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Original Article

Prognostic value of RNF113A shows a correlation with immune infiltrates in colorectal cancer

Minghui Chen[1,2,3*], Xiaoyu Song[4*], Huizhen Lin[3*], Wenli Cheng[1,2]

[1]Department of Blood Transfusion, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou 510655, Guangdong, China; [2]Biomedical Innovation Center, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou 510655, Guangdong, China; [3]Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, Guangdong, China; [4]Central Laboratory, Guangzhou Panyu Central Hospital, Guangzhou 511400, Guangdong, China. [*]Equal contributors.

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Abstract: Objective: To explore the prognostic role of RNF113A in colorectal cancer (CRC) and its relationship with immune infiltration. Methods: Data from publicly available datasets were collected and analyzed to evaluate RNF113A expression in different tumors compared with normal samples and investigate the relationship between RNF113A and CRC survival. The protein expression of RNF113A among colorectal cancer cell lines (HCT116, Caco2, Colon3) and human colorectal mucosa cell (FHC) was detected as well. Pathway enrichment analysis was performed to identify signaling pathways associated with RNF113A. The diagnostic and prognostic values of RNF113A expression in CRC and its correlation with cancer immune characteristics were analyzed by using the TIMER and TISIDB databases. Results: RNF113A is predominantly overexpressed in CRC, which has diagnostic and prognostic value. The protein expression of RNF113A in Colon3 cells was significantly higher than that of FHC cells ($P<0.05$). The rRNA processing signaling pathway-related gene SNU13 was positively correlated with RNF113A ($R=0.245$, $P<0.001$). The area under the ROC curve (AUC) of RNF113A expression for diagnosis of CRC was 0.885. The nomogram showed that RNF113A expression outperformed traditional clinical features such as age in predicting prognosis. RNF113A expression was negatively correlated with the infiltration level of memory B cells, NK cells, Th2 cells, and $CD8^+$ T cells. Moreover, RNF113A expression was negatively correlated with the expression of CCL4, CXCL16, CCR5, and CXCR4. Conclusion: RNF113A may regulate CRC through the rRNA processing pathway and negatively correlate with the infiltration level of immune cells, serving as a prognostic biomarker for CRC.

Keywords: RNF113A, prognosis, immune infiltrate, rRNA processing pathway, colorectal cancer

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related mortality and the third most prevalent cancer worldwide, with an increasing prevalence among people under 50 years of age [1]. Although there has been improvement in screening and therapy, the prevalence and mortality of CRC remain high. The survival rate for CRC patients at advanced stage is low even after active treatments including surgery, chemotherapy, radiation therapy, or targeted therapy [2]. Thus, it is imperative to screen for more effective biomarkers to facilitate novel therapeutic methods.

Ring finger protein 113A (RNF113A) is located on chromosome Xq24.9 [3]. It contains a C3H1-type zinc finger domain and a C3HC4 ring-type zinc finger domain. The ring-type zinc finger domain was identified in various tumor suppressors, DNA repair genes, and cytokine receptor-associated molecules, and is probably involved in mediating protein-protein interactions [4, 5]. The C3H1-type RNF113A zinc finger domain is often found in RNA-binding proteins involved in splicing, while zf-C3HC4 is a ubiquitin-related structural domain and is often found in E3 ubiquitin ligases [6, 7]. Several studies have indicated that there are close associations between ubiquitin ligase and the occurrence, development, and metastasis of cancer [8]. Recent studies have shown that RNF113A promotes the proliferation, migration, and invasion of esophageal squamous cell carcinoma (ESCC) cell lines [9]. In research on cervical

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cancer (CC), high expression of RNF113A dramatically promoted proliferation and suppressed autophagy both in vitro and in vivo [10]. However, few studies have reported on the role of RNF113A in CRC.

Therefore, we investigated the relationship between RNF113A expression and prognosis in colorectal cancer, with the hope to provide more insight for research on the diagnosis and treatment of CRC.

Materials and methods

Public datasets

Profiles of TCGA-COAD (Colon adenocarcinoma) and TCGA-READ (Rectal adenocarcinoma) were downloaded from TCGA database (https://portal.gdc.cancer.gov). The mRNA expression data of patients with adenocarcinoma (including 698 colorectal cancer samples and 51 paracancerous samples) were extracted using TPM format, excluding clinical and duplicate information and processed as $\log_2$(value+1). We also downloaded the following gene expression profiles from the GEO database (https://www.ncbi.nlm.nih.gov/geo/): GSE20842 (including 65 CRC samples and 65 paired adjacent nontumor samples), GSE89076 (including 39 CRC samples and 39 paired adjacent nontumor samples), GSE37364 (including 37 CRC samples and 28 adjacent nontumor samples), GSE9348 (including 70 CRC samples and 12 adjacent nontumor samples), GSE35279 (including 74 CRC samples and 5 adjacent nontumor samples) [11-15]. Data were normalized again with the limma package's Normalize Between Arrays function. Data visualization was realized using the ggplot2 package. The Human Protein Atlas (HPA) database (http://www.proteinatlas.org/) was used to verify the expression of RNF113A in CRC at the protein level.

Functional enrichment analysis

Based on the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) interactions, we used the R cluster Profiler package to enrich the 100 genes with the highest correlation with RNF113A in colorectal cancer [16-18]. The protein interaction network of RNA113A was constructed using the String online database (https://string-db.org/).

Immunocorrelation analysis

The relationship between RNF113A expression and the abundance of tumor-infiltrating immune cells (TIICs) in the TCGA-COAD and TCGA-READ datasets was analyzed using the TIMER 2.0 online database (https://www.proteinatlas.org/) [19]. We further used the "chemokine" module in the TISIDB database (http://cis.hku.hk/TISIDB/) to analyze and evaluate the correlation between RNF113A expression and chemokine/chemokine receptor expression levels of TIICs [18].

Cell culture

Human colorectal mucosa FHC cells and colorectal cancer cell lines (HCT116, Caco2 and Colon3) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, C1199-5500BT) or Roswell Park Memorial Institute (RPMI) 1640 (Gibco, 11875119) medium containing 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco, 15140122) in a CO_2 incubator at 37°C.

Western blot

Cells were washed with cold PBS twice. Cold cell lysis buffer (Beyotime, P0013) with protease inhibitors was added and incubated on ice for 30 min. Lysate supernatant was collected after centrifugation (12000 g, 4°C, 10 min). The protein lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and imprinted on polyvinylidene fluoride (PVDF) membranes (Millipore, IPVH00010) for analysis. Anti-RNF113A (1:1000 dilution, Proteintech, 27018-1-AP) and anti-GAPDH (1:5000 dilution, Proteintech, 60004-1) were incubated overnight at 4°C. Horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000 dilution, Beyotime, A0181) were added for 1 h at room temperature. Western blot analysis was interpreted ImageJ software.

Statistical analysis

R version 4.2.1 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism 8.4 (GraphPad Software, Inc., San Diego, CA) were applied for statistical analyses. CRC patients were classified into two groups according to median RNF113A gene expression

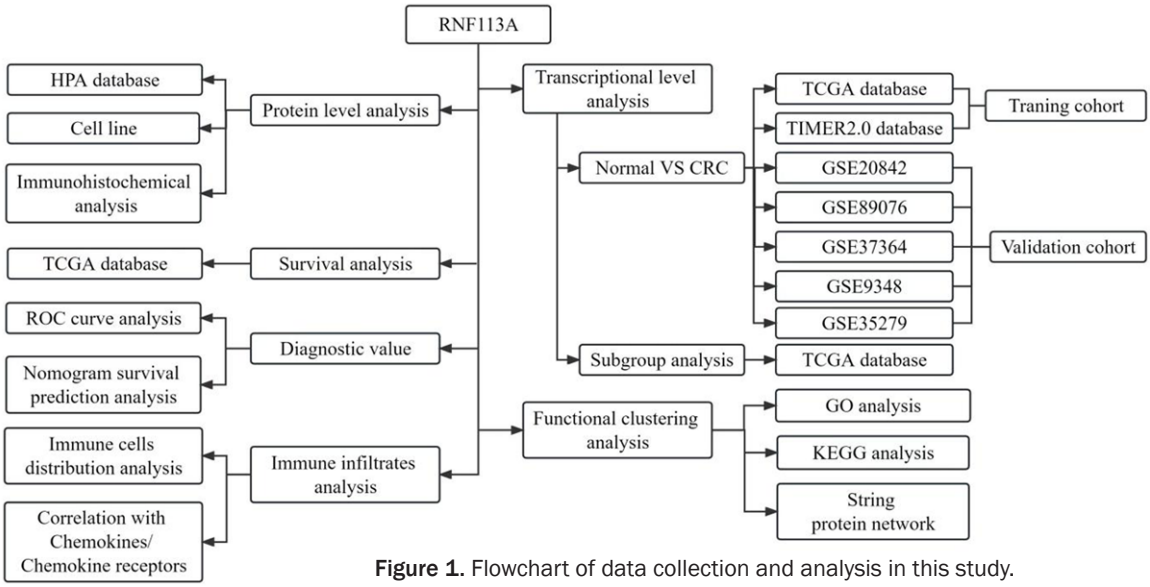

Figure 1. Flowchart of data collection and analysis in this study.

level based on TCGA database: a low RNF113A expression group and a high RNF113A expression group. Overall survival of the two groups was analyzed by Kaplan-Meier curves and Wilcoxon log-rank tests. Cox regression models were employed to perform univariate and multivariate analyses, while Spearman correlation was utilized for evaluating correlation of RNF113A expression with other genes. A *P*-value of less than 0.05 was considered significant.

Results

RNF113A is upregulated in colorectal cancer

The whole process of this study is shown in **Figure 1**. RNA-seq data from TIMER database showed that the mRNA expression of RNF113A was significantly upregulated in the tumor tissues of various cancers, including colon cancer, rectal cancer, bladder cancer, breast cancer, bile duct cancer, esophageal cancer, kidney cancer, hepatocellular carcinoma, lung cancer, prostate cancer, pancreatic cancer, and gastric cancer (**Figure 2A**). Further investigation of RNF113A level in normal tissues and CRC tissues revealed that the expression of RNF113A in CRC tissues was significantly upregulated compared to that of normal tissues (P<0.01) (**Figure 2B**). Similarly, the same expression trend was confirmed in CRC tissues and paired para-cancerous tissues (**Figure 2C**). To analyze the correlation between RNF113A expression and clinical features in CRC patients, we analyzed the mRNA expression of RNF113A in patients with different clinical categories in the TCGA database. The results showed that a high expression of RNF113A was significantly correlated with the clinical TNM stage, pathologic stage, gender, tumor type, and DSS events (**Figure 2D-J**). These data indicate that RNF113A is significantly upregulated in CRC.

Validation using independent external databases and clinical specimens

To further explore the expression of RNF113A in CRC, we analyzed seven other profiles from GEO datasets to determine the expression of RNF113A mRNA in CRC tissues and non-cancerous tissues (**Figure 3A-G**). The results showed that the transcription level of RNF113A in colorectal cancer tissues was significantly elevated compared to unmatched para-cancerous tissues. Similarly, the trend of RNF113A expression in colorectal cancer was consistent across matched sample analyses in two of the datasets. We further investigated RNF113A protein levels among colorectal cancer cell lines (HCT116, Caco2, Colon3) and compared it with that of human colorectal mucosa FHC cells. The results indicated the protein levels of RNF113A were increased in cancer cells (**Figure 3H**, **3I**). Moreover, the expression level of RNF113A in CRC was higher than that in normal colon tissue (**Figure 3J**). These data confirmed a high expression of RNF113A in CRC.

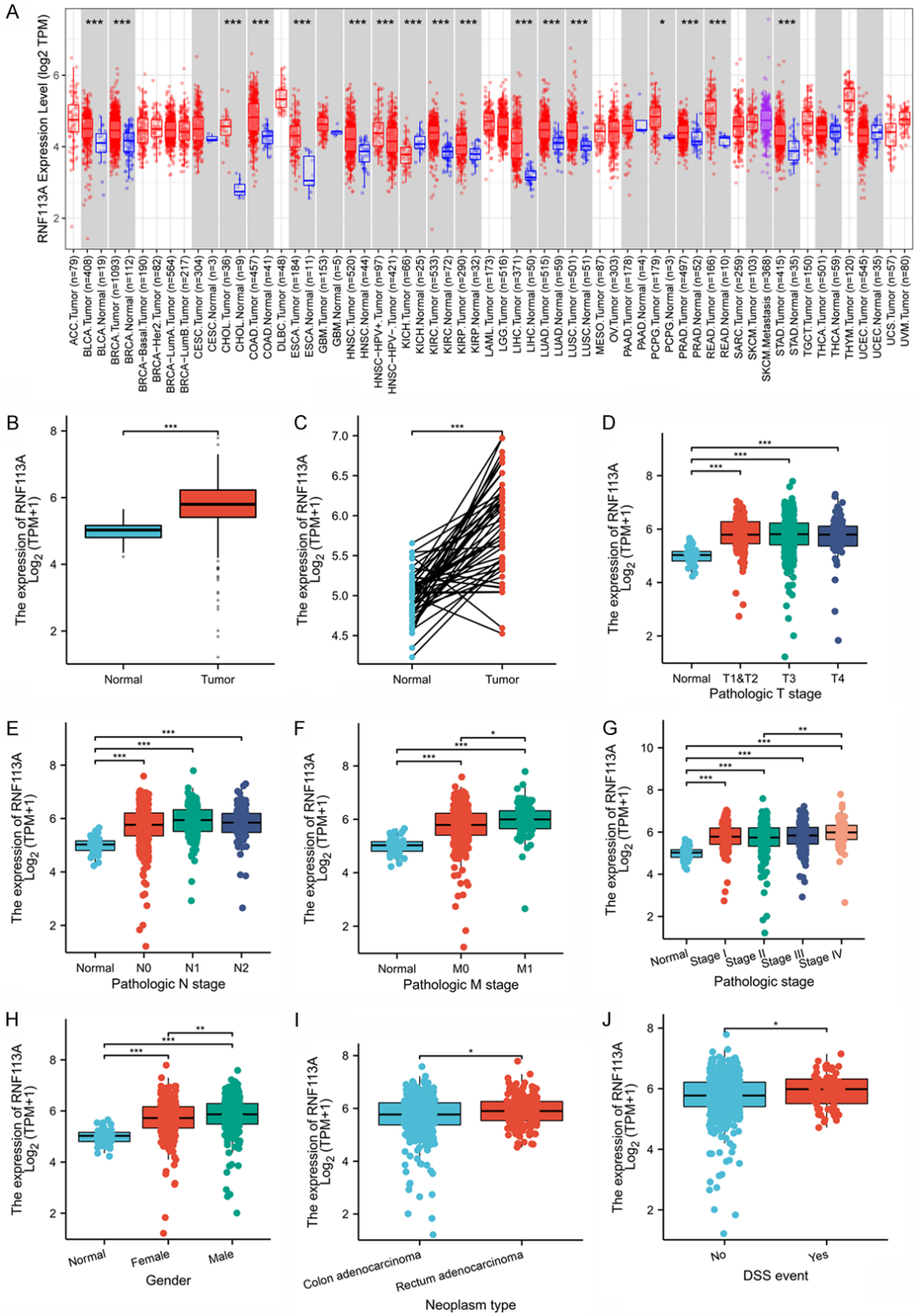

Figure 2. Expression of RNF113A in different human cancers. (A) TIMER database was used to detect the expression levels of RNF113A in different tumors; (B-J) Expression level of RNF113A in normal tissues (B), paired adjacent tissues (C), and the tumor tissues from patients with different clinical characteristics in the TCGA database [T stage (D), N stage (E), M stage (F), clinical stage (G), Gender (H), Neoplasm type (I), and DSS event (J)]. Compared to normal group, *P<0.05, **P<0.01, ***P<0.001.

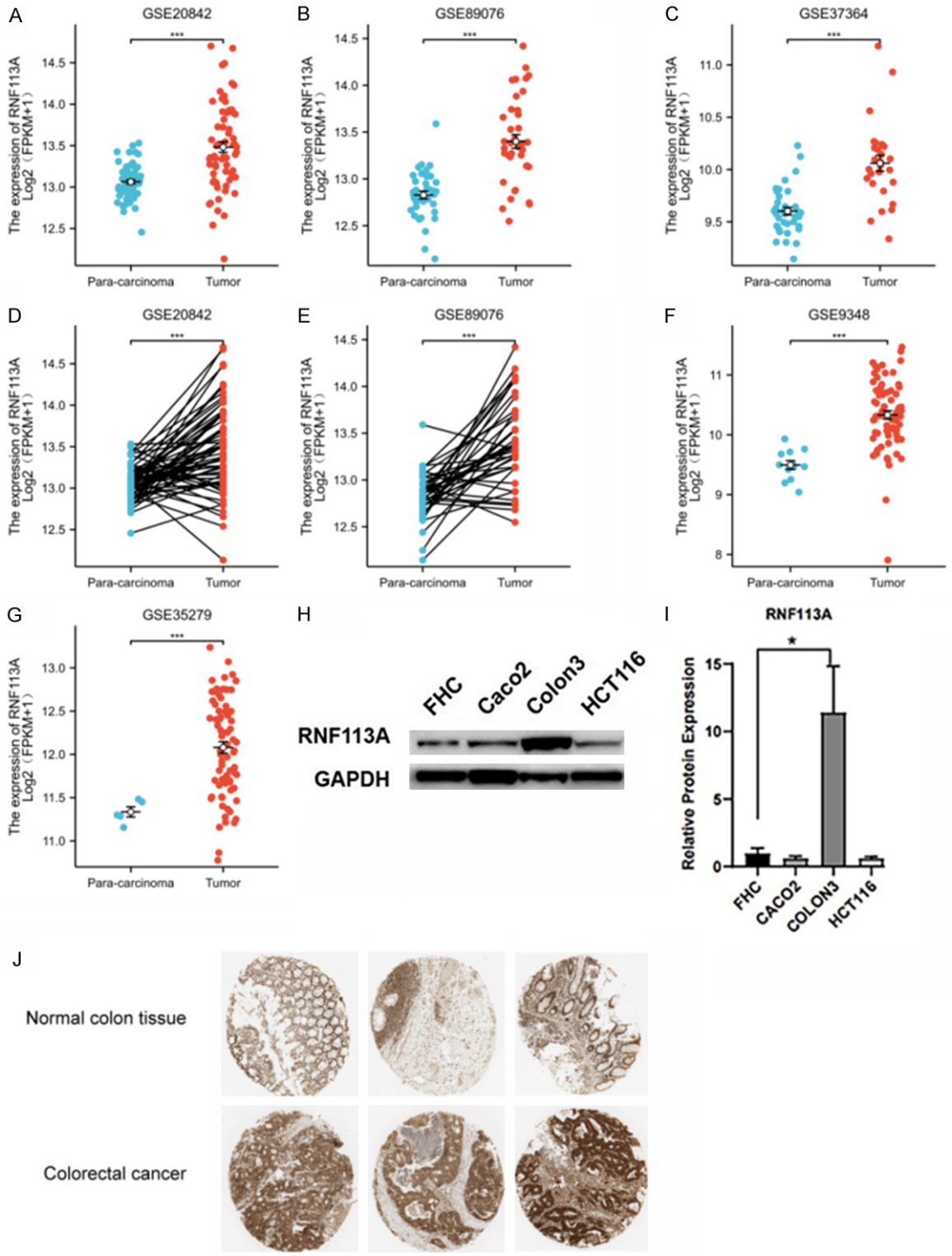

Figure 3. The expression of RNF113A in CRC was validated using the Gene Expression Omnibus (GEO) database, cell lines, and the Human Proteome Atlas (HPA) online database. A-E: Expression of RNF113A in tumor and unpaired para-carcinoma tissues of the GSE20842, GSE89076, GSE37364, GSE9348, and GSE35279 datasets in the GEO database; F, G: Expression level of RNF113A in tumor and paired adjacent tissues in the GSE20842 and GSE89076 of the GEO database. H, I: Protein expression of RNF113A in colorectal mucosa cell lines and CRC cell lines; J: Validation of the expression level of RNF113A in CRC using the HPA database. Compared with para-carcinoma group, $^{*}P<0.05$, $^{***}P<0.001$.

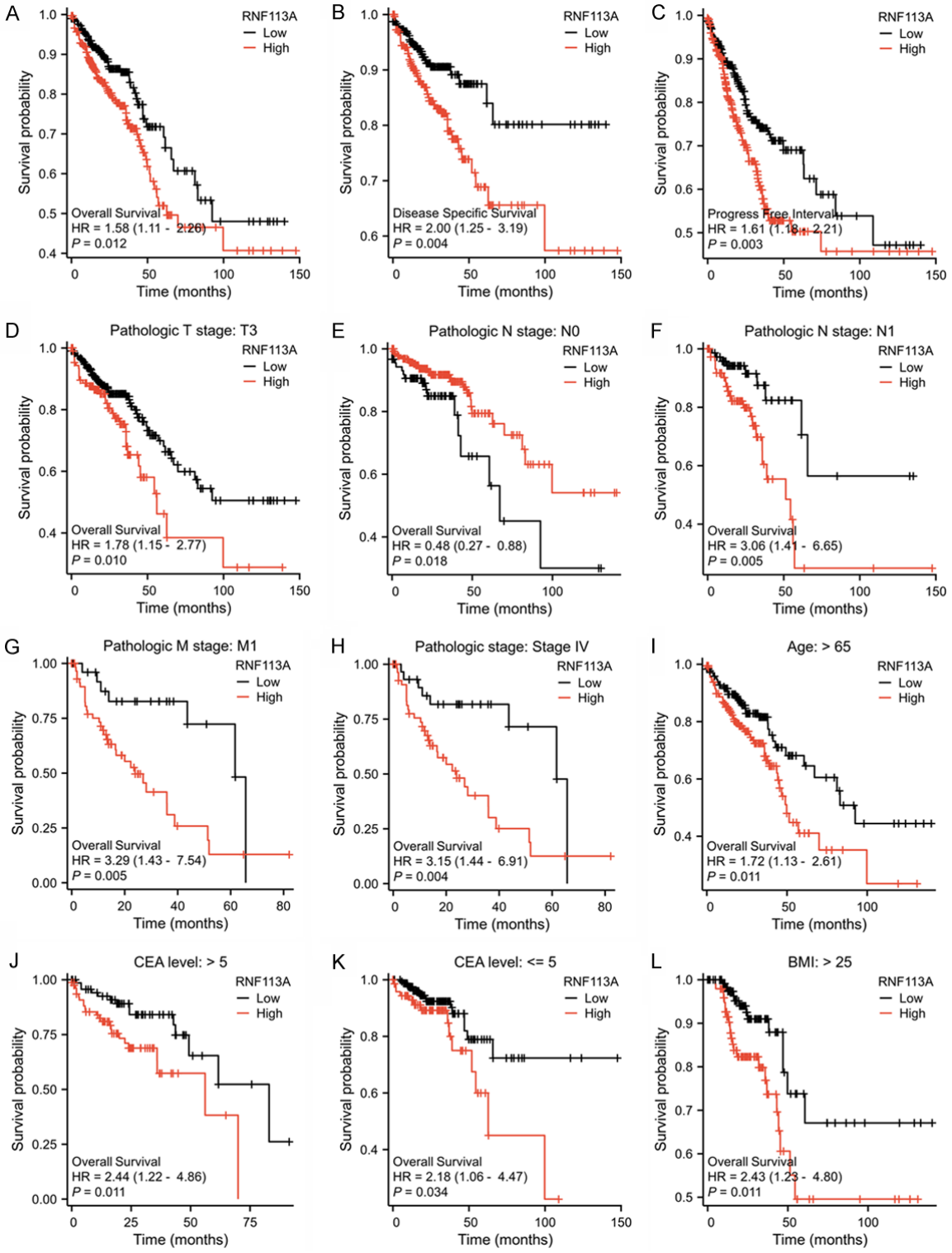

Figure 4. Kaplan-Meier survival analysis of the prognostic significance of RNF113A in CRC. (A-C) Kaplan-Meier estimates of the survival probability [overall survival probability (A), disease-specific survival probability (B) and progression-free interval survival probability (C)] of TCGA patients in all CRC patients; (D-L) Subgroup analysis based on different clinicopathologic characteristics: T3 (D), N0 (E), N1 (F),M1 (G),stage IV (H), age greater than 65 years (I), CEA greater than 5 (J), CEA smaller than 5 (K), and BMI greater than 25 (L).

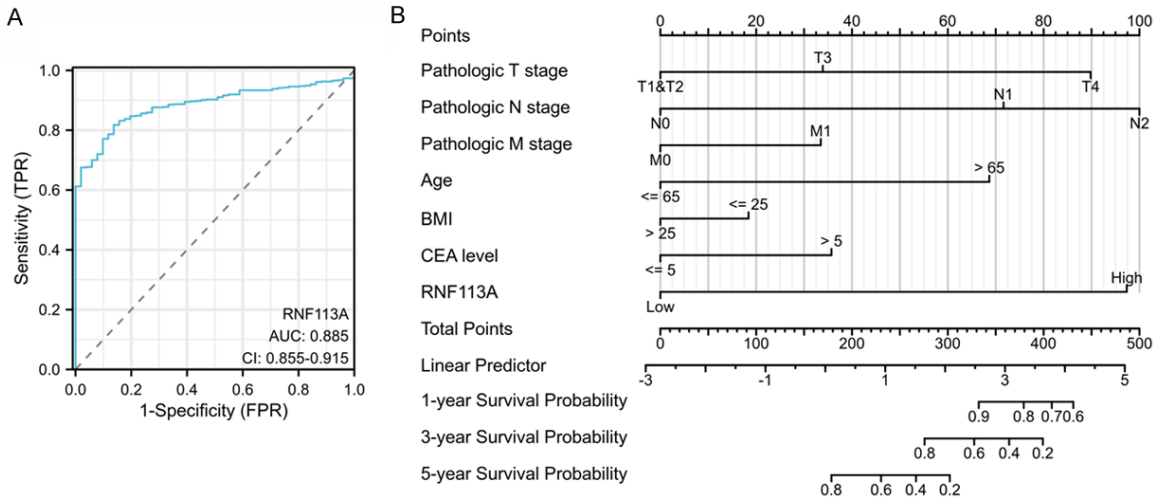

Figure 5. Diagnostic value of RNF113A in CRC. A: Receiver operating characteristic (ROC) curve analysis for RNF113A expression in CRC and adjacent tissues; B: Nomogram survival prediction chart for predicting the 1-year, 3-year, and 5-year overall survival rates.

High expression of RNF113A predicted poor clinical outcome of CRC

To determine whether the expression of RNF113A is associated with patient prognosis, we divided the CRC cases in the TCGA database into two groups according to RNF113A level: a high RNF113A expression group and a low RNF113A expression group. Kaplan-Meier survival analysis showed that high expression of RNF113A was associated with poor prognosis, including overall survival, disease-specific survival and progression-free survival, in CRC patients (**Figure 4A-C**). Further analyses on the survival of patients with high/low RNF113A expression across different pathologic characteristics showed that high expression of RNF113A was significantly associated with poor prognosis of CRC patients in the following groups: T3, N0, N1, M1, pathological stage IV, patients over 65 years old, CEA level less than 5, and BMI over 25 (**Figure 4D-L**). These data suggest that high expression of RNF113A is an independent prognostic factor for OS in CRC patients.

To analyze the diagnostic value of RNF113A expression in CRC, a receiver operating characteristic (ROC) curve was drawn, and the results showed that the area under the ROC curve (AUC) of RNF113A for diagnosing CRC was 0.885, suggesting high diagnostic performance (**Figure 5A**). We combined expression levels of RNF113A with clinical variables to construct a nomogram to predict patient survival probabilities at 1, 3, and 5 years. The nomogram showed that RNF113A expression level was superior to traditional clinical features such as age in predicting CRC prognosis (**Figure 5B**). In summary, RNF113A may be a candidate prognostic biomarker for OS in CRC patients.

RNF113A is closely related to rRNA processing - related genes in CRC cells

To explore the biological function of RNF113A in colorectal cancer, the co-expression pattern of RNF113A in CRC was analyzed using TCGA database. According to the highest Spearman correlation coefficient, 100 positively correlated genes and 100 negatively correlated genes were screened, and functional enrichment analyses of GO and KEGG were performed using the R clustering Profiler software package. The results showed that the rRNA and ribosome processing related pathways in colorectal cancer were enriched in these genes, and the rRNA processing pathway was most significantly enriched (**Figure 6A**).

A heat map was constructed to determine whether RNF113A is associated with the 205 rRNA processing associated genes in CRC, and the results showed that RNF113A upregulation in colorectal cancer was significantly correlated with these genes (**Figure 6B**). In addition, a protein-protein interaction network of these associated genes was constructed

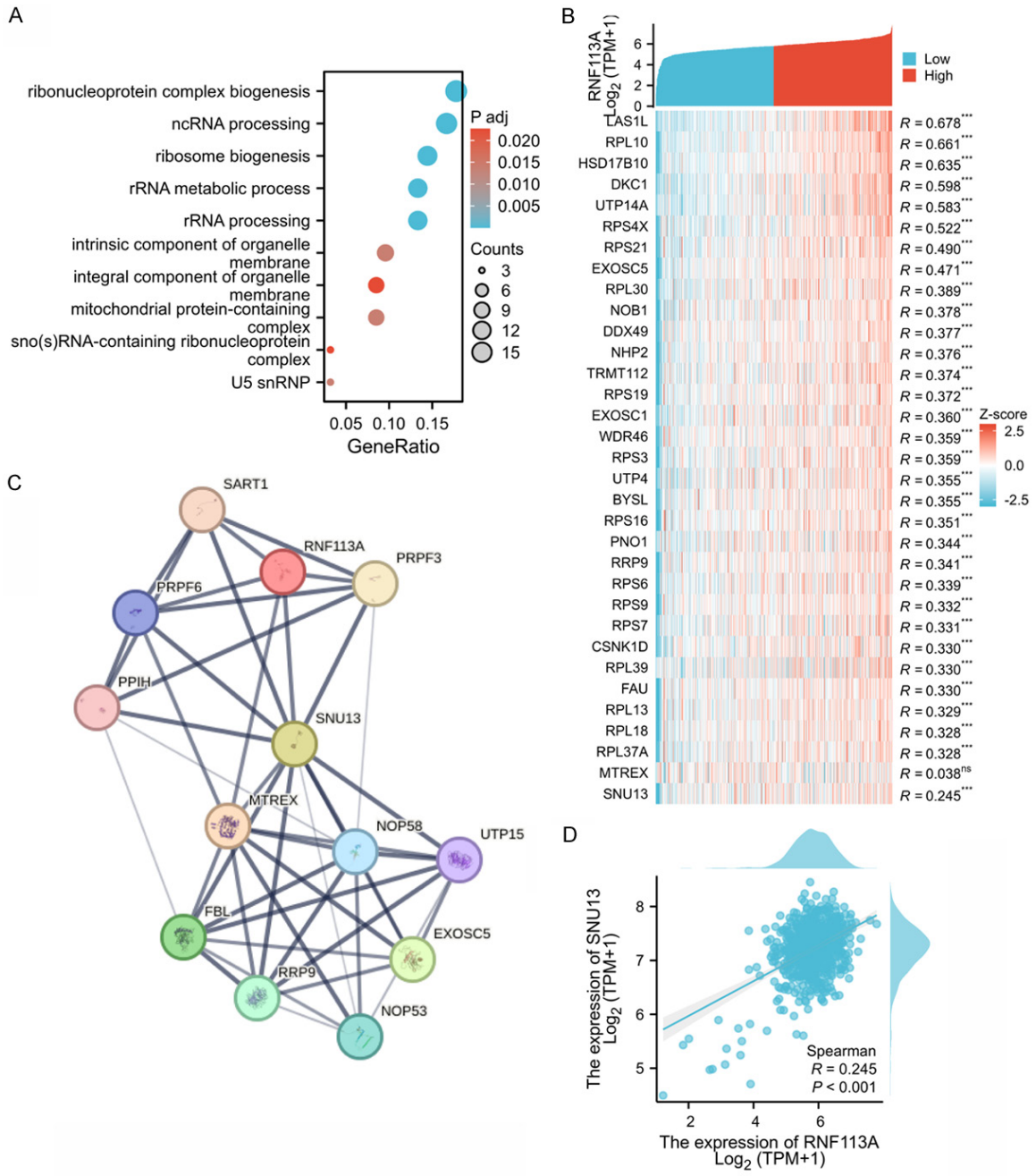

Figure 6. RNF113A functional clustering and interaction network analysis of RNF113A-related genes. A: Gene Ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of RNF113A-related genes in colorectal cancer (CRC); B: rRNA processing related genes that significantly associated with RNF113A as shown by heatmap. Red represents positively-correlated genes and blue represents negatively correlated genes; ***P<0.001; C: The string online database predicts the protein interaction network of RNF113A; D: Correlation between the expression of RNF113A and SNU13 in CRC.

(**Figure 6C**). Results showed that rRNA processing signaling pathway-related gene SNU13 was positively correlated with RNF113A (R=0.245, P<0.001) (**Figure 6D**). In summary, RNF113A is closely related to the regulation of rRNA processing signaling pathways in colorectal cancer.

Correlation of RNF113A expression with immune characteristics

Infiltration of immune cells in the tumor microenvironment can affect tumor progression. To explore the relationship between RNF113A expression and tumor immune response, we

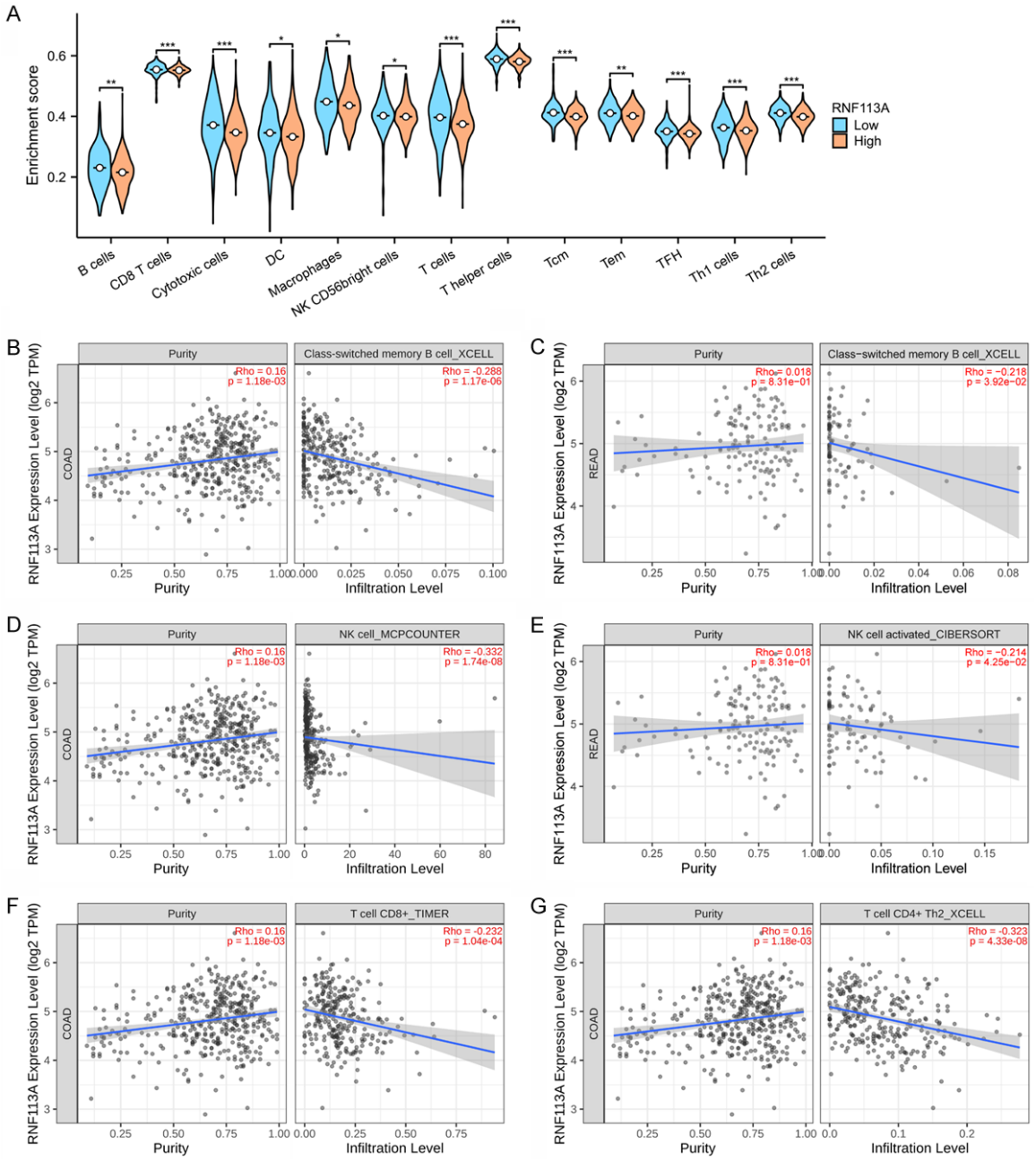

Figure 7. Correlation analysis of RNF113A expression and immune infiltration in CRC. (A) Differential distribution of immune cells in patients with high and low expression of RNF113A; (B-G) Correlation between the expression level of RNF113A and tumor purity in B cells (B, C), NK cells (D, E), and T cells (F, G). Compared with low RNF113A expression group, *P<0.05, **P<0.01, ***P<0.001.

used TIMER database to study immune cell infiltration in CRC patients with different RNF-113A expression levels. The results showed that the infiltration levels of B cells, $CD8^{+}$ T cells, dendritic cells (DCs), macrophages, central memory T cells, effective memory T cells, and follicular helper T cells in CRC patients with high RNF113A expression were significantly lower than those in patients with low RNF113A expression (**Figure 7A**). We further analyzed the correlation between the RNF113A expression in CRC and immune infiltration, and the results showed that the expression level of RNF113A was negatively correlated with the infiltration level of memory B cells, NK cells, Th2 cells, $CD8^{+}$ T cells (**Figure 7B-G**). Therefore, these

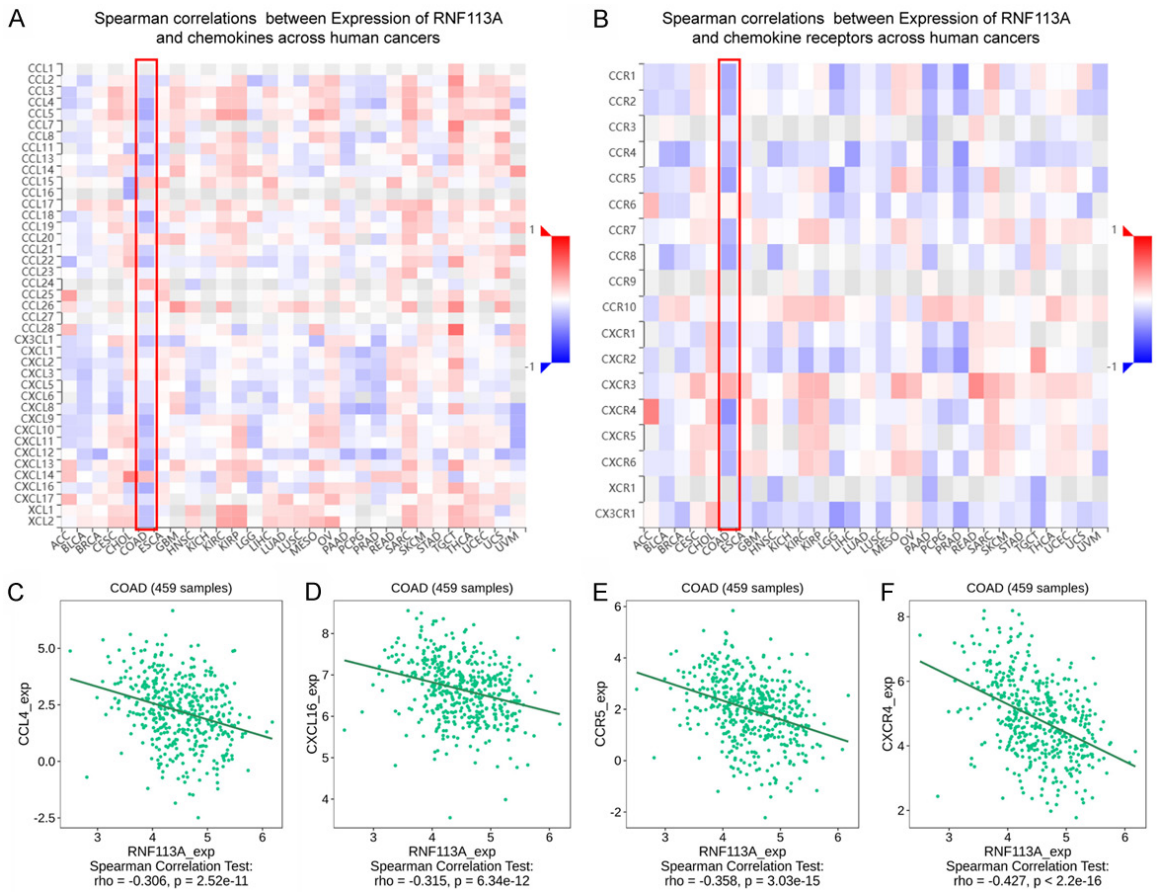

Figure 8. Correlation between RNF113A expression and chemokine/chemokine receptor expression levels of TIICs was analyzed by TISIDB database. (A, B) Correlation between chemokine (A)/chemokine receptor (B) expression levels and RNF113A expression in different cancers; (C-F) Correlation between CCL4 (C), CXCL16 (D), CCR5 (E), and CXCR4 (F) expression levels and RNF113A expression.

data suggest that RNF113A may inhibit the tumor immune response of CRC by negatively regulating $CD8^+$ T cells, B cells, DC, and macrophage infiltration into tumors.

Chemokines and chemokine receptors are necessary for immune cells to infiltrate the tumor. Therefore, we analyzed the correlation between the expression level of RNF113A in CRC and immune cell chemokines and chemokine receptors. The heat map results displayed a significant relationship between RNF113A level and chemokines and chemokine receptors in CRC (**Figure 8A**, **8B**). To further elucidate the relationship between RNF113A expression and immune cell migration, we comprehensively analyzed the correlation between RNF113A expression and chemokine/chemokine receptor. The results showed that RNF113A expression was negatively correlated with CCL4, CXCL16, CCR5, and CXCR4 (**Figure 8C-F**). Subsequently, we analyzed the expression of RNF113A with immune-inhibitors and immune-stimulators in different types of human cancers using the TISIDB database (**Figure 9A**, **9B**). Interestingly, RNF113A was negatively correlated with the expression of both immune-inhibitors and immune-stimulators, including PDCD1, CTLA4, CXCR4, and IL2RA (**Figure 9C-F**). These results suggest that the RNF113A gene plays an important role in tumor immunity.

Discussion

Ring finger protein 113A (RNF113A) has a RING domain, which is often found in E3 ubiquitin ligases and is involved in the regulation of the stability of E2 and E3 family proteins [6, 7]. In addition, RNF113A has a C3HC4 zinc finger domain, which is found in E3 ubiquitin ligase

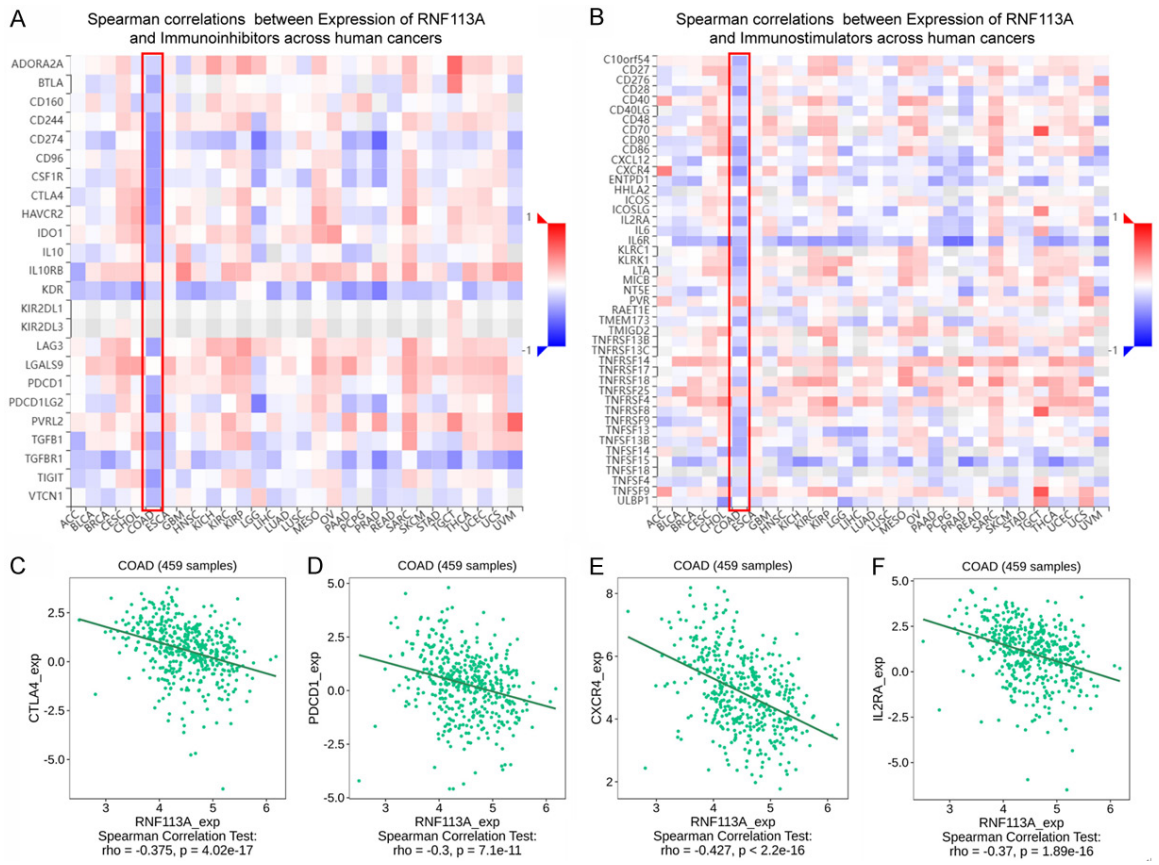

Figure 9. The correlation between RNF113A expression and immunoinhibitors/immunostimulators expression levels in TIICs was analyzed by TISIDB database. (A, B) Correlation between (A) immunoinhibitors' or (B) immunostimulators' expression levels and RNF113A expression in different cancers. (C-F) Correlation between immunoinhibitors/immunostimulators expression levels and RNF113A expression [CTLA4 (C), PDCD1 (D), CXCR4 (E), IL2RA (F)].

and is involved in tumorigenesis [20]. Increasing studies have shown that RNF113A is involved in many biologic processes, such as cell proliferation, survival, and differentiation. In recent years, the role of RNF113A in tumor has received increasing attention. RNF113A has been found to be associated with metastasis and poor prognosis in cervical cancer, esophageal cancer, and lung cancer. However, there were few studies on the role of RNF113A in CRC. In this study, the biological activity of RNF113A and its possible regulatory pathways were comprehensively investigated through bioinformatic analysis. RNF113A was found to be up-regulated in CRC, and its high expression was associated with poor clinicopathologic features. In addition, RNF113A was useful for diagnosis and prognosis of CRC. These findings strongly suggest that RNF113A is an oncogene and prognostic biomarker.

To analyze the molecular mechanism of RNF113A-mediated CRC progression and poor prognosis, we performed GO functional enrichment and KEGG pathway analysis, and found that RNF113A was closely related to the regulation of rRNA processing signaling pathways in colorectal cancer. In addition, we also found that RNF113A was positively correlated with rRNA processing signaling pathway-related gene SNU13, a key component in assembling the spliceosome [21]. Our findings are consistent with other studies. Research has found that RNF113A is important on spliceosome activation, mediating interactions among the core components [22]. Other studies have also shown that RNF113A is an RNA-binding protein which regulates the splicing of multiple candidates involved in cell survival [23]. Pre-messenger RNA (mRNA) splicing is an essential step in the control of eukaryotic gene expression. Therefore, RNF113A may influence tumor

cell survival by regulating the splicing of related genes.

The development of tumor is related to its surrounding environment and tumor microenvironment (TME), in which immune cell infiltration affects the process of tumor occurrence, development, and metastasis [24]. This study investigated a correlation between the expression of RNF113A and the level of immune infiltration in CRC. Our results showed a negative correlation between high RNF113A expression and the infiltration level of memory B cells, NK cells, Th2 cells, and $CD8^+$ T cells. In addition, chemokines and their receptors play an important role in the directed migration of immune cells [25]. We further used the TISIDB database to analyze the correlation between the expression level of RNF113A in CRC and the expression of chemokines and chemokine receptors in immune cells. The results showed that RNF113A expression was negatively correlated with CCL4, CXCL16, CCR5, and CXCR4. These results suggest that high expression of RNF113A may inhibit the migration of immune cells into the TME and play a regulatory role in the tumor immune microenvironment of CRC.

We also found that the expression level of RNF113A was negatively correlated with the expression of both immune-inhibitors and immune-stimulators, including PDCD1 (PD1), CTLA4, CXCR4, and IL2RA (CD25). Both PDCD1 and CTLA4 are critical immune checkpoint proteins [26]. Antibodies targeting PCDC1 and its ligand PD-L1 can reactivate exhausted T cells to restore the immune response against cancer cells, and their use in clinical trials has been a success [27]. CTLA4 is highly expressed in regulatory T cells (Tregs), which play a vital role in the immune suppression of tumor development [28]. Moreover, IL2RA (CD25) is another maker for Tregs and participates in the regulation of immune tolerance by controlling the activity of Treg cells, so as to inhibit the activation and proliferation of effector T cells [29]. A high expression level of CXCR4 has been reported in more 23 kinds of tumors, and CXCL12/CXCR4 axis targeted therapy has shown promising value in cancer treatment [30]. Our findings indicate that the high expression of RNF113A may negatively impact the immunotherapy response in CRC.

In summary, the expression of RNF113A is significantly upregulated and strongly associated with poor prognosis in CRC patients. RNF113A has value for the diagnosis and prognosis of CRC. RNF113A may influence the progression of CRC by regulating protein processing and immune infiltration. It may serve as a novel prognostic biomarker for CRC patients. However, there are some limitations of this study, which we hope to overcome soon. First, the comparability and reproducibility of microarray data generated in different laboratories remains controversial, which can lead to systematic bias. Second, to improve the reliability of our findings, we need to conduct more *in vivo/in vitro* experiments to demonstrate an effect of RNF113A on tumor cells. Third, there is no direct evidence that RNF113A influences the prognosis of CRC by participating in immune invasion, although a close relationship between RNF113A expression and immune invasion and the prognosis of CRC was observed in this study. The impact of RNF113A expression on chemotherapy also deserves further evaluation.

Disclosure of conflict of interest

None.

Address correspondence to: Wenli Cheng, Department of Blood Transfusion, The Sixth Affiliated Hospital, Sun Yat-sen University, No. 26, Erheng Road, Yuan Village, Tianhe District, Guangzhou 510655, Guangdong, China. Tel: +86-13533-189135; E-mail: chengwl@mail.sysu.edu.cn

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