Am J Cancer Res 2025;15(4):1540-1558
www.ajcr.us /ISSN:2156-6976/ajcr0161817

Original Article

SEMA6B promotes thyroid tumorigenesis and chemoresistance via WNT/β-catenin signaling in response to doxorubicin

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Received November 7, 2024; Accepted April 8, 2025; Epub April 15, 2025; Published April 30, 2025

Abstract: Thyroid cancer (THCA) is the most common endocrine malignancy and typically has a favorable prognosis. However, aggressive subtypes, particularly anaplastic thyroid carcinoma, present significant treatment challenges due to their high metastatic potential and resistance to conventional therapies. Therefore, a better understanding of the underlying mechanisms is essential for improving treatment strategies. Herein, we aimed to investigate the role of SEMA6B in THCA progression and explore its associated molecular mechanisms. Differentially expressed genes (DEGs) in THCA was screened using RNA sequencing data from paired THCA and normal tissues of 10 patients. The expression and functional role of SEMA6B in THCA were further examined using datasets from TCGA and GEO. Functional assays were conducted to evaluate the effects of SEMA6B overexpression and knockout on THCA cell proliferation. *In vivo* xenograft assays were performed to validate these findings. Additionally, the impact of SEMA6B on the WNT/β-catenin signaling pathway was verified. SEMA6B was highly expressed in THCA and significantly associated with poor clinical outcomes. SEMA6B overexpression significantly increased cell proliferation and colony formation, whereas its knockout reduced cell proliferation and enhanced sensitivity to Doxorubicin. Mechanistically, SEMA6B was found to promote activation of the WNT/β-catenin signaling pathway. In conclusion, these data reveal a novel oncogenic role for the SEMA6B/WNT/β-catenin signaling pathway in THCA, providing new insights and potential avenues for therapeutic intervention.

Keywords: THCA, SEMA6B, WNT/β-catenin signaling, chemoresistance, doxorubicin, differentially expressed genes (DEGs), tumor microenvironment, prognostic biomarker

Introduction

Thyroid cancer (THCA) is a common malignancy that arises from the follicular epithelial cells of the thyroid gland. With advances in medical technology, the incidence of THCA has steadily increased [1]. Nevertheless, individuals with advanced or poorly differentiated THCA often face significant challenges such as lymph node metastasis and unfavorable survival rates, despite receiving treatments like lymph node dissection and radioactive iodine therapy [2, 3]. Given the multifactorial nature of thyroid cancer progression, including genetic, environmental factors, and disruptions in oncogenes and tumor suppressor genes, it is imperative to investigate the molecular pathways associated with metastasis.

Next-generation sequencing (NGS) has revolutionized our understanding of molecular landscape of THCA, enabling the identification of key genetic alterations such as BRAF, RAS, RET/PTC, and TERT mutations, all of which are implicated in tumor initiation and progression [3-5]. In the present study, we utilized NGS to analyze gene expression patterns in THCA tissues versus normal thyroid tissues. Our results revealed significant alterations in the expression of several genes, with SEMA6B emerging as a potential key regulator in THCA progression.

https://doi.org/10.62347/BIUC4913

SEMA6B, a member of the semaphorin family, has recently garnered attention for its role in cancer biology [6]. Initially identified as an axon guidance molecule in the nervous system, SEMA6B has since been implicated in various physiological and pathological processes, including immune regulation, angiogenesis, and tumor progression [7]. Recent studies have highlighted its oncogenic potential in several cancers, including breast cancer [8], colorectal cancer [9], and gastric cancer [10], where it has been shown to promote tumor growth, invasion, and chemoresistance. However, the functional role of SEMA6B in thyroid cancer remains largely unexplored.

In this study, we aim to investigate the effect of SEMA6B on the chemosensitivity of THCA cells to Doxorubicin, a commonly used chemotherapy drug for aggressive THCA cases [11, 12]. By elucidating the molecular mechanisms through which SEMA6B contributes to THCA progression and therapeutic resistance, this study seeks to provide novel insights that could inform the development of targeted treatments and improve patient outcomes.

Materials and methods

RNA sequencing and differential expression analysis

In October 2020, a total of 6 paired samples of THCA tissues and matched normal thyroid tissues were collected from patients at Quanzhou First Hospital Affiliated to Fujian Medical University. This study was approved by the hospital's Ethics Committee (Ethical No. 2020256).

Inclusion Criteria: Patients with histologically confirmed THCA based on pathological diagnosis; Availability of sufficient tumor tissue and corresponding normal thyroid tissue for molecular analysis; Patients who had not undergone prior chemotherapy or radiation therapy to ensure that the tumor samples were unaffected by treatment; Patients who provided written informed consent for the use of their tissue samples in this research.

Exclusion Criteria: Patients with incomplete clinical or pathological data; Cases with insufficient tissue samples or degraded RNA/DNA quality following extraction; Patients with other concurrent malignancies to avoid confounding results; Patients who declined to participate or withdrew their consent.

Total RNA was extracted from the collected samples and subjected to RNA sequencing, which was performed by Tianyi Huiyuan, Biotechnology Co., Ltd., Wuhan, China. Differential gene expression analysis was conducted using Limma and DESeq2 packages. Data normalization was carried out using the Trimmed Mean of M-values (TMM) method. Genes with an absolute log2 fold change ($|log2FC| > 1$) and a false discovery rate ($FDR < 0.05$) were considered differentially expressed genes (DEGs). Results were visualized using hierarchical clustering, heatmaps, and volcano plots for comprehensive data presentation.

Analysis of SEMA6B in THCA using TCGA and GEO datasets

The Cancer Genome Atlas (TCGA) dataset was utilized to assess SEMA6B expression across multiple cancer types, including THCA, using R software (version 3.6.3) [10]. Gene expression levels were normalized using either Reads Per Kilobase of transcript per Million mapped reads (RPKM) or Transcripts Per Million (TPM) methods. To further validate SEMA6B expression in THCA, data from Gene Expression Omnibus (GEO) datasets (GSE24551 and GSE41258) were analyzed [13]. The diagnostic utility of SEMA6B in differentiating tumor tissues from normal tissues was assessed using Receiver Operating Characteristic (ROC) curve analysis, with the area under the curve (AUC) calculated to quantify diagnostic accuracy.

Gene set enrichment analysis (GSEA)

Gene Set Enrichment Analysis (GSEA) was performed using GSEA software to identify biologically relevant pathways enriched in the ranked gene list based on the log2 fold change.

Survival and correlation analysis using TCGA data

Patients in the TCGA dataset were stratified into SEMA6B-high and SEMA6B-low groups based on SEMA6B expression levels. Kaplan-Meier survival curves were constructed to evaluate overall survival (OS) and disease-specific survival (DSS), with statistical significance determined using the log-rank test. Univariate

and multivariate survival analyses were conducted using the Cox proportional hazards model to calculate hazard ratios (HRs) and 95% confidence intervals (CIs).

Correlation analysis of SEMA6B and CTNNB1 (catenin beta 1) gene expression across various cancer types

In a pan-cancer analysis, the Spearman correlation coefficient (r) was calculated for each cancer type to assess the relationship between SEMA6B and CTNNB1 expression across multiple cancer types.

Generation of SEMA6B overexpression and knockout THCA cell lines

To generate THCA cells overexpressing SEMA6B, the SEMA6B gene was cloned into a pHAGE-Flag vector and transfected into CAL-62 and FTC-133 cells using Lipofectamine 3000. Stable cell lines were selected using puromycin (2 μg/mL), and successful overexpression of SEMA6B was confirmed via Western blot analysis using an anti-Flag antibody.

For SEMA6B knockout in THCA cell lines, the CRISPR/Cas9 gene-editing system was utilized. Specific guide RNAs (sgRNAs) targeting the SEMA6B gene (5'-CCGGATGACATCCTCAACTTT-3') were designed and inserted into the lenti-CRISPR v2 vector and Cas9. The recombinant plasmid was transfected into TPC-1 and B-CPAP cells using Lipofectamine 3000. Stable clones were selected using puromycin (2 μg/mL), followed by single-cell isolation and expansion. Successful SEMA6B knockout was validated through Western blot analysis.

Luciferase reporter assay

Following the manufacturer's instructions, the Cignal GRE reporter assay kit (Qiagen, China) was used to evaluate the activation of SEMA6B-induced signaling pathways in 293T cells. Briefly, 293T cells were transfected with Cignal reporter vectors along with SEMA6B expression plasmids or control vectors. After 24-48 hours, luciferase activity was quantified using a dual-luciferase reporter assay system, with Firefly luciferase activity normalized to Renilla luciferase to account for transfection efficiency. Furthermore, TOP/FOP luciferase reporter vectors were employed to measure the impact of SEMA6B on β-catenin-driven transcriptional activity.

Flow cytometry

Flow cytometry was performed to evaluate the effect of SEMA6B deletion on the sensitivity of TPC-1 cells to Doxorubicin. TPC-1 cells and SEMA6B knockout clones (KO1 and KO2) were treated with either DMSO (control) or Doxorubicin for 24 hours. Following drug exposure, the cells were collected and stained with Annexin V-FITC and Propidium Iodide following the manufacturer's protocol. This staining allowed for quantifying apoptotic and necrotic cell populations, providing insight into the impact of SEMA6B knockout on doxorubicin-induced cell death.

In vivo assays

Four-week-old NOD/SCID mice were obtained from the Animal Experiment Center of Wuhan University. The mice were housed in a pathogen-free environment with controlled temperature (22-24°C), humidity (40-60%), and a 12-hour light/dark cycle. The animals had free access to autoclaved standard rodent chow and water throughout the experiment.

A xenograft model was established to examine the impact of SEMA6B knockout on tumor growth *in vivo*. TPC-1 cells (2 × 10^6) were subcutaneously injected into the flanks of the mice. The experiment consisted of three groups: wild-type (WT) cells, SEMA6B knockout clone cells, KO1 and KO2, with 6 mice per group. Tumor growth was monitored by measuring tumor length (L) and width (W) every three days using a calliper, and tumor volumes were calculated using the formula: Tumor Volume = 0.5 × L × W^2.

After 30 days, the mice were humanely euthanized using CO_2 asphyxiation. The tumors were removed, photographed, and weighed. All animal experiments were conducted following institutional guidelines and approved by the Institutional Animal Care and Use Committee (IACUC). This study was approved by the ethics committee at the Huaqiao University School of Medicine (ethics code: A2022060).

CCK-8 cell proliferation assay

Experimental cells were seeded in 96-well plates at a density of 2 × 10^3 cells per well in 100 μL of complete DMEM medium. Cell proliferation was measured 24-, 48-, 72-, and 96-hours post-seeding using the CCK-8 assay (Dojindo, Japan). At each time point, CCK-8 reagent was added to each well, and the plates were incubated for 2 hours at 37°C. The absorbance was recorded at 450 nm using a microplate reader to quantify cell viability.

Colony formation assay

Experimental cells were seeded in 6-well plates at 500 cells per well. After 10-14 days of incubation, the cells were fixed with 4% paraformaldehyde for 15 minutes and stained with 0.5% crystal violet (Beijing Baiaolaibo Technology Co., Ltd., China) for 30 minutes at room temperature. Colonies containing more than 50 cells were manually counted under a light microscope.

Western-blot

Experimental cells were harvested and lysed in RIPA buffer (Thermo Fisher Scientific, USA) supplemented with protease and phosphatase inhibitors (Roche, USA). Protein concentrations were quantified using the BCA Protein Assay Kit (Yeason, China), and 20 μg of protein from each sample was separated on a 10% SDS-PAGE gel. Proteins were transferred onto a PVDF membrane (Millipore) using a wet transfer system. The membrane was blocked with 5% non-fat dry milk in TBST for 1 hour at room temperature, followed by overnight incubation at 4°C with primary antibodies against SEMA6B (cat#ab204423, 1:1000) and GAPDH (cat#ab8245, 1:5000) diluted in TBST containing 5% BSA. The membrane was then incubated with HRP-conjugated secondary antibodies (1:5000 dilution) for 1 hour at room temperature. Protein bands were detected using an enhanced chemiluminescence (ECL) system (Thermo Fisher Scientific, USA) and imaged using a ChemiDoc XRS+ system (Bio-Rad).

Statistical analyses

Statistical analyses were performed using GraphPad Prism and R software. Data were expressed as mean ± standard deviation (SD). Comparisons between the two groups were conducted using unpaired or paired Student's t-tests, depending on the experimental design. For comparisons involving multiple groups, one-way or two-way ANOVA was applied, followed by Tukey's post-hoc test for pairwise comparisons. Kaplan-Meier survival curves were analyzed using the log-rank test. Multivariate survival analyses were performed using the Cox proportional hazards model to estimate hazard ratios (HRs) and 95% confidence intervals (CIs). Correlations between variables were assessed using Pearson or Spearman correlation coefficients, depending on the data distribution. The diagnostic accuracy was assessed by receiver operating characteristics (ROC) curve analysis. Statistical significance was defined as a *p*-value less than 0.05.

Results

Identification and validation of differentially expressed genes in thyroid tumorigenesis

Initially, we analyzed differentially expressed genes (DEGs) in 10 paired THCA and adjacent noncancerous tissues using RNA sequencing. The expression of DEGs was visualized through heat maps (**Figure 1A**) and volcano plots (**Figure 1B**). Analysis of DEGs using Limma and DESeq2 revealed 1,016 overlapping DEGs (**Figure 1C**, **1D**). Further comparison of upregulated DEGs with those associated with poor prognosis in the TCGA dataset identified three overlapping genes: SEMA6B, SPRED3, and NKD2 (**Figure 1E**). We subsequently analyzed the expression of these three genes in paired normal and tumor tissues and confirmed their significant upregulation in tumor tissues (**Figure 1F-H**). Given the limited research on SEMA6B's role in THCA, we selected it as the primary focus for further investigation.

Pan-cancer and THCA expression analysis of SEMA6B

To investigate the role of SEMA6B in cancers, we analyzed its expression across various cancer types, with a specific focus on THCA. Our results revealed a significant increase in SEMA6B expression in THCA, as well as in breast cancer (BRCA), liver hepatocellular carcinoma (LIHC) (**Figure 2A**), highlighting its potential relevance in cancer biology. Paired analyses of normal and tumor samples further con-

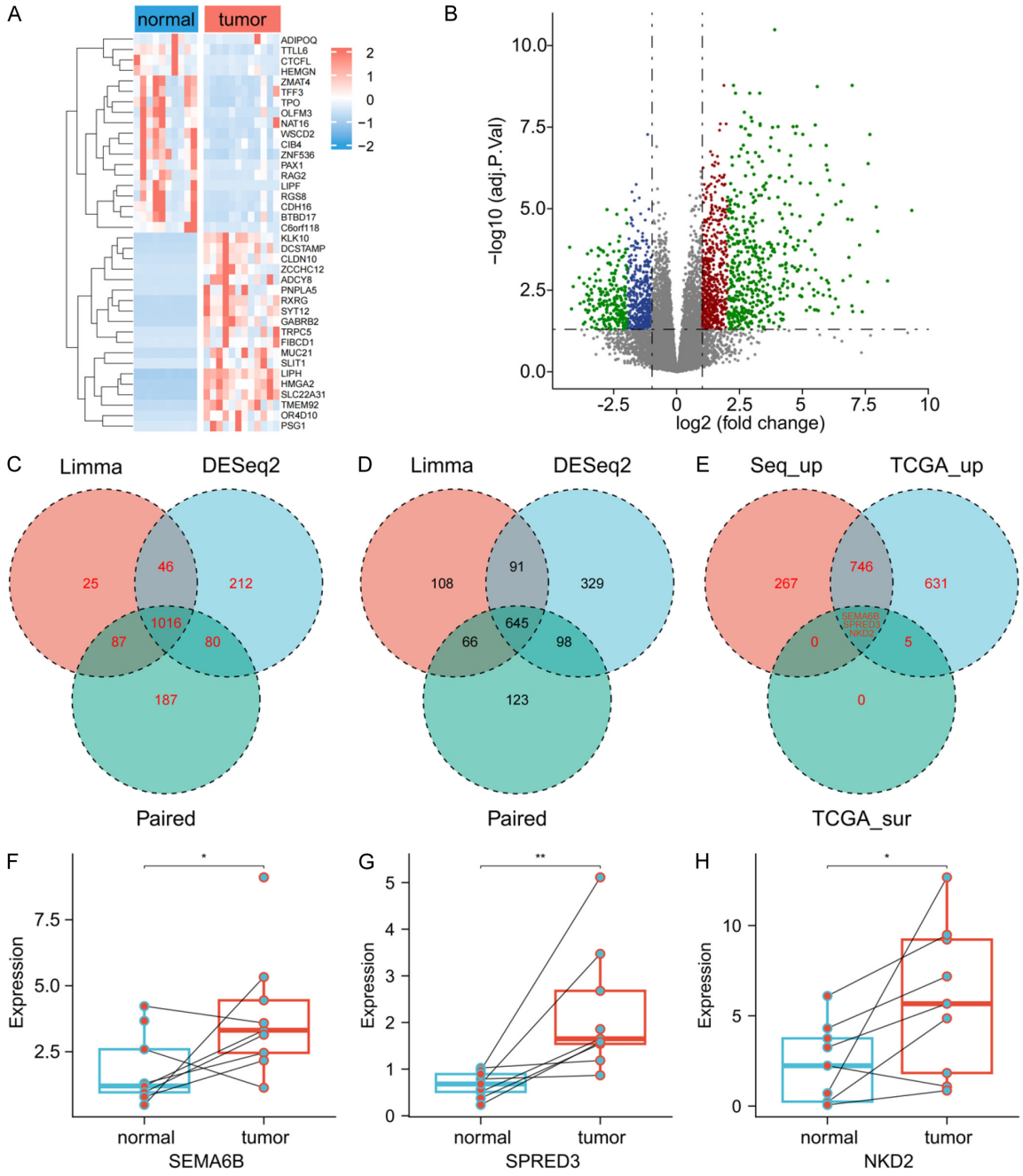

Figure 1. Identification and validation of differentially expressed genes in tumorigenesis. A. Heatmap of differentially expressed genes (DEGs) between normal and tumor tissues. B. Volcano plot illustrating the distribution of DEGs based on log2 fold change and -log10 adjusted *p*-value. C. Venn diagram showing the overlap of DEGs identified by Limma and DESeq2 methods in paired samples. D. Venn diagram comparing DEGs identified by Limma and DESeq2 across datasets. E. Venn diagram comparing upregulated DEGs with those correlated with poor prognosis in the TCGA dataset. F-H. Box plots of SEMA6B, SPRED3, and NKD2 expression levels in paired normal and tumor tissues, *P < 0.05, **P < 0.01.

firmed SEMA6B overexpression in THCA, and liver hepatocellular carcinoma (LIHC) (**Figure 2B**). Consistent with this, SEMA6B expression was significantly elevated in tumor tissues compared to normal tissues in two independent THCA microarray datasets (GSE24551 and GSE41258) (**Figure 2C**, **2D**), suggesting its implication in thyroid tumorigenesis. Addition-

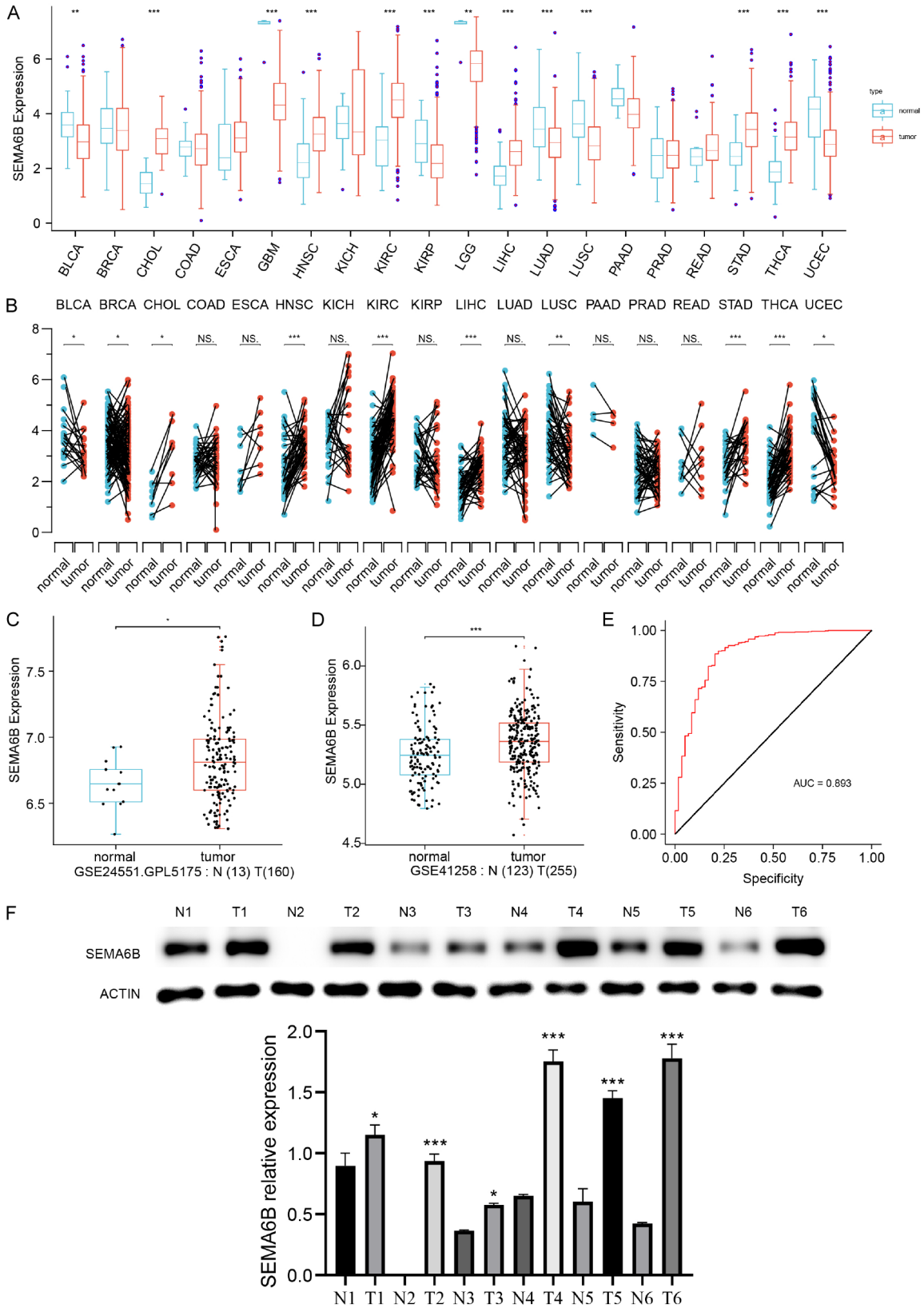

Figure 2. SEMA6B expression analysis in pan-cancer and THCA with diagnostic evaluation. A. Box plots showing SEMA6B expression levels in typical versus tumor tissues across various cancer types (pan-cancer analysis). *P < 0.05, **P < 0.01, ***P < 0.001. B. Paired analysis of SEMA6B expression in matched normal and tumor tis-

sues across different cancers. C. SEMA6B expression in normal versus thyroid tumor tissues (GSE24551 dataset). D. SEMA6B expression in normal versus thyroid tumor tissues (GSE41258 dataset). ***P < 0.001. E. ROC curve assessing the diagnostic potential of SEMA6B expression in THCA. The AUC of 0.893 indicates high diagnostic accuracy. F. Western blot analysis of SEMA6B expression in 6 paired THCA tissues and normal tissues.

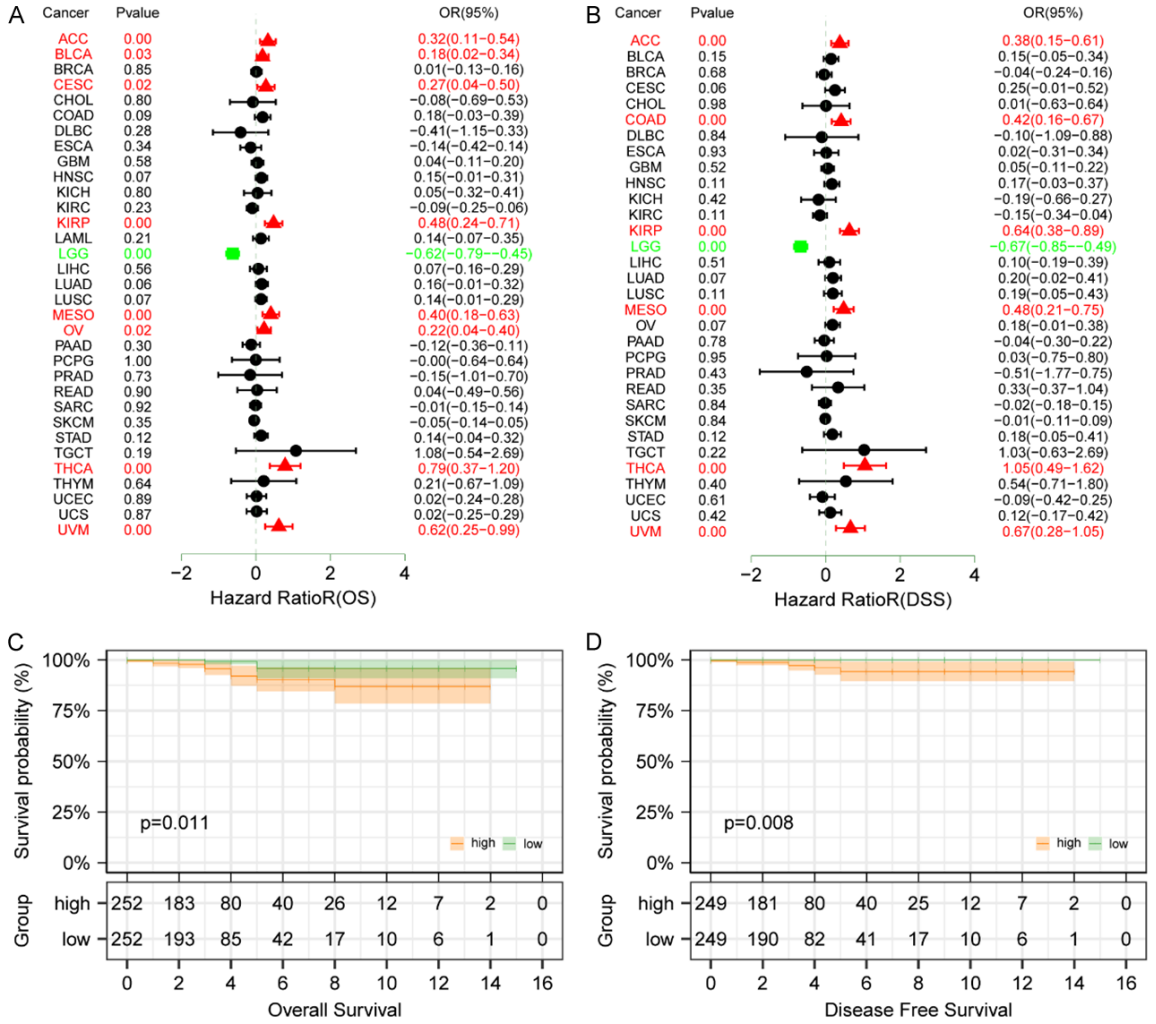

Figure 3. Prognostic significance of SEMA6B in THCA based on TCGA database. A. Forest plot showing the hazard ratios (HRs) for overall survival (OS) associated with SEMA6B expression across various cancers, including THCA (THCA). B. Forest plot depicting the hazard ratios (HRs) for disease-specific survival (DSS) linked to SEMA6B expression across different cancer types. C. Kaplan-Meier curve comparing overall survival between high and low SEMA6B expression groups in THCA patients. D. Kaplan-Meier curve for disease-free survival in THCA, comparing high and low SEMA6B expression groups.

ally, SEMA6B demonstrated strong potential as a diagnostic biomarker for THCA, with an area under the curve (AUC) of 0.893 in ROC curve analysis (**Figure 2E**), indicating high diagnostic accuracy. Supporting these findings, western blot analysis confirmed higher SEMA6B protein expression levels in THCA tissues than in normal tissues (**Figure 2F**). These results underscore the critical role of SEMA6B in multiple cancers and highlight its potential as a diagnostic biomarker, particularly in THCA.

Prognostic significance of SEMA6B in THCA based on TCGA database

Forest plots for overall survival (OS) and disease-specific survival (DSS) in THCA revealed that SEMA6B expression demonstrated a hazard ratio (HR) of 0.79 (95% CI: 0.37-1.20) for OS and 1.05 (95% CI: 0.49-1.62) for DSS (**Figure 3A**, **3B**). Kaplan-Meier survival analysis further demonstrated that patients with high SEMA6B expression had significantly reduced survival

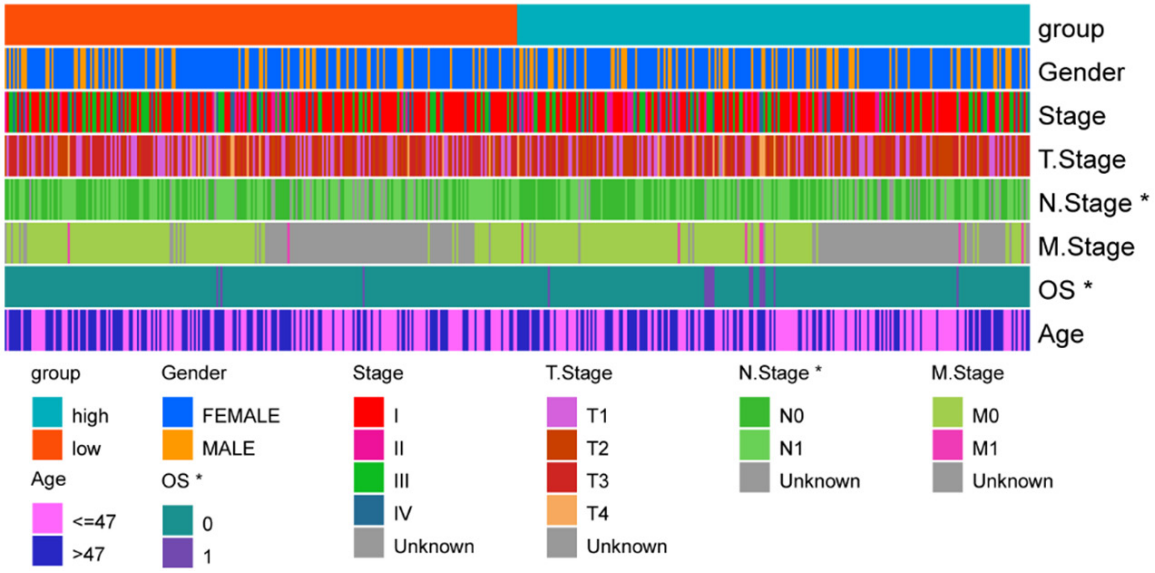

Figure 4. Heatmap showing the association of SEMA6B expression with clinical parameters in THCA.

rates, with lower overall survival (P = 0.011, **Figure 3C**) and disease-free survival (P = 0.008, **Figure 3D**). Additionally, high SEMA6B was associated with inferior OS and advanced N stage (**Figure 4**).

Influence of SEMA6B expression on tumor microenvironment, tumor heterogeneity, and survival outcomes in cancer

The tumor microenvironment plays a pivotal role in invasiveness of THCA. To better understand the connection between SEMA6B and the tumor microenvironment, we examined its association with immune-related scores and cell infiltration. Notably, higher SEMA6B expression was significantly correlated with lower immune score and increased stromal scores (**Figure 5A**). Moreover, elevated SEMA6B expression was also linked to higher levels of CD8_T cells, and Endnothelia cells as well as the lower levels of B cells and CD4 T cells (**Figure 5B**), suggesting that SEMA6B may contribute to remodeling the tumor microenvironment. Further analyses using the MCP-counter and Immunophenoscore (IPS) systems demonstrated that higher SEMA6B expression was associated with cytotoxic lymphocytes, NK cells, Monocytic lineage, Neutorphils, Endothelia cells and Fibroblasts, indicating that SEMA6B may contribute to an immunosuppressive microenvironment (**Figure 5C**, **5D**). Consistent with these findings, the TIMER, QuanTIseq and CIBERSORT algorithms showed that tumors with high SEMA6B expression exhibited a tight linkage with CD8 T cells, Marcrophage, NK cells, plasma cells, resting memory T cells (**Figure 5E-G**), further supporting its involvement in shaping the immune landscape of the tumor.

Tumors are inherently heterogeneous, and this heterogeneity contributes to aggressiveness, treatment resistance, and poor survival outcomes. To explore the relationship between SEMA6B and tumor heterogeneity, we analyzed its impact on various tumor characteristics. Our findings revealed that elevated SEMA6B expression was associated with a lower tumor mutation burden (TMB) (**Figure 6A**), while no significant correlation was observed with intratumor heterogeneity (MATH score) (**Figure 6B**). Additionally, higher SEMA6B expression was linked to reduced Basal Response Score (BRS), and increased Tumor Differentiation Score (TDS) and ERK score (**Figure 6C**). Correlation analyses further confirmed these findings, showing a negative association between SEMA6B expression and BRS and positive correlations with TDS and ERK pathway activity (**Figure 6D-F**). These results suggest that SEMA6B is critical in regulating tumor progression by modulating the microenvironment and key signaling pathways.

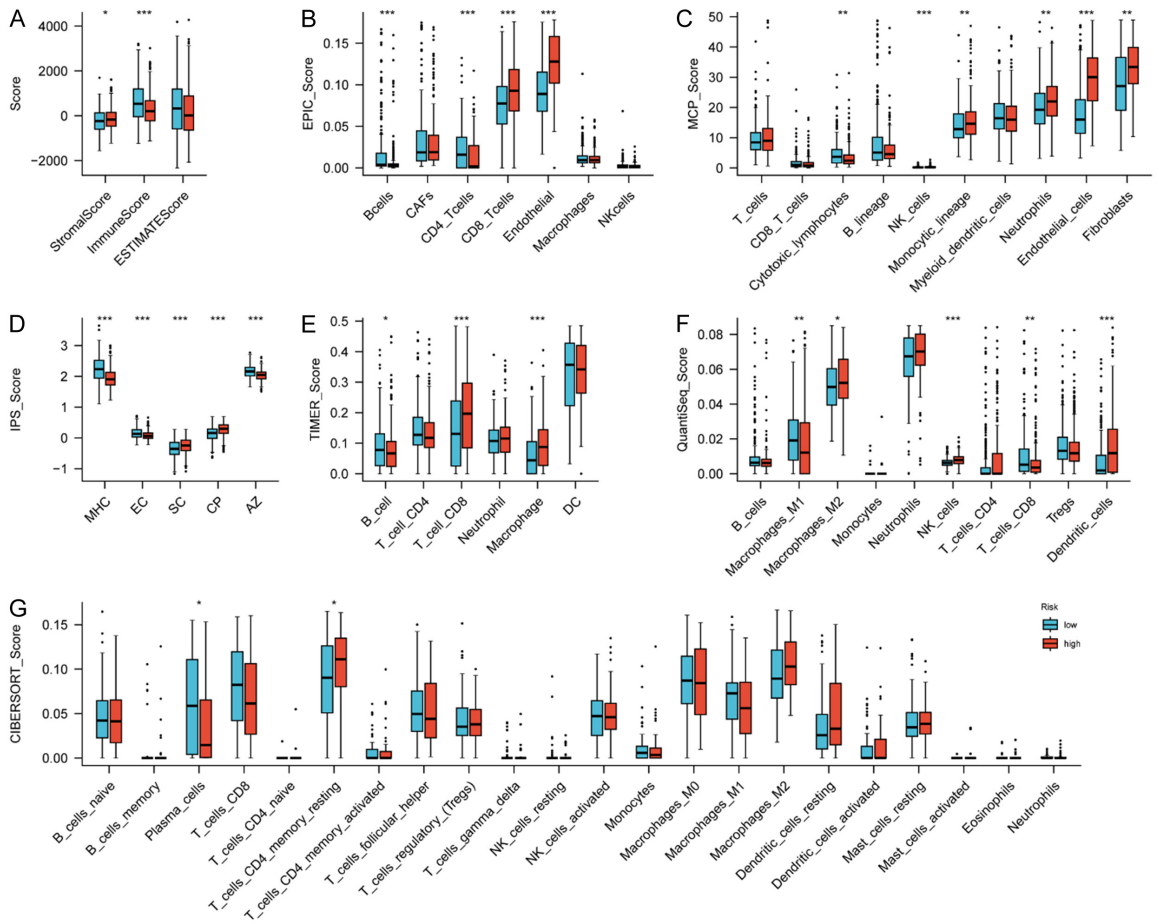

Figure 5. Immune cell infiltration and tumor microenvironment profiles associated with high and low SEMA6B expression. A. Box plots showing the association of SEMA6B expression with ESTIMATE immune and stromal scores, indicating higher immune and stromal cell infiltration in tumors with elevated SEMA6B expression. B. Analysis using the EPIC algorithm demonstrates that higher SEMA6B expression correlates with the increased presence of cancer-associated fibroblasts (CAFs), endothelial cells, and macrophages. C. MCP-counter scores show the relationship between SEMA6B expression and various immune cell populations, including T cells, macrophages, and fibroblasts. D. Immunophenoscore (IPS) analysis indicates that higher SEMA6B expression is associated with a more immunosuppressive tumor microenvironment. E. TIMER algorithm results, showing increased infiltration of immune cells such as macrophages, T cells, and dendritic cells in tumors with higher SEMA6B expression. F. QuanTIseq algorithm analysis further validates the association between elevated SEMA6B expression and increased immune cell infiltration. G. CIBERSORT analysis linking high SEMA6B expression with various immune cell populations, including CD8+ T cells and M2 macrophages.

Single-cell analysis of SEMA6B expression across cancer types

Single-cell analysis across various cancer types revealed distinct patterns of SEMA6B expression, particularly within the tumor microenvironment. SEMA6B expression was significantly elevated in endothelial cells and fibroblasts across multiple cancer datasets (**Figure 7A**), suggesting a potential role in the tumor stroma. In THCA specifically, SEMA6B was predominantly expressed in endothelial cells, as shown by the UMAP plot and contour plot overlay (**Figure 7B**, **7C**). A violin plot further confirmed a significant increase in SEMA6B expression in endothelial cells within the THCA tumor microenvironment (**Figure 7D**). These findings suggest that SEMA6B may play a key role in vascular or stromal components, potentially influencing tumor progression and providing opportunities for targeted therapeutic interventions.

SEMA6B promoted THCA cell proliferation and tumor growth

To investigate the role of SEMA6B expression in THCA, we first analyzed its levels in various THCA cell lines using the CCLE database. Our

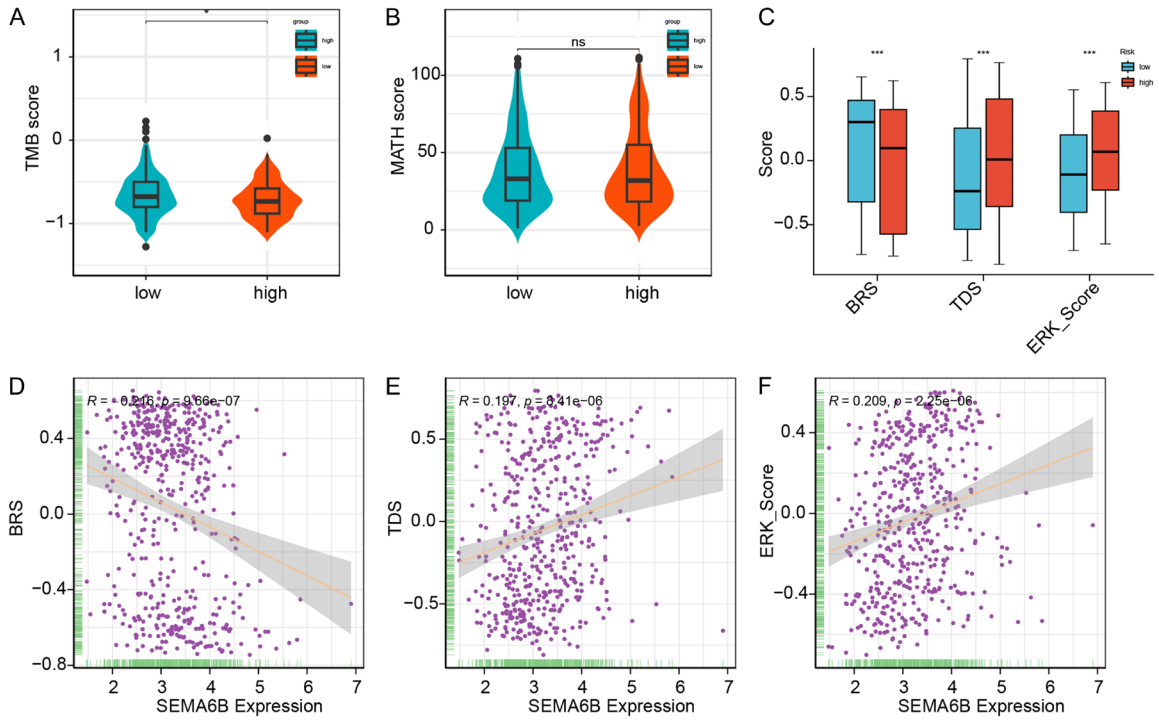

Figure 6. Association of SEMA6B expression levels with tumor mutation burden, tumor heterogeneity, and key pathway activities. A. Violin plot illustrating the association between SEMA6B expression and tumor mutation burden (TMB), with higher expression linked to lower TMB. B. Violin plot showing that SEMA6B expression does not significantly correlate with intratumor heterogeneity (MATH score). C. Box plots showing the association of high SEMA6B expression with worse outcomes in BRS, TDS, and ERK survival scores. D-F. Scatter plots illustrate the correlations between SEMA6B expression and BRS, TDS, and ERK scores, with higher SEMA6B expression linked to poorer prognosis and increased ERK pathway activity. MHC: MHC molecules, EC: effector cells, SC: suppressor cells, AZ: Adaptive Zone.

analysis revealed that SEMA6B expression varied across different THCA cell lines (**Figure 8A**), with relatively low expression in CAL-62 and FTC-133 cells and high expression in TPC-1 and B-CPAP cells (**Figure 8B**), promoting the selection of these cell lines for subsequent studies.

Overexpression of SEMA6B in these cells was confirmed by Western blot analysis (**Figure 8C**). Colony formation assay demonstrated that SEMA6B overexpression significantly increased the colony formation of CAL-62 cells and FTC-133 cells (**Figure 8D**, **8E**, **8G**, **8H**). Additionally, CCK-8 assays demonstrated that SEMA6B overexpression significantly enhanced cell proliferation in CAL-62 and FTC-133 cells (**Figure 8F**, **8I**).

To further explore the functions of endogenous SEMA6B, CRISPR/Cas9 genomic editing was employed to knock out SEMA6B in TPC-1 and B-CPAP cells (**Figure 9A**, **9E**). SEMA6B knockout markedly reduced cell proliferation (**Figure 9B**, **9F**) and colony formation (**Figure 9C**, **9D**, **9G**, **9H**), indicating a loss of tumorigenic potential.

In a mouse xenograft model, SEMA6B knockout significantly suppressed tumor growth, resulting in smaller tumor volumes and weights than wild-type controls (**Figure 9I-K**). These findings underscore the pivotal role of SEMA6B in driving THCA progression, providing strong evidence that SEMA6B promotes THCA cell proliferation and tumor growth.

SEMA6B modulated WNT/β-catenin signaling pathway activity

To explore the molecular mechanisms underlying the tumor-promoting effects of SEMA6B in THCA, we compared mRNA expression profiles between high and low SEMA6B expression groups. The volcano plot and heatmap (**Figure 10A**, **10B**) highlight distinct patterns of gene expression clustering between these groups.

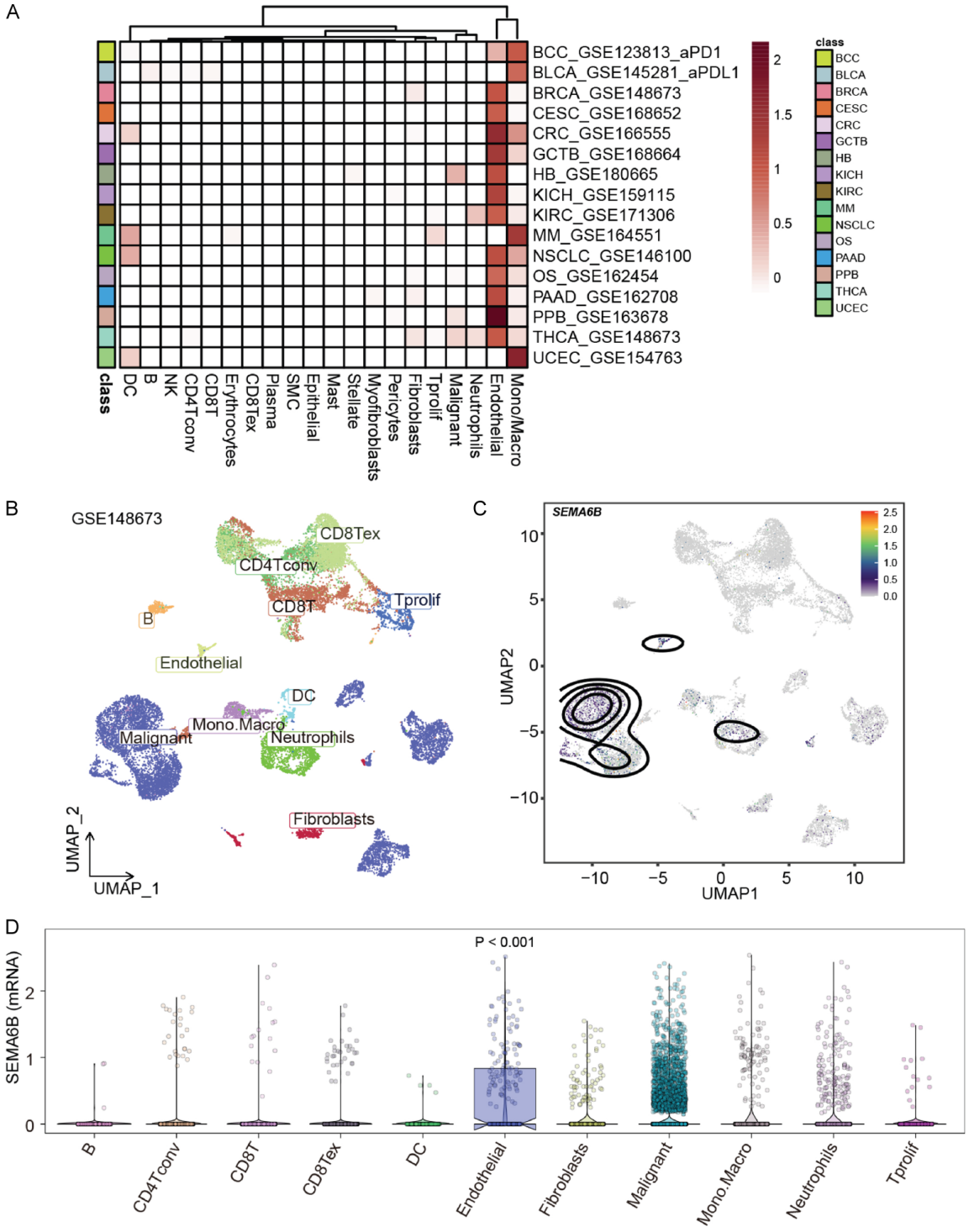

Figure 7. Single-cell analysis of SEMA6B expression inTHCA. A. Heatmap showing SEMA6B expression across different cell types in multiple cancer datasets. The expression is notably enriched in endothelial cells and fibroblasts, suggesting a role in the tumor stroma. B. UMAP clustering from the GSE148673 dataset, depicting the distribution of various cell types, including T cells, B cells, fibroblasts, and endothelial cells. SEMA6B expression is remarkably heterogeneous, with higher levels in stromal cells. C. UMAP plot highlighting the specific localization of SEMA6B expression, primarily in regions associated with endothelial cells and fibroblasts, indicating a role in modulating the tumor microenvironment. D. Box plot quantifies SEMA6B expression across various cell populations, with significantly higher expression in endothelial cells than other cell types ($P < 0.001$), supporting its involvement in angiogenesis and tumor stroma formation.

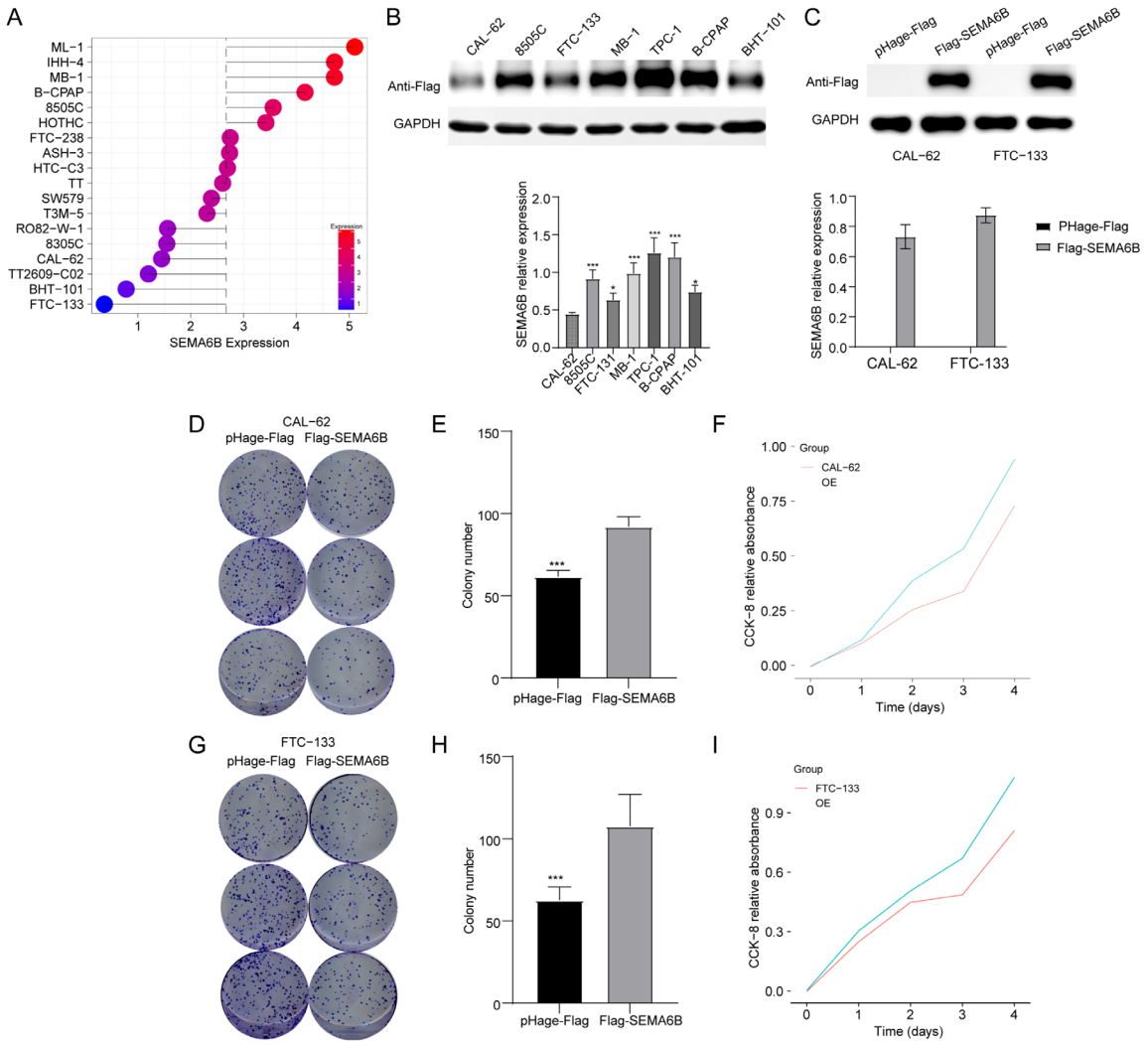

Figure 8. Overexpressing SEMA6B promotes THCA cell proliferation. (A) SEMA6B expression levels across various THCA cell lines, with higher expression observed in more aggressive cell lines. (B) Schematic representation of the overexpression of SEMA6B in CAL-62 and FTC-133 cells. (C) Western blot analysis confirming SEMA6B overexpression in CAL-62 and FTC-133 cells. (D and G) Clonogenic assays showed increased colony formation in CAL-62 (D) and FTC-133 (G) cells after SEMA6B overexpression. (E and H) Quantification of colony numbers in CAL-62 (E) and FTC-133 (H) cells, showing a significant increase with SEMA6B overexpression (***P < 0.001). (F and I) CCK-8 assays demonstrating enhanced proliferation in CAL-62 (F) and FTC-133 (I) cells with SEMA6B overexpression.

To gain further insights into the biological pathways associated with these differentially expressed genes, we performed GSEA. The high SEMA6B expression significantly enriched in pro-tumorigenic pathways, including mesenchymal transition, angiogenesis, and WNT/β-catenin signaling (**Figure 10C**). Moreover, **Figure 10D** confirms the enrichment of key oncogenic pathways, such as WNT/β-catenin and KRAS signaling, in the high SEMA6B expression group. In contrast, pathways such as estrogen response and protein secretion exhibited negative enrichment, indicating that SEMA6B may differentially regulate cancer-related processes. These findings suggest high SEMA6B expression is associated with a pro-tumorigenic gene expression profile.

To further investigate the role of SEMA6B in THCA, dual-luciferase reporter assays were conducted to assess its impact on oncogenic signaling pathways. Overexpression of SEMA6B significantly activated the WNT/β-catenin signaling pathway compared to the control group

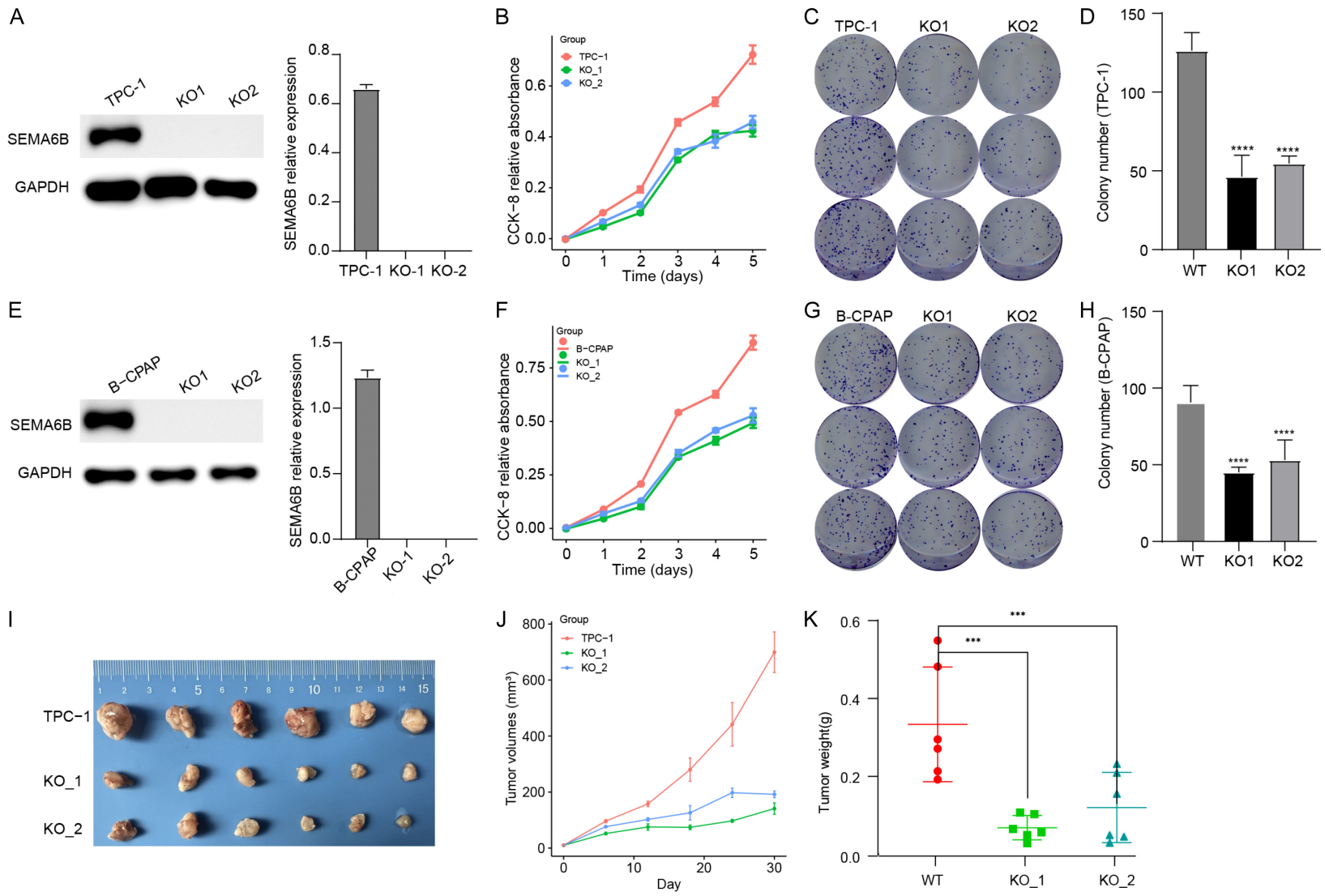

Figure 9. Knockout SEMA6B lessen thyroid tumorigenesis. A. Western blot validation of SEMA6B knockout in TPC-1 cells. B. CCK-8 assay indicating reduced proliferation in TPC-1 cells following SEMA6B knockout. C and D. Clonogenic assays and quantification show a significant decrease in colony formation in TPC-1 cells after SEMA6B knockout, ****P < 0.0001. E. Western blot validation of SEMA6B knockout in B-CPAP cells. F. CCK-8 assay showing reduced proliferation in B-CPAP cells following SEMA6B knockout. G and H. Clonogenic assays and quantification show a significant decrease in colony formation in B-CPAP cells after SEMA6B knockout, ****P < 0.0001. I. Representative images of tumors from the xenograft model, comparing tumor sizes between wild-type and SEMA6B knockout groups. J. Tumor growth curves in the xenograft model, indicating reduced tumor growth in SEMA6B knockout groups compared to wild-type. K. Final tumor weights are significantly lower in the SEMA6B knockout groups, ***P < 0.001.

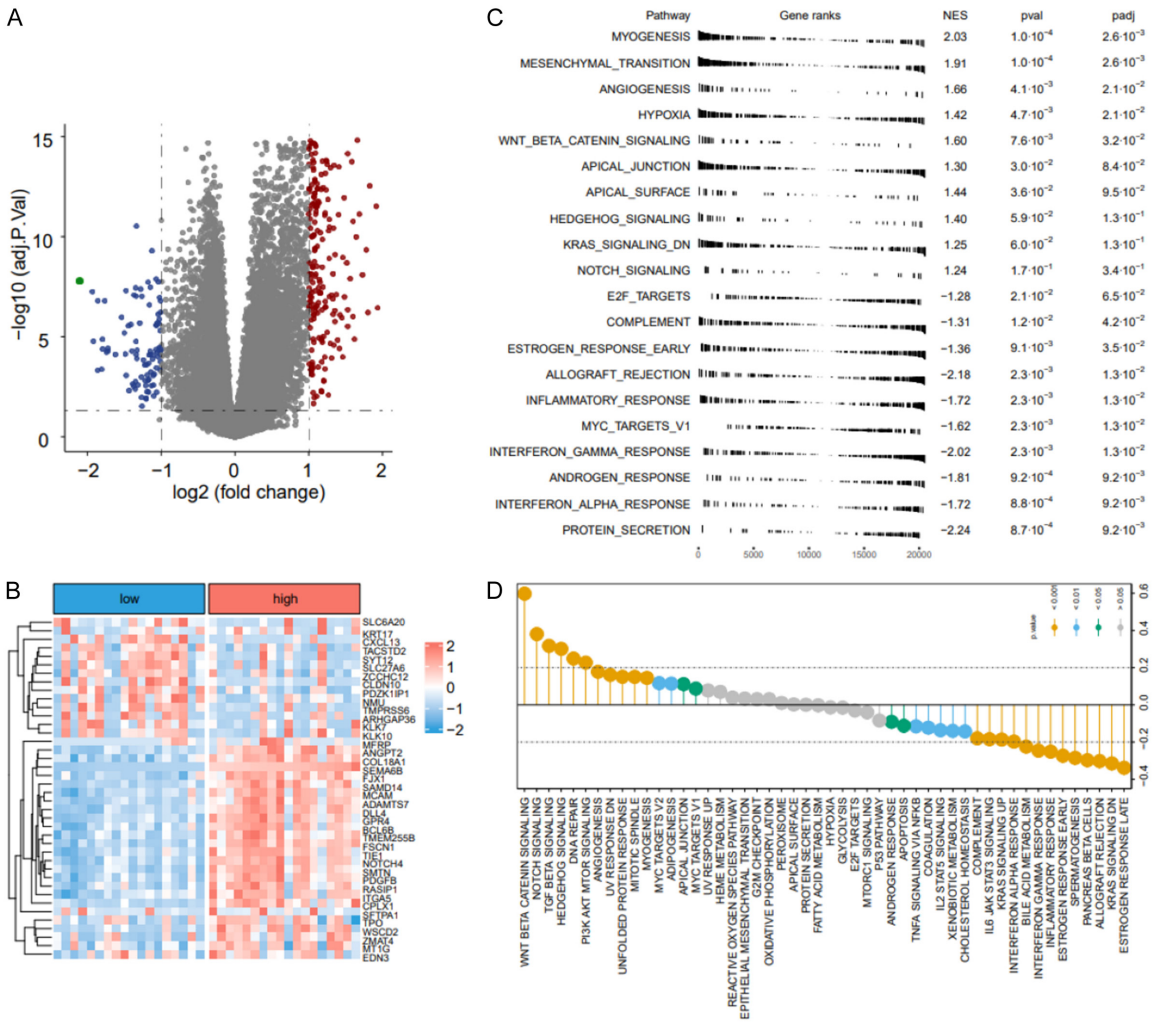

Figure 10. Differential gene expression and pathway enrichment analysis. A. Volcano plot showing differentially expressed genes between high and low SEMA6B expression groups. B. Heatmap of the top differentially expressed genes stratifying the high and low SEMA6B expression groups. C. Gene Set Enrichment Analysis (GSEA) highlighting the top enriched pathways in the high SEMA6B expression group. D. Ranked enrichment scores across various pathways in the high SEMA6B expression group.

(**Figure 11A**), suggesting that SEMA6B is critical for regulating key oncogenic pathways. Additionally, a strong positive correlation was observed between SEMA6B and CTNNB1 (β-catenin) expression across various cancer types, including THCA (**Figure 11B**), indicating that SEMA6B may influence cancer progression through modulation of β-catenin activity. Functional validation using a TOP/FOP flash luciferase reporter assay confirmed that SEMA6B overexpression increased β-catenin transcriptional activity in 293T cells (**Figure 11C**). Conversely, SEMA6B knockout in B-CPAP cells resulted in a marked reduction in β-catenin activity. These results underscore SEMA6B's pivotal role as a regulator of the WNT/β-catenin signaling pathway, contributing to its oncogenic functions in THCA.

SEMA6B knockout enhanced chemosensitivity in THCA cells

Next, we evaluated whether blocking SEMA6B could enhance the sensitivity of THCA cells to doxorubicin treatment. Flow cytometry analysis of apoptosis demonstrated a significant increase in the percentage of apoptotic cells in SEMA6B knockout THCA cells compared to wild-type TPC-1 cells following doxorubicin treatment (**Figure 12**). These findings suggest that targeting SEMA6B may overcome doxoru-

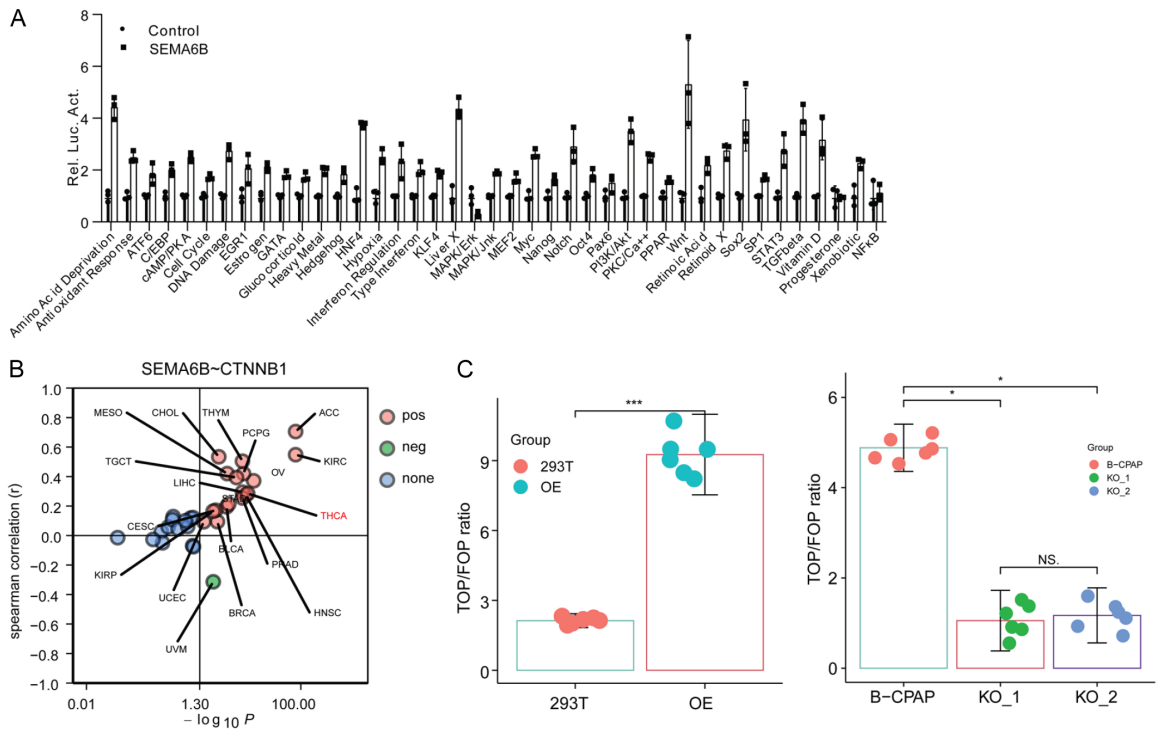

Figure 11. SEMA6B modulates WNT/β-catenin signaling pathway activity. A. Dual-luciferase reporter assay showing the activation of various signalling pathways, including WNT/β-catenin, in response to SEMA6B overexpression. B. Correlation analysis between SEMA6B and CTNNB1 (β-catenin) expression across different cancer types. C. TOP/FOP flash luciferase reporter assay demonstrating the impact of SEMA6B overexpression/knockout on β-catenin transcriptional activity in 293T cells.

bicin resistance by enhancing apoptotic responses in THCA cells.

Discussion

Our findings demonstrate that SEMA6B upregulation is associated with poor prognosis and heterogeneous microenvironment constituents such as immune and stromal cells. Overexpression of SEMA6B stimulated THCA cell growth, while SEMA6B knockout reduced tumor progression and enhanced the sensitivity of THCA cells to chemotherapy via the WNT/β-catenin signaling pathway (**Figure 13**).

SEMA6B, a member of the semaphorin family, was initially identified as a guidance factor for axon growth in neurons, playing a crucial role in neural development. Studies have revealed its involvement in various physiological and pathological processes, including immune regulation, angiogenesis, tumor growth, and cell migration [14, 15]. In THCA, SEMA6B has emerged as a promising predictive biomarker for prognosis and response to immunotherapy [16, 17]. Consistently, our study confirms that SEMA6B is upregulated in THCA and is linked to poorer survival outcomes. These findings indicate that semaphorins can modulate immune cell recruitment and tumor-stroma interactions, further underscoring the multifaceted role of SEMA6B in tumor progression.

Furthermore, we identified that THCA patients with high SEMA6B expression exhibited lower TMB and reduced immunogenicity. These findings suggest that SEMA6B may allow tumors to evade immune detection by modulating the microenvironment, creating a more permissive environment for tumor growth and progression [18]. This highlights the potential predictive value of SEMA6B for prognosis and guiding immunotherapy strategies in THCA. The observed immune modulation warrants further investigation to determine how SEMA6B interacts with immune checkpoints, such as PD-1/PD-L1, and whether targeting SEMA6B could synergize existing immunotherapies. Our *in vitro* and *in vivo* experiments confirmed that SEMA6B promotes tumor growth and resis-

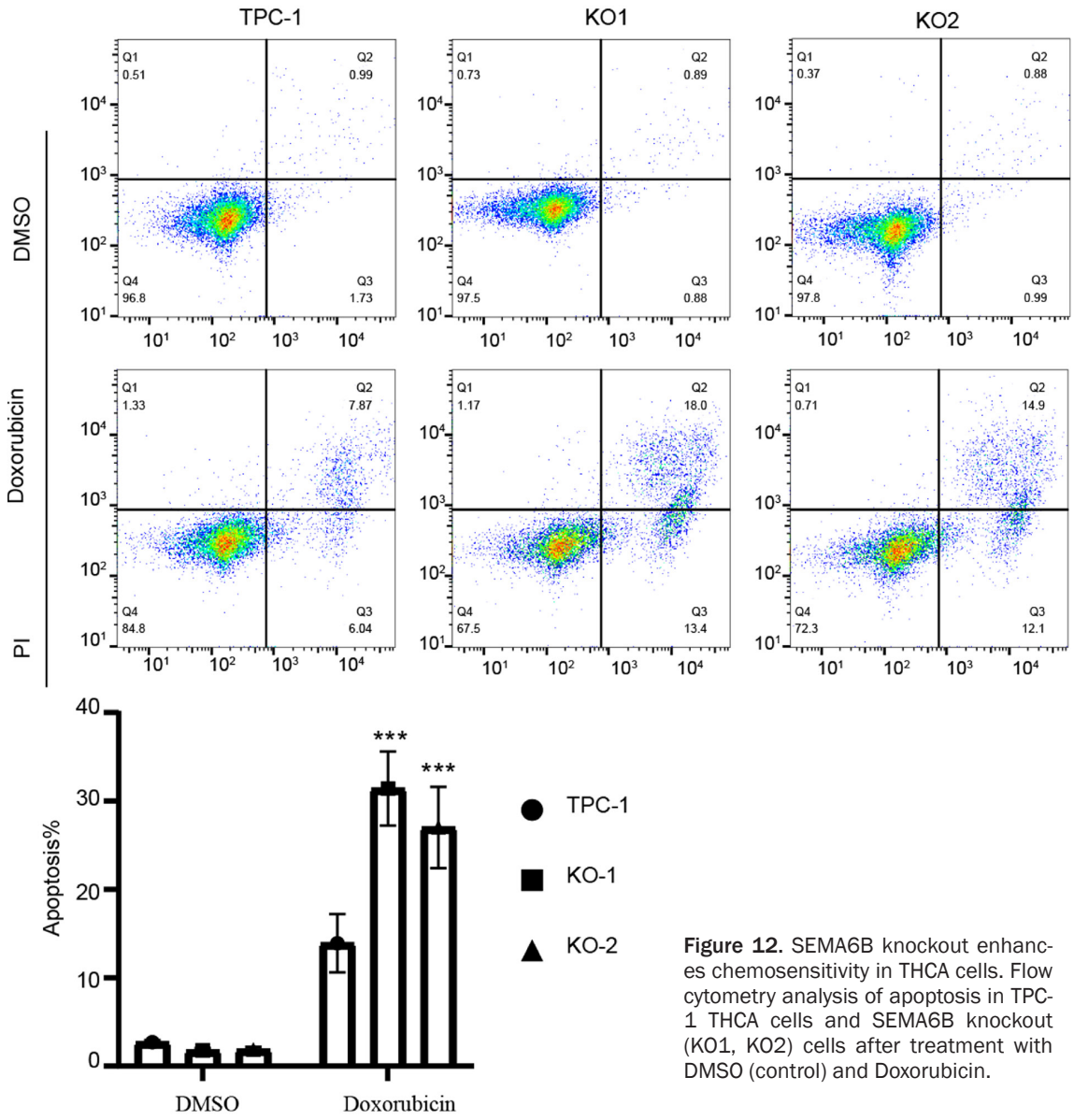

Figure 12. SEMA6B knockout enhances chemosensitivity in THCA cells. Flow cytometry analysis of apoptosis in TPC-1 THCA cells and SEMA6B knockout (KO1, KO2) cells after treatment with DMSO (control) and Doxorubicin.

tance to chemotherapy. In particular, the deletion of SEMA6B significantly sensitized THCA cells to Doxorubicin, a commonly used chemotherapy drug. This is consistent with prior studies [12, 19] indicating that semaphorin family members can regulate chemotherapy resistance by modulating intracellular signaling pathways.

The WNT/β-catenin signaling pathway is a well-established regulator of various physiological and pathological processes. Its activation begins with the inactivation of GSK-3β, a kinase responsible for phosphorylating β-catenin and targeting it for proteasomal degradation [20, 21]. Inhibition of GSK-3β prevents β-catenin degradation, resulting in its cytoplasmic accumulation and subsequent translocation into the nucleus [22, 23]. Within the nucleus, β-catenin interacts with the Tcf/Lef (T cell factor/lymphoid enhancer factor) family of transcription factors, activating genes involved in critical cellular processes such as proliferation, stem cell self-renewal, and metabolic regulation [21, 24]. Beyond these canonical interactions, β-catenin associates with other transcription factors, including AP-1, E2F, and Smad, expanding its functional roles to differentiation, apoptosis, and cell migration [25, 26]. Under normal physiological conditions, the

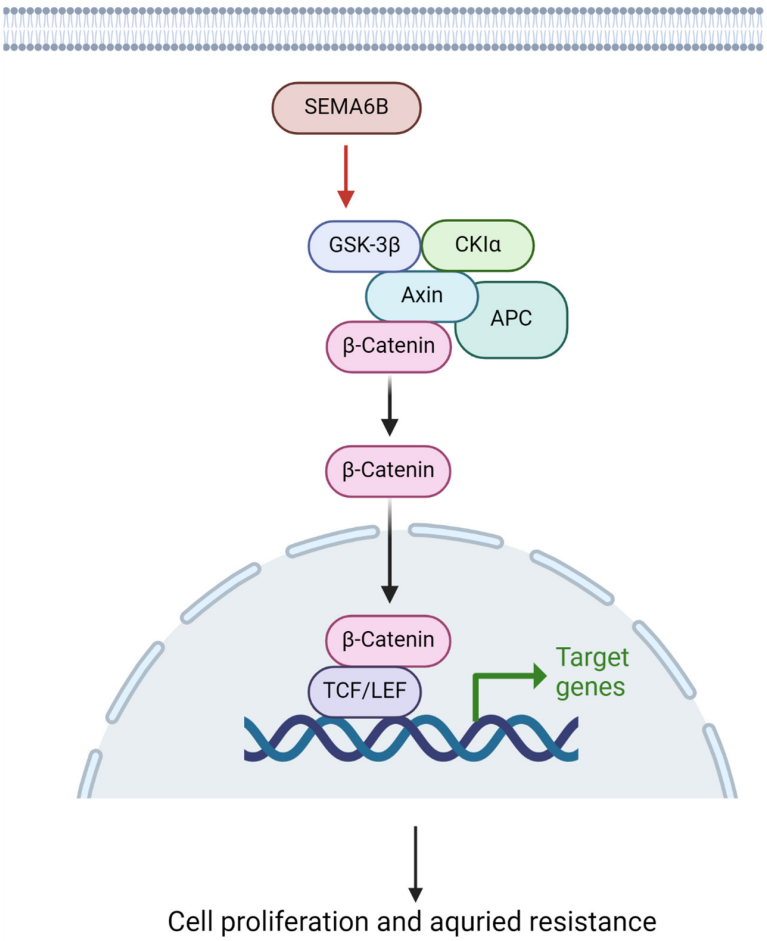

Figure 13. Graphic abstract of molecular mechanisms of SEMA6B promoting tumor progression in THCA.

WNT/β-catenin pathway is tightly regulated to maintain tissue homeostasis. However, dysregulation has been implicated in numerous diseases, including cancer. Aberrant activation of this pathway drives tumorigenesis by promoting cancer cell survival, proliferation, and metastasis [20, 27]. In THCA specifically, the WNT/β-catenin pathway has been shown to play pivotal roles in cancer progression, including cell proliferation, chemotherapy resistance, and the maintenance of cancer stem cell-like properties. These characteristics are critical contributors to disease progression and therapeutic failure, highlighting the pathway's significance in THCA biology.

Furthermore, we observed that SEMA6B overexpression significantly enhanced β-catenin transcriptional activity, providing novel evidence that SEMA6B acts as a positive regulator of the WNT/β-catenin signaling pathway in THCA. This finding is highly significant, as it elucidates a potential mechanism through which SEMA6B contributes to the aggressive phenotype of THCA cells. By enhancing β-catenin activity, SEMA6B may promote a transcriptional program that supports tumor growth, immune evasion, and resistance to therapy. Moreover, the WNT/β-catenin signaling pathway is widely recognized for mediating drug resistance across multiple cancer types. For instance, prior studies have demonstrated that inhibiting this pathway can effectively reduce doxorubicin resistance in triple-negative breast cancer cells [28, 29]. Similarly, in THCA, the activation of β-catenin by SEMA6B may enhance cancer cell survival under chemotherapeutic stress, thereby facilitating the development of resistance to Doxorubicin. This aligns with our observation that SEMA6B knockout significantly increased the sensitivity of THCA cells to Doxorubicin-induced apoptosis, suggesting that SEMA6B may contribute to chemoresistance by modulating the WNT/β-catenin pathway.

While our study provides novel insights into the role of SEMA6B in THCA, several limitations should be acknowledged. First, our analysis of patient samples was limited to a small cohort of 10 pairs of THCA and normal tissues. Although we validated our findings using publicly available datasets (e.g., TCGA and GEO), future studies with larger patient cohorts must be conducted to confirm the clinical significance of SEMA6B as a prognostic biomarker. Second, while we demonstrated the role of SEMA6B in modulating the WNT/β-catenin pathway, the precise molecular mechanism underlying this interaction remains unclear. For instance, whether SEMA6B directly interacts with upstream regulators of WNT signaling or influences β-catenin activation through indirect

mechanisms. Further studies are required to elucidate these molecular details. Third, our *in vivo* experiments relied on subcutaneous xenograft models in NOD/SCID mice, which lack a fully functional immune system. This limits our ability to evaluate the interaction between SEMA6B and the immune microenvironment. Immunocompetent mouse models or patient-derived organoid systems could provide a more comprehensive understanding of these interactions.

In conclusion, our study demonstrates a strong correlation between SEMA6B expression in THCA and poor prognosis as well as increased immune and stromal cells within the tumor microenvironment. This suggests that SEMA6B plays an oncogenic role in THCA. Functionally, SEMA6B promotes THCA growth and confers resistance to Doxorubicin by activating the WNT/β-catenin signaling pathway. Therefore, targeting the SEMA6B/WNT/β-catenin axis could be a promising specific treatment strategy for THCA.

Acknowledgements

This project was supported by Natural Science Foundation of Quanzhou Science and Technology Bureau (2022J01146).

Disclosure of conflict of interest

None.

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