European Review for Medical and Pharmacological Sciences 2020; 24: 5481-5492

SNHG3 silencing suppresses the malignant development of triple-negative breast cancer cells by regulating miRNA-326/integrin α5 axis and inactivating Vav2/Rac1 signaling pathway

P. WANG[1], G.-Z. LIU[1], J.-F. WANG[2], Y.-Y. DU[2]

[1]Department 1 of Breast Surgery, [2]Department 2 of Breast Surgery; Linyi Cancer Hospital, Linyi, Shandong, China

Abstract. – OBJECTIVE: Long non-coding RNA small nucleolar RNA host gene 3 (SNHG3) has been shown to participate in several tumorigenesis. Triple-negative breast cancer (TNBC) is an aggressive type of breast cancer, which is the first leading cause of new cancer diagnoses in women globally. However, the role of SNHG3 remains little known in breast cancers, especially in TNBC.

MATERIALS AND METHODS: Expression of SNHG3, miRNA-326-5p (miR-326) and integrin α5 (ITGA5) was detected using Real Time-PCR and Western blotting. Cell viability, apoptosis, migration, and invasion were measured by methyl thiazolyl tetrazolium assay, flow cytometry, and transwell assays, respectively. Vav2/Rac1 signaling pathway was evaluated by Western blotting by analyzing Vav2 and Rac1 levels. The interaction among miR-326, SNHG3 and ITGA5 was confirmed by Dual-Luciferase reporter assay.

RESULTS: We found that the expression of SNHG3 and ITGA5 was upregulated and miR-326 was downregulated in TNBC tumors and cell lines (MDA-MB-231, BT-549, MDA-MB-468 and SUM159). Functionally, both SNHG3 silencing and miR-326 overexpression enhanced cell apoptosis, but depressed cell viability, migration and invasion in MDA-MB-231 and BT-549 cells, as well as inhibited Vav2 and Rac1 expression. Notably, miR-326 deletion could abolish the tumor-suppressive role of SNHG3 silencing; meanwhile, the similar anti-tumor effect of miR-326 overexpression was abrogated by ITGA5 restoration. Mechanically, SNHG3 silencing downregulated ITGA5 expression by functioning as a molecular "sponge" for miR-326.

CONCLUSIONS: Silencing of SNHG3 suppressed the malignant development of TNBC cells, at least partially, through miR-326/ITGA5 axis and inhibiting Vav2/Rac1 signaling pathway.

Key Words:
SNHG3, TNBC, MiR-326, Integrin α5 (ITGA5), Vav2/Rac1 signaling pathway.

Introduction

Triple-negative breast cancer (TNBC) is a subtype of breast cancer with high aggressiveness. TNBC is featured with estrogen receptor negative (ER-), progesterone receptor negative (PR-) and human epidermal growth factor receptor 2 negative (HER2-), which resulted in useless of conventional targeted therapy for TNBC patients[1]. Generally, TNBC accounts for almost 15% of all types of breast cancers and has a worse prognosis than other breast cancers[2]. Due to its high metastasis and early recurrence, chemotherapy remains an effective adjuvant therapy for TNBC patients[3]. Therefore, it is of great significance to search for available therapeutic targets for TNBC. Even though many molecular changes have been found in TNBC, the molecular biological mechanism remains largely unclear to date.

The abnormal expression of non-coding RNAs has been comprehensively analyzed in TNBC, including long non-coding RNAs (lncRNAs) and short non-coding RNAs (microRNAs, miRNAs)[4]. Moreover, plenty of lncRNAs and miRNAs have been identified to play crucial roles in the cell fate of TNBC, thus serving as new biomarkers and targets[5,6]. LncRNA small nucleolar RNA host gene 3 (SNHG3) is a new member of SNHGs, which has acknowledged roles in the pathogenesis of breast cancers[7]. SNHG3 has been identified to correlate with malignant status and poor prognosis in sev-

Corresponding Author: Yangyang Du, MD; e-mail: btklct@163.com

eral types of cancers[8-10]. In breast cancer, SNHG3 was declared to be highly expressed in TNBC than other subtypes of breast cancer[7]. However, its cellular functions and molecular mechanisms are poorly understood.

It is hypothesized that SNHG3 may regulate message RNA (mRNA) expression or function as a competing endogenous RNA (ceRNA) for miRNAs[11]. MiR-326 was first identified in neurons[12]. After that, it has been suggested that miR-326 can involve in the development of malignant tumors[13]. In breast cancer, cell development and drug resistance have been modulated by aberrant expression of miR-326 as well[14,15]. Whereas, the comprehension of its underlying mechanism is gloomy in breast cancer.

Integrin α5 (ITGA5) is a subunit of integrin and takes an essential part in stemness and metastasis in TNBC through Vav2/Rac1 signaling pathway[16], which is an emerging pathway participating in several tumorigenicity including breast cancer[16-18]. In this study, we aimed to figure out the dysregulation of SNHG3 and miR-326 in TNBC tumors and cell lines, and to confirm the roles of them in the malignant development of TNBC cells *in vitro*, as well as the hidden molecular mechanism.

Materials and Methods

Clinical Tissue Collection

This study consisted of 30 women with TNBC tumors (immunohistochemically: ERα-, PR- and HER2-) without any evidence of distant metastasis at the time of surgery at the Linyi Cancer Hospital. All patients gave informed consent and the protocol was approved by the Clinical Research Ethics Committee of the Linyi Cancer Hospital. None of the patients received any anti-cancer therapy before mastectomy procedure. The tissue samples, including TNBC tumors and the matched adjacent normal tissues, were collected during the operation and transferred in liquid nitrogen until use.

Cell Culture

Human normal mammary epithelial cell line MCF-10A (CRL-10137) and TNBC cell lines MDA-MB-231 (HTB-26), BT-549 (HTB-122), MDA-MB-468 (HTB-132) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA), and SUM159 (SIDM01452) cells were obtained from Cell Model Passports (Hinxton, Cambridgeshire, UK). BT-549 cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; HyClone, South Logan, UT, USA) and the other cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; HyClone). All the cell cultures were supplemented with 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) and 50 μg/mL gentamicin, and the cells were maintained in 5% CO_2 at 37°C.

Real Time-PCR

Total RNA was isolated by mirVANA qRT-PCR miRNA Detection Kit (Ambion, Austin, TX, USA). After determining the RNA concentration, the reverse transcription was performed using HiFiScript cDNA Synthesis Kit (CWBIO, Beijing, China), and miRNA qRT-PCR Starter kit (RIOBOBIO, Guangzhou, China) was used for reverse transcription of miRNA. Then, SYBR Green Master Mix (CWBIO) was utilized to amplify complementary DNA (cDNA) on the default program of 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The expression of gene was calculated with $2^{-\Delta\Delta CT}$ method and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; for SNHG3 and ITGA5) and U6 small nuclear RNA (U6; for miR-326). The primers used were listed as follows: SNHG3, 5'-GCCCAGGAGTGACCTATACTCAAA-3' (sense) and 5'-GGTATCCACGTTGGAATGC TCA-3' (anti-sense)[19]; miR-326, 5'-ACTGTCC TTCCCTCTGGGC-3' (sense) and 5'-AATG-GTTGTTCTCCACTCTCTCTC-3' (anti-sense); ITGA5, 5'-GTCGGGGGCTTCA ACTTAGAC-3' (sense) and 5'-CCTGGCTGGCTGGTATT-AGC-3' (anti-sense); GAPDH: 5'-TATGATGA-TATCAAGAGG GTAGT-3' (sense) and 5'-TG-TATCC AAACTCATTGTCATAC-3' (anti-sense); U6, 5'-CTCGCTTCGGCAGCACA-3' (sense) and 5'-AACGCTTCACGAATTTGCGT-3' (anti-sense).

Cell Transfection

Small interfering RNAs against SNHG3 (si-SNHG3), miR-326 mimics and 2'-O-methyl-modified miR-326 inhibitors (anti-miR-326) were purchased from RIBOBIO (Guangzhou, China), as well as their negative controls. For overexpression, the full length of coding domain sequence of human ITGA5 (NM_002205) was inserted into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). In the transfection procedures, 50 pmol/mL of RNA oligonucleotides and 2 μg of vectors were transfected into BT-549 and

MDA-MB-231 cells using Lipofectamine RNAi MAX (Invitrogen, Carlsbad, CA, USA). The sequence of siRNAs was as follows: si-SNHG3#1, 5'-GCCAAGAATCTGAATGGTA-3'; and si-SNHG3#2, 5'-GGTGACATCAGCCAAGAAT-3'.

Methyl Thiazolyl Tetrazolium (MTT) Assay

Transfected BT-549 and MDA-MB-231 cells were transferred into 96-well plates (2 000 cells/well). Briefly, 20 μL MTT (5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) was added in fresh culture medium without FBS after transfection for 0 d, 1 d, 2 d, and 3 d. The cells were incubated with MTT for 4 h, and then, the absorbance at 490 nm was measured and the result of each group was the average of 3 independent treatment. Cell growth curve was drawn.

Flow Cytometry

BT-549 and MDA-MB-231 cells were transfected for 24 h. The cells and the supernatant were collected and washed with ice-cold phosphate buffer solution (PBS). After centrifugation, cell pellets were re-suspended in 500 μL of staining buffer solution and incubated with 5 μL of Annexin V-fluorescein isothiocyanate (FITC; Keygen Biotech, Nanjing, China) and 5 μL of propidium iodide (PI; Keygen Biotech, Nanjing, China) for 15 min in the dark. The fluorescent intensities were analyzed using Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) equipped with FlowJo 10 software (BD Biosciences, Franklin Lakes, NJ, USA). The apoptotic rate was recorded as percentage of apoptotic cells in Annexin V+/PI- and Annexin V+/PI+ quadrants.

Transwell Assay

The invasion assay was measured using transwell chamber (8 μm pore size; Chemicon, Temecula, CA, USA) coated with matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Transfected HCC-70 and MB231 cells (4×10^5 cells) in 200 μL medium without FBS was filled in the upper, and 500 μL of medium with 10% FBS was in the low chamber. For cell migration analysis, the chamber was introduced without matrigel and the other operations were the same to invasion analysis. All transwell systems were stayed in 37°C for 24 h. After removing cells on the upper surface of the membrane, the migrated and invaded cells on the lower surface were fixed in 70% ethanol, and stained with 0.2% crystal violet. The pictures were photographed under a microscope (Olympus, Tokyo, Japan) in five random fields (×200). The numbers of migrated and invaded cells were counted with Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA). Three independent experiments were carried out.

Western Blotting

Transfected BT-549 and MDA-MB-231 cells were subjected to extract total protein using M-PER mammalian protein extraction reagent (Pierce, Rockford, IL, USA). Generally, 20 μg protein samples were adopted to undergo the standard procedures for Western blotting assay. The primary antibodies utilized in this study were purchased from Cell Signaling Technology (Danvers, MA, USA) and listed as follows: anti-Vav2 (#2502, 1:1 000), anti-Rac1 (#4651, 1:1 000), anti-ITGA5 (#4705, 1:1 000) and anti-β-actin (#4967, 1:1 000). The protein column ratios were determined using β-actin as calibration. The protein expression levels were quantified by Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA) and compared to the control group.

Dual-Luciferase Reporter Assay

BT-549 and MDA-MB-231 cells were transferred in 24-well plate for 24 h. The full length of SNHG3 (NR_036473) and the 3' untranslated region (3' UTR) of ITGA5 (NM_002205) containing the complementary binding site of miR-326 were mutated using Site-Directed Mutagenesis Kit (SBS Genetech, Beijing, China). The wild type and mutant type of SNHG3 and ITGA5 3' UTR (namely SNHG3-WT/MUT and ITGA5-WT/MUT) were amplified by PCR method and cloned into downstream of pmirGLO Dual-Luciferase vector (Promega, Madison, WI, USA). The cells were co-transfected with miR-326 mimics or miR-NC mimics with either SNHG3-WT/MUT or ITGA5-WT/MUT for 24 h. The Firefly Luciferase activity was examined using GloMax LUMINOMETER (Promega, Madison, WI, USA) first normalized to Renilla Luciferase activity (the internal control), and then, normalized to control group. All operations were carried out at least three times.

Statistical Analysis

The data were expressed as the mean ± standard deviation (SD) from three independent experiments. Statistical analysis was performed using two-tailed Student *t*-test and one-way analysis of variance followed by Tukey's test on GraphPad Prism 5 (GraphPad, San Diego, CA, USA). *p*-value < 0.05 was considered as significant difference.

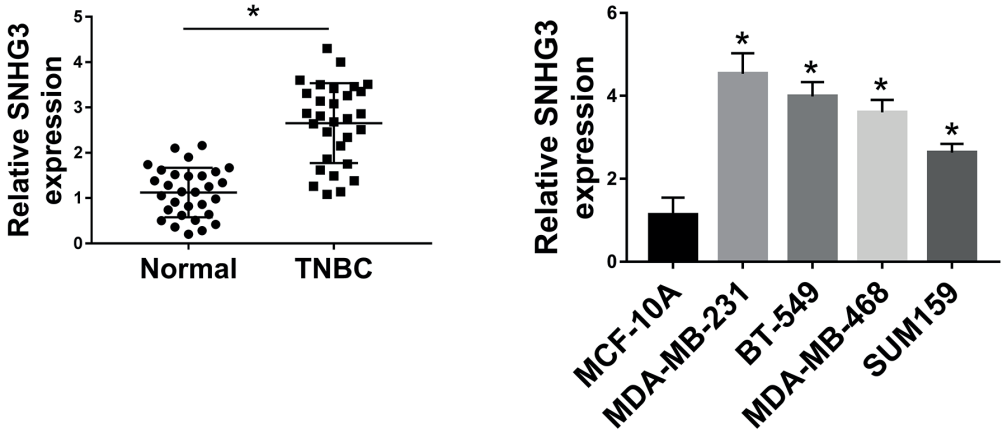

Figure 1. LncRNA small nucleolar RNA host gene 3 (SNHG3) was highly expressed in triple-negative breast cancer (TNBC). **A,** Real Time-PCR detected SNHG3 expression level in tumor tissues and matched adjacent normal tissues from patients (n=30) with TNBC. **B,** Real Time-PCR examined SNHG3 level in human TNBC cell lines (MDA-MB-231, BT-549, MDA-MB-468 and SUM159) and normal mammary epithelial cell line MCF-10A. Relative SNHG3 expression was presented as fold change normalized to internal control GAPDH. *$p < 0.05$.

Results

SNHG3 Was Highly Expressed in TNBC Tumors and Cell Lines

To identify the divergence of SNHG3 expression in TNBC, we performed Real Time-PCR to examine SNHG3 expression. As shown in Figure 1A, level of SNHG3 was significantly higher in tumor tissues isolated from 30 TNBC patients than that in the matched adjacent normal tissues. Moreover, SNHG3 expression was overall dramatically upregulated in TNBC cell lines, including MDA-MB-231, BT-549, MDA-MB-468 and SUM159 compared to the normal mammary epithelial cell line MCF-10A (Figure 1B). Notably, SNHG3 level was the highest in MDA-MB-231 and BT-549 cells. These outcomes showed that SNHG3 was highly expressed in TNBC tumors and cell lines.

Silencing of SNHG3 Suppressed the Malignant Development of TNBC Cells In Vitro and Inhibited Vav2/Rac1 Signaling Pathway

In order to elucidate the role of SNHG3 in TNBC cell progression, we silenced SNHG3 using special siRNAs in MDA-MB-231 and BT-549 cells. Both si-SNHG3#1 and #2 caused marked knockdown efficiency in MDA-MB-231 and BT-549 cells (Figure 2A). Subsequently, si-SNHG3#1-transfected cells were used for functional experiments. MTT assay results revealed a striking inhibition on cell viability in MDA-MB-231 and BT-549 cells transfected with SNHG3 silencing (Figure 2B and 2C). On the contrary, apoptotic rate was significantly increased due to downregulation of SNHG3 as depicted by flow cytometry (Figure 2D), indicating a pro-apoptosis role of SNHG3 deletion. In terms of metastasis ability *in vitro*, transwell assays were carried out. Migrated cells and invaded cells were decreased in SNHG3-downregulated MDA-MB-231 and BT-549 cells (Figure 2E and 2F). Previous studies indicated that Vav2/Rac1 signaling pathway could promote tumorigenicity of breast cancer. Here, protein expression of Vav2 and Rac1 was significantly reduced by SNHG3 downregulation in MDA-MB-231 and BT-549 cells (Figure 2G and 2H). These results demonstrated a tumor-suppressive role of SNHG3 silencing in TNBC cells *in vitro*.

Expression of MiR-326 Was Inhibited in TNBC and MiR-326 Was Sponged by SNHG3

Mechanically, SNHG3 was investigated to function as a molecular "sponge" for miRNAs, and we searched StarBase2.0 database (http://starbase2/lncRNA-miR-326Interaction) to find potential target miRNAs. As shown in Figure 3A, there existed a complementary binding region (in box) between SNHG3 and miR-326. To further confirm this putative targeting site, Dual-Luciferase reporter assay was performed. We generated recombinant reporter pmirGLO plasmids expressing SNHG3-WT or SNHG3-MUT,

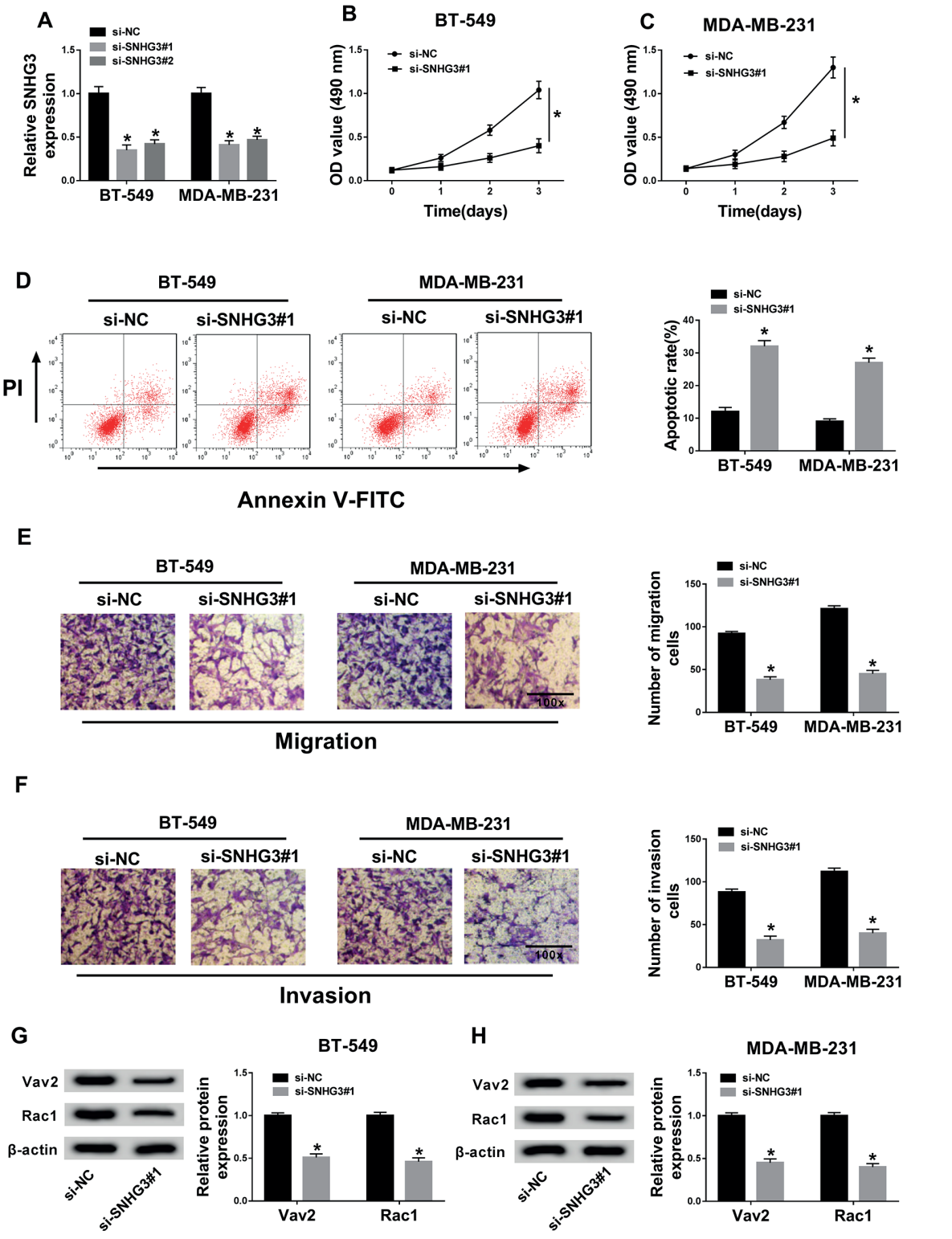

Figure 2. Silencing of SNHG3 suppressed the malignant development of TNBC cells *in vitro* and inhibited Vav2/Rac1 signaling pathway. **A,** Levels of SNHG3 were determined in MDA-MB-231 and BT-549 cells after transfected with small interfere RNAs (siRNAs) targeting SNHG3 (si-SNHG3#1 and #2) or its scrambled siRNA (si-NC). **B-H,** MDA-MB-231 and BT-549 cells were transfected with si-SNHG3#1 or si-NC. **B-C,** MTT assay tested cell viability after transfection for 1 d, 2 d and 3 d. The optical density (OD) value was recorded at 490 nm. **D,** Annexin V-FITC Apoptosis Detection kit assessed apoptotic rate on flow cytometry after 24 h-transfection. Data was presented as percentage of cells in Annexin V+/PI and Annexin V+/PI+ quadrants. **E-F,** Transwell assays determined migration and invasion cells after 24 h-transfection. Data was number of migration/invasion cells (100×). **G-H,** Western blotting evaluated expression of key proteins in Vav2/Rac signaling pathway. The protein bands of Vav2 and Rac1 were quantified by densitometry and the relative expression level of proteins was presented as fold change with normalization to β-actin. *$p < 0.05$.

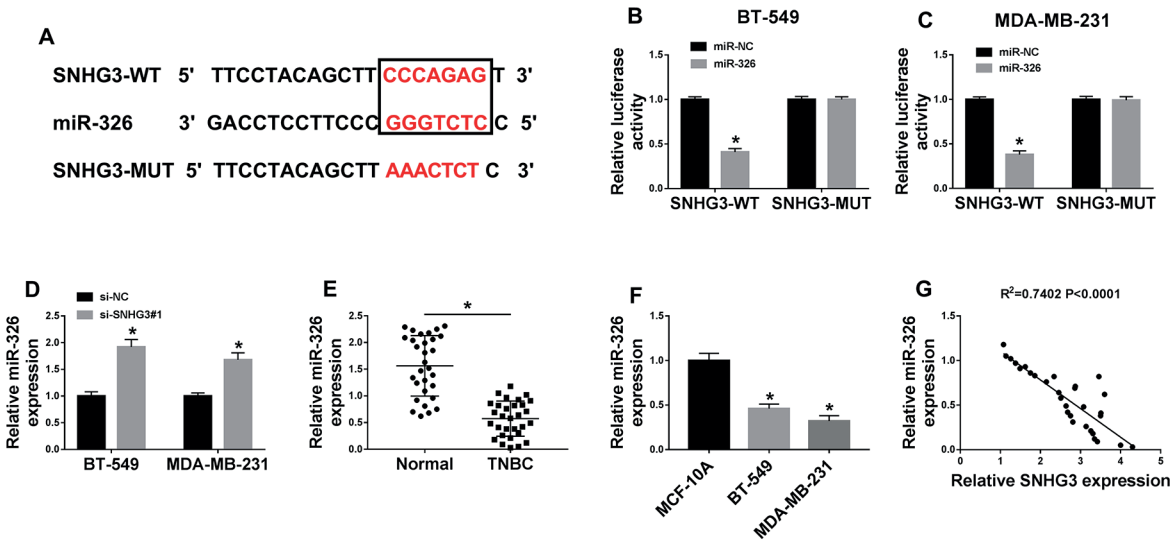

Figure 3. MiRNA-326 (miR-326) was lowly expressed in TNBC and its expression was sponged by SNHG3. **A,** StarBase 2.0 predicted a complementary binding region (in box) between SNHG3 and miR-326. The putative bases were artificially mutated. **B-C,** Dual-Luciferase reporter assay examined the luciferase activity of SNHG3-WT/MUT in BT-549 and MDA-MB-231 cells when co-transfected with miR-326 mimics (miR-326) or its control (miR-NC). Data were presented as the relative Firefly luciferase activity normalized to the internal Renilla luciferase activity. **D,** Changes of miR-326 expression were measured by real-time PCR in BT-549 and MDA-MB-231 cells transfected with si-SNHG3#1 or si-NC. **E-F,** Real Time-PCR tested miR-326 expression in tissues from TNBC patients (n=30) and TNBC cell lines MDA-MB-231 and BT-549, as well as in normal mammary epithelial cell line MCF-10A. **G,** Pearson correlation analysis identified a negative correlation between SNHG3 and miR-326 expression in TNBC tumors (n=30). Relative miR-326 expression was presented as fold change normalized to internal control U6. *$p < 0.05$.

and found that relative Luciferase of SNHG3-WT was significantly declined in BT-549 and MDA-MB-231 cells co-transfected with miR-326 mimics (Figure 3B and 3C); meanwhile, there was little difference in SNHG3-MUT groups whether transfected with miR-326 mimics or miR-NC mimics. Real Time-PCR analysis also detected a negative regulatory effect of SNHG3 on miR-326 expression in these cells (Figure 3D). In TNBC, the basic expression of miR-326 was lower in tumors and cell lines (BT-549 and MDA-MB-231) (Figure 3E and 3F). Furthermore, we observed that miR-326 expression was negatively correlated to SNHG3 in this cohort of TNBC patients (Figure 3G; Pearson correlation analysis, R^2=0.7402, $p < 0.0001$). These data manifested that miR-326 was lowly expressed and was sponged by SNHG3 in TNBC.

The Function of SNHG3 Silencing in TNBC Cells Was Abolished by MiR-326 Deletion

After transfection with si-SNHG3#1 together with anti-miR-326, the upregulation of miR-326 induced by SNHG3 silencing was decreased in BT-549 and MDA-MB-231 cells (Figure 4A). Subsequently, MTT assay results revealed a prominent improvement of cell viability by miR-326 deletion in SNHG3 silenced-cells (Figure 4B and 4C). On the contrary, flow cytometry depicted that the increased apoptotic rate was remarkably dropped due to downregulation of miR-326 (Figure 4D). In terms of metastasis ability of MDA-MB-231 and BT-549 cells, transwell assays showed that migrated cells and invaded cells were decreased when SNHG3-was downregulated, and this effect was attenuated when miR-326 was deleted simultaneously (Figure 4E and 4F). With miR-326 deletion, SNHG3-mediated inhibition on Vav2 and Rac1 levels was significantly rescued in MDA-MB-231 and BT-549 cells (Figure 4G and 4H). These results demonstrated the tumor-suppressive role of SNHG3 silencing in TNBC cells depended on promoting miR-326.

ITGA5 Was Targeted by MiR-326 and Was Upregulated in TNBC Tissues and Cell Lines

Next, the downstream target genes of miR-326 were searched on TargetScan database (http://www.targetscan/vert_71/miR-326&ITGA5). ITGA5 was predicted to have four base

pairing regions of miR-326, and the conserved one was shown in Figure 5A. To further confirm this putative targeting site, Dual-Luciferase reporter assay was conducted. As a result, the relative Luciferase of vectors containing ITGA5-WT was significantly declined in BT-549 and MDA-MB-231 cells when co-transfected with miR-326 mimics (Figure 5B and 5C). Real Time-PCR and Western blotting data also detected a downregulatory effect of miR-326 on ITGA5 expression in BT-549 and MDA-MB-231 cells (Figure 5D and 5E). In TNBC, the basic expression of ITGA5 was higher in tumors (Figure 5F and 5G) and cell lines (Figure 5H and 5I). Furthermore, we observed that ITGA5 mRNA expression was negatively correlated to miR-326 in this cohort of TNBC patients (Figure 5J; Pearson correlation analysis, R^2=0.3550, $p < 0.0001$). These data indicated that ITGA5 was highly expressed and was targeted by miR-326 in TNBC.

The Effect of MiR-326 Overexpression in TNBC Cells Was Abrogated by ITGA5 Restoration

After transfection with miR-326 mimics combined with pcDNA-ITGA5 (ITGA5), the downregulation of ITGA5 induced by miR-326 over-

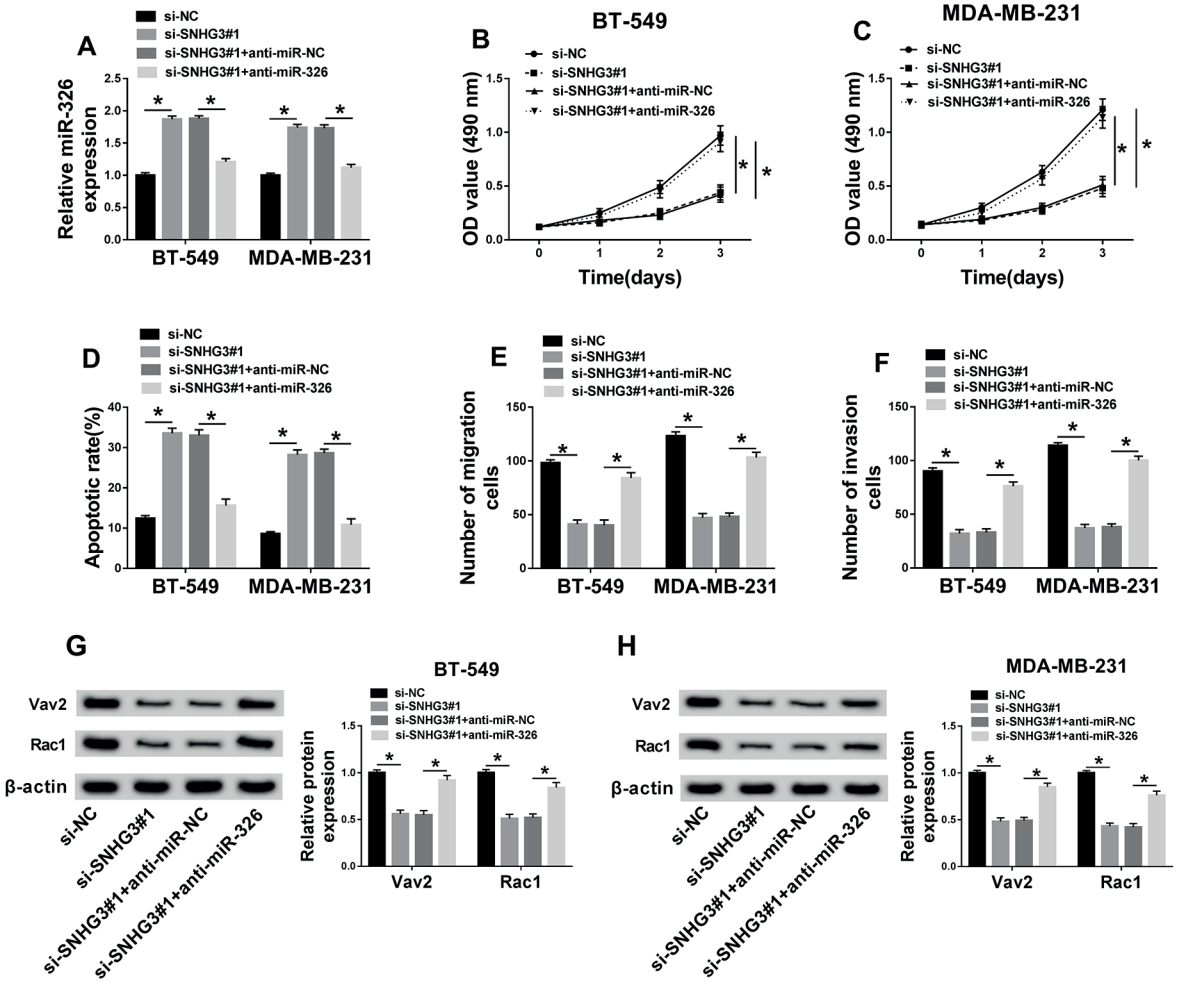

Figure 4. The function of SNHG3 silencing in TNBC cells was abolished by miR-326 deletion. **A,** Real-time PCR measured relative miR-326 expression in BT-549 and MDA-MB-231 cells transfected with si-SNHG3#1 or si-NC, and co-transfected with si-SNHG3#1 and 2'-O-methyl-modified miR-326 inhibitors (anti-miR-326) or its control (anti-miR-NC). **B-C,** MTT assay tested OD490 values after transfection for 1 d, 2 d and 3 d. **D,** Flow cytometry analyzed apoptotic rate after 24 h-transfection. **E-F,** Transwell assays determined migration and invasion cells after 24 h-transfection. **G-H,** Western blotting evaluated relative expression of Vav2 and Rac1 after 24 h-transfection. *$p < 0.05$.

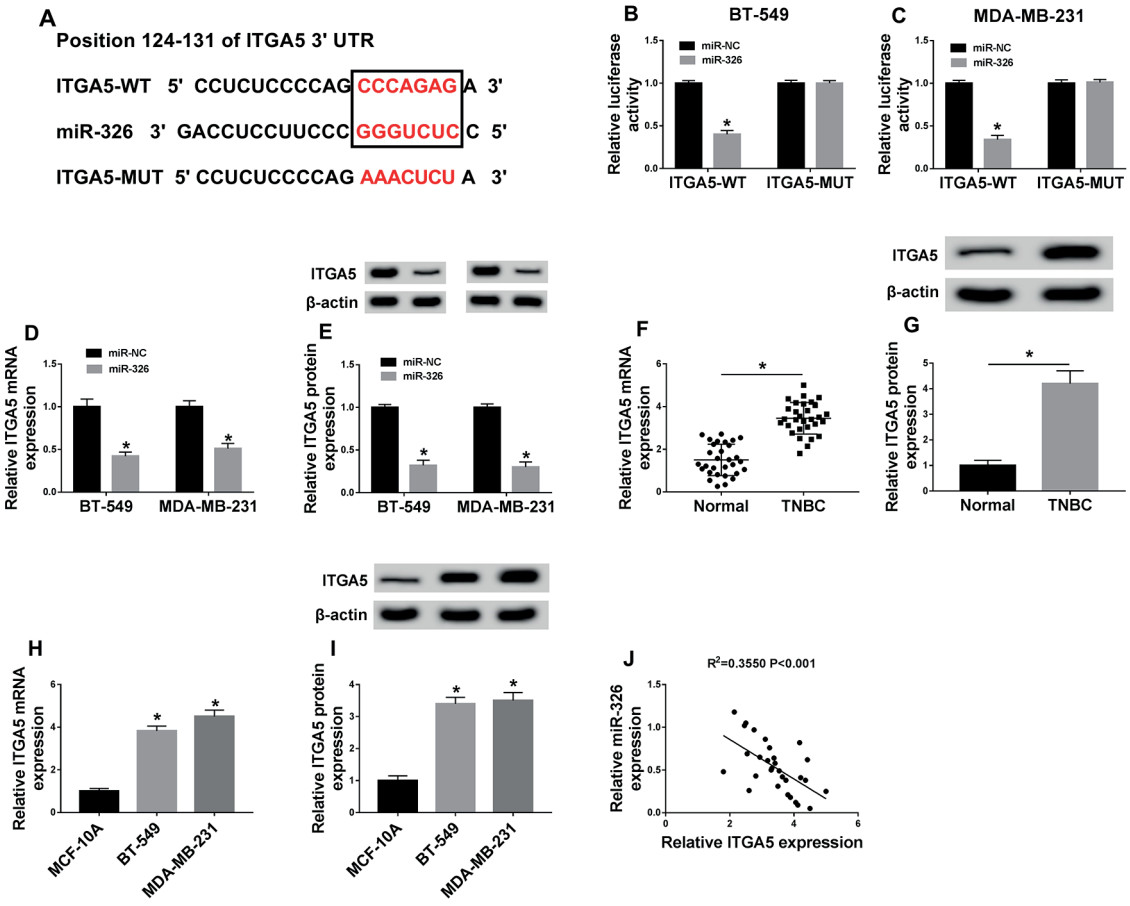

Figure 5. Integrin α5 (ITGA5) was targeted by miR-326 and was upregulated in TNBC. **A,** TargetScan predicted a complementary binding region (in box) between ITGA5 3' untranslated region and miR-326. The putative bases were artificially mutated. **B-C,** Dual-Luciferase reporter assay examined the relative luciferase activity of ITGA5-WT/MUT in BT-549 and MDA-MB-231 cells when co-transfected with miR-326 or miR-NC. Changes of ITGA5 message RNA (mRNA) expression and protein expression were measured by real-time PCR and western blotting in **D-E,** BT-549 and MDA-MB-231 cells transfected with miR-326 or miR-NC, **F-G,** tissues from TNBC patients (n=30) and **H-I,** normal mammary epithelial cell line (MCF-10A) and TNBC cell lines (MDA-MB-231 and BT-549). **J,** Pearson correlation analysis identified a negative correlation between ITGA5 and miR-326 expression in TNBC tumors (n=30). Relative ITGA5 protein expression was presented as fold change normalized to internal control β-actin. *$p < 0.05$.

expression was increased in BT-549 and MDA-MB-231 cells (Figure 6A and 6B). Subsequently, MTT assay results revealed a prominent enhancement of cell viability in miR-326 mimics-transfected cells with ITGA5 restoration (Figure 6C and 6D). On the contrary, flow cytometry described that the increased apoptotic rate was remarkably dropped due to ITGA5 upregulation (Figure 6E). Transwell assays showed that migrated cells and invaded cells were decreased by miR-326 elevation, which was attenuated by ITGA5 boosting, simultaneously (Figure 6F and 6G). With ITGA5 restoration, miR-326-mediated inhibition on Vav2 and Rac1 expression was significantly rescued in MDA-MB-231 and BT-549 cells (Figure 6H and 6I). These results demonstrated the tumor-suppressive role of miR-326 upregulation in TNBC cells depended on depressing ITGA5 expression.

SNHG3 Silencing Downregulated ITGA5 Expression by Sponging MiR-326

Considering that miR-326 directly interacted with SNHG3 and ITGA5, it was unclear that whether there occurred a SNHG3/miR-326/ITGA5 axis in the malignant development of TNBC cells. Therefore, MDA-MB-231 and BT-549 cells were transfected with si-SNHG3#1 or si-NC, and co-transfected with si-SNHG3#1 and anti-miR-326 or anti-miR-NC, followed by analysis of ITGA5 expression. As Figure 7A and 7B

showed, mRNA and protein expression of ITGA5 were lower when SNHG3 silencing, whereas anti-miR-326 transfection led to the recovery of ITGA5 level. This outcome suggested ITGA5 expression was downregulated by SNHG3 silencing through acting as a "sponge" for miR-326.

Discussion

As a ceRNA, SNHG3 has been introduced to sponge several miRNAs in human malignancies. In fact, Huang et al[9] asserted that SNHG3 upregulation promoted colorectal cancer cell proliferation *in vitro* and tumor growth *in vivo* by sponging miRNA-182-5p and correlating with c-myc. Li et al[20] boldly speculated that there was a SNHG3-miRNA-186-5p/miRNA-590-3p interaction network in regulating energy metabolism of ovarian cancer. In osteosarcoma, miRNA-196-5p and miRNA-151a-3p were competitively bound by SNHG3 to alter cell growth, migration and invasion[10,21]. However, SNHG3-miRNAs network is still poorly annotated, especially in breast cancer. Herewith, we investigated role of SNHG3 in TNBC malignant development, and its underlying mechanism by acting as a molecular "sponge" was further researched.

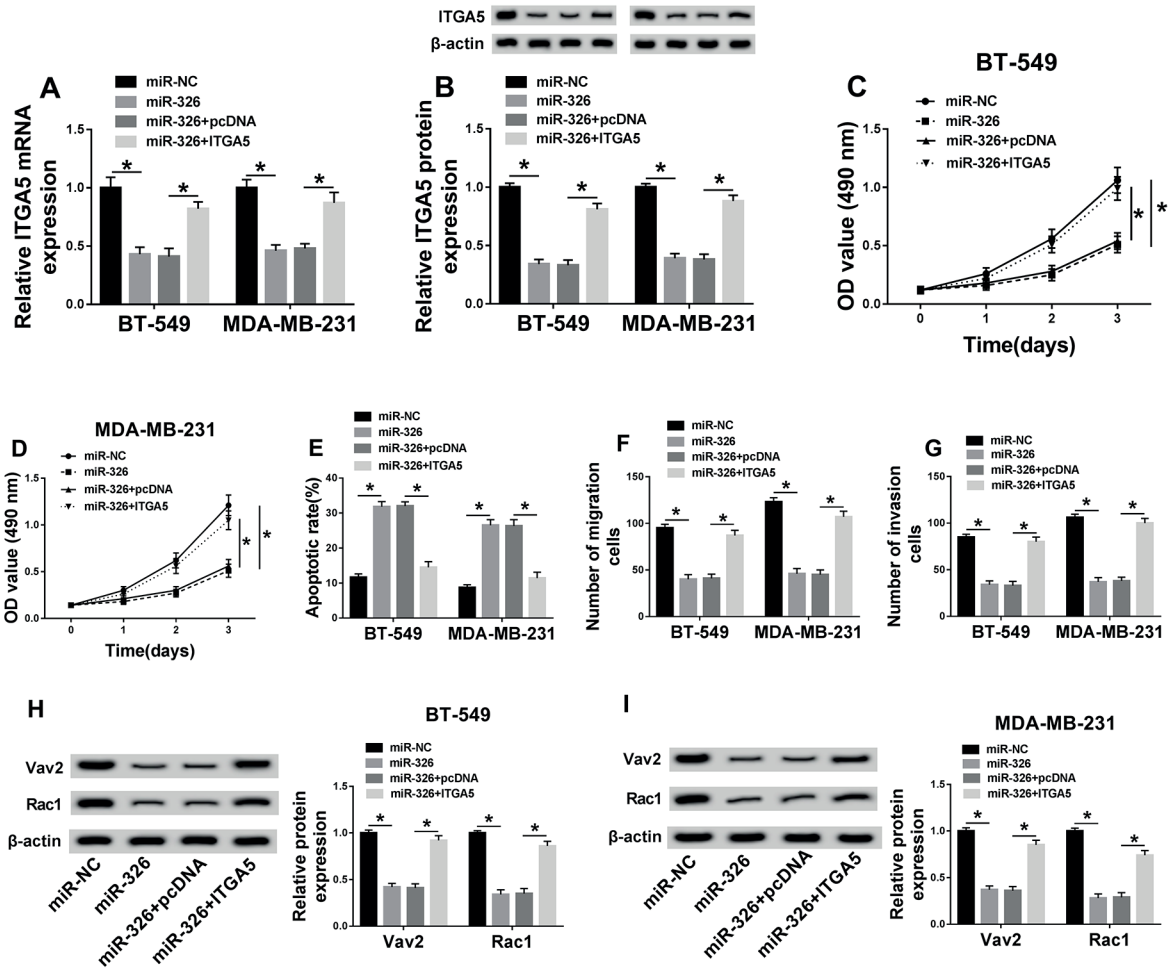

Figure 6. The effect of miR-326 overexpression in TNBC cells was abolished by ITGA5 restoration. **A-B,** Real Time-PCR and Western blotting measured relative ITGA5 expression in BT-549 and MDA-MB-231 cells transfected with miR-326 or miR-NC, and co-transfected with miR-326 and pcDNA-ITGA5 (ITGA5) or its control (pcDNA). **C-D,** MTT assay tested OD490 values after transfection for 1 d, 2 d and 3 d. (E) Flow cytometry analyzed apoptotic rate after 24 h-transfection. (F, G) Transwell assays determined migration and invasion cells after 24 h-transfection. **H-I,** Western blotting evaluated relative expression of Vav2 and Rac1 after 24 h-transfection. *$p < 0.05$.

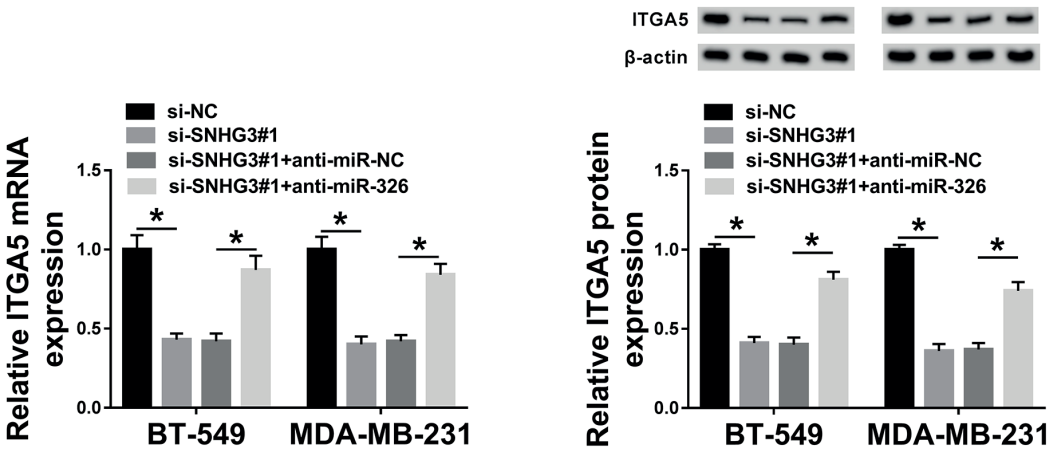

Figure 7. SNHG3 silencing downregulated ITGA5 expression through sponging miR-326. **A,** Relative ITGA5 mRNA expression and (**B**) ITGA5 protein expression in MDA-MB-231 and BT-549 cells, following transfection with si-SNHG3#1 or si-NC, and co-transfection with si-SNHG3#1 and anti-miR-326 or anti-miR-NC. *$p < 0.05$.

In this study, we observed a higher expression of SNHG3 in TNBC tumors and cell lines (MDA-MB-231, BT-549, MDA-MB-468 and SUM159) comparing to normal paired normal tissues and mammary epithelial cells MCF-10A, which was in accordance with the previous study[7]. Loss-of-functional experiments showed a loss of cell viability, migration and invasion, but an increase of cell apoptosis of MDA-MB-231 and BT-549 cells when SNHG3 was knocked down. Mechanically, SNHG3 presumably sponged miR-326, thus positively affecting ITGA5 expression in TNBC cells *in vitro*. Nonetheless, we did not further discuss the association between SNHG3 expression and clinicopathologic characteristics, such as distance metastasis and overall survival. This essential issue should be further detected. Notably, we noticed that several SNHGs namely SNHG1, 3, 12, 14, and 18 were predicted to have binding site of miR-326 according to bioinformatics algorithms of starBase v2.0 and DINAN LncBase Predicted v.2. However, only SNHG1 was further identified as miR-326 "sponge"[22,23]. It would be meaningful and intriguing to verify these putative target binding regions.

MiR-126 could be sponged by different lncRNAs in different cancers, such as lncRNA PCAT1 in oesophageal squamous cell carcinoma[24], lncRNA TDRG1 in cervical cancer[25], lncRNA H19 in hepatocellular carcinoma and glioblastoma[26,27] and lncRNA EWSAT1 in nasopharyngeal carcinoma[28]. Unluckily, there is barely evidence on lncRNAs-miR-326 interplay in breast cancer, even though the vital role of this miRNA has been acknowledged in breast cancer[14,15,29]. Ghaemi et al[29] found that miR-326 was downregulated in breast cancer tissues and cells, and its expression was even lower in BRCA (breast invasive carcinoma) tissues and cell lines than less-aggressive tissues and cell lines; also, it acted as a tumor-suppressor in breast cancer, which was consistent with our findings. In this study, we draw a conclusion that the overexpression of miR-326 suppressed TNBC cell viability, migration and invasion *in vitro* as evidenced by MTT assay and transwell assays, coupled with apoptosis promotion. Except these similar outcomes, miR-326 overexpression also inhibited epithelial-mesenchymal transition, cell cycle and colony formation in breast cancer cells[14,29]. Collectively, miR-326 exerted universal functions in breast cancer development, including TNBC. Meanwhile, miR-326-related molecular mechanisms remains to be further documented. Subsequently, the downstream target of miR-326 was explored. We disclosed ITGA5 was targeted and downregulated by miR-326. Besides, ITGA5 was positively modulated by SNHG3 through miR-326. The expression of ITGA5 was boosted in TNBC tissues and cells (MDA-MB-231 and BT-549), and this high expression could partially abolish the anti-tumor activity of miR-326 upregulation in MDA-MB-231 and BT-549 cells, hinting ITGA5 as an oncogene in TNBC. This finding has been reported in previous researches. In particular, Cimino et al[30] indicated that miRNA-148b-mediated ITGA5 attenuation could contribute to breast cell apopto-

sis stimulated by chemotherapies, such as anoikis and paclitaxel. In tumor metastasis respect, silencing ITGA5 was announced to remit skeletal tumor stress of ER-/PR-negative breast cancer cells[31]. Orso et al[32] also revealed that the dissemination of primary breast cancer or metastatic melanoma was impaired by deregulating miRNA-214 or miRNA-148b, which could be overridden by ITGA5 overexpression. ITGA5 served as oncogenic role in TNBC and its knockout suppressed the metastasis and stemness traits[16,33]; besides, its upregulation was associated with poor survival. Notably, miRNA-205/ITGA5 axis could regulate Vav2/Rac1 signaling pathway to affect breast cancer stemness and tumor metastasis[16]. Vav2/Rac1 signaling pathway was also implicated in cell proliferation and growth in breast cancer cells and gastric cancer cells both *in vitro* and *in vivo*[17,34]. Our study also demonstrated that SNHG3/miR-326/ITGA5 axis regulated TNBC cell development via modulating Vav2/Rac1 signaling pathway. In addition, cyclin D1, RhoA, and CDC42 were the other biological effectors of Vav2[35]. However, these corresponding molecules remain to be further investigated.

Conclusions

Briefly, we observed that SNHG3 was highly expressed in TNBC tumors and cell lines, and its silencing could suppress malignant development of TNBC cells *in vitro* presumably through miR-326/ITGA5 axis and inhibiting Vav2/Rac1 signaling pathway. This result might render SNHG3 as a potential biomarker in TNBC tumorigenesis.

Conflict of Interests

The Authors declare that they have no conflict of interests.

Ethics Approval and Consent to Participate

The present study was approved by the Ethical Review Committee of Linyi Cancer Hospital. Written informed consent was obtained from all enrolled patients.

References

1) Rakha EA, Ellis IO. Triple-negative/basal-like breast cancer: review. Pathology 2009; 41: 40-47.
2) Dawood S. Triple-negative breast cancer: epidemiology and management options. Drugs 2010; 70: 2247-2258.
3) Amos KD, Adamo B, Anders CK. Triple-negative breast cancer: an update on neoadjuvant clinical trials. Int J Breast Cancer 2012; 2012: 385978.
4) Yang R, Xing L, Wang M, Chi H, Zhang L, Chen J. Comprehensive analysis of differentially expressed profiles of lncRNAs/mRNAs and miRNAs with associated ceRNA networks in triple-negative breast cancer. Cell Physiol Biochem 2018; 50: 473-488.
5) Rodriguez Bautista R, Ortega Gomez A, Hidalgo Miranda A, Zentella Dehesa A, Villarreal-Garza C, Avila-Moreno F, Arrieta O. Long non-coding RNAs: implications in targeted diagnoses, prognosis, and improved therapeutic strategies in human non- and triple-negative breast cancer. Clin Epigenetics 2018; 10: 88.
6) Mathe A, Scott RJ, Avery-Kiejda KA. MiRNAs and other epigenetic changes as biomarkers in triple negative breast cancer. Int J Mol Sci 2015; 16: 28347-28376.
7) Taherian-Esfahani Z, Taheri M, Dashti S, Kholghi-Oskooei V, Geranpayeh L, Ghafouri-Fard S. Assessment of the expression pattern of mTOR-associated lncRNAs and their genomic variants in the patients with breast cancer. J Cell Physiol 2019; 234: 22044-22056.
8) Zhang T, Cao C, Wu D, Liu L. SNHG3 correlates with malignant status and poor prognosis in hepatocellular carcinoma. Tumour Biol 2016; 37: 2379-2385.
9) Huang W, Tian Y, Dong S, Cha Y, Li J, Guo X, Yuan X. The long non-coding RNA SNHG3 functions as a competing endogenous RNA to promote malignant development of colorectal cancer. Oncol Rep 2017; 38: 1402-1410.
10) Chen J, Wu Z, Zhang Y. LncRNA SNHG3 promotes cell growth by sponging miR-196a-5p and indicates the poor survival in osteosarcoma. Int J Immunopathol Pharmacol 2019; 33: 2058738418820743.
11) Lu W, Yu J, Shi F, Zhang J, Huang R, Yin S, Songyang Z, Huang J. The long non-coding RNA Snhg3 is essential for mouse embryonic stem cell self-renewal and pluripotency. Stem Cell Res Ther 2019; 10: 157.
12) Kim J, Krichevsky A, Grad Y, Hayes GD, Kosik KS, Church GM, Ruvkun G. Identification of many microRNAs that copurify with polyribosomes in mammalian neurons. Proc Natl Acad Sci U S A 2004; 101: 360-365.
13) Jadideslam G, Ansarin K, Sakhinia E, Babaloo Z, Abhari A, Ghahremanzadeh K, Khalili M, Radmehr R, Kabbazi A. Diagnostic biomarker and therapeutic target applications of miR-326 in cancers: A systematic review. J Cell Physiol 2019; 234: 21560-21574.
14) Du Y, Shen L, Zhang W, Ding R, Li Q, Li S, Zhang H. Functional analyses of microRNA-326 in breast cancer development. Biosci Rep 2019; 39 (7). pii: BSR20190787.
15) Liang Z, Wu H, Xia J, Li Y, Zhang Y, Huang K, Wagar N, Yoon Y, Cho HT, Scala S, Shim H. Involvement

of miR-326 in chemotherapy resistance of breast cancer through modulating expression of multidrug resistance-associated protein 1. Biochem Pharmacol 2010; 79: 817-824.

16) Xiao Y, Li Y, Tao H, Humphries B, Li A, Jiang Y, Yang C, Luo R, Wang Z. Integrin alpha5 down-regulation by miR-205 suppresses triple negative breast cancer stemness and metastasis by inhibiting the Src/Vav2/Rac1 pathway. Cancer Lett 2018; 433: 199-209.

17) Lee KT, Chang CL, Li CY, Song H, Shan YS, Lai MD. The oncogenic role of MST3 in human gastric cancer. Am J Cancer Res 2018; 8: 2130-2139.

18) Patel V, Rosenfeldt HM, Lyons R, Servitja JM, Bustelo XR, Siroff M, Gutkind JS. Persistent activation of Rac1 in squamous carcinomas of the head and neck: evidence for an EGFR/Vav2 signaling axis involved in cell invasion. Carcinogenesis 2007; 28: 1145-1152.

19) Kato L, Begum NA, Burroughs AM, Doi T, Kawai J, Daub CO, Kawaguchi T, Matsuda F, Hayashizaki Y, Honjo T. Nonimmunoglobulin target loci of activation-induced cytidine deaminase (AID) share unique features with immunoglobulin genes. Proc Natl Acad Sci U S A 2012; 109: 2479-2484.

20) Li N, Zhan X, Zhan X. The lncRNA SNHG3 regulates energy metabolism of ovarian cancer by an analysis of mitochondrial proteomes. Gynecol Oncol 2018; 150: 343-354.

21) Zheng S, Jiang F, Ge D, Tang J, Chen H, Yang J, Yao Y, Yan J, Qiu J, Yin Z, Ni Y, Zhao L, Chen X, Li H, Yang L. LncRNA SNHG3/miRNA-151a-3p/RAB22A axis regulates invasion and migration of osteosarcoma. Biomed Pharmacother 2019; 112: 108695.

22) Wang J, Cao L, Wu J, Wang Q. Long non-coding RNA SNHG1 regulates NOB1 expression by sponging miR-326 and promotes tumorigenesis in osteosarcoma. Int J Oncol 2018; 52: 77-88.

23) Tan H, Zhao L, Song R, Liu Y, Wang L. The long noncoding RNA SNHG1 promotes nucleus pulposus cell proliferation through regulating miR-326 and CCND1. Am J Physiol Cell Physiol 2018; 315: C21-C7.

24) Huang L, Wang Y, Chen J, Wang Y, Zhao Y, Wang Y, Ma Y, Chen X, Liu W, Li Z, Zhao L, Shan B, Dong X, Li D, Shao S, Song Y, Zhan Q, Liu X. Long noncoding RNA PCAT1, a novel serum-based biomarker, enhances cell growth by sponging miR-326 in oesophageal squamous cell carcinoma. Cell Death Dis 2019; 10: 513.

25) Jiang H, Liang M, Jiang Y, Zhang T, Mo K, Su S, Wang A, Zhu Y, Huang G, Zhou R. The lncRNA TDRG1 promotes cell proliferation, migration and invasion by targeting miR-326 to regulate MAPK1 expression in cervical cancer. Cancer Cell Int 2019; 19: 152.

26) Wei L Q, Li L, Lu C, Liu J, Chen Y, Wu H. Involvement of H19/miR-326 axis in hepatocellular carcinoma development through modulating TWIST1. J Cell Physiol 2019; 234: 5153-5162.

27) Fawzy MS, Ellawindy A, Hussein MH, Khashana MS, Darwish MK, Abdel-Daim MM. Long non-coding RNA H19, and not microRNA miR-326, is over-expressed and predicts survival in glioblastoma. Biochem Cell Biol 2018.

28) Song P, Yin SC. Long non-coding RNA EWSAT1 promotes human nasopharyngeal carcinoma cell growth in vitro by targeting miR-326/-330-5p. Aging (Albany NY) 2016; 8: 2948-2960.

29) Ghaemi Z, Soltani BM, Mowla SJ. MicroRNA-326 functions as a tumor suppressor in breast cancer by targeting ErbB/PI3K signaling pathway. Front Oncol 2019; 9: 653.

30) Cimino D, De Pittà C, Orso F, Zampini M, Casara S, Penna E, Quaglino E, Forni M, Damasco C, Pinatel E, Ponzone R, Romualdi C, Brisken C, De Bortoli M, Biglia N, Provero P, Lanfranchi G, Taverna D. miR148b is a major coordinator of breast cancer progression in a relapse-associated microRNA signature by targeting ITGA5, ROCK1, PIK3CA, NRAS, and CSF1. FASEB J 2013; 27: 1223-1235.

31) Croset M, Pantano F, Kan CWS, Bonnelye E, Descotes F, Alix-Panabières C, Lecellier CH, Bachelier R, Allioli N, Hong SS, Bartkowiak K, Pantel K, Clézardin P. miRNA-30 family members inhibit breast cancer invasion, osteomimicry, and bone destruction by directly targeting multiple bone metastasis-associated genes. Cancer Res 2018; 78: 5259-5273.

32) Orso F, Quirico L, Virga F, Penna E, Dettori D, Cimino D, Coppo R, Grassi E, Elia AR, Brusa D, Deaglio S, Brizzi MF, Stadler MB, Provero P, Caselle M, Taverna D. miR-214 and miR-148b targeting inhibits dissemination of melanoma and breast cancer. Cancer Res 2016; 76: 5151-5162.

33) Li Y, Xiao Y, Lin HP, Reichel D, Bae Y, Lee EY, Jiang Y, Huang X, Yang C, Wang Z. In vivo beta-catenin attenuation by the integrin alpha5-targeting nano-delivery strategy suppresses triple negative breast cancer stemness and metastasis. Biomaterials 2019; 188: 160-172.

34) Cho CY, Lee KT, Chen WC, Wang CY, Chang YS, Huang HL, Hsu HP, Yen MC, Lai MZ, Lai MD. MST3 promotes proliferation and tumorigenicity through the VAV2/Rac1 signal axis in breast cancer. Oncotarget 2016; 7: 14586-14604.

35) Abe K, Rossman KL, Liu B, Ritola KD, Chiang D, Campbell SL, Burridge K, Der CJ. Vav2 is an activator of Cdc42, Rac1, and RhoA. J Biol Chem 2000; 275: 10141-10149.