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MicroRNA-548-3p and MicroRNA-576-5p enhance the migration and invasion of esophageal squamous cell carcinoma cells via NRIP1 down-regulation

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Nuclear receptor interacting protein (NRIP1), also known as RIP140, is a transcriptional co-regulator required for the maintenance of energy homeostasis and ovulation. Although several studies have identified roles for NRIP1 in various cell processes, the biological functions of NRIP1 in esophageal squamous cell carcinoma (ESCC) remain unknown. Herein, we demonstrate that NRIP1 inhibits the migration and invasion of ESCC cells. Further mechanistic studies revealed that NRIP1 is directly targeted by miR-548-3p and miR-576-5p. We then identified that miR-548-3p and miR-576-5p regulate the migration and invasion of ESCC cells by inhibiting NRIP1 expression. Interestingly, the expression of miR-548-3p and miR-576-5p in ESCC cell lines and ESCC tissues is up-regulated and NRIP1 is down-regulated relative to controls. A statistically significant inverse association was found between the expression levels of miR-548-3p/miR-576-5p and NRIP1. These combined results reveal novel functions for miR-548-3p, miR-576-5p, and NRIP1 in regulating ESCC cell migration and invasion which are important functions for the metastatic process in esophageal cancer.

Key words: esophageal squamous cell carcinoma, microRNA-548, microRNA-576-5p, NRIP1, migration and invasion

Esophageal squamous cell carcinoma (ESCC) is the predominant histological type of esophageal cancer and one of the most aggressive and lethal malignancies worldwide [1, 2]. Despite the development of multi-modal therapies, surgery still remains the first choice of treatment for resectable ESCC [3]. Outcomes are generally fairly poor and depend on the extent of the disease and the presence of medical conditions such as the metastasis of lymphadenopathy, hepatomegaly and pleural effusion in the late stages [4, 5]. Treatment is also complicated by the absence of early symptoms, thus resulting in delayed treatment. The 5-year overall survival rate is less than 20%, and more than half the patients develop recurrence within 2–3 years of surgical treatment [6, 7]. Metastasis is the major cause of ESCC-related death [8]. Therefore, exploring the molecular mechanism underlying carcinogenesis, progression and metastasis of ESCC will present clinical advantage in the treatment of this disease.

Nuclear receptor interacting protein 1 (NRIP1), also known as RIP140, is expressed in specific cell types in many tissues, and plays an essential role in the control of gene networks regulating female fertility and energy homeostasis [9–12]. NRIP1 interacts with many nuclear receptors, including the estrogen receptor (ER), Vitamin D receptor, peroxisome proliferator-activated receptors, estrogen-related receptors, thyroid receptors and glucocorticoid receptors [13–16]. NRIP1 in most cases acts as a co-repressor to inhibit target gene transcription not only by competing with co-activators but also by active repression through recruitment of histone deacetylases and carboxy-terminal-binding proteins [17, 18]. However, NRIP1 has also been reported to function as a co-activator of inflammatory gene expression.

Despite the many reports of NRIP1function, its role in ESCC has not been determined. Here, we report that NRIP1 inhibits the migration and invasion of ESCC cells and further study showed that both miR-548-3p and miR-576-5p enhance the migration and invasion of ESCC cells by targeting the 3'-UTR of NRIP1. Understanding the molecular correlation between miR-548-3p/miR-576-5p/NRIP1 activity and the migration and invasion of ESCC cells could lay the basis for future development of new therapeutic protocols in ESCC.

Patients and methods

Patients. Samples from a total of 52 ESCC patients, including the tumors and their adjacent tissues, were obtained from Zhongnan Hospital of Wuhan University.

All patients had squamous cell carcinomas of the thoracic esophagus but no history of any other cancers. All specimens were divided into two parts. One part was used to determine the histopathological type of the tumor, the grading, and the TNM classification according to the 1997 UICC criteria of pathological assessment. The other part was immediately snap-frozen in liquid nitrogen and stored in liquid nitrogen until RNA extraction. Informed consent was obtained from each subject and anonymity was protected. The characteristics of the patients are described in Table S1. The study was approved by the institute research ethics committee of the First Affiliated Hospital of Wenzhou Medical University (project license no. AHWM0401492) and was performed in accordance with the Helsinki declaration.

Cell lines and cell culture. All cell lines were obtained from the Cell Bank of Shanghai (Shanghai, China). SHEE SHEEC, and EC109 cells were cultured in 199 medium (Gibco BRL) with 10% new bovine serum. KYSE150, KYSE450. TE3 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco BRL) with 10% fetal bovine serum and 293T cells were cultured in Dulbecco modified Eagle medium (DMEM; Gibco BRL). All cells were maintained in a humidified 5% CO_2 incubator at 37 °C.

Plasmids. An NRIP1 construct was created by RT-PCR amplification of the open reading frame from KYSE450 cells. To create the NRIP1-encoding vector, the NRIP1 gene was amplified using the primers NRIP1 sense 5'-GTGTCGACATGACTCATGGAGAAGAGCTT-3' and NRIP1 antisense 5'-GTGGGCCCTTATTCTGATTCTTTCTTTATCG-3', in which Acc I and Apa I sites were introduced, respectively. The PCR product was cloned into Acc I and Apa I sites of pCMV-tag2B (Stratagene) to generate the plasmid pCMV-NRIP1, in which NRIP1 was tagged with FLAG. The 3'-UTR of NRIP1 was amplified by genomic PCR: sense 5'-TTACGCGTAATGTACCTGCCATCCAGTTTTG-3' and antisense 5'-GGCTCGAGGACAATGCATTTTTTCTCATTTA-3'. The PCR fragment was inserted in the MluI-XhoI sites of pGL3-Basic (Promega). All constructs were confirmed by DNA sequencing.

Cell migration and invasion assay. Cell migration and invasion assays were performed using 24-well inserts (Falcon cell culture inserts (8-μm pore size; BD Biosciences) with or without matrigel Biocoat (BD Biosciences) according to the manufacturer's instructions. In brief, 1×10^5 cells were suspended in 200 μl of serum free media and seeded in the upper chamber. A medium containing 10% FBS was added to the lower wells. After 24 h of incubation at 37 °C, the inserts were removed, and the upper surface of the membrane was wiped with cotton swabs to remove non-migrated cells. The cells were fixed and stained with 0.2% crystal violet solution and then counted by ImageJ cell counting plug-in (National Institutes of Health, Bethesda, MD, USA).

Establishment of stable NRIP1 knock-down cells. To generate stably transfected cells, EC 109 cells were transfected with shRNA against NRIP1 using Tfx-20 (Promega, Madison, WI, USA) according to the manufacturer's protocol. Monoclonal cell colonies were selected with puromycin at a concentration of 6mg/mL; (Sigma) for 14 days and real-time PCR analysis determined the efficiency of the knock-down cells. The short hairpin RNA (shRNA)-based RNAi expression vectors (shNRIP1 and shCtrl) were generated by Genepharma (Shanghai, China). Finally, the target sequence is given in Table S2.

Western blot. All protein extracts were prepared and quantified by protein assay kit (Bio-Rad). Polypeptides from cell lysates were separated on SDS/12% polyacrylamide gels cross-linked with N,N-methylenebisacylamide and electrophoretically transferred to nitrocellulose membranes. Nonspecific sites were blocked with 5% nonfat dried milk before being incubated with a specific antibody targeting the proteins assessed in this study. Abs against human NRIP1 (ab53740) and β-actin (ab8227) were purchased from Abcam. Blots were developed using SuperSignal Chemiluminescent reagent (Pierce, Rockford, IL, USA) and the stained membranes were analyzed by LAS-4000 image document instrument (FujiFilm, Tokyo, Japan).

Cell transfection and luciferase reporter gene assay. The indicated plasmids and miRNAs were transfected into the cells using SuperFect transfection reagent (QIAGEN, USA) according to the manufacturer's protocol. At 48 h post-transfection, cells were washed with PBS and lysed in lysis buffer (Promega) and the protein concentration of the lysate was determined using the Bio-Rad method. Five micrograms of lysate were mixed with 30 μl luciferase assay substrate (Promega, Madison, WI, USA) and luciferase activity was immediately detected by luminometer (GloMax 20/20; Promega).

RNA extraction and real-time PCR. Total cellular RNA was extracted with Trizol reagent (Invitrogen) and reverse transcribed using the Reverse Transcription System (Takara). The relative expression of miR-548-3p and miR-576-5p was measured by TaqMan microRNA assay (Applied Biosystems) according to the manufacturer's protocol. snRNA U6 was used as the endogenous control for qPCR of miRNA, and the relative mRNA expression levels were detected using the SYBR green qPCR assay (BioRad, Hercules, USA) according to the manufacturer's protocol and with β-actin as the endogenous control. The primers used are as follows: β-actin, sense 5'-GAGCACAGAGCCTCGCCTTT-3', antisense 5'-AGAGGCGTACAGGGATAGCA-3'. NRIP1, sense 5'-GAGCACTCCACCTTTACTTACAT-3', anti-sense 5'-CAATCATACCTATCGGTTTATCTG-3'.

Statistical analysis. Data is presented as mean ± standard deviation (SD), unless otherwise stated. The paired Student t-test determined the level of significance between the different groups and Spearman's correlation tested the significance of association between miR-548-3p, miR-576-5p expression and NRIP1 RNA expression. Statistical analysis was performed on SPSS software for Windows 7.0 (SPSS, Inc., Chicago, IL) and $p<0.05$ was considered statistically significant.

Results

NRIP1 inhibits the migration and invasion of ESCC cells. To determine the role of NRIP1 in the migration and invasion of ESCC cells, we performed a transwell migration and invasion assay. As shown in Figure 1A, over-expression of NRIP1 inhibited the migration and invasion of EC109 cells. We next designed 4 human NRIP1-specific short hairpin RNAs (shRNA) and tested their efficiency. Results from qRT-PCR and Western blot analysis showed that the shRNA#2 plasmids markedly inhibited the expression of NRIP1 (Figure 1B). In cell migration and invasion experiments, the knock-down of NRIP1 expression enhanced migration and invasion of ESCC cells (Figure 1C). The degree of enhancement correlated with the efficiencies of the shRNA-NRIP1 plasmid (Figure 1C). We then constructed NRIP1-knockdown EC109 cells using NRIP1 shRNA in a lentiviral system to ensure specific and stable gene silencing (Figure 1D). The NRIP1-knock-down cells showed increased migration and greater invasion capabilities than the control cells (Figure 1E), but these effects were blocked by transfection of NRIP1 over-expressed plasmids (Figure 1E). These observations strongly suggest that NRIP1 plays an important role in the migration and invasion of ESCC cells.

Identification of candidate miRNAs targeting NRIP1. We investigated microRNAs (miRNAs) role in the mechanism of NTIP1 activity. miRNAs are small, endogenous, non-coding RNAs which negatively regulate the stability of their target mRNAs at 3'-UTR. The PicTar, TargetScan, miRanda and miRGen programs were used to identify these miRNAs. Two high-scoring candidates, miR-548-3p and miR-576-5p, were selected. To examine whether these miRNAs affect the function of the 3'-UTR of NRIP1, the wild type NRIP1 3'-UTR and a mutated version with ablation of the miR-548-3p predicted target sites were cloned into a firefly luciferase reporter plasmid (Figure 2A). Reporter assays indicated that the miR-548-3p effectively reduced luciferase activity controlled by the wild type NRIP1 3'-UTR and showed no effect when the 3'-UTR sequence was mutated (Figure 2B). The miR-548-3p inhibitor increased luciferase activity controlled by the NRIP1 3'-UTR (Figure 2C) and had no effect on the mutated 3'-UTR construct (Figure 2C).

Similarly, we investigated whether miR-576-5p targets the 3'-UTR of NRIP1 mRNA. The potential target sites of miR-576-5p and corresponding mutated sites in the 3'-UTR of NRIP1 sequences are shown in Figure 2D. As shown in Figures 2E and F, the miR-576-5p mimic decreased luciferase activity in the wild-type reporter and the miR-576-5p inhibitor increased luciferase activity. However, in the experiments with the mutant NRIP1 3'-UTR luciferase reporter plasmids, the luciferase activity was not significantly changed by either over-expression or inhibition of miR-576-5p (Figures 2E and F). These effects are consistent with miR-548-3p and miR-576-5p directly targeting the NRIP1 3'-UTR.

We then determined the effect of miR-548-3p and miR-576-5p on the endogenous expression of NRIP1. Results from qRT-PCR and Western blot analysis show that NRIP1 mRNA and protein level were efficiently reduced by miR-548-3p and miR-576-5p, while higher levels of NRIP1 mRNA and protein were observed in miR-548-3p and miR-576-5p inhibitor transfected cells (Figures 2G and H).

To examine the expression pattern of miR-548-3p, miR-576-5p, and NRIP1 in ESCC cell lines, we initially examined the mRNA levels in a panel of six ESCC cell lines, a human immortalized esophageal epithelial cell line (SHEE), and the 293T cell line. Results from qRT-PCR analysis indicate that miR-548-3p and miR-576-5p are expressed at a relatively high level and NRIP1 is expressed at a lower level in ESCC cell lines compared to the levels detected in the SHEE

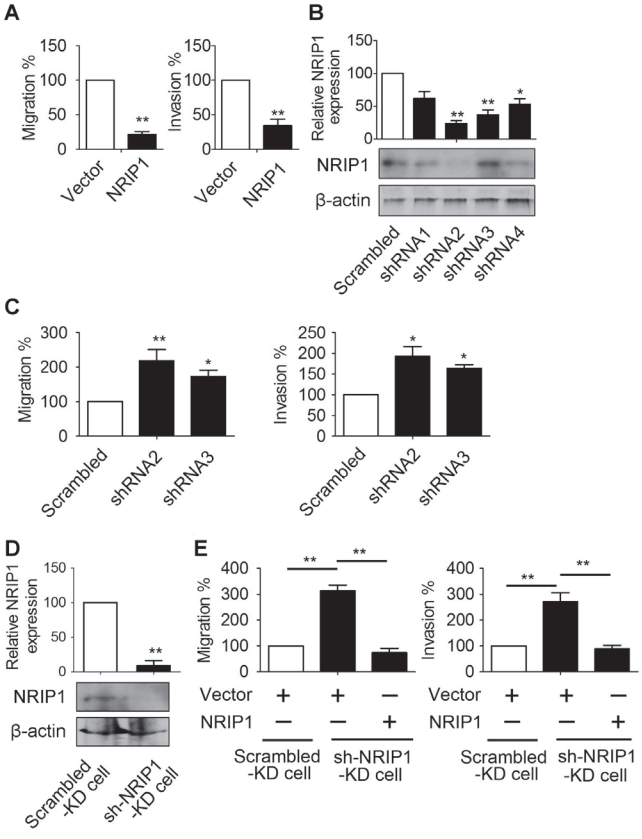

Figure 1. The effect of NRIP1 on the migration and invasion of ESCC cells. (A) EC109 cells were transfected with pCMV-NRIP1 or the vector control for 48 h prior to transwell migration and invasion analyses. (B) EC109 cells were transfected with the indicated NRIP1 shRNAs for 48h prior to qRT-PCR and Western blot analysis. (C) Experiments were performed as in A, except the indicated NRIP1 shRNAs or the shRNA control were used. (D) qRT-PCR analysis and Western blot were used to determine the expression of NRIP1 2 days after infection of EC109 cells with lentiviral vector pLKO.1 carrying shRNA for NRIP1 (shNRIP1-KD cell) or control shRNA (Scramble-KD cell). (E) Scramble-KD cells and shNRIP1-KD cells were transfected with the indicated expression plasmids or indicated shRNAs for 48 h prior to transwell migration and invasion analyses. Data are expressed as means ± S.D. (n = 3–5) (*, p<0.05 vs. control; **, p<0.01 vs. control).

and 293T cell lines (Figures S1 A–C). These combined results provide strong evidence that NRIP1 is a direct target gene of miR-548-3p and miR-576-5p.

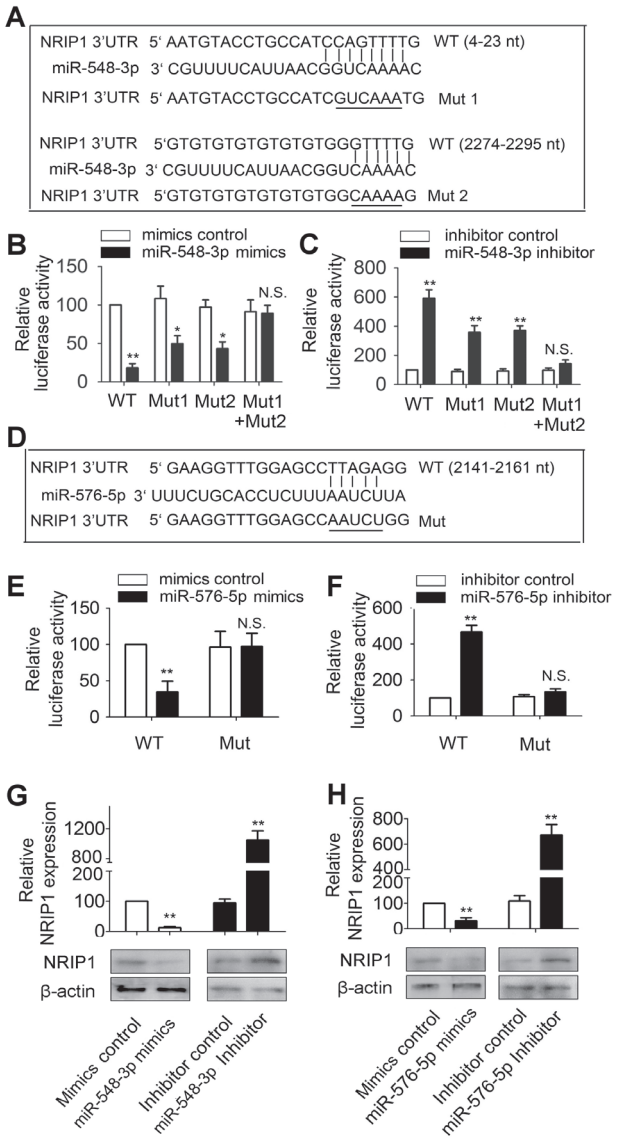

Figure 2. Identification of miRNAs targeting the 3'-UTR of NRIP1. (A) The predicted miR-548-3p targeting site in the NRIP1 3'-UTR and the UTR mutant. Perfect matches in the seed regions are indicated by a line. Mutations (underlined) were generated in the binding sites of 3'-UTR and miR-548-3p seed region for the reporter gene assay. (B) EC109 cells were co-transfected with WT or MUT NRIP1 3'-UTR reporter plasmids and miR-548-3p mimic for 48 hours prior to luciferase assays. (C) Experiments were performed as in B, except the miR-548-3p inhibitor or inhibitor control were used as indicated. (D) The predicted miR-576-5p targeting site in the NRIP1 3'-UTR and the UTR mutant. (E, F) Experiments were performed as in B and C, except the miR-576-5p mimic or miR-576-5p inhibitor were used indicated. (G) EC109 cells were transfected with miR-548-3p mimic or inhibitor for 48h prior to qRT-PCR and Western blot analysis. (H) Experiments were performed as in G, except miR-576-5p mimic or miR-576-5p inhibitor were used as indicated. Data are expressed as means ± S.D. (n = 3–5) (*, p<0.05 vs. control; **, p<0.01 vs. control).

miR-548-3p and miR-576-5p promote migration and invasion of ESCC cells. Since miR-548-3p and miR-576-5p directly target the 3'-UTR of NRIP1, we determined whether miR-548-3p and miR-576-5p regulate the migration and invasion of ESCC cells. In cell migration and invasion experiments, the miR-548-3p-mimic significantly up-regulated the migration and invasion of EC109 cells, and conversely, the miR-548-3p inhibitor down-regulated these effects (Figures 3A and B). Similar results were also obtained using the miR-576-5p-mimic and inhibitor-transfected cells (Figures 3C and D). This combined data indicates that miR-548-3p and miR-576-5p can induce ESCC cell migration and invasion.

miR-548-3p and miR-576-5p promote ESCC cell migration and invasion via NRIP1. To verify whether miR-548-3p and miR-576-5p affect the migration and invasion of ESCC cells via NRIP1, EC109 cells were co-transfected with the NRIP1 expression vector (or an empty vector) and the miR-548-3p or miR-576-5p mimics (or miR-Ctrl). Transwell migration and invasion assays showed that miR-548-3p and miR-576-5p mimics both induced migration and invasion in EC109 cells, but NRIP1 over-expression abolished mimic effect (Figure 4A). Consistently, the knock-down of NRIP1 induced the effect of the mimics on cell migration and invasion (Figure 4B). The effect of the miR-548-3p/miR-576-5p/NRIP1 axis on cell migration and invasion was then further evaluated using miR-548-3p and miR-576-5p inhibitors. We found that the inhibitors reduced the migration and invasion of EC109 cells, and that NRIP1 over-expression synergistically inhibited cell migration and invasion (Figure 4C). Conversely, high levels of migration and invasion were observed in EC109 cells when NRIP1 expression was inhibited by knock-down. This indicates that knock-down of NRIP1 expression is responsible for the ability of miR-548-3p and miR-576-5p to promote ESCC cell invasion and migration.

miR-548-3p and miR-576-5p expression was up-regulated and NRIP1 expression was down-regulated in ESCC cells and tissues. We further examined expression of miR-548-3p, miR-576-5p and NRIP1 in 52 pairs of ESCC tissue samples and adjacent normal tissues. As shown in Figures 5 A–C, the miR-548-3p and miR-576-5p expression was up-regulated and the NRIP1 expression was down-regulated in ESCC tissues compared to the expression in adjacent normal tissues. Further study revealed that both miR-548-3p and miR-576-5p inversely correlate with NRIP1in ESCC tissues (Figures 5D and E). This combined data indicates that altered miR-548-3p, miR-576-5p and NRIP1 expression correlates with ESCC.

Discussion

We investigated the role of NRIP1 in the migration and invasion of ESCC cells, and study revealed that NRIP1 is an important mediator of ESCC cell migration and invasion

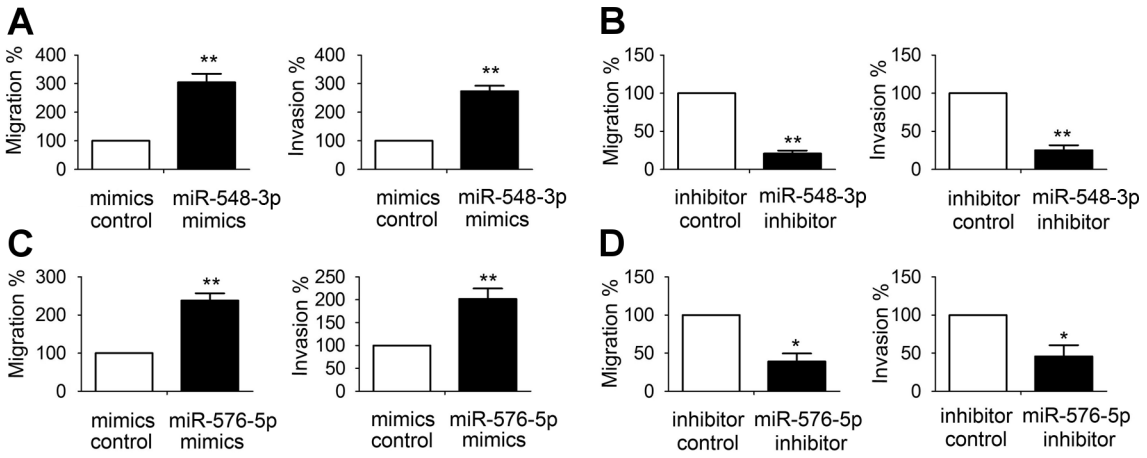

Figure 3. The effect of miR-548-3p and miR-576-5p on the migration and invasion of ESCC cells. (A) EC109 cells were transfected with miR-548-3p mimic or mimic control for 48 h prior to transwell migration and invasion analyses. (B) Experiments were performed as in A, except miR-548-3p inhibitor or inhibitor control were used. (C, D) Experiments were performed as in A and B, except miR-576-5p mimic or miR-576-5p inhibitor were used as indicated. Data are expressed as means ± S.D. (n = 3–5; *, p<0.05 vs. control; **, p<0.01 vs. control).

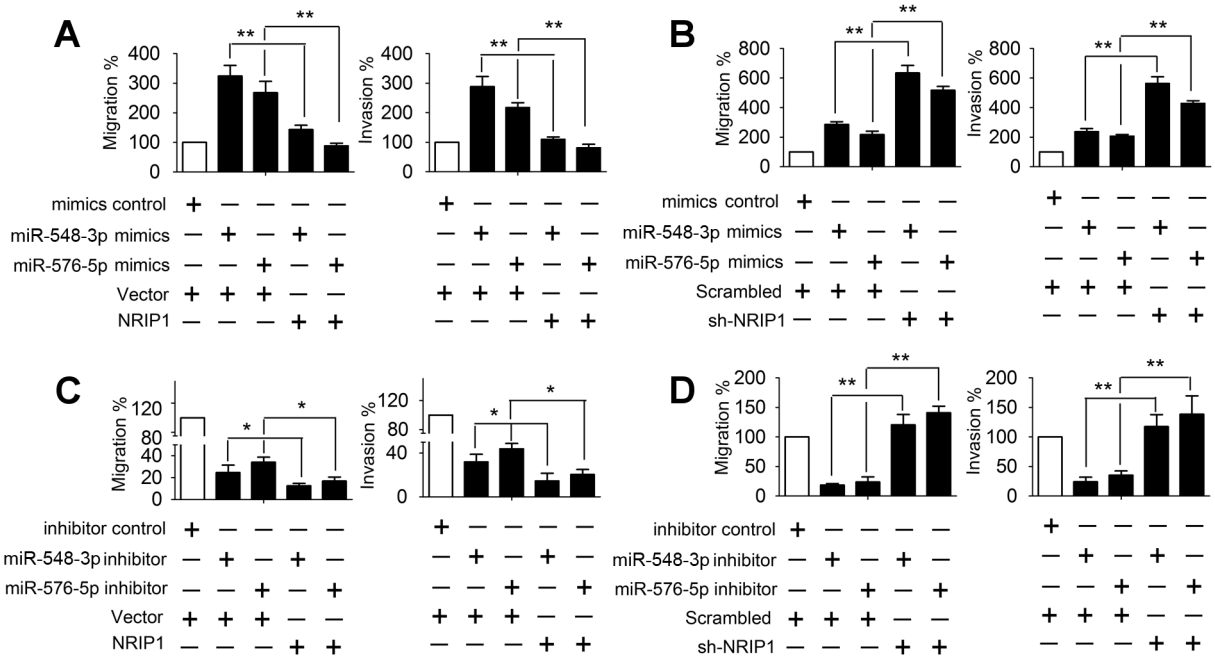

Figure 4. miR-548-3p and miR-576-5p promote the migration and invasion of EC109 cells through NRIP1 inhibition. (A) EC109 cells were transfected with the indicated miRNA mimic and plasmid for 48 h prior to transwell migration and invasion analyses. (B) Experiments were performed as in A, except NRIP1 shRNAs or shRNA control were used as indicated. (C, D) Experiments were performed as in A and B, except miR-548-3p inhibitor or miR-576-5p inhibitor were used as indicated. Data are expressed as means ± S.D. (n = 3–5; *, p<0.05 vs. control; **, p<0.01 vs. control).

because its over-expression and loss severely changed ESCC cell migration and invasion. We found that NRIP1 is targeted by two miRNAs (miR-548-3p and miR-576-5p) and demonstrated that miR-548-3p and miR-576-5p promote migration and invasion via NRIP1.

NRIP1, first identified in human breast cancer cells through its interaction with estrogen receptor α has been shown to interact with transcription factors, including ER and the following nuclear receptors; the vitamin D receptor and peroxisome proliferator-activated, estrogen-related, thyroid and glucocorticoid receptors [19, 20].

We then explored the role of NRIP1 in ESCC and found that NRIP1 inhibits the migration and invasion of ESCC cells. However, whether those nuclear receptors and transcrip-

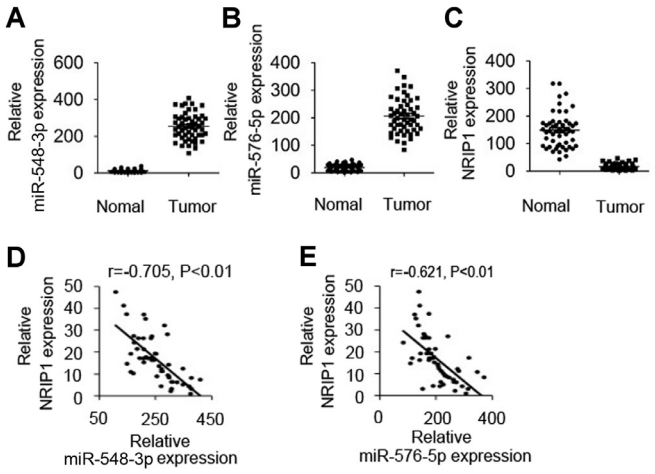

Figure 5. MiR-548 and miR-576-5p are negatively correlated with NRIP1 RNA expression in ESCC tissues. (A-C) The expression levels of miR-548 (A), miR-576-5p (B) and NRIP1 (C) were examined in tumor tissue (n=52) and adjacent tissue (n=52). (D, E) Pearson's correlation scatter plot of the fold change of miR-548 (D), miR-576-5p (E) and NRIP1 mRNA in tumor tissue (n=52). (*, p<0.05 vs. control; **, p<0.01 vs. control).

tion factors associated with NRIP1 also participate in ESCC progression remains an important question for future investigation. Although not explicitly examined here, protein modification including phosphorylation, arginine methylation and acetylation have been shown to have important roles in NRIP1 function [21–23]. Determining if the post-translational modification of NRIP1 also influences ESCC metastasis is a further direction for future research.

We sought to determine the potential molecular mechanism responsible for NRIP1 reduced cell migration and invasion. Here, we identified the two miR-548-3p and miR-576-5p miRNAs that inhibit NRIP1 expression by directly targeting its 3'-UTR. These microRNAs induced ESCC cell migration and invasion through NRIP1. While a number of reports have identified miRNAs' role in tumor proliferation, apoptosis, differentiation, invasion and metastasis [24–26], there are few reports of the function of miR-548 and miR-576. To our knowledge, there are two applicable studies of miR-548 and two studies that report the function of miR-576.

These are: (1) Li. et al found that miRNA-548 directly targeted the 3'-UTR of IFN-λ1, leading to down-regulation of the host antiviral response [27]: (2) Shi. et al found that miR-548-3p suppressed the proliferation of breast cancer cells by ECHS1 [28]: (3) Liang. et al reported that miR-576-3p inhibits proliferation in bladder cancer cells by binding the 3'-UTR of Cyclin D1 [29] and (4) Yarbrough. et al found that miR-576-3p, which is induced by IRF3, down-regulated interferon expression, to prevent excessive innate immune signaling [30]. In this manuscript, we identify a novel role for miR-548-3p and miR-576-5p in ESCC.

In conclusion, our study identified the role of miR-548-3p, miR-576-5p and NRIP1 in the migration and invasion of ESCC cells for the first time. Although precise details of miR-548-3p, miR-576-5p and NRIP1 molecular mechanisms still require elucidation, our results suggest that the two miRNAs and their target have exciting potential for future esophageal squamous cell carcinoma therapy.

Supplementary information is available in the online version of the paper.

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MicroRNA-548-3p and MicroRNA-576-5p enhance the migration and invasion of esophageal squamous cell carcinoma cells via NRIP1 down-regulation

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Supplemental Material

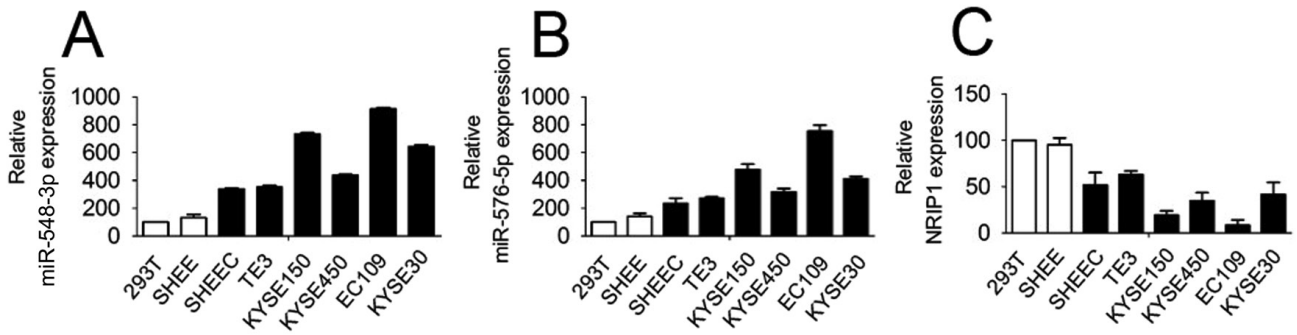

Figure S1. MiR-548 and miR-576-5p are negatively correlated with NRIP1 RNA expression in ESCC cell lines. (A-C) The expression of miR-548 (A), miR-576-5p (B) and NRIP1 (C) were measured in a panel of cell lines by qRT-PCR analysis. Six ESCC cell lines (SHEEC, TE3, KYSE150, KYSE450, EC109, and KYSE30), a human immortalized esophageal epithelial cell line (SHEE), and the 293T cell line were analyzed. Data are expressed as means ± S.D. (n = 3–5; *, p<0.05 vs. control; **, p<0.01 vs. control).

Table S1. Correlation of miR-548, miR-576-5p and NRIP1 expression according to clinicopathological characteristics.

Clinicopathologic parameters	Case no.	miR-548 expression (folds)	p-value	miR-576-5p expression (folds)	p-value	NRIP1 expression (folds)	p-value
Tissue type							
Normal tissue	52	12.44±4.77	<0.01	19.36±7.9	<0.01	148.87±62.34	<0.01
Tumor tissues	52	254±72.03		206.29±63.81		16.56±7.93	
Sex (only tumor tissues)							
Male	30	266.51±58.1	ns	221.23±48.91	ns	18.93±6.31	ns
Female	22	241.49±43.29		191.35±40.33		14.19±5.76	
Age (only tumor tissues)							
≤60	28	248.24±50.23	ns	214.31±39.24	ns	15.07±5.39	ns
>60	24	259.76±46.82		198.27±42.81		18.05±6.77	
T stage							
T1	13	154.21±24.33	<0.01	140.28±29.74	<0.01	31.24±10.82	<0.01
T2	10	207.62±35.07		181.52±34.18		18.28±7.11	
T3	11	243.22±37.15		207.33±41.07		9.18±4.39	
T4	18	351.25±40.39		296.03±44.25		3.24±2.71	
N stage							
N1	21	173.36±41.71	<0.05	107.25±32.52	<0.05	27.18±8.19	<0.01
N0	31	334.51±51.28		305.47±47.88		5.94±4.55	

Table S2. The target sequence of NRIP1 shRNAs.

miRNAs	target sequence (5' to 3')
NRIP1#1	GTACTACAGCTTATTGACAT
NRIP1#2	GAAAAATCAGAATCTGACC
NRIP1#3	AGATACCTCAAAGAATTCTA
NRIP1#4	ACAGCTTATTGACATCAGTA
siRNA-control	GTTCTCCGAACGTGTCACGT