RNF24 is a Novel Biomarker of Prognosis and Immunological Cell Infiltration in Cancers

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Research Article

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RNF24 is a Novel Biomarker of Prognosis and Immunological Cell Infiltration in Cancers

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Abstract

Background: The RNF family engaged in diverse biological and pathological processes, including tumorigenesis and cancer advance. However, studies about Ring Finger Protein 24 (RNF24) were limited and have not been reported in cancer. A systematic analysis in pan-cancer is a benefit to understand the function of RNF24.

Methods: RNF24 expression was evaluated in pan-cancer based on the data from The Cancer Genome Atlas (TCGA) analyzed by TIMER, UALCAN, GEPIA, and HPA. Then, the effect of RNF24 on the prognostic value was assessed by clinical survival data in Kaplan–Meier Plotter and GEPIA. And mutation burden and related survival of RNF24 was observed in cBioPortal. Furthermore, protein-protein interaction (PPI) networks of RNF24 and pathway enrichment analysis were explored on multiple websites. Lastly, relationships between RNF24 expression and immune cells infiltration were analyzed in the TIMER2 online database with various algorithms.

Results: The mRNA and protein levels of RNF24 were significantly upregulated in most types of cancer compared to normal tissues. And RNF24 was a reliable biomarker to predict prognosis in at least 10 types of cancer, including liver hepatocellular carcinoma (LIHC). In addition, we showed the

genetic alteration, PPI networks, and functional pathway of RNF24. Moreover, immune cell infiltration exhibited RNF24 expression negatively linked to CD8+ T cells, but positively to Tregs, MDSCs, HSC, and macrophages in pan-cancer.

Conclusions: Our pan-cancer analysis revealed RNF24 as an oncogene and its expression predicted OS in multiple human cancers, especially in LIHC. RNF24 might predict the immunotherapy response for cancer patients based on its expression with infiltration of immune and immunosuppressive cells.

Introduction

Cancer has been a leading reason for death and the burden of cancer is quickly expanding in the global world according to Global Cancer Statistics in 2020 [1]. Primary liver cancer ranks sixth in diagnosed cancer and third in cancer-related fatality based on the latest data [1]. There is still a great need to find out new biomarkers and targeted to separate patients into distinct risk groups and gain effective treatment. And novel genes with pan-cancer analysis are helping to better understand the extremely complex process of tumorigenesis and to confirm which cancer to target may gain more benefit.

T cell dysfunction that is influenced by multiple suppressive signals has been known to be the main cause of immune escape of cancer cells and limit the efficiency of cancer immunotherapy. Blockade of PD-1, or its ligand PD-L1, has been verified to effectively improved T cell function [2]. And antibodies targeting PD-1/PD-L1 have achieved a prominent clinical outcome in various cancer, such as non-small-cell lung cancer (NSCLC) [3], head and neck squamous cell cancer (HNSCC) [4]. However, the objective response rates (ORR) were limited (range from 15% to 34%) in these cancers with immune therapy[5]. Many cancers respond poorly to immune checkpoint blockade, including liver cancer[6]. In addition, immunosuppressive cell accumulation in the tumor microenvironment (TME) is the other major tumor escape mechanism [7]. For example, regulatory T cells (Tregs) are important immunosuppressive cells that facilitate tumor advance by impeding T cell functions [8]. Myeloid-derived suppressor cells (MDSCs) as another immunosuppressive cell have been proved to accumulate in cancer patients [8]. MDSCs take part in tumor progression by inhibiting the effector immune response [8, 9]. Thus, it is important to identify key bio-markers that screen out immune benefit patients with precision medicine.

The RNF family belongs to families of E3 ubiquitin ligase, which are engaged in various biological and pathological processes, including taking part in cell signaling [10], tumorigenesis [11], cell epithelial-mesenchymal transition (EMT), stemness [12] control of antiviral innate immunity [13], and protein degradation by ubiquitination [14]. RNF24 is a 148 amino-acid long protein that includes a RING-H2 domain. RNF24 has drawn attention as an interactor with all TRPCs [15]. However, the research about RNF24 is little. It was only one paper in cancer that showed its expression was upregulated in esophageal adenocarcinoma (EAC) so far.

Here, we investigated the function of RNF24 using a pan-cancer analysis in various cancer types. We reported that the RNF24 expression level was abnormal in many tumors, and its expression had a prognostic value, especially in LIHC. We also analyzed its mutation status and possible signal pathway of function in TCGA pan-cancer. Moreover, we showed significant associations between RNF24 and immunosuppressive cell accumulation in the TME. This study firstly demonstrated the prognostic value of RNF24 in pan-cancer and its correlation with immunosuppressive cells, which may benefit to offer a new drug target for antitumor strategy.

Results

RNF24 expression is significantly up-regulated in most human cancers compared to normal tissues

To contrast the expression of RNF24 between normal tissues and tumors, we first explored it in the Oncomine database. The result showed the mRNA level of RNF24 was significantly higher in some cancers, such as colorectal cancer (Fig.1A). We further examined in TCGA [16], we found that RNF24 tended to be significantly upregulated in bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), head and neck squamous cell carcinoma (HNSC), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), rectum adenocarcinoma (READ), stomach adenocarcinoma (STAD), thyroid carcinoma (THCA), uterine corpus endometrial carcinoma (UCEC), but downregulated in kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP) (Fig.1B). While the number of normal tissue data is smaller than tumor analyzed by UALCAN [16], we again evaluated the expression of RNF24 between tumor and normal tissues by the TIMER database [19]. Most of the results were consistent between TCGA and TIMER database, besides BRCA and esophageal carcinoma (ESCA) (Fig.1C).

In addition, we showed the analysis data of RNF24 with notable changes from the GEPIA website. However, about half of the results were inconsistent with the first two databases (Fig.1D). To detect the protein expression of RNF24, we checked IHC results presented on the HPA website (Fig.2A). We found that the protein expression ratios of liver cancer, pancreatic cancer, endometrial cancer, ovarian cancer, breast cancer, and glioma were more than 50%, and liver cancer owned the highest ratios with RNF24 upregulated (Fig.2A-B). The above results indicated that RNF24 was overexpressed in most cancer tissues, except in kidney relative cancers, and might have a function in the process of cancer.

RNF24 has a prognostic value for pan-cancer

To explore whether the abnormal expression of RNF24 had clinical value in human cancers, we performed survival analysis according to RNF24 expression. We firstly investigated in GEPIA website[17] and found that higher expression of RNF24 was associated with poorer overall survival (OS) in LIHC, sarcoma (SARC), adrenocortical carcinoma (ACC), UVM, and KIRP (Fig.3A-F). And disease-free survival (DFS) of RNF24 showed that the high expression group had a poor prognosis in 7 cancers, including LIHC, ACC, and UVM (Fig.3G-J). We then observed the prognostic value of RNF24 in 18 types of cancer through Kaplan–Meier Plotter (Fig.4A). The results exhibited that high expression of RNF24 was a risk factor of OS in LIHC, SARC, KIRP, PAAD, PCPG, UCEC, STAD, LUSC (Fig.4B-I). Taken together, these findings implied that the expression level of RNF24 was closely related to the prognosis of multiple cancers.

RNF24 is a prognostic biomarker of LIHC patients

To show the exactly prognostic value of RNF24 in specific cancer, we picked up LIHC for further evaluation. We inspected several databases to study the RNF24 expression in the development of LICH. The expression level of RNF24 was gradually increased from grade 1 to 4 as well as from stage 1 to 4 (Fig.5A-B). In addition, RNF24 was upregulated in LIHC metastasis (Fig.5C-D). There was no difference in women and men about RNF24 expression (Fig.5E). Hepatocellular carcinoma (HCC) is the most common type of LIHC, it always development in chronic inflammation and cirrhosis [22, 23]. We noticed RNF24 expression raised in HCC relative to cirrhosis base on HCCDB [24] (Fig.5F). The methylation level in promoter area of genes always affects themselves expression [25], and the RNF24 methylation level in its promoter was gradually declined (Fig.5G). It was a serious possibility that RNF24 expression was regulated by methylation level in LIHC, which similar

to many DNA methylations always repressed transcription [25]. Then, we detected the prognostic value of RNF24 based on pathological grading, basic information, and risk factors of LIHC patients (Fig.6A). The data analysis in Kaplan-Meier Plotter revealed that high expression of RNF24 had a poor survival in almost pathology, besides grade 3. Similar conclusions were gained from patients with or without a risk factor, such as alcohol consumption, hepatitis virus. However, there was no prognostic value between RNF24 high- and low- groups in women (Fig.6A). It is about 90% of primary liver cancers is hepatocellular carcinoma (HCC) [22], we furthermore evaluated the OS by RNF24 expression in tumor or adjacent tissues. The result was similar to Fig.3B and Fig.4B in tumor, but it was no significant in adjacent tissues (Fig.6B-C). Finally, we discovered the OS with two factors, RNF24 expression combine with grades or sex, in LIHC patients by UALCAN websites. The consequences showed that RNF24 was a prognostic biomarker regardless of patient grades (Fig.6D). And RNF24 was a remarkable biomarker for OS of men but not women (Fig.6E), which resemble to Fig.6A. These above results proved RNF24 was a meaningful biomarker for predicted survival of LIHC patients.

Genetic alteration analysis of RNF24

Due to genome instability is an important factor in cancer development [26], we explored the RNF24 genetic alterations in pan-cancer samples. Based on our analysis, the frequency of RNF24 alteration is highest in ovarian carcinoma with “amplification” for the main type (Fig.7A). Both PAAD and UCEC had a higher incidence of “mutation” type, and we showed the main mutations and their location (C104Y) within RNF24 (Fig.7A-B). As RNF24 protein contains a RING-type zinc finger, we displayed its 3D protein structure (Fig.7C). There are at least 21 types of mutation in RNF24, and 5 types within the RING-H2 domain (Fig.7A). We also presented genes with the highest frequency of mutations in the RNF24 alter group compared to the unaltered group, including MAVS, AP5S1, PANK2, SMOX, CENPB, CDC25B, SIGLEC1, LINC01433, SPEF1, ADRA1D (Fig.7C). To make sure the relationship between genetic alterations of RNF24 and the clinical survival prognosis of cancer patients, we analyzed it on the cBioPortal website. The results revealed that patients with genetic alteration of RNF24 had a poorer prognosis in PFS (P=0.0122), OS (P=0.008917), and DFS (P=0.0358), compared to patients without RNF24 alterations (Fig.7E-G). In a word, these outcomes suggested that RNF24 genetic alterations might be considered as a risk factor for cancer development.

Functional enrichment analysis of RNF24

The reported about the function of RNF24 is little, especially in disease. Protein-protein interaction (PPI) networks might help us to clearly understand its molecular mechanisms. Here, we aimed to gain a network of RNF24 related protein interactions by using GeneMANIA online database [27]. The network is mainly consisted of physical interactions, co-expression, co-localization, and shared protein domains (Fig.8A). TRPC6 as the physical interactions predicted in GeneMANIA has been verified interacted with RNF24 through GST pull-down and co-immunoprecipitation assays [15]. While RNF24 also interacted specifically with other members of the TRPC family [15], it is not shown in GeneMANIA. We again explored it in the STRING websites [28], which including known and predicted interactions (Fig.8B). FpClass was another PPIs website used in our paper, as it obtained better agreement with experimental results [29]. The outcomes demonstrated in table 1, and involving cancer advance molecular LGALS1[30], PSG9 [31], CD44 [32]. To study the molecular mechanism of RNF24, we selected the known RNF24-interacting proteins and the three websites predicted genes for the pathway enrichment analyses using the DAVID online tool [21] (Fig.8C-D). And the analysis data showed that most of these genes are linked to take party in calcium ion transport and protein polyubiquitination in the BP (biological process) category, plasma membrane in the CC (cellular component) category, and calcium channel activity in the MF (molecular function) category (Fig.8C-E). We also detected in Metascape [20] and observed enrichment in second messengers, thyroid cancer, metastasis, immune cell change pathway (Fig.8F)

Immune cells infiltration of RNF24 in patients with cancer

Taking into account the role of RNF24 described above, we hypothesized that abnormal RNF24 expression level or its genetic change might influence the infiltrating immune cells response in cancer. Studies have proven that immune cells infiltration, especially immunosuppressive cells accumulation in the TME has a great influence on immunotherapy [7]. Consequently, we searched the relationship between RNF24 expression and immune-related cells infiltration in human cancers (Fig.9, Table 2-3). Firstly, we executed exploration of the link between immune T cell of CD 8+ infiltration and expression of RNF24 across various cancers by TIMER2, EPIC, MCPCOUNTER, CIBERSORT, CIBERSORT-ABS, QUANTISEQ, XCELL, naïve_XCELL, central memory_XCELL, and effector memory_XCELL with mutiple algorithms (Fig.9A). In most cancers, a negative correlation was discovered between RNF24 expression level and infiltration of CD8+ T cell, for example, HNSC (Head and Neck squamous cell carcinoma), THYM (Thymoma), UCEC. It's worth noting that the negative relationship was existed in metastasis but not primary cancer in SKCM

(Skin Cutaneous Melanoma). While the positive correlation was only observed in UVM (Uveal Melanoma). It is widely known that some immunosuppressive cells, for instance, Tregs[33], MDSCs [34, 35], or HSC, are recruited by tumors and having antitumor immunosurveillance. Then, we observed their correlation with RNF24 in diverse websites. A positive correlation was observed between RNF24 and Tregs in LICH, COAD, PCPG (Pheochromocytoma and Paraganglioma), and THCA (Thyroid carcinoma) (Fig.9B). In addition, 20 types of cancer had a strikingly favorable association between RNF24 and MDSCs (Fig.9B). Tumor-associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs) were closely related to the occurrence and development of cancer [36]. Lastly, we further investigated their relationship and the results showed a significant positive association between RNF24 expression and infiltration level of CAFs and TAMs in the database (Fig.9C-D). According to the above results, we hypothesized that RNF24 might be a potential pan-cancer biomarker and predicted the immunotherapy response based on its expression.

RNF24 expression is associated with immune-related cells infiltration and survival in LIHC

To evidence the value of the expression level of RNF24 in immune-associated cells infiltration, we examined it in LIHC. First, we explored the association between RNF24 expression and immune cells infiltrated in TME in the 371 patients of LIHC by TIMER (Fig.10A, Table 2). As shown in Figure 10A and Table 2, the six kinds of immune cells were positively correlated with RNF24, including B cells, CD4+ T cell, CD8+ T cell, macrophage, neutrophil, and dendritic cell. TAMs, mostly M2 polarized, as one important of infiltrating immune cell types in the tumor. Our results showed RNF24 expression positively correlated with TAMs and M2 (Fig.10B). The correlation of T cell (general) is higher than CD8+ T cell presented in table 2, and the link between in CD8+ T cell infiltrated and RNF24 in LIHC was inconsistent with 6 datasets (Fig.9A). Subsequently, we made a correlated analysis between RNF24 and gene markers of different types of T cells in TIMER (Table 3). The most associated type was Tregs, which was similar to the analysis of CIBERSORT, CIBERSORT-ABS, QUANTISEQ (Fig.9B). Moreover, we investigated the accurate prognostic value of RNF24 in 11 types of immune cells. The results exhibited that RNF24 high expression groups had poor OS no matter in the enriched or decreased group of 6 immune cells, including B-cells, CD4+ memory T-cells, CD8+ T-cells, macrophages, natural killer T-cells, type 2 T-helper cells. As the immune cells of basophils and eosinophils were down-regulated in most LIHC patients, we analyzed in decreased group and RNF24 expression also had a prognostic value. While RNF24 expression predicted survival only in the decreased group of mesenchymal stem cells (Fig.10E) as

well as enriched groups of Treg and type 1 T-helper cells (Fig.10F-G). In a word, RNF24 expression was relative with immune cell infiltration, especially to TAMs and Tregs, and had a prognostic value in LIHC.

Discussion

RNF family played important roles in cancer progression, including EMT, proliferation, and metastasis [10, 37]. For example, RNF38 is a key driver of HCC by ubiquitinating and degrading AHNAK, a TGF-β signaling inhibitor, and promoting EMT [10]. And RNF122, as the paralog gene of RNF24, mediated RIG-I degradation and was implicated in the control of antiviral innate immunity [13]. However, it was our first reported that RNF24 abnormal expression in pan-cancer, and its expression level had noteworthy prognostic value in multiple cancers. In our study, RNF24 was a significantly different expression in 15 types of cancer proved in 2 datasets and 3 types (CHOL, KICH, STAD) in 3 datasets compared to normal tissues. And its expression was upregulated in most cancer but downregulated in kidney-related cancers. It indicated that RNF24 had diverse biological functions in different types of cancer. Additionally, enhanced expression of RNF24 predicted poor prognoses in at least 10 types of cancer, including KIPR. These results strongly indicated RNF24 as a potential prognostic biomarker in cancers.

In HEK 293T cells, RNF24 had an interaction with all TRPCs and caused their intracellular retention [15]. In our study, RNF24 might interact with the other 31 proteins predicted by 3 PPI websites. Some of them were reported closely with cancer progression, similar outcomes were gained in functional enrichment analysis. While the specific mechanism of RNF24 in cancers needs to further explore by experiments.

Immunosuppressive cells gathered in TME is a key mechanism of tumor cell's escape. The components of TME, such as CAFs, are also used by tumor cells to evade immune surveillance. Here, we surveyed the correlation between RNF24 and immune cells, immunosuppressive cells, and CAFs. The expression level of RNF24 could forecast the infiltration of CD8+ T cells in 12 kinds of cancer with consistent results by 5 datasets. In contrary to CD8+ T cell, RNF24 expression negative with infiltration of immunosuppressive cells and CAFs. These results revealed that RNF24 might benefit tumor progression by regulating tumor-infiltrating lymphocytes (TILs) and predicted the immunotherapy response. However, molecular mechanisms are still required to investigate.

In addition, our findings provided novel understandings to the prognostic evaluation of LIHC and presented valuable information for deeper study on immunity escape of LIHC. The prognosis but not expression level of RNF24 had gender differences and the specific whether exited in others cancer yet to evidence. DNA hypomethylation is high frequently discovered in the genome of tumors tissues compare to normal [38]. Hypomethylation of DNA is always a mechanistic implication to gene activation [39]. We also revealed hypomethylation in DNA promoters arear was the possible reason for RNF24 overexpression in cancer tissues. While the adverse effects of RNF24 during cancer progression require further cancer-type-specific analysis.

Conclusions

In our study, RNF24 was a significantly abnormal expression in multiple cancers detected in 3 datasets compared to normal tissues. And its expression was upregulated in most cancer. We also assessed RNF24 protein expression by IHC with stain intensity in 16 kinds of cancer. Moreover, RNF24 was a reliable biomarker for predicting the OS in 10 cancers. In addition, we revealed the genetic alteration of RNF24 in pan-cancer. Furthermore, PPI networks of RNF24 involved cancer-related proteins, and functional enrichment analysis of RNF24 included calcium ion transport, protein polyubiquitination, and some pathways of cancers. Moreover, immune cell infiltration demonstrated RNF24 expression negative correlation with CD8+ T cells, but positive with immunosuppressive cells, such as Tregs, MDSCs, HSC, and TAMs in pan-cancer. Finally, we examined RNF24 in LIHC. In brief, the results showed RNF24 was a prognostic biomarker and might predict the immunotherapy response in most cancers, especially for LIHC patients.

Declarations

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Availability of data and material

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

Code availability Not applicable.

Authors' contributions

Mei Ma and Bin Yu performed the experimental design, data analysis, and wrote the manuscript. Both authors read and approved the final manuscript.

Ethics approval The study was approved by the Ethics Committee of The First Affiliated Hospital of Nanchang University.

Consent to participate Not applicable.

Consent for publication Not applicable.

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Materials and methods

UALCAN

UALCAN (http://ualcan.path.uab.edu/) [16] is a useful web resource to identify biomarkers by analyzing cancer OMICS data. We carried out RNF24 gene expression analysis in pan-cancer, predicted epigenetic regulation of RNF24 expression by promoter methylation. We also detected survival information of RNF24 combine with sex or grade in LIHC.

GEPIA2

GEPIA2 [17] (Gene Expression Profiling Interactive Analysis) was used to examine RNF24 mRNA expression from TCGA and GTEx datasets in various cancers. We also detected OS and DFS in pan-cancer based on RNF24 mRNA expression into high or low groups.

Kaplan–Meier Plotter

Kaplan–Meier plotter [18] as a website platform is designed to uncover and verify the OS of diverse cancers. To evaluate the prognostic value of RNF24, the patients in cohorts were divided into high- and low- groups based on the expression. Here, the OS of RNF24 was presented in pan-cancer. Moreover, we investigated the prognostic value of RNF24 in 6 types of immune cells, including B-cells, CD4+ memory T-cells, CD8+ T-cells, macrophages, natural killer T-cells, Type 2 T-helper cells, according to their enrichment in TME.

TIMER2.0

TIMER2.0 [19] is applied to examine immune cells infiltration in many cancers, including LIHC. It provides an effective method to analyze TCGA samples data and benefit to evaluate the correlation between RNF24 expression and infiltrating immune cells. In this study, we estimated the relationship of RNF24 with immune cells infiltration by TIMER, CIBERSORT, quanTIseq, xCell, MCP-counter, and EPIC algorithms.

The Human Protein Atlas

The Human Protein Atlas (HPA, https://www.proteinatlas.org/) was used to compare the expression of RNF24 protein, which was detected by immumohistochemical staining in 16 types of cancer. And we acquired representative IHC images in LIHC and adjacent normal tissues from the HPA website.

cBioPortal

cBioPortal (https://www.cbioportal.org/) was employed to check the mutation status of RNF24 in diversity cancers from TCGA datasets. We showed mutation features, sites, and relative number of RNF24 in specific cancer by cBioPortal. In addition, the highest frequency of molecular mutations, PFS, OS, and DFS were exhibited in the RNF24 alter group compared to the unaltered group.

Metascape and DAVID Bioinformatics Resources 6.8

Metascape[20] and DAVID (The Database for Annotation, Visualization, and Integrated Discovery) [21] were used to enrichment analysis of RNF24. Metascape is designed to provide an extensive gene list annotation and to analyze resources for users. Enrichment analysis was the essential part of Metascape. Here, we selected the prominently related genes of RNF24 for enrichment analysis by custom analysis. DAVID is a useful online tool to identify enriched biological themes. We searched enrichment analyses of RNF24-interacting proteins into BP, CC, MF categories using the DAVID online tool.

Figures 1-10 and Figure Legends

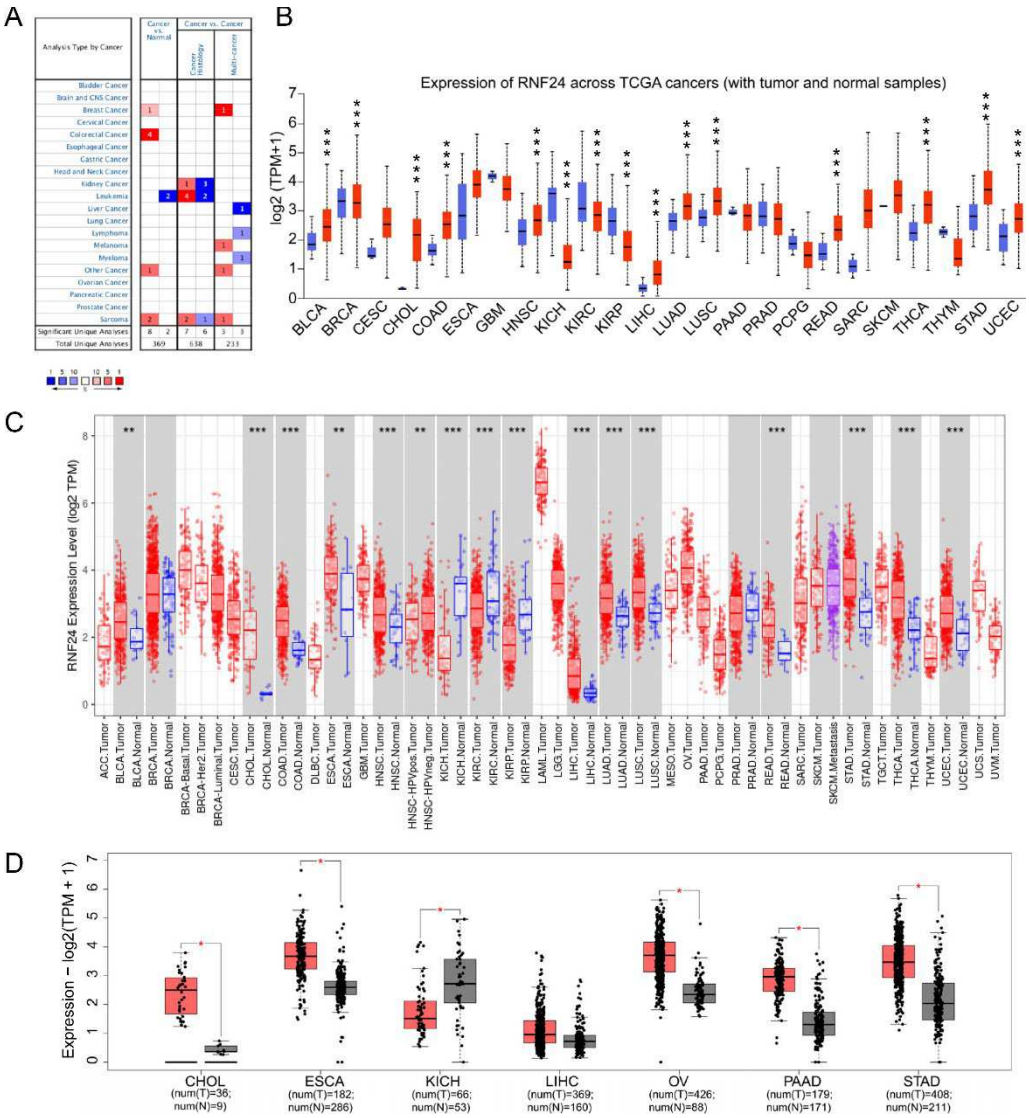

Fig. 1. RNF24 gene abnormal expression in Pan-cancer.

(**A**) Oncomine database revealed the mRNA expression patterns of RNF24, including the numbers of datasets with significant changes. Red indicated overexpression and blue for under-expression.

(**B**) Using UALCAN website detected RNF24 mRNA expression in pan-cancer with tumor and normal tissues. ***P < 0.001

(**C**) Expression level of RNF24 in TCGA tumors compared to adjacent tissues analyzed by TIMER2. **P < 0.01; ***P < 0.001.

(**D**) Boxplot exhibited the expression level of RNF24 comparison in CHOL, ESCA, KICH, LIHC, OV, PAAD, and STAD according to the corresponding normal tissues (TCGA normal and GTEx database). *P < 0.05.

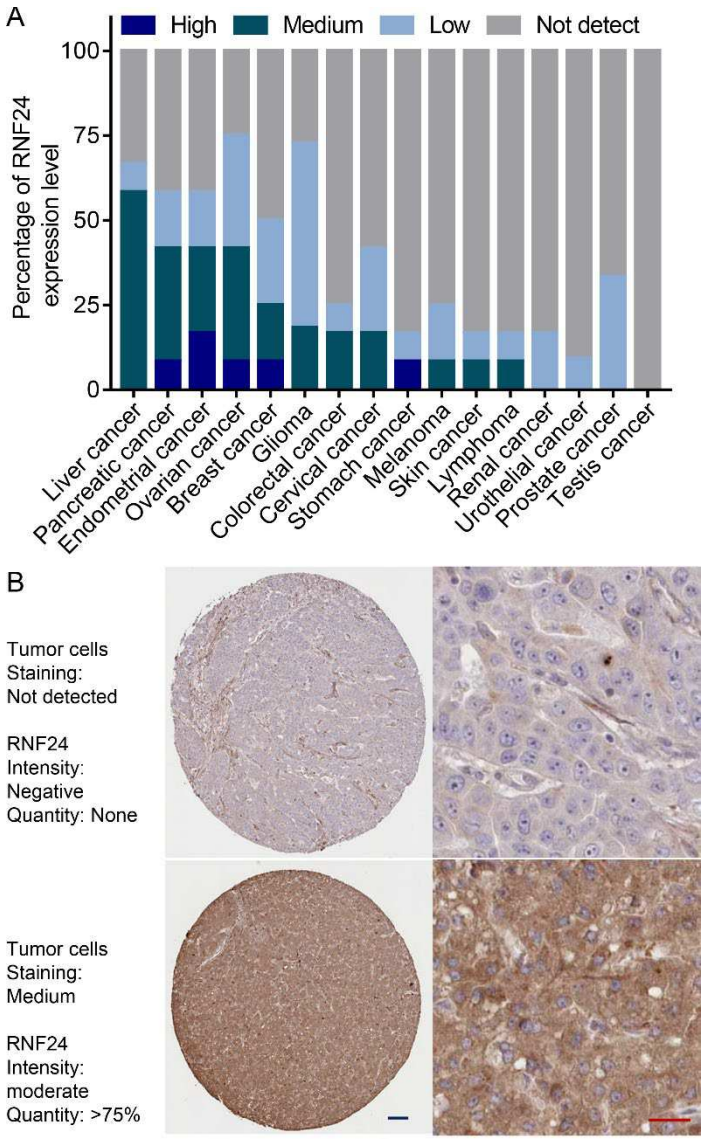

Fig. 2. RNF24 protein expression derived from the HPA database.

(**A**) Comparison of RNF24 protein expression detected by immumohistochemical staining in 16 types of cancer.

(**B**) Representative immunohistochemistry images in LIHC and adjacent normal tissues acquired from the HPA website.

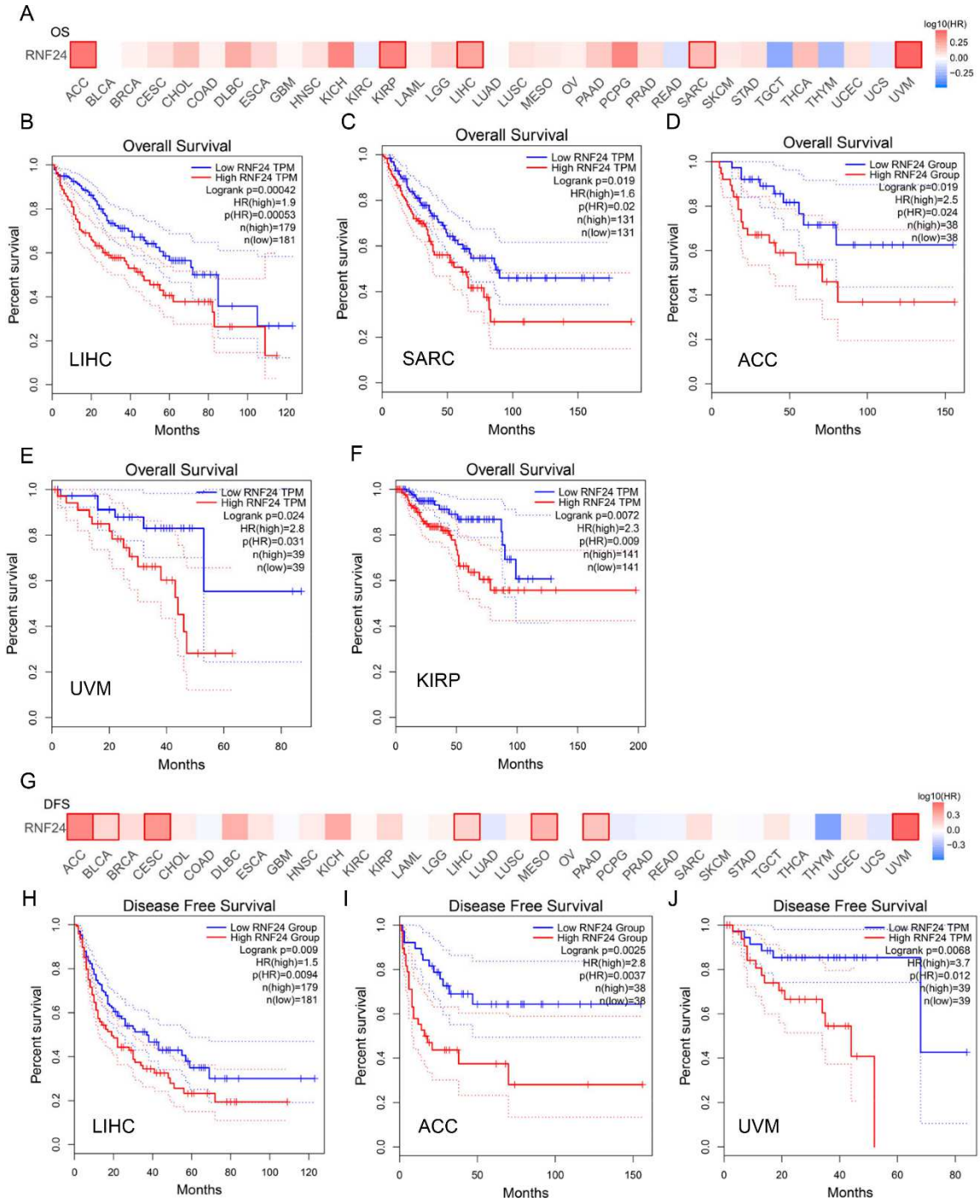

Fig. 3. Survival prognosis of RNF24 gene expression in high- and low- groups in pan-cancer analyzed using GEPIA2.

(**A**) Overall survival (OS) map of RNF24 in 33 types of cancer.

(**B-F**) Kaplan–Meier survival analysis based on RNF24 expression significantly difference in LIHC (B), SARC (C), ACC (D), UVM (E), and KIRP (F).

(**G**) Disease-free survival map of RNF24 in 33 types of cancer and significant difference in 7 cancers.

(**H-J**) The disease-free survival rate of LIHC (H), ACC (I), and UVM (J).

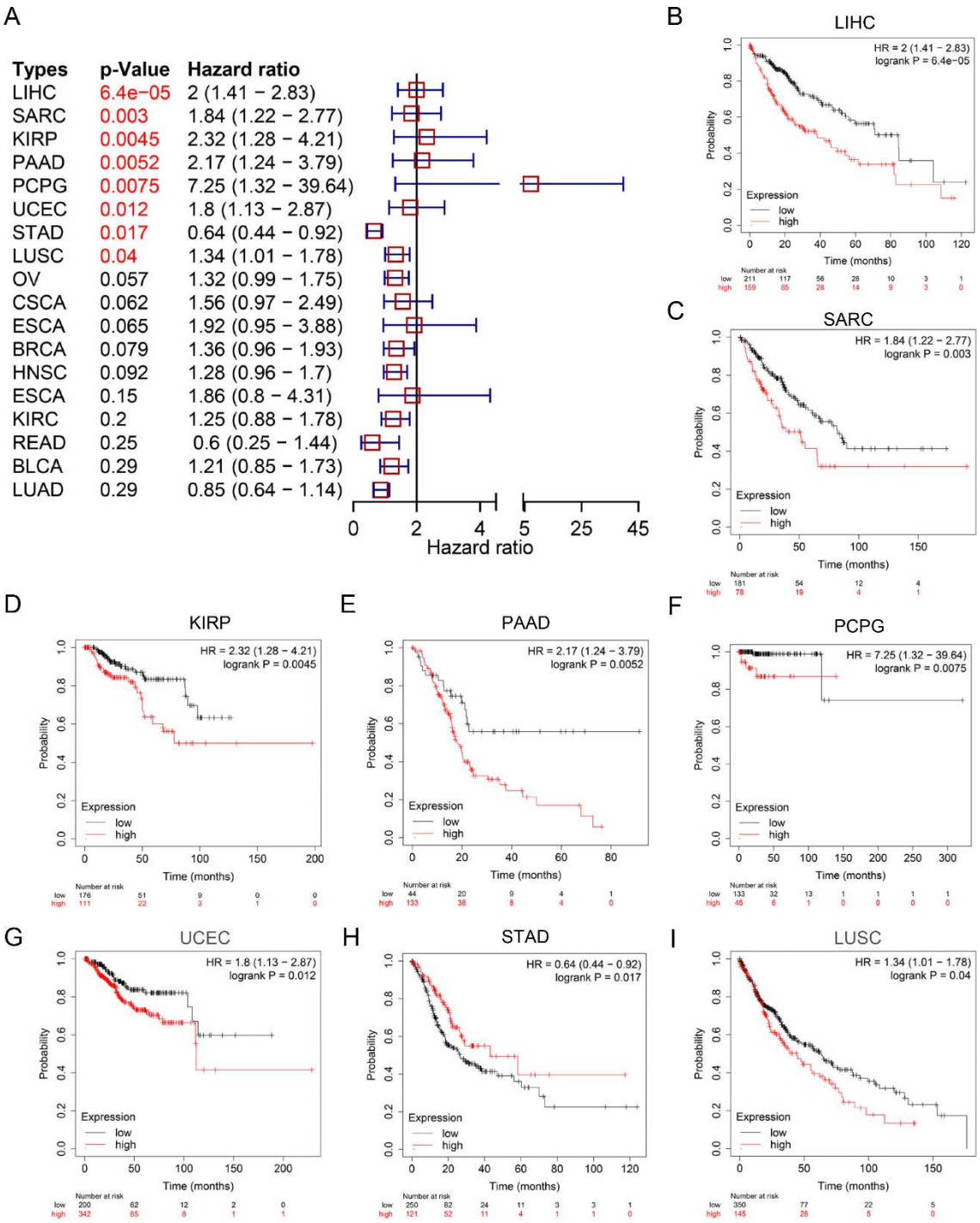

Fig. 4. Prognosis value of RNF24 from databases of GEO, EGA, and TCGA analyzed through KM plotter website.

(**A**) The forest map showed the OS based on RNF24 expression in 18 types of cancer.

(**B-I**) Kaplan–Meier survival analysis according to RNF24 expression in LIHC (B), SARC (C), KIRP (D), PAAD (E), PCPG (F), UCEC (G), STAD (H), and LUSC (I). Only tumor types with log-rank p < 0.05 were displayed.

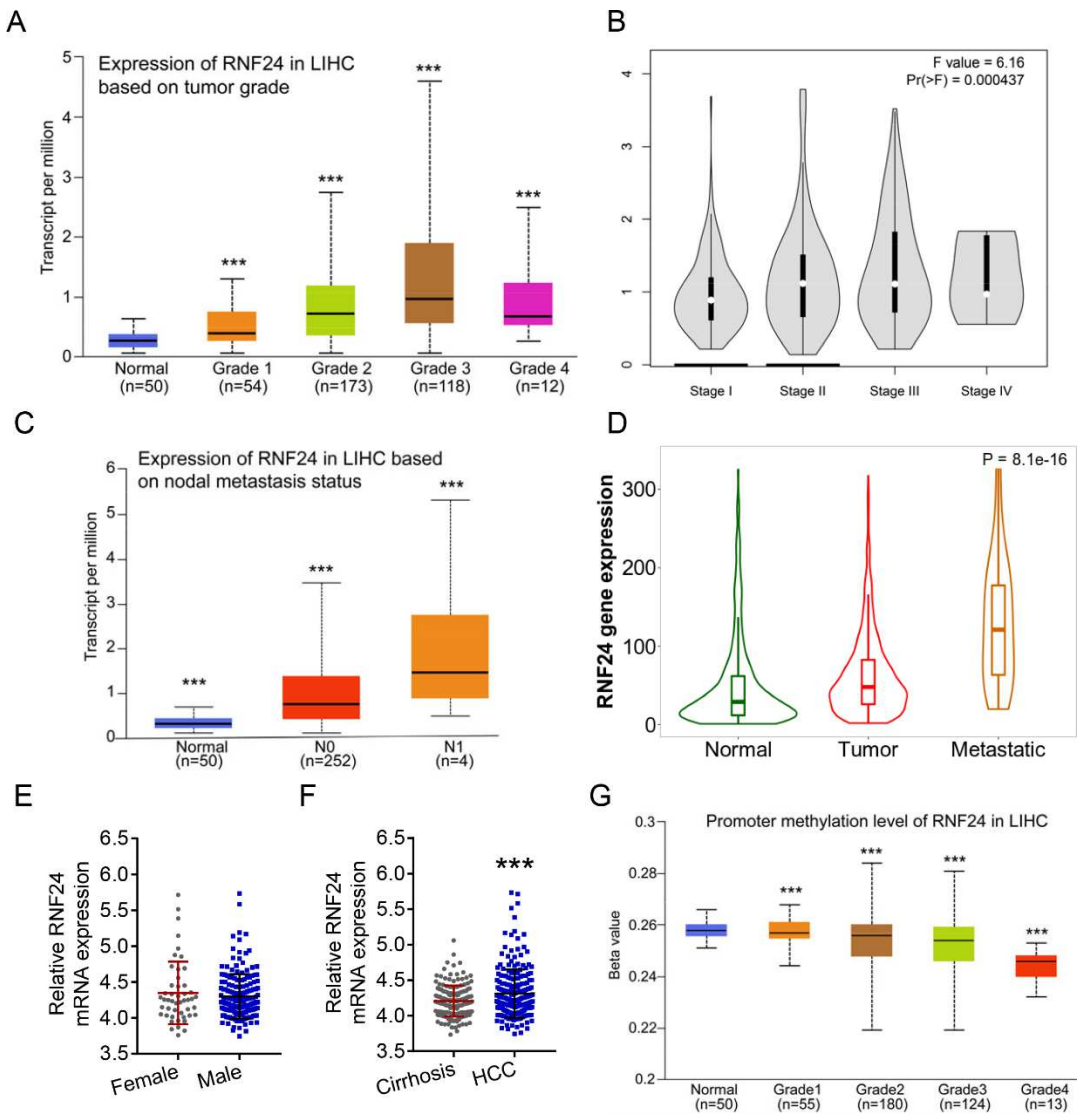

Fig. 5. RNF24 expression in LIHC patients with different pathological and clinical features.

(**A**) RNF24 expression outstandingly increased from normal to tumor grade 4 analyzed by UALCAN. ***P < 0.001.

(**B**) RNF24 expression remarkably increased from stage I to stage IV analyzed by GEPIA.

(**C**) RNF24 expression prominently increased with the nodal metastasis status. ***P < 0.001.

(**D**) Expression of RNF24 gene in LIHC based on metastasis status analyzed by TNMplot. Normal = 379, Tumor = 806, Metastatic = 24.

(**E**) RNF24 expression had no significant difference in females and males in data of HCCDB.

(**F**) RNF24 expression of HCC patients upregulated compared to cirrhosis situation. ***P < 0.001.

(**G**) Promoter methylation of RNF24 noteworthy decreased in LIHC with tumor advance. ***P < 0.001.

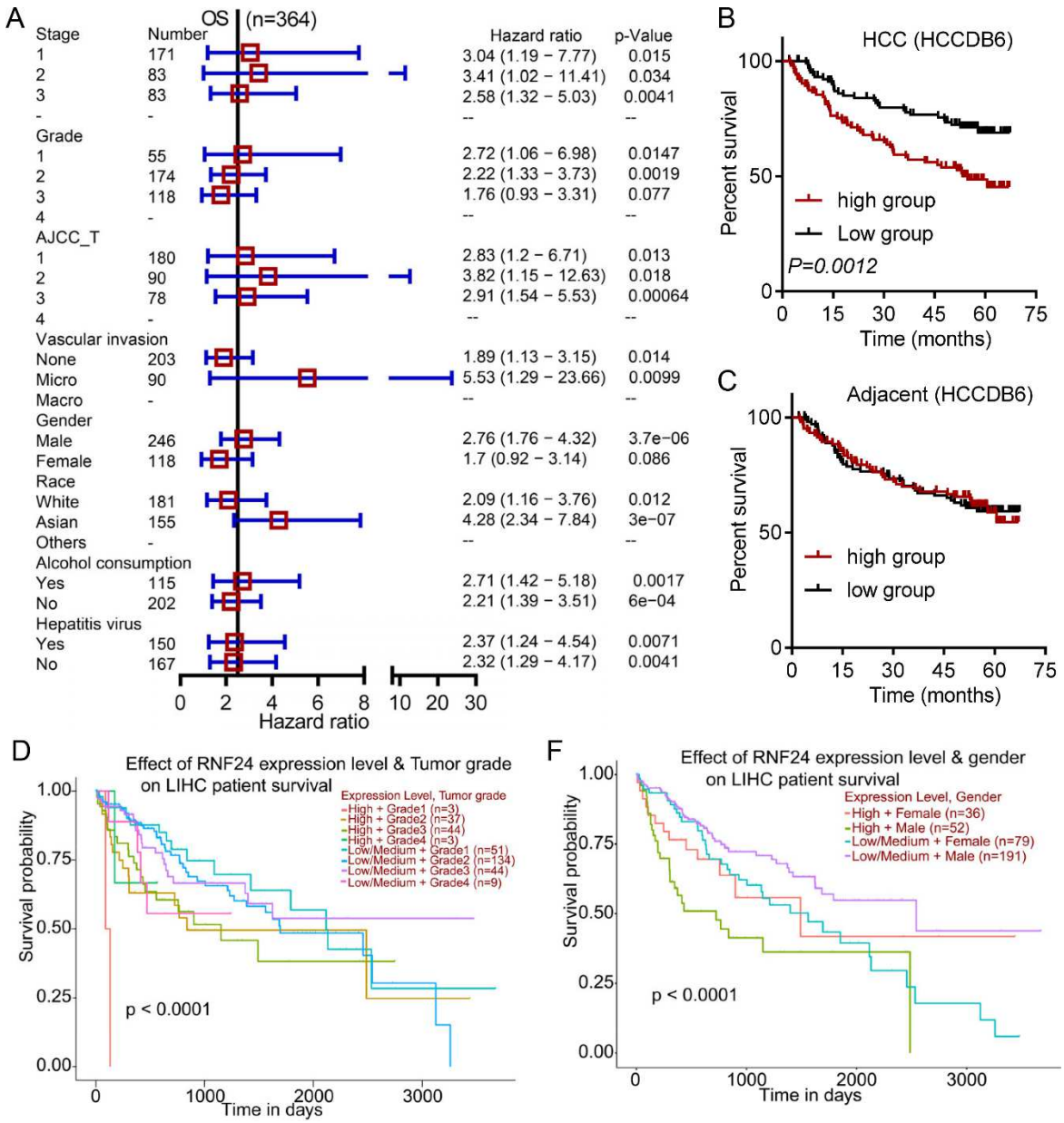

Fig. 6. RNF24 is a prognostic biomarker of LIHC patients.

(**A**) Prognostic value of RNF24 based on pathological grading, basic information, and risk factors of LIHC patients

(**B-C**) OS of LIHC patients according to RNF24 expression in tumor (B) or adjacent tissues (C).

(**D-F**) OS observed in RNF24 expression combine with grades (D) or sex (F) in LIHC patients by UALCAN websites.

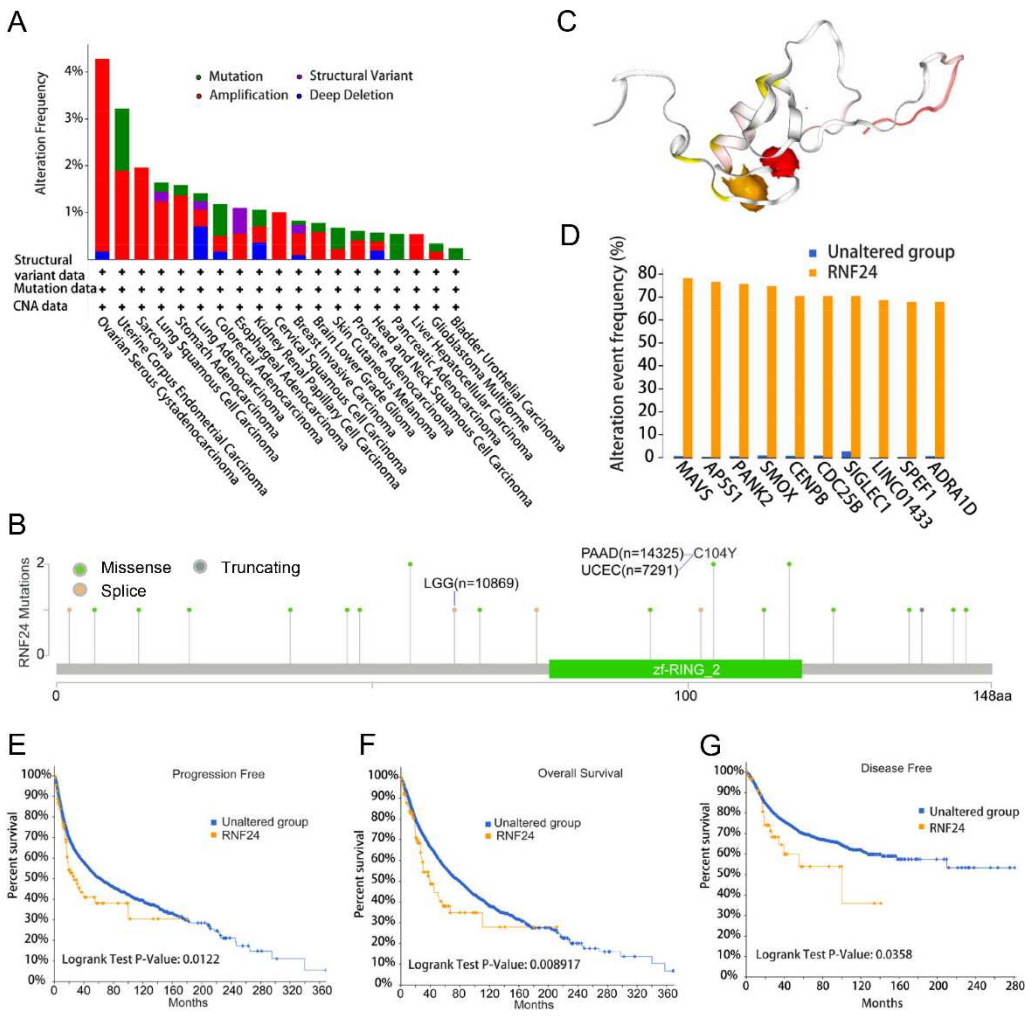

Fig. 7. Mutation status of RNF24 in various cancers.

(**A**) Mutation features of RNF24 in TCGA were analyzed using the cBioPortal tool.

(**B**) Mutation sites and the greatest change sites of RNF24.

(**C**) 3D structure of RNF24.

(**D**) Highest frequency of molecular mutations in RNF24 alter group compared to the unaltered group.

(**E-G**) PFS (Progression-free survival), OS, DFS (Disease-free survival) of RNF24 in mutation group and unaltered group.

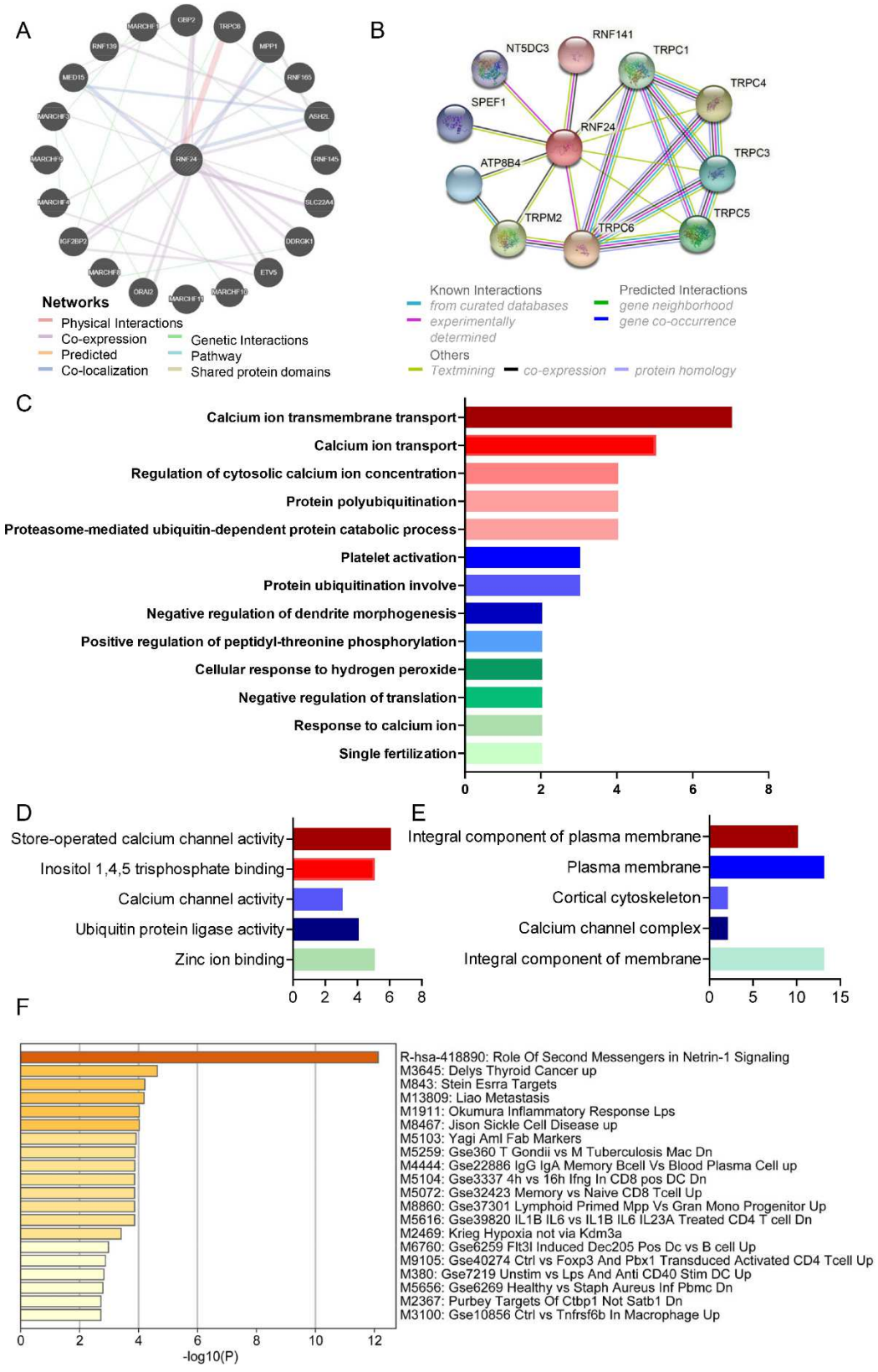

Fig. 8. Functional enrichment analysis of RNF24.

(**A**) Prediction of RNF24 related protein interactions by using GeneMANIA.

(**B**) STRING websites showed RNF24 interactions proteins.

(**C-E**) Enrichment analyses of RNF24-interacting proteins into BP (C), CC (D), MF (E) category using the DAVID online tool. The red colors represent p<0.001, the blue colors represent 0.001 <p<0.05, the green colors represent p>0.05.

(**F**) Metascape website showed enrichment analyses of RNF24 related interaction proteins.

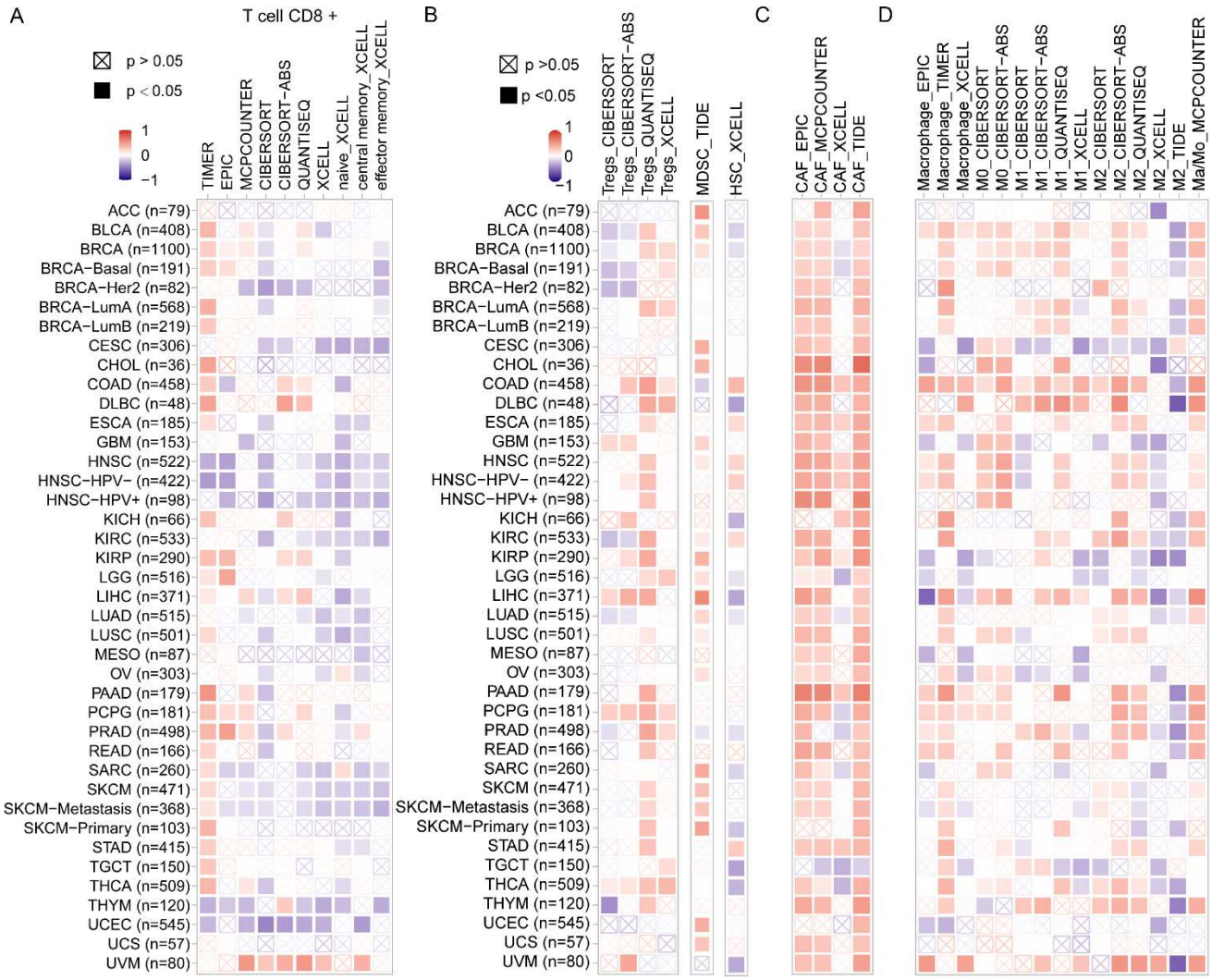

Fig. 9. Immune cells infiltration of RNF24 in patients with cancer.

(**A**) Correlative analysis from 6 datasets showed the expression levels of RNF24 were negatively associated with CD8+ T cell filtration. The red color represents a positive correlation, the blue color represents a negative correlation.

(**B-D**) Diversity websites revealed RNF24 expression had a significantly positive association with the infiltration of Tregs (B), CAFs (C), and macrophages (D).

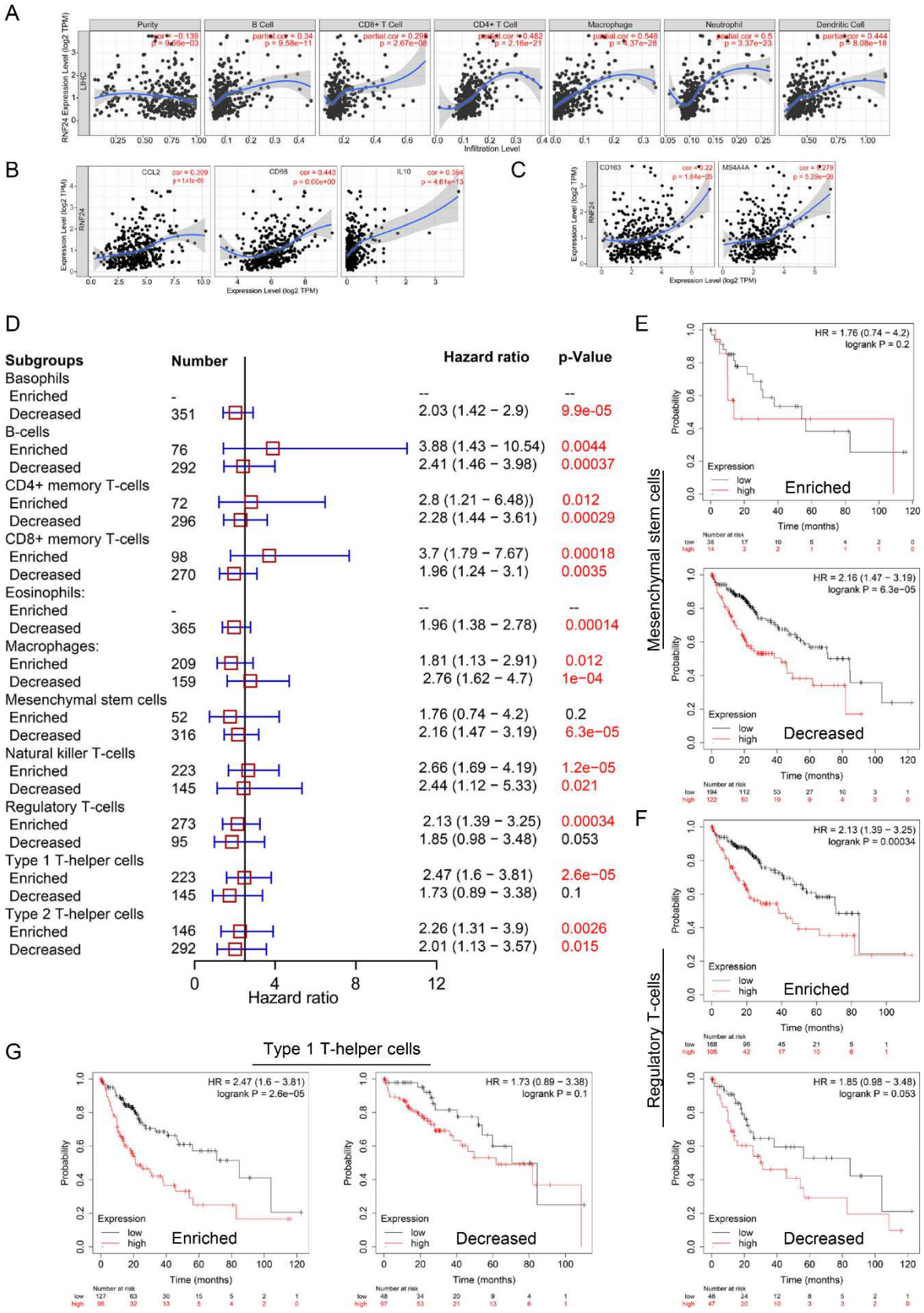

Fig. 10. RNF24 expression was associated with immune-related survival in LIHC.

(A) Immune cells, including B cells, CD4+ T cell, CD8+ T cell, macrophage, neutrophil, and dendritic

cell, were positively correlated with RNF24 expression.

(**B-C**) RNF24 expression positively correlated with TAMs (B) and M2 (C).

(**D**) Prognostic value of RNF24 based on 11 types of immune-related cells enriched or decreased.

(**E**) RNF24 expression predicted OS in the decreased or enriched group of mesenchymal stem cells.

(**F-G**) RNF24 expression predicted OS in the enriched but not decreased groups of regulatory T-cells (F) and type 1 T-helper cells (G).

Tables 1-3

Table 1 In silico prediction of physical protein interactions of RNF24 using FpClass.

Predicted Partner Symbol	Total Score	Domain Score	Physio-Chemical Score	Localization Score	Function Score	Co-expression Score
LGALS1	0.4493	0	0.0011	0.0032	0	0.4449
PSG9	0.3394	0	0.001	0.0007	0.0008	0.337
BMP15	0.3225	0.0008	0.001	0	0	0.3208
CD44	0.305	0.0008	0.0009	0.0029	0.0007	0.2997
HRG	0.2948	0	0.0008	0.003	0.0007	0.2903
KCNMB4	0.2811	0.001	0.001	0	0	0.2791
CA12	0.2541	0.001	0.001	0.0007	0.0008	0.2506

Table 2 Correlation analysis between RNF24 and gene markers of immune cells in TIMER.

Description	Gene markers	None Cor	p		Purity Cor	p	
B cell	CD19	0.254713	6.63E-07	***	0.221074	3.43E-05	***
	CD79A	0.227321	9.79E-06	***	0.18806	0.000445	***
T cell (general)	CD3D	0.374739	1.07E-13	***	0.35465	1.16E-11	***
	CD3E	0.328919	1.07E-10	***	0.310242	3.92E-09	***
	CD2	0.320123	3.47E-10	***	0.30005	1.31E-08	***
CD8+ T cell	CD8A	0.258688	4.81E-07	***	0.224327	2.61E-05	***
	CD8B	0.223199	1.43E-05	***	0.186779	0.000488	***
Monocyte	CD86	0.449935	0	***	0.456099	3.97E-19	***
	CSF1R	0.382924	2.43E-14	***	0.376781	4.43E-13	***
TAM	CCL2	0.309257	1.41E-09	***	0.273181	2.55E-07	***
	CD68	0.443108	0	***	0.420893	3.03E-16	***
	IL10	0.363951	4.61E-13	***	0.341456	7.21E-11	***
M1	IRF5	0.404486	4.90E-16	***	0.418281	4.81E-16	***
	PTGS2	0.375432	7.30E-14	***	0.370347	1.18E-12	***

	NOS2	0.068273	0.189478		0.053317	0.323435	
M2	CD163	0.220385	1.84E-05	***	0.184905	0.000557	***
	VSIG4	0.304719	2.50E-09	***	0.2844	7.67E-08	***
	MS4A4A	0.27899	5.29E-08	***	0.259758	1.00E-06	***
Neutrophils	CEACAM8	0.094042	0.070409		0.085449	0.11313	
	ITGAM	0.436121	0	***	0.425701	1.28E-16	***
	CCR7	0.234009	5.23E-06	***	0.200647	0.000176	***
Natural killer cell	KIR2DL1	0.023139	0.656868		-0.00161	0.976224	
	KIR2DL3	0.173405	0.000796	***	0.14051	0.008965	***
	KIR2DL4	0.167201	0.001227	***	0.145849	0.006653	***
	KIR3DL1	0.008314	0.873188		-0.01405	0.794827	
	KIR3DL2	0.068372	0.188834		0.037863	0.483315	
	KIR3DL3	0.005975	0.908691		0.000549	0.991889	
	KIR2DS4	0.037327	0.473505		0.030975	0.566381	
Dendritic cell	HLA-DPB1	0.325362	1.73E-10	***	0.295456	2.23E-08	***
	HLA-DRA	0.320683	3.23E-10	***	0.293841	2.68E-08	***

HLA-DPA1	0.314546	7.19E-10	***	0.290349	3.97E-08	***
CD1C	0.256279	5.63E-07	***	0.221423	3.33E-05	***
NRP1	0.514008	0	***	0.498488	4.52E-23	***
ITGAX	0.46216	0	***	0.472766	1.30E-20	***

Table 3 Correlation analysis between RNF24 and gene markers of different types of T cells in TIMER.

Description	Gene markers	None Cor	p		Purity Cor	p	
Th1	TBX21	0.145389	0.005018	***	0.109286	0.042498	*
	STAT4	0.356748	1.95E-12	***	0.348136	2.89E-11	***
	STAT1	0.401421	0	***	0.399641	1.16E-14	***
	TNF	0.383317	1.97E-14	***	0.384094	1.43E-13	***
	IFNG	0.231341	1.80E-10	***	0.208717	3.07E-08	***
Th1-like	HAVCR2	0.489498	5.75E-46	***	0.509122	7.67E-47	***
	CXCR3	0.327667	1.27E-10	***	0.29376	2.70E-08	***
	BHLHE40	0.274441	8.81E-08	***	0.288576	4.84E-08	***
	CD4	0.265779	2.27E-07	***	0.23388	1.14E-05	***
Th2	STAT6	0.224657	1.25E-05	***	0.226076	2.25E-05	***
	STAT5A	0.461013	6.38E-21	***	0.450552	1.19E-18	***
Treg	FOXP3	0.105255	0.000436	***	0.097579	0.001655	**
	CCR8	0.459472	4.99E-40	***	0.459399	2.25E-37	***
	TGFB1	0.529113	0	***	0.507801	5.16E-24	***

Resting Treg	IL2RA	0.456531	1.68E-20	***	0.455319	4.64E-19	***
Effector Treg T-cell	TNFRSF9	0.418965	3.35E-17	***	0.419993	3.56E-16	***
Effector T-cell	CX3CR1	0.285605	2.48E-08	***	0.279436	1.31E-07	***
	FGFBP2	-0.13606	0.00869	***	-0.15183	0.00471	**
	FCGR3A	0.32973	9.57E-11	***	0.313734	2.56E-09	***
Naïve T-cell	CCR7	0.234009	1.09E-10	***	0.200647	1.04E-07	***
	SELL	0.285704	2.10E-15	***	0.259955	3.90E-12	***
Effector memory T-cell	DUSP4	0.544193	0	***	0.539621	1.84E-27	***
	GZMK	0.139122	0.007281	**	0.088827	0.099525	
	GZMA	0.163442	0.001584	**	0.118478	0.027779	*
Resident memory T-cell	CD69	0.272891	9.28E-08	***	0.245269	4.03E-06	***
	CXCR6	0.277805	5.32E-08	***	0.250289	2.51E-06	***
	MYADM	0.528712	0	***	0.517575	4.92E-25	***

memory T-cell	IL7R	0.309771	1.08E-09	***	0.299694	1.37E-08	***
Exhausted T-cell	LAG3	0.198141	0.000126	***	0.167458	0.001801	**
	CXCL13	0.208297	5.28E-05	***	0.192832	0.000315	***