The Role of CXCL10 in Prognosis of Patients with Colon Cancer and Tumor Microenvironment Remodeling

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Abstract

Background: In the past few years, tumor microenvironment (TME) has gradually become a hot topic in tumor research, which has important significance in the diagnosis, prevention and prognosis of tumors. Importantly, the immune system is a major contributing factor in TME, and studies have shown that tumors are partially infiltrated with various immune cell subsets. The immune characteristics of the TME play an essential role in evaluating the prognosis of patients. The immune scoring system based on the distribution of tumor local immune cell subsets and cell density has been an essential indicator in the evaluation of patient prognosis and has been verified in various tumor studies. TME is indispensable in the occurrence and development of Colorectal cancer (CRC). However, understanding the dynamic regulation of immunity and matrix components in TME of CRC is still a challenge and should be investigated further.

Methods: In this study, we collected transcriptome RNA-seq data of 521 Colon adenocarcinoma (COAD) patients from The Cancer Genome Atlas (TCGA) data portal. We then estimate the fraction of stromal and immune cells in COAD cases by ESTIMATE algorithms [1]. A total of 1109 stromal-immune score-related differentially expressed genes (DEGs) were identified and used to generate a high-confidence protein–protein intersection (PPI) network and univariate COX regression analysis. Intersection analysis of the data from PPI network and univariate COX regression analysis showed the core gene. Then we performed Gene set enrichment analysis (GSEA), survival analysis and clinical analysis for CXCL10, and applied CIBERSORT algorithms to estimate the tumor-infiltrating immune cells (TICs) proportion in COAD cases.

Results: The proportion of immune and stromal components in TME are associated with the progression of COAD. For example, tumor metastasis is inversely proportional to immune score. A total of 1109 DEGs were obtained by analyzing the low-score shared genes and the high-score shared genes by intersection analysis which might be the determinant of TME status. The GO enrichment analysis indicated that DEGs are associated with immune-related terms. KEGG pathway enrichment analysis showed that these DEGs are mainly enriched in cytokine cytokine receptor signaling pathway etc. Therefore, DEGs are related to immune regulation, which indicates that the participation of immune factors is the main characteristic of TME in COAD. Moreover, the expression level of CXCL10 has significantly connection with the prognosis of patients and the progression of COAD.

Conclusion: Taken together, we conducted a comprehensive analysis of the TME in COAD, and predicted a prognostic indicator for COAD, which provided a novel insight for therapeutics of COAD.

Background

Colon cancer, also known as Colorectal cancer (CRC), is one of the common malignant gastrointestinal tumors with high morbidity and mortality worldwide[2], of which the most common form is Colon adenocarcinoma (COAD). In recent years, better achievements have been gained in the field of CRC

research. A molecular cluster named miR-371-373 which is mainly responsible for the regulation of cancer metastasis in CRC patients has been found by *Pit Ullmann et al.*[3], which could help researchers develop new therapies that effectively inhibit tumor growth. *Mara Roxana Rubinstein et al.*[4] reported that fusobacterium nucleatum, a Gram-negative oral anaerobe, accelerates CRC. This study might make it easier for researchers to identify and treat malignant CRC, besides it helps explain why some patients' disease progresses much faster than others, which may be due to the presence of this bacteria in the mouth. *Vipul C. Chitalia et al.* [5] discovered a new target protein, c-Cbl, which might improve survival of CRC patients. In CRC, the signaling pathway that drives tumor progression is a promising target for systemic therapy. In addition to the WNT and MAPK pathways, activation of NOTCH signaling pathway has also been found in most tumors. *Eva Marina Schmidt et al.*[6] found that high NOTCH activity marks a subpopulation of colon cancer cells with low levels of WNT and MAPK signals and a significant epithelial phenotype. Drugs targeting the MAPK signaling pathway have a limited inhibitory function on tumor growth, since it will cause the expand of NOTCH highly activated cancer cells, but if NOTCH signaling pathway is targeted, cancer cells with high MAPK activity will increase. They found that there is a high adaptability between tumor cell subpopulations, which is also a mechanism that causes tumor drug resistance. The combined therapy targeting NOTCH and MAPK signaling can produce a very significant tumor suppressive effect in vivo.

At present, the treatment of COAD is mainly by surgical treatment, supplemented by comprehensive treatment such as chemotherapy, radiotherapy or targeted therapy. With the development of surgical techniques, the survival rate of colon cancer has been improved, but the metastasis and recurrence of the tumor still caused a poor prognosis to patients. Thus, it is necessary to investigate the carcinogenesis and therapeutics of COAD.

In recent years, tumor microenvironment (TME) has gradually become an important research topic to overcome cancer. TME is the internal environment in which tumor cells are produced and live. Since tumors are neither just composed of malignant cells nor independent of the environment. They could recruit and corrupt other non-malignant cells, such as fibroblasts and immune cells associated with cancer, cytokines and chemokines etc, creating TME. Tumor growth depends not only on genetic changes in malignant tumor cells, but also on changes in the TME such as stroma, blood vessels, and infiltrating inflammatory cells. TME has long been a hot topic in tumor research, which has important significance to assist us to better understand the occurrence, development, and metastasis of tumors. Furthermore, it also plays an important role in the diagnosis, prevention and prognosis of tumors.

The immune system is a major contributing factor in TME, and studies have shown that tumors are partially infiltrated with various immune cell subsets. In tumor colonization or early stages of tumor growth, these immune cells and related matrix components recruited and activated by tumor cells form a tumor-inhibiting inflammatory microenvironment that blocks tumorigenesis and development.

However, after continuous tumor antigen stimulation and immune activation, the relevant effector cells in TME are in a state of exhaustion or remodeling, which can not perform normal functions or even promote

the malignant characterization of tumors, creating an immunosuppressive microenvironment. Since the tumor types are different, the infiltrating immune cells are also different, and even among the different patients with same pathological type tumors, infiltrating immune cell subsets are not the same. Therefore, how to establish an effective immune evaluation system, and take advantages of TME-targeted immunotherapy strategies to stimulate or restore the inherent tumor suppressive ability of the immune system and reshape the active immune microenvironment has become a burning question.

In this paper, we performed Estimate algorithms to calculate the fraction of stromal and immune cells in COAD samples from The Cancer Genome Atlas (TCGA) database, and applied CIBERSORT algorithms to estimate the tumor-infiltrating immune cells (TICs) proportion in COAD samples. Finally, the predictive marker C-X-C motif chemokine 10 (CXCL10) has been identified. CXCL10 belongs to CXC chemokine family which binds to CXCR3 receptor to mediate immune response on activated leukocytes[7]. It has an essential role in kinds of human diseases, such as tumor development, metastasis and dissemination infectious diseases, chronic inflammation, immune dysfunction etc. In addition, it has been identified as a major biological marker in various disease, such as chronic graft-versus-host disease (cGVHD)[8], Kawasaki disease (KD)[9], and heart failure and left ventricular dysfunction[10] etc. Here, we identified the emerging role of CXCL10 in TME and its potential role as a prognostic indicator for COAD and Biomarkers in Cancer Therapy.

Methods

Data Download

Transcriptome RNA-seq datasets and clinical data of 521 COAD cases (normal samples, 41 cases; tumor samples, 480 cases) were downloaded from TCGA database (<https://portal.gdc.cancer.gov/>).

Survival Analysis

The R package named survival and survminer were used to carry out survival analysis. Log-rank test were used to analyze the survival-related data and Kaplan–Meier plots was used to assess the survival curve.

Identification of DEGs

The R package named estimate used ESTIMATE algorithm to predict tumor purity, and the content of immune and stromal cells in tumor tissues by using gene expression data, represented by ImmuneScore and StromalScore, respectively. ESTIMATEScore represents the sum of both. 521 TCGA-COAD tumor samples were divided into high and low score group depending on the median value of ImmuneScore and StromalScore, respectively.

The R package named limma was applied to perform differentiation analysis of the gene expression, and DEGs were obtained by comparing the high-score group and the low-score group, with the threshold of false discovery rate (FDR) <0.05 and fold change (FC) >1.

Gene Ontology (GO) and KEGG Pathway Enrichment Analysis

GO and KEGG pathway enrichment analysis were used for DEGs using ggplot2, enrichplot, clusterProfiler R package with $p < 0.05$ and $q < 0.05$.

PPI network

The Search Tool for the Retrieval of Interacting Genes (STRING) online database was used to construct the PPI network of DEGs. Then we used Cytoscape software, a confidence score of interactive relationship > 0.95 was set as the cut-off criterion for building network.

COX Regression Analysis and Heatmaps

Survival R package was used for univariate Cox survival analysis, and pheatmap R package was used for constructing heatmaps of DEGs.

Gene set enrichment analysis (GSEA)

The gene set from Molecular Signatures Database “c5.all.v7.0 symbols.gmt (Gene Ontology)” were downloaded using the software gsea-3.0. A normalized enrichment score (NES) was calculated, and only an FDR-normalized q -value < 0.05 were considered as significant.

CIBERSORT and assessment of TICs

CIBERSORT, a computational method, was used to estimate the fractions of multiple cell types in the gene expression profiles of admixtures. The TIC abundance profile of tumor samples can be estimated based on standardized gene expression data. In this study, the proportion and composition of 22 kinds of TICs in tumor were analysed.

Results

Association Of Scores With Pathology and Prognosis of COAD patients

From TCGA databases, we collected transcriptome RNA-seq data of 521 COAD patients. The clinicopathological characteristics of these COAD patients are shown in (Table 1). We then estimated the fraction of stromal and immune cells in COAD samples by ESTIMATE algorithms [1]. A total of 1109 stromal-immune score-related differentially expressed genes (DEGs) were identified and used to generate a high-confidence protein–protein interaction (PPI) network and univariate COX regression analysis. Intersection analysis of the data from PPI network and univariate COX regression analysis showed CXCL10 is the core gene. Then we performed GSEA, survival analysis and clinical analysis for CXCL10 (Figure 1A).

Based on Immune, Stromal, and ESTIMATE Score, Kaplan–Meier survival analysis was used to determine the potential association between prognoses and elevated scores. ImmuneScore showed a potential

negative correlation with the overall survival rate, although there is no statistically significant difference ($P = 0.059$) (Figure 2.E). StromalScore ($P = 0.133$) and ESTIMATE scores ($P = 0.476$) were not correlated with the overall survival rate (Figure 1B-D).

The relationship of Stromal and Immune scores with COAD patient pathologic characteristics was analyzed by calculating the score distributions among different tumor stages, and histology classifications (TNM stages). As shown in Figure 2A, significant negative correlation was observed between immune scores and tumor stage, the immune scores decreased in the late tumor stage. Also, the immune score has a different distribution in M and N classifications of TMN stages (Figure 2 C,D). The above results indicated that the distribution of immune components was associated with the progress of COAD.

DEGs of Immune Scores and Stromal Scores

To identify the relationship of gene expression profiles with immune scores and stromal scores, we analyzed gene microarray data of the 521 COAD cases obtained from TCGA. By comparing the high score group and low score group, we obtained 1322 DEGs from immune score and 1692 DEGs from stromal score. Heatmap showed distinct gene expression profiles of top 50 DEGs of low score and high score immune or stromal groups (Figure 3A, 3B). Among immune score, 1290 up-regulated genes and 32 down-regulated genes were obtained (Figure 3C). Similarly, from stromal score 1681 genes were up-regulated and 11 genes were down-regulated (Figure 3D). The intersection analysis of Venn diagrams showed 1103 co-upregulation genes in high immune scores and stromal scores groups and 6 co-downregulation genes in low score groups. Then these 1109 DEGs were performed Gene Ontology (GO) enrichment analysis to identify the potential function of these DEGs. The enriched GO terms found mainly include T-cell activation, leukocyte migration, regulation of lymphocyte activation, which are mainly immune related GO terms (Figure 3E). In addition, the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathways are mainly enriched in chemokine signaling pathway, cytokine– cytokine receptor interaction, and chemokine signaling pathway, cell adhesion molecules which are related to immune response (Figure 3F)

Intersection Analysis of Protein–protein interaction network (PPI) and univariate COX

Based on the STRING database and Cytoscape software, we screened 1109 DEGs into PPI network (Figure 4A), the top 30 genes ranked by number of nodes was shown in (Figure 4B). To explore the association of these 1109 DEGs and survival prognosis of COAD patients, we performed univariate COX regression analysis (Figure 4C). Then we intersected the leading 50 nodes in PPI network and the top 31 significant factors in univariate COX regression. Only one overlapping gene, CXCL10 can be observed according to the above analysis (Figure 4D).

The expression of CXCL10 in COAD patients are associated with the TNM staging system and survival prognosis

CXCL10 is secreted by leukocytes and tissue cells in response to IFN- γ [11][12], which is highly expressed in a variety of human diseases such as infectious diseases, immune dysfunction, inflammatory diseases, and cancer [13][14][15][16]. To analyze the potential relationship between the expression of CXCL10 and the overall survival of the COAD samples, we divided COAD samples into CXCL10 high expression group and low expression group according to the median expression value. The survival curve showed that COAD patients with high expression of CXCL10 has a lower survival rate than the low expression group (Figure 5A). And as shown in Figure 5B, the expression of CXCL10 in the tumor samples was significantly increased than that in the normal samples. Since the number of tumor samples is inconsistent with the number of normal samples, we performed matched paired analysis to identify the expression of CXCL10 in normal and tumor tissues derived of the same patient. Consistent with previous results, CXCL10 is highly expressed in tumor samples (Figure 5C). There were significant differences in the expression of CXCL10 of the four tumor stages, from low to high: Stage IV < Stage III < Stage II and Stage I (Figure 5D). From the aspect of tumor infiltration depth (T), there was no significant difference in the CXCL10 expression of the four stages (Figure 5E). From the aspect of metastasis (M), the relationships between CXCL10 expression of the two stages were: M0 > M1, (with statistical significance) (Figure 5F). Finally, from the aspect of lymph node staging (N), CXCL10 expression is in the same order: N0 > N1 > N2, with statistical significance (Figure 5G). These data indicated that CXCL10 expression in TME was negative correlation with the prognosis of COAD patients. Moreover, there is a negative correlation between the expression of CXCL10 and the TNM staging system. To analyze the potential mechanisms of CXCL10 in association with COAD, GSEA of TCGA was performed to retrieve biological process enriched in CXCL10 with highly and lowly expressed samples. Figure 5H presents that the genes in CXCL10 high-expression group were mainly enriched in “antigen processing and presentation” “autoimmune thyroid disease” “chemokine signaling pathway” “leishmania infection” “systemic lupus erythematosus”. For CXCL10 low-expression group, the genes were enriched in “maturity onset diabetes of the young” (Figure 5I).

The relevance of CXCL10 With the Proportion of TICs

To estimate the relative proportion of Tumor-infiltrating immune cells (TICs) in COAD samples, CIBERSORT algorithm was used to analyse 521 cases from the TCGA dataset [17] (Figure 6A). Then correlation analysis among CXCL10 and TICs was performed (Figure 6B). Figure 7A shows that the proportion of 22 types of TICs in 521 cases of COAD was calculated and in each samples the proportion of TICs is different. Furthermore, as shown in Figure 6C, strong correlation with each other between different 22 TICs in COAD microenvironment can be seen. For example, macrophages M0 is the most positively correlated with T cell CD8, which indicated that these two types of immune cells have a synergistic effect in COAD patients. And T cells CD8 is negatively correlated with T cells CD4 memory activated illustrating that these two types of immune cells have an antagonistic effect in COAD patients. The violin plot in Figure 7A showed that ratio differentiation of 22 TICs between COAD tumor samples with low or high CXCL10 expression. Then we performed correlation analysis between CXCL10 expression and TICs proportion (Figure 7B). Finally the intersection analysis between difference analyses and correlation analyses showed that 13 kinds of TICs were correlated with CXCL10 expression (Figure 7C). In these 13 kinds of TICs, there is a positive correlation between 9 kinds of TICs and CXCL10

expression, the remaining were negatively correlated with CXCL10 expression. The above data indicated that the expression levels of CXCL10 accosicated with the immune activity of TME.

Discussion

CRC is the most prevalent cancer in the world, with high morbidity and mortality. There are approximately 1.3 million new cases each year, and almost 700,000 people died from this cancer.

With the development of precision medicine, it holds great promise for CRC patients. However, the high rate of metastasis and invasiveness of CRC has been the bottleneck to reduce mortality, resulting in its 5-year survival rate is still unsatisfactory[18]. Therefore, understanding the biological mechanism behind cancer progression and metastasis has very important clinical significance for clinical treatment of patients.

In this paper, we collected transcriptome RNA-seq data and clinical data of COAD patients from TCGA database. TME-related DEGs which are associated with the survival and the classification of TNM stages in COAD patients are identified. Finally, by intersection analysis CXCL10 was identified, which might be an new indicator for the status of TME in COAD patients. Tumor metastasis is the main cause of cancer treatment failure and patient death. Its molecular mechanism is complex, involving multiple steps, multiple stages, and multiple gene changes. As a place for tumor cells to survive, TME plays a vital role in the process of tumor metastasis. In TME, immune cells and stromal cells are two main types of non-tumor components, which have been proposed to be valuable for tumor diagnosis and prognostic evaluation. By analyzing the database from TGCA-COAD, we found the proportion of immune and stromal components in TME are not related to the prognosis of patients but are associated with the progression of COAD. For example, tumor metastasis is inversely proportional to immune score. These data emphasize the importance of exploring the interaction between different types of cells in TME and will help provide new immunotherapy options. A recent important paper report was published in *The Lancet*[19],and scientists have confirmed that the immune score can more effectively and accurately predict the risk of CRC progression. Infiltration of immune cells in the tumor can be used as a good reference indicator to indicate the development of CRC, which is a potential prognostic indicator. On the basis of traditional TNM staging, combined with immune score, which can further improve tumor prognosis assessment. Immune scores provide accurate and reliable indicators for recurrence risk assessment of CRC patients, and can be used as a new component of cancer classification. Recurrence risk classification based on immune scores can be used to improve individualized treatment strategies, especially to guide the adjustment of chemotherapy. They showed that the immune score, as an analysis of tumor immune responsiveness, can better guide individualized and accurate anti-cancer, which will likely change the clinical research design ideas. Eventually, it will help improve the prevention and control level of the global cancer.

To determine the exact changes in gene expression profiles related to immune and stromal components in TME, a differential analysis was performed on samples with high scores and low scores. A total of

1109 DEGs were obtained by analyzing the low-score shared genes and the high-score shared genes by intersection analysis which might be the determinant of TME status. The GO enrichment analysis indicated that DEGs are associated with immune-related terms. KEGG pathway enrichment analysis showed that these DEGs are mainly enriched in cytokine cytokine receptor signaling pathway and so on. Therefore, DEG is related to immune regulation, which indicates that the participation of immune factors is the main characteristic of TME in COAD. Then, we found that the expression level of CXCL10 has significantly connection with the prognosis of patients and the progression of COAD. Therefore, these analyses indicated that CXCL10 have a potential to be a prognostic indicator and a therapeutic target for TME in COAD.

Nicola Giuliani et al.[20] found that CXCL10 can directly bind to the CXCR3 receptor of cancer cells to play an anti-proliferative effect. And it can interfere with the expression of oncogenic factors, such as E6 and E7, which caused increased expression of p53 and promoted the death of cancer cells[21]. CXCL10 also exerts an anti-tumor effect by regulating immunity, it can recruit a variety of immune cells to directly kill tumor cells. *Liu et al.* [22] showed that esophageal squamous cell carcinoma secreted CXCL10 protein, which recruited CD8 + T lymphocytes into esophageal squamous cell carcinoma tissues and caused cancer tissue damage. The overall survival rate of patients with this type of cancer has been improved. Tumor immune escape mechanism might partially be caused by the defective expression of CXCL10[23]. Clinically, there are few studies on the correlation between CXCL10 and the therapeutic effect of tumors. Studies have shown that in patients treated with tamoxifen, the survival rate of breast cancer patients with high expression of CXCL10 is higher than that with low CXCL10 expression, which can be used as a useful predictor of tamoxifen therapy[24]. Studies on rectal cancer have shown that the positive expression rate of CXCL10 in neoadjuvant radiochemotherapy (pCR) is 73.7%, which is significantly higher than that of pathological incomplete remission (npCR) 38.6%, indicating that high expression of CXCL10 has higher treatment sensitivity, and CXCL10 is related to the efficacy and prognosis of rectal cancer[25]. CXCL10 mainly exerts its biological effects by binding to its receptor CXCR3. On one hand, it can antagonize the tumor by promoting the chemotaxis of immune cells, inhibiting angiogenesis, and promoting tumor cell apoptosis, on the other hand, it can also promote the development of tumors by suppressing immunity, damaging immune responses, promoting tumor angiogenesis, and inhibiting apoptosis[26][27][28][29]. The mechanism of its role in the occurrence and development of tumors needs to further be investigated.

In our study, upon further analysis of the association between the expression of CXCL0 and TME, we performed GSEA enrichment analysis and found CXCL10 high- expression group were mainly enriched in immune-related signaling pathways indicating that CXCL10 might participate the regulation of immune signaling pathway in TME.

In recent years, finding new specific therapeutic targets has become a hot spot in the research of tumor treatment. The multiple characteristics of CXCL10 are expected to make it a new strategy for targeted treatment of tumors, and become a indicator of clinical tumor prognosis evaluation.

Abbreviations

CRC: Colorectal cancer

TME: Tumor Microenvironment

COAD: Colon adenocarcinoma

DEGs: Differentially expressed genes

TICs: Tumor-infiltrating immune cells

Declarations

Availability of data and materials

The data supporting the article are available in the TCGA database (<https://portal.gdc.cancer.gov>).

Author information

HLY and WWS contributed equally to this work.

Contributions

HLY, WWS, and CA designed the entire study, and HLY and WWS contributed equally to this work. CGH, QTM, BW, XY ,JT and ZSJ were partly involved in the experimental process and data analysis. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

This research was approved by the Beijing Tsinghua Changgung Hospital.

Consent for publication

This manuscript is approved by all authors for publication.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 The clinicopathological characteristics of COAD patients			
Clinical characteristics	Type	Total(452)	%
Age	<=65	185	40.90%
	> 65	267	59%
Gender	Male	238	52.60%
	Female	214	47.30%
Stage	Stage I	77	17%
	Stage II	178	39.40%
	Stage III	125	27.70%
	Stage IV	62	13.70%
T classification	T1	10	2.20%
	T2	77	17%
	T3	281	62%
	T4	83	18.40%
M classification	M0	334	74%
	M1	62	13.70%
N classification	N0	269	59.50%
	N1	103	22.80%
		79	17.50%

Figures

Figure 1

(A) Workflow of this study. (B-D) Kaplan–Meier survival analysis for COAD patients divided into high or low score determined by the comparison with the median.

Figure 2

Association with ImmuneScore StromaScore EstimateScore and clinicopathologic characteristics. (A) Clinical stage (B) T classification (C) M classification (D) N classification.

Figure 3

Identification of DEGs. (A) Heatmap for DEGs via comparing the high score samples to the low score samples in ImmuneScore and StromalScore separately. DEGs were determined by Wilcoxon rank sum test with $q = 0.05$ and fold-change >1 as the Statistically significant.

Figure 4

Intersection Analysis of PPI and univariate COX. (A) PPI Interaction network of DEGs, and the interaction confidence value setting is greater than 0.95. (B) The top thirty genes sorted by the number of nodes. (C) Univariate COX regression analysis with $p < 0.03$ setting. (D) Intersection analysis of PPI and univariate COX.

Figure 6

The distribution of immune infiltration in COAD. (A)The workflow of this part analysis. (B)The proportions of different immune cells in each COAD sample. (C) Correlation of all type of immune cells. Blue plot indicated negatively related immune cells, red plot indicated positive related immune cells.

Figure 7

The correlation of TICs proportion with CXCL10 expression. (A) High or low expression of CXCL10 in different immune cells. (B) The correlation of TICs proportion with the CXCL10 expression, $p < 0.05$ as statistically significant. (C) Intersection analysis.