ORIGINAL ARTICLE

SNHG16/miR-605-3p/TRA6/NF-κB feedback loop regulates hepatocellular carcinoma metastasis

Yi-Lin Hu1,2 | Ying Feng1,2 | Yu-Yan Chen1,2 | Jia-Zhou Liu1,2 | Yang Su3 | Peng Li1 | Hua Huang4 | Qin-Sheng Mao1 | Wan-Jiang Xue1,2

Abstract

The mechanism by which miR-605-3p regulates hepatocellular carcinoma (HCC) metastasis has not been clarified. In this study, we found that miR-605-3p was down-regulated in HCC and that low miR-605-3p expression was associated with tumour thrombus and tumour satellites. HCC patients with low miR-605-3p expression showed shorter overall survival and disease-free survival after surgery. Overexpression of miR-605-3p inhibited epithelial-mesenchymal transition and metastasis of HCC through NF-κB signalling by directly inhibiting expression of TRAF6, while silencing of miR-605-3p had the opposite effect. We also found that SNHG16 directly bound to miR-605-3p as a competing endogenous RNA. Mechanistically, high expression of SNHG16 promoted binding to miR-605-3p and inhibited its activity, which led to up-regulation of TRAF6 and sustained activation of the NF-κB pathway, which in turn promoted epithelial-mesenchymal transition and metastasis of HCC. TRAF6 increased SNHG16 promoter activity by activating NF-κB, thereby promoting the transcriptional expression of SNHG16 and forming a positive feedback loop that aggravated HCC malignancy. Our findings reveal a mechanism for the sustained activation of the SNHG16/miR-605-3p/TRA6/NF-κB feedback loop in HCC and provide a potential target for a new HCC treatment strategy.

KEYWORDS

feedback loop, hepatocellular carcinoma, metastasis, miR-605-3p, NF-κB signalling

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide, with nearly half of all cases occurring in China. The recurrence and metastasis rates of HCC after surgery remain high, and the prognosis of HCC patients is poor. Epithelial-mesenchymal transition (EMT) is an important cause of HCC metastasis. However, its underlying mechanism remains unclear. Therefore, exploring the molecular mechanism of EMT in HCC is not only critical for the further understanding of metastasis in HCC, but...
may also provide clues towards identifying early diagnostic markers of HCC and new therapeutic targets.

Non-coding RNAs are a class of RNAs that lack protein-coding ability and are involved in regulating several cellular processes, including malignant biological activity in cancers. These molecules are classified as short non-coding RNAs or long non-coding RNAs (lncRNAs) according to their length. MicroRNAs (miRNAs) are a class of short non-coding RNAs of 21-23 bp in length that mediate RNA-induced silencing complex (RISC) formation at the 3′-untranslated region (UTR) of target genes, which results in target gene degradation or translational inhibition. Previous studies have shown that miRNAs are involved in the regulation of cellular processes such as organ formation, fat metabolism, cell proliferation and apoptosis. Furthermore, studies have shown that abnormal miRNA regulation may be a critical factor leading to HCC metastasis.

LncRNAs are non-coding RNAs of more than 200 bp in length that regulate chromatin modification, transcription, miRNAs, mRNA stability, protein function and other important regulatory functions. LncRNAs exert important functions as critical regulators of various cell processes, and their abnormal expression may play an important role in cancer metastasis.

Competitive endogenous RNAs (ceRNAs) represent a novel gene expression regulatory mechanism for lncRNA-miRNA-mRNA interactions. Recent studies showed that ceRNAs are involved in regulating various cellular activities such as HCC metastasis. LncRNAs act as miRNA sponges and competitively bind to common miRNAs through the miRNA response element, which attenuates the miRNA-mediated inhibition of target genes and increases target gene expression. The target gene in turn regulates the progression of cancer metastasis. miRNAs are the core RNA molecules in the ceRNA hypothesis.

miR-605-3p is a recently discovered miRNA with tumour suppressor functions. Overexpression of miR-605-3p inhibits the migration and invasion of bladder cancer and glioma cells. However, its expression and function in HCC have not been reported.

In this study, we revealed a tumour-suppressive function of miR-605-3p in human HCC for the first time. Mechanistically, miR-605-3p targets TRAF6, a signal transducer in the NF-κB signalling pathway that has a crucial role in activating NF-κB signalling to suppress TRAF6 expression and thereby repress NF-κB signalling. We further found that SNHG16 is overexpressed in HCC cells and can directly bind to and affect miR-605-3p function, which leads to up-regulation of TRAF6 and continuous NF-κB activation in HCC. In turn, TRAF6 up-regulates SNHG16 expression via NF-κB/p65.

2 | MATERIALS AND METHODS

2.1 Cell lines, cell culture and reagents

HCCLM3, MHCC97L and MHCC-97H cell lines were gifts from the Liver Cancer Institute, ZhongShan Hospital. A normal hepatocyte cell line (L02) and two HCC cell lines (Hep3B and HepG2) were purchased from GeneChem. All cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% foetal bovine serum with 100 U/mL penicillin and 100 mg/mL streptomycin in a humidified incubator at 37°C containing 5% CO₂. SN-50 was purchased from MedChem Express.

2.2 Patients and tissue samples

All HCC tissues and matched adjacent normal tissues were collected from the Affiliated Hospital of Nantong University. A total of 78 HCC tumour samples and matched adjacent normal tissues were obtained between 2004 and 2009 at the Department of General Surgery, and a panel of 16 fresh HCC cancer tissues and adjacent normal tissues, including eight metastasis-free tissues and eight intrahepatic metastasis tissues, were obtained between 2012 and 2018. All patients with clear HCC pathology had never received neo-adjuvant chemotherapy, radiation therapy or immunotherapy before surgery. The demographic and clinical characteristics of the HCC patients are shown in Table 1. Follow-up was completed by August 2015. Approval was obtained from the Human Research Ethics Committee of Nantong University Affiliated Hospital, and written informed consent was obtained from each patient.

2.3 Online bioinformatics analysis

Putative miR-605-3p target genes were predicted by TargetScan (http://www.targetscan.org/vert_72/). Putative lncRNAs targeting miR-605-3p were predicted by Starbase (http://starbase.sysu.edu.cn/). Putative transcription factors that regulate SNHG16 expression were predicted by PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), JASPAR (jaspar.genereg.net/) and LASAGNA (https://biogrid-lasagna.engr.unc.edu/lasagna_search/). The GEPIA database (http://geopia2.cancer-pku.cn/) was used to analyse the expression correlation between SNHG16 and RELA (NF-κB/p65), SNHG16 expression and the prognostic significance of SNHG16 expression in the liver hepatocellular carcinoma data set of TCGA database.

2.4 Chromatin immunoprecipitation (ChIP) assays

Cells were cross-linked with 1% formaldehyde and quenched in glycin solution. ChIP assays were performed using a Pierce Magnetic ChIP Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer’s protocol. Anti-p65 antibody and normal IgG (MultiSciences) were used for immunoprecipitation. ChIP-enriched DNA samples were analysed by qRT-PCR to quantify the putative p65-binding sites in the SNHG16 promoter region. The data are shown as relative enrichment normalized to control IgG. Primer sequences for ChIP assays were as follows: forward, 5′-CCTGGTAAGTGCTATGAAGT-3′; reverse, 5′-TCTATCCCTGCAAACATAGT-3′.
For NF-κB luciferase assays, $3 \times 10^4$ HCC cells/well were seeded in 48-well plates and cultured for 24 hours. A pNF-κB-luciferase plasmid (GeneChem), control luciferase plasmid, pRL-TK Renilla plasmid (GeneChem) and miR-605-3p agomir or miR-605-3p agomir control were cotransfected into HCC cells using Lipofectamine 3000 (Invitrogen). After 48 hours, firefly and Renilla luciferase activities were measured with a Dual Luciferase Reporter Assay Kit (Beyotime).
To observe the interactions between miR-605-3p and TRAF6, the 3′-UTR of TRAF6 and the 3′-UTR containing a mutated putative binding site were cloned into the pGL3 luciferase reporter plasmid (GeneChem). TRAF6-WT or TRAF6-MUT was cotransfected with miR-605-3p agomir or miR-605-3p agomir control. To observe the interactions between SNHG16 and miR-605-3p, SNHG16 and the mutated putative binding site of miR-605-3p in SNHG16 were cloned into the pGL3 luciferase reporter plasmid (GeneChem). SNHG16-WT or SNHG16-MUT was cotransfected with miR-605-3p agomir or miR-605-3p agomir control. At 48 hours post-transfection, a Dual Luciferase Assay (Beyotime) was used to determine the luciferase reporter activities according to the manufacturer’s instructions.

Putative binding sites of transcription factors were analysed using the JASPAR, PROMO and LASAGNA databases (assembly Grch38/hg38). The predictive SNHG16 promoter region was chr17:76555764-76557863. The promoter region was chr17:76555900-76555909, which included the binding site for NF-κB/p65 (GGGAAATTCC). The wild-type SNHG16 promoter and a promoter with mutated NF-κB-binding sites were constructed by GeneChem. SNHG16-WT or SNHG16-MUT was cotransfected with pcDNA3.1 vector or pcDNA3.1 NF-κB/p65 (GeneChem). A dual-luciferase reporter assay was conducted to measure the functional NF-κB/p65 binding site in the SNHG16 promoter. Firefly luciferase activity was normalized to Renilla luciferase activity and presented as relative luciferase activity.

**FIGURE 1** Low expression of miR-605-3p is related to poor prognosis of hepatocellular carcinoma (HCC) patients. A, miR-605-3p expression was detected using real-time qPCR in 78 HCC tissues and matched adjacent normal tissues. miR-605-3p expression was normalized to U6. B, Relative expression of miR-605-3p was detected using real-time qPCR in 16 HCC tissues and matched adjacent normal tissues. C, Relative expression of miR-605-3p was detected using real-time qPCR in 16 HCC tissues with or without intrahepatic metastasis. D, Typical image of miR-605-3p expression detected by fluorescence in situ hybridization in 16 HCC tissues with or without intrahepatic metastasis and their adjacent normal tissues (scale bar, 25 μm). E and F, Correlation between miR-605-3p expression and overall survival (E) and disease-free survival (F) by Kaplan-Meier analysis of HCC patients with high or low miR-605-3p expression. *P < .05, ***P < .001
The details for RNA extraction, qRT-PCR, RNA fluorescence in situ hybridization, subcellular fractionation, Western blotting, immunohistochemistry, cell transfection, lentivirus production and transduction, wound healing assay, Matrigel invasion assay, immunofluorescence, RNA immunoprecipitation assays, in vivo animal experiments and statistical analyses are described in the Supplementary Materials.

### RESULTS

#### 3.1 miR-605-3p is down-regulated in HCC and is associated with poor prognosis

To investigate the role of miR-605-3p in HCC, we first detected the expression levels of miR-605-3p in 78 HCC and paired adjacent normal tissues. The results showed that miR-605-3p expression was down-regulated in HCC tissues (Figure 1A). Analysis of the clinicopathological characteristics showed that low miR-605-3p expression was positively associated with tumour thrombus and tumour satellites in the 78 HCC patients (Table 1). qRT-PCR (Figure 1B, C) and fluorescence in situ hybridization (Figure 1D) data indicated that the relative expression of miR-605-3p was significantly lower in the intrahepatic metastasis group compared with the intrahepatic metastasis-free metastasis group. Kaplan-Meier analysis showed that HCC patients with low miR-605-3p expression had worse overall survival (OS) and disease-free survival (DFS) rates than those with high miR-605-3p expression (Figure 1E, F). Multivariate analysis also showed that low expression of miR-605-3p was an independent predictor of OS and DFS (Table 2). Taken together, these results suggest that miR-605-3p may protect against HCC.

| Variable                            | OS Univariate analysis | OS Multivariable analysis | DFS Univariate analysis | DFS Multivariable analysis |
|-------------------------------------|------------------------|---------------------------|-------------------------|---------------------------|
|                                    | P>|z|                   | HR(95%CI)                   | P>|z|                        | HR(95%CI)                   |
| miR-605-3p expression                |                        |                           |                          |                           |
| Low (n = 39) vs. high (n = 39)       | .001*                  | .002*                     | .002*                    | .005*                     |
|                                    |                        | 0.383 (0.210-0.702)       | 0.452 (0.260-0.783)       |
| Gender                             |                        |                           |                          |                           |
| Male (n = 60) vs. female (n = 18)   | .131                   |                           | .205                     |                           |
| Age (years)                        |                        |                           |                          |                           |
| ≤54 (n = 41) vs. >54 (n = 37)       | .833                   |                           | .537                     |                           |
| Grade of differentiation            |                        |                           |                          |                           |
| Low (n = 39) vs. middle-high (n = 39)| .101                   |                           | .148                     |                           |
| Tumour diameter (cm)                |                        |                           |                          |                           |
| ≤5 (n = 43) vs. >5 (n = 35)         | .200                   |                           | .194                     |                           |
| Liver function (Child-Pugh stage)   |                        |                           |                          |                           |
| A (n = 63) vs. B or C (n = 15)      | .182                   |                           | .507                     |                           |
| Hepatocirrhosis                    |                        |                           |                          |                           |
| Absent (n = 26) vs. present (n = 52)| .425                   |                           | .176                     |                           |
| Hepatitis B virus                   |                        |                           |                          |                           |
| Absent (n = 38) vs. present (n = 40)| .0177                  |                           | .553                     |                           |
| Tumour thrombus                     |                        |                           |                          |                           |
| Absent (n = 66) vs. present (n = 12)| .011*                  |                           | .008*                    |                           |
| AFP (ng/ml)                         |                        |                           |                          |                           |
| ≤20 (n = 42) vs. >20 (n = 36)       | .060                   |                           | .107                     |                           |
| BCLC stage                          |                        |                           |                          |                           |
| I (n = 57) vs. II, III, or IV (n = 21)| .151                   |                           | .763                     |                           |
| Envelope                            |                        |                           |                          |                           |
| Absent (n = 46) vs. present (n = 32)| .189                   |                           | .168                     |                           |
| Tumour satellite                    |                        |                           |                          |                           |
| Absent (n = 62) vs. present (n = 16)| .068                   |                           | .069                     |                           |

*P < .05.
3.2 | miR-605-3p suppresses HCC cell metastasis in vitro and in vivo

qRT-PCR analyses showed that miR-605-3p expression was highest in HepG2 cells and lowest in HCCLM3 cells among the five HCC cell lines examined in this study (Figure S1A). Therefore, HCCLM3 and HepG2 cells were chosen for subsequent experiments. We confirmed the efficiency of overexpression or silencing of miR-605-3p in both HCC cell lines (Figure S1B). Wound healing and Matrigel invasion assays showed that overexpression of miR-605-3p specifically suppressed HCCLM3 cell migration and invasion (Figure 2A, B), while miR-605-3p silencing led to increased HepG2 cell migration and invasion (Figure 2C, D).

We further investigated whether miR-605-3p expression regulated the metastatic ability of HCC cells in vivo. We found that the number and size of metastatic colonies on the lung surface of mice were largely decreased in the HCCLM3/miR-605-3p group (Figure 2E). We also detected metastatic colonies in the HepG2/anti-miR-605-3p group, while no metastatic colonies were found in the HepG2/anti-miR-NC group (Figure 2F). These results indicated that miR-605-3p inhibited the metastatic ability of HCC cells in vitro and in vivo.

3.3 | miR-605-3p inhibits EMT in HCC

EMT is a critical process involved in cancer metastasis. To investigate whether miR-605-3p regulates EMT in HCC cells, immunofluorescence and Western blotting assays were conducted. The results showed that up-regulation of miR-605-3p in HCCLM3 cells increased E-cadherin expression and decreased vimentin expression (Figure 3A, C). Conversely, down-regulation of miR-605-3p in HepG2 cells decreased E-cadherin expression and increased vimentin expression (Figure 3B, D).

3.4 | miR-605-3p inhibits NF-κB activation in HCC

NF-κB is an important pathway that regulates EMT and metastasis in HCC. Thus, we examined whether miR-605-3p regulates activation of the NF-κB pathway. Luciferase reporter assays indicated that NF-κB-induced activity was reduced in miR-605-3p-overexpressing cells (Figure 3E) but increased in miR-605-3p-silenced cells (Figure 3F). Immunofluorescence assays showed that NF-κB/p65 nuclear expression was decreased

**FIGURE 2** miR-605-3p inhibits HCC cell migration and invasion in vitro and in vivo. A–D, The effects of miR-605-3p overexpression in HCCLM3 cells (A, B) and miR-605-3p silencing in HepG2 cells (C, D) on migration and invasion were analysed by wound healing and Matrigel transwell assays. (scale bar, 100 μm) E and F, miR-605-3p inhibited HCC cell metastasis in vivo. (Left) Representative bright-field images of lungs. (Right) Haematoxylin and eosin (H&E) staining of lung serial sections. Arrows indicate lung lesions. (Panel) Numbers of nodules on the lungs of mice (n = 5 per group) at 6 weeks after tail vein injection of HCCLM3/miR-NC or HCCLM3/miR-605-3p cells (scale bar, 25 μm) (E) and HepG2/anti-miR-NC or HepG2/anti-miR-605-3p cells (F). *P < .05, **P < .01. NS: no significance.
in miR-605-3p-overexpressing cells (Figure 3G) but increased in miR-605-3p-silenced cells (Figure 3J). Western blotting analyses showed that up-regulation of miR-605-3p expression reduced the phosphorylation of IKK-β and IκBα (Figure 3H) but increased their phosphorylation in miR-605-3p-silenced cells (Figure 3J). Additionally, miR-605-3p up-regulation decreased nuclear p65 expression (Figure 3H), while miR-605-3p down-regulation increased nuclear p65 expression (Figure 3J).

**FIGURE 3** miR-605-3p inhibits epithelial-mesenchymal transition (EMT) in HCC cells and attenuates NF-κB signalling activation. A–D, Immunofluorescence and Western blotting were conducted to examine the effects of miR-605-3p overexpression on EMT in HCCLM3 cells (scale bar, 25 μm) (A, C) and miR-605-3p silencing on EMT in HepG2 cells (scale bar, 25 μm) (B, D). E–J, NF-κB luciferase reporter activity, immunofluorescence and Western blotting analyses were performed to detect the effects of miR-605-3p overexpression on NF-κB signalling activation in HCCLM3 cells (scale bar, 25 μm) (E, G, H) and miR-605-3p silencing on NF-κB signalling activation in HepG2 cells (scale bar, 25 μm) (F, I, J). *P < .05, **P < .01.
3.5 | miR-605-3p targets TRAF6 in HCC

We next predicted the target genes of miR-605-3p by TargetScan. Among the identified genes, TRAF6 was identified as a gene of interest, as previous studies showed that TRAF6 regulates the activation of NF-xB signalling and affects NF-xB-mediated EMT in carcinogenesis and cancer development. Therefore, we first investigated whether miR-605-3p regulates TRAF6 through binding to the 3′-UTR of TRAF6. WT-TRAF6 or MUT-TRAF6 3′-UTR luciferase reporter vectors (Figure 4A) along with miR-605-3p or miR-NC were cotransfected into HCCLM3 and HepG2 cells. Activity of the WT-TRAF6 3′-UTR vector was significantly downregulated in the miR-605-3p group compared with the miR-NC group in HCCLM3 and HepG2 cells (Figure 4B, C). Conversely, miR-605-3p had no impact on the MUT-TRAF6 3′-UTR reporter. Furthermore, qPCR and Western blot assays showed that miR-605-3p overexpression downregulated TRAF6 expression, while miR-605-3p knockdown upregulated TRAF6 expression in HCCLM3 (Figure 4D, F) and HepG2 cells (Figure 4E, G).

We next investigated whether TRAF6 is involved in the effects of miR-605-3p on HCC cell migration. The suppressive effects of miR-605-3p overexpression on cell migration and invasion were partly abolished by TRAF6 overexpression in HCCLM3 cells (Figure 4H, J, L, N). Furthermore, silencing of TRAF6 blocked the effects of downregulated TRAF6 expression on the migration and invasion of miR-605-3p-silenced HepG2 cells (Figure 4I, K, M, O). We also found that exogenous expression of TRAF6 abolished miR-605-3p-induced inhibition of NF-xB-mediated EMT in HCCLM3 cells (Figure 4P, R), while silencing of TRAF6 blocked miR-605-3p-induced activation of NF-xB-mediated EMT in HepG2 cells (Figure 4Q, S). The efficiency of TRAF6 overexpression or silencing in HCC cell lines was confirmed (Figure 5C, D).

3.6 | Reciprocal negative regulation between miR-605-3p and SNHG16

Accumulating evidence has revealed that lncRNAs can function as ceRNAs for miRNAs. To determine whether any lncRNAs regulate TRAF6 expression through miR-605-3p, we searched for potential lncRNAs that bind to miR-605-3p using an online bioinformatic tool (Starbase). The small nucleolar RNA host gene (SNHG) family caught our attention. Among the predicted SNHG family members, SNHG16 expression was highest in HCC tissues and predominantly located in the cytoplasm of HCC cells (Figure S2A; Figure 5A, B). SNHG16 expression was upregulated in the 78 HCC tissues compared with adjacent non-tumour tissues (Figure 5C, D) as well as in HCC cell lines (Figure S2B) and in the liver HCC data set of TCGA database (Figure S2C). SNHG16 expression was also negatively correlated with miR-605-3p expression in the 78 HCC tissues (Figure 5E, F). High SNHG16 expression was not only positively associated with tumour diameter, tumour thrombus, presence of an envelope and tumour satellites in the 78 HCC patients (Table S1), but also served as an independent prognostic indicator for both OS and DFS (Figure S3A–D; Table S2).

To validate miR-605-3p as a target for SNHG16, dual-luciferase reporter assays were performed (Figure 5G). The results showed that cotransfection of WT-SNHG16 and miR-605-3p in HCCLM3 cells (Figure 5H) and MHCC-97H cells (Figure 5I) decreased luciferase activity compared with the control group. However, the luciferase activity in the MUT-SNHG16 group was not affected.

We further examined whether SNHG16 expression was regulated by ectopic miR-605-3p expression or inhibition. The results showed that up-regulation of miR-605-3p in HCCLM3 and MHCC-97H cells downregulated SNHG16 expression, while downregulation of miR-605-3p elevated SNHG16 expression (Figure 5J, 5K, Figure S2D, E). The expression of miR-605-3p was increased by SNHG16 silencing (Figure 5J, 5K, Figure S2D, E).

To examine whether SNHG16 could sponge miR-605-3p expression in an RISC-dependent manner, RNA immunoprecipitation assays were conducted. SNHG16 and miR-605-3p were more abundant in the AGO2 pellet compared with the IgG pellet (Figure 5N, 5O). These data indicated that SNHG16 may act as a ceRNA by sponging miR-605-3p.

3.7 | Down-regulation of SNHG16 suppresses HCC metastasis, EMT and NF-xB activation by interacting with miR-605-3p in vitro and in vivo

We found that SNHG16 knockdown inhibited EMT and NF-xB signalling activity in HCC cells (Figure S4A–R). When HCC cells were cotransfected with miR-605-3p and sh-SNHG16, the effects of SNHG16 silencing on HCC cell metastasis (Figure S5A–H), EMT and TRAF6 expression (Figure S5J, I) were partly abolished.
**FIGURE 5** SNHG16 directly binds to miR-605-3p. A and B, Cell fractionation and real-time qPCR were performed to detect SNHG16 cytoplasmic and nuclear expression levels in HCCLM3 and MHCC-97H cells. U6 was used as a nuclear control, and GAPDH was used as a cytoplasmic control. C and D, The relative expression of SNHG16 was detected by real-time qPCR in 78 HCC tissues and adjacent normal tissues. E and F, SNHG16 was negatively related to the expression of miR-605-3p. G, Predicted potential SNHG16 binding sites and mutated nucleotides in the potential binding sequence of miR-605-3p in SNHG16. H and I, Dual-luciferase reporter assays were performed to detect the effects of miR-605-3p overexpression on the luciferase activity of reporters driven by WT and MUT SNHG16 in HCCLM3 and MHCC-97H cells. J and K, Effects of SNHG16 silencing on miR-605-3p expression in HCCLM3 and MHCC-97H cells. L and M, Effects of miR-605-3p silencing on SNHG16 expression in HCCLM3 and MHCC-97H cells. N, O, RNA-binding protein immunoprecipitation assays with anti-AGO2 antibodies were performed in HCCLM3 and MHCC-97H cells transiently transfected with miR-605-3p. *P < .05, **P < .01. NS: no significance.
Additionally, the suppressive effect of SNHG16 silencing on NF-κB promoter luciferase reporter activity was partly reversed by miR-605-3p silencing (Figure 5K, L). These data showed that the effect of SNHG16 on promoting HCC cell metastasis was mediated by miR-605-3p.

We then investigated whether SNHG16 expression regulates the metastatic ability of HCC cells in vivo by interacting with miR-605-3p. HCCLM3/sh-NC, HCCLM3/sh-SNHG16, HCCLM3/sh-SNHG16/anti-miR-605-3p, MHCC-97H/sh-NC, MHCC-97H/sh-SNHG16 or MHCC-97H/sh-SNHG16/anti-miR-605-3p were injected into the tail vein of
nude mice. We found that SNHG16 silencing decreased the number and size of metastatic colonies from HCCLM3 cells (Figure 6A) and MHCC-97H cells (Figure 6B) on the lung surface of mice. However, the effect of SNHG16 silencing was partly abolished by miR-605-3p silencing.

A subcutaneously implanted tumour model in nude mice showed that SNHG16 silencing decreased both the weight and growth rate of mice compared with mice injected with control HCCLM3 or MHCC-97H cells (Figure S6A–E). The effects of SNHG16 silencing in HCCLM3 and MHCC-97H cells were partly reversed by miR-605-3p silencing. We examined E-cadherin, vimentin, TRAF6 and p65 expression in the subcutaneous tumours by IHC and found that SNHG16 silencing increased E-cadherin expression, decreased vimentin expression, decreased TRAF6 expression and decreased p65 nuclear signals and that these effects were partly abolished by miR-605-3p silencing (Figure 6C, D).

### 3.8 | SNHG16 and NF-κB form a positive feedback loop

We further examined whether any of the identified factors act upstream of SNHG16 in HCC. We analysed the promoter sequence of SNHG16 using the PROMO, JASPAR and LASAGNA databases and found that RELA (NF-κB/p65) was identified as a potential binding factor to the SNHG16 gene promoter in all three databases (Figure 7A). We also found a positive correlation between the expression of SNHG16 and NF-κB/p65 in HCC using the GEPIA database (Figure 7B; R = .26, P < .001). These findings suggest that the promoter region of SNHG16 may contain a binding motif for NF-κB/p65 (Figure 7C, D). SNHG16 expression was up-regulated in HCCLM3 and MHCC-97H cells in response to ectopic expression of NF-κB/p65 compared with the empty vector group (Figure 7E). As NF-κB is a downstream target of TRAF6, we examined whether TRAF6 overexpression significantly elevated SNHG16 expression (Figure 7F). However, treatment with the NF-κB inhibitor SN-50 largely abolished the effect of TRAF6 on SNHG16 expression (Figure 7G). We next examined whether NF-κB/p65 interacted with promoter region of SNHG16 via the predicted binding site. ChIP assays demonstrated that NF-κB/p65 binds the SNHG16 promoter (Figure 7H). Dual-luciferase reporter assays showed that ectopic expression of NK-κB/p65 enhanced luciferase activity driven by the WT-SNHG16 promoter (Figure 7I). When the NF-κB-binding sequence in the SNHG16 promoter was mutated, luciferase expression was significantly decreased. These results indicate that NF-κB/p65 interacted with the SNHG16 promoter via the predicted binding site.

### 4 | DISCUSSION

In the present study, we revealed that the putative tumour suppressor miR-605-3p was frequently silenced in HCC cell lines and tissues. Overexpression of miR-605-3p inhibited HCC cell metastasis in vitro and in vivo via the NF-κB pathway. More importantly, low miR-605-3p expression was associated with malignant clinicopathological characteristics and poor survival outcome in HCC patients. Thus, examination of miR-605-3p expression by qRT-PCR could be used as an additional tool for distinguishing HCC patients at high risk of metastasis and may provide useful information for clinicians to optimize individual therapy management for HCC.

We also determined the underlying mechanism involved in miR-605-3p regulation of the NF-κB pathway. miRNAs play multiple cellular roles by targeting different genes. Bioinformatics analyses confirmed that TRAF6, a signal transducer for inflammatory NF-κB signalling activation, is a direct target of miR-605-3p that affects HCC metastatic malignancy. TRAF6 acts as an E3 ubiquitin ligase that activates IκB kinase (IKK), which leads to IκB degradation and p65 nuclear translocation, which suggests an important role for TRAF6 in NF-κB activation. Additionally, studies have shown that TRAF6 has an important role in the development of various cancers. Activation of signalling downstream of TRAF6, including NF-κB, has a significant role in TRAF6-mediated tumorigenesis.

In the present study, we showed that TRAF6 participates in the abnormal expression of miR-605-3p and induces metastasis and EMT promotion in HCC. Co-expression of miR-605-3p and TRAF6 largely reversed the tumour-suppressive effects of miR-605-3p up-regulation alone, which suggests that miR-605-3p inhibits the malignant behaviour of HCC cells by suppressing TRAF6 expression. Our findings also showed that miR-605-3p inhibited HCC metastasis by inhibiting NF-κB activation and that this effect of miR-605-3p was dependent on TRAF6, which indicates that the miR-605-3p/TRAF6/NF-κB axis functions in the regulation of HCC metastasis.

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**FIGURE 7** TRAF6 promotes SNHG16 expression through NF-κB signalling. A, Only RELA (NF-κB/p65) was identified to possibly bind to the SNHG16 gene promoter through the intersection of the JASPAR, PROMO and LASAGNA databases. B, Relative expression of SNHG16 and NF-κB/p65 in HCC was determined using the GEPIA database. C, JASPAR logo of NF-κB/p65-binding sites in the SNHG16 promoter. E, Real-time qPCR was performed to determine the expression of SNHG16 after NF-κB/p65 overexpression in HCCLM3 and MHCC-97H cells. F, Real-time qPCR was performed to determine the expression of SNHG16 after TRAF6 overexpression in HCCLM3 and MHCC-97H cells. G, Real-time qPCR was performed to determine the expression of SNHG16 after TRAF6 overexpression in HCCLM3 and MHCC-97H cells treated with or without SN-50 (NF-κB signalling inhibitor). H, ChIP assays were performed to detect NF-κB/p65 on the SNHG16 promoter in HCCLM3 and MHCC-97H cells. I, Luciferase reporter assays were performed to confirm NF-κB activation of the SNHG16 promoter through the NF-κB/p65-binding sites in HCCLM3 and MHCC-97H cells. J, Schematic illustration showing the positive feedback loop. SNHG16 up-regulates TRAF6 expression by directly binding to miR-605-3p as a ceRNA. TRAF6 promotes SNHG16 expression by activating NF-κB signalling. *P < .05, **P < .01
The ceRNA hypothesis proposes that numerous non-coding RNAs may function as molecular sponges for miRNAs and thus functionally liberate RNA transcripts that are targeted by these miRNAs. Through bioinformatics analysis and PCR validation, SNHG16 was determined to likely function as a ceRNA with miR-605-3p and affects the expression of TRAF6. SNHG16 has been identified as an oncogene in many cancers. Up-regulation of SNHG16 predicts poor prognosis and induces sorafenib resistance in HCC. High SNHG16 not only contributes to the promotion of HCC proliferation through the miR-302a-3p/FGF19 axis, but also sponges miR-4500 and targets STAT3, and sponges miR-195 to aggravate the tumorigenesis and development of HCC. In line with these previous studies, our results suggest that SNHG16 may function as an oncogene in HCC by promoting cell migration and invasion. To ascertain whether there was direct binding between SNHG16 and miR-605-3p, we examined the subcellular localization of SNHG16 by nucleoplasm separation and found that miR-605-3p and SNHG16 were both mainly localized in the cytoplasm. Additionally, we conducted luciferase reporter assays and RNA-IP analyses. We verified that SNHG16 directly binds to miR-605-3p via a putative MRE and that the RISC was involved in this ceRNA regulatory network. Taken together, these results suggest that there is reciprocal repression between SNHG16 and miR-605-3p mediated by the RISC and that SNHG16 likely binds to other miRNAs as well as miR-605-3p.

One of the most interesting findings in this study was that NF-κB can also regulate the expression of SNHG16. While SNHG16 has been identified as an oncogene in many cancers including HCC, the biological functions of SNHG16 and its underlying mechanisms in HCC are not fully understood. Positive feedback regulation is common in the regulation of many biological functions, especially sustained activation of cancer-promoting signalling pathways. Multiple studies have shown that abnormal activation of the NF-κB pathway occurs in various cancers. However, the mechanism for its continued activation in HCC remains unclear. We found that SNHG16 acts as a ceRNA to compete for miR-605-3p, thereby protecting TRAF6 from miR-605-3p and ultimately activating the NF-κB pathway. Zhou et al found that SNHG16 expression was elevated in LPS-induced WI-38 cells, while Wang et al found that SNHG16 reversed the effects of LPS. Furthermore, SNHG16 is known as an NF-κB pathway activator. We speculated whether NF-κB, as a transcription factor, could promote SNHG16 expression by binding to its promoter region. Thus, we analysed the promoter sequence of SNHG16 using the PROMO algorithm and JASPAR. The results revealed the presence of a putative binding site for NF-κB within the SNHG16 promoter region. Moreover, we found a positive correlation between the expression of SNHG16 and p65 in HCC in the GEPIA database. Taken together with the ChIP and dual-luciferase reporter assays, these results demonstrated that activation of the NF-κB signalling pathway promotes SNHG16 expression.

In summary, the present study demonstrates the tumour-suppressive role of miR-605-3p in HCC metastasis for the first time and indicates the importance of the interactions between SNHG16, miR-605-3p, TRAF6 and the NF-κB pathway in the regulation of HCC cell malignancy. Decreased expression of SNHG16 promotes miR-605-3p expression, which down-regulates TRAF6 and NF-κB signalling and thereby inhibits a series of metastatic effects in HCC cells. In turn, activated NF-κB can up-regulate SNHG16 expression to form a positive feedback loop. Thus, targeting the SNHG16/miR-605-3p/TRA6/NF-κB loop may be a potential new therapeutic strategy to improve the treatment and survival of HCC patients (Figure 7).

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CONFLICT OF INTEREST
The authors confirm that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS
WJX and QSM conceived and designed the experiments. YLH, YF and YYC participated in the experiments and drafted the manuscript. PL and HH performed the statistical analyses. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT
The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

ORCID
Wan-Jiang Xue https://orcid.org/0000-0002-0137-7331

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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