BIOENGINEERED
2021, VOL. 12, NO. 1, 7446–7458
https://doi.org/10.1080/21655979.2021.1975969

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Long non-coding RNA SNHG22 facilitates hepatocellular carcinoma tumorigenesis and angiogenesis via DNA methylation of microRNA miR-16-5p

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ABSTRACT

Hepatocellular carcinoma (HCC) is considered as a common malignancy worldwide. Considerable evidence has illustrated that abnormally expressed long noncoding RNAs (lncRNAs) are in a close correlation with the initiation and progression of various tumors, including HCC. LncRNA small nucleolar RNA host gene 22 (SNHG22) has been reported to play important roles in tumor initiation, but its role and mechanism are little known in HCC. In our report, we discovered the high level of SNHG22 in HCC tissues and cells, and the high expression of SNHG22 was correlated with unfavorable clinical outcome in HCC patients. Functional assays implied that SNHG22 deficiency suppressed cell proliferation, migration, invasion, and angiogenesis in vitro. Additionally, it was also confirmed that silenced SNHG22 suppressed tumor growth and angiogenesis in vivo. Mechanistic exploration revealed that SNHG22 recruited DNMT1 to miR-16-5p DNA promoter through EZH2 and inhibited miR-16-5p transcription via DNA methylation. Finally, we verified that the suppression of miR-16-5p countervailed the suppressive effect of SNHG22 deficiency on HCC cell proliferation, migration, invasion, and angiogenesis. Conclusively, this study clarified the SNHG22/EZH2/DNMT1/miR-16-5p axis and revealed that SNHG22 could be an underlying biomarker for HCC.

KEYWORDS

SNHG22; miR-16-5p; dnmt1; ezh2; hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is known as the sixth common primary malignancy and is regarded as the third major reason of tumor-correlated death all over the world [1]. Extensive studies showed that the occurrence of HCC is involved in many risk factors; among which, cirrhosis and viral hepatitis B are identified as the common events in tumor incidence [2,3]. In the past few decades, massive financial resources were put into the research of therapeutic methods for HCC, which led to continuous progress in HCC treatment [4]. However, it remains relatively poor in the clinical outcome of HCC patients [5]. One reason is that a lot of patients are usually definitely diagnosed at the later stage that correlated with metastasis [6]. Thus, it is important to explore molecular mechanisms for the finding of more effective biomarkers underlying HCC progression.

With the continuous improvement in sequencing techniques, many new kinds of genes were recognized as key factors in physiological and pathological development [7]. Long noncoding RNAs (LncRNAs) are a cluster of transcripts exceeding 200 nucleotides and lacking the ability of protein-coding [8]. In recent years, extensive evidence has revealed that lncRNAs with aberrant expression are dysregulated in cancer progression. For example, lncRNA MAFG-AS1 promotes tumor progression in bladder urothelial carcinoma by regulating HuR/PTBP1 axis [9]. LINC00460 is highly expressed in colon cancer and upregulates ANXA2 to facilitate cell invasion, proliferation, epithelial–mesenchymal transition (EMT), and tumor growth through binding to miR-433-3p [10]. LncRNA GATA6-AS1 is downregulated and restrains lymph node metastasis (LNM) and EMT in gastric cancer via downregulating FZD4 expression to inactivate Wnt/β-catenin Signaling [11]. Previous study has also validated

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some HCC-related lncRNAs, such as LINC01224 [12], HOXA11-AS [13], and ID2-AS1 [14]. Therefore, it is valuable to inspect more HCC-relevant lncRNAs and investigate novel regulatory mechanisms for HCC therapy.

Small nucleolar RNA host gene 22 (SNHG22) is discovered as an oncogenic lncRNA and plays a vital role in some tumors. As reported, SNHG22 is found to enhance malignant phenotypes via the miR-324-3p/SUDS3 axis in triple-negative breast cancer [15]. Additionally, SNHG22 is upregulated and functions as an oncogene in epithelial ovarian carcinoma [16]. Nevertheless, it has not been elaborated the detailed role and mechanism of SNHG22 in HCC. The main objective of our research was to investigate its biological role and probable mechanism in HCC.

Aberrant DNA methylation and histone methylation have been identified in various human cancers [17]. EZH2 is a histone methyltransferase that specifically modulates histone H3 lysine 27 trimethylation (H3K27me3) [18], while DNMT1 is a DNA methyltransferase that catalyzes the process of cytosine base methylation [19]. Increasing evidence indicated that lncRNAs could directly interact with EZH1 or DNMT1 and epigenetically silence miRNA expression in HCC. For example, lncRNA HOTAIR suppressed miR-122 expression in HCC via DNMTs-mediated DNA methylation [20]. PVT1 epigenetically inhibited miR-214 expression by binding with EZH2 to promote the progression of HCC [21]. Moreover, miR-16-5p has been reported to act as a tumor suppressor in various types of cancers [22,23] and can inhibit tumorigenesis and chemoresistance of HCC [24,25]. However, the regulatory mechanisms of miR-16a need to be further elucidated.

The present study aimed to investigate the role and molecular mechanism of SNHG22 in HCC and the results demonstrated that SNHG22 contributed to HCC tumorigenesis and angiogenesis via DNA methylation of miR-16-5p, suggesting SNHG22 might serve as an effective target for HCC treatment.

Materials and methods

Clinical samples

Sixty paired tumor and adjacent non-tumor specimens were collected from HCC patients at Jianhu People's Hospital. The clinicopathological characteristics of HCC patients were presented in Table 1, and it was found that high SNHG22 or low miR-16-5p expression was associated with tumor size, lymph node metastasis, and clinical stage, while there was no significant association with sex or age in patients with HCC (Table 1). Before the surgery, all patients had not received chemo- or radio- therapy, and each of them has provided written informed consent. In liquid nitrogen, the tissues were sharply frozen and then preserved at −80°C after surgical resection. For this study, the permission from Ethics Committee of Jianhu People's Hospital was obtained.

Cell lines

Chinese Academy of Sciences Cell Bank (Shanghai, China) provided THLE-3 cell line (human normal liver cell line) and HCC cell lines (Huh7, HCCLM6, MHCC97H and SNU-398). All cells were maintained in DMEM with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS, Gibco, USA) and cultured at 37 °C in a humidified incubator containing 5% CO_2. In addition, Huh7 and HCCLM6 cell lines were treated with different concentrations (0, 1, and 5 μM) of 5-Aza-2 -deoxycytidine (5-Aza-dC; cat. no. S3196; Selleck Chemicals) for 48 h at 37 °C.

Cell transfection

Short hairpin RNA (sh-RNA) targeting SNHG22 (sh-SNHG22; 5′-GGGAGAGTCATCCAAGGAA -3′), DNMT1 (sh-DNMT1; 5′-GCUACGAGA UCGAGUUCAUTT-3′), DNMT3A (sh-DNM T3A; 5′-GTGCAGAAACATCGAGGACAT-3′) and DNMT3B (sh-DNMT3B; 5′- AAGTGGA AAAGTACATTGCCT-3′) and matched control shRNA (sh-NC; 5′-UUCUCCGAACGUGUC ACGUTT-3′) were provided by GenePharma (Shanghai, China). MiR-16-5p inhibitor (5′-CGCCAAUAUUUACGUGCUGCUA-3′) and NC inhibitor (5′-UUGUACUACACAAAAGUACUG -3′) were also obtained from GenePharma. The above-mentioned plasmids (50 nM) were transfected into Huh7 and HCCLM6 cells by Lipofectamine 3000 (Invitrogen, Carlsbad, CA,

Table 1. Association between SNHG22 or miR-16-5p expression and clinicopathological characteristics in HCC.

		SNHG22			miR-16-5p		
Features	No.	High	Low	P-value	High	Low	P-value
No.	60	32	28		29	31	
Sex							
Male	39	20	19	0.915	17	22	0.793
Female	21	12	9		12	9	
Age (years)							
<60	26	14	12	0.815	13	13	0.942
≥60	34	18	16		16	18	
Tumor size (cm)							
<5	41	15	26	0.001	26	15	0.003
≥5	19	17	2		3	16	
Lymph node metastasis							
Absence	37	15	22	0.007	24	13	0.005
Presence	23	17	6		5	18	
Clinical stage							
I + II	40	18	22	0.008	23	17	0.007
III + IV	20	14	6		6	14	

USA). Then, the transfection efficiency of these plasmids was confirmed by RT-qPCR after the incubation of 48 h.

RT-qPCR analysis

TRIzol reagent (Invitrogen) was used for the extraction of total RNA. Utilizing reverse-transcription system kit (Takara, Otsu, Japan), the total RNA was reverse transcribed into cDNA, and qPCR analysis was carried out by the standard SYBR Green PCR kit (Takara). Through a miRNA First-Strand cDNA Kit (TIANGEN, Beijing, China), the reverse transcription of miRNA was performed, and qPCR analysis was conducted with miRcute Plus miRNA qPCR Kit (Tiangen) Relative quantification analysis was carried out by $2^{-\Delta\Delta CT}$ method. GAPDH (for lncRNA analysis) or U6 (for miRNA expression analysis) was taken as the negative control. The primers were as follows: SNHG22 forward, 5′-AGGAGAGCTGCTCTTCACAGG-3′ and reverse, 5′-TCCTAGGCTGAGTGTGTCTCC-3′; miR-16-5p forward, 5′-GGAAGATGAGGAGGTCGCTG-3′ and reverse, 5′-GACTTGACTGGAAGGGTGGG-3′; GAPDH forward, 5′-CCTGGCACCCAGCACAAT-3′ and reverse, 5′-GGGCCGGACTCGTCATCG-3′, U6 forward, 5′-GATTTCTCCCTCATCGCTTACAG-3′ and reverse, 5′-CTGCTTCATGATCGTTGTTGCTTG-3′.

Western blot

Total protein was isolated using RIPA buffer (Beyotime), exposed to 10% SDS-PAGE, and transferred onto PVDF membranes (Millipore). Then, the membranes were incubated with anti-VEGF (ab150375; Abcam), anti-endoglin (ab252345; Abcam), and anti-GAPDH (ab8245; Abcam) overnight at 4 °C. Following the incubation of secondary antibody for 1 h. The protein signals were analyzed by the ECL system kit (Pierce, Rockford, USA) [26].

CCK-8 assay

The transfected HCC cells were seeded into 96-well plates (4×10^3 cells/well) and incubated for 0, 24, 48, and 72 h. Later, 10 µl of CCK8 reagent (Dojindo Laboratories) were added to incubate for another 2 h. At last, a microplate reader was used to measure the viability of cells [27].

Transwell assay

Transwell assay was used for assessing the migratory and invasive abilities of transfected HCC cells [28]. The transwell chambers (pore size, 8 µm) were precoated with Matrigel (BD Biosciences, NJ, USA) for invasion assay and were not precoated with Matrigel for migration assay. In brief, culture medium with 10% FBS was seeded into bottom chambers, and cells (5×10^4) in 100 µl serum-free medium were placed into top chambers. After incubation at 37°C for 24 h, non-migrated or non-invaded cells were removed by a cotton swab. Cells migrated or invaded into the bottom chamber were in fixation and staining with paraformaldehyde (4%) and crystal violet (0.1%). Under an optical microscope, cell numbers were counted.

Tube formation assay

As previously described, the standard Matrigel assay was applied to assess angiogenesis activity in vitro through the quantification of tube formation [29]. In brief, the conditioned medium was the suspension from the transfected Huh7 and HCCLM6 cells for 48 h. Then, the conditioned

medium was cultured in 24-well plates with 6×10^4 human umbilical vein endothelial cells (HUVECs), and growth-factor-reduced Matrigel (50 µl, BD Biosciences) was coated in each well. After 24 h, Zeiss inverted microscope (Carl Zeiss) was used to acquire capillary-like structures.

Xenograft study

Male BALB/c nude mice aged 4-week-old were from Shi Laike Company (Shanghai, China), and fed in specific pathogen-free (SPF) conditions. For imitating tumor growth in vivo, mice were inoculated with Huh7 cells (1×10^6) with transfection of sh-SNHG22 or sh-NC by subcutaneous injection. Every 4 days, tumor volume was recorded, and mice were sacrificed after 4 weeks. Then, tumors were weighted. The Animal Ethics Committee of Jianhu People's Hospital approved animal studies.

Immunohistochemistry (IHC)

Tumor tissues acquired from mice were processed by paraformaldehyde (4%) for fixation, and then the fixed tissues were dehydrated and embedded in paraffin. Later, sections (4-mm-thick) were acquired via intersecting the paraffin-embedded tissues. Next, the sections were deparaffinized and incubated at 4°C overnight with Ki67 antibody (Abcam, MA, USA). After incubation with secondary antibody, sections were stained by hematoxylin and photographed under a microscope.

Microvessel density (MVD) detection

MVD in the xenografts was detected IHC. Based on MVD, the microvascular density was determined as previously described [30]. Briefly, the neovascular structure was defined by yellow/brown colored cells or areas which could distinguish from grands or proximal microvessel. The unconnected branches of neovascularization were also identified as a neovascular structure. For counting, five views were chosen at random on each slide and average value was utilized to show MVD of each slide. The average value more than MVD threshold was defined as MVD positive, and the average value was considered as MVD negative.

Methylation-specific PCR

As the manufacturer's protocol, Monarch genomic DNA-purification kit (NEB) was applied to isolate the genomic DNA from Huh7 and HCCLM6 cells [31]. Then, the conversion of DNA samples was performed by Methylation-Gold kit (Zymo Research). Later, Taq 2× master mix (NEB) was used perform methylation-specific PCR and routine PCR for analyzing the methylation of miR-16-5p. Finally, to ensure that proper PCR products were acquired, PCR products were sequenced.

Subcellular fractionation assay

By Life Technologies' PARIS kits (Hongfu Biotech, China), the isolated RNAs of cytoplasmic and nuclear were performed [32]. Briefly, 5×10^6 Huh7 or HCCLM6 cells were collected, washed, and placed in a fractionation buffer (350 µl). Then, the samples were centrifuged after incubation for 15 min at 4°C. Later, cytoplasmic section was removed from the nuclear fraction. Then, the lysis of nuclear fraction was conducted in cell disruption buffer, and then RNA isolation was performed by 2× Lysis/Binding Solution. At last, RNAs from nuclear and cytoplasmic fractions were assessed by qPCR, U6 (for nuclear transcript) or GAPDH (for cytoplasmic transcript) was used as the negative control.

Chromatin immunoprecipitation (ChIP) assay

Millipore Magna ChIP kit (YuSheng Biotech) was used for ChIP assay [33]. Cells were collected and treated with formaldehyde to cross-link DNAs and proteins. Next, the formaldehyde was quenched by placing the cells into glycine buffer. Later, DNA fragments (200–400 bp) produced by sonication were precipitated by anti-DNMT1 antibody (ab188453; Abcam), and anti-IgG antibody (as negative control; ab181236; Abcam). At last, level of precipitated DNAs was validated by RT-qPCR analysis.

RNA immunoprecipitation (RIP) assay

Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) was used for RIP assay [33]. In the lysis buffer, cells were lysed, and then cell lysates were immunoprecipitated with anti-IgG (Abcam) or anti-EZH2 (ab283270; Abcam) antibody. After the extraction and purification, immunoprecipitated RNA was reverse transcribed to cDNA for RT-qPCR using SNHG22-specific primers.

Co-IP assay

For Co-IP assay, pre-cleared cell lysate was centrifuged for 15 minutes at 12,000 rpm. Then, cell lysates and primary antibodies were co-incubated at 4 °C overnight. Later, protein Agarose beads (100 μL, GE Healthcare, USA) were added and incubated for 2 h at room temperature with gentle rotation. Using RIPA buffer, the precipitates were washed and then boiled in a loading buffer. Finally, the precipitates were analyzed by western blot, and normal rabbit IgG was taken as the internal control.

Statistical analysis

Statistical analyses were conducted by using SPSS 20.0 (SPSS, Chicago, IL, USA), and data were exhibited as mean ± SD. For evaluating statistical significance in two or multiple groups, the Student's t-test or one-way ANOVA was employed. The Kaplan-Meier method was used to calculate the overall survival curve. $P < 0.05$ was considered as statistical significance.

Results

SNHG22 was highly expressed and correlated with unfavorable prognosis

At first, we used RT-qPCR to identify SNHG22 expression in HCC tissues and adjacent non-tumor tissues. It indicated that SNHG22 was considerably upregulated in HCC tissues compared with non-cancerous specimens (Figure 1(a)). Then, SNHG22 expression in THLE-3 cells and HCC cell lines was measured by RT-qPCR. As a result, SNHG22 also showed a higher level in HCC cell lines (Figure 1 (b)). Moreover, we applied Kaplan–Meier analysis and discovered that HCC patients with high SNHG22 level presented shorter survival time than those with low SNHG22 level (Figure 1(c)). Taken together, SNHG22 was up-regulated and predicted poor prognosis.

SNHG22 silencing inhibited cell proliferation, invasion, and angiogenesis *in vitro*

To further investigate the role of SNHG22, we designed SNHG22 shRNA (sh-SNHG22) to silence SNHG22 expression in Huh7 and HCCLM6 cell lines, and its transfection efficiency was confirmed by RT-qPCR (Figure 2(a)). Then, CCK-8 assay was carried out to assess the effect of SNHG22 on HCC cell proliferative ability. We discovered SNHG22 silencing remarkably prohibited the proliferation of Huh7 and HCCLM6 cells (Figure 2

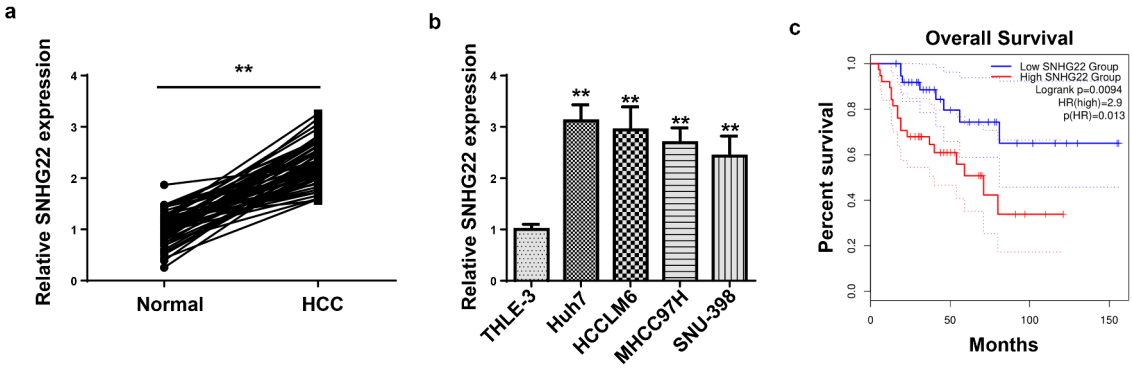

Figure 1. Relative SNHG22 expression in HCC tissues and cells and its correlation with patients' survival. (a) RT-qPCR analysis of relative SNHG22 expression in HCC tissues and adjacent non-cancerous tissues. (b) relative SNHG22 expression in HCC cell lines and THLE-3 cell line was detected by RT-qPCR. (c) correlation between SNHG22 expression and HCC patients' survival. **p < 0.01.

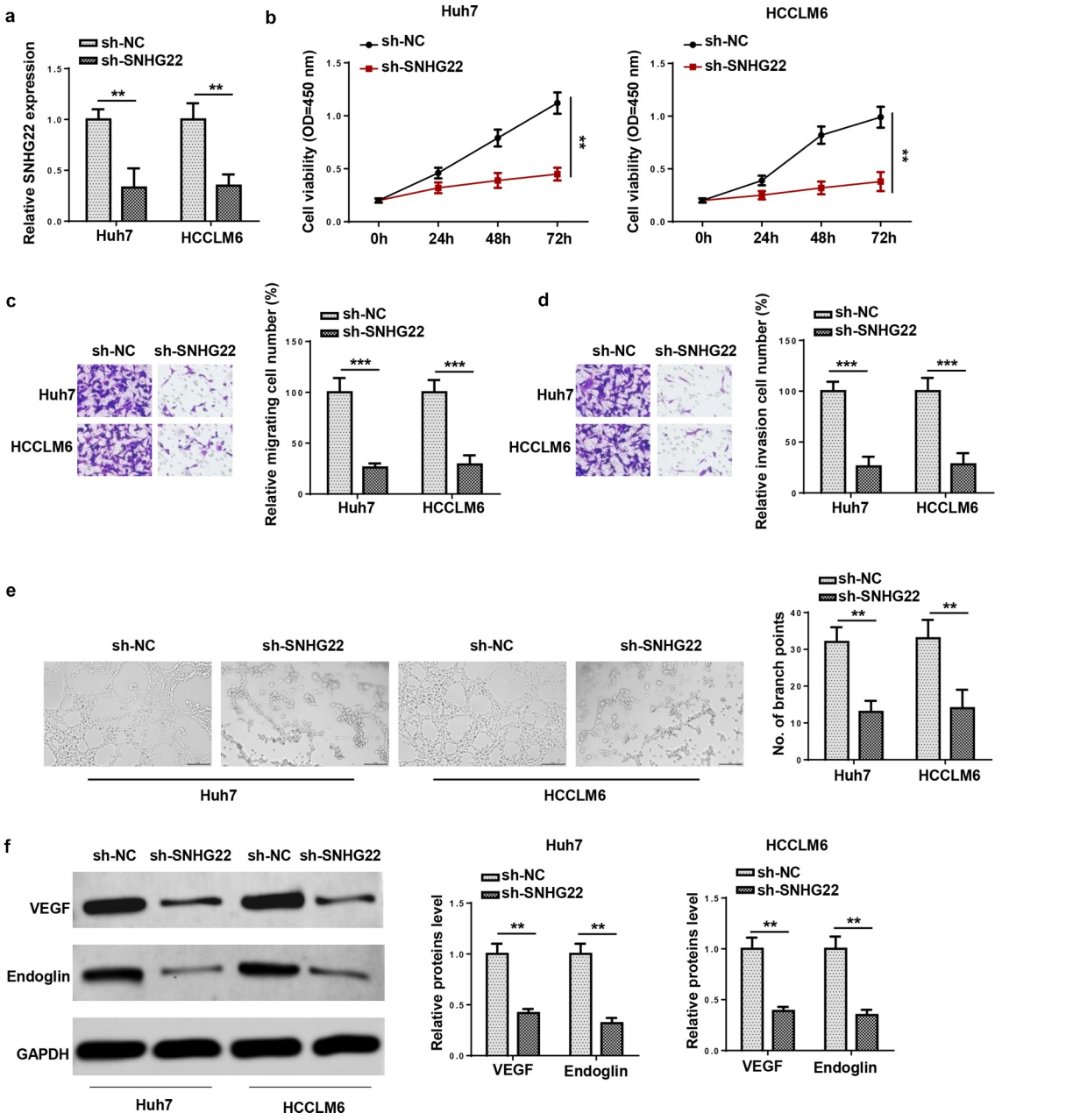

Figure 2. The effect of SNHG22 knockdown on cell proliferation, migration, invasion and angiogenesis. (a) the transfection efficiency of sh-SNHG22 in Huh7 and HCCLM6 cells was examined by RT-qPCR. (b) The proliferative ability of Huh7 and HCCLM6 cells transfected with sh-SNHG22 was determined by CCK-8 assay. (c and d) transwell assay was done for detecting the migration and invasion of transfected Huh7 and HCCLM6 cells. (e) The effect of SNHG22 knockdown on angiogenesis of Huh7 and HCCLM6 cells. (f) western blot showed the protein levels of VEGF and endoglin in HCC cells transfected with sh-NC and sh-SNHG22. **p < 0.01, ***p < 0.001.

(b)). Moreover, angiogenesis of HUVECs in sh-SNHG22 group was considerably inhibited compared with sh-NC group (Figure 2(e)). In addition, through examining the angiogenesis factors, it was found that the silencing of SNHG22 decreased the protein levels of VEGF and endoglin in HCC cell lines (Figure 2(f)). Collectively, HCC cell proliferation, invasion, and angiogenesis were restrained by SNHG22 deficiency in vitro.

SNHG22 deficiency suppressed tumor growth and angiogenesis *in vivo*

Subsequently, the biological function of SNHG22 in tumorigenesis *in vivo* was evaluated. Then, Huh7 cells with stable transfection of sh-SNHG22 or sh-NC were inoculated into nude mice via subcutaneous injection. As we observed, compared to the control group, tumors in sh-

SNHG22 group were smaller (Figure 3(a)). Moreover, SNHG22 deficiency significantly inhibited the volume of tumors (Figure 3(b)). In addition, the weight of tumors from SNHG22-knockdown group was lighter than those from sh-NC group (Figure 3(c)). According to immunohistochemical staining, we observed that the silencing of SNHG22 reduced Ki67 positivity compared to control group (Figure 3(d)). Furthermore, it was found that the tumor MVD was decreased after Huh7 cells transfected with sh-SNHG22 (Figure 3 (e)), indicating that SNHG22 knockdown inhibited angiogenesis. To be concluded, SNHG22 deficiency inhibited HCC tumor growth and angiogenesis in *vivo*.

SNHG22 decreased miR-16-5p expression through DNA promoter methylation

In this section, we studied the regulatory mechanism of SNHG22 in HCC. Considerable evidence has proved that lncRNAs often play a biological role in tumors via regulating or binding to microRNAs [34,35]. Previously, miR-16-5p has been reported in HCC. In our study, we further confirmed that miR-16-5p expression was down-regulated in HCC tissues and cells (Figure 4(a)). After the transfection of sh-SNHG22, we discovered that miR-16-5p expression was augmented (Figure 4(b)). Usually, lncRNAs modulate miRNAs via acting as 'miRNA sponges'. However, through analyzing the sequences between SNHG22 and miR-16-5p, we did not find their potential-binding sites. This indicated that SNHG22 might regulate miR-16-5p expression via other approaches. DNA methylation was a vital pattern to modulate gene expression [36]; thus, we conjectured that miR-16-5p expression suppressed by SNHG22 might be attributed to DNA promoter methylation. Furthermore, methylation-specific PCR was applied to testify the function of SNHG22 on miR-16-5p promoter methylation. The results implied that SNHG22 deficiency attenuated methylation of miR-16-5p (Figure 4(c)). To further verify the function of DNA methylation in regulating miR-16-5p expression, 5-aza-dC (DNA methylation inhibitor) was used. It indicated that 5-aza-dC treatment

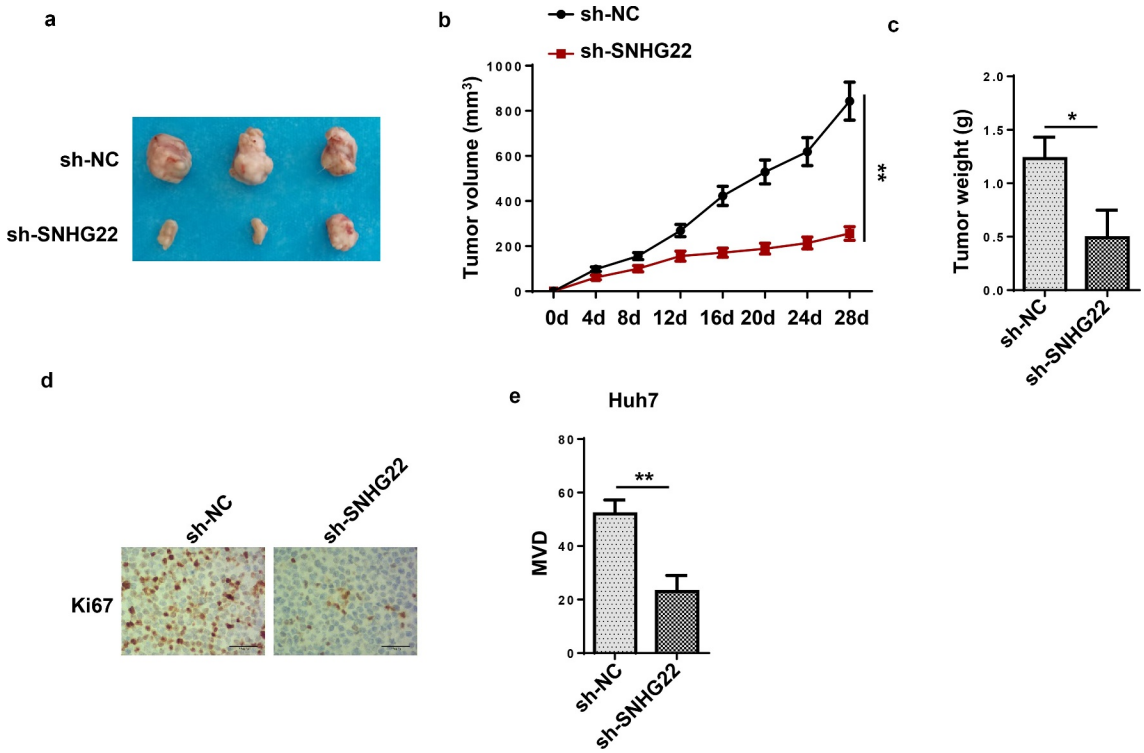

Figure 3. SNHG22 deficiency inhibited tumor growth and angiogenesis. (a) the tumors excised from the nude mice in sh-SNHG22 group or sh-NC group. (b) The volume of tumors in two groups was assessed. (c) the tumor weight from sh-SNHG22 group or sh-NC group. (d) IHC was used for detecting Ki67 expression in each group. (e) MVD was assessed in each group. *$p < 0.05$, **$p < 0.01$.

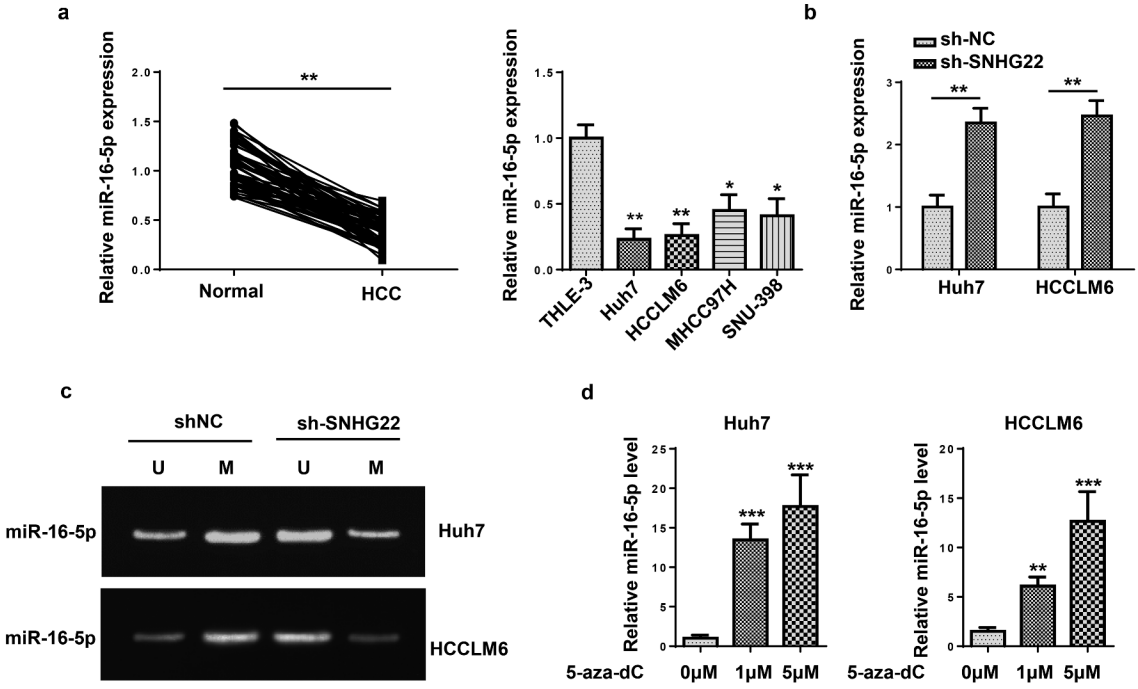

Figure 4. SNHG22 facilitated DNA promoter methylation to inhibit miR-16-5p. (a) The expression of miR-16-5p in HCC tissues and cells. (b) MiR-16-5p expression in HCC cells transfected with sh-SNHG22 or sh-NC. (c) Methylation-specific PCR was conducted to analyze the effect of SNHG22 on miR-16-5p methylation. (d) relative miR-16-5p expression after 5-aza-dC treatment in HCC cells. *p < 0.05, **p < 0.01, ***p < 0.001.

remarkably augmented miR-16-5p expression (Figure 4(d)). All data suggested that SNHG22 inhibited miR-16-5p expression by enhancing DNA promoter methylation.

SNHG22 recruited DNMT1 to promote miR-16-5p DNA promoter methylation via EZH2

Subsequently, we inspected the molecular mechanism by which SNHG22 facilitated miR-16-5p DNA promoter methylation. At first, the localization of SNHG22 in Huh7 and HCCLM6 cells was determined by the subcellular fractionation assay. Results revealed that SNHG22 showed higher expression in the nucleus than the cytoplasm in HCC cell lines (Figure 5(a)), indicating that SNHG22 might exert function and regulate gene expression via directly binding to RNA-binding proteins at the transcriptional level. It has been reported that DNA methyltransferases (DNMTs), including DNMT3A, DNMT3B, and DNMT1, played a crucial role in DNA promoter methylation [37]. To verify whether the DNMT regulated miR-16-5p DNA promoter methylation, sh-RNAs for DNMT1, DNMT3A, and DNMT3B were designed. RT-qPCR analysis manifested that only the deficiency of DNMT1 augmented miR-16-5p expression in Huh7 and HCCLM6 cells (Figure 5(b)). Therefore, we considered that DNMT1 might execute the DNA promoter methylation of miR-16-5p. In addition, we conducted ChIP assay to validate whether SNHG22 was necessary for DNMT1 interacting with miR-16-5p promoter region. The result showed that SNHG22 knockdown weakened the binding ability of DNMT1 to miR-16-5p promoter region (Figure 5(c)). Previous report has confirmed that lncRNAs locating in the nucleus could interact with EZH2 (a catalytic subunit of PRC2), and then modulate downstream target genes [38]. Importantly, it was also reported that lncRNAs could recruit DNMT1 to promote DNA promoter methylation of miRNAs via EZH2 [39]. According to RIP assay, we found that SNHG22 directly interacted with EZH2 in HCC cells (Figure 5(d)). In addition, Co-IP assay indicated that EZH2 could bind to DNMT1, and the binding was hampered when SNHG22 was knocked down (Figure 5(e)). All

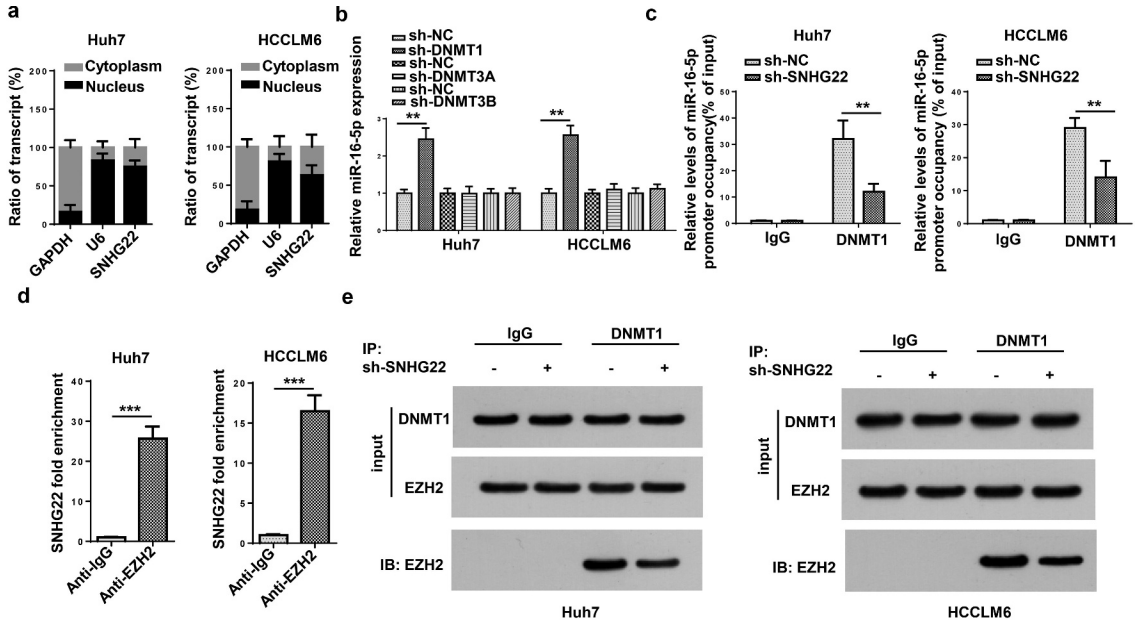

Figure 5. SNHG22 recruited DNMT1 via EZH2 to promote miR-16-5p DNA promoter methylation. (a) the subcellular location of SNHG22 in Huh7 and HCCLM6 cells. (b) RT-qPCR analysis for miR-16-5p expression in cells transfected with sh-DNMT1, sh-DNMT3A and sh-DNMT3B. (c) ChIP-RT-qPCR analysis testified the effect of SNHG22 silencing on the binding of DNMT1 to miR-16-5p promoter. (d) The interaction between SNHG22 and EZH2 was verified by RIP assay. (e) The binding capacity between DNMT1 and EZH2 was validated by Co-IP assay when SNHG22 was silenced. **p < 0.01, ***p < 0.001.

results validated that SNHG22 enhanced the methylation of miR-16-5p DNA promoter through recruiting DNMT1 via EZH2.

SNHG22 promoted cell proliferation, invasion, and angiogenesis via miR-16-5p

Finally, rescue assays were performed to verify whether SNHG22 modulated HCC cell growth and angiogenesis via miR-16-5p. Firstly, the transfection efficiency of miR-16-5p inhibitor was detected, and the result showed that miR-16-5p expression was decreased with miR-16-5p inhibitor transfection (Figure 6(a)). CCK-8 assay confirmed that miR-16-5p inhibition rescued the suppressed proliferative ability caused by SNHG22 knockdown (Figure 6(b)). Based on the transwell assay, it was discovered that the inhibited migration and invasion of sh-SNHG22-transfected HCC cells were restored by miR-16-5p inhibition (Figure 6(c-d)). Furthermore, the angiogenesis inhibited by SNHG22 silencing was also counteracted with miR-16-5p inhibitor transfection (Figure 6(e)). Collectively, SNHG22 executed its function in HCC cells via miR-16-5p.

Discussion

The majority of mammalian genomes have been identified to be transcribed into non-coding RNAs [40]. Recently, more and more attention has been focused on lncRNA due to its regulatory role in gene expression [7]. It was reported that lncRNAs are involved in the immune microenvironment [41], cellular metabolism [42], metastasis [43], proliferation [44], invasion [45], chemotherapy resistance [46], and other characteristics of tumor cells. In HCC, some lncRNAs have been inspected and confirmed effectively in tumorigenesis [12,13,47]. LncRNA SNHG22 has been previously reported as a tumor facilitator in triple-negative breast cancer [15], epithelial ovarian carcinoma [16], and papillary thyroid cancer [48]. In this research, we validated that SNHG22 was upregulated in HCC, and its upregulation was correlated with dissatisfactory prognosis. Moreover, SNHG22 silencing suppressed cell proliferation, migration, invasion, and angiogenesis in vitro. In addition, the tumor growth and angiogenesis in *vivo* were also restrained by SNHG22 knockdown. This indicated that SNHG22 might function as an oncogene in HCC.

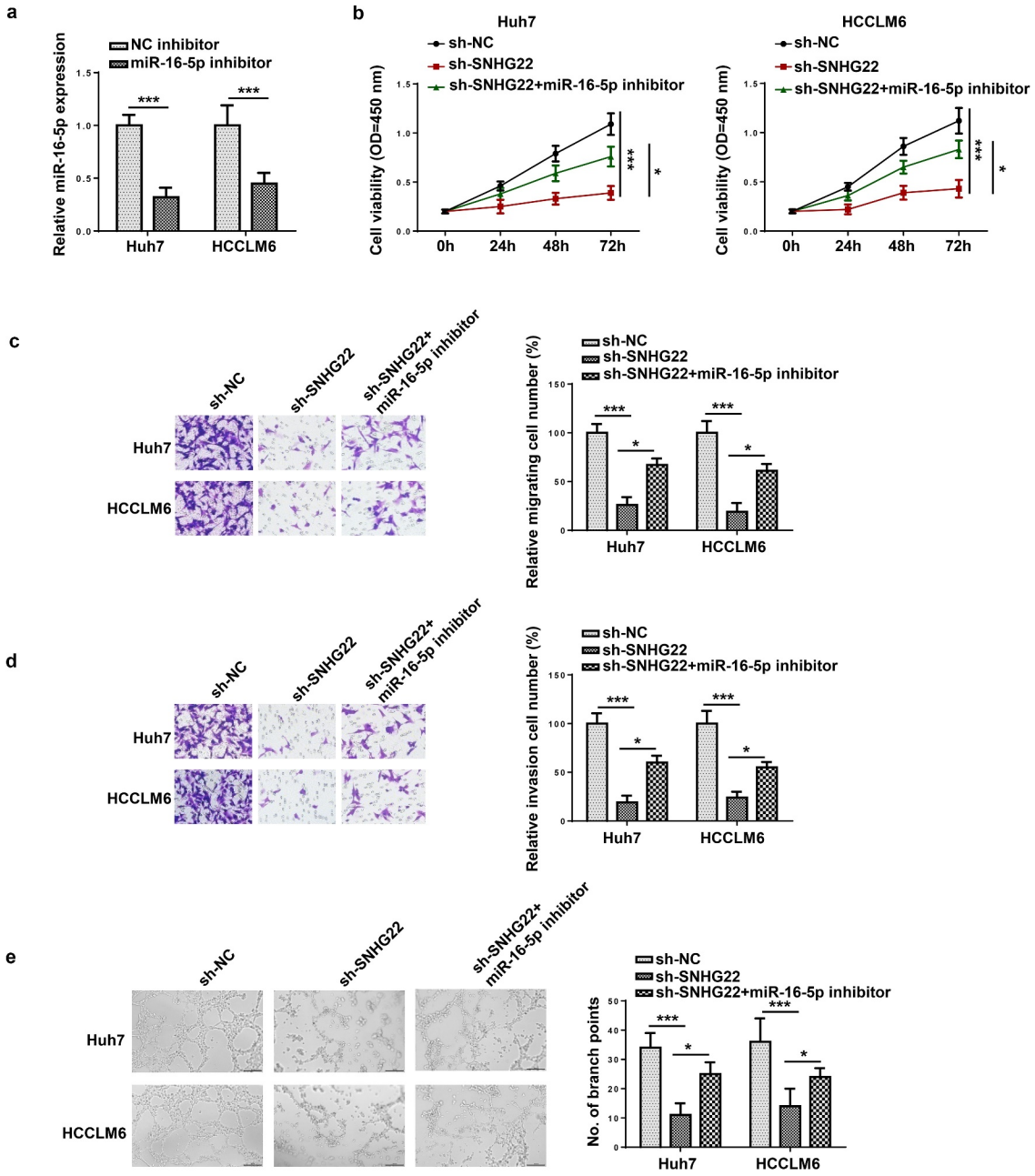

Figure 6. SNHG22 promoted cell proliferation, migration, invasion and angiogenesis via miR-16-5p. (a) RT-qPCR analysis was performed to test the transfection efficiency of miR-16-5p inhibitor. (b) The proliferation of each group was assessed by CCK-8 assay. (c and d) The migration and invasion of the transfected cells were evaluated by transwell assay. (e) Tube formation assay was conducted to verify angiogenesis in each group. *$p < 0.05$, ***$p < 0.001$.

As a pattern of epigenetic regulation, DNA methylation is important in tumor occurrence and development [36,49]. It has been proved that lncRNA could modulate miRNA via DNA methylation [20]. MiR-16-5p has been previously validated to exert inhibitory function in colorectal cancer [50], neuroblastoma [51], and HCC [52]. In our study, miR-16-5p was lowly expressed in HCC, and its expression or DNA promoter methylation was inhibited after SNHG22 knockdown. Additionally, the treatment of 5-aza-dC lifted miR-16-5p expression. These results proved that SNHG22 could regulate miR-16-5p expression via DNA methylation.

It was well-known that the catalysis of DNA methylation process could be attributed to DNMTs, such as DNMT1, DNMT3A, and DNMT3B [37]. Different DNMT exerts an effect

on methylation at different stages, and the DNMT also changes in diverse gene modulation [53]. In our research, we first confirmed SNHG22 was chiefly distributed in the nucleus. Then, we found that it was DNMT1 leading to the DNA promoter methylation of miR-16-5p by RT-qPCR. Importantly, SNHG22 increased the binding capacity between DNMT1 and miR-16-5p promoter. A former study supported that lncRNA POU3F3 could interact with EZH2 to recruit DNMTs [54]; thus, we performed RIP assay and confirmed the direct binding of EZH2 to SNHG22. More importantly, it was identified that SNHG22 could increase the binding of EZH2 to DNMT1. Collectively, SNHG22 could recruit DNMT1 to promote miR-16-5p DNA promoter methylation by EZH2.

Conclusion

Our study demonstrated that SNHG22 facilitated HCC progression by modulating miR-16-5p methylation. Our discovery revealed the underlying role and mechanism of SNHG22 and validated SNHG22/EZH2/DNMT1/miR-16-5p regulatory axis in HCC, which provided the valuable theoretical basis for HCC treatment. However, the present study has several limitations, which remain to be addressed. For example, other downstream effectors may also be important in SNHG22-regulated phenotypes of HCC, which needs to be further investigated.

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