TGFβ1: an Indicator for Tumor Immune Microenvironment (TIME) of Colon Cancer from a Comprehensive Analysis of TCGA

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Research

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Abstract

Background: Tumor microenvironment (TME) and tumor-infiltrating immune cells (TIC) greatly participated in the genesis and development of colon cancer (CC). However, there are few researches exploring the dynamic modulation of TME.

Methods: In our study, we analyzed the proportion of immune/stromal component and TIC in TME of 473 CC samples and 41 normal samples from The Cancer Genome Atlas (TCGA) database through ESTIMATE and CIBERSORT algorithm. Correlation analysis was carried out to evaluate the association between immune/stromal component in TME and clinicopathological characteristics of CC patients. The difference analysis was performed to obtain the differentially expressed genes (DEGs). These DEGs were further analyzed by gene ontology (GO), kyoto encyclopedia of genes and genomes (KEGG) enrichment analyses, protein–protein interaction (PPI) network construction and COX regression analysis. Transforming growth factor β1 (TGFβ1) was finally overlapping from the above analysis. Furthermore, TGFβ1 was analyzed by paired analysis, Gene Set Enrichment Analysis (GSEA). The intersection between the difference analysis and correlation analysis was also conducted to learn the association between TGFβ1 and TICs.

Results: Our result showed that immune component in TME was negatively related with the stages of CC. GO and KEGG enrichment analysis revealed that 1110 DEGs obtained from difference analysis were mainly enriched in immune-related activities. The intersection analysis between PPI network and COX regression analysis indicted that TGFβ1 was significantly associated with the communication of genes in PPI network and the Hazard Ratio (HR) of CC patients' survival. In addition, TGFβ1 was up-regulated in the tumor samples and significantly related with poor prognosis of CC patients. Further GSEA suggested that genes in TGFβ1 up-regulated group were primarily enriched in immune-related activities and the function of TGFβ1 might depend on the communications with TICs, including T cells CD4 naïve and T cells regulatory (Tregs).

Conclusions: The expression of TGFβ1 might be an indicator for tumor immune microenvironment (TIME) of CC and sever as a prognostic factor of CC. Drugs targeting TGFβ1 might be a potential immunotherapy for CC patients in the future.

Background

Colon cancer (CC) is one of the most common causes of cancer-associated mortality in the United States [1]. Although the overall incidence and mortality continue to decline, the incidence in young and middle-aged adults keeps rising [2]. Although considerable efforts have been made to improve the clinical outcomes of CC patients, CC is still a fatal disease [3, 4]. Additionally, curative effect of multiple treatments, including chemotherapy, immunotherapy and targeted therapy, are obviously reduced by drug resistance [5]. Hence, it is urgent to further explore the detailed molecular mechanism of CC and to identify the vital prognosis biomarkers of CC.

Recently, accumulating researches have been focusing on understanding the role of tumor microenvironment (TME) in the genesis and development of cancers. TME is composed of multiple immune cells, stromal cells, extracellular matrix, and kinds of cytokines and chemokines [6]. These components in the TME are in a dynamic process, greatly participate in tumor growth, invasion, metastasis and drug resistance [7–10]. The activation of tumor-infiltrating immune cells is an important parameter that acts as a prognostic biomarker and affects various tumor biological processes [11, 12]. For instance, CD8-positive (CD8+) tumor-infiltrating lymphocytes (TILs) in the peri-tumoral microenvironment are significantly correlated with poor clinical outcome of salivary gland carcinoma patients [13]. Mechanically, interleukin-38 advances tumor growth by affecting CD8 + TILs in the TME of lung cancer [14]. In addition, dual suppression of both PI3K-γ and colony stimulating factor-1/colony stimulating factor-1 receptor (CSF-1/CSF-1R) pathways in tumor associated macrophages (TAM) could remodel tumor immune microenvironment (TIME) and synergistically activate antitumor immune responses in pancreatic cancer [15].

In recent ten years, tumor-infiltrating immune cells (TIC) have emerged as potential therapeutic targets. The novel therapeutic strategy, known as immune checkpoint inhibition, focuses on inhibiting the molecular communication between tumor cells and immune cells. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1), commonly express on activated T-cells, have been recognized as the most reliable targets for the immunological therapy of multiple cancers [16–18]. According to the abundant clinic trials, PD-1 blocker alone, or combined with CTLA-4 have been proved with good curative effect in various cancer types, including CC [19, 20]. However, there are still significant proportion of cancer patients, who do not respond or initially respond and later develop tumor progression, indicating the existence of immune resistance [21]. Fortunately, researches have suggested that TME and infiltrating immune cells are specific to different cancer types and might explain the immunotherapeutic responsiveness of cancers [22, 23]. As a result, further exploration of immune infiltration in CC TME is essential in clarifying the mechanisms underlying the progression of CC.

In this study, to investigate potential signatures for CC patients, we obtained a list of TME-related genes of prognostic value using immune/stromal scores after ESTIMATE algorithm-processing in multiple cohorts. Functional annotations and immune infiltration correlation were analyzed for significant hub genes. We hypothesized that TGFβ1 was correlated with poor prognosis, might act as an indicator for tumor immune microenvironment of CC and potential immune therapies targeting TGFβ1 might provide new hope to colon patients

Methods

Data collection based on TCGA

We collected the transcriptome RNA-seq profiling, the clinical data of CC tissues and normal colon tissues from the TCGA database (https://portal.gdc.cancer.gov/). Ultimately, 514 CC cases (473 tumor samples

and 41 normal samples) and the corresponding clinical data were included.

Calculation of Immune Score, Stromal Score, and ESTIMATE Score

R language version 3.6.3 (https://www.r-project.org/) was used to analyze the proportion of immune/stromal component in TME of each tumor sample through ESTIMATE algorithm. Immune Score, Stromal Score, and ESTIMATE Score reflected the corresponding ratio of immune, stromal, and the sum of both in TME. The higher score represented the larger ratio of immune/stromal component in TME.

Survival Analysis

Survival and survminer packages in R were used for the survival analysis. Kaplan–Meier plot and log-rank tests were conducted to evaluate the relationship between survival rates and differentially expressed genes (DEGs). $P < 0.05$ was considered to be statistically significant.

Differential analysis of Scores With Clinicopathological Characteristics

The differential analysis was performed by R language. Wilcoxon rank sum and Kruskal–Wallis rank sum test were based on the number of TNM stages for comparison.

Affirmation of DEGs Between High-Score and Low-Score Groups and Heatmaps

A total of 473 tumor samples were classified into the high-score group and the low-score group relying on the comparison with the median score. Data analysis was performed by package limma in R. The fold change was calculated by log2 (high-score group/low-score group). A fold change (FC) > 1 and false discovery rate (FDR) <0.05 were set up to screen DEGs. Heatmaps of DEGs were generated by pheatmap package in R.

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

GO and KEGG enrichment analyses of 1110 DEGs were carried out by clusterProfiler, enrichplot, and ggplot2 packages in R. $P< 0.05$ was considered to be statistically significant.

Protein–Protein Interaction (PPI) Network and Gene Set Enrichment Analysis

PPI network was constructed by the Search Tool for Retrieval of Interacting Genes/Proteins (STRING) database (version 11.0). Nodes with confidence of interactive relationship greater than 0.95 were applied. And the network was further reconstructed with Cytoscape of version 3.6.1. A functional profile of the gene set derived from the PPI was further retrieved by using Gene Set Enrichment Analysis (GSEA) 4.1.0. $P< 0.05$ was considered to be statistically significant.

COX Regression Analysis

Univariate COX regression was performed by package survival in R.

Tumor-infiltrating immune cell Profile

TIC abundance profile in CC tumor samples was estimated by using CIBERSORT computational method. Finally, a total of 473 CC patients' tumor samples were included for further analysis with $p < 0.05$.

Results

Analysis Process

We first downloaded the transcriptome RNA-seq profiling, the clinical data of CC tissues and normal colon tissues from the TCGA database. And then ESTIMATE algorithms were used to analyze the proportion of immune/stromal component in TME. Correlation analysis was carried out to evaluate the association between immune/stromal score and the clinic–pathological staging of CC Patients. The intersection analysis was used to obtain DEGs shared by immune score and stromal score. PPI network and univariate COX regression analysis were further conducted. The intersection analysis was carried out to find the DEGs which were both the top leading nodes in PPI network and the top factors of univariate COX regression. Finally, transforming growth factor β1 (TGFβ1) was obtained, and we focused on TGFβ1 for the subsequent series of analysis, such as expression pattern analysis, survival analysis, clinic–pathological features correlation analysis, COX regression, GSEA, and correlation analysis with tumor-infiltrating immune cell (TIC). The specific analysis process of our study was displayed in Figure 1.

Scores Were Associated With the Clinic–Pathological features of CC Patients

In order to explore the underlying associations between the ratio of immune/stromal components and the clinic-pathological features (Supplement Table 1), we analyzed the TNM stages of CC patients regarding Immune Score, Stromal Score, and ESTIMATE Score. Interestingly, There were significant differences in the Immune Score in stage I compared with stage II, III, and IV (Figure 2a, $p = 0.0099, 0.0021, 0.039$). In particular, Immune Score was negatively related with M classification of TNM stages (Figure 2d, $p = 0.0019$). However, Stromal Score and ESTIMATE Score had nothing to do with the TNM stages of CC patients (Figure 2e-l, $p>0.05$). The above results indicated that the proportion of immune components might play an important role in the advance of CC, especially diatant metastasis.

DEGs Shared By Immune Score and Stromal Score Were Significantly Associated With Immune-Related Activities

In order to acquire the detailed gene profile in TME, the difference analysis between high score and low score tumor samples were conducted. The results displayed that a total of 1313 DEGs were acquired from immune score group (high score tumor samples vs. low score tumor samples), among which 1280 DEGs were up-regulated and 33 DEGs were down-regulated when compared to the median (Figure 3a). In addition, 1697 DEGs were acquired from stromal score group, including 1684 up-regulated genes and 13 down-regulated genes (Figure 3b). Furthermore, the intersection analysis was carried out to obtain the up-regulated or down-regulated genes both in immune score and stromal score. Venn plot displayed that

1103 genes were up-regulated and 7 genes were down-regulated in both immune score and stromal score (Figure 3c-d). These DEGs, a total of 1110 genes, might play a significant role in regulating the status of TME. Therefore, GO enrichment analysis and KEGG enrichment analysis were used to evaluate the functions of these DEGs. GO enrichment analysis revealed that these DEGs were closely associated with immune-related GO terms, including T cell activation, leukocyte migration, positive regulation of cytokine production, and so on (Figure 3e-g). Besides, KEGG enrichment analysis indicated that 1110 DEGs were significantly related with cytokine-cytokine receptor interaction, chemokine signaling pathway, positive regulation of cytokine production, mononuclear cell proliferation and so on (Figure h-j). From above, the functions of these DEGs seemed to be significantly associated with immune-related activities and might be a predominant characteristic of TME in CC.

Intersection analysis between PPI Network and Univariate COX Regression

In order to further explore the underlying mechanisms regarding these 1110 DEGs, we first constructed PPI network by using STRING database and Cytoscape software. The detailed interactions between 1110 DEGs were displayed in Fig. 4a. The top 50 DEGs ranked by the number of nodes were shown in Fig. 4b. Univariate COX regression analysis was conducted to find the most significant DEGs regarding the survival of CC patients (Fig. 4c). Finally, the intersection analysis was carried out to find the DEGs which were both the top 50 leading nodes in PPI network and the top 14 factors of univariate COX regression. Transforming growth factor β1 (TGFβ1) was overlapping from the above analysis (Fig. 4d).

TGFβ1 was Associated with the Survival and Clinic–Pathological Staging of CC Patients

TGFβ1 was a pleiotropic cytokine and played a vital role in immune reconstruction [24, 25]. A. L. Teixeira, et al. [26] discovered that TGFβ1 affected non-small cell lung cancer (NSCLC) susceptibility with impact in cellular microenvironment. In our study, the pairing analysis revealed that TGFβ1 was up-regulated in the tumor samples than that in the paired normal samples from the same patients (Fig. 5a, $p = 0.0025$). And then we divided CC samples into two groups, including TGFβ1 high-expression group and TGFβ1 low-expression group. The survival analysis revealed that CC patients with up-regulated TGFβ1 had shorter survival than that of down-regulated TGFβ1 (Fig. 5b, $p = 0.036$). Specifically, the up-regulated TGFβ1 was related with lymph node stage of CC patients (Fig. 5c). In conclusion, TGFβ1 was negatively associated with the prognosis of CC patients.

TGFβ1 might participate in the modulation of TME

Considering that the expression of TGFβ1 were negatively associated with the survival and lymph node stages of CC patients, GSEA was carried out in the up-regulated and the down-regulated groups compared with the median level of TGFβ1 expression, respectively. The genes in TGFβ1 up-regulated group were primarily enriched in immune-related activities, including cell adhesion molecules, chemokine signaling pathway, complement and coagulation, cytokine-cytokine receptor interaction and so on (Fig. 5d). Nonetheless, few genes were enriched in the TGFβ1 down-regulated group. The above results indicated that TGFβ1 might participate in the modulation of TME.

TGFβ1 was connected with T cells CD4 naïve and T cells regulatory (Tregs) in TIME

To further affirm the connection between TGFβ1 and the immune microenvironment, we applied CIBERSORT algorithm to examine the ratio of tumor-infiltrating immune cells in CC (Fig. 6a-b). The difference test indicated that 5 kinds of TICs were significantly related with the expression of TGFβ1, such as T cells CD4 naïve, T cells CD4 memory activated, Tregs, NK cells resting and eosinophils (Fig. 6c). The correlation test revealed that neutrophils, Tregs, T cells CD8 were positively related with the expression of TGFβ1, and T cells CD4 naïve was negatively related with the expression of TGFβ1 (Fig. 6d). The intersection between the difference test and correlation test suggested that T cells CD4 naïve and Tregs were potentially associated with the expression of TGFβ1 (Fig. 6e, Supplement Table 2). These results further indicated the potential role of TGFβ1 in the immune activity of TME.

Discussion

In recent years, great advances have been made in the exploration of the CC treatment. It gradually moves away from chemotherapy, which has been the standard treatment of CC for decades, toward immunotherapy that modulates immune responses against tumor cells [27, 28]. In the last few years, TME was found to play a vital role in the initiation and progression of tumorigenesis [29–32]. Researches indicated that TIME was closely related with the prognosis of cancers, including hepatocellular carcinoma [33], lung adenocarcinoma, lung squamous cell carcinoma [34] and so on. Therefore, it is of great benefit to identify the vital potential biomarkers associated with the remodeling of TME and prognosis, and develop more specific drugs against CC.

In this study, we attempted to comprehensively assess the TME of CC and identify TME-associated genes that related with the survival and clinic–pathological features of CC patients from the TCGA database. Firstly, we evaluated the proportion of immune/stromal component in TME and found out that immune/stromal component was associated with the clinic–pathological staging of CC patients. Secondly, DEGs, shared by immune score and stromal score, were found out and further explored by PPI network and univariate COX regression analysis. Thirdly, TGFβ1 came to our eyes and we carried out survival analysis, clinic–pathological features correlation analysis, COX regression, GSEA, and TIC correlation analysis. Finally, based on the above researches, we concluded that TGFβ1 played a significant role in the TIME of CC potential through communicating with T cells CD4 naïve and Tregs. In conclusion, TGFβ1 might be a potential indicator for the status of TIME in CC patients.

TGFβ1 was a multi-functional cytokine and regulated a variety of biologic processes in the host, such as cell proliferation, apoptosis, differentiation, migration, invasion and angiogenesis[35]. In addition, it was aberrantly activated in the late-stages of tumorigenesis and the dysregulation of TGFβ1 signaling pathway contributed to various aspects of cancer progression [36, 37]. Recently, studies indicated that TGFβ1 greatly participated in numerous immune regulatory functions, such as tumor immune suppression and escape [38]. It was also significantly associated with the function of kinds of TICs, such as tumor-associated macrophages [39], dendritic cells [40, 41], neutrophils [42], T cells [43] and natural killer

cells [44]. However, there are no studies exploring the role of TGFβ1 in the TIME of CC and whether it could interact with TICs.

Conclusion

Our study implied that TGFβ1 greatly participated in the modulation of colon cancer TIME through communicating with T cells CD4 naïve and Tregs. Furthermore, TGFβ1 might be a potential indicator of the status of TIME in CC patients. Therefore, further investigations are needed to clarify the detailed mechanisms between TGFβ1, and T cells CD4 naïve or Tregs. Drugs targeting TGFβ1 might be a potential treatment for CC patients in the future.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets generated and analyzed in this research are available in TCGA database (https://portal.gdc.cancer.gov)

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

Conceptualization, J.W.(Jinyan Wang) and Q.Z.; methodology, J.W.(Jinqiu Wang); software, Q.G.; validation, Q.Z.; formal analysis, J.W.(Jinyan Wang); investigation, J.W.(Jinqiu Wang).; resources, Q.G.; data curation, Q.G..; writing—original draft preparation, J.W.(Jinyan Wang), J.W.(Jinqiu Wang) and Q.Z.; writing—review and editing, J.W.(Jinyan Wang) and Q.Z.; supervision, Y.Y. and J.Z; project administration, Y.M; funding acquisition, J.W.(Jinyan Wang), Y.Y. and Q.Z. All authors have read and agreed to the published version of the manuscript.

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Figures

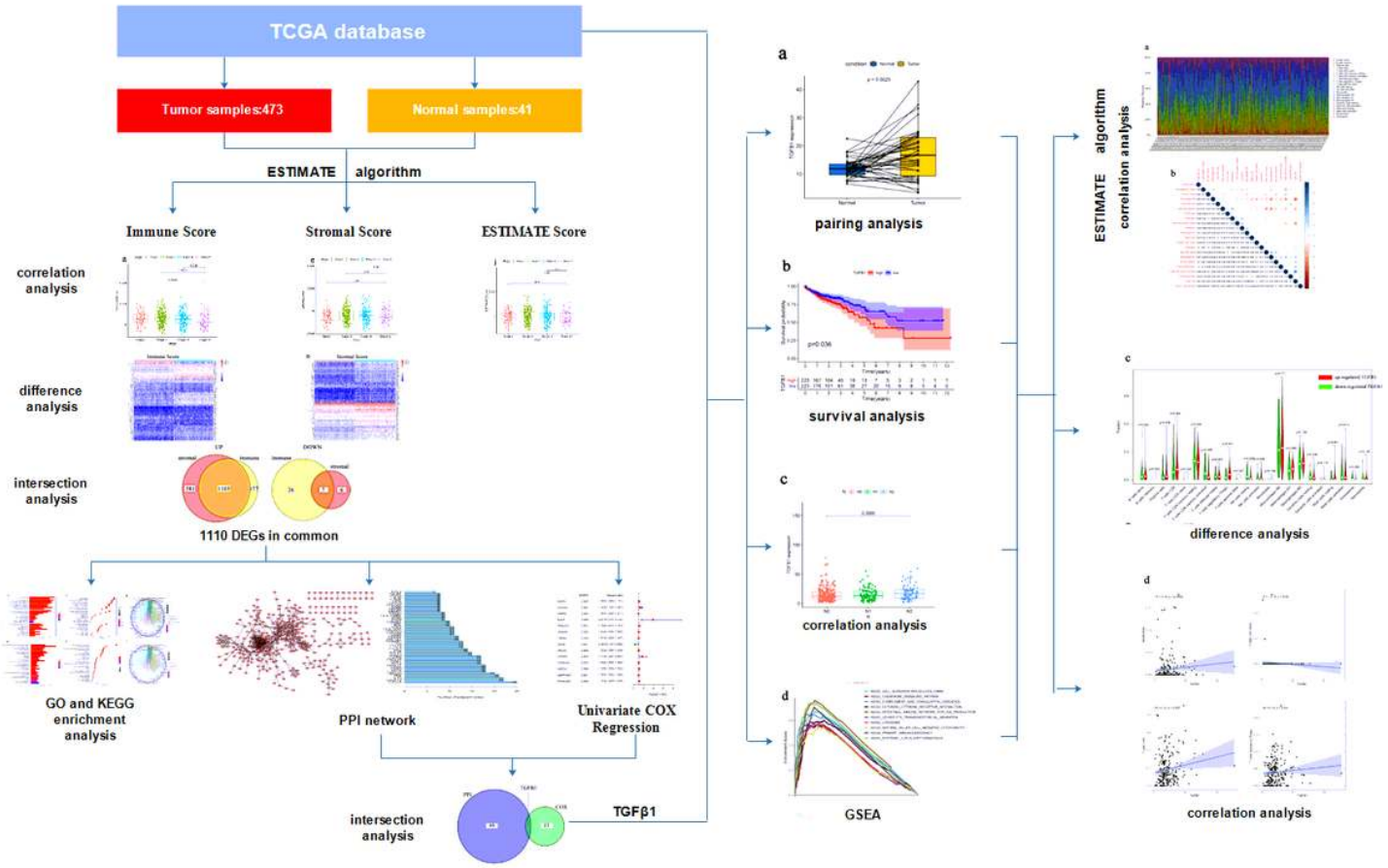

Figure 1

Analysis process of this study.

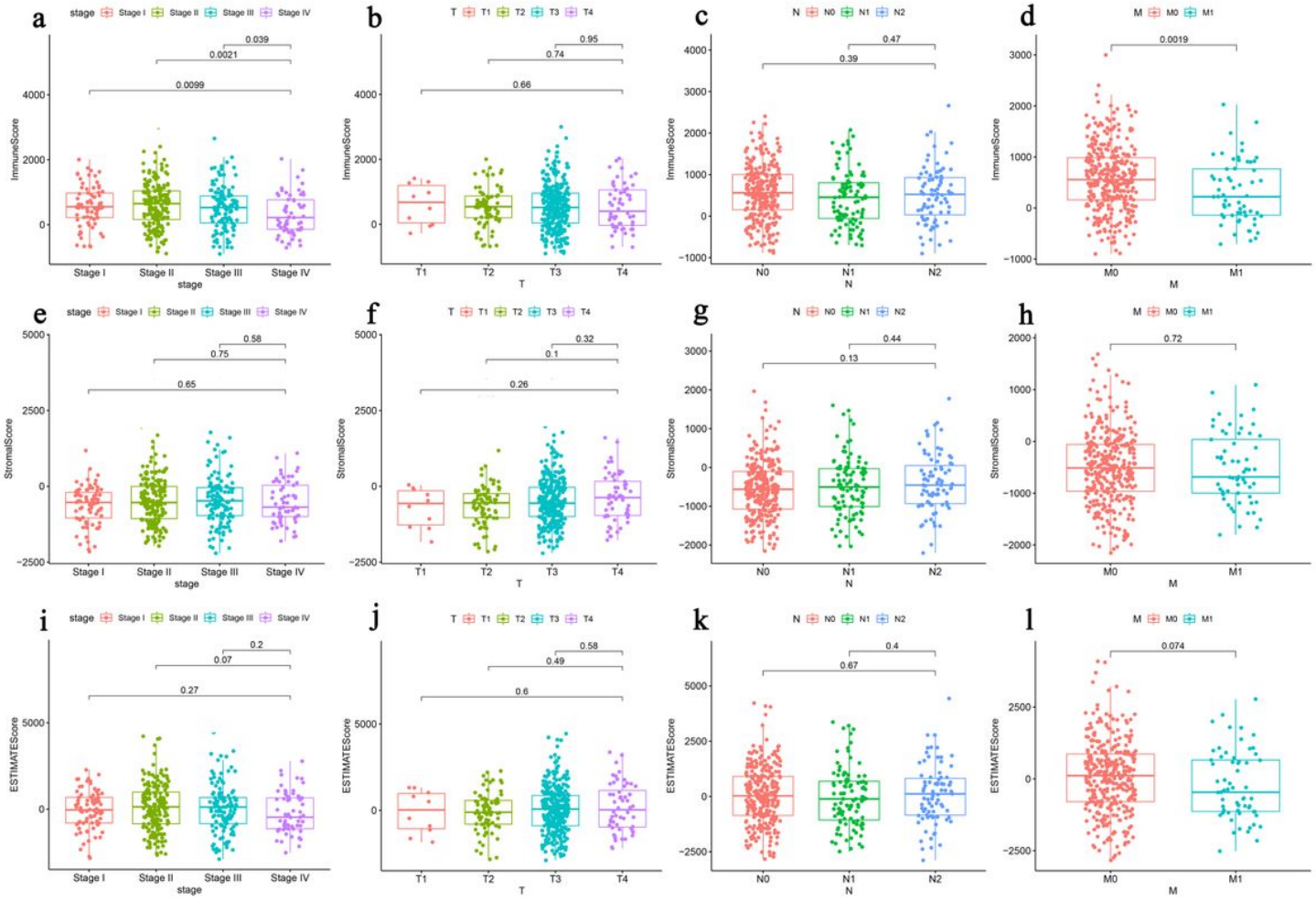

Figure 2

Association of Immune Score, Stromal Score and Estimate Score with the clinic–pathological features of CC Patients. (a-d) Association of Immune Score with stages and TNM classification by Kruskal–Wallis rank sum test; (e-h) Association of Stromal Score with stages and TNM classification Kruskal–Wallis rank sum test; BP: biological process; CC: cell component; MF: molecular function; (i-l) Association of Estimate Score with stages and TNM classification Kruskal–Wallis rank sum test; BP: biological process; CC: cell component; MF: molecular function.

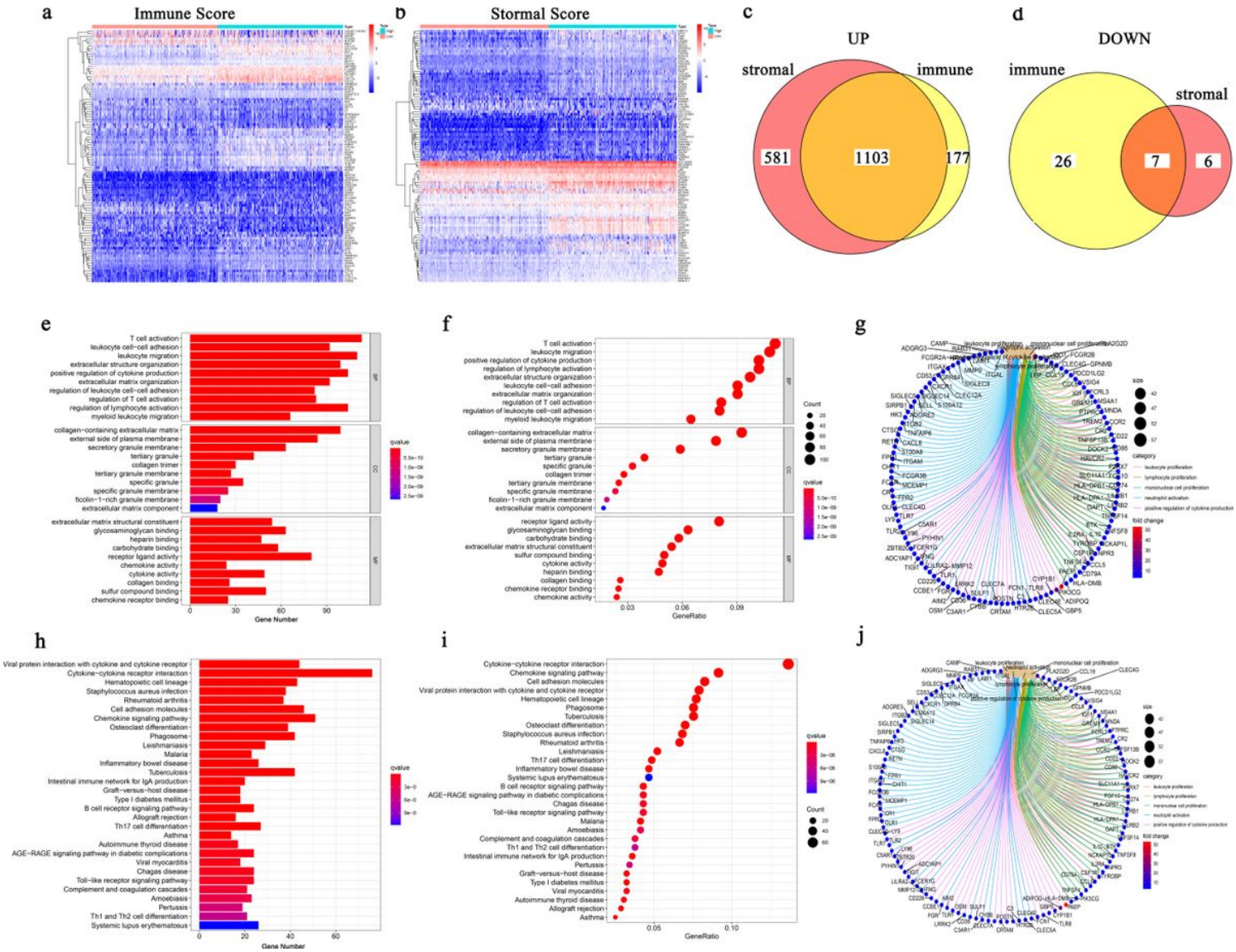

Figure 3

Heatmaps, Venn plots, and enrichment analysis of GO and KEGG for 1110 DEGs. (a) Heatmap for DEGs conducted by comparing high Immune Score tumor samples with low Immune Score tumor samples. Row name: the gene name. Column name: the ID of samples which not shown in plot. DEGs were examined by Wilcoxon rank sum test with q = 0.05 and log2fold-change >1 as the significance threshold. (b) Heatmap for DEGs conducted by comparing high Stromal Score tumor samples with low Stromal Score tumor samples. Row name: the gene name. Column name: the ID of samples which not shown in plot. DEGs were examined by Wilcoxon rank sum test with q = 0.05 and log2fold-change >1 as the significance threshold; (C,D) Venn plots plots common overexpressed and downexpressed DEGs shared by Immune Score and Stromal Score; (e-g) GO enrichment analysis for 1110 DEGs, p< 0.05 was considered to be enriched significantly; (h-j) KEGG enrichment analysis for 1110 DEGs, p< 0.05 was considered to be enriched significantly.

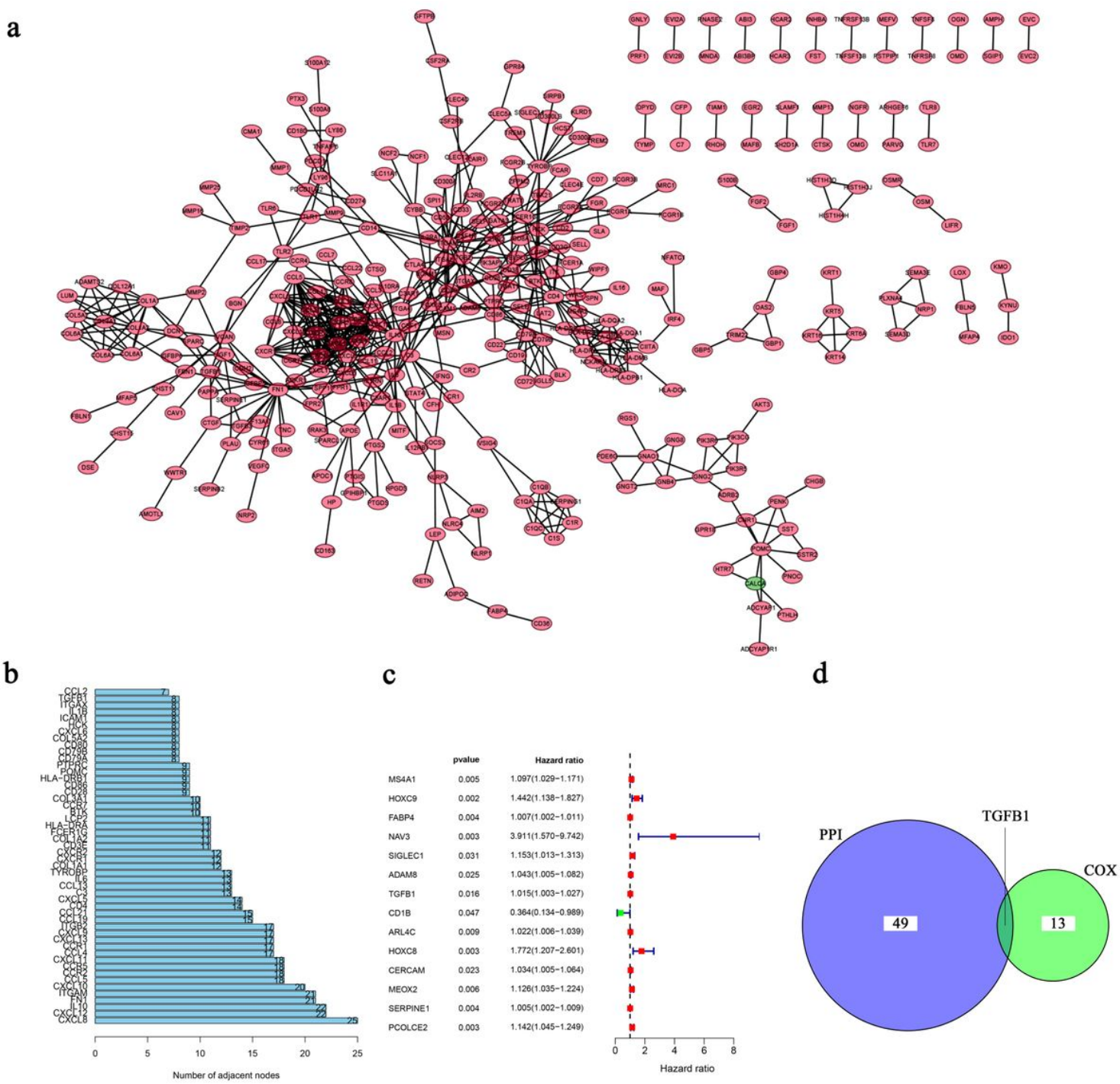

Figure 4

PPI network and univariate COX regression analysis. (a) PPI network was carried out with the nodes with interaction confidence value >0.95; (b) The top 50 genes ranked by the number of nodes. (c) Univariate COX regression analysis with DEGs. $p < 0.05$ was considered to as the significance threshold; (D) Venn plot displays the common genes shared by l the top 50 genes in PPI and top significant genes in univariate COX regression analysis.

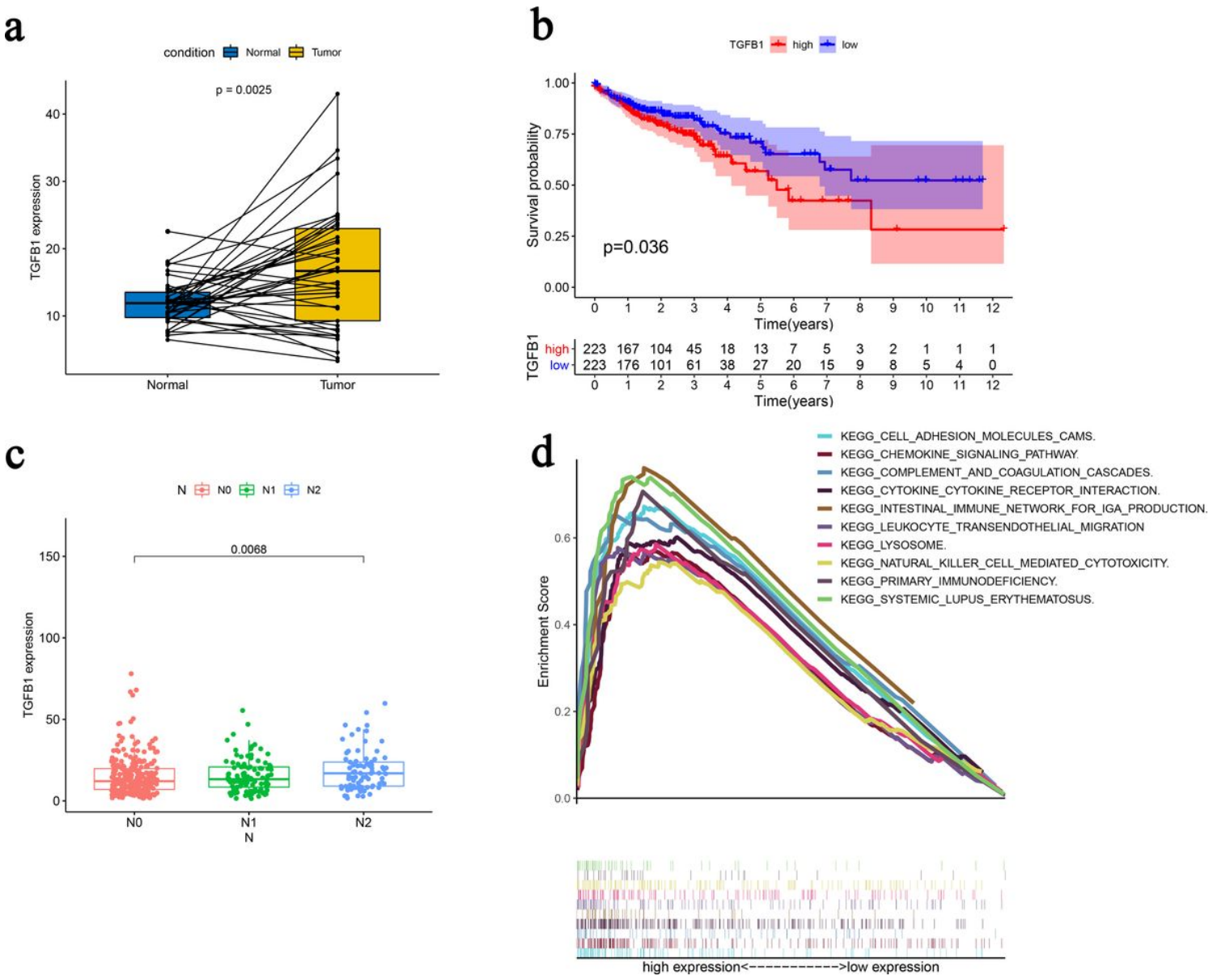

Figure 5

The expression of TGFβ1 in CC patients and the association with survival, TNM classification and GSEA. (a) Paired differentiation analysis for expression of TGFβ1 in the colon cancer samples and the paired normal samples deriving from the same patient (p=0.0025 by Wilcoxon rank sum test); (b) Survival analysis for colon cancer patients with high expression or low expression of TGFβ1. p = 0.036 by log-rank test; (c) Association of the expression of TGFβ1 with N stage by Kruskal–Wallis rank sum test; p=0.0068; (d) GSEA for tumor samples with high expression or low expression of TGFβ1

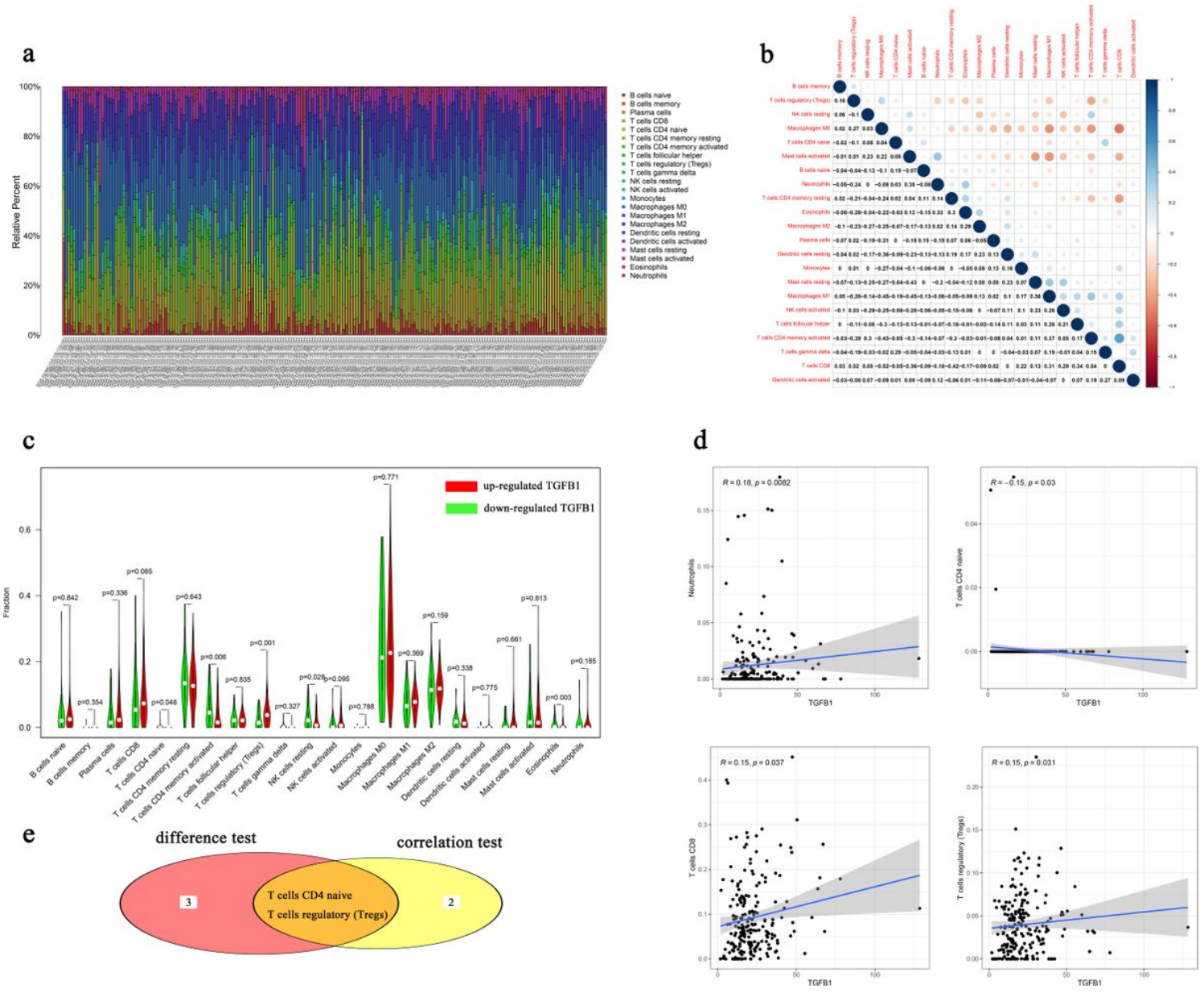

Figure 6

TIC profile and correlation analysis. (a) Barplot displays the ratio of 22 kinds of TICs in colon cancer samples. Column names: sample ID. (b) Heatmap displays the association between 22 kinds of TICs; each spot represents p value of correlation between two kinds of cells; Pearson coefficient was carried out for significance test; (c) Violin plot displays the differentiation of 22 kinds of TICs between colon cancer samples with high or low expression of TGFβ1; Wilcoxon rank sum was carried out for the significance test; (d) Scatter plot displays the association between 4 kinds of TICs and the expression of TGFβ1; Pearson coefficient was carried out for the correlation test; $p < 0.05$ was considered to as the significance threshold and ploted; (e) Venn plot displays two kinds of TICs shared by difference and correlation tests showed in violin and scatter plots, respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementTable2.docx
- SupplementTable1.docx
- manuscript.pdf