Functional & Integrative Genomics (2024) 24:210
https://doi.org/10.1007/s10142-024-01493-x

RESEARCH

Multi-omics analysis of the biological function of the VEGF family in colon adenocarcinoma

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Received: 27 August 2024 / Revised: 10 October 2024 / Accepted: 29 October 2024

Abstract

The vascular endothelial growth factor (VEGF) family plays a crucial role in cancer progression, but the prognostic significance and biological functions of VEGF family members in colon adenocarcinoma (COAD) remain unclear. Using data from The Cancer Genome Atlas, Gene Expression Omnibus, Gene Set Cancer Analysis, cBioPortal, GeneMANIA, String, MethSurv and starBase database, we identified vascular endothelial growth factor B (VEGFB) as a key gene associated with COAD prognosis, with its abnormal expression linked to methylation dysregulation. In vitro experiments confirmed VEGFB expression was significantly higher in colon cancer tissues compared to normal tissues, as shown by Real-time quantitative PCR and immunohistochemistry. Cell Counting Kit-8 and colony formation assay showed that decreased VEGFB expression in SW480 cells resulted in decreased cell viability and proliferation ability. Scratch assay showed that VEGFB downregulation impaired SW480 cell migration. In addition, our research suggests that VEGFB not only promotes angiogenesis but is also involved in the tumor microenvironment and immune regulation. The SHNG17-miR-375-VEGFB regulatory axis provides a potential therapeutic target for COAD, highlighting VEGFB's role in immune activation during anti-angiogenic therapy and potential reversal of drug resistance.

Keywords VEGFB · COAD · Multi-omics analysis · Immune infiltration

Introduction

Colon cancer is a prevalent malignant tumor of the digestive tract that imposes a heavy burden on society and families worldwide. According to the latest global cancer statistics(Bray et al. 2024), colorectal cancer ranks third in incidence and second in mortality among all malignancies. In developing nations such as China, the number of colon cancer cases and deaths is on the rise, and the trend of younger patients is becoming increasingly apparent(Han et al. 2024). Colon adenocarcinoma (COAD) is the most common histologic subtype of colon cancer, accounting for more than 90% of all cases(Fleming et al. 2012). At the time of diagnosis, approximately 20% of COAD patients have developed distant metastases due to the disease's insidious onset and high invasiveness, and the prognosis is relatively poor(Dekker et al. 2019). The identification of biomarkers for early diagnosis and prognosis prediction is advantageous for the hierarchical management of COAD patients and the advancement of precision medicine. In addition, COAD patients are highly resistant to conventional chemotherapy(Hu et al. 2016), and an increasing number of researchers are looking for safer and more effective alternatives. Immunotherapy, including immune checkpoint inhibitors, and targeted therapies that target specific molecules have been shown to be effective in the treatment of patients with advanced colon cancer(Wu 2018). However, neither approach is beneficial for all patients. In an effort to improve the prognosis of COAD patients, we therefore sought to identify molecules with promising applications in both targeted therapy and immunotherapy.

By targeting the vascular endothelial growth factor receptor (VEGFR)(Lohela et al. 2009), the vascular endothelial growth factor (VEGF) family of proteins can promote

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Published online: 11 November 2024

angiogenesis and lymphangiogenesis. VEGF can be secreted under physiological conditions or pathologically by tumor cells or stroma(Carmeliet 2005; Lohela et al. 2009). VEGFA (NM_001025366.3), VEGFB (NM_001243733.2), VEGFC (NM_005429.5), VEGFD (NM_004469.5), and placental growth factor (PGF) (NM_002643.4) are the six isoforms of the VEGF family. The VEGF family is highly expressed in a variety of solid tumors, and its elevated expression level is associated with tumor progression, according to studies(Yanase et al. 2014; Blank et al. 2015).

Anti-angiogenesis therapy has been demonstrated to be one of the most effective treatments for COAD(Lopes-Coelho et al. 2021), but it is ineffective for some patients. This is primarily due to acquired drug resistance during treatment (Itatani et al. 2018). Recent research indicates that the combination of antiangiogenic therapy and immunotherapy has the potential to alter the tumor microenvironment and enhance the therapeutic response(Song et al. 2020). Therefore, it is essential to investigate the VEGF family members for immune correlation analysis in order to enhance the anti-angiogenic therapy's resistance. In addition, prior research has demonstrated that high VEGF expression is associated with a worse stage, poor prognosis, and postoperative recurrence in COAD patients(Martini et al. 2018; Wojtukiewicz et al. 2020). However, the majority of these studies are still restricted to the VEGF family and its receptors, thereby ignoring the heterogeneity and interaction between the members(Kazemi et al. 2016). Our investigation is the first to investigate comprehensively the expression, mutation, diagnostic efficacy, prognostic efficacy, and correlation of all VEGF family isoforms. VEGFB was found to be closely associated with the prognosis of COAD patients and the most promising target for targeted therapy. In addition to immunohistochemical analysis, enrichment analysis, methylation analysis, immune cell infiltration, and tumor microenvironment analysis of VEGFB, we also constructed a long non-coding RNA (lncRNA)-microRNA (miRNA)-mRNA regulatory network. We believe that these findings will assist in elucidating the role of the VEGF family in the etiology of COAD and provide new insights for the early diagnosis, prognostication, and treatment of COAD patients.

Materials and methods

Data acquisition

First, we downloaded COAD gene expression data (HTSeq - TPM) from The Cancer Genome Atlas Program (TCGA) database (https://portal.gdc.cancer.gov/) and obtained a total of 521 samples (480 tumors vs. 41 normal groups). Simultaneously, the clinical information of corresponding patients, including survival time, survival status, age, gender, race, tumor differentiation, T stage, N stage, M stage, pathological stage, perineural invasion, lymphatic invasion, serum CEA level, etc., was downloaded. Then, we downloaded from the Gene Expression Omnibus (GEO) database the microarray data set GSE44076, which included 98 primary COAD samples and paired adjacent normal tissues. The microarray data set's platform file was GPL13667[HG-U219](Moreno et al. 2018). Using R software's "limma" package, data were combined and compiled (x64 4.1.1).

mRNA expression analysis

The "GTEx Expression" module of the Gene Set Cancer Analysis (GSCA) database (http://bioinfo.life.hust.edu.cn/web/GSCALite/) (Liu et al. 2018a)was utilized to observe the mRNA expression of VEGF family members in various human tissues, with the results presented as heat maps for visualization purposes. On the basis of TCGA-COAD data, VEGF family expression was observed in unpaired samples (41 tumors versus 480 normal groups) and paired samples (41 tumors vs. 41 normal groups). Using 98 paired COAD samples from the microarray dataset GSE44076, the aforementioned results were confirmed. For unpaired samples, the Wilcoxon rank sum test was used to observe differences in expression, while the t-test was used for paired samples. A P value of less than 0.05 was statistically significant.

Genetic alterations and interactions network

VEGF family member mutations were observed in 640 samples from the "Colon Cancer (CPTAC-2 Prospective, cell 2019)" module of the cBioPortal Database(Cerami et al. 2012). The "Survival" module was utilized to analyze the difference in patient survival between mutant and wild-type individuals. VEGF family network was drawn using GeneMANIA Database(Warde-Farley et al. 2010). String database (V.11.5)(Szklarczyk et al. 2021), with the following parameters: organism ("Homo sapiens"), network type ("full STRING network"), and minimum required interaction score, was used to generate the protein-protein interaction (PPI) network between the VEGF family member ("Medium confidence 0.400"). On the basis of the TCGA-COAD data, the co-expression heat map for VEGF families was created using Spearman correlation analysis. A P value of less than 0.05 was statistically significant.

Analysis of prognostic and diagnostic value

Initially, the area under the curve (AUC) of the receiver operating characteristic (ROC) curve was utilized to assess the diagnostic utility of VEGF family members in COAD patients. Then, univariate and multivariate Cox regression analyses were conducted utilizing the "Survival" package in

R software to identify clinicopathological characteristics and VEGF family members that could be utilized as independent risk factors for COAD patients. Overall survival (OS), disease-specific survival (DFS), and the progression-free interval (PFI) were compared between VEGFB gene high-expression and VEGFB gene low-expression groups using Kaplan-Meier survival curves. The Wilcoxon rank sum test was utilized to determine the relationship between VEGFB and clinicopathological characteristics. The "rms" package was then used to draw a nomogram that can predict the 1-, 3-, and 5-year survival probability of COAD patients, and the calibration curve was used to evaluate the accuracy of the nomogram. A P value of less than 0.05 was statistically significant.

DNA methylation and tumor mutational burden analysis of VEGFB

Spearman correlation analysis was used to evaluate the relationship between VEGFB Expression level and DNA Methylation level in COAD using the "Methylation & Expression" module of the GSCA database. Finally, the "Gene Visualization" function of the MethSurv database(Modhukur et al. 2018) was utilized to generate a methylation map of each CpG site within the VEGFB Gene in COAD tissues. Simultaneously, the "Single CpG" module was used to perform survival analysis for each CpG site of the VEGFB gene, and univariate Cox analysis was performed to assess the prognostic value of the various CpG sites. The methylation level of genes was determined using beta values ranging from 0 (no methylation) to 1 (complete methylation) (complete methylation). A P value of less than 0.05 was statistically significant.

Function enrichment analysis

COAD patients from the TCGA database were divided into a high expression group and a low expression group based on the median VEGFB expression level. Using the "DESeq2" R package, differentially expressed genes (DEGs) between the two groups were identified. The "ClusterProfiler" package was then employed to conduct Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses on the differentially expressed genes. The following screening criteria for DEGs were established: the absolute value of log2 fold change values ≥ 1.0, $P < 0.05$.

Tumor microenvironment and immune-related analysis

Using the "ESTIMATE" package of the R programming language, the ratio of immune and matrix components in the tumor microenvironment (TME) of each TCGA-COAD sample was estimated. Immune Score, Stromal Score, and ESTIMATE Score represent immunity, matrix, and the sum of the two, respectively. The ratio of components in the corresponding TME is greater the higher the score. The R package "Reshape2" was utilized to analyze the correlation between tumor-infiltrating immune cell (TIIC) levels and VEGFB expression levels. The R package "Corrplot" was used to identify immune checkpoints closely related to VEGFB expression levels.

Construction of the lncRNA-miRNA-mRNA network

The "miRNA-mRNA" module of the starBase database (Li et al. 2014)was utilized to predict miRNAs that interact with VEGFB, whereas the "miRNA-lncRNA" module was utilized to predict lncRNAs that compete with miRNAs to bind mRNA. To validate the candidate miRNA and lncRNA, expression difference analysis, survival analysis, immune infiltration analysis, immune checkpoint correlation analysis, and molecular correlation analysis were performed on the TCGA-COAD cohort. Spearman's analysis of correlation was utilized for correlation analysis. A P value of less than 0.05 was statistically significant.

Cell line and culture conditions

The SW480 human colon adenocarcinoma cell line was procured from Procell Life Science & Technology Co., Ltd. (China). These cells were grown in DMEM medium (Gibco), supplemented with 10% bovine serum (Gibco), and incubated at 37 °C in a humidified incubator containing 5% CO_2.

Real-time quantitative PCR (RT-qPCR)

This study was approved by the Ethics Committee of Shandong Provincial Hospital, with all participants providing written informed consent. Six pairs of colon adenocarcinoma tissues and adjacent normal tissues were collected for RT-qPCR and immunohistochemical analysis. RNA was extracted using TRIzol reagent (#15596018, Thermo Fisher Scientific). cDNA was synthesized from the isolated RNA using PrimeScript™ RT Master Mix (RR036A; Takara). Quantitative PCR was subsequently carried out using 2× Taq PCR MasterMix (KT201; TIANGEN). β-actin served as an internal control for normalizing mRNA levels. Relative gene expression was determined using the 2-ΔΔCt method. The primers for GAPDH were: forward, 5'-CACCCAGCACAATGAAGATCAAGAT-3'; reverse, 5'-CCAGTTTTTAAATCCTGAGTCAAGC-3'. The primers for VEGFB were: forward, 5'-GAGATGTCCCTGGAAGAACACA-3'; reverse, 5'-GAGTGGGATGGGTGATGTCAG-3'.

Immunohistochemistry (IHC) staining

Samples were paraffin-embedded and fixed in 10% formalin. Four-micrometer sections were prepared, and VEGFB expression was detected using a monoclonal antibody (Abcam, ab51867). The study's methodology is detailed in the manuscript.

siRNA knockdown and transfection

Cells were seeded into six-well plates and transfected according to the Lipofectamine™ 3000 protocol (Invitrogen, New York, USA). After a 6-hour incubation, complete medium was added. The siRNA sequences targeting VEGFB were synthesized by GENERAY (Shanghai, China), with the sequence for VEGFB siRNA being 5′- GAAAGUGGUGUC AUGGAUATT − 3′.

Cell viability assay

For the cell viability assay, 1,000 cells were seeded into each well of a 96-well culture plate. Cell viability was assessed using the Cell Counting Kit-8 (DOJINDO), with 10 µL of the reagent added to each well for a 3-hour incubation. Optical density at 450 nm was then measured.

Colony arrangement assay

To evaluate the proliferative capacity of the cells, a colony formation assay was conducted. 1,000 cells were seeded in 6-well plates and incubated at 37 °C in a 5% CO2 environment. After 2 weeks, colonies were fixed with 4% paraformaldehyde and stained with crystal violet. Images were captured using a digital camera.

Cell migration assay

Cell migration was assessed by seeding cells in 6-well plates until they reached approximately 90% confluence. A vertical scratch was made in the center of each well, and images were taken at 0 and 48 h to monitor cell migration.

Statistical analysis

The significance of gene expression differences was assessed using the rank-sum and T-tests. Prognostic survival analysis was conducted through univariate and multivariate Cox regression models, along with the log-rank test. Spearman correlation was employed to evaluate the relationship between two variables. For clinical feature analysis, the Kruskal-Wallis test, T-test, and univariate analysis were applied. Experimental data are presented as the mean ± standard deviation from at least three independent experiments. Paired samples were compared using a paired T-test, with statistical significance defined as two-tailed $P<0.05$.

Results

Gene expression of VEGF family members

Using the GTEx database, we initially investigated the expression of VEGF family members in normal human tissues. As shown in Fig. 1A, VEGFA and VEGFB are more abundantly expressed in various normal human tissues than the other members. No family members showed significantly elevated expression levels in normal colon tissue. Using the TCGA-COAD cohort, a pan-cancer analysis revealed that members of the VEGF family exhibited distinct expression patterns in tumor tissues versus normal tissues. VEGFA, VEGFB, VEGFC, VEGFD, and PGF were expressed differently in 13, 17, 16, 19, and 18 tumors and normal samples, respectively (Fig. 1B). The expression of VEGF family members was then analyzed in COAD using paired TCGA-COAD samples (41 cases of the tumor group vs. 41 cases of the normal group). The results indicated that VEGFA, VEGFB, and PGF were highly expressed in tumors, whereas VEGFD expression was low in COAD (Fig. 2A). A total of 98 paired samples from the GSE44076 microarray in the GEO database were utilized for validation analysis, and the results were largely consistent with those of the TCGA-COAD cohort (Fig. 2B, C).

Genetic alterations and gene and protein networks of VEGF family members

Using the cBioportal database, we evaluated the gene mutation status of the VEGF family in 110 colon cancer samples and found that the mutation frequency of VEGF family members varied significantly. VEGFA had the highest mutation frequency (6%), followed by VEGFB and VEGFC (VEGFB 2.9% and VEGFC 2.9%), while VEGFD and PGF were relatively conservative (VEGFD, 1.0% and PGF, 1.0%) (Fig. S1A). Moreover, the VEGF gene mutations did not significantly affect the overall survival of COAD (Fig. S2). Then, we constructed the gene-gene interaction network and protein-protein interaction (PPI) network of the VEGF family using the GeneMANIA and STRING databases, respectively. The gene-gene interaction network uncovered twenty potential VEGF family target genes (Fig. S1B). According to the outcomes of the protein-protein interaction network, there are close interactions between VEGF family genes (PPI enrichment p-value = 4.22e-13) (Fig. S1C). Lastly, correlation analysis based on the TCGA-COAD cohort revealed

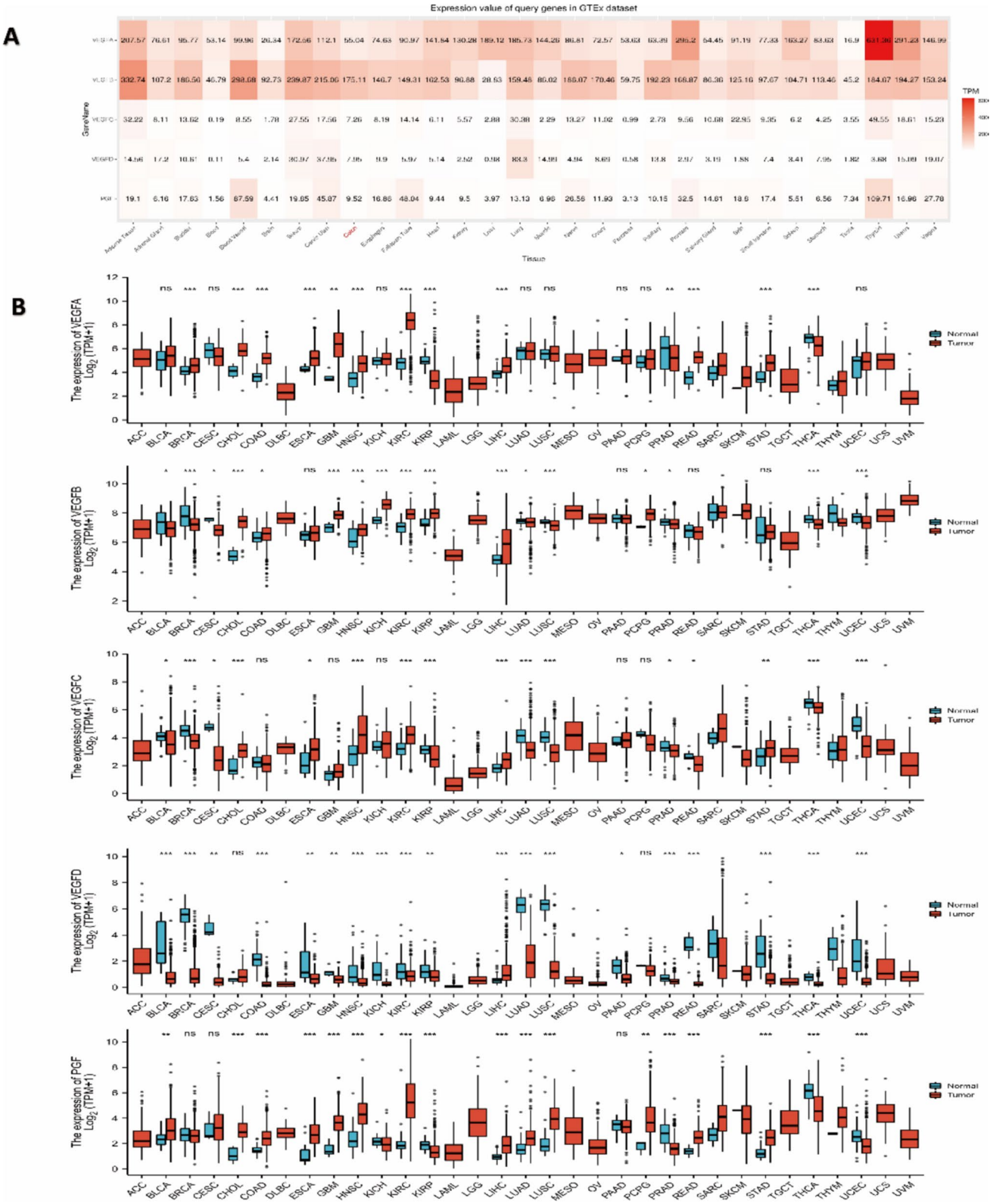
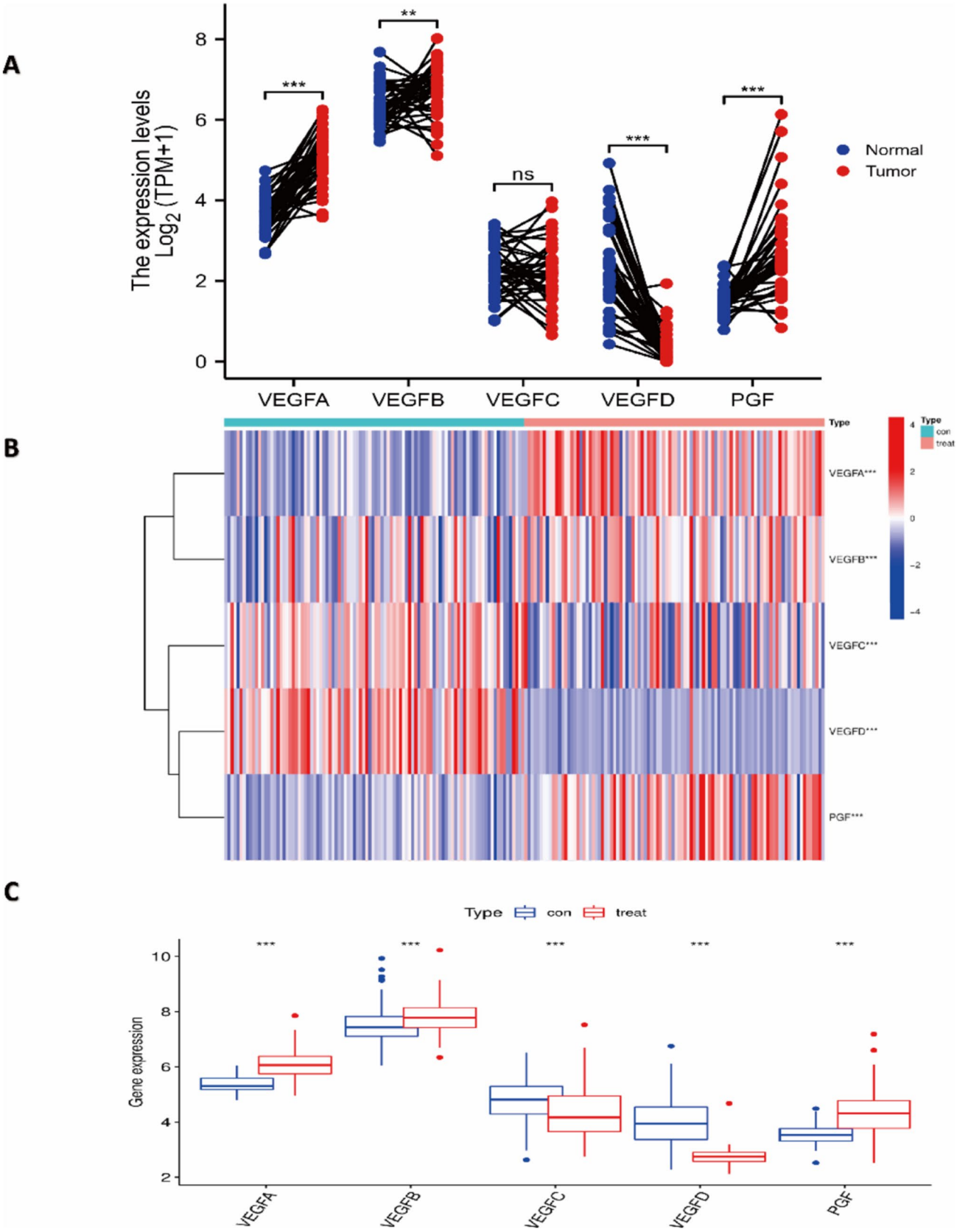


Fig. 1 The mRNA expression of VEGF family in normal and tumor tissues (A) The expression level of VEGF family member in human normal tissues based on GTEx database(B) The expressing levels of VEGF members across diverse cancer types


A
The expression levels
Log2 (TPM+1)
Normal
Tumor
VEGFA
VEGFB
VEGFC
VEGFD
PGF
B
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con
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VEGFD***
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VEGFA
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VEGFD
PGF

◂**Fig. 2** Differential mRNA expression of VEGF members in paired samples based on TCGA and GEO database. **A** Analysis of VEGF members expression in 41 pairs of COAD and adjacent normal tissues. **B** The heatmap of VEGF members expression in GSE44067 datasets. **C** Analysis of VEGF members expression in COAD and adjacent normal tissues based on GSE44067 datasets. *P < 0.05; ***P < 0.001

that all VEGF family members were positively correlated (Fig. S1D).

Survival, prognostic and clinical correlation analysis of the VEGF family in COAD

Using univariate and multivariate Cox regression analyses, the risk factors associated with the prognosis of COAD patients were identified. Age, T stage, N stage, M stage, pathologic stage, CEA level, and VEGFB expression level were identified as risk factors for the prognosis of COAD patients by univariate Cox regression analysis (Fig. 3A, B). The AUC of the ROC curve indicated that VEGFA, VEGFB, VEGFC, and VEGFD had good predictive efficacy in the VEGF family (their respective AUC values were 0.777, 0.736, 0.707, and 0.969). (Fig. 3C). Age, pathologic stage, and the expression level of VEGFB were identified as independent prognostic markers for COAD patients by multivariate Cox analysis (Fig. 3D). High mRNA expression of VEGFB was associated with poor overall survival (OS) ($P = 0.008$), disease-specific survival (DSS) ($P = 0.004$), and progression-free survival (PFS) ($P = 0.041$) in COAD patients as determined by the Kaplan-Meier survival curve (Fig. 3E). In addition, our study demonstrated that high VEGFB expression was associated with adverse clinicopathological characteristics of COAD patients, including advanced tumor stage, increased lymph node and distant metastasis, and poor prognosis (Fig. 3F).

VEGFB family diagnosis and significance in COAD

We developed a nomogram to predict the 1-, 3-, and 5-year prognosis of COAD patients by integrating the independent risk factors (patient age, pathological stage, and VEGFB expression level) identified by multivariate Cox regression analysis (Fig. 4A). The patient's prognosis becomes direr as the score calculated by the nomogram increases. The calibration curve demonstrated that the constructed nomogram had excellent predictive performance (Fig. 4B).

Function enrichment analysis of VEGFB in COAD

Using the median VEGFB expression level, samples from the TCGA-COAD cohort were separated into VEGFB high and low expression groups. Between the two groups, a total of 7055 DEGs were identified, including 724 up-regulated genes and 6331 down-regulated genes (Fig. 5A, B). The analyses of GO and KEGG were used to determine the biological functions and pathways associated with DEGs. GO enrichment analysis of up-regulated genes revealed that these genes were mainly related to various protein complexes, extracellular matrix and muscle contraction (Fig. 5C). KEGG enrichment analysis revealed that these genes were primarily involved in the regulation of vascular smooth muscle contraction and the cAMP/Wnt signaling pathway (Fig. 5D). GO enrichment analysis of down-regulated genes revealed that they were primarily involved in mRNA binding, receptor activation, DNA complexes, and chromosome and nucleosome assembly (Fig. 5E), whereas KEGG enrichment analysis revealed that they were primarily involved in RNA transport and viral carcinogenesis (Fig. 5F).

DNA methylation and and tumor mutational burden analysis of VEGFB in COAD

We investigated the methylation status of VEGFB in the COAD using the GSCA database. The results indicated that the methylation level of VEGFB decreased gradually as VEGFB expression increased (r= -0.33, P 0.001). (Fig. 6A). Utilizing the MethSurv database, we then drew the methylation site expression heat map of VEGFB, yielding a total of seven methylation sites (Fig. 6B). The methylation sites cg05492845 and cg18872604 are significantly associated with colon cancer patients' prognosis (Fig. 6C). The preceding findings suggest that VEGFB methylation may play a role in the development of COAD. The correlation analysis between VEGFB expression level and tumor mutation burden showed that with the increase of VEGFB expression level, the level of tumor mutation burden decreased, and there was a negative correlation between them (Fig. 6D).

Immune-related analysis of VEGFB in COAD

Analyzing and comprehending the tumor immune microenvironment will improve immunotherapy's efficacy. We therefore investigated the connection between VEGFB and the immune microenvironment. There were statistically significant differences between the VEGFB high expression group and the low expression group in terms of immune score, stromal score, and estimate score (Fig. 7A). We hypothesize that VEGFB may exert its biological function by regulating the immune system. Next, we analyzed the difference in immune cell composition between the group with high VEGFB expression and the group with low VEGFB expression. In the group with a high level of VEGFB expression, the proportion of regulatory T cells and M0 macrophages was greater. T cells CD4 memory activated, T cells gamma delta, resting NK cells, activated Dendritic cells, and

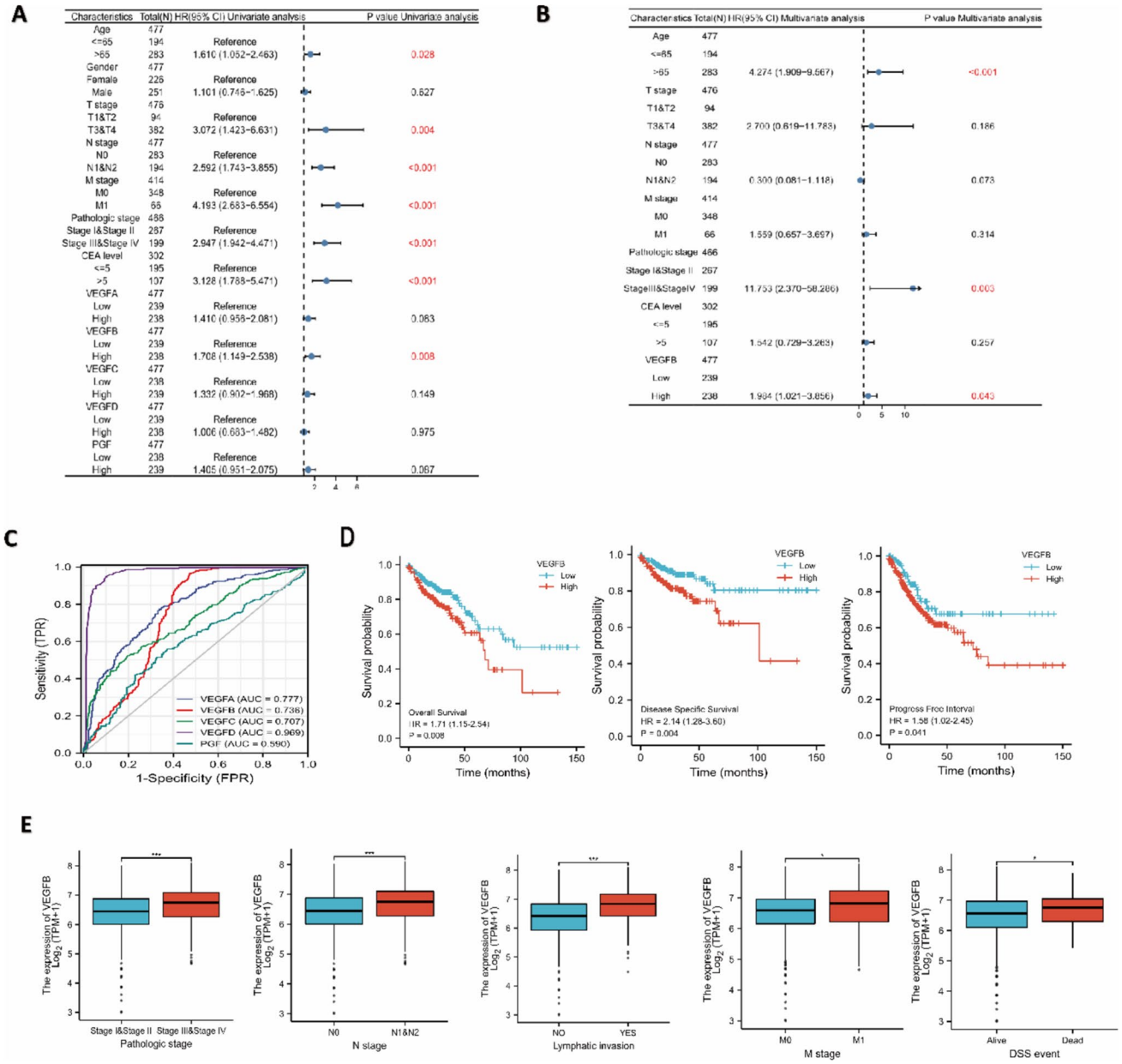

Fig. 3 Survival, prognostic and clinical correlation analysis of the VEGF family in COAD. **A** ROC analysis of VEGF family members. **B** Univariate Cox regression analyses of VEGF family and clinical Parameters in COAD. **C** Multivariate Cox regression analyses of VEGF family and clinical Parameters in COAD. **D** Representative images of VEGFB expression in COAD tissues and normal controls. Original magnifications 50× and 100×; **E** Kaplan-Meier survival curves comparing the high and low expression of VEGFB in COAD patients, including overall survival, disease specific survival and progress free interval. **F** The mRNA expression of VEGFB in COAD based on pathologic stage, N stage, lymphatic invasion, M stage and DSS event

Eosinophils were even less prevalent in the VEGFB high expression group (Fig. 7B). In addition, correlation analysis between VEGFB and immune cells revealed that VEGFB was positively correlated with regulatory T cells (Tregs) and Macrophages and negatively correlated with eosinophils, dendritic cell activation, T cell gamma delta, NK cells resting, T cell CD4 memory activated, and T cell CD4 memory resting (Fig. 7C, D, E). The abnormal expression and function of immune checkpoint molecules is one of the primary causes of the development and occurrence of numerous tumors. Consequently, we conducted a correlation analysis to identify the immune checkpoints closely associated with VEGFB. Included in the analysis are the following checkpoints: ADORA2A, BTLA, BTNL2, C10orf54, CD160, CD200, CD200R1, CD244, CD27, CD274, CD276, CD28, CD40, CD40LG, CD44, CD48, CD70, CD80, CD86,

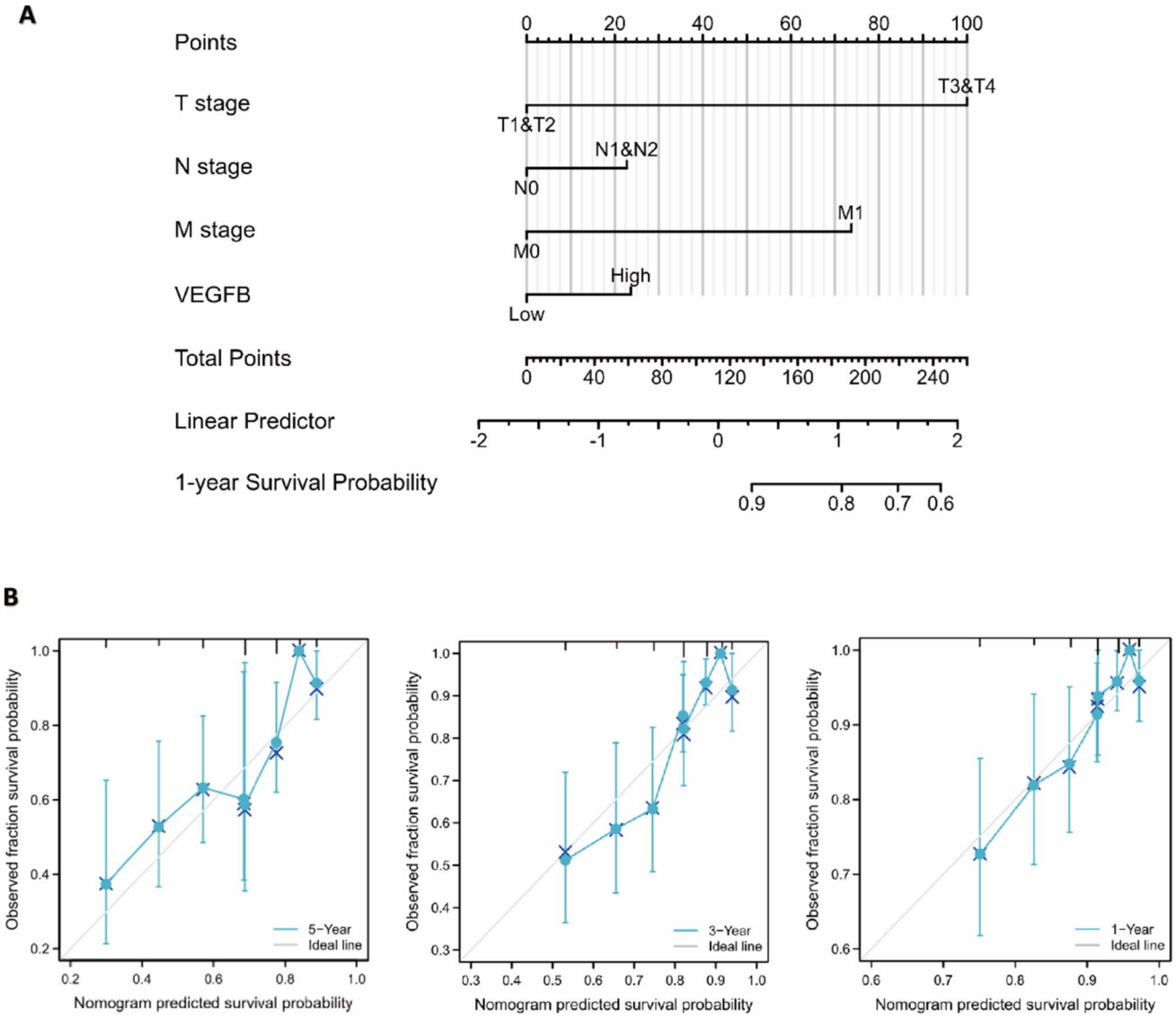

Fig. 4 Construction and validation of the nomogram. **A** The nomogram to predict the 1 -, 3 -, and 5-year prognosis of COAD patients; **B** The calibration curve of nomogram

CTLA4, HAVCR2, HHLA2, ICOS, ICOSLG, IDO1, IDO2, KIR3DL1, LAG3, LAIR1, LGALS9, NRP1, PDCD1, PDCD1LG2, TIGIT, TMIGD2, TNFRSF14, TNFRSF18, TNFRSF25, TNFRSF4, TNFRSF8, TNFRSF9, TNFSF14, TNFSF15, TNFSF18, TNFSF4, TNFSF9, and VTCN1. VEGFB was positively correlated with the immune checkpoints TNFRSF4, TNFRSF8, and TNFRSF18 (Fig. 8A–C).

SHNG17-miR-375-VEGFB regulatory axis construction

We attempted to determine the regulatory network upstream of VEGFB's miRNA. Through the "miRNA-mRNA" module of the StarBase database, 57 potential miRNA genes were identified. Given that miRNAs typically inhibit mRNA expression, the target miRNA should have a negative correlation with VEGFB, be lowly expressed in tumor tissues, and be associated with a favorable prognosis. The correlation analysis revealed that, among the 57 candidate miRNAs, 17 were significantly negatively correlated with VEGFB (Table S1). Expression analysis revealed that six microRNAs were significantly overexpressed in normal colon tissues (Fig. S3). We then conducted a survival analysis to narrow down the candidate miRNAs. Only miR-375 was significantly associated with a favorable prognosis for COAD patients, according to the results. (Fig. S4). We concluded that miR-375 is the most likely upstream miRNA of VEGFB in COAD based

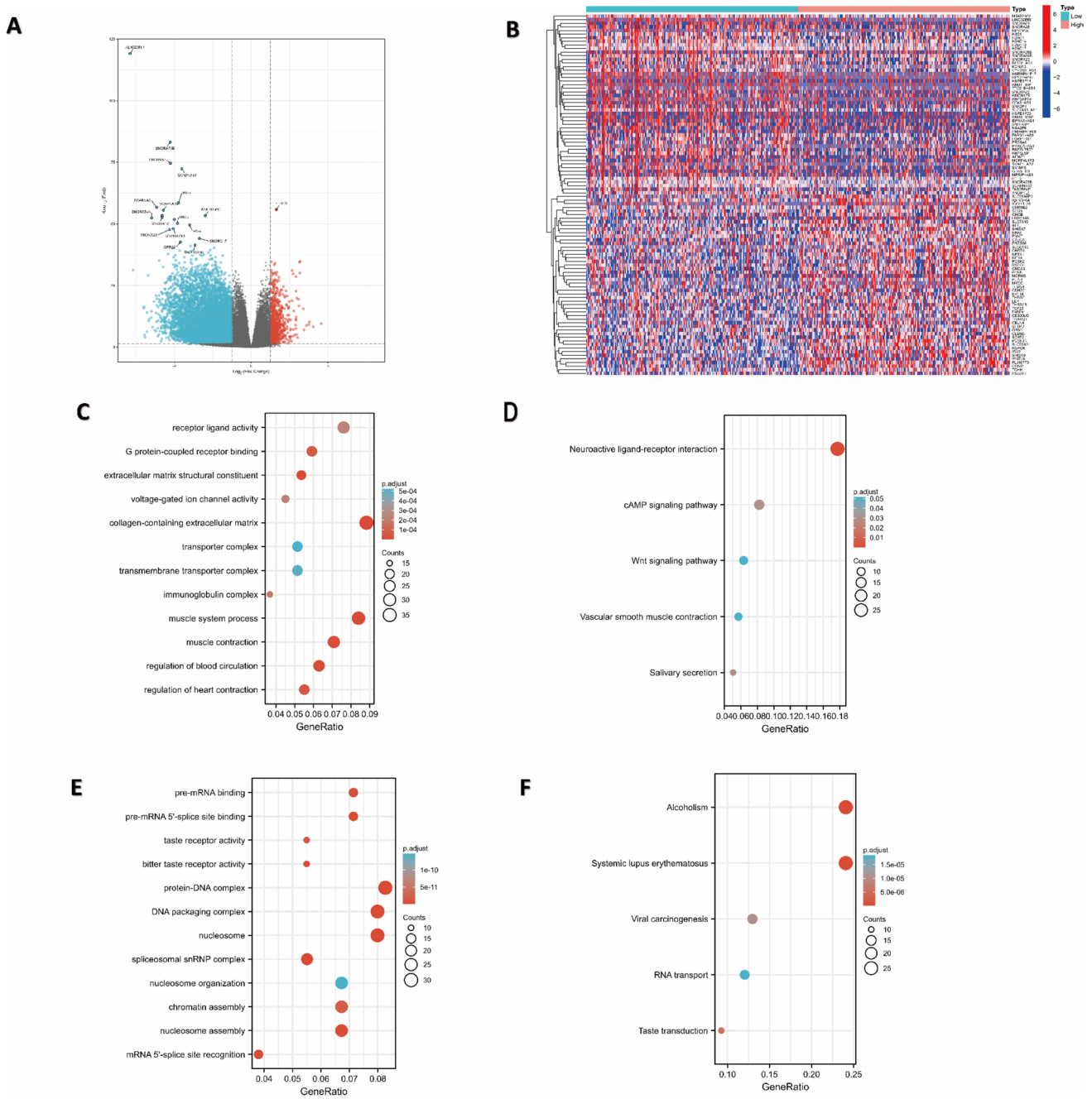

Fig. 5 Function enrichment analysis and DNA methylation of VEGFB in COAD. **A** Differentially expressed genes (DEGs) for high expression of VEGFB vs. low expression of VEGFB in COAD were shown in the volcano plot, with red dots representing significantly up-regulated genes and blue dots representing significantly down-regulated genes in with high expression of VEGFB. **B** The heatmap exhibits the expression level. **C** Enrichment analysis for GO term of up-regulated genes. **D** Enrichment analysis for GO pathway of down-regulated genes

on correlation, expression, and survival analyses (Fig. 9A, B). We analyzed its association with immune infiltrating cells and immune checkpoints in greater detail. There is a significant positive correlation between hsa-miR-375 and VEGFB (TNFRSF4, TNFRSF8, and TNFRSF18) and a negative correlation between it and macrophages and immune checkpoints (Fig. 9C-E). The aforementioned results confirmed miR-375 as the upstream miRNA of VEGFB. We utilized the same method to examine the upstream lncRNAs of miR-375 in COAD. Through the "miRNA-lncRNA" module, eight lncRNAs were extracted initially. lncRNAs should be negatively correlated with

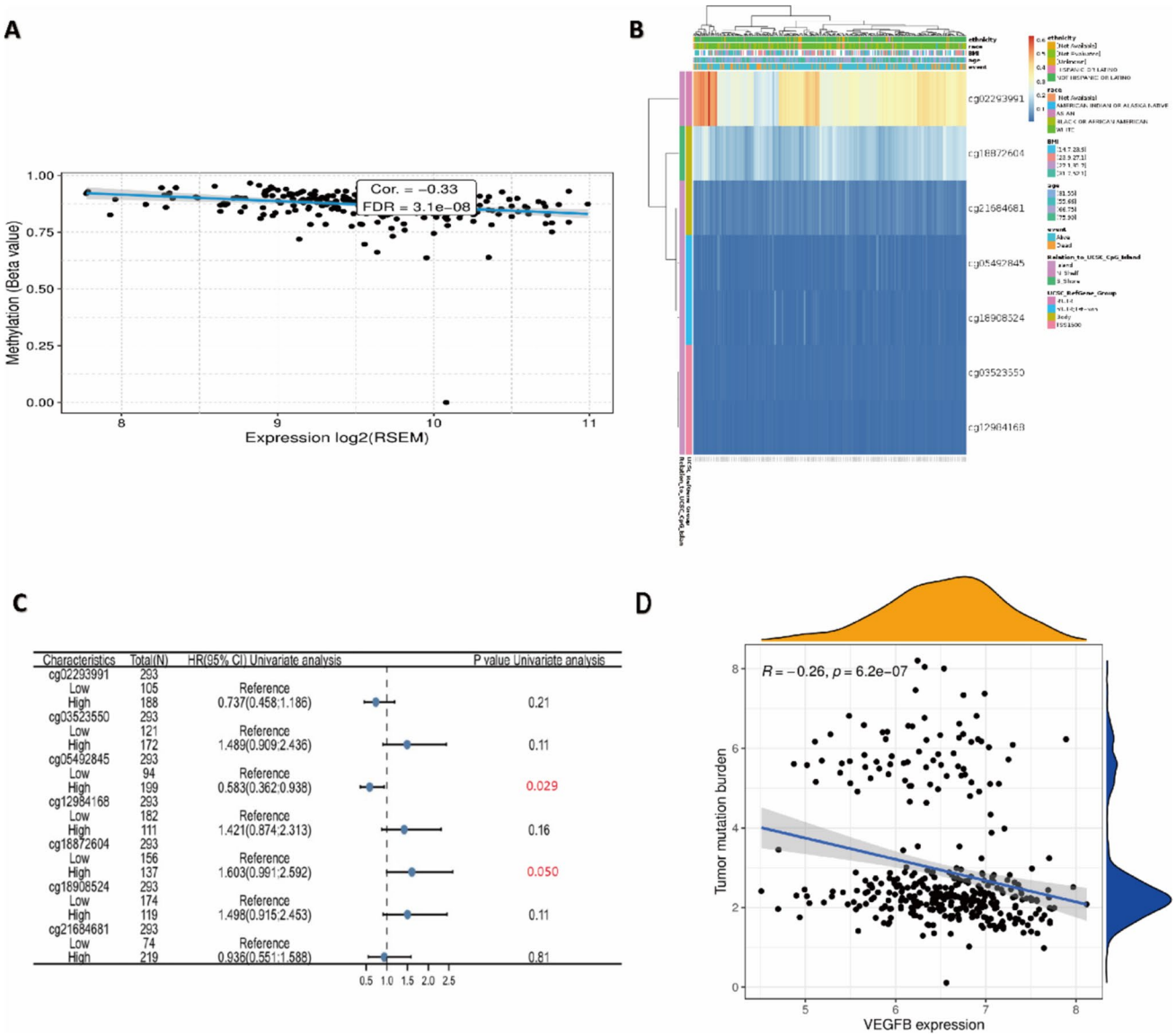

Fig. 6 The DNA methylation analysis of VEGFB in COAD. **A** Correlation between the VEGFB mRNA expression and DNA methylation levels in COAD. **B** The heatmap of DNA methylation of VEGFB in COAD obtained from MethSurv database. **C** The prognostic value of DNA methylation of VEGFB in COAD with different CpG sites. **D** The relationship between VEGFB expression levels and tumor mutational burden

miRNAs and positively correlated with mRNAs, given that they compete with miRNAs for mRNA binding. Only SNHG17 satisfies the aforementioned two requirements (Fig. 10A). According to the correlation analysis, SNHG17 had a negative correlation with miR-375 and a positive correlation with VEGFB (Fig. 10B). Expression and survival analysis confirmed the reliability of SNHG17 as an upstream lcnRNA. The results demonstrated that SNHG17 was highly expressed in COAD tissues and was associated with a dismal prognosis for patients (Fig. 10C, D). Finally, we constructed a predictive model for the SNHG17-miR-375-VEGFB axis in relation to COAD prognosis and progression (Fig. 10E).

VEGFB enhance the proliferation and migration of colon cells

The expression level of VEGFB in colon cancer tissues was significantly higher than that in adjacent normal tissues, both RT-qPCR and immunohistochemistry confirmed (Figs. 11A, B). We further transfected SW480 cells with siRNA and successfully down-regulated the expression

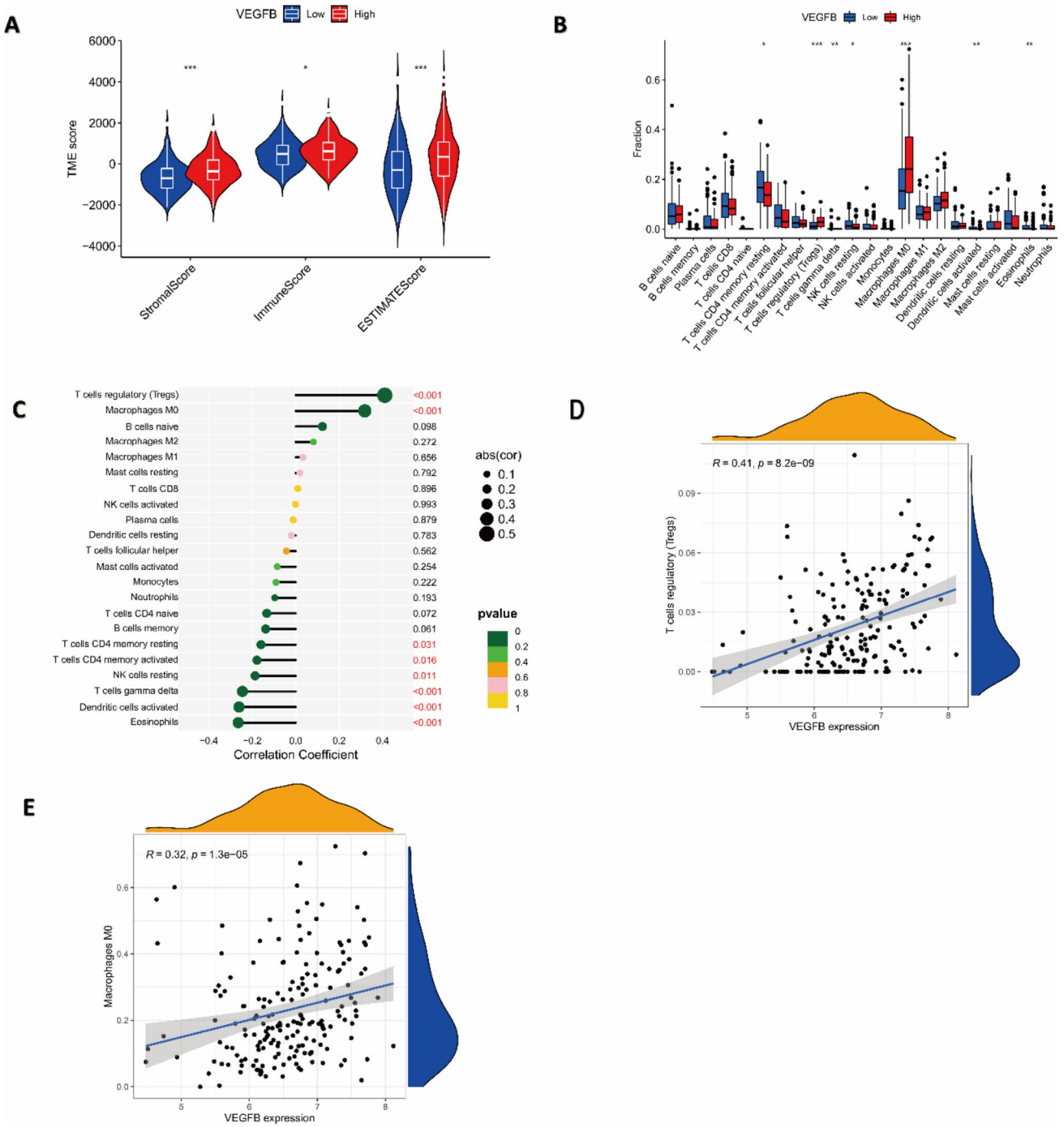

Fig. 7 Tumor microenvironment and immune-related analysis of VEGFB in COAD. **A** The relationship between VEGFB expression and tumor microenvironment. **B** The difference of the levels of 22 TIICs between high and low VEGFB expression groups. **C** The relationship between the abundance of 22 immune cells and VEGFB mRNA expression. The transcription level of VEGFB was significantly positively associated with the levels of Tregs cells and macrophages infiltration in COAD tissues. **D** Correlation analysis between VEGFB expression level and Tregs infiltration. **E** Correlation analysis between VEGFB expression level and macrophage cells infiltration

of VEGFB, and SiRNA NC was also transfected into SW480 cells (Fig. 11C). The results of CCK-8 assay and clonal formation assay showed that down-regulation of VEGFB expression reduced cell viability and proliferation (Figs. 11D, E). Scratch assay was used to examine the effect of VEGFB on the migration ability of sw480 cells (Fig. 11F), and the results showed that down-regulation of VEGFB expression reduced the migration of SW480 cells.

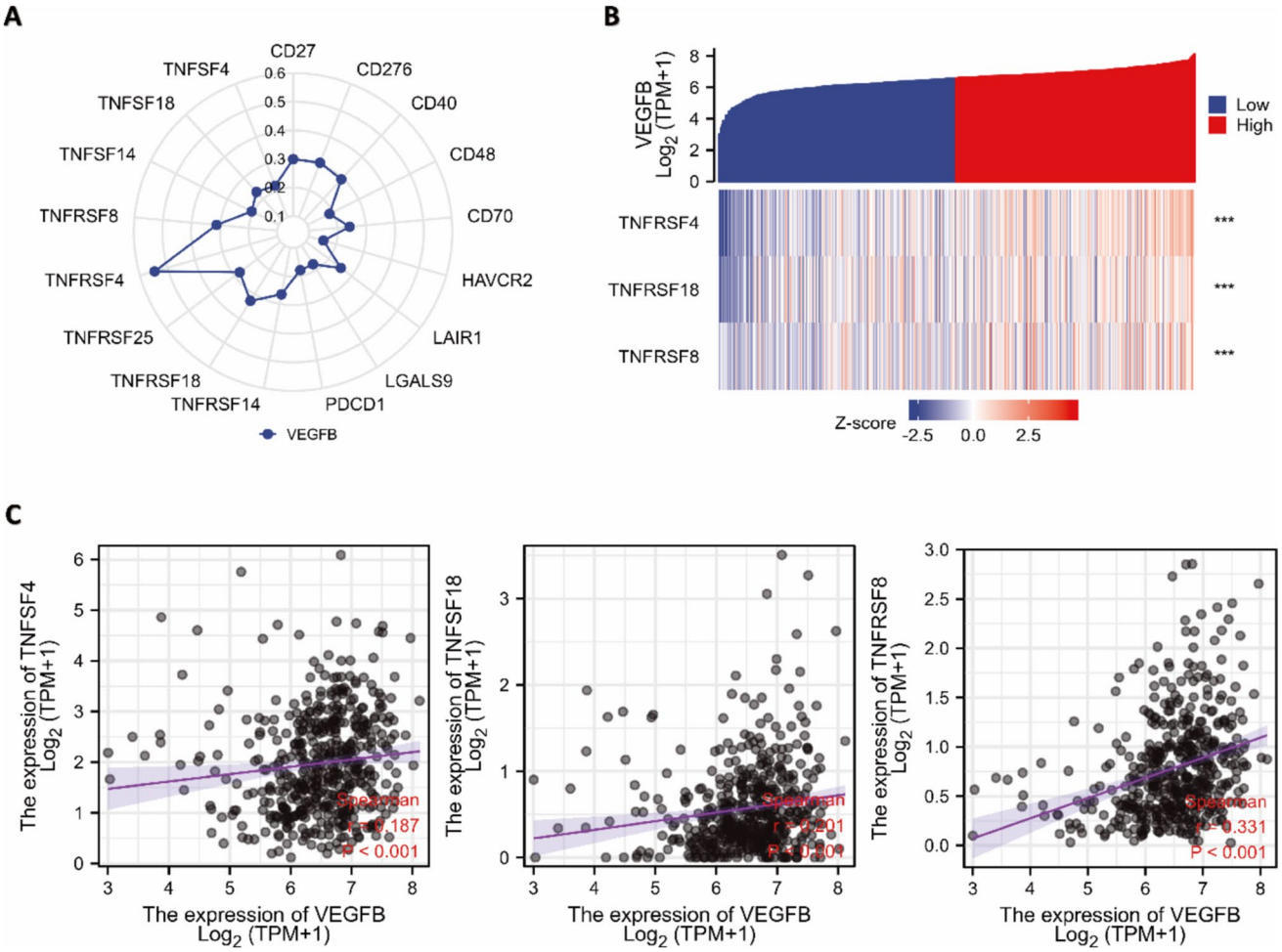

Fig. 8 Correlation between the VEGFB expression and immune checkpoints in COAD. **A** Radar chart evaluating the relationship of immune checkpoint molecules in COAD. **B** The co-expression heatmap of VEGFB and the expression of TNFRSF4, TNFRSF8 and TNFRSF18. **C** Scatter diagrams showed that TRPV3 mRNA expression was significantly positively correlated with the expression of TNFRSF4, TNFRSF8 and TNFRSF18. ***$P < 0.001$

Discussion

The discovery of the VEGF family has transformed our knowledge of the regulatory mechanisms of angiogenesis and lymphangiogenesis (Ferrara and Adamis 2016). The VEGF family is a type of cytokine that can bind specifically to VEGFR (VEGFR-1, VEGFR-2, and VEGFR-3) after secretion and promote the formation of blood vessels and lymph vessels by acting as stimulating factors. This change, whose primary purpose is to increase the supply of oxygen and nutrients while removing metabolic waste and carbon dioxide, can occur under both physiological and pathological circumstances. As the tumor continues to grow, particularly when the tumor diameter exceeds 2 mm, the oxygen content in the tumor microenvironment is insufficient to support exponential growth of tumor tissue, and the tumor cells and surrounding stroma secrete VEGF to promote pathological angiogenesis and lymphangiogenesis (8, 9). Previous research has demonstrated that the VEGF family plays a crucial role in the occurrence and progression of a variety of solid tumors. Overexpression of VEGFA, for instance, is associated with a poor prognosis in patients with oral cancer and with tumor invasion and lymphatic metastasis in esophageal squamous cell carcinoma(Kudelski et al. 2020). A poor prognosis is also indicated by the high expression of VEGFC and VEGFD in patients with gastric cancer(Blank et al. 2015). Nonetheless, the prognostic and biological functions of VEGF family members in COAD patients have not been exhaustively and methodically elucidated. Therefore, we conducted a comprehensive analysis of the expression and mutations of the VEGF family and assessed its diagnostic and prognostic value. In addition, we performed functional clustering, DNA methylation analysis, tumor microenvironment, immune cell infiltration, and correlation analysis of immune checkpoints for VEGFB. We also predicted the upstream miRNA and lncRNA of VEGFB and established

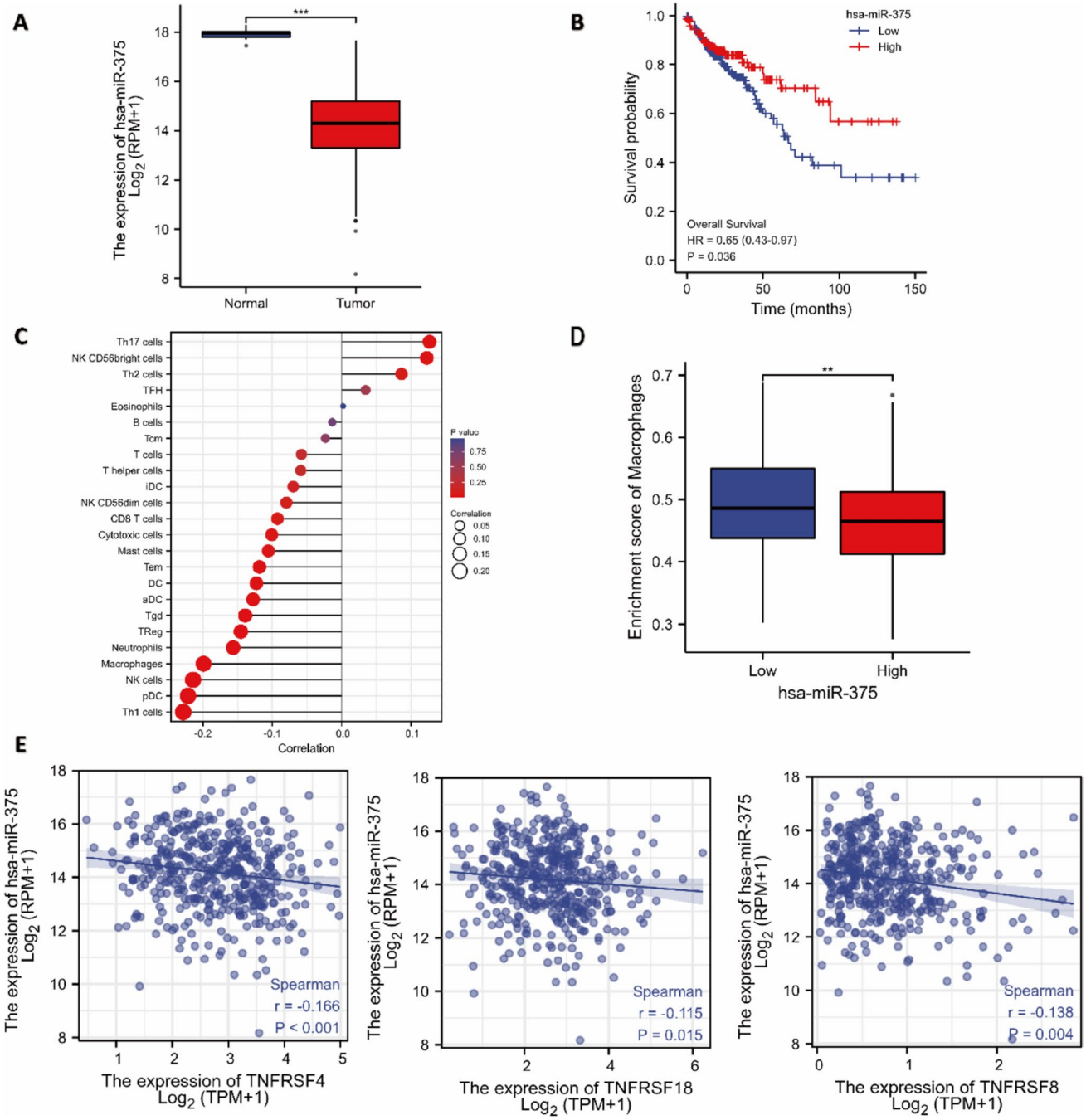

Fig. 9 Identification of miR-375 as a potential upstream miRNA of VEGFB in COAD. SHNG17-miR-375-VEGFB Regulatory Axis Construction. **A** miR-375 was downregulated in COAD. ***$P < 0.001$. **B** COAD patients had a better survival with high expression of miR-375. **C** The relationship between the abundance of 24 immune cells and miR-375 expression. **D** The level of Macrophages was significantly down-regulated in the miR-375 high-expression group compared to the low-expression group. ***$P < 0.001$. **E** Scatter diagrams showed that miR-375 expression was significantly negatively correlated with the expression of TNFRSF4, TNFRSF8 and TNFRSF18

the lncRNA-miRNA-mRNA regulatory network based on the aforementioned findings.

Our study analyzed the expression of VEGF family members using immunohistochemical techniques and multiple databases. Consistent with the findings of previous research, pan-cancer analysis revealed that the VEGF family is highly expressed in the majority of tumor tissues(Kerbel 2008). In COAD, the expression levels of VEGFA, VEGFB, and PGF were significantly higher than in normal tissues, whereas the expression level of

A

lncRNA id	lncRNA name	R value (VEGFB)	P value (VEGFB)	R value (hsa-miR-375)	P value (hsa-miR-375)
ENSG00000273004	AL078644.2	0.061	0.186	-0.058	0.224
ENSG00000278978	AC092611.3	0.191	<0.001	0.018	0.705
ENSG00000244349	AL662791.1	0.048	0.291	0.039	0.411
ENSG00000239569	KMT2E-AS1	0.154	<0.001	0.060	0.211
ENSG00000285338	AC091564.7	-0.005	0.910	-0.023	0.636
ENSG00000246223	LINC01550	-0.035	0.447	0.233	<0.001
ENSG00000196756	SNHG17	0.223	<0.001	-0.157	<0.001
ENSG00000196741	LINC01560	-0.021	0.650	-0.161	<0.001

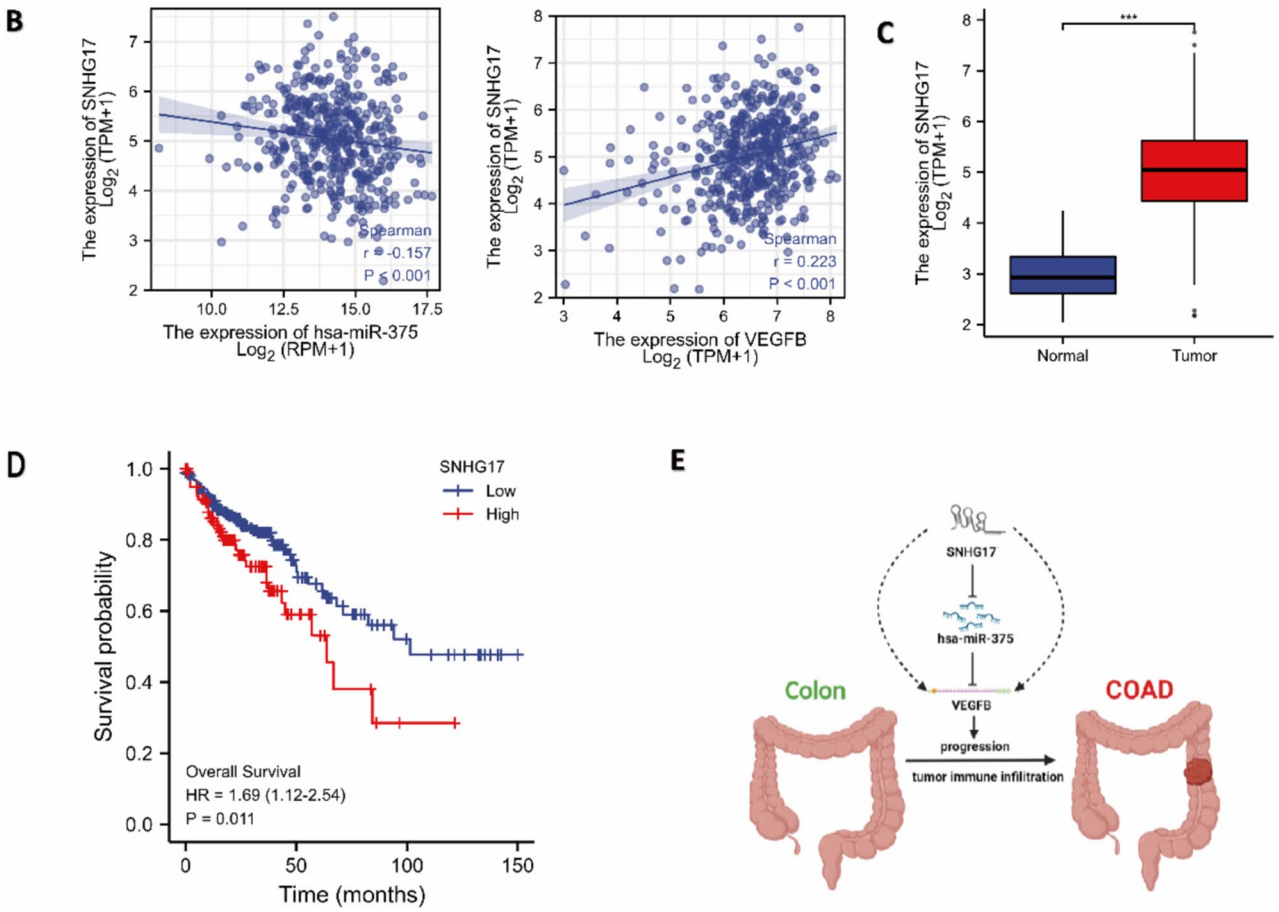

Fig. 10 Identification of SNHG17 as potential upstream lncRNAs of has-miR-375 in COAD (**A**) Correlation of VEGFB and miR-375 with candidate lncRNAs. **B** SNHG17 was negatively correlated with miR-375 while positively correlated with VEGFB. **C** SNHG17 were upregulated in COAD. ***$P < 0.001$. **D** High expression of SNHG17 predicted a worse survival of COAD patients. **E** The model of SNHG17-miR-375-VEGFB axis in carcinogenesis of COAD

VEGFD was significantly lower. In addition, we evaluated the mutation frequency of the VEGF family in COAD tissues and determined that the family members are relatively epigenetically conserved. In addition, there was no difference in survival between patients with the wild-type and those with mutations, indicating that targeted therapy for the VEGF family may be ineffective.

Our research suggests that mutations in the VEGF gene family do not significantly impact the prognosis of COAD (colon adenocarcinoma) patients. Several factors may

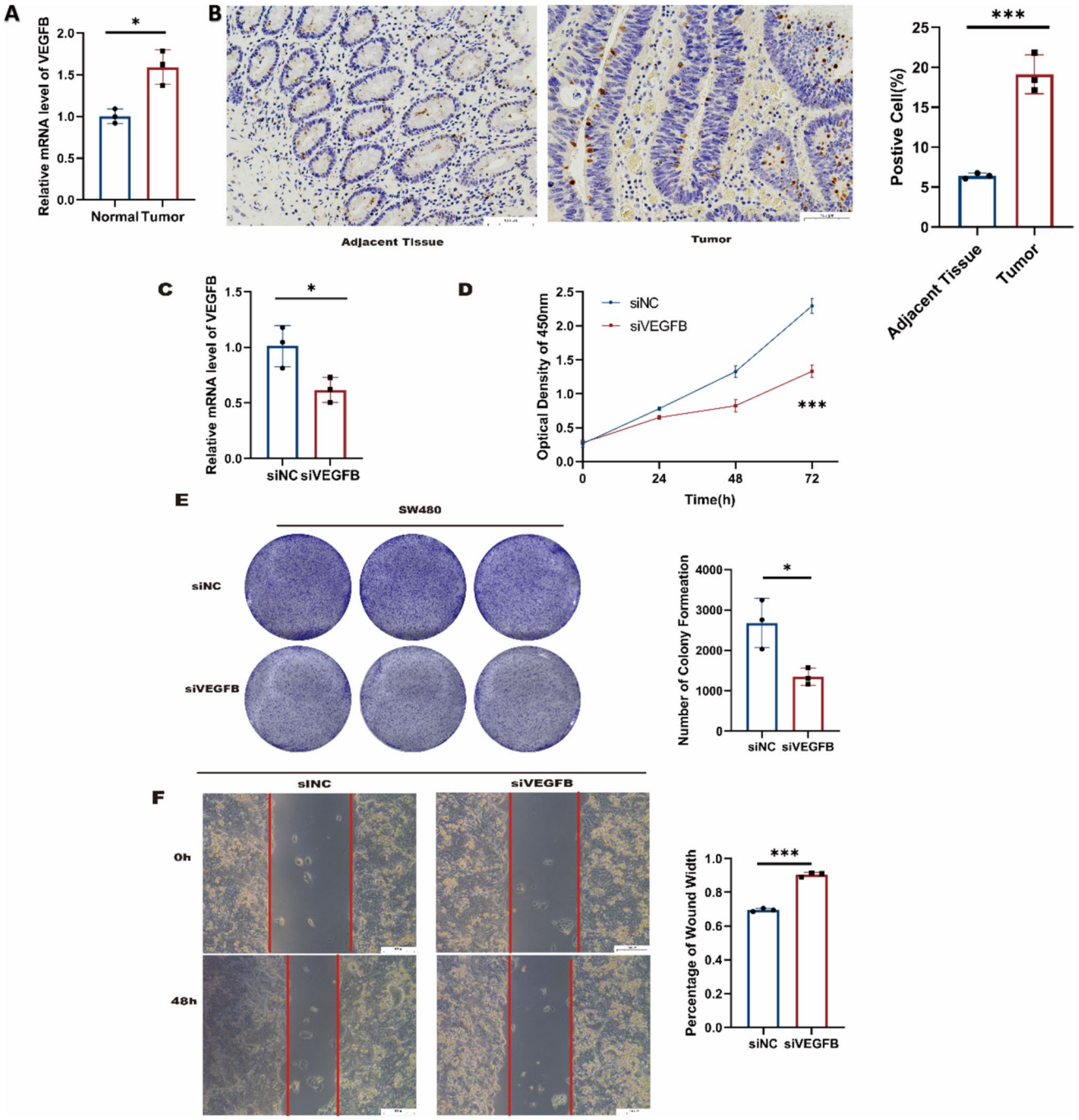

Fig. 11 VEGFB is highly expressed in colon cancer and its activity is necessary for tumor cell proliferation and migration. **A** The expression of VEGFB in tumor tissues was significantly higher than that in adjacent normal tissues verified by RT-q-PCR; **B** Immunohistochemistry confirmed that the expression of VEGFB in tumor tissues was significantly higher than that in adjacent normal tissues; **C** Treatment of SW480 cells with control siRNA or VEGFB-targeting siRNA confirmed that VEGFB was successfully inhibited by RT-q-PCR analysis. **D** The CCK-8 showed that inhibition of VEGFB expression could reduce cell viability. **E** The colony arrangement assay confirmed that the expression of VEGFB was inhibited and the proliferation ability of cells was reduced. **F** The scratch assay confirmed that the expression of VEGFB was decreased and the cell migration ability was decreased

contribute to this finding: First of all, not all mutations are "gain-of-function" mutations. Some may lead to loss of function or have minimal effects on gene activity. In such cases, the mutation neither enhances the oncogenic activity of the VEGF signaling pathway nor significantly impairs angiogenesis, thereby having little influence on patient prognosis(Papachristos et al. 2019). Secondly, our analysis focused on the mutations in the VEGF gene family, which

includes multiple members (such as VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PGF). Each of these members plays a distinct role in tumor progression. For instance, mutations in VEGF-A or VEGF-B may be strongly associated with increased tumor aggressiveness, while mutations in other family members may counterbalance the effects of VEGF-A/B, reducing their impact on prognosis.

Early diagnosis of COAD patients can significantly improve their prognosis; therefore, it is crucial to identify biomarkers with diagnostic and prognostic utility for COAD patients. Previous research has demonstrated that VEGF is more sensitive than carcinoembryonic antigen (CEA) in diagnosing colorectal cancer, and that the combination of VEGF and CEA is more sensitive than either indicator alone(Celen et al. 2004). In addition, these studies demonstrate a positive correlation between VEGF expression levels and clinicopathological variables such as tumor size and vascular invasion in colorectal cancer(Wang et al. 2021). In our study, VEGFA, VEGFB, VEGFC, and VEGFD all demonstrated strong predictive ability (AUC values of 0.777, 0.736, 0.707, and 0.969, respectively). Cox analysis and survival analysis demonstrated that, within the VEGF family, high VEGFB expression is an independent risk factor for COAD patients and is positively correlated with poor OS, PFI, and DSS. The clinical correlation analysis suggested that the level of VEGFB expression was elevated in patients with an advanced clinical stage, lymphatic metastasis, and distant metastasis. Based on the aforementioned findings, we can conclude that VEGFB is highly expressed in COAD and has excellent prognostic value. VEGFB is an independent risk factor for COAD patients, and it may play a role in cancer promotion by promoting lymphatic and distant metastasis of tumors.

Due to the fact that VEGFB was discovered after VEGFA and PGF, it is one of the VEGF family members with the least well-defined mechanism of action(Olofsson et al. 1996). VEGFB is broadly expressed in diverse tissues. It can promote the formation of capillaries in the matrix by stimulating the growth and migration of endothelial cells and boosting their mitogenic activity(Ikuta et al. 2000). However, the role of VEGFB in tumor formation remains debatable. VEGFB has been discovered to be highly expressed in colorectal, ovarian, and prostate cancers(Gunningham et al. 2001; Hanrahan et al. 2003). VEGFB was found to be associated with advanced stage, tumor diversity, and vascular invasion in HCC patients(Kanda et al. 2008). Additionally, VEGFB was found to be associated with prognosis in patients with node-positive breast cancer(Mylona et al. 2007).

Given the significance of VEGFB in COAD tissue, we chose VEGFB as the primary research subject for this study's continuation. There are currently few studies on VEGFB(Shen et al. 2018; Ling et al. 2021). Most researchers believe that VEGFB's role in angiogenesis and lymphangiogenesis is not as significant as VEGFA's (Fischer et al. 2008). According to studies, an increase in its expression level also contributes to the progression of tumors, and its possible mechanism is to aid the blood vessels and lymphatic vessels of existing tumor tissues in maintaining their viability (Melincovici et al. 2018).

One of the epigenetic mechanisms used by cells to regulate gene expression is DNA methylation. Hypomethylation of proto-oncogene promoters can induce activation of proto-oncogenes and cell carcinogenesis(Chen et al. 2022) (Koch et al. 2018). Studies demonstrate that VEGFB methylation increases the risk of progression to high-grade serous ovarian cancer (Dai et al. 2013). The methylation level of the VEGFB gene decreased as its expression level increased, according to our study. Methylation profiles revealed that the methylation sites cg05492845 and cg18872604 were significantly associated with the prognosis of colon cancer patients, indicating that VEGFB methylation may play a role in the development of COAD.

As is now common knowledge, tumors grow and evolve through a constant crosstalk with the surrounding microenvironment, and there is emerging evidence that angiogenesis and immunosuppression frequently occur simultaneously in response to this crosstalk(Gnoni et al. 2019). TME is defined as a complex environment in which tumor cells thrive and proliferate. Immune cells and stromal cells in the TME also play a crucial role in tumor progression. To determine whether the proangiogenic effect of VEGFB is accompanied by immunosuppression, we compared the immune microenvironment between groups with high and low VEGFB expression. In stromal components, immune components, and the sum of the two, the amount of VEGFB in the high expression group was significantly greater than that in the low expression group. The increase of stromal components in tumor tissue is associated with an increase in VEGFB expression levels, which may be associated with an increase in VEGFB secretion in tumor stroma under hypoxia. Interestingly, the immune component was also associated with the level of VEGFB expression, suggesting that VEGFB may regulate tumor progression via immune regulation.

Previous research has demonstrated that capecitabine has an antitumor effect by inhibiting the synthesis of DNA and RNA in tumor cells, and that it can also increase immune cells' resistance to tumors(Derakhshani et al. 2021). This study suggests that anticancer drugs may exert their antitumor effects via multiple mechanisms. Therefore, we investigated the association between VEGFB and immune cell infiltration further. First, in terms of immune cell infiltration, the VEGFB high expression group had a greater proportion of regulatory T cells (Tregs) and macrophage M0. VEGFB was positively correlated with Tregs and macrophages M0, and negatively correlated with eosinophils, dendritic

cell activation, T cell gamma delta, NK cell resting, T cell CD4 memory activation, and T cell CD4 memory resting, according to the correlation analysis. According to studies, the endothelial cells of tumor blood vessels can regulate the activity of immune cells to influence immunity(De Sanctis et al. 2018). Tregs and tumor-associated macrophages are the primary effector cells in the immunosuppressive microenvironment that can play an immunosuppressive role in promoting tumor progression(McAllister and Weinberg 2014; Kitamura et al. 2015). Consequently, VEGF-targeting anti-angiogenic therapy can have additional effects on immunity by altering endothelial cell function and, consequently, the infiltration level of effector cells. Our study demonstrates that VEGFB is highly correlated with Tregs and macrophages associated with tumors, providing new evidence that anti-angiogenic drugs can activate immunity to exert anti-tumor effects.

Given the possibility of immunosuppression resulting from VEGFB overexpression, we investigated its association with immune checkpoints in greater depth. The results revealed a positive correlation between VEGFB and the immune checkpoints TNFRSF4, TNFRSF8, and TNFRSF18. Resistance to anti-vascular therapy frequently results in treatment failure and recurrence of disease(Itatani et al. 2018). Vascular co-option, intussusception microvascular growth (IMG), and vasculogenic mimicry (VM) all contribute to the development of anti-angiogenic therapy resistance (Ribatti et al. 2021). Combining anti-angiogenic therapy with immune checkpoint inhibitors has been shown to reduce drug resistance induced by long-term anti-angiogenic therapy(Hodi et al. 2014). This phenomenon may be related to the further downregulation of VEGF expression and diminution of its pro-angiogenic effect following activationof immune checkpoints(Zheng et al. 2018). Our study's identification of a close relationship between immune checkpoints and VEGFB is an excellent illustration of this concept. Combining TNF inhibitors with anti-angiogenic drugs that target VEGFB could be a promising future treatment for COAD patients.

The competing endogenous RNA (ceRNA) hypothesis holds that lncRNA can compete with miRNA for mRNA binding(Chiu et al. 2018). This mechanism is prevalent in tumors and can regulate the expression of tumor-related genes during tumor proliferation, invasion, and metastasis(Wang et al. 2020). Consequently, we attempt to determine the ceRNA regulatory network of VEGFB in COAD tissues. The StarBase database provided us with 57 potential miRNAs. Through comprehensive expression analysis, survival analysis, and immune correlation analysis, the upstream miRNA of VEGFB was identified as miR-375. Multiple studies have demonstrated that miR-375 inhibits colorectal cancer cell proliferation and metastasis(Cui et al. 2016; Elshafei et al. 2017; Liu et al. 2018b). Our study revealed that miR-375 was lowly expressed in COAD and that its high expression level was associated with a favorable prognosis for patients, which was a useful addition to previous research. Next, the upstream lncRNAs of miR-375 were predicted. Among the eight potential lncRNAs identified by the StarBase database, SNHG17 was deemed the most probable. Previous research has suggested that high SNHG17 expression in colorectal cancer contributes to tumor cell proliferation and metastasis and is associated with a poor prognosis for patients(Ma et al. 2017; Liu et al. 2020; Bian et al. 2021). The results of our analysis of SNHG17 expression and prognostic analysis were consistent with those of previous research. In conclusion, our research uncovered an SNHG17-miR-375-VEGFB regulatory axis. This will provide patients with COAD with effective biomarkers and therapeutic targets.

This study, like all others, has limitations that must be considered when determining future research directions: (1) Our findings may not be applicable to patients with other subtypes of colon cancer because our study was limited to adenocarcinoma, the most prevalent subtype of colon cancer. In addition, in the "post-genome" era, genomics-based network stratification has emerged as a novel classification method for cancer. Based on the similarity of their molecular profiles, tumors can be subdivided into subtypes with identical clinical and biological significance. Increasing numbers of studies are based on the analysis of tumor methylation, immunity, and tumor microenvironment subtypes, leading to more reliable results. (2) The ROC curve is generated by continuously adjusting the "threshold" to produce a sequence of key points. Consequently, the sensitivity and specificity of ROC curves vary across a spectrum of possible thresholds. Future research should contemplate the incorporation of more precise and individualized prediction methods.

In conclusion, the expression of VEGFA/VEGFB/PGF was upregulated in COAD tissues, while the expression of VEGFD was downregulated in COAD tissues. VEGFA / VEGFB/ VEGFC/ VEGFD are potential diagnostic biomarkers for patients with COAD, whereas VEGFB is a potential prognostic biomarker. The abnormal expression of VEGFB in COAD tissues may be related to methylation regulation abnormalities. In addition to promoting angiogenesis, VEGFB is closely associated with the tumor microenvironment, immune cell infiltration, and immune checkpoints, which provides substantial evidence for explaining the phenomenon of immune activation in anti-angiogenic therapy and the reversal of drug resistance. The SHNG17-miR-375-VEGFB regulatory axis may offer a novel approach for investigating the occurrence and progression of COAD.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10142-024-01493-x.

Acknowledgements We acknowledge the GSCA, cBioPortal, GeneMANIA, String, MethSurv and starBase databases for free use.

Author contributions Y.J and L.C: Conceptualization. W.Z: methodology. Y.J: software. L.C: validation. Y.J: formal analysis and investigation. Y.J and L.C: writing-original draft preparation and visualization. J.K: writing-review and editing, supervision and project administration. All authors reviewed the manuscript.

Funding This study was supported by the National Natural Science Foundation of China (52372262).

Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics statement The studies involving human participants were reviewed and approved by Institutional Research Ethics Committee of Shandong provincial hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Competing interests The authors declare no competing interests.

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