The KIT ligand KITLG promotes portal vein tumor thrombosis by up-regulating COL4A1 through STAT3-SMAD2 signaling in hepatocellular carcinoma

Graphical abstract

Highlights

- The platelet activator COL4A1 is much more highly expressed in PVTT tissues than HCC primary tumors.
- KITLG plays a crucial role in regulating COL4A1 expression through STAT3-SMAD2 signaling.
- KITLG promotes COL4A1 expression and subsequently induces PVTT formation by activating platelets.

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In brief
This research delves into how the c-KIT ligand KITLG boosts COL4A1 expression through the STAT3/SMAD2 signaling pathway, ultimately aiding in platelet activation during PVTT formation.

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The KIT ligand KITLG promotes portal vein tumor thrombosis by up-regulating COL4A1 through STAT3-SMAD2 signaling in hepatocellular carcinoma

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ABSTRACT

Portal vein tumor thrombosis (PVTT), a severe complication of hepatocellular carcinoma (HCC), markedly influences patient prognosis by fostering a hypercoagulable state. However, its molecular underpinnings remain largely unexplored. This study sheds light on the critical role of the KIT ligand (KITLG) in modulating expression of the collagen gene COL4A1 via the STAT3-SMAD2 signaling pathway, thereby influencing platelet activation and PVTT development. Extensive analysis of PVTT tissue samples, alongside in vitro and in vivo experiments including cell-platelet interaction assays and PVTT animal models, revealed the mechanism through which KITLG regulates COL4A1 expression, as well as its downstream effects on platelet behavior and the coagulation cascade. Our findings revealed that marked upregulation of COL4A1 expression, mediated by KITLG through the STAT3-SMAD2 pathway, led to increased platelet activation and PVTT formation. KITLG and COL4A1 expression was markedly higher in PVTT tissues than primary HCC tissues, thus highlighting their critical role in the pathophysiological trajectory leading to thrombosis. The finding that the KITLG-COL4A1 signaling axis is a crucial mediator in PVTT development may offer promising new directions for developing targeted diagnostic and therapeutic strategies. This study underscores the importance of the KITLG-COL4A1 axis in PVTT formation and its potential as a therapeutic target in HCC treatment protocols.

Keywords: Hepatocellular Carcinoma, Portal Vein Tumor Thrombosis, Platelets, KITLG, COL4A1

1. INTRODUCTION

Hepatocellular carcinoma (HCC), the fifth most prevalent cancer globally and the third leading cause of cancer-related deaths [1-3], often extends into the portal vein branches and culminates in portal vein tumor thrombosis (PVTT). The occurrence of PVTT among patients with HCC has been documented to range from 44% to 62.2% [4, 5]. As a critical determinant of prognosis, PVTT exacerbates tumor metastasis, impairs liver functionality, and induces portal hypertension, thus contributing to diminished survival outcomes in patients with HCC, for which the median overall survival (OS) is 2.7–4.0 months without intervention [4, 6, 7]. Despite the endorsement of sorafenib and lenvatinib as targeted therapeutic options in Western guidelines, and the preference for a multidisciplinary approach—including surgery, transcatheter arterial chemoembolization (TACE), FOLFOX-based hepatic artery infusion chemotherapy, radiotherapy, targeted pharmacotherapy, immunotherapy and living
donor liver transplantation [8, 9] among Asian experts, particularly those from China, consensus regarding the optimal management strategy for HCC with PVTT remains elusive [10]. This difference in recommendations stems primarily from the limited understanding of the molecular underpinnings governing the development of PVTT.

PVTT is a tumor thrombus that infiltrates the segmental branches or higher levels of the portal vein, and exhibits both neoplastic and thrombotic properties. This condition is a multifaceted pathophysiological phenomenon influenced by a myriad of factors. Clinically, PVTT correlates with factors such as compromised portal vein circulation; increased tumor burden, characterized by larger sizes and greater numbers of tumors; advanced tumor grade; deteriorated liver function, as indicated by the Child-Pugh classification; and elevated serum alpha-fetoprotein (AFP) levels [7, 11, 12]. A substantial proportion of patients with HCC experience portal hypertension, a condition that decreases the velocity of blood flow through the portal vein. This deceleration facilitates the lodging and adhesion of shed cancer cells to the portal vein walls and culminates in the formation of PVTT. From a genetic perspective, next-generation sequencing has revealed associations between mutations in genes including KDM6A, CUL9, FGD6, AKAAP3, and RNF139 and the emergence of PVTT in HCC casesassociated with chronic hepatitis B infection [13, 14]. Furthermore, PVTT development is influenced by various cytokines. Among the predisposing factors for PVTT, a state of hypercoagulability—wherein the blood composition shifts toward excessive accumulation of platelets and fibrin—plays a critical role in promoting thrombosis within the portal vein, thus potentially resulting in both arterial and venous thrombotic events [15-18].

Prior research has indicated that tumor cells can either secrete or display specific surface markers that actively contribute to the activation of platelets and the coagulation cascade. Notably, microparticles endowed with tumor-derived tissue factor and phosphatidylserine have been identified as key promoters of intravascular coagulation within the cancer milieu [19, 20]. Additionally, cancer cells produce platelet agonists such as ADP [21, 22], thrombin, and podoplanin (PDPN), which directly bind their receptors—purinergic receptor P2Y12 (P2RY12) [23, 24], protease-activated receptors 1 and 4 (PAR1/4), and C-type lectin-like receptor 2 (CLEC-2), respectively—on platelets, and consequently facilitate platelet activation and aggregation [25, 26]. Plasminogen activation inhibitor-1 (PAI-1), which is prevalent in various cancers, is essential for preventing fibrinolysis, and consequently further contributes to the coagulopathy observed in patients with cancer [18, 27]. Tumors also indirectly promote thrombosis by releasing inflammatory cytokines and other agents that foster a procoagulant endothelial phenotype and the formation of neutrophil extracellular traps (NETs). NETs, comprising DNA, histones, and neutrophil-derived proteases, form a network that traps platelets, neutrophils, and erythrocytes, and subsequently leads to thrombus formation [28, 29]. Moreover, cancer cells enhance coagulation through interactions with platelets mediated by adhesion molecules, such as integrins and mucins [30, 31]. Tumor cell-induced platelet aggregation, a phenomenon observed across diverse cell lines—including those derived from pancreatic, colorectal, and renal cancers [30, 32, 33]—is associated with propensity for metastasis, thereby underscoring the intricate relationship between cancer cell biology and the coagulation system.

Although numerous studies have demonstrated the critical roles of platelets in thrombus development, direct evidence linking platelet activity to PVTT formation remains scarce. To elucidate the influence of tumor cells on platelet activation during the process of PVTT formation, we conducted a comprehensive transcriptomic analysis, based on microarray technology, to compare PVTT with adjacent primary tumors from three patients with HCC with PVTT. Despite similar gene expression patterns suggesting PVTT’s potential origin from metastatic HCC nodules, distinct gene sets were observed between PVTT and the corresponding tumors. Our transcriptomic investigation revealed notable overexpression of collagen type IV alpha 1 chain (COL4A1), a component of type IV collagen (COL4), in PVTT tissues compared with primary tumors and non-tumorous tissues.

Normal hemostasis relies partially on platelet signal transduction initiated by platelet adherence to collagen fibers via the integrin α2β1 and glycoprotein VI (GPVI) at sites of vascular injury [34-36]. The COL4 molecule, forming an intricate network and serving as a primary constituent of basement membranes, acts as a scaffold in the extracellular matrix and engages with various integrin and non-integrin receptors in performing multifaceted roles, including the induction of platelet aggregation [37]. Specifically, COL4A1 constructs the α1 chain of COL4 and, alongside another α2 chain, assembles into the complete COL4 molecule [α1(IV),α2(IV)] [38].

Our research demonstrated that COL4A1 was markedly overexpressed in PVTT tissues and in the CSQT-2 cell line, which is derived from PVTT and has notable metastatic capabilities. Notably, COL4A1 stimulates CSQT-2 cells to activate platelets, thereby facilitating coagulation processes both in vitro and in nude mouse models. The c-KIT ligand, also known as KITLG or stem cell factor (SCF) [39], plays a critical role in this process by modulating COL4A1 expression through the phosphorylation of the transcription factors STAT3 and SMAD2, and subsequently leads to platelet activation and thrombosis. The activation of c-KIT signaling, initiated after KITLG’s binding to its receptor, triggers a cascade involving the PI3K/AKT, SRC, and JAK/STAT pathways [40].

These findings suggest that KITLG and COL4A1 expressed in PVTT cells may serve as novel therapeutic targets or pathways for effectively managing PVTT.
2. MATERIALS AND METHODS

2.1 Cell lines, culture, and plasmids

The human liver tumor cell lines HepG2 (ATCC, cat. HB-8065), Hep3B (ATCC, cat. HB-8064), and Huh7 (BeNa Culture Collection; cat. BNC2337690) were cultured under standard conditions in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum and antibiotics penicillin (100 U/mL) and streptomycin (100 U/mL), as well as antimycoplasm agent (100 U/mL) in a humidified 5% CO₂ incubator at 37°C. The identification and viability of the cell lines were determined by third-party biology services (Beijing HuaKe Gene Technology Co., Ltd., Beijing, China). The PVTT cell line CSQT-2 was derived from a portal vein tumor thrombus of HCC and was established in our laboratory [41]. cDNAs of COL4A1 and SMAD2 were obtained through RT-PCR, and the fragments were separately cloned into a lentiviral vector and pcDNA3.1(+) (+). Active mutants of SMAD2-2D (S465D/S467D) were subsequently generated through a PCR-based method.

2.2 Tissue samples

This investigation used tissue samples from adult patients, including primary HCC tissues, corresponding adjacent normal tissues, and associated PVTT tissues. These samples were collected from individuals who underwent surgical resection at the Eastern Hepatobiliary Surgery Hospital. For inclusion, patients were required to have a (1) definitive histopathological diagnosis of primary HCC, with or without PVTT, and (2) absence of preoperative treatment or other neoplastic conditions. All procured tissue samples were carefully preserved at −80°C until analysis. The Research Ethics Committee of the Eastern Hepatobiliary Surgery Hospital approved the study protocols involving human participants. Moreover, informed consent was obtained from all participating patients.

2.3 Immunohistochemistry and hematoxylin-eosin staining

Immunohistochemistry was performed as previously described [42] with a VECTASTAIN Elite ABC Kit (Universal) (Vector Laboratories, cat. PK-7200) and DAB Substrate Kit (Vector Laboratories, cat. SK-4100). Counterstaining was performed with hematoxylin (Vector Laboratories, cat. H-3404). Primary antibodies to KITLG (Abcam, cat. ab52603) and COL4A1 (Abcam, cat. ab6586) were used for immunohistochemistry. Two methods were used for quantification: (1) positivity, defined as >30% of immunohistochemically positive tumor cells, and (2) the Allred scoring system [43], adapted for KITLG and COL4A1. The proportion of positive tumor cells (PS) was as follows: 0%, 0 points; <1%, 1 point; 1%–10%, 2 points; 11%–33%, 3 points; 34%–67%, 4 points; and >67%, 5 points. The intensity score of positive tumor cells (IS) was as follows: none, 0 points; weak, 1 point; intermediate, 2 points; and strong, 3 points. The total score (TS) was calculated as PS + IS, and positivity was defined as TS > 2. For hematoxylin-eosin (HE) staining, the slices were stained for 3 min and 5 s after dewaxing.

2.4 RNA microarrays

Three pairs of PVTT tissues and matched primary and non-tumorous tissues were used for RNA microarray analysis. Total RNA was isolated with a Qubit RNA Assay Kit (Thermo Fisher Scientific, cat. Q32852) according to the manufacturer’s protocol. RNA microarray and data analyses were performed by Shanghai KangChen Bio-Tech (Shanghai, China).

2.5 siRNA transfection and shRNA stable cell line

To knock down KITLG and COL4A1, we used two small interfering RNAs (siRNAs) targeting KITLG or COL4A1, which were generated and tested by GenePharma (Shanghai, China). The human siRNA target sequences were as follows: siKITLG-1: sense-5′-GCAGGAACUGUGACUAATT-3′; antisense-5′-UUAUGUACACAUGUACUGTTT-3′; siKITLG-2: sense-5′-GCACUGAAACACUGUGAATT-3′; antisense-5′-AUCAUCUAGUUACGAGUGTTT-3′; siCOL4A1-1: sense-5′-CCAGGAUGCAUGCCACAAATT-3′; antisense-5′-UUGUGCCAUUGCAUCCUGTTT-3′; and siCOL4A1-2: sense-5′-GCAAGAUCUUUUGCGGCAATT-3′; antisense-5′-UCGACUCUAAUACUGCUGTTT-3′. To knock down STAT3 and SMAD2, we used siRNAs targeting STAT3 or SMAD2, which were generated and tested by HanHeng (Shanghai HanHeng Co., Ltd., Shanghai, China). The human siRNA target sequences were as follows: siSTAT3: sense-5′-GGGCUCAAGCUUACACATT-3′; and siSMAD2: sense-5′-CUCCUUAGGUUACUCCAAUUGTT-3′. The cells were transfected with 20 nmol/L siRNA with Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, cat. 13778150) according to the manufacturer’s protocol. ShRNAs were designed and inserted into the pHBLV-U6-MCS-CMV-ZsGreen-PGK-PURO vector by Han Heng Biotechnology (Shanghai), which was subsequently transfected into cells to establish stable cell lines.

2.6 Cell proliferation assays

We performed CCK8 assays to detect cell proliferation (Cell Counting Kit-8, Dojindo Molecular Technologies Inc., cat. CK04), according to the manufacturer’s protocol. Cells were plated in 96-well plates in triplicate at a density of 2 x 10³ to 5 x 10⁴ cells/well and cultured in growth medium. The cells were treated with siRNAs, and the number of cells per well was measured at (450 nm) at the indicated time points.

2.7 Plate colony formation assays

The colony formation rate indicated the independent proliferation and viability of the cells. Logarithmic growth cells were plated in six-well plates at a density of 4,000 cells/well. The culture medium was replaced when the generative cells adhered to the wall, and the plate
was incubated for 7–10 days until the colonies were clearly visible to the naked eye. Subsequently, the culture medium was discarded, and cells in six-well plates were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 30 min, with washing with PBS after every step. Fifty cells were considered to be equivalent to one colony. The colonies were counted in five random visual fields and photographed.

2.8 Transwell migration and invasion assays
Logarithmic phase cells were digested with trypsin and resuspended in FBS-free DMEM after being washed once with PBS and twice with FBS-free DMEM. A cell suspension (200 μL, 1 × 10⁵ cells) was added to the Transwell upper chamber coated with Matrigel membrane in duplicate. Subsequently, 600 μL of DMEM containing 20% FBS was added to the lower chamber of each well, and cells were cultured at 37°C for 24 h. Non-invasive cells on the upper membrane surface were removed and immobilized with a fixative for 20 min. Invasive cells were stained with 100 μL crystal violet and subsequently examined in five random high-power fields. Transwell migration assays were conducted in the same manner as the invasion assays, except in the absence of Matrigel coating. The cells were stained and counted as described previously.

2.9 RNA extraction and quantitative real-time polymerase chain reaction
Total RNA was extracted from tissues and cells with TRIzol reagent (Thermo Fisher Scientific, cat. 15596018) and used for reverse transcription with PrimeScript™ RT Master Mix (Takara, cat. RR036A). The genes of interest were amplified with quantitative real-time PCR (qRT-PCR) primers designed according to the National Center of Biotechnology Information (NCBI) sequences: KITLG-forward: 5′-CAGAGTCAGTGTCACAAAACCATT-3′, KITLG-reverse: 5′-TTGGCCTTCCTATTACTGCTACTG-3′, COL4A1-forward: 5′-AACTCTTTTGTGATGCACACCA-3′, COL4A1-reverse: 5′-AGCTGTAAGCGTTTGCGTA-3′, GAPDH-forward: 5′-AGAAGGCTGGGCTATTGG-3′, GAPDH-reverse: 5′-AGGGGCCATCCACAGTCTTC-3′. qRT-PCR was performed with SYBR Premix Ex Taq II (TaKaRa, cat. RR820A) on an ABI 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific). The mRNA levels were calculated with the 2−ΔΔCt method. All samples were normalized to endogenous levels of GAPDH.

2.10 Western blot analysis
The total cellular protein content was assessed through western blot analysis. Samples underwent separation by SDS-PAGE and were then transferred onto polyvinylidene difluoride membranes (Millipore). These protein samples, once blocked, were incubated overnight with primary antibodies at 4°C. After incubation with secondary antibodies, the membranes were analyzed via immunoblotting with an ECL immunoblotting kit (Epizyme, cat. SQ201, Shanghai, China), strictly according to the manufacturer’s guidelines. The intensity of each band was normalized to that of GAPDH for comparative analysis.

2.11 Platelet preparation, aggregation, PAC-1 binding, and P-selectin exposure
Human platelets were washed, prepared, and stimulated according to previously established protocols [44, 45]. To evaluate platelet activation in patients with HCC and PVTT, we prepared 300 μL platelets at a concentration of 3 × 10⁹/mL for aggregation and analyzed post-α-thrombin stimulation. The platelets were incubated with either FITC-conjugated anti-PAC1 or PE-conjugated anti-P-selectin, then stimulated with 0.1 U/mL α-thrombin for 20 minutes at ambient temperature. Subsequent analysis of PAC1 binding and P-selectin exposure was performed with flow cytometry (LSR Fortessa/FACSCalibur, BD Biosciences).

2.12 Fibrin clot retraction
Platelet-rich plasma was pre-activated with 0.05 U/mL thrombin or varying concentrations of CaCl₂. The resultant cancer cells and PRP mixtures were then left unstirred at 37°C in siliconized tubes. The degree of clot retraction was visually monitored, and the area of clots was quantified with ImageJ (National Institutes of Health, Washington, DC, USA). Clot retraction is reported as percentages reflecting the ratio of the clot area to the reaction mixture’s total volume. These results, presented as the mean ± standard deviation (SD) for three independent experiments, were statistically analyzed via unpaired Student’s t-test. The area ratio method was analogous to the traditionally used weight ratio method.

2.13 Animal studies
The Animal Care and Use Committee of the Second Military Medical University approved all animal experiments conducted in this study. Male athymic BALB/c nude mice 4 weeks of age were sourced from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in a specific-pathogen-free environment. CSQT-2 cells genetically modified to express KITLG-shRNA, COL4A1-shRNA, or a control scrambled shRNA were prepared for the study. Through a technique developed by our research team [46], 1 × 10⁶ cells/mL of CSQT-2 cells were intrasplenically injected into the mice. After 24 days, the animals were euthanized via cervical dislocation, and their liver tissues were harvested for the detection of micro-metastases through HE staining.

2.14 Reagents and antibodies
Human recombinant KITLG (cat. 255-SC-010) was used at concentrations of 50 or 100 nM. Primary antibodies to the following targets were used for western blotting: KITLG (Cell Signaling, cat. 2093, USA), COL4A1 (SAB Signalway antibody, cat. 40773, USA), STAT3 (cat. 9139,
USA), p\textsuperscript{Ser423/425}SMAD2 (Bioss, cat. bs-3425R, China), TGF-β1 (cat. bsm-33287M, China), and GAPDH (cat. 5174, USA).

2.15 Statistical analysis
Dichotomous variables were assessed with the χ\textsuperscript{2} test or Fisher’s exact test. Student’s t-test was performed to compare continuous variables between groups. The Kaplan–Meier test was used to estimate the survival rate, and multivariate Cox regression analysis with a backward stepwise approach was used to test for independent prognostic factors. The relationships in the relevant parametric data were evaluated with Spearman’s analysis. Differences were considered statistically significant at P < 0.05. Statistical differences between groups were analyzed in SPSS 22.0, and plotting was performed in GraphPad Prism 8.0.

2.16 Data availability
The RNA microarray data generated in this study are available on request. The publicly available databases and reference genome assemblies used herein were as follows: GEO (GSE77509), TCGA database, STRING [47], and Human Genome Assembly GRCh38 NCBI_p7_201606.

3. RESULTS

3.1 Enhanced platelet aggregation and secretion in response to agonists in patients with HCC with PVTT
PVTT, a distinct form of metastasis in HCC, substantially compromises patient survival. Platelet-derived signals have been reported to enhance tumor cells’ invasive and metastatic capabilities. Moreover, tumor cells release cytokines that activate platelets in the bloodstream, thereby escalating thrombotic incidents and augmenting the malignancy of tumor cells. To assess the involvement of platelets in PVTT development, we measured the expression of the platelet activation markers CD62P (P-selectin) and fibrinogen (Fg) in PVTT tissues through immunohistochemical staining (Supplementary Figure S1). The expression levels of CD62P and Fg were notably higher in PVTT tissues than primary tumors and non-tumorous tissues, and localized primarily in areas rich in fibrin but sparsely populated by tumor cells. We subsequently evaluated the platelet aggregation capacity in portal vein blood, as triggered by the platelet agonist thrombin. Patients with rather than without PVTT exhibited significantly greater platelet aggregation in response to thrombin (Figure 1A).

P-selectin, a transmembrane protein within the α-granule membranes of resting platelets, relocates to platelet surfaces during activation through a secretory mechanism. P-selectin released from platelets is a crucial marker of platelet activation. After stimulation with thrombin, we explored the surface expression of P-selectin on platelets via flow cytometry. Platelet secretion was significantly intensified in patients with PVTT after exposure to thrombin, thus indicating heightened platelet activity (Figure 1B).

3.2 COL4A1, a subunit of type IV collagen, is upregulated in PVTT tissues and is associated with PVTT progression

The intricate interaction between HCC tumor cells and their surrounding environment is increasingly acknowledged as a critical factor influencing malignancy progression. In this context, platelets have been found to enhance the proliferation and invasion of HCC cells. However, the effects of HCC, particularly PVTT tumor cells, on platelets have not been thoroughly explored. To examine the potential direct effects of PVTT tumor cells on platelets, we performed microarray analysis of gene expression profiles across PVTT tissues, corresponding primary tumors, and non-tumorous tissues. The variations in gene expression among non-tumorous tissues, tumors, and PVTT tissues are depicted in a heatmap (Figure 1C, D) and UpSet diagram (Supplementary Figure S2A). With a significance threshold of P < 0.05, we identified that four collagen-associated genes, COL5A2, COL4A3BP, COL4A1, and COL15A1, exhibited an increase in expression from non-tumorous tissues to tumors, and notably in PVTT tissues, as determined through series of cluster analysis of differentially expressed genes (DEGs) (Supplementary Figure S2B, Tables S1 and S2). Subsequent analysis revealed that COL4A1, a subunit of COL4, had the highest expression in PVTT tissues, followed by primary tumors, and the lowest expression in non-tumorous tissues (Figure 1C, D and Supplementary Figure S2B, Table S1).

Given the role of COL4 in facilitating platelet aggregation under normal hemostasis conditions, we sought to determine its contribution to PVTT formation. The presence of COL4A1 in PVTT tissues was confirmed through immunohistochemical staining of PVTT tissues from 63 patients. The findings, in agreement with the mRNA microarray findings, indicated elevated COL4A1 protein levels in these tissues (partial data shown in Figure 1E, F and Supplementary Figure S3). Furthermore, analysis of TCGA database gene expression data from patients with HCC allowed us to segregate patients into groups with low or high COL4A1 expression levels. This stratification revealed that higher COL4A1 expression significantly correlated with shorter overall survival (OS, P < 0.05, 364 patients with HCC) and recurrence-free survival (RFS, P < 0.05, 316 patients with HCC), thus serving as a negative prognostic indicator for HCC (Figure 2A, B). These observations underscored the clinical relevance of COL4A1 expression and highlighted its potential as a prognostic biomarker for patients with HCC with PVTT.
Figure 1 | Platelet aggregation and secretion are enhanced in patients with HCC with PVTT, and the platelet activator COL4A1 is associated with PVTT progression.

A. Aggregation of washed human platelets collected from patients with HCC with or without PVTT in response to 1 U/mL thrombin (n = 5; 3 men). B. P-selectin exposure of washed human platelets from patients with HCC with or without PVTT stimulated with 0.1 U/mL thrombin (n = 5, 3 men). C, D. Heatmap of differentially expressed mRNAs among nontumorous tissues (N), paired primary tumors (T), and PVTT tissues, according to RNA microarrays (P < 0.05 and |log₂FC| > 1). C. Top 20 upregulated genes between three pairs of PVTT tissues. D. Top 20 downregulated genes in PVTT tissues. E. RNA expression levels of COL4A1 in various tissues, according to RNA microarrays. Fold changes are presented. F. COL4A1 proteins are highly expressed in PVTT tissues. Immunohistochemistry staining of COL4A1 was performed in paired PVTT samples (PVTT), primary tumors (T), and normal liver tissues (N). Representative images from 126 patients with HCC with PVTT are shown. *P < 0.05, **P < 0.01.
3.3 COL4A1 enhances the growth, migration, and invasion ability of PVTT cells in vitro

Initially, we assessed COL4A1 expression across various HCC cell lines through qRT-PCR and western blot analyses. These evaluations revealed that the PVTT cell line CSQT-2, derived from a PVTT biopsy of patients with HCC, exhibited significantly greater levels of COL4A1, at both the RNA and protein levels, than observed in the other cell lines (Figure 3A-C). Although the Huh7 cell line also demonstrated substantial RNA expression of COL4A1, low protein level of COL4A1 may be a result of either impaired protein translation or enhanced protein ubiquitination degradation. To further examine the specific roles of COL4 and COL4A1 in the biological behaviors and formation of PVTT, we generated two cell lines with stable COL4A1 knockdown. The first cell line, characterized by superior knockdown efficiency, was selected for subsequent analyses (Figure 3D, E). The shRNA-mediated decrease in COL4A1 resulted in slightly lower viability of PVTT cells than control cells (Figure 3F, G). Importantly, the knockdown of COL4A1 markedly inhibited these cells’ migratory and invasive properties, according to Transwell assays (with or without Matrigel) (Figure 3H-K). Nevertheless, after overexpression of COL4A1 in Hep3B cells, a notable increase was observed in proliferation (Figure 3L, M), migration, and invasion.

Figure 2 | Five-year OS and RFS of COL4A1 and KITLG, according to TCGA database.

A. COL4A1 5-year OS

B. COL4A1 5-year RFS

C. KITLG 5-year OS

D. KITLG 5-year RFS
3.4 KITLG regulates COL4A1 expression in PVTT cells

Transforming growth factor-beta (TGF-β) typically functions as a tumor suppressor in normal and non-malignant tissues. However, TGF-β may exhibit paradoxical effects within malignancy by enhancing cancer cell proliferation. The TGF-β/SMAD signaling pathway is critically involved in regulating COL4 expression via remodeling of the extracellular matrix. To explore the potential direct involvement of the TGF-β/SMAD pathway in PVTT development through COL4, we analyzed TGF-β1 expression across primary tumors, non-tumorous tissues, and PVTT tissues, by using microarray data (Figure 1C, D). Intriguingly, differential gene expression analysis revealed higher TGF-β1 expression in primary tumors and PVTT tissues than non-tumorous tissues, and notably lower expression in PVTT tissues than in primary tumors (Figure 4A-C, E). Moreover, we examined the relationship between TGF-β1 and COL4A1 expression in HCC tissues from TCGA database (Supplementary Figure S2C). Pearson correlation analysis demonstrated a modest correlation between TGF-β1 and COL4A1 expression (R = 0.263, P < 0.001). In contrast, KITLG [the ligand for the type III receptor tyrosine kinase KIT (c-KIT)] exhibited pronounced expression in PVTT tissues (Figure 4A-D) and a stronger correlation with COL4A1 in HCC samples from TCGA database (R = 0.351, P < 0.001) (Supplementary Figure S2D). The relationship between KITLG and COL4A1 expression in PVTT tissues in the GSE77509 dataset (20 patients with HCC with PVTT) from the GEO database, revealed a higher Pearson correlation coefficient (R = 0.42) than observed in non-tumorous and primary tumor tissues (Figure 4F). Furthermore, the levels of both COL4A1 and KITLG expression in PVTT were found to be positively associated with their expression in primary tumors, thereby indicating that these genes were highly expressed in HCC and were also elevated in corresponding PVTT tissues (Figure 4G). These findings suggested that KITLG might critically regulate COL4A1 expression in PVTT tissues.

KITLG, also known as stem cell factor (SCF), is expressed by cancer and immune cells, and plays a major role in tumor cell proliferation and migration. These findings underscored the critical role of COL4A1 in facilitating the proliferation, migration, and invasion of PVTT cells.
Figure 4 | COL4A1 expression correlates with KITLG in HCC.

A-C. Volcano plot showing differentially expressed genes (DEGs). (A) DEGs between paired primary tumors (T) and nontumorous tissues (N). B. DEGs between paired PVTT tissues and nontumorous tissues (N). C. DEGs between paired PVTT tissues and primary tumors (T). D, E. RNA expression levels of KITLG (D) and TGFBI (E) in various tissues, according to RNA microarrays. Fold changes are presented. F. Correlation of COL4A1 and KITLG expression in nontumorous tissues (N), primary tumors (T), and PVTT tissues, according to GSE77509 (20 patients with HCC and PVTT) from the GEO database. G. Correlation of COL4A1 and KITLG expression between primary HCC tumors and PVTT tissues, according to GSE77509. H. KITLG protein is highly expressed in PVTT tissues. Immunohistochemistry staining of KITLG was performed in paired PVTT samples (PVTT), primary tumors (T), and normal liver tissues (N). Representative images are shown. I-K. RNA (I) and protein (J) expression levels of KITLG in the indicated HCC cell lines and the PVTT cell line CSQT-2. K. Quantification of protein expression in (J). *P < 0.05, **P < 0.01.
role in tumor growth, metastasis, and cell stemness via the KITLG/c-KIT signaling pathway. The relative expression levels of KITLG in non-tumorous tissues, primary tumors, and PVTT tissues were assessed. Like COL4A1, KITLG showed the highest expression in PVTT, according to immunohistochemical staining (Figure 4H and Supplementary Figure S4). Analysis of patients with HCC from TCGA database revealed that higher KITLG levels were significantly associated with shorter overall survival (OS, \( P < 0.05 \), 364 patients with HCC) and recurrence-free survival (RFS, \( P < 0.05 \), 316 patients with HCC) (Figure 2C, D).

Furthermore, we assessed the mRNA (Figure 4I) and protein levels (Figure 4J, K) of KITLG in PVTT cell lines, and observed that the CSQT-2 cell line exhibited the highest KITLG expression. A CSQT-2 cell line with stable KITLG-knockdown was subsequently established, and effective knockdown was verified by qRT-PCR and western blotting (Figure 5A, B). Subsequent functional analyses revealed that KITLG knockdown significantly decreased CSQT-2’s cell viability, and migratory and invasive capacity (Figure 5C-H). We next investigated KITLG’s role in modulating COL4A1 expression and PVTT formation, and observed a substantial decrease in COL4A1 mRNA and protein levels with KITLG-knockdown in CSQT-2 cells (Figure 5I, J). However, the introduction of soluble human recombinant KITLG in the cells’ culture medium restored COL4A1 expression (Figure 5J). Our data indicated that KITLG is a key regulator of COL4A1 expression in PVTT cell lines.

3.5 Development of a CSQT-2 cell fibrin clot retraction model mimicking PVTT formation

Platelet activation leads to spreading, secretion from granules containing pro-thrombotic factors, and enhanced affinity of the fibrinogen receptor integrin \( \alpha_{IIb}\beta_3 \) for fibrinogen. These “inside-out” signals cause platelet aggregation through bivalent binding to fibrinogen. After fibrinogen binding, the phosphorylation of the \( \beta(3) \) integrin subunit’s cytoplasmic tail triggers an “outside-in” signal and subsequently generates a secondary activation wave crucial for the formation of irreversible platelet thrombi. The in vitro clot retraction study is a straightforward and reliable method for evaluating platelet functionality. Plasma clots typically retract from the walls of a glass tube within several hours, thereby facilitating the rapid analysis of “outside-in” signaling via platelet integrin \( \alpha_{IIb}\beta_3 \) (48). The degree of clot retraction indicates platelet activity and functionality.

To directly examine whether KITLG and COL4A1 might facilitate PVTT formation in vitro by CSQT-2 cells, we created a thrombus model through the co-incubation of CSQT-2 cells with platelet-rich plasma (PRP) in a fibrin clot retraction assay. The CSQT-2 cells were washed with a solution of sodium chloride, to mitigate the influence of the cell culture medium. Subsequently, the cells were mixed with PRP and simultaneously activated by a high concentration of calcium chloride, and the resulting mixtures were left undisturbed in siliconized tubes. In contrast to PRP alone, the combination of CSQT-2 cells and PRP exhibited significantly enhanced contraction capability under the influence of a potent stimulant (Figure 6A, B).

Further experiments were conducted to explore whether other HCC cell lines with diminished KITLG and COL4A1 expression might similarly induce clot retraction. Notably, without any stimulants, none of the HCC cell lines, including CSQT-2, initiated clot retraction. However, under low calcium chloride concentrations, only the PVTT cell line CSQT-2 showed induction of clot retraction (Figure 6C, D). Additionally, HE staining of the clot in the CSQT-2-PRP clot retraction assay revealed numerous cell clusters, thereby indicating the potential of PVTT cells to continue growing and increasing within the fibrin clot microenvironment (Figure 6E). Typically, normal cells’ anchorage-independent growth is inhibited by a specific type of apoptosis known as anoikis. However, transformed cells can grow and divide independently of substrate attachment (49). On the basis of these findings, PVTT-derived CSQT-2 cells can thrive and persist within this specialized PVTT-mimicking fibrin clot environment.

3.6 KITLG and COL4A1 promote PVTT formation both in vitro and in vivo

Subsequently, we used stable KITLG-knockdown and COL4A1-knockdown CSQT-2 cells in the fibrin clot retraction assays. In the presence of low calcium chloride concentrations, the knockdown-negative-control CSQT-2 cells immediately induced fibrin clot retraction, in contrast to the no-stimulant condition (Figure 7A). Incubation of KITLG-knockdown or COL4A1-knockdown CSQT-2 cells with PRP significantly restricted plasma retraction, and thin, transparent, and irregular fibrin clots formed, which were particularly prominent in the COL4A1-knockdown group. The clots contracted and precipitated significantly over time, yet the knockdown groups failed to form sufficient clots (Figure 7B, C). Notably, after 2 weeks, the plasma in the wild-type and knockdown-negative-control groups had fully coagulated, thus indicating platelet activation by CSQT-2 cells (Figure 7D).

The integrin \( \alpha_{IIb}\beta_3 \), a platelet adhesion receptor, orchestrates bidirectional platelet activation signaling. Under agonist stimulation, \( \alpha_{IIb}\beta_3 \) undergoes a conformational change that facilitates soluble fibrinogen binding platelets (inside-out signaling) and leads to platelet aggregation (44). The PAC1 monoclonal antibody, which identifies activated human \( \alpha_{IIb}\beta_3 \), served as a tool for assessing inside-out signal-driven \( \alpha_{IIb}\beta_3 \) activation. After 24-hour incubation of platelets from healthy volunteers with either KITLG-knockdown or COL4A1-knockdown CSQT-2 cells, PAC1 binding to platelets was significantly less than that observed with incubation with wild-type CSQT-2 cells. This finding
Figure 5 | KITLG promotes the growth, migration, and invasion of PVTT cells.

A, B. Detection of KITLG knockdown efficiency by qRT-PCR (A) and western blotting (B), respectively. C, D. KITLG knockdown inhibits cell proliferation, according to CCK8 assays (C) and colony formation assays (D). E-H. Lack of COL4A1 inhibits cell migration (E, F) and invasion (G, H), according to Transwell assays without or with Matrigel. I. Knockdown of KITLG decreases the mRNA level of COL4A1. J. Lack of KITLG downregulates the protein level of COL4A1, whereas this effect is rescued by exogenous KITLG treatment. Data are presented as means ± standard deviation. Student’s t test and two-way ANOVA followed by Tukey’s multiple comparisons test, *P < 0.05, **P < 0.01, ***P < 0.001.
indicated that KITLG and COL4A1 play crucial roles in regulating the activation of signaling by integrins such as \(\alpha_{IIb}\beta_3\) (Figure 7E-H).

We successfully established an in vitro thrombus model by co-culturing PVTT cells with PRP, thereby demonstrating that KITLG and COL4A1 are essential in regulating fibrin clot retraction and hence platelet activation. Moreover, PAC1 binding assays confirmed that KITLG and COL4A1 influence \(\alpha_{IIb}\beta_3\)-mediated inside-out signaling.

Through intrasplenic injection of CSQT-2 cells, we developed a stable and realistic animal model for PVTT that simulates the entire progression of PVTT formation in primary HCC and clarifies various PVTT phenotypes [46]. Intrasplenic injection with wild-type, KITLG-knockdown, or COL4A1-knockdown CSQT-2 cells yielded results echoing our prior findings: CSQT-2 cells originating from PVTT induced multiple tumor nodules (Figure 8A). In agreement with the in vitro PVTT model outcomes, the ability of the KITLG-knockdown or COL4A1-knockdown CSQT-2 cells to trigger thrombus tissue located around the blood vessel wall was markedly diminished, thus indicating that KITLG and COL4A1 indeed facilitated PVTT development (Figure 8B, C).

3.7 KITLG regulates COL4A1 expression through STAT3-SMAD2 signaling

Although the TGF-\(\beta\)/SMAD signaling pathway has been extensively documented to govern COL4 expression, our findings indicated a more critical role of KITLG, the ligand for c-KIT, in the regulation of COL4A1, thereby significantly affecting the progression of PVTT in HCC. The interaction between KITLG and its receptor c-KIT activated downstream signaling pathways, including PI3K/AKT, SRC, and JAK/STAT. A previous study has highlighted that the interplay between canonical TGF-\(\beta\) and c-KIT signaling creates a positive feedback loop promoting tumor growth through the KITLG/c-KIT/STAT3/TGF-\(\beta\)/SMAD2-axis; in contrast, the disruption of this loop restores the tumor-suppressing function of TGF-\(\beta\) in HCC [40].

To verify that KITLG’s influence on COL4A1 was mediated through STAT3-SMAD2 signaling, we measured the expression of \(\text{p}^{\text{Tyr705}}\text{STAT3}, \text{p}^{\text{Syr684}}\text{STAT5}, \text{p}^{\text{Ser465/467}}\text{SMAD2}, \text{p}^{\text{Ser423/425}}\text{SMAD3}, \text{and TGF-}\beta_1\) in CSQT-2 cells treated with KITLG siRNA or recombinant human KITLG cytokine. In line with our prior observations, the introduction of additional KITLG cytokine markedly
Figure 7 | KITLG and COL4A1 promote PVTT formation in vitro and influence integrin mediated inside-out signaling.

A-D. Incubation of WT or KITLG knockdown or COL4A1 knockdown PVTT cells (CSQT-2) with platelet-rich plasma (PRP). The fibrin clot retraction was recorded at 15 min (A), 24 h (B), 48 h (C), and 1 week (D). Upper panel: no additional calcium chloride; lower panel: low concentration of calcium chloride. The 24-hour clot retraction ratio is shown in (B). E-H. Incubation of healthy volunteers’ platelets with wild-type CSQT2 cells (E, F), KITLG-knockdown (G), or COL4A1 knockdown (H) CSQT2 cells for 24 hours, and stimulation of platelets with 0.1 U/mL α-thrombin for 20 minutes at room temperature. The signal of PAC1 binding platelets was detected by flow cytometry. *P < 0.05, **P < 0.01.
Figure 8 | KITLG and COL4A1 contribute to PVTT formation in vivo, and KITLG regulates COL4A1 expression through STAT3-SMAD2 signaling.

A-C. Mouse PVTT model based on intrasplenic injection with wild-type, KITLG-knockdown, or COL4A1-knockdown PVTT cells CSQT-2. (A) Wild-type PVTT cells induce numerous tumor nodules, and lack of KITLG or COL4A1 decreases tumor nodule formation. (B, C) HE staining of liver tissue from mice, indicating PVTT formation and metastasis. Arrows indicate the locations of tumor cells. (C) Quantitative data of PVTT numbers in (B).

D. Protein expression of COL4A1, pY705STAT3, STAT3, pY623/625SMAD2, and SMAD3 in PVTT cells treated with KITLG siRNA, and 50 nM or 100 nM recombinant KITLG cytokine for 48 hours.

E. Protein expression of TGFB1, pY623/625SMAD2, pY623/625SMAD3, and SMAD3 in PVTT cells treated with KITLG siRNA, and 50 nM or 100 nM recombinant KITLG cytokine for 48 hours.

F. Protein expression of COL4A1 in the presence of STAT3 and SMAD2 knockdown by siRNAs and the active mutant form of SMAD2-2D (S465D/S467D). **P < 0.01.
suppressed and restored COL4A1 expression, and both pTyr705STAT3 and total STAT3 levels changed accordingly. Notably, the pTyr694STAT5 signal was activated only under elevated KITLG levels, thus suggesting that STAT5 does not have a crucial role in the KITLG-COL4A1 pathway (Figure 8D). Although a positive feedback loop between TGF-β and c-KIT signaling has been suggested, TGF-β1 expression showed only a minor decrease. Both SMAD2 and SMAD3, because of their structural similarity, are considered crucial in TGF-β signaling; however, our phospho-signal analysis revealed that only the pSer465/467SMAD2 signal was significantly altered, and pSer423/425SMAD3 levels remained unaffected (Figure 8E). To further confirm the involvement of STAT3 and SMAD2 in COL4A1 expression, we individually silenced STAT3 and SMAD2 in CSQT2 cells. The absence of either STAT3 or SMAD2 resulted in altered COL4A1 expression, which was restored by introduction of the active mutant form of SMAD2-2D (S465D/S467D) (Figure 8F).

In conclusion, our study demonstrated a substantial increase in platelet activity among patients with HCC with PVTT. Using a PVTT cell-fibrin clot retraction model and an intrasplenic injection mouse model for PVTT, we established that KITLG and COL4A1, a subunit of COL4, are key regulators of platelet activation. Specifically, KITLG enhances COL4A1 expression via the STAT3/SMAD2 signaling pathway, thus facilitating platelet activation in patients with HCC with PVTT (Figure 9).

4. DISCUSSION

Herein, we identified that COL4A1 is markedly upregulated in PVTT tissues and initiates coagulation both in vitro and in vivo. Notably, the c-KIT ligand KITLG was found to modulate COL4A1 expression via the transcription factors STAT3 and SMAD2. Our findings suggest that KITLG and COL4A1 expressed in PVTT cells may serve as novel therapeutic targets or pathways for treating PVTT.

PVTT, an advanced manifestation of HCC, is the most frequent form of macrovascular invasion observed in HCC cases. PVTT severity has significant prognostic value, correlating with nearly a twofold increase in mortality risk [13]. Nonetheless, effective therapeutic options for patients with HCC presenting with PVTT are scarce. Investigating the molecular intricacies of PVTT development could pave the way to innovative treatment strategies. In pursuit of this goal, our team pioneered the development of the first HCC-derived portal vein tumor thrombus cell line, CSQT-2, which can be used to induce PVTT in nude mice through intrasplenic injection [41, 46]. Using this cell line, we designed an in vitro CSQT-2

Figure 9 | Schematic diagram of KITLG's upregulation of COL4A1 expression through the STAT3/SMAD2 axis, and induction of platelet activation in HCC and PVTT formation.
cell-PRP thrombosis model and conducted fibrin clot retraction assays.

The CSQT-2 cell line, unlike typical HCC cell lines, induced clot retraction in vitro under minimal calcium chloride concentrations. This observation underscored the CSQT-2 cell line’s enhanced ability to activate platelets and the coagulation cascade. Platelets substantially contribute to tumor angiogenesis and cancer progression, shield cancer cells from the cytotoxic effects of chemotherapy, and preserve the structural integrity of tumor blood vessels. Elevated platelet counts have been identified as indicators of cancer in individuals with undiagnosed malignancies [50] and have consistently been associated with improved progression-free and overall survival rates in various cancers, including ovarian [51, 52], colorectal cancer [53], lung cancer [54], stomach cancer [55, 56], and breast cancers. The role of platelets in the progression of HCC is particularly notable. An analysis performing univariate Kaplan–Meier survival and multivariate Cox regression on data from 216 patients with HCC has revealed a significant correlation between platelet count and prognosis in patients with PVTT. Specifically, patients with thrombocytopenia exhibit extended periods of tumor-free and overall survival [57], thus highlighting platelet count as an independent prognostic factor for patients with HCC with portal vein tumor thrombosis.

Beyond platelet count, platelet activity substantially contributes to cancer progression. Our study revealed heightened platelet aggregation and secretion in patients with HCC with PVTT, thereby indicating elevated platelet activity during PVTT development. Platelets may engage in pro-fibrinogenic signaling, influence the hepatic immune response, facilitate interactions within the tumor stroma, and consequently enhance tumor growth and metastasis [58]. For instance, platelets can release CXCL5 and CXCL7, which in turn encourage granulocyte recruitment and the establishment of an early metastatic niche. Moreover, activated platelets secrete growth factors and metalloproteases, which in turn lead to extracellular matrix degradation and metastatic niche formation [30].

Although various mechanisms of tumor cell-mediated platelet activation have been identified, our research highlights the COL4A1 subunit of type IV collagen as a critical element in activating platelets and coagulation by PVTT cells. Notably, this process is regulated primarily by KITLG, the ligand for the c-KIT receptor, via STAT3-SMAD2 signaling in PVTT cells rather than by TGF-β1. Whereas TGF-β1 typically suppresses tumors through canonical SMAD signaling, TGF-β1/SMAD2 activation has also been implicated in invasive tumor phenotypes [40]. Here, we established that KITLG, acting through the c-KIT receptor, is essential in controlling COL4A1 expression via STAT3-SMAD2 signaling, thereby ultimately triggering platelet activation and thrombosis both in vitro and in vivo. Despite our creation of in vitro and in vivo models to simulate PVTT formation, an ideal model that fully replicates this complex process remains to be developed. A comprehensive understanding of PVTT formation, including the roles of hemodynamic factors and immune cell activity, remains an area for future investigation. Moreover, platelets not only activate but also support tumor cell growth and dissemination; therefore, further study is necessary to determine platelets’ roles in tumor advancement.

In summary, by developing an in vitro CSQT-2 cell-PRP thrombus model and an in vivo PVTT animal model, we examined the mechanisms underlying PVTT formation. Our findings indicated that KITLG, rather than TGF-β, is crucial in regulating COL4A1 expression and subsequent platelet activation. Given platelets’ diverse roles in the tumor microenvironment, particularly within PVTT tissue, targeting KITLG and COL4A1 in tumor cells may serve as a promising therapeutic strategy for PVTT management.

**ABBREVIATIONS**

HCC, hepatocellular carcinoma; PVTT, portal vein tumor thrombosis; COL4A1, collagen type IV alpha 1 chain; KITLG, KIT ligand; SCF, stem cell factor; PRP, platelet-rich plasma; STAT3, signal transducer and activator of transcription 3; SMAD2, SMAD family member 2; OS, overall survival; RFS, recurrence free survival; TACE, transcatheter arterial chemoembolization; AFP, alpha-fetoprotein; KDM6A, lysine demethylase 6A; CUL9, Cullin 9; FGDF6, FYYE, Rhe GEF and PH domain containing 6; AKA3P, A-kinase anchoring protein 3; RNF139, ring finger protein 139; ADP, adenosine diphosphate; P2Y12, purinergic receptor P2Y12; NETs, neutrophil extracellular traps; GPV1, glycoprotein VI; Fg, fibrinogen; TGF-β, transforming growth factor beta.

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**CONFLICTS OF INTEREST**

The authors declare no conflicts of interest for this work.

**DATA AND MATERIAL AVAILABILITY**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

This research was approved by the Research Ethics Committee of the Eastern Hepatobiliary Surgery Hospital. The animal experiments were approved by the Animal Care and Use Committee of Second Military Medical University.
CONSENT FOR PUBLICATION

All authors give consent for the publication of the manuscript.

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