

Original Research

ANK3 Is Regulated by Recursive Splicing and Inhibits Hepatocellular Carcinoma Metastasis by Inhibiting E-Cadherin Protein Degradation

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Abstract

Background: Ankyrin G (*ANK3*), belonging to the ankyrin family, contributes to cellular structural integrity by linking the cytoskeleton to the plasma membrane. Abnormal *ANK3* expression has been reported across several human malignancies, yet the regulatory mechanisms involved are still poorly understood. The process of dividing introns into several steps is referred to as recursive splicing (RS). RS can control the quality of transcripts produced by regulating the retention of the RS-exon. Hundreds of annotated RS-exons in human mRNAs are attributed to the inhibition of RS by the exon junction complex (EJC). **Methods:** In this study, we demonstrated that *ANK3* is reduced in hepatocellular carcinoma (HCC) and suppresses HCC metastasis. We then analyzed the multiple splicing methods of *ANK3*, confirming that RS exists in *ANK3* transcript variant 4 (*ANK3-TV4*) and that RS was weakened in HCC. **Results:** Mechanistically, *ANK3* inhibited HCC metastasis, which may be partly attributed to inhibition of the Wnt pathway. *ANK3* binds to E-cadherin via its N-terminal ankyrin repeat domain to regulate E-cadherin expression. *ANK3* knockdown activates the Wnt pathway, downregulates E-cadherin expression, and promotes its degradation. Conversely, *ANK3-TV4* overexpression inhibited the Wnt signaling pathway, upregulated E-cadherin protein expression, and inhibited E-cadherin degradation. *RBM8A*, a core EJC factor, regulates the RS of *ANK3-TV4*. **Conclusions:** Knockdown of *RBM8A* promoted RS of *ANK3-TV4* and upregulated its expression. We investigate the role of RS in HCC, providing a novel therapeutic perspective and identifying potential targets for intervention.

Keywords: ankyrin G; recursive splicing; hepatocellular carcinoma

1. Introduction

Liver cancer ranks as the sixth most prevalent malignancy globally and is the third leading cause of cancer-related mortality [1]. Hepatocellular carcinoma (HCC) constitutes nearly 80% of primary liver cancer cases [2]. Patients with HCC generally face poor clinical outcomes and limited effective treatment options [3]. Therefore, elucidating the pathogenic mechanism of HCC and its key regulatory molecules is essential for developing novel therapeutic strategies aimed at its prevention and treatment. Several studies have highlighted alternative splicing (AS) as an important source of new prognostic biomarkers and therapies for HCC [4–10].

More than 90 percent of human genes generate different transcripts through AS, thereby forming diversified transmissions of genetic information [5,11–14]. Recursive splicing (RS) is a noncanonical splicing mechanism in which introns are cut in several steps. In general, an RS-exon exists in the introns of genes that produce RS. This exon contains a premature termination codon (PTC), which commonly activates the nonsense-mediated mRNA decay (NMD) and terminates translation. RS removes the RS-exon, which can prevent the production of abnormal N-terminal mutant or truncated proteins, thereby maintaining normal gene expression and cellular functions [15,16]. Several studies have shown that the Exon Junction Complex

(EJC) suppresses RS and is an important factor in abnormal RS [17,18]. It is worth emphasizing that, as a rare AS pattern, RS has seldom been studied; moreover, its underlying mechanisms and implications in cancer remain poorly understood.

Cytoskeletal rearrangement is necessary for multiple pathological processes, such as the migration and invasion of tumor cells, and plays a key role in tumor metastasis [19]. Therefore, in-depth research on this topic is of great significance for identifying the intrinsic mechanisms and targets of tumor metastasis and developing new targeted therapies. Ankyrin 3 (*ANK3*), a member of the Ankyrin family, plays a crucial role in maintaining cell stability by linking the cytoskeleton to the plasma membrane [20,21]. *ANK3* typically comprises three major functional regions: an N-terminal membrane-interacting domain, a central domain that engages pectin/fodrin, and a regulatory C-terminal domain [20,22]. Current studies have reported that there are multiple transcripts of *ANK3* [16,23,24] and it is abnormally expressed across various tumor types [21]. Nonetheless, the association between *ANK3* expression and AS in HCC patients has not yet been elucidated.

In our study, we first demonstrated that *ANK3* was downregulated in HCC and inhibited HCC metastasis, confirming that RS exists in *ANK3* transcript variant 4 (*ANK3-TV4*) and that RS is weakened in HCC. Mechanistically,



ANK3 inhibited HCC metastasis, which may be partly attributed to inhibition of the Wnt pathway. *ANK3* binds to E-cadherin via its N-terminal ankyrin repeat domain to regulate E-cadherin expression. *ANK3* knockdown activates the Wnt pathway, downregulates E-cadherin expression, and promotes its degradation. Conversely, overexpression of *ANK3-TV4* had opposite effects. *RBM8A*, a core EJC factor, regulates the RS of *ANK3-TV4*. *RBM8A* knockdown promotes the RS of *ANK3-TV4*. We explored the involvement of RS in HCC, offering a new strategy for HCC treatment and identifying promising targets.

2. Materials and Methods

2.1 Cell Culture

SNU449, SK-Hep1, and Huh7 cells were purchased from the Type Culture Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured following the protocols provided by the supplier. All the cells in this study were purchased from the Type Culture Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines were validated by STR profiling and tested negative for mycoplasma.

2.2 Transfection

Small interfering RNAs (siRNAs) used in this study were commercially synthesized by GenePharma (Shanghai, China). Transfection was carried out using HiPerfect for siRNAs and Attractene for plasmids (Qiagen, Hilden, Germany), as per the supplier's guidelines. Details of the siRNA sequences can be found in **Supplementary Table 1**.

2.3 RNA Extraction and PCR (Including RT-PCR and qRT-PCR)

Total RNA was isolated using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's standard protocol. We performed RT-PCR by first generating cDNA with the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TRAN, Beijing, China). The subsequent amplification step utilized the Golden Star T6 Super PCR Mix (TSINGKE, Beijing, China), as per the manufacturer's instructions. Primer sequences are listed in **Supplementary Table 2**.

cDNA synthesis for real-time quantitative PCR (qRT-PCR) was carried out using murine leukemia virus reverse transcriptase (Invitrogen, Life Technologies, Carlsbad, CA, USA), followed by amplification with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Gene expression levels were normalized to β -actin, with corresponding primer sequences shown in **Supplementary Tables 3,4**.

2.4 Cell Proliferation

To assess cell proliferation, an 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-

tetrazolium (MTS) assay was performed according to the established method [25]. Briefly, 2500 cells were inoculated in a 96-well plate, and cell proliferation rates were measured using the CellTiter 96 AQ Ueous Nonradioactive Cell Proliferation Assay (MTS) (Promega, Madison, WI, USA) according to the manufacturer's instructions [26].

2.5 Lentivirus Transduction

To knock down ANK3 or RBM8A, we utilized the pLKO.1 lentivirus vector (Addgene, Cambridge, Massachusetts, USA). Target cells were transduced with viral particles in the presence of polybrene (2 mg/mL; Sigma-Aldrich, St. Louis, MO, USA), and puromycin (Sigma-Aldrich) was applied for the selection of positive cells. Refer to **Supplementary Table 1** for the short hairpin RNAs (shRNAs) sequences.

2.6 Cell Migration and Invasion Experiment

Cell migration and invasion experiments were performed as described previously [25]. For the wound-healing experiment, cells were cultured in 6-well plates to achieve a confluent monolayer. A sterile pipette tip was used to induce scratches. Cell migration into the scratch area was then monitored and photographed. To assess cell migration and invasion, transwell assays were performed. 5×10^4 cells per well in serum-free medium were plated in the upper chamber. For the invasion assay, the membrane was pre-coated with a substrate matrix, while it was left uncoated for migration. Following a 24-hour incubation, cells on the lower membrane surface were stained with crystal violet for microscopic observation [26].

2.7 Immunoblotting

Western blot analysis was performed on total protein extracts using standard protocols [27]. Respective primary antibodies against E-Cadherin (1:1000, 3195, Cell Signaling Technology, Danvers, MA, USA), β -Catenin (1:1000, 9562, Cell Signaling Technology, Danvers, MA, USA), β -actin (1:1000, 20536-1-AP, Proteintech, Wuhan, China), β -Tubulin (1:1000, 10094-1-AP, Proteintech, Wuhan, China), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000, 10494, Proteintech, Wuhan, China), and ANK3 (1:1000, 27980-AP, Proteintech, Wuhan, China) were utilized for detection.

2.8 Histology and Immunostaining

We performed immunohistochemical (IHC) staining on formalin-fixed, paraffin-embedded tissue sections. The sections were incubated with an anti-ANK3 antibody (1:2000, 27980-AP, Proteintech, Wuhan, China) following the manufacturer's instructions.

2.9 Human Liver Samples

Paired clinical specimens, comprising liver tumors and their matched adjacent normal tissues, were obtained

from West China Hospital of Sichuan University. Total RNA was extracted from these tissues with Tri-Reagent and subsequently analyzed via qRT-PCR. This study obtained ethical approval from the Ethics Committee of West China Hospital of Sichuan University (2020, no. 196) for the use of human samples. All participants provided written informed consent prior to inclusion.

2.10 Statistical Analysis

Data are presented as mean $\pm$ SEM from a minimum of three independent replicates. The threshold for statistical significance was set at $p < 0.05$, marked by asterisks (Non-significant (NS) for $p > 0.05$, * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$). Group comparisons were conducted using two-way ANOVA, Student's *t*-test, or paired *t*-test, as applicable.

3. Results

3.1 *ANK3* is Downregulated in HCC and Inhibits the Migration and Invasion of HCC Cells In Vitro

To elucidate the role of *ANK3* in HCC, we used GSE20017 data from the NCBI to analyze the expression of *ANK3*, and found that *ANK3* expression in patients with microvascular invasion was markedly lower than that in patients without microvascular invasion (Fig. 1a), suggesting that *ANK3* may be involved in regulating HCC metastasis. We investigated *ANK3* expression in human HCC tissues. *ANK3*-total mRNA levels were lower in HCC tissues (Fig. 1b), consistent with publicly available data (Fig. 1c). Western blot (WB) results showed that the patterns of the different sizes of *ANK3* proteins were inconsistent. The protein, approximately 150 kDa (*ANK3-TV2*), was significantly downregulated in HCC tissues, while the expression pattern of the protein, approximately 250 kDa (*ANK3-TV3/4/5*), was uncertain (Fig. 1d). IHC analysis further confirmed reduced *ANK3* levels in HCC samples (Fig. 1e).

We next assessed whether *ANK3* contributes to liver tumorigenesis. *ANK3* knockdown by siRNA had no impact on the growth of SNU449 or SK-Hep1 cells (Supplementary Fig. 1), indicating that *ANK3* does not regulate HCC proliferation. Conversely, *ANK3* knockdown markedly promoted the migration ability of SNU449 and SK-Hep1 cells (Fig. 1f,g), which was confirmed by a wound-healing experiment (Fig. 1h). Collectively, these *in vitro* findings establish that *ANK3* functions primarily to suppress the migratory and invasive capabilities of HCC cells.

3.2 Recursive Splicing of *ANK3-TV4* Exists in Human Liver Tissue

With reference to NCBI, we analyzed the splicing methods existing in *ANK3* (Fig. 2a), including alternative promoters and exon jumping, which resulted in *ANK3* producing five transcripts. The five transcriptional variants (TVs) of human *ANK3* in the NCBI

database encoded three proteins of different sizes. First, through an alternative promoter, *ANK3* produces four transcription start sites that produce four transcripts: transcript variant 3 (*TV3*) (NM_001204403), transcript variant 4 (*TV4*) (NM_001204404), transcript variant 1 (*TV1*) (NM_020987), and transcript variant 2 (*TV2*) (NM_001149). As a result of exon jumping, *TV1* produces another transcription variant named transcript variant 5 (*TV5*) (NM_001320874), which lacks exon 37. Another study reported that *TV1* also underwent alternative 5' splicing and retained a part of exon 37 [28]. In addition, RS has been reported in *ANK3* [16]. Sequence alignment and analysis revealed that RS may exist in *ANK3-TV4*. We first used primers from the literature for RT-PCR [16] but failed to detect RS-exon in human liver tissue. We then re-designed the primers and performed a nested PCR. Primer a was used for the first round of PCR, b/c/d/e primers were used for the second round of PCR in lanes 1/2/3/4, respectively (Fig. 2b,c). Theoretically, there were two bands in lane 1 at 103 bp (RS exon skipping) and 255 bp (RS exon retention). However, in lane 1, only the 103 bp band was visible to the naked eye, suggesting that only a small proportion of transcripts retained the RS-exon. The product sizes in lanes 2 and 3 (using primers c and d, respectively) were the same as expected. Collectively, our results demonstrate that the RS and RS-exon exist in *ANK3-TV4* in the human liver and hepatoma tissues.

3.3 RS of *ANK3-TV4* is Weakened in HCC, and *ANK3-TV4* Suppresses the Migration and Invasion of HCC In Vitro

As illustrated in Fig. 1, *ANK3* expression was markedly reduced in HCC. We designed primers specific to different *ANK3* transcripts to detect their expression in human HCC tissues. All transcripts were significantly downregulated in HCC (Fig. 2d), which was consistent with the publicly available data (Fig. 2e,f). In addition, the results showed that in *ANK3-TV4*, the transcript with RS-exon skipping was significantly downregulated in HCC (Fig. 3a,b), whereas the transcript with RS-exon retention showed no significant pattern (Fig. 3c). Similar results were obtained by semi-quantitative PCR (Fig. 3d). This suggests that the RS of *ANK3-TV4* was weakened in HCC.

Next, we explored the role of *ANK3-TV4* in liver tumorigenesis. Because of the particularity of the *ANK3-TV4* sequence, only one pair of siRNAs could be specifically designed. Specific knockdown of *ANK3-TV4* by siRNAs markedly promoted the migration ability of SNU449 cells, as demonstrated by transwell and invasion assays as well as a wound-healing assay (Fig. 3e,f), suggesting that cell migration and invasion are suppressed by *ANK3-TV4* *in vitro*.

3.4 *ANK3* Significantly Inhibits HCC Metastasis Through its Ankyrin Repeat Domain

Structurally, *ANK3* is organized into three functional domains: the N-terminal membrane domain (including

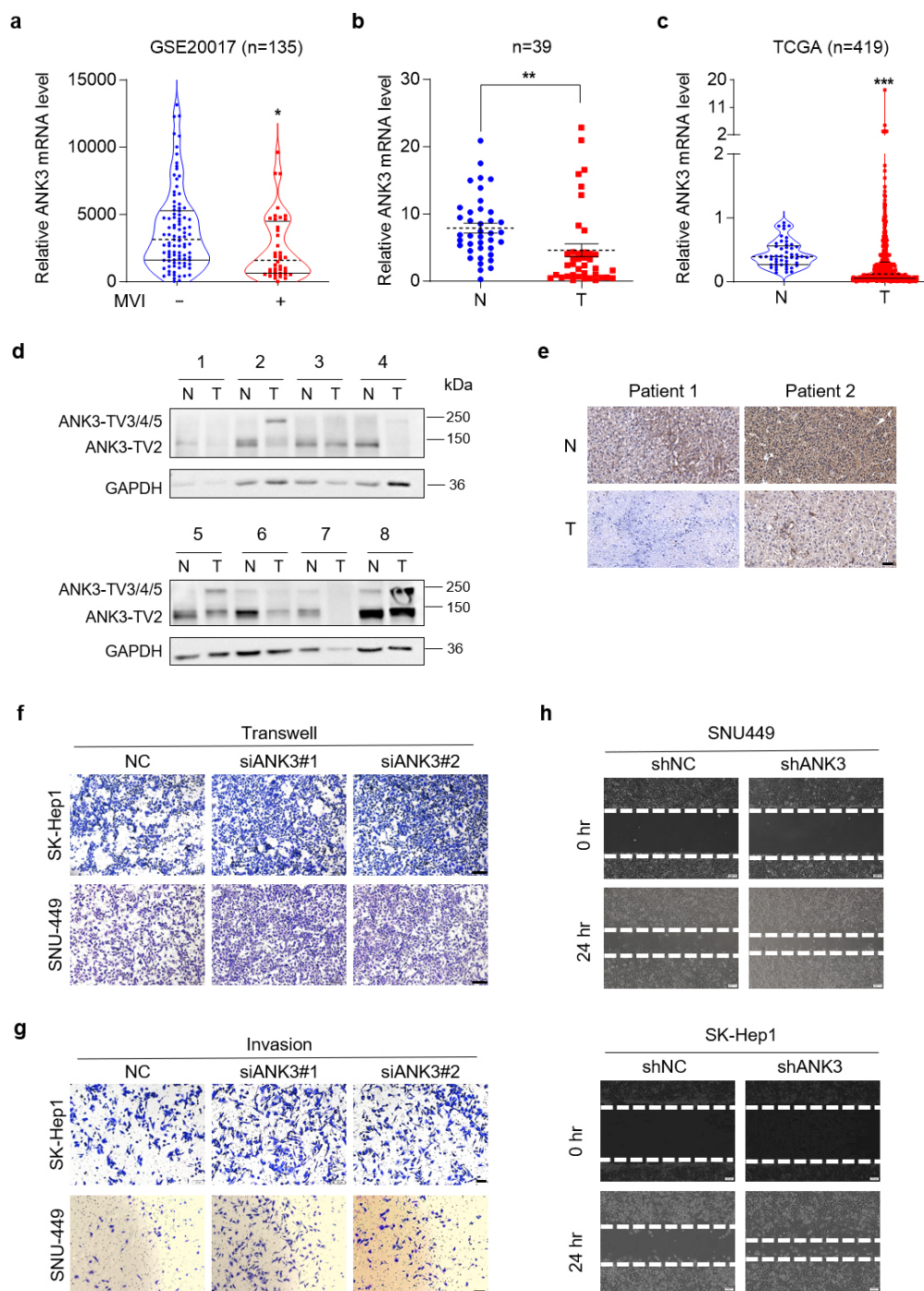


Fig. 1. Ankyrin 3 (ANK3) is significantly down-regulated in hepatocellular carcinoma (HCC). Knockdown of *ANK3* promotes HCC metastasis. (a) Dot plots depicting *ANK3* expression in HCC patients stratified by microvascular invasion (MVI) status (GSE20017 dataset). (b) Total *ANK3* mRNA levels in 39 paired HCC (T) and adjacent non-tumorous (N) tissues. (c) *ANK3* expression levels in HCC (T) and adjacent non-tumorous (N) tissues from the The Cancer Genome Atlas (TCGA) cohort. (d) Representative western blot (WB) images of *ANK3* protein in 8 paired HCC (T) and adjacent non-tumor (N) samples, with quantitative analysis relative to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) shown below. (e) IHC staining of *ANK3* in representative HCC and matched adjacent tissue. (f) Transwell assay of SK-Hep1 (top) and SNU449 (bottom) cells transfected with negative control (NC) or siANK3. (g) Invasion assay of SK-Hep1 (top) and SNU449 (bottom) cells transfected with NC or siANK3. (h) Wound-healing assay of SNU449 (top) and SK-Hep1 (bottom) cells transfected with shNC or ANK3 shRNA (shANK3). Scale bar: 50 μ m (e), 100 μ m (f,g), 200 μ m (h). Data are presented as mean $\pm$ SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 (Student's paired t test).

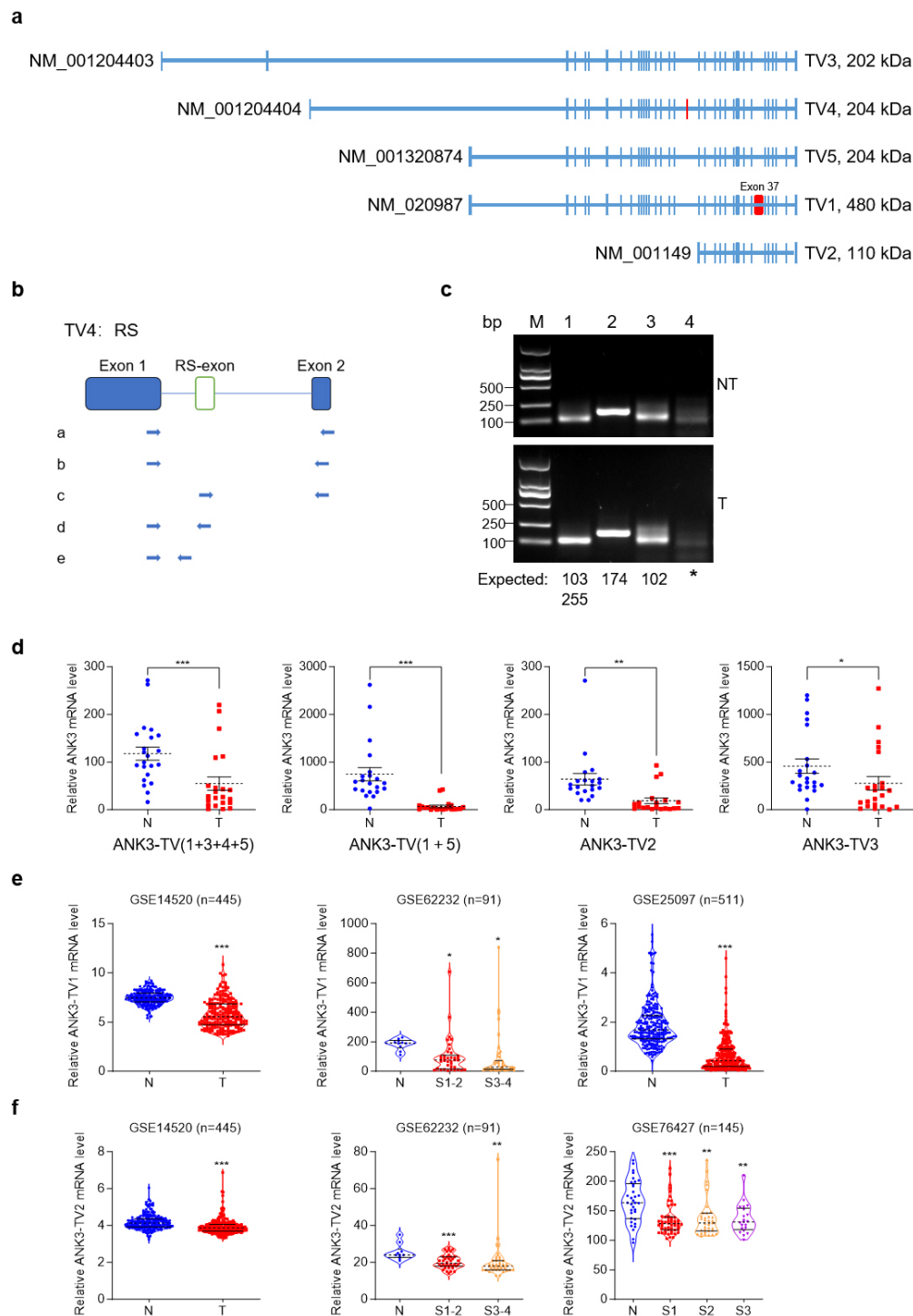


Fig. 2. Transcriptome structure diagram of *ANK3*. *ANK3* transcript variant 4 (*TV4*) undergoes recursive splicing (RS). (a) *ANK3* from the NCBI database underwent alternative promoter and exon jumps, producing five TVs. The blue boxes represent exons. The red boxes represent the unique exons of *TV1* and *TV4*, respectively. (b) Schematic diagram of the nested PCR primers. (c) *ANK3-TV4* undergoes RS. Using the nest-PCR assay, *ANK3-TV4* was recursively spliced in both HCC tumours (T, down) and adjacent non-tumor (NT, up). Primer a was used for the first round of PCR. b/c/d/e primers were used for the second round of PCR in lanes 1/2/3/4, respectively. PCR product sizes are annotated below each lane. (d) mRNA levels of different transcripts in T and N tissues. (e) mRNA level of *ANK3-TV1* in available HCC databases. (f) mRNA level of *ANK3-TV2* in available HCC databases. Data are presented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's paired t test).

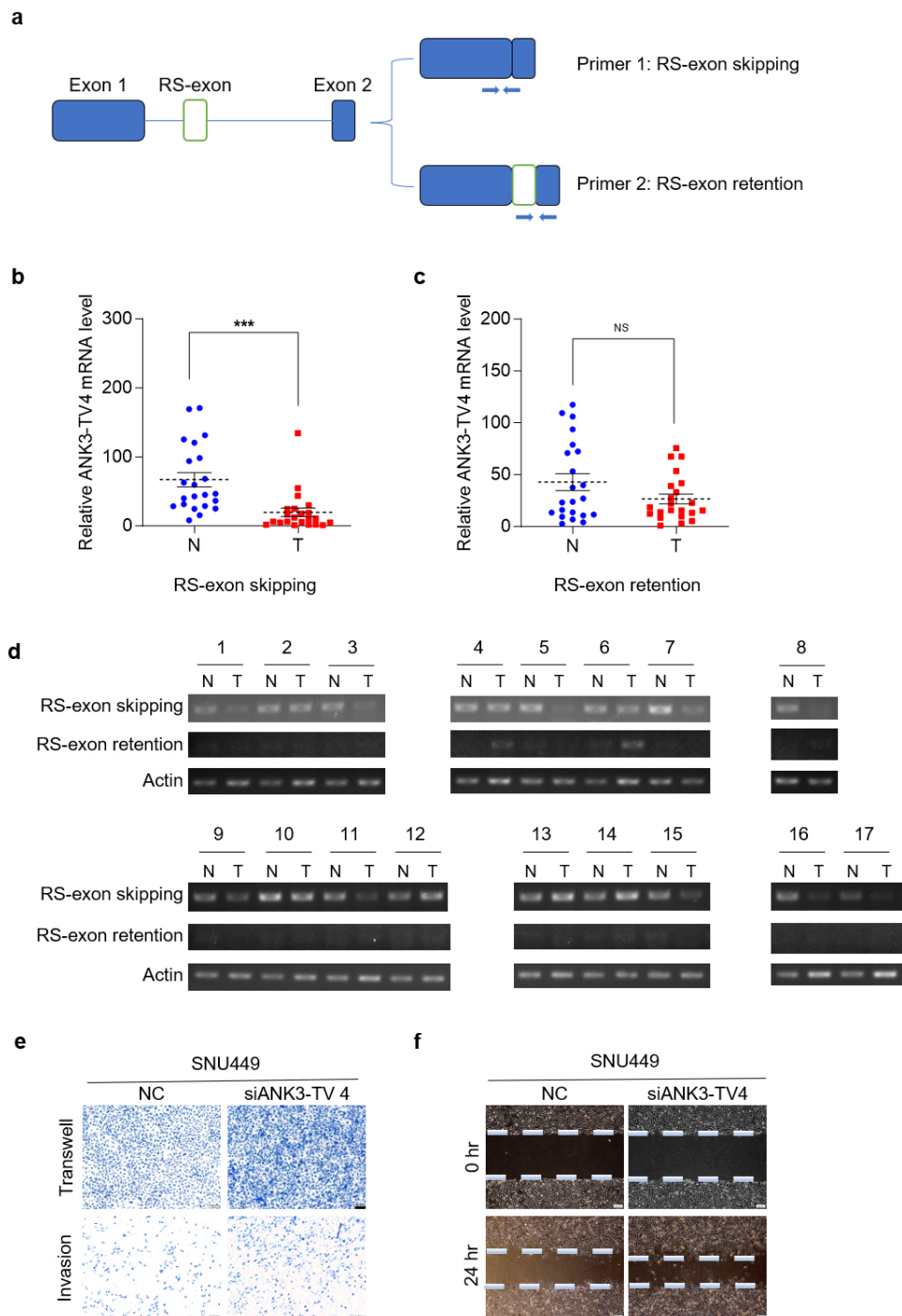


Fig. 3. RS of *ANK3-TV4* is weakened in HCC, and *ANK3-TV4* inhibits the metastasis of HCC cells. (a) Primer 1 was designed across the primary/secondary exon of *ANK3-TV4*, specifically quantifying the transcripts with RS (RS-exon skipping). Primer 2 was designed in the *ANK3-TV4* RS-exon and the second exon, respectively, specifically quantifying the transcripts with RS-exon retention. (b) Quantification of *ANK3-TV4* (RS-exon skipping) mRNA in HCC (T) and paired non-cancerous tissues (N). (c) mRNA abundance of *ANK3-TV4* (RS-exon retention) in HCC samples (T) relative to adjacent controls (N). (d) Semi-quantitative PCR analysis of both *ANK3-TV4* splicing variants in 24 clinical sample pairs (Cycle parameters: Actin-25, *ANK3-TV4*-40). (e) Transwell and invasion assays of SNU449 cells following *ANK3-TV4* knockdown. (f) Wound-healing assay of SNU449 cells transfected with NC or siANK3-TV4. Scale bar: 100 μ m (e), 200 μ m (f). Data are presented as mean $\pm$ SEM, NS: $p > 0.05$, *** $p < 0.001$ (Student's paired t test).

24 anchoring protein repeat sequences responsible for binding the entire membrane protein), a central spectrum

protein/fodrin-binding domain that connects the anchoring protein to the actin-based cytoskeleton through spectrum

protein isomers, and a C-terminal regulatory domain [20]. All other transcripts contained the three domains, except for *ANK3-TV2*, which completely lacked the ankyrin repeat domain. To explore the influence of various *ANK3* domains on HCC migration, *ANK3-TV2* was initially overexpressed in stable *ANK3*-total knockdown cells to assess its impact on metastatic behavior. Overexpression of *ANK3-TV2* did not markedly restore the promoting effect of *ANK3* knockdown on cell migration (Fig. 4a,b). Since *ANK3-TV2* lacks while *ANK3-TV4* contains an ankyrin repeat domain, we compared the effects of overexpressing *ANK3-TV2* and the ankyrin repeat-containing *ANK3-TV4* (Supplementary Fig. 2a–c). The results clearly showed that only *ANK3-TV4* significantly inhibited HCC cell invasion and migration (Fig. 4c–e), suggesting that *ANK3* significantly inhibits HCC metastasis through its ankyrin repeat domain.

3.5 Knocking Down *ANK3* Activates the Wnt Pathway and Promotes E-cadherin Protein Degradation

To explore the molecular mechanism of *ANK3* regulation of cell migration, transcriptomic sequencing was performed on RNA extracted from stable *ANK3* knockdown SNU449 cells. Gene Ontology Enrichment Analysis (GO) showed that *ANK3* participates in the regulation of the Wnt signaling pathway (Fig. 5a–d). We initially searched for proteins that might interact with *ANK3* in the BioGrid database and found that *ANK3* might interact with 151 proteins (Supplementary Fig. 3a). We screened the proteins associated with metastasis and summarized them in Supplementary Fig. 3b. At the same time, we reviewed the literature on these proteins. E-cadherin, located in the cytomembrane, has been reported to interact with *ANK3* and β -catenin in the cytoplasm to form ternary complexes. *ANK3* knockdown facilitates the dissociation of β -catenin from E-cadherin, and its subsequent translocation into the nucleus, thereby activating the canonical Wnt signaling pathway [29].

Based on the transcriptome sequencing results, BioGrid database search, and literature review, we selected E-cadherin as the research object and conducted subsequent experimental verification. The schematic diagram of the *ANK3* domain is shown in Fig. 6a. The expression of HA-tagged control and E-cadherin plasmid in 293T cells is shown in Fig. 6b. Western Blot assay showed that the E-cadherin antibody could precipitate *ANK3* containing an ankyrin repeat domain with a size of approximately 240 kDa and E-cadherin, but could not precipitate *ANK3-TV2* lacking the ankyrin repeat domain with a size of 110 kDa (Fig. 6c), indicating that endogenous E-cadherin interacts with the ankyrin repeat domain of *ANK3*. A co-immunoprecipitation (Co-IP) assay confirmed that *ANK3-TV4* could, while *ANK3-TV2* couldn't, interact with E-cadherin (Fig. 6d,e). *ANK3-TV4* overexpression significantly upregulated E-cadherin expression in Huh7 cells (Fig. 6f).

Transcriptomic sequencing suggests that knockdown of *ANK3* can activate the Wnt pathway. We first examined the potential role of *ANK3* in regulating the Wnt signaling pathway. Both qPCR and WB experiments confirmed that *ANK3* knockdown significantly upregulated the expression of several Wnt pathway components (Fig. 7a), while *ANK3-TV4* overexpression inhibited the expression of these genes (Fig. 7b).

The pro-metastatic function of Wnt/ β -catenin signaling is well-established across multiple cancer types and is attributed to the unique transcriptional program induced by the β -catenin/TCF complex [30]. Within this pathway, the TCF/LEF family transcription factor *TCF4* (also known as *TCF7L2*) plays a pivotal role. Utilizing its HMG domain for DNA binding, *TCF4* activates the expression of Wnt target genes, a function shared across the four members of the TCF/LEF family (*TCF1*, *TCF3*, *TCF4*, and *LEF*) [31]. In stable *ANK3*-total knockdown cells, *TCF4* and β -catenin were knocked down instantaneously, both of which could restore the promoting effect of *ANK3* knockdown on cell migration (Fig. 7c–f). These data suggested that *ANK3* participates in HCC progression by influencing the Wnt pathway.

ANK3 binds to E-cadherin via its N-terminal ankyrin repeat domain to regulate E-cadherin expression. *ANK3* knockdown activates the Wnt pathway, downregulates E-cadherin expression, and promotes its degradation. Conversely, *ANK3-TV4* overexpression inhibited the Wnt pathway, upregulated E-cadherin protein expression, and inhibited its degradation (Fig. 6g,h). Finally, we analyzed the correlation between E-cadherin and *ANK3* proteins in clinical samples from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) database of The Cancer Genome Atlas (TCGA) and found that E-cadherin protein positively correlated with *ANK3* protein in both liver cancer and pancreatic samples, which was consistent with our previous conclusions (Fig. 6i).

3.6 Knockdown of *RBM8A* Promoted RS of *ANK3-TV4*

Multiple annotated RS-exons in human mRNAs can be attributed to the inhibition of RS by EJC. The EJC is generally composed of core EJC factors, including *eIF4A3*, *MAGOY*, and *RBM8A*, and peripheral factors such as *PNN* and *RNPSI* [17]. Screening of candidate factors in clinical HCC samples revealed that *RBM8A* was the sole factor significantly overexpressed (Fig. 8a,b), consistent with publicly available data (Fig. 8c). Kaplan-Meier analysis of overall survival in the TCGA liver cancer cohort (n = 182) demonstrated a significant reduction in survival time for patients with high *RBM8A* expression (Fig. 8d). Elevated *RBM8A* expression was significantly correlated with reduced disease-free survival in HCC patients from the TCGA-LIHC cohort (Fig. 8e), underscoring its negative impact on patient prognosis.

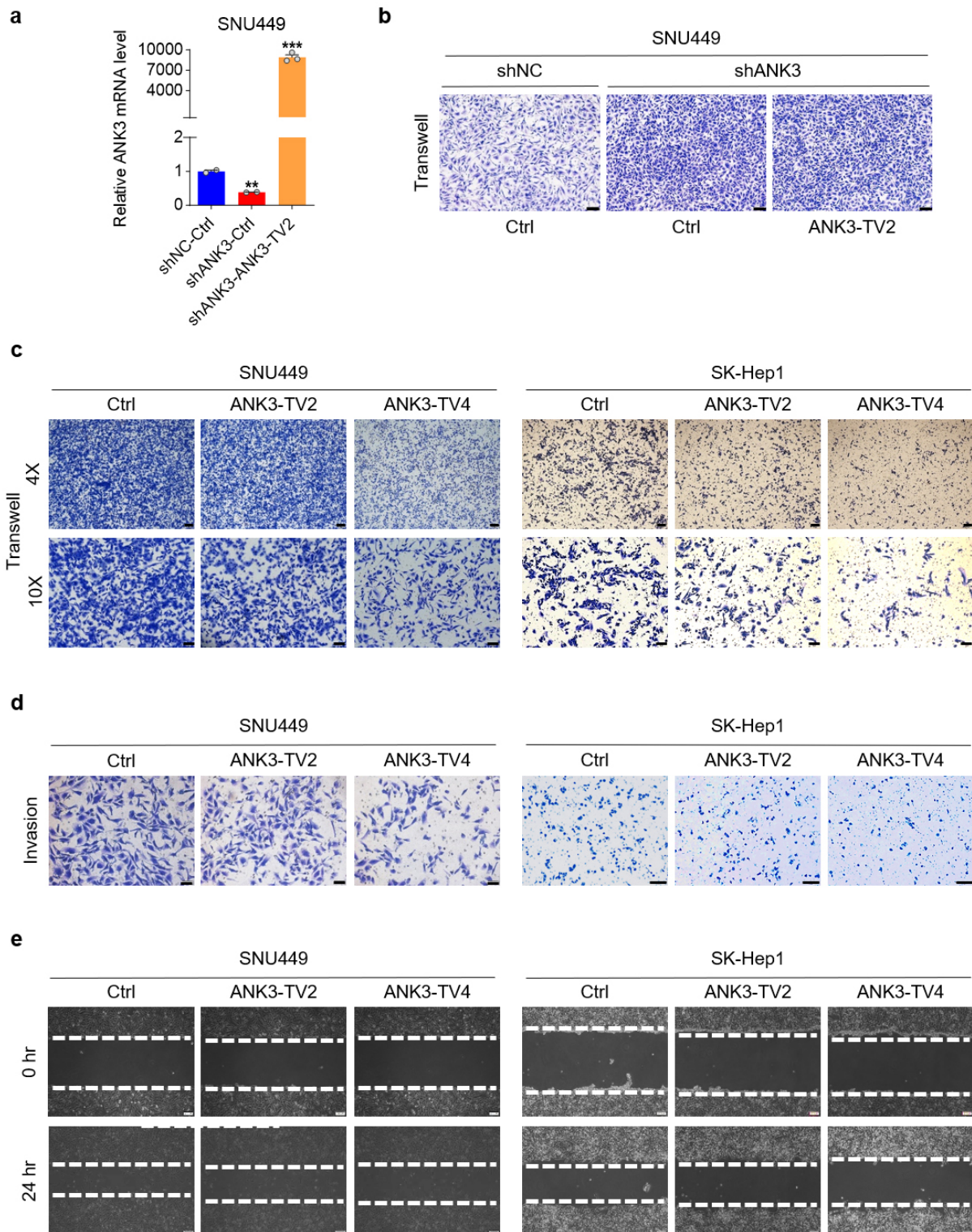


Fig. 4. Ankyrin repeats of *ANK3* are critical in cell migration. (a) *ANK3* mRNA expression in SNU449 cells with stable ANK3-total knockdown, following transfection with an empty vector (Ctrl) or an *ANK3-TV2* overexpression vector. (b) Transwell assay of *ANK3-TV2* overexpression in stable cell lines with ANK3-total knockdown. (c) Transwell assay of SNU449 (left) or SK-Hep1 (right) cells transfected with ctrl (Empty Vector) or *ANK3-TV2/ANK3-TV4* overexpression vectors. (d) Invasion assay of SNU449 (left) or SK-Hep1 (right) cells transfected with ctrl (Empty Vector) or *ANK3-TV2/ANK3-TV4* overexpression vectors. (e) Wound-healing assay of SNU449 (left) or SK-Hep1 cells (right) transfected with ctrl (Empty Vector) or *ANK3-TV2/ANK3-TV4* overexpression vectors. Scale bar: 100 μ m (b), 200 μ m (c 4 $\times$), 100 μ m (c 10 $\times$), 100 μ m (d), 200 μ m (e). Data are shown as mean $\pm$ SEM, Student's paired *t* test, ***p* < 0.01, ****p* < 0.001.

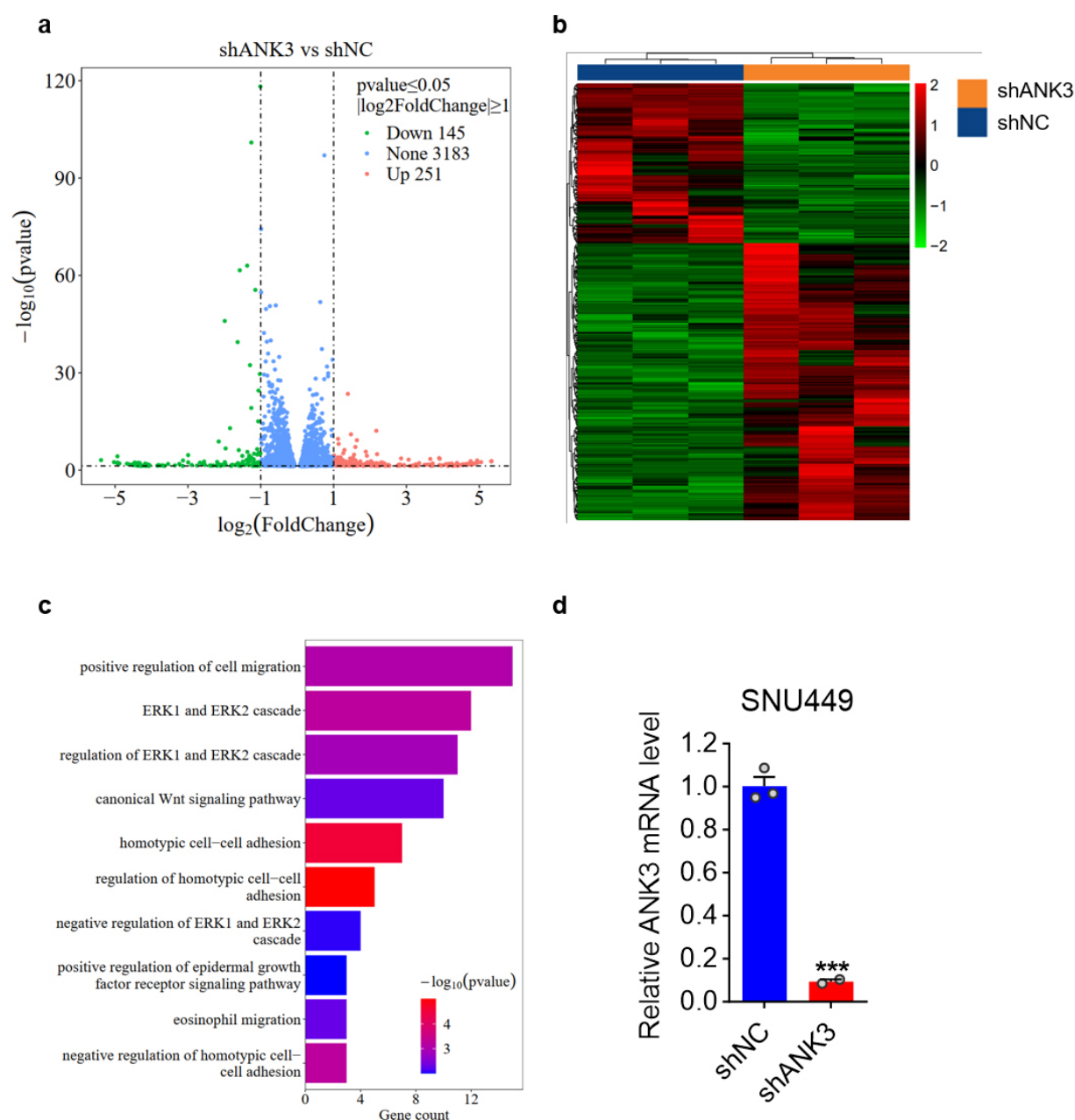


Fig. 5. Enrichment analysis of differentially expressed protein-coding genes in SNU449 cells with stable *ANK3* knockdown. (a) A volcano plot visualization of the data. The X-axis represents the magnitude of expression change ($\log_2$ fold change), while the Y-axis indicates the statistical significance ($-\log_{10} p\text{-value}$) of that change. Genes are highlighted as down-regulated (green), up-regulated (red), or unchanged (blue). (b) Cluster heat maps: Each column represents a sample, and each group has 3 biologically duplicated samples. Each line represents a protein-coding gene. Fragments Per Kilobase Million (FPKM) values were used for clustering, with red representing high-expression genes and green representing low-expression genes, and the darker the color, the greater the degree of up-regulation or down-regulation. (c) Gene Ontology Enrichment Analysis (GO): The vertical axis is the name of the GO pathway, and the horizontal axis represents the number of genes enriched into that pathway. The darker the color, the smaller the p value, indicating that the enrichment significance of differentially expressed genes in this pathway is more reliable. (d) qRT-PCR analysis of *ANK3* expression in SNU449 cells infected with shNC or shANK3 lentivirus. Data are presented as mean $\pm$ SEM, *** $p < 0.001$ (Student's paired t test).

We next investigated the functional contribution of *RBM8A* to liver tumorigenesis. Functional interrogation of *RBM8A* in liver tumorigenesis revealed that its siRNA-

mediated knockdown attenuated the proliferation of SK-Hep1 and SNU449 cells (Fig. 8h), and markedly impaired the migration and invasion of SK-Hep1 cells (Fig. 8f).

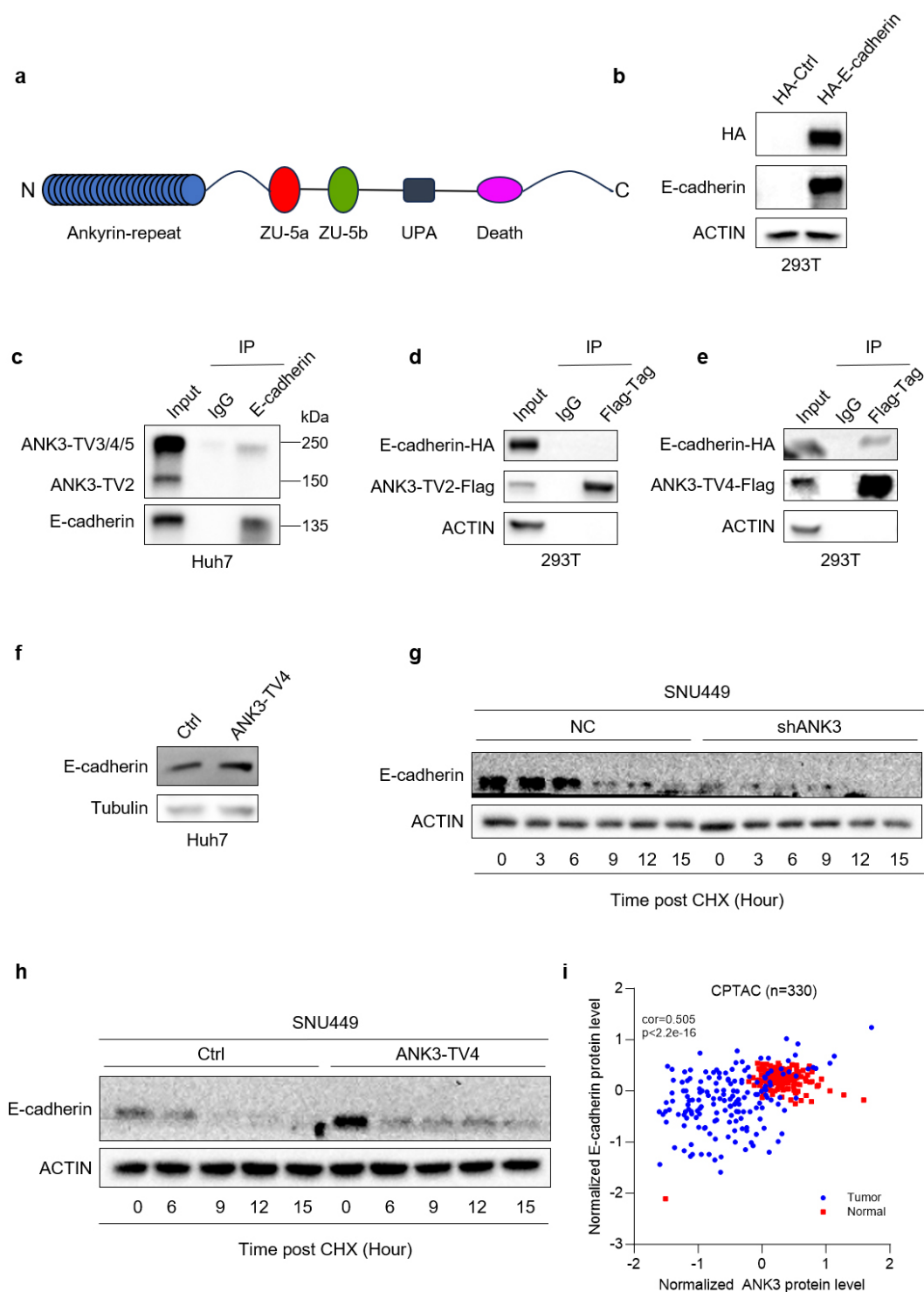


Fig. 6. ANK3 interacts with E-cadherin, and knockdown of ANK3 promoted E-cadherin protein degradation. (a) Schematic diagram of the ANK3 domain. (b) Validation of HA-tagged control and E-cadherin plasmid expression in 293T cells by WB. (c) Endogenous interaction between ANK3 and E-cadherin in Huh7 cells, confirmed by co-immunoprecipitation. (d,e) Exogenous interaction of E-cadherin with ANK3-TV2 (d) or ANK3-TV4 (e) in 293T cells, assessed by co-immunoprecipitation. (f) Overexpression of ANK3-TV4 significantly upregulated E-cadherin. (g) Knockdown of ANK3 promoted E-cadherin protein degradation. (h) Overexpression of ANK3-TV4 inhibited E-cadherin protein degradation. (i) E-cadherin protein and ANK3 protein were positively correlated in HCC in the CPTAC database.

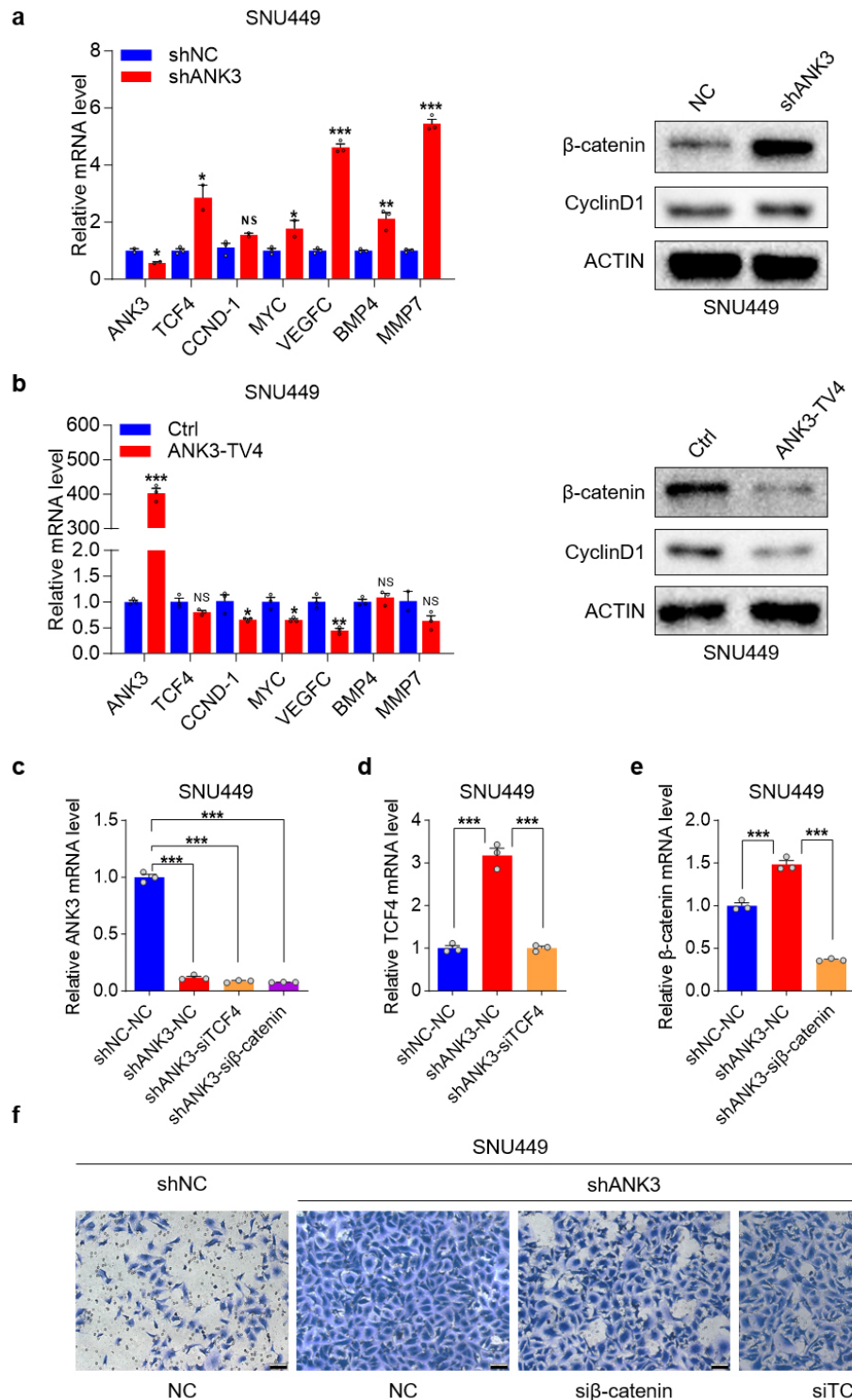


Fig. 7. Knockdown of *ANK3* activates the Wnt pathway. (a) qRT-PCR and WB analysis of Wnt pathway factor expression upon *ANK3* knockdown in SNU449 cells. (b) qRT-PCR and WB analysis of Wnt pathway components in response to *ANK3-TV4* overexpression in SNU449 cells. (c) qRT-PCR analysis of *ANK3* expression in response to transient *TCF4*/ β -catenin knockdown in stable *ANK3*-knockdown cell lines. (d) *TCF4* expression level was detected following transient knockdown of *TCF4* in stable cell lines with *ANK3*-total knockdown. (e) The expression level of β -catenin was detected after transient knockdown of β -catenin in stable cell lines with *ANK3*-total knockdown. (f) Transwell assay after transient knockdown of *TCF4*/ β -catenin in stable cell lines with *ANK3*-total knockdown. Scale bar: 100 μ m. Data are presented as mean $\pm$ SEM, NS: $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's paired t test).

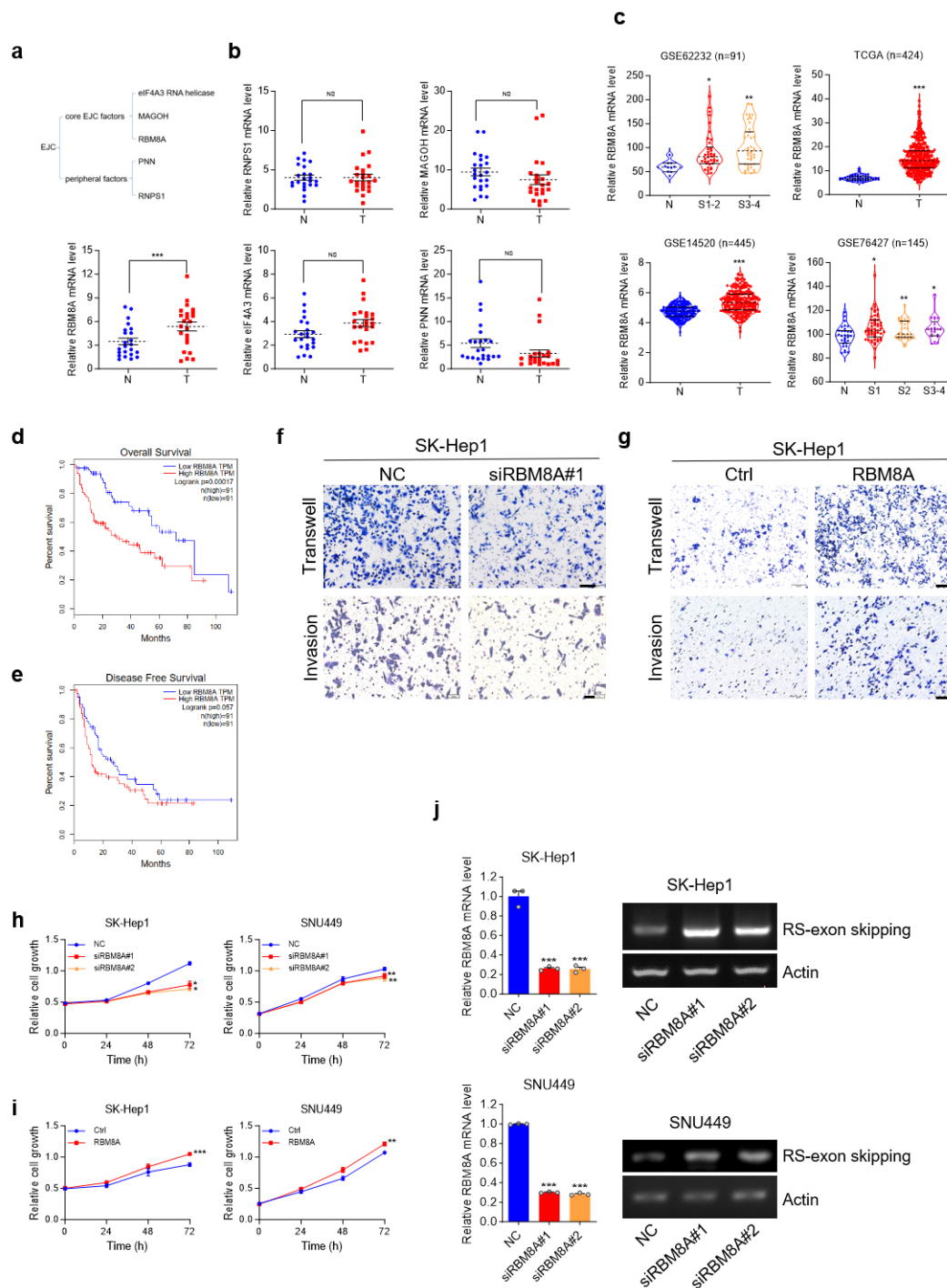


Fig. 8. *RBM8A* is upregulated in HCC. Knockdown of *RBM8A* inhibits the proliferation and metastasis of HCC cells, and knockdown of *RBM8A* promotes RS of *ANK3-TV4*. (a) Schematic of exon junction complex (EJC). (b) qRT-PCR analysis of EJC factors identifies *RBM8A* as the sole significantly upregulated member in HCC. (c) *RBM8A* mRNA levels in HCC and normal livers from published cohorts. (d) Kaplan-Meier analysis of overall survival data from TCGA cohort (n = 182), stratified by *RBM8A* expression. (e) Kaplan-Meier analysis of disease free survival in the TCGA-LIHC cohort (n = 182). (f) Impact of *RBM8A* depletion on the migratory and invasive capacity of SK-Hep1 cells. (g) Effect of *RBM8A* overexpression on the migratory and invasive capacity of SK-Hep1 cells. (h) MTS analysis of SK-Hep1 (left) and SNU449 (right) cells transfected with NC or siRBM8A. (i) MTS analysis of SK-Hep1 (left) and SNU449 (right) cells transfected with ctrl (Empty Vector) or *RBM8A* overexpression vector. (j) The semi-quantitative RT-PCR proved that knocking down *RBM8A* promotes RS of *ANK3-TV4* in SK-Hep1 or SNU449 cells. Actin: 25 cycles; *ANK3-TV4* with RS-exon skipping: 42 cycles. Scale bar: 100 μ m. Data are presented as mean $\pm$ SEM, NS: $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's paired t test).

Conversely, *RBM8A* overexpression enhanced these malignant properties, including migration, invasion, and growth (Fig. 8g,i). Collectively, these findings establish *RBM8A* as a promoter of HCC cell proliferation, migration, and invasion *in vitro*.

The role of *RBM8A* in modulating the RS of *ANK3-TV4* was assessed. Transcripts with RS-exon skipping were quantified using primers crossing the first and second exons of *ANK3-TV4*. The data indicate that reducing *RBM8A* levels promotes RS of *ANK3-TV4* (Fig. 8j), which promoted the jump of the RS-exon, reducing the NMD of *ANK3-TV4*. Thus, normal functional *ANK3-TV4* was upregulated.

4. Discussion

In 1998, Hatton *et al.* [32] first discovered the RS in *Drosophila melanogaster Ubx* and identified a new splicing site between the first and second exons of the *Ubx* gene. Researchers have conducted in-depth studies on the *Ubx* gene and found that the first intron of the gene is 73 kb long, and the removal of the intron involves three splicing steps [33]. In 2015, Sibley *et al.* [16] conducted deep RNA sequencing of vertebrate neurons and found that RS phenomena are widespread in vertebrate neurons, including in genes such as *ANK3* and *CADM2*. Emmett [34] found that in the human brain tissue, seven genes associated with RS are closely related to neurological diseases. Given the established link between aberrant long-gene expression and neurological disorders [35–37], it is plausible that mutations or deletions at RS sites constitute another mechanism for human genetic diseases [16]. RS events, observed in diverse cell types including neurons [34] and endodermal cells [38], are associated with a spectrum of human diseases spanning neurological (e.g., Parkinson's) and circulatory (e.g., retinal sclerosis, hypertension) disorders [39]. In addition, Srndic [40] detected over 12,000 RS events in multiple human tissues and found that RS might play a role in nonsense-mediated RNA decay.

ANK3 was originally identified in the axonal initial segment and nodes of Ranvier [41,42]. It has been extensively characterized and is now recognized as an established risk gene for several neuropsychiatric disorders, including bipolar disorder, schizophrenia, and autism spectrum disorder [21,43–45]. A relevant study has reported that *ANK3* undergoes RS [16], but did not indicate the transcript in which the RS is present. *ANK3* is perversely expressed in various human cancers; however, the underlying mechanism remains unclear. Currently, only studies have reported the function of *ANK3* in prostate cancer [26], and colorectal cancer [46].

Referring to the study by Blazquez *et al.* [17], who found that the EJC suppressed hundreds of annotated RS, mainly constitutive RS-exons, and they investigated the role of EJC in regulating the RS of *CADM2*, mainly by RT-PCR. Given this context, we sought to determine whether the EJC, particularly its key components, regulates *ANK3*

in HCC. Systematic examination of EJC factors in clinical HCC samples revealed *RBM8A* as the sole significantly upregulated member. Subsequent functional characterization confirmed the oncogenic properties of *RBM8A*. Therefore, we explored the effect of *RBM8A* on *ANK3* RS. However, we did not successfully detect RS by RT-PCR using the primers used in the study by Sibley *et al.* [16]; thus, we redesigned the primers to specifically detect the expression of RS transcripts by semi-quantitative PCR. Knockdown of *RBM8A* significantly promoted the RS of *ANK3-TV4*, which promoted a jump in the RS-exon, reducing the NMD of *ANK3-TV4*. Thus, normally functional *ANK3-TV4* is upregulated, inhibiting cell migration. Of course, *RBM8A*'s regulation on the RS of the *ANK3-TV4* is only slight, and this regulatory process must involve many other factors and more complex pathways. Our study merely preliminarily links RS to HCC. However, it is not yet clear whether the anti-metastasis effect of *RBM8A* depletion is specifically mediated by *ANK3-TV4* RS.

Durak *et al.* [29] demonstrated that *ANK3* regulates canonical Wnt signaling by modulating the subcellular localization and availability of β -catenin in proliferating cells. They found that *ANK3* deficiency disrupts the β -catenin/E-cadherin interaction, leading to increased nuclear translocation of β -catenin, which in turn enhances Wnt signaling and promotes neural progenitor cell proliferation. However, the study did not address the subsequent fate of E-cadherin. Our results indicated that *ANK3* knockdown downregulates E-cadherin, promotes E-cadherin degradation, and activates the Wnt pathway, thus promoting HCC metastasis. Our study did not compare the Wnt inhibition mediated by *ANK3* with other reported Wnt regulators in HCC (such as AXIN2, APC), nor did it test whether *ANK3* affects EMT markers (such as N-cadherin, vimentin, Snail), which are the key downstream effects of E-cadherin regulation. This is one of the deficiencies of this study. This study initially indicates that *ANK3* is involved in regulating the Wnt signaling pathway. The mechanism by which it participates in HCC pathogenesis by regulating the Wnt pathway requires further elucidation.

The growing recognition of introns as critical regulators of gene expression, driven by advances in RNA sequencing, highlights the importance of deciphering RS in this process. HCC is the third most common cause of cancer mortality worldwide [47], creating an urgent need to define its molecular drivers, particularly for metastasis [26]. Investigating RS may therefore yield novel therapeutic targets for this lethal malignancy. We studied the effects of *ANK3* on HCC development via its regulation by RS. This study only explored the regulation of *RBM8A* on the RS of *ANK3-TV4* at the mRNA level, but there was no discussion on the level of *ANK3* protein. Therefore, it is difficult to link it with the downstream mechanism of *ANK3* regulation. In addition, the puzzling thing is that the expression pattern of the protein near 250 kDa (*ANK3-TV3/4/5*) in HCC is un-

certain, which is not completely consistent with the tumor suppressive function of *ANK3*. Therefore, more complex regulatory mechanisms at the protein level need to be explored. Further *in vivo* experiments are required to assess the antitumor efficacy of *ANK3*.

5. Conclusions

In summary, we found that *ANK3* was downregulated in HCC and inhibited HCC metastasis, confirming that RS exists in *ANK3-TV4* and that RS is weakened in HCC. *RBM8A*, a core EJC factor, regulates the RS of *ANK3-TV4*. Mechanistically, *ANK3* regulates HCC progression by simultaneously controlling the expression of E-cadherin and the Wnt signaling pathway. Our study on RS regulation of *ANK3* is superficial, but it provides a new idea for introns to regulate gene expression and participate in disease, and an in-depth study of RS, *ANK3*, and EJC may pave the way for developing targeted therapies against HCC.

Availability of Data and Materials

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

Author Contributions

YG, XF and YT designed the research study. YG performed the research. YG analyzed the data. YG wrote the manuscript. YT provided help on the revision of the article. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was conducted in accordance with the Declaration of Helsinki. The research protocol was approved by the Ethics Committee of Institutional Review Committee of West China Hospital of Sichuan University (Ethic Approval Number: 2020, no. 196) and all of the participants provided signed informed consent.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/FBL46013>.

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