and CCL25 showed genetic alterations in a mere 0.5% of the COAD samples (Supplementary Figure 4A). These findings indicate that mutations in CXCL10, CXCL12, CXCL16, and CCL25 are rare occurrences in COAD and suggest that other mechanisms may contribute to the dysregulation of these genes in the disease.

Our analysis of mutual exclusivity revealed that CXCL10, CXCL12, CXCL16, and CCL25 frequently exhibit dysregulation in conjunction with one another in the context of COAD (Supplementary Figure 4B). Specifically, CXCL10 demonstrates mutual co-expression with CXCL12, CXCL16, and CCL25 (Supplementary

Figure 4B). Likewise, CXCL12 exhibits mutual co-expression with CXCL10, CXCL16, and CCL25. CXCL16 displays mutual co-expression patterns with CXCL10, CXCL12, and CCL25 (Supplementary Figure 4B). Lastly, CCL25 also exhibits mutual co-expression with CXCL10, CXCL12, and CXCL16 (Supplementary Figure 4B).

Following that, we employed the "TIMER" tool to investigate the connections between the infiltration of immune cells (CD4+ T cells, CD8+ T cells, and macrophages) and the expression of the hub genes (CXCL10, CXCL12, CXCL16, and CCL25). Our analysis revealed a noteworthy positive correlation between the expression levels of CXCL10, CXCL12, CXCL16, and CCL25 hub genes and the abundance of CD4+ T cells, CD8+ T cells, and macrophages immune cells (Supplementary Figure 4C). These findings suggest that the dysregulation of these hub genes in COAD may play a role in modulating the infiltration of immune cells, specifically CD4+ T cells, CD8+ T cells, and macrophages, within the tumor microenvironment.

Functional enrichment analysis

GO and KEGG enrichment analyses of hub genes (CXCL10, CXCL12, CXCL16, and CCL25) were done with the help of the DAVID tool. In this study, "Nucleocytoplasmic transport complex, CBM complex, and External side of plasma membrane" were the major CC of the hub genes (Supplementary Figure 5A). "CCR10 chemokine receptor binding, CXCR3 chemokine receptor binding, CXCR chemokine receptor binding, and Guanylate kinase activity etc.", BP were mainly associated with hub genes (Supplementary Figure 5B), while "Neg. reg. of leukocyte tethering and rolling, Neg. reg. of leukocyte adhesion to vascular endothelial cell, and Neg. reg. of extracellular extravasation etc.", were the primary MFs of the hub genes (Supplementary Figure 5C). Moreover, KEGG pathways for the identified hub genes are highlighted in Supplementary Figure 5D, and "Intestinal immune network for IgA production, viral protein interaction with cytokine and cytokine receptor, Chemokine signaling pathways etc.", were found to be involved in the pathogenesis of COAD.

miRNA-mRNA interaction network

Using ENCORI and Cytoscape, we generated co-regulatory networks for CXCL10, CXCL12, CXCL16, and CCL25 involving miRNAs and mRNAs. Within these networks, a total of 85 miRNAs and 4 mRNAs were identified (Supplementary Figure 6). Interestingly, among these networks, we identified a specific miRNA (hsa-mir-744-5p) that simultaneously targets all the hub genes. This intriguing discovery leads us to speculate that the hsa-mir-744-5p and the hub genes (CXCL10, CXCL12, CXCL16, and CCL25) may collectively contribute as potential regulators involved in the development of COAD.

Drug prediction analysis of CXCL10, CXCL12, CXCL16, and CCL25

In the context of COAD treatment, the primary approach usually involves medical intervention, making the identification of appropriate candidate drugs crucial. In our research, we employed the DrugBank database to explore potential drugs with the capability to reverse the gene expression patterns of the identified hub genes for COAD treatment. **Table 1** displays a variety of drugs that show potential in modulating the expression of CXCL10, CXCL12, CXCL16, and CCL25 during COAD treatment. However, the effectiveness and suitability of these drugs require further experimental testing to validate their therapeutic potential for COAD patients.

Discussion

The intricate etiology and genetic heterogeneity of COAD contribute to the limited understanding of its molecular basis [53]. Despite extensive research efforts aimed at elucidating its pathogenesis and identifying prognostic biomarkers, the prognosis for advanced COAD remains discouraging. Hence, the primary objective of this study was to explore pivotal hub genes linked to the initiation, advancement, and prognosis of COAD. By focusing on these key genes, we aim to enhance our comprehension of COAD and potentially pave the way for improved detection and therapeutic strategies.

In our current investigation, we adopted a comprehensive approach to explore the diagnostic and prognostic implications of the complete CC and CXC chemokine families in COAD. Within this context, our analysis pinpointed CXCL10, CXCL12, CXCL16, and CCL25 as the central genes in the CC and CXC chemokine families. These genes were identified as hubs due to

Table 1. DrugBank-based hub genes-associated drugs

Sr. No	Hub gene	Drug name	Effect	Reference	Group
1	CCL25	Methotrexate	Decrease expression of CCL25 mRNA	A23201	Approved
		Acetylcysteine		A20451	
2	CXCL10	Acetaminophen	Decrease expression of CXCL10 mRNA	A20426	Approved
		Acteoside		A20456	
		Cyclosporine		A20661	
		Polydatin		A20456	
3	CXCL12	Belinostat	Increase expression of CXCL12 mRNA	A21037	Approved
		Decitabine		A21958	
		Mestranol		A21106	
4	CXCL16	Cyclosporine	Decrease expression of CXCL16 mRNA	A20661	Approved
		Estradiol		A21424	

CXCL10 = C-X-C motif chemokine ligand 10, CXCL12 = C-X-C motif chemokine ligand 12, CXCL16 = C-X-C motif chemokine ligand 16, CCL25 = CC motif chemokine ligand 25.

their crucial roles and potential significance in COAD. Uncovering these hub genes highlights their essential contributions to the initiation and progression of the disease, offering insights into their potential involvement in the underlying mechanisms of COAD. Furthermore, across COAD clinical samples and TCGA datasets, we observed significant up-regulation of CCL25, CXCL10, and CXCL16, while CXCL12 exhibited a notable decrease when compared to normal specimens. Additionally, our results demonstrated that these hub genes, identified through our study, can effectively serve as a reliable prognostic model for predicting the OS of COAD patients.

The chemokine CXCL10, which has a vital role in cancer biology, plays a critical role in modulating immune responses [54]. It is produced in response to stimulation by interferon-gamma and functions by binding to its receptor CXCR3, which is expressed in both immune cells and tumor cells [55]. Research findings have indicated that CXCL10 exhibits abnormal expression patterns in various cancer types such as breast, colorectal, lung, and pancreatic cancer [56, 57]. Increased levels of CXCL10 have been linked to tumor growth, angiogenesis, immune evasion, and the spread of cancer cells to distant sites [58]. By facilitating the recruitment of immune cells like T cells and natural killer cells to the tumor microenvironment, CXCL10 plays a role in modulating the immune responses against tumors [59]. Furthermore, CXCL10 has emerged as a promising candidate for cancer prognosis and predicting therapeutic response [60].

Playing a pivotal role in cancer progression and metastasis, CXCL12 assumes significant importance [61]. This chemokine is synthesized by stromal cells and exerts its effects by binding to its receptor CXCR4, which is expressed in cancer cells [61]. Investigations have revealed that CXCL12 contributes to cancer cell survival, migration, and invasion by activating signaling pathways associated with cellular proliferation and cytoskeletal rearrangement [62].

Furthermore, it promotes the migration of cancer cells expressing CXCR4 to particular organs, referred to as pre-metastatic niches, thereby facilitating the establishment of metastasis [63]. Moreover, CXCL12 has the capacity to impact the tumor microenvironment by regulating immune responses, fostering immunosuppression, and influencing the recruitment and activity of immune cells [63]. In colorectal cancer, the abnormal regulation of CXCL12 is implicated in tumor growth, angiogenesis, and unfavorable prognosis [64]. Likewise, in lung cancer, dysregulation of CXCL12 is associated with enhanced invasion, migration, and angiogenesis [65]. Additionally, in pancreatic cancer, CXCL12 dysregulation is observed, leading to increased tumor growth, invasion, and metastasis [66]. Furthermore, dysregulated expression of CXCL12 in prostate cancer is linked to an aggressive phenotype [67].

CXCL16, an integral membrane chemokine, exerts a diverse range of functions in human cancer [68]. It is expressed by multiple cell types, including tumor cells, endothelial cells, and immune cells [68]. CXCL16 operates

through its receptor, CXCR6, and participates in tumor growth, invasion, angiogenesis, and modulation of immune responses [69]. Numerous investigations have demonstrated the upregulation of CXCL16 expression in various cancer types, including breast, colorectal, lung, pancreatic, and prostate cancer. Heightened levels of CXCL16 have been linked to aggressive tumor characteristics, such as enhanced invasiveness and increased likelihood of metastasis [70, 71]. Furthermore, CXCL16 participates in tumor angiogenesis, facilitating the development of new blood vessels to sustain tumor growth [72]. Moreover, CXCL16 has the ability to regulate immune responses by attracting immune cells to the tumor microenvironment, potentially impacting antitumor immunity [72].

The role of CCL25 in the progression and spread of cancer is of utmost importance [73]. This chemokine is predominantly synthesized in the small intestine and operates by binding to its corresponding receptor, CCR9, thereby controlling the movement and recruitment of immune cells to the intestinal mucosa [74]. Nevertheless, abnormalities in the CCL25/CCR9 pathway have been documented in different types of cancers, such as colorectal, breast, and pancreatic cancer [75, 76]. Research studies have shown that up-regulation of CCL25 facilitates the proliferation, invasion, and angiogenesis of tumor cells, whereas inhibiting or blocking this chemokine results in decreased tumor growth and metastasis [77]. For instance, in the case of breast cancer, the involvement of the CCL25/CCR9 interaction has been demonstrated in activating the Akt pathway, which contributes to the development of cisplatin resistance in cancer cells [78]. In pancreatic cancer, the CCL25/CCR9 axis has been linked to the promotion of cell proliferation, invasion, and metastasis [79]. Moreover, the expression of CCR9 has been identified as a prognostic marker for stage III colon cancer patients receiving adjuvant chemotherapy, indicating its potential as a target for therapeutic interventions [75].

In relation to the mutational and methylation profiles of the CXCL10, CXCL12, CXCL16, and CCL25 genes, it was noticed that these genes rarely experience genetic alterations in COAD. Nevertheless, abnormal promoter methylation was linked to increased expression of CCL25, CXCL10, and CXCL16, and decreased expression of CXCL12. Previous studies have also indicated that CXCL10, CXCL12, CXCL16, and CCL25 genes tend to exhibit stability and do not undergo substantial genetic mutations [80, 81]. In accordance with earlier investigations, our findings support the idea that CXCL10, CXCL12, CXCL16, and CCL25 display a relatively low occurrence of genetic alterations in COAD patients. These results reinforce the existing body of evidence indicating a diminished prevalence of mutations in these particular genes among cancer patients.

In our study, a significant finding emerged regarding the regulatory impact of hsa-mir-744-5p miRNA on the expression of CXCL10, CXCL12, CXCL16, and CCL25 hub genes in COAD patients. We observed that these genes were collectively regulated by hsa-mir-744-5p, and their expression levels displayed a significant correlation with the infiltration of immune cells, including CD4+ T cells, CD8+ T cells, and macrophages.

The dysregulation of hsa-mir-744-5p has emerged as a significant molecular factor in the progression of cancer [82]. Extensive research has demonstrated the involvement of hsa-mir-744-5p in various cancer types, such as breast, lung, colorectal, and ovarian cancer [73-76, 83-86]. The dysregulated expression of hsa-mir-744-5p promotes tumor growth by targeting critical genes involved in regulating the cell cycle, apoptosis, and metastasis. Gaining a comprehensive understanding of the intricate mechanisms underlying hsa-mir-744-5p dysregulation and its downstream effects in cancer provides promising avenues for the development of targeted therapies and diagnostic biomarkers. This study provides initial evidence highlighting the potential cancer-promoting role of hsa-mir-744-5p miRNA in relation to the hub genes CXCL10, CXCL12, CXCL16, and CCL25 in COAD. It is the first of its kind to shed light on the probable involvement of hsa-mir-744-5p in driving cancer-related processes specifically associated with these hub genes. By uncovering this novel association, our findings contribute to the growing understanding of the complex molecular mechanisms underlying the development of COAD. Further investigations are warranted to fully elucidate the functional

significance of hsa-mir-744-5p and its potential implications for targeted therapies in COAD.

Conclusion

Based on our extensive study, we have put forth a model consisting of four hub genes (CXCL10, CXCL12, CXCL16, and CCL25) from the CC and CXC gene families, which have demonstrated significant involvement in the onset and advancement of COAD. These hub genes exhibit promising potential as dependable biomarkers for diagnosing, prognosing, and treating COAD patients. However, it is crucial to conduct further comprehensive investigations to unravel the precise pathogenic roles of these genes in COAD. By deepening our understanding of their mechanisms and functions, we can pave the way for more targeted and efficacious approaches to manage COAD and enhance patient outcomes.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Statistics of CXCL10 transcriptomic expression analysis in normal individual and COAD patients of various clinicopathological features via UALCAN

Transcriptomic expression analysis statistics of CXCL10 in normal individuals and COAD patients						
Parameters	Quartile and *P* values					
	Minimum value	1st quartile	2nd quartile	3rd quartile	Maximum value	*P*-value
Normal	0.672	2.442	4.427	7.67	17.509	6.20e-04
Primary tumor	0.466	5.675	16.601	33.308	101.927	
Transcriptomic expression analysis statistics of CXCL10 based on COAD tumor's stage						
Normal vs Stage 1	1.948	7.565	18.157	29.585	94.2	8.99e-04
Normal vs Stage 2	0.446	7.242	22.042	46.918	114.625	1.80e-03
Normal vs Stage 3	0.52	3.618	13.015	22.503	81.985	8.28e-02
Normal vs Stage 4	0.854	3.24	7.109	17.997	42.996	4.21e-02
Transcriptomic expression analysis statistics of CXCL10 based on COAD patients' race						
Normal vs Caucasian	0.52	7.925	19.505	48.244	128.374	1.67e-03
Normal vs African-American	0.446	2.205	6.878	14.762	39.366	3.10e-03
Normal vs Asian	1.843	5.993	9.376	26.951	75.68	1.41e-02
Transcriptomic expression analysis statistics of CXCL10 based on COAD patients' gender						
Normal vs Male	0.52	5.175	16.515	40.071	120.467	6.50e-03
Normal vs Female	0.446	6.941	17.164	30.116	81.995	6.36e-04
Transcriptomic expression analysis statistics of CXCL10 based on COAD patients' age group						
Normal vs 21-40 yrs	2.156	11.201	15.824	47.019	90.462	8.87e-03
Normal vs 41-61 yrs	0.446	5.391	11.531	20.993	54.43	9.76e-04
Normal vs 61-80 yrs	0.52	4.933	17.681	40.324	120.467	7.54e-03
Normal vs 81-100 yrs	1.117	10.132	23.893	54.03	118.99	5.86e-01

First quartile: the lowest 25% of numbers. Second quartile: between 25.1% and 50% (up to the median). Third quartile: 51% to 75%. COAD = Colon adenocarcinoma.

Supplementary Table 2. Statistics of CXCL12 transcriptomic expression analysis in normal individual and COAD patients of various clinicopathological features via UALCAN

Transcriptomic expression analysis statistics of CXCL12 in normal individuals and COAD patients						
Parameters	Quartile and *P* values					
	Minimum value	1st quartile	2nd quartile	3rd quartile	Maximum value	*P*-value
Normal	34.679	64.923	75.278	90.27	166.136	2.12e-10
Primary tumor	0.337	4.827	8.667	13.913	43.112	
Transcriptomic expression analysis statistics of CXCL12 based on COAD tumor's stage						
Normal vs Stage 1	0.832	4.742	9.093	12.153	29.294	1.55e-10
Normal vs Stage 2	0.861	4.14	7.505	12.49	33.784	1.43e-10
Normal vs Stage 3	0.832	5.678	9.885	18.431	46.951	3.98e-10
Normal vs Stage 4	0.337	4.879	7.26	11.495	32.518	5.81e-11
Transcriptomic expression analysis statistics of CXCL12 based on COAD patients' race						
Normal vs Caucasian	0.832	5.267	10.318	17.54	50.102	2.44e-10
Normal vs African-American	0.377	4.144	6.286	9.415	35.811	1.70e-10
Normal vs Asian	0.918	3.434	6.255	9.19	15.447	1.04e-11
Transcriptomic expression analysis statistics of CXCL12 based on COAD patients' gender						
Normal vs Male	0.337	4.993	8.543	15.611	44.84	1.64e-10
Normal vs Female	1.155	4.681	9.051	12.976	36.825	1.91e-10
Transcriptomic expression analysis statistics of CXCL12 based on COAD patients' age group						
Normal vs 21-40 yrs	0.337	6.566	11.961	42.919	72.981	1.91e-10
Normal vs 41-61 yrs	0.918	5.401	8.667	13.385	36.746	4.59e-10
Normal vs 61-80 yrs	0.832	4.324	8.442	15.907	43.885	1.04e-10
Normal vs 81-100 yrs	1.369	4.805	9.722	11.957	17.294	8.14e-11

First quartile: the lowest 25% of numbers. Second quartile: between 25.1% and 50% (up to the median). Third quartile: 51% to 75%. COAD = Colon adenocarcinoma.

Supplementary Table 3. Statistics of CXCL16 transcriptomic expression analysis in normal individual and COAD patients of various clinicopathological features via UALCAN

Transcriptomic expression analysis statistics of CXCL12 in normal individuals and COAD patients						
Parameters	Quartile and *P* values					
	Minimum value	1st quartile	2nd quartile	3rd quartile	Maximum value	*P*-value
Normal	28.254	36.971	41.714	46.197	61.003	<1e-12
Primary tumor	8.504	57.812	85.324	112.919	200.297	
Transcriptomic expression analysis statistics of CXCL16 based on COAD tumor's stage						
Normal vs Stage 1	21.577	69.454	94.53	130.061	222.359	8.45e-11
Normal vs Stage 2	8.504	56.056	87.944	112.86	194.37	<1e-12
Normal vs Stage 3	22.043	61.725	82.871	112.29	179.959	1.62e-12
Normal vs Stage 4	34.785	56.829	78.434	104.588	151.023	1.35e-08
Transcriptomic expression analysis statistics of CXCL16 based on COAD patients' race						
Normal vs Caucasian	8.504	59.648	86.83	115.887	206.92	1.62e-12
Normal vs African-American	9.57	56.133	83.241	111.506	165.976	1.66e-11
Normal vs Asian	26.056	56.634	104.459	125.462	155.154	4.15e-03
Transcriptomic expression analysis statistics of CXCL16 based on COAD patients' gender						
Normal vs Male	8.504	57.475	78.887	107.336	179.959	1.62e-12
Normal vs Female	9.57	60.181	93.736	126.167	233.131	<1e-12
Transcriptomic expression analysis statistics of CXCL16 based on COAD patients' age group						
Normal vs 21-40 yrs	39.926	66.022	111.375	138.386	216.505	1.48e-03
Normal vs 41-61 yrs	9.57	58.818	87.651	116.148	179.555	1.62e-12
Normal vs 61-80 yrs	8.504	58.068	79.101	104.82	179.959	<1e-12
Normal vs 81-100 yrs	36.934	63.6	92.955	135.723	233.131	4.02e-08

First quartile: the lowest 25% of numbers. Second quartile: between 25.1% and 50% (up to the median). Third quartile: 51% to 75%. COAD = Colon adenocarcinoma.

Supplementary Table 4. Statistics of CCL25 transcriptomic expression analysis in normal individual and COAD patients of various clinicopathological features via UALCAN

Transcriptomic expression analysis statistics of CCL25 in normal individuals and COAD patients						
Parameters	Quartile and *P* values					
	Minimum value	1st quartile	2nd quartile	3rd quartile	Maximum value	*P*-value
Normal	0	0	0.066	0.128	0.332	2.31e-04
Primary tumor	0	0	0.062	0.181	0.951	
Transcriptomic expression analysis statistics of CCL25 based on COAD tumor's stage						
Normal vs Stage 1	0	0	0.072	0.145	0.555	8.45e-04
Normal vs Stage 2	0	0	0.07	0.154	0.859	2.11e-05
Normal vs Stage 3	0	0.034	0.103	0.212	0.525	1.62e-8
Normal vs Stage 4	0	0	0.077	0.184	0.673	1.35e-04
Transcriptomic expression analysis statistics of CCL25 based on COAD patients' race						
Normal vs Caucasian	0	0	0.73	0.179	0.833	1.62e-04
Normal vs African-American	0	0	0.075	0.152	0.927	1.66e-103
Normal vs Asian	0	0	0.03	0.204	3.84	4.15e-03
Transcriptomic expression analysis statistics of CCL25 based on COAD patients' gender						
Normal vs Male	0	0	0.70	0.199	1.251	1.62e-04
Normal vs Female	0	0	0.77	0.154	0.722	2.11e-03
Transcriptomic expression analysis statistics of CCL25 based on COAD patients' age group						
Normal vs 21-40 yrs	0	0	0.197	0.532	1.354	1.48e-03
Normal vs 41-61 yrs	0	0	0.070	0.159	0.859	1.62e-04
Normal vs 61-80 yrs	0	0	0.070	0.213	1.099	1.11e-04
Normal vs 81-100 yrs	0	0	0.077	0.111	0.338	4.02e-08

First quartile: the lowest 25% of numbers. Second quartile: between 25.1% and 50% (up to the median). Third quartile: 51% to 75%. COAD = Colon adenocarcinoma.

Supplementary Table 5. Statistics of CXCL10, CXCL12, CXCL16, and CCL25 transcriptomic expression analysis in normal individual and COAD patients via GEPIA

Transcriptomic expression analysis statistics of CXCL10 in normal individuals and COAD patients						
Parameters	Quartile and *P* values					
	Minimum value	1st quartile	2nd quartile	3rd quartile	Maximum value	*P*-value
Normal	0.7	1.93	3.8	2.5	8.0	0.00061
Primary tumor	0	2.6	3.8	4.5	8.97	
Transcriptomic expression analysis statistics of CXCL12 in normal individuals and COAD patients						
Normal	0	2.2	3.0	4.0	7.1	0.000043
Primary tumor	4.8	5.6	5.8	6.4	8.1	
Transcriptomic expression analysis statistics of CXCL16 in normal individuals and COAD patients						
Normal	4.5	4.8	5.2	5.4	6.1	0.00062
Primary tumor	2.7	5.5	6.1	6.5	8.0	
Transcriptomic expression analysis statistics of CCL25 in normal individuals and COAD patients						
Normal	0.01	0,08	0.1	0.12	7.95	0.0021
Primary tumor	0.4	0.44	0.9	1.2	8.33	

First quartile: the lowest 25% of numbers. Second quartile: between 25.1% and 50% (up to the median). Third quartile: 51% to 75%. COAD = Colon adenocarcinoma.

Supplementary Table 6. Statistics of CXCL10, CXCL12, CXCL16, and CCL25 transcriptomic expression analysis in normal individual and COAD patients via OncoDB

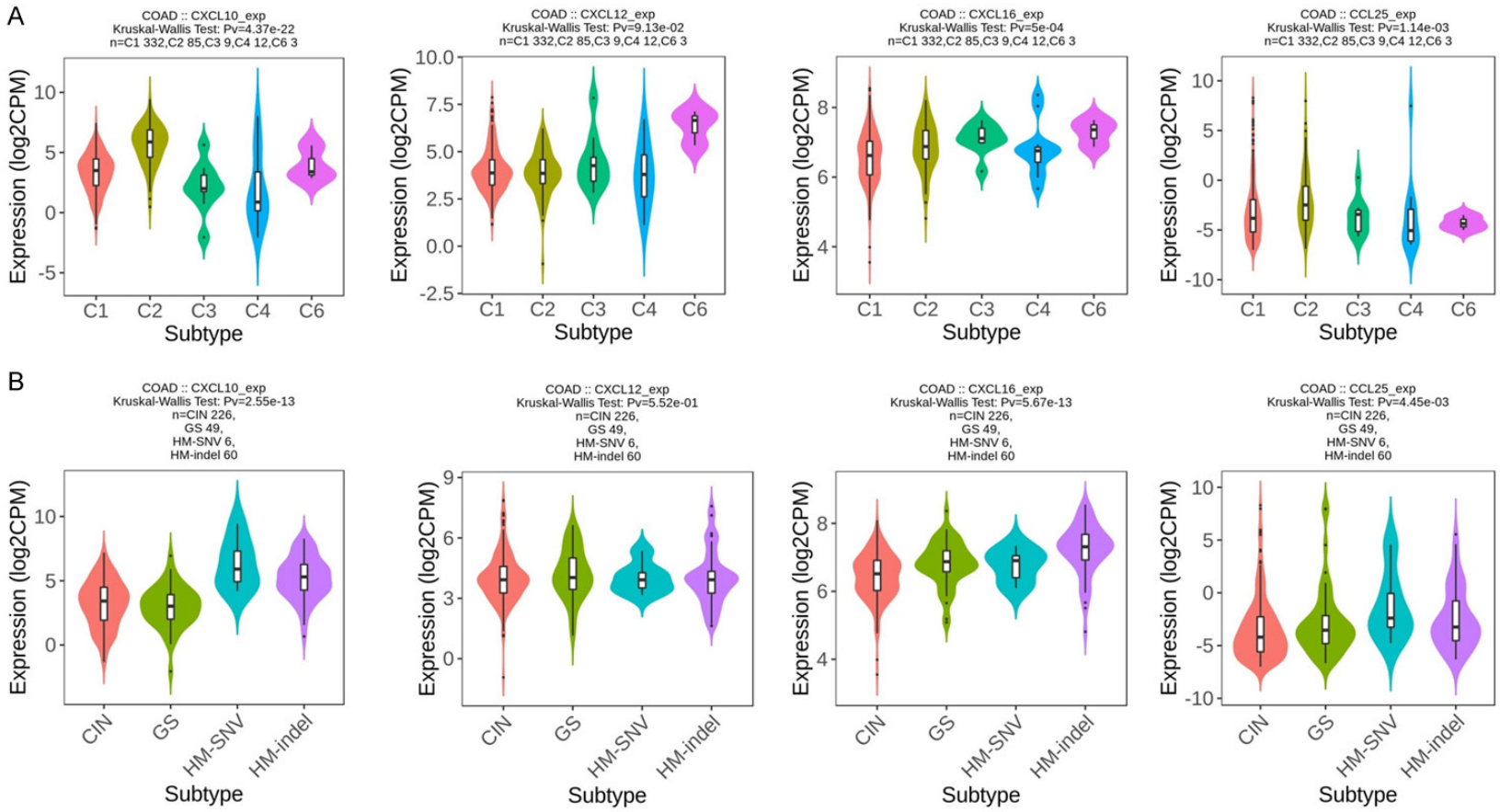
Transcriptomic expression analysis statistics of CXCL10 in normal individuals and COAD patients						
Parameters	Quartile and *P* values					
	Minimum value	1st quartile	2nd quartile	3rd quartile	Maximum value	*P*-value
Normal	0	20	45	82	1960	2.1e-3
Primary tumor	0	15	20	25	889	
Transcriptomic expression analysis statistics of CXCL12 in normal individuals and COAD patients						
Normal	45	99	110	179	350	3.7e-10
Primary tumor	0	10	20	41	348	
Transcriptomic expression analysis statistics of CXCL16 in normal individuals and COAD patients						
Normal	20	40	50	60	98	1.3e-29
Primary tumor	5	90	110	160	455	
Transcriptomic expression analysis statistics of CCL25 in normal individuals and COAD patients						
Normal	0	0	0	0	410	4.8e-3
Primary tumor	55	57	58	61	579	

First quartile: the lowest 25% of numbers. Second quartile: between 25.1% and 50% (up to the median). Third quartile: 51% to 75%. COAD = Colon adenocarcinoma.

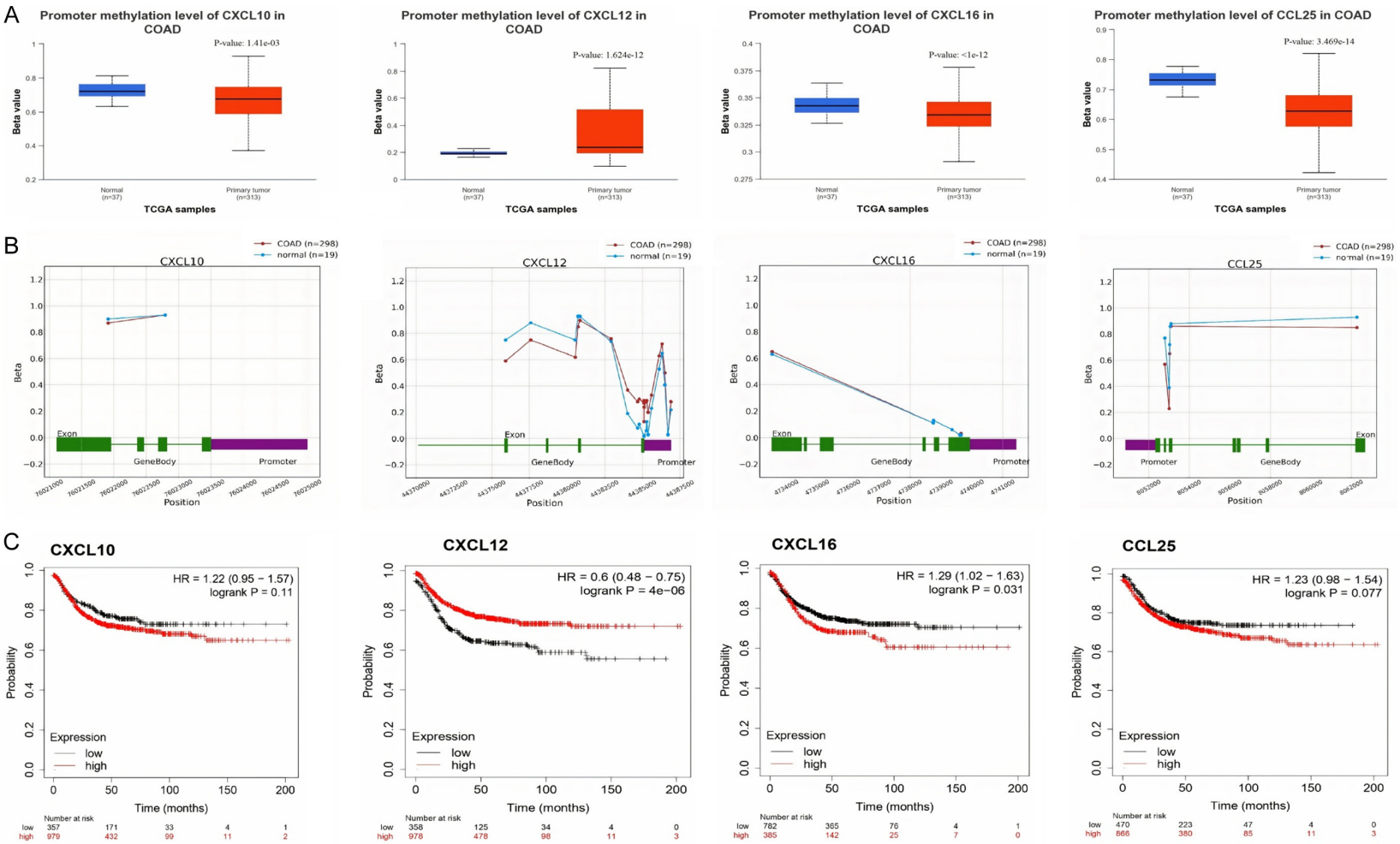
Supplementary Table 7. Statistics of CXCL10, CXCL12, CXCL16, and CCL25 transcriptomic expression analysis in normal individual and COAD patients in GSE17538

Transcriptomic expression analysis statistics of CXCL10 in normal individuals and COAD patients						
Parameters	Quartile and *P* values					
	Minimum value	1st quartile	2nd quartile	3rd quartile	Maximum value	*P*-value
Normal	0.4	0.5	2.39	3.89	4.19	0.0089
Primary tumor	2.6	2.69	4.13	4.4	5.05	
Transcriptomic expression analysis statistics of CXCL12 in normal individuals and COAD patients						
Normal	4.29	4.65	5.9	6.4	6.59	0.00032
Primary tumor	3.91	4.18	4.41	4.68	6.55	
Transcriptomic expression analysis statistics of CXCL16 in normal individuals and COAD patients						
Normal	2.71	4.31	4.62	5.99	6.21	0.0000012
Primary tumor	6.08	6.49	6.62	6.81	8.21	
Transcriptomic expression analysis statistics of CCL25 in normal individuals and COAD patients						
Normal	4.09	4.2	4.97	4.78	5.03	0.0029
Primary tumor	6.61	7.97	8.18	8.48	10.07	

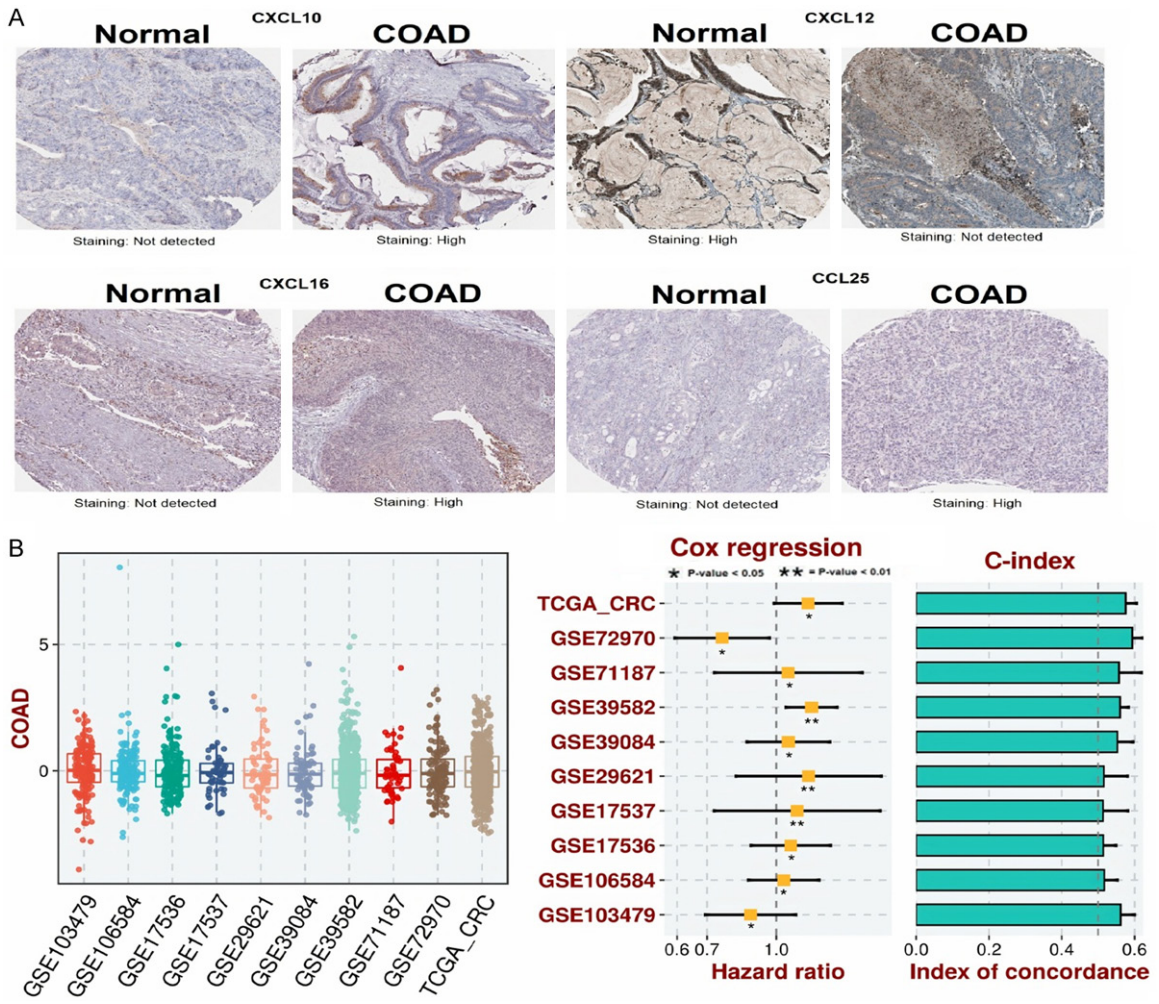
First quartile: the lowest 25% of numbers. Second quartile: between 25.1% and 50% (up to the median). Third quartile: 51% to 75%. COAD = Colon adenocarcinoma.



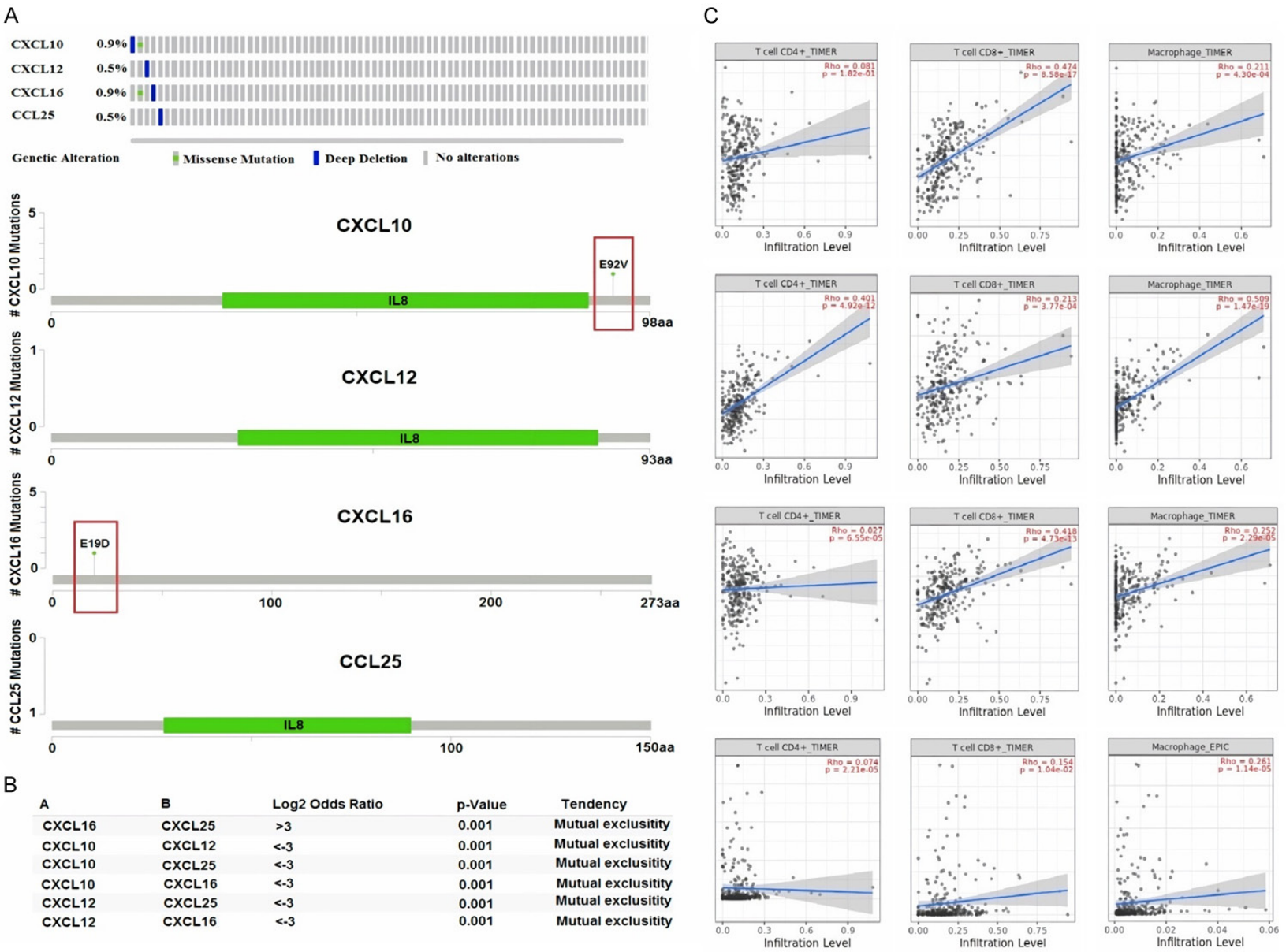
Supplementary Figure 1. Correlation analysis of CXCL10, CXCL12, CXCL16, and CCL25 with different immune subtypes as well as immune molecular subtypes of COAD. (A) Correlation with different immune subtypes, and (B) Correlation with different molecular subtypes. COAD = Colon Adenocarcinoma.



Supplementary Figure 2. Promoter methylation and survival analyses of CXCL10, CXCL12, CXCL16, and CCL25. (A) Promoter methylation analysis via UALCAN, (B) Promoter methylation analysis via OncoDB, and (C) Survival analysis via KM plotter. KM = Kaplan Meier.



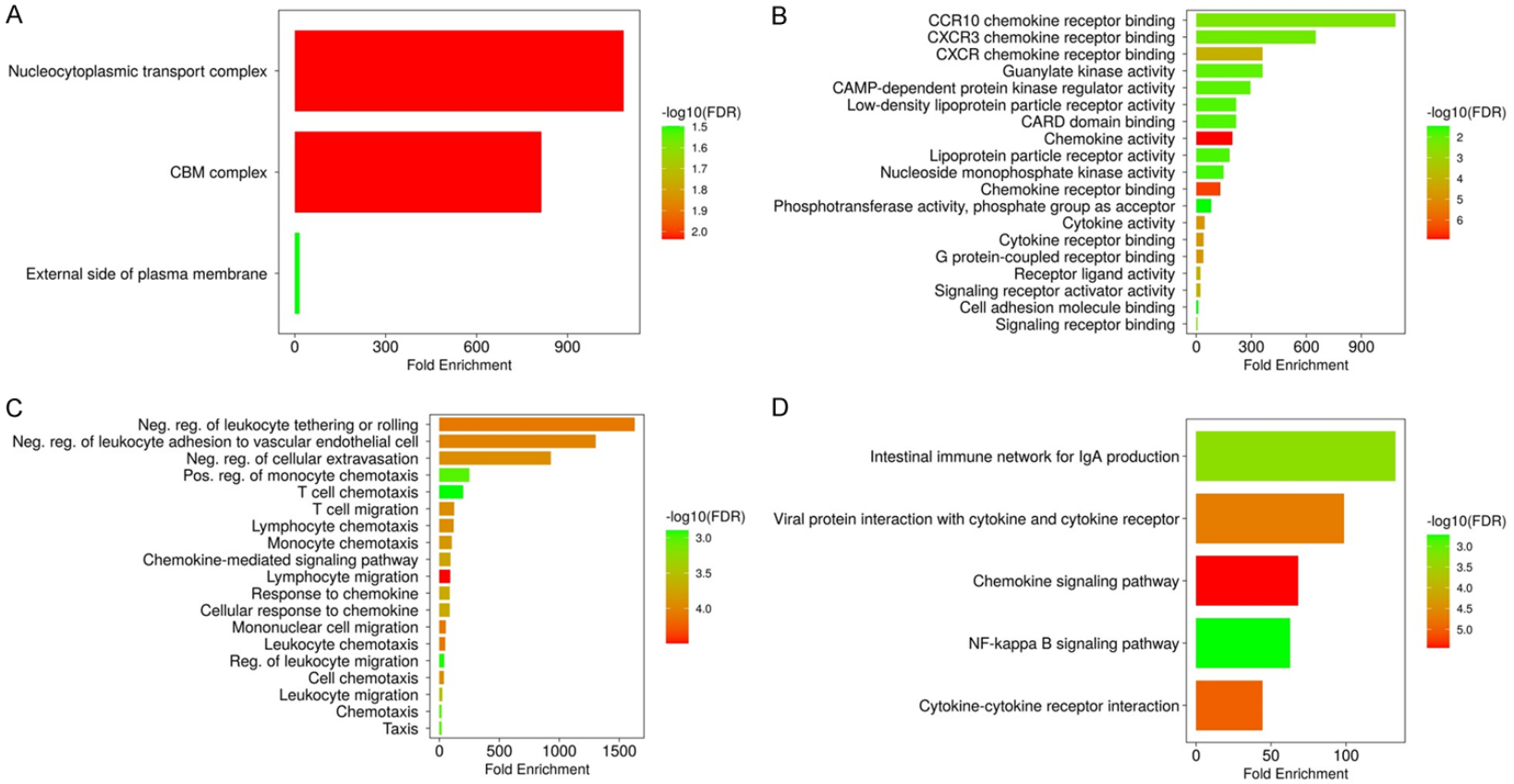
Supplementary Figure 3. Protein expression analysis and development of prognostic model based on the CXCL10, CXCL12, CXCL16, and CCL25 genes in COAD. (A) Protein expression analysis of CXCL10, CXCL12, CXCL16, and CCL25 via HPA database, and (B) Development of prognostic model based on the CXCL10, CXCL12, CXCL16, and CCL25 genes. COAD = Colon Adenocarcinoma.



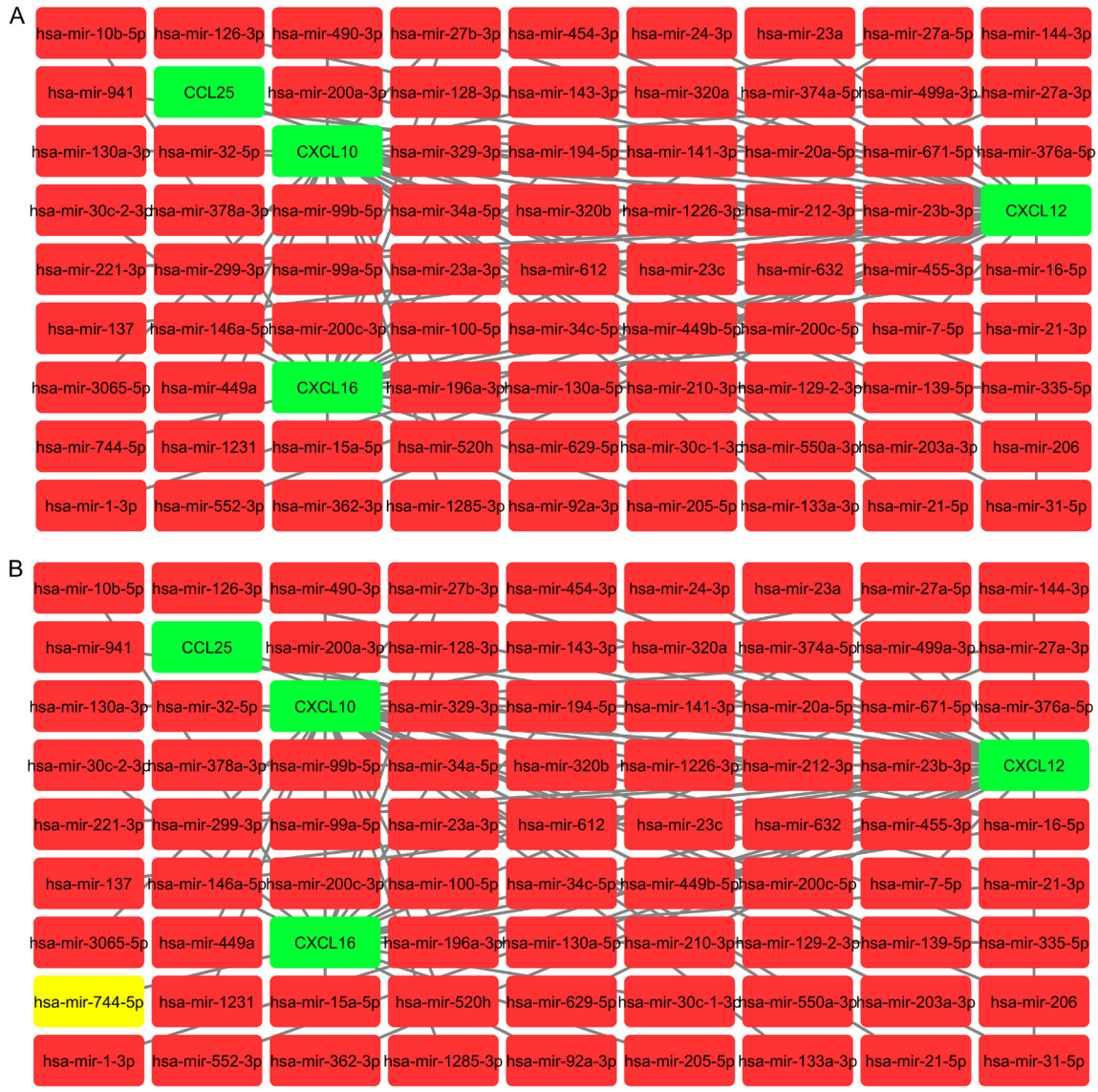
B

A	B	Log2 Odds Ratio	p-Value	Tendency
CXCL16	CXCL25	>3	0.001	Mutual exclusitity
CXCL10	CXCL12	<-3	0.001	Mutual exclusitity
CXCL10	CXCL25	<-3	0.001	Mutual exclusitity
CXCL10	CXCL16	<-3	0.001	Mutual exclusitity
CXCL12	CXCL25	<-3	0.001	Mutual exclusitity
CXCL12	CXCL16	<-3	0.001	Mutual exclusitity

Supplementary Figure 4. Genetic alteration and immune cell infiltration analyses of CXCL10, CXCL12, CXCL16, and CCL25 gene. (A) Genetic alteration analysis results via cBioPortal, (B) Mutual exclusivity of hub gene expression levels, and (C) Immune cell infiltration analysis results via TIMER2 database.



Supplementary Figure 5. Gene enrichment analysis of CXCL10, CXCL12, CXCL16, and CCL25. (A) CXCL10, CXCL12, CXCL16, and CCL25 associated CC terms, (B) CXCL10, CXCL12, CXCL16, and CCL25 associated MF terms, (C) CXCL10, CXCL12, CXCL16, and CCL25 associated BP terms, and (D) CXCL10, CXCL12, CXCL16, and CCL25 associated KEGG terms. CC = Cellular component, MF = Molecular function, BP = Biological Process, KEGG = Kyoto Encyclopedia of Genes and Genomes.



Supplementary Figure 6. miRNA-mRNA co-regulatory network of CXCL10, CXCL12, CXCL16, and CCL25 hub genes. (A) A PPI of miRNAs targeting hub genes, and (B) A PPI highlighting most important miRNA (hsa-mir-744-5p) targeting all hub genes. PPI = Protein protein interaction.