Antisense lncRNA NNT-AS1 Promoted Esophageal Squamous Cell Carcinoma Progression by Regulating Its Sense Gene NNT Expression

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Abstract

Background

Antisense lncRNAs were endogenous productions from the antisense strand of coding genes and transcribed in the opposite direction of sense gene. This study aimed to systematically evaluate the roles and functions of antisense lncRNAs in esophageal squamous cell carcinoma (ESCC).

Methods

Differentially expressed antisense lncRNAs were initially screened using transcriptome data from 119 paired ESCC samples in GSE53624, and were further validated in 6 paired ESCC samples from our institution. Log-rank test was adopted to identify ESCC prognosis associated lncRNAs. Finally, functional assays were performed to reveal the functions of our identified antisense lncRNAs.

Results

In total, 174 antisense lncRNAs were differentially expressed in both GSE53624 and JSPH samples. Five of them were significantly associated with ESCC prognosis (NNT-AS1, NKILA, CCDC18-AS1, SLCO4A1-AS1 and AC110619.1). The upregulation of NNT-AS1 was validated in ESCC cell lines. Knockdown of NNT-AS1 inhibited ESCC cell proliferation, migration, and promoted ESCC cells apoptosis and induced cell cycle arrest in G2/M stage. NNT-AS1 expression was significantly correlated with its sense gene NNT and NNT-AS1 knockdown could suppress NNT expression. Inhibition of NNT suppressed ESCC cell proliferation and migration. Mechanically, NNT-AS1 served as a competing endogenous RNA to sponge the miR-382-5p, which could repress NNT expression. Pathway enrichment analysis and western blot assay indicated that NNT-AS1 and NNT could regulate the cell cycle pathway.

Conclusion

Antisense lncRNA NNT-AS1 promoted ECSS progression by targeting NNT through sponging miR-382-5p. This study provided us a deeper insight into the roles of antisense lncRNAs in ESCC and identified potential therapeutic targets.

Background

Esophageal carcinoma (ESCA) is one of the major malignant tumors threatening global health. Esophageal cancer incidence ranks the seventh of all malignancies, with mortality ranking the sixth [1, 2]. Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (ESAD) account for the major histological subtypes of esophageal cancer [3]. ESCC has a particularly high incidence in eastern Asia, eastern and southern Africa [4]. Through decades of clinical advances, the five-year survival rate of ESCC remains low [5, 6]. Therefore, it is urgent to identify novel molecular targets to improve the prognosis of ESCC patients.

Long non-coding RNAs (lncRNAs), which were thought to be transcriptional ‘noises’ formerly, have been investigated to play crucial roles in tumor progression via varied mechanisms in tumors [7, 8]. LncRNAs can be classified into several categories based on their positions relative to protein-coding regions: long intergenic noncoding RNAs, natural antisense transcripts, overlapping transcripts, bidirectional lncRNAs, and sense intronic lncRNAs [9]. Antisense lncRNAs are endogenous productions in nature that formed from the antisense strand of coding genes and transcribed in the opposite direction. They overlapped with sense genes or regulatory regions and could function both in cis or trans [10, 11]. Antisense lncRNAs could regulate protein-coding sense genes through the following mechanisms: transcriptional collision, gene recombination, promotor inactivation, alternative splicing, miRNA binding sites blocking and endogenous siRNA formation [12, 13, 14]. For example, FOXP4-AS1 served as a sponge to sequester miR-3184-5p to upregulate FOXP4 in prostate cancer [15]. SATB2-AS1 could cis-activate SATB2 transcription by histone H3K4me3 deposition and DNA demethylation in the promoter region of SATB2 through binding to WDR5 and GADD45A [16].

In ESCC, a few antisense lncRNAs have been identified contributing to the tumorigenesis or progression in previous studies. For example, ZEB1-AS1 down-regulation suppressed the proliferation and invasion by inhibiting ZEB1 expression [17]. EZR-AS1 interacted with SMYD3 to promote SMYD3 binding to the EZR gene, accelerating ESCC cell growth in vitro and in vivo [18]. Upregulation of ZNF667-AS1 increased ZNF667 expression by interacting with TET1 and UTX, which could decrease histone H3K27 trimethylation to activate ZNF667, suppressing ESCC proliferation and migration [19]. KLF3-AS1 suppressed ESCC cell invasion and migration by decreasing miR-185-5p-mediated inhibition of KLF3 [20]. All these studies suggested the important roles of antisense lncRNAs and their sense genes in ESCC. However, the roles of antisense lncRNAs in ESCC progression have not been systematically evaluated.

In this study, we aimed to systematically evaluate the roles and functions of antisense lncRNAs in the progression of ESCC. Firstly, differentially expressed antisense lncRNAs (Annotated using Gencode V29) were screened using transcriptome data from 119 paired ESCC samples in GSE53624, and were further validated in 6 paired ESCC samples from our Jiangsu Province Hospital (JSPH, Jiangsu, China) database. Then, the log-rank test was adopted to assess the associations between promising antisense lncRNAs and ESCC prognosis. Finally, functional assays, including MTT assays, colony formation assays, EdU assays, transwell assays and Flow cytometry assays were performed to reveal the functions of our identified antisense lncRNA.

Materials And Methods

Tissue samples and transcriptome sequencing

Six paired ESCC and normal tissues were collected from patients who received surgical treatment at JSPH. Total RNA was extracted from the samples. Then ribosomal RNAs were removed by TruSeq StrandedTotal RNA with Ribo-Zero Gold Kit (Illumina, California, USA) and the residual RNAs were broken into short fragments. Using the broken RNA as a template, the first strand of cDNA was synthesized with

arbitrary primers, and then the second-strand synthesis reaction system was prepared to synthesize the second strand of cDNA, during which dTTPs were replaced by dUTPs. One strand containing dUTP was digested using the UNG method, and the other strand was purified. After purification, the end of cDNA was repaired and poly-A tail was added as well as the sequencing connector was connected to the cDNA. Finally, the RNA library was established by PCR amplification and assessed by Agilent2100Bioanalyzer. The sequencing was performed using the Illumina sequenator (California, USA) The current study was approved by the ethic committee of the first affiliated hospital of Nanjing medical university and the written informed consent was acquired by each patient.

Cell culture and transfection

Human ESCC cell lines (Eca-109, Kyse-30, and TE-1) and human normal esophageal epithelial cell line (HEEC) were purchased from iCell Bioscience (Shanghai, China). RPMI-1640 medium (Gibco, Rockville, USA) that contained 10% fetal bovine serum (FBS; Biological Industries, Israel), penicillin-G (100 U/ml) and streptomycin (100 g/ml) (Gibco, Rockville, USA) was used for Kyse-30 and TE-1 incubation. DMEN (Gibco, Rockville, USA) that contains the same ingredients was used for Eca-109 and HEEC. All the cell lines were cultured at 37℃ and 5% CO2. The sequence of transfected RNAs together with the name of suppliers were listed in **Table S1**. For overexpression of NNT, the cDNA encoding NNT was amplified and cloned into pcDNA3.1 vector (Invitrogen, Carlsbad, USA). Transfections were carried out using Lipofectamine3000 (Invitrogen, Carlsbad, USA) in opti-MEM medium (Gibco, Rockville, USA) according to the manufacturer's suggestions.

Quantitative Real-time PCR (qRT-PCR)

The cell lines at the period of logarithmic growth were transfected for 48 h. Then the cultured cells were collected to extract total RNA by using TRIzol reagent (Invitrogen, Carlsbad, USA). The extracted RNAs were reversely transcribed to cDNAs using PrimeScriptTM II Reverse Transcriptase (Takara, Tokyo, Japan). QRT-PCR analysis was carried out to determine the gene expression through $2^{-\Delta\Delta CT}$ method using SYBR Green PCR Kit (Takara, Tokyo, Japan) on QuantStudio 7 (Thermo Fisher, California, USA). Primer sequences were illustrated in **Figure S1**.

Western blot assay

The proteins were extracted using ice-cold RIPA lysis buffer (Beyotime, Shanghai, China) with Protease Inhibitor Cocktail (MCE, Shanghai, China), The protein concentration was measured by the BCA Protein Assay kit (Thermo Scientific, Massachusetts, USA). The proteins with loading buffer (volume ratio = 1:4) were added in lanes of 4%-20% SurePAGE (GeneSript, Nanjing, China). After electrophoresis, the protein gel was transferred to PVDF membranes (Millipore, Massachusetts, USA) and then the membranes were blocked in 5% skim milk for 2 hours. Following, the membranes were incubated in specific antibodies as follows: NNT (1:500 dilution), CDK1 (1:1000 dilution), CDK2 (1:1000 dilution), CCNB1 (1:1000 dilution), CCNB2 (1:1000 dilution), and GAPDH (1:2000 dilution). All the antibodies mentioned above were purchased from Abcolonal, Wuhan, China. Clarity Western ECL Substrate (Bio-Rad, California, USA) was employed to detect the bands through GelDoc XR+ ((Bio-Rad, California, USA).

5-Ethynyl-2'-deoxyuridine (EdU) assay

1×10^5 transfected cells were added evenly in 96-well plate per well and incubated for 2 days. EdU assays were performed using YF®555 Click-iT EdU Imaging Kits (US EVERBRIGHT, Suzhou, China) under the instruction of the manufacturer. The treated cells were observed under an inverted fluorescence Microscope and the graphs were captured simultaneously.

MTT assay

2×10^3 transfected cells were planted in 96-well plate per well. At different checking points (0, 24, 48, 72, and 96h), the cells were incubated in complete medium with 20μL MTT (concentration: 0.5 mg/mL) for 4 h. Then, the medium was removed and DMSO (150 μL per well) was added to dilute the formazan that formed from MTT. The cell viability was measured by the absorbance at 490 nm.

Colony forming assay

2×10^3 transfected cells were planted in 6-well plate per well and incubated in complete medium until the colonies could be detected by naked eyes, during which the medium was replaced every 5 days. Then the culture medium was discarded and formed colonies were washed with normal saline (NS) and fixed with 4% paraformaldehyde at room temperature for 15 min. After fixation, the colonies were stained with 0.5% crystal violet staining. The number of colonies was detected by ImageJ software.

Flow cytometry assays

5×10^5 cells were cultured in 6-well plate and transfection was performed 24h later. 48h after transfection, cells were collected and then fixed in 75% ice-cold ethanol followed by staining with PI to detect the cell cycle distribution. Cell apoptosis assay was carried out according to the protocol of YF®488-Annexin V and PI Apoptosis Kit (US EVERBRIGHT, Suzhou, China). The results were assessed by FACSCalibur (BD, New Jersey, USA). Data were analyzed with FlowJo software version 10.8.0 (BD, New Jersey, USA).

Animal experiment

Stably transfected cell lines with NNT-AS1 silence (shNNT-AS1) or negative control (shCtrl) were constructed for this experiment. Eca-109 cells were incubated in 6-well plates for 24h and then infected with lentivirus-coated shRNA, which contained the green fluorescent protein (GFP). The cells that had not been successfully transfected were killed by puromycin (1:10 000). The transfection efficiency was determined by observing the fluorescence intensity under a fluorescence microscope and qRT-PCR was utilized for accurate evaluation.

Six Nude male mice (6 weeks old) were purchased from Weitonglihua (Beijing, China). The stably transfected Eca-109 cells were injected into the back of the mice, right for shNNT-AS1 and left for shCtrl respectively. Tumor volume was measured and recorded every 3 days basing on the formulas: V = larger diameter × (smaller diameter)^2/2. The nude mice were euthanized on the 15th day, then the tumors were dissected and weighted.

Dual-luciferase assay

The potential binding sites of miR-382-5p and NNT-AS1 or NNT were predicted by Starbase (http://starbase.sysu.edu.cn/). The binding site of miR-382-5p in NNT-AS1 and NNT 3′UTR were cloned in the pmirGLO vector (Promega, Madison, USA) and generated corresponding luciferase reporter vectors (NNT-AS1 WT, NNT-AS1 MUT, NNT WT, and NNT MUT). MiRNAs and luciferase reporter vectors were co-transfected in Eca-109 cells respectively using Lipofectamine 3000 (Invitrogen, Carlsbad, USA). Luciferase assays were carried out using the Dual-Luciferase Reporter Assay Kit (Vazyme, Nanjing, China) according to the protocols.

Transwell assay

Transwell assays were carried out using Transwell apical chamber (Corning Life Sciences, Corning, NY, USA). 5×10^4 transfected cells with 300μl serum-free medium were added into the upper compartment while 700μl complete medium into the lower part. After 24h, the cells were washed twice and fixed with 4% paraformaldehyde at room temperature for 15 min. Following fixation, the cells were stained with 0.5% crystal violet staining. The cells on the inner surface of the upper compartment were scrubbed by the cotton swab. The number of migratory cells was counted by ImageJ software.

Statistical analysis

In this study, differential expression analysis was performed using paired student's t test and the false discovery rate (FDR) was used to correct the *P* values. Log-rank test was adopted to evaluate the associations between lncRNAs expression and ESCC prognosis. Differences between experimental groups were determined using the student's t-test. The relationship between NNT-AS1 expression and NNT expression was assessed using Pearson correlation. All these analyses were performed based on R 3.6.0 and Graphpad Prism 8.0. $P < 0.05$ was considered statistically significant.

Results

1. NNT-AS1 was aberrantly upregulated in ESCC and associated with a poorer prognosis

Firstly, the differentially expressed antisense lncRNAs were screened in the GSE53624 database that contained 119 paired ESCC and adjacent tissues. Antisense lncRNAs annotation was performed based on Gencode V29. In total, 1,386 promising antisense lncRNAs were initially identified. Further, 174 of them were validated in our JSPH samples (**Figure S1**). The survival analysis showed that 5 of 174 antisense lncRNAs were significantly associated with poorer ESCC prognosis: NNT-AS1, NKILA, CCDC18-AS1, SLCO4A1-AS1, AC11069.1 (Fig. 1a), of which NNT-AS1 showed the most significant association with ESCC prognosis. The normalized NNT-AS1 expression was aberrantly up-regulated in ESCC tumor tissues in both GSE53624 (Fig. 1b) and the JSPH database (Fig. 1c). The high expression of NNT-AS1 was significantly associated with a poorer prognosis of ESCC patients (Fig. 1d). Likewise, the expression of NNT-AS1 was upregulated in ESCC cell lines (Eca-109, Kyse-30, TE-1) compared with HEEC (Fig. 1e).

2. Knockdown of NNT-AS1 suppressed ESCC cell proliferation, migration,

induced cell cycle arrest, and promoted cell apoptosis.

We designed two siRNAs to knock down the expression of NNT-AS1. The knockdown efficiency was shown in Fig. 2a. Colony formation assays, MTT assays, and EdU assays were conducted to assess the capacity of cell proliferation. The number of colonies was reduced after NNT-AS1 knockdown (Fig. 2b) and the cell viability of Eca-109 and Kyse-30 was inhibited as well (Fig. 2c**)**. NNT-AS1 silence resulted in the decrease of cell proliferation rate according to the EdU assays (Fig. 2d). We then carried out flow cytometry cell cycle assays. As speculated, the depletion of NNT-AS1 significantly decreased the proportion of the cells in the S phase and induced the G2/M arrest in both cell lines (Fig. 2e). In addition, cell apoptosis assays showed that the apoptosis rate was increased after silencing NNT-AS1 (Fig. 2f). Furthermore, transwell assays revealed that downregulation of NNT-AS1 suppressed migration abilities of ESCC cell lines (Fig. 2g). Taken together, NNT-AS1 knockdown restrained ESCC cell proliferation, migration, and induced ESCC cell cycle arrest and promoted cell apoptosis.

3. NNT-AS1 positively regulated the expression of its sense gene NNT, which served as an oncogene in ESCC as well.

As shown in Fig. 3a, NNT-AS1 expression was significantly positively correlated with its sense gene NNT expression (Fig. 3a, r = 0.915, $P < 0.001$). NNT was also up-regulated in ESCC tumor tissues (Fig. 3b-c, GSE53624 and JSPH). Similarly, NNT expression showed significant association with the prognosis of ESCC patients (Fig. 3d, HR = 1.44 (1.01–2.11), $P = 0.031$, GSE53624 + GSE53622). Consistent with tissue samples, the NNT expression in ESCC cell lines was higher than that in HEEC (Fig. 3e). As expected, inhibition of NNT-AS1 suppressed the expression of NNT in mRNA level (Fig. 3f) and such a tendency was reflected in the protein level **(**Fig. 3g).

Three siRNAs were synthesized to knock down NNT (Fig. 4a). MTT (Fig. 4b), colony formation (Fig. 4c) and EdU (Fig. 4d) assays indicated that NNT knockdown significantly inhibited the proliferation of ESCC cells. Transwell assay showed that inhibition of NNT suppressed ESCC cells migration. All these results revealed that NNT functioned as an oncogene in the tumorigenesis and progression of ESCC.

4. NNT-AS1 could serve as a ceRNA to sponge the miR-382-5p in ESCC.

LncRNA-miRNA-mRNA axis was a crucial mechanism in ESCC progression [21, 22], thus we predicted the NNT-mediated miRNA using ENCORI database and 49 miRNAs were screened. Subsequently, we analyzed the differentially expressed miRNAs based on the GSE114110 database and 6 miRNAs of 49 candidates were markedly downregulated, including miR-26a-5, miR-26b-5p, miR-186-5p, miR-382-5p, miR-130a-3p, miR-582-5p. Among them, the foldchange of miR-382-5p ranks first. Additionally, miR-382 has been verified to functions as a tumor suppressor against ESCC [23]. Thus, we hypothesized that NNT-AS1 might regulate the expression of NNT through sponging miR-382-5p. The binding site of NNT-AS1 and

miR-382-5p is illustrated in Fig. 5a. As anticipated, miR-382-5p was downregulated in both ESCC tissues and cell lines (Fig. 5b-c).

MiR-382-5p mimics significantly upregulated the expression of miR-382-5p while the inhibitor suppressed miR-382-5p expression (Fig. 5d). We found that miR-382-5p mimics downregulated the expression of NNT-AS1 in Eca-109 and Kyse-30 (Fig. 5e) and transfected with si-NNT-AS1 upregulated miR-382-5p expression (Fig. 5f). In addition, dual-luciferase reporter assays indicated that miR-382-5p upregulation weakened the luciferase activity in the NNT-AS1-WT group but failed to affect that of NNT-AS1-Mut. Furthermore, the rescue assays demonstrated that miR-382-5p inhibition partially rescued the decline of proliferation and migration abilities mediated by NNT-AS1 knockdown (Fig. 5h-i). These findings suggested that NNT-AS1 could serve as a ceRNA to sponge the miR-382-5p in ESCC.

5. NNT was negatively regulated by miR-382-5p and NNT overexpression retrieved the decreased cell functions mediated by NNT-AS1 silence.

The potential binding site of miR-382-5p and NNT was exhibited in Fig. 6a. When co-transfected with NNT-WT and miR-382-5p mimics, the luciferase activity was weakened compared to miR-NC in Eca-109, while NNT-Mut failed to induce such a reduction. In contrast, co-transfection of NNT-WT and miR-382-5p inhibitor resulted in the increased luciferase activity in comparison with the miR-NC (Fig. 6b). Furthermore, miR-382-5p partly rescued the downregulation of the NNT mRNA and protein expression mediated by NNT-AS1 knockdown (Fig. 6c-d). NNT overexpression enhanced ESCC cells proliferation and migration. As expected, overexpression of NNT could countervail the suppressed ESCC cells proliferation and migration induced by NNT-AS1 inhibition (Fig. 6e-g).

6. NNT-AS1 regulated ESCC cell growth in vivo and regulated cell cycle signaling pathway.

Oncogenesis assay in nude mice showed that Eca-109 cells stably transfected with shNNT-AS1 had a smaller tumor volume than the shCtrl group (Fig. 7a-b). The average tumor weight measured on the 15th day was distinctly lower in the shNNT-AS1 group (Fig. 5c). Moreover, the positivity for Ki-67 was markedly decreased in tumors formed from Eca-109 cells that stably transfected with shNNT-AS1 (Fig. 7d). All the findings suggested that the silence of NNT-AS1 could suppress tumor growth in vivo.

Pathway enrichment analysis suggested that NNT might modulate multiple signaling pathways, including the cell cycle pathway, one of the central pathways that contributed to cancers (Fig. 7e). This result was consistent with the above flow cytometry tfindings. Western blot assays indicated that when transfected with si-NNT-AS1 and si-NNT, the expression levels of cyclin B1, cyclinB2, CDK1 and CDK2 were suppressed (Fig. 7f). Taken together, NNT regulated the functions of ESCC cells through the cell cycle pathway, at least partly.

Discussion

In this study, we performed a systematic analysis to evaluate the roles of antisense lncRNAs in the tumorigenesis and progression of ESCC. As a result, NNT-AS1 was found aberrantly up-regulated in ESCC tumor tissues and was significantly associated with a poorer ESCC prognosis. Functionally, we found that NNT-AS1 might contribute to ESCC progression by positively regulating its sense gene NNT expression by sponging miR-382-5p.

NNT-AS1, located in 5p12, has been verified to play a carcinogenic role in various malignant tumors [24]. For instance, NNT-AS1 promoted lung squamous cell carcinoma progression by regulating the miR-22/FOXM1 axis [25]. NNT-AS1 promoted cell proliferation and invasion through the Wnt/β-catenin signaling pathway in cervical cancer [26]. Consistent with previous studies, we found the significant upregulation of NNT-AS1 in ESCC, which was associated with a poorer prognosis for the first time. Functionally, the depletion of NNT-AS1 inhibited cell proliferative and migratory abilities, induced cell cycle arrest, and boosted cell apoptosis. NNT, located in 5p12 as well, is the sense transcript of NNT-AS1 that encodes an integral protein of the inner mitochondrial membrane. Some studies have demonstrated the tumorigenic role of NNT in malignant tumors. For example, the knockdown of NNT significantly suppressed gastric cancer growth and metastasis through the oxidative stress pathway [27]. NNT regulated mitochondrial metabolism in non-small cell lung cancer through maintenance of Fe-S protein function [28]. Nevertheless, the roles that NNT plays and the regulatory mechanism between NNT and NNT-AS1 in ESCC remain unclear. As our study demonstrated, both NNT-AS1 and NNT were upregulated in ESCC tissues and cell lines. NNT-AS1 knockdown downregulated the expression of NNT in mRNA and protein levels. Functionally, the silence of NNT exerted inhibitory effects on the growth and migration of ESCC cells, while NNT overexpression could partially retrieve the decreasing function that NNT-AS1 silence mediated. Additionally, tumor xenograft experiment in nude mice exhibited that knockdown of NNT-AS1 suppressed tumor growth in vivo.

lncRNAs could serve as molecular sponges for miRNAs, ulteriorly resulted in the degradation and translation inhibition of target genes [29, 30]. In this study, we found the evident downregulation of miR-382-5p in ESCC. Through dual luciferase assays, we verified that miR-382-5p could bind to NNT-AS1 and the 3’UTR of NNT. MiR-382-5p inhibition retrieved the downregulation of NNT expression that NNT-AS1 knockdown mediated in ESCC cell lines. Functionally, rescue experiments further verified that downregulation of miR-382-5p reversed the suppressive effects of NNT-AS1 knockdown on ESCC progression. Additionally, miR-382-5p was reported as a tumor suppressor in ESCC and was associated with a favorable prognosis in previous studies [23, 31], which was consistent with the present study. All the findings above suggested that NNT-AS1 acted as a ceRNA by sponging miR-382-5p to regulate its sense gene NNT.

Previous studies have identified that knockdown of oncogenic lncRNAs induced G2/M arrest through the cell cycle pathway in many malignancies [32, 33]. In this study, NNT-AS1 knockdown induced the G2/M arrest in ESCC cell lines. Besides, pathway enrichment analysis hinted that the cell cycle pathway might be the pathway through that NNT modulated the ESCC progression. Western blot assays confirmed that inhibition of NNT and NNT-AS1 suppressed the expression levels of CDK1, CDK2, CCNB1, and CCNB2. All

these findings suggested that NNT-AS1 and NNT could promote ESCC progression through regulating cell cycle signaling pathway.

Conclusions

Antisense lncRNA NNT-AS1 could promote ESCC progression by targeting its sense gene NNT through competitively sponging miR-382-5p. This study provided us a deeper insight into the roles of antisense lncRNAs in the progression of ESCC and identified novel potential therapeutic targets for ESCC.

Abbreviations

ESCA
Esophageal carcinoma
ESCC
Esophageal squamous cell carcinoma
ESAD
Esophageal adenocarcinoma
lncRNA
Long non-coding RNA
JSPH
Jiangsu Province Hospital
NNT
Nicotinamide nucleotide transhydrogenase
NNT-AS1
Nicotinamide nucleotide transhydrogenase antisense 1
EdU
5-Ethynyl-2'-deoxyuridine
UNG
Uracil-N-Glycosylase
PCR
Polymerase chain reaction
RT-qPCR
Quantitative reverse transcription PCR
HEEC
Human normal esophageal epithelial cell line
PVDF
Polyvinylidene difluoride
CDK
Cyclin-dependent kinase
CCN

Cyclin

GAPDH

Glyceraldehyde-3-phosphate dehydrogenase

NS

Normal saline

MiRNA

MicroRNA

SiRNA

Small interfering RNA

ShRNA

short hairpin RNA

GFP

Green fluorescent protein

FDR

False discovery rate

UTR

Untranslated regions

CeRNA

Competing endogenous RNA

Declarations

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Ethics approval and consent to participate

All the experiments were approved by the ethic committee of the first affiliated hospital of Nanjing medical university and the informed consent forms were acquired by each patient.

Consent for publication

Not applicable.

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions:

PXL and WQ: Conceptualization, Writing-original draft, Methodology, Formal analysis. YY: Data curation, Methodology, Formal analysis, Resources. WWB: Supervision, Resources, Funding acquisition. CL: Data curation, Methodology, Conceptualization. WW and LZH: Conceptualization, Writing-original draft, Project administration, Resources, Funding acquisition.

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Figures

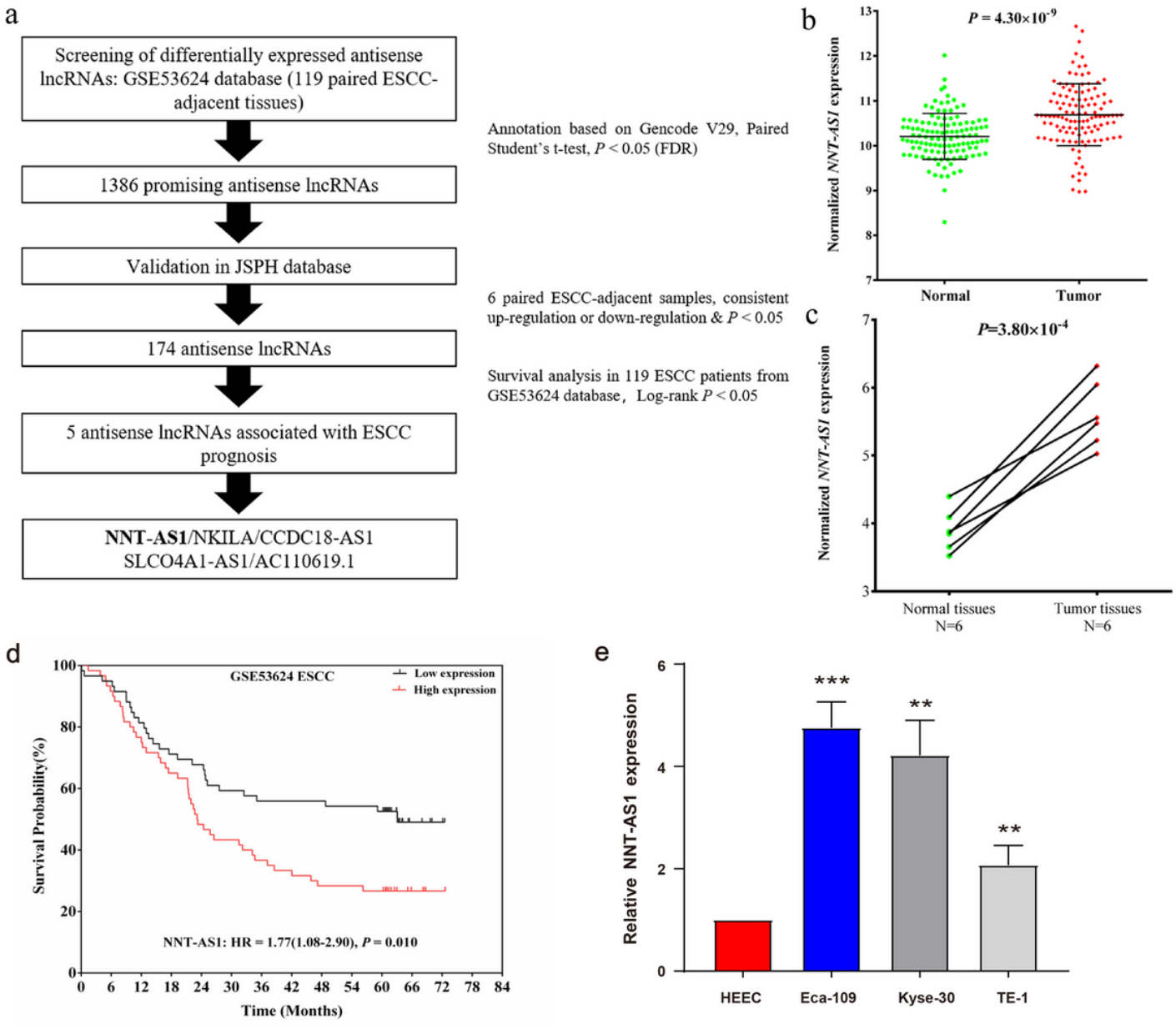

Figure 1

NNT-AS1 was aberrantly up-regulated in ESCC and associated with poorer ESCC prognosis. a: The screening process of NNT-AS; b-c: NNT-AS1 expression was up-regulated in ESCC tissues (b: GSE53624; c: JSPH); d: High expression of NNT-AS1 was significantly associated with an inferior ESCC prognosis; e: The expression level of NNT-AS1 in ESCC cell lines (Eca-109, Kyse-30, and TE-1) was significantly higher than that in human normal esophageal epithelial cell line (HEEC).

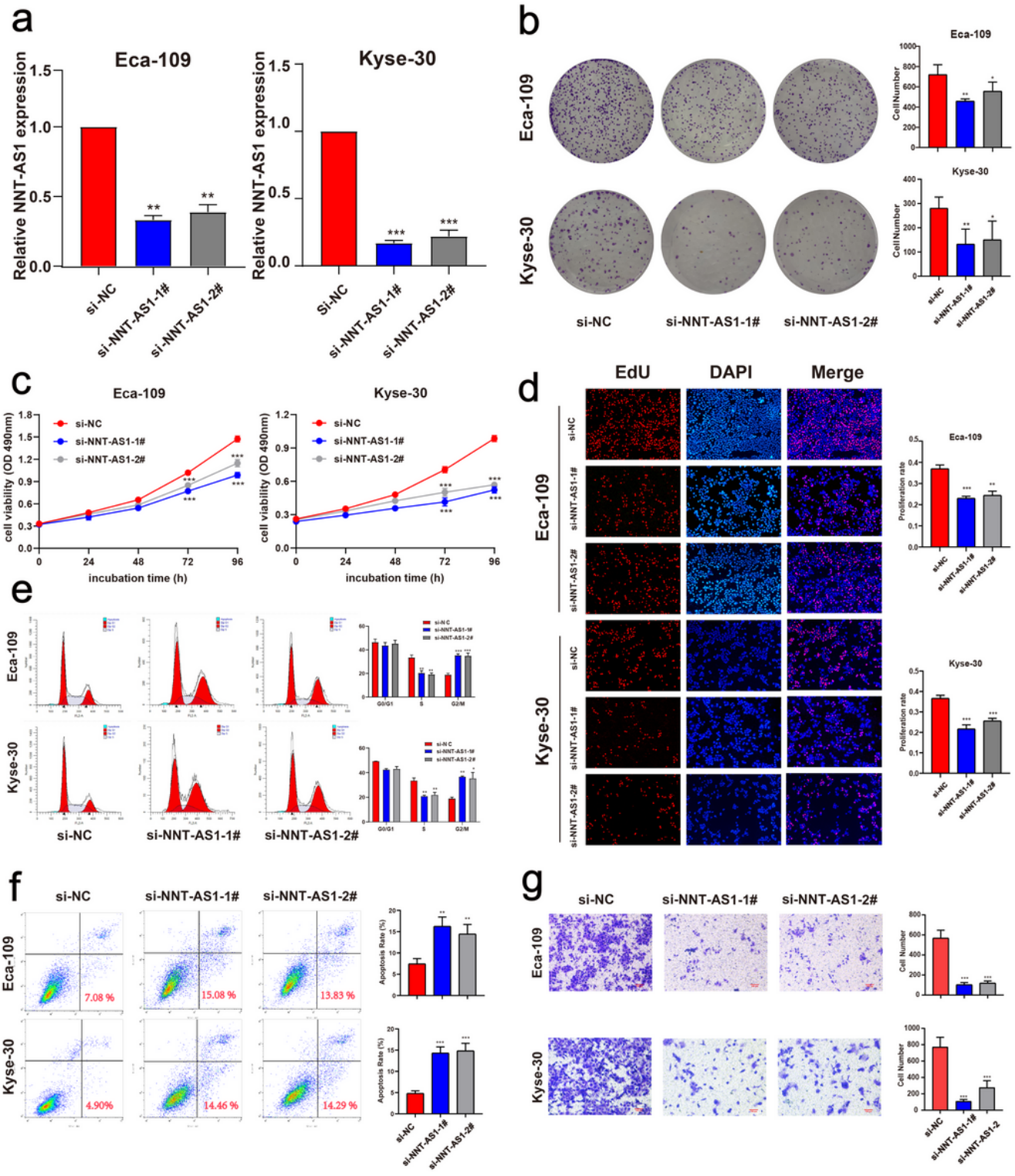

Figure 2

Knockdown of NNT-AS1 inhibited ESCC cells proliferation, migration, and induced cell cycle arrest and promoted cell apoptosis. a: The knockdown efficiency of short interfering RNAs for NNT-AS1; b-d: Knockdown of NNT-AS1 significantly inhibited the proliferation of Eca-109 and Kyse-30 cells using colony forming assays (b), MTT assays (c), and EdU assays (d); e: Cell cycle assays were employed to detect the

distribution of cells in different phases; f: Knockdown of NNT-AS1 increased the apoptotic rate of both cell lines; g: Knockdown of NNT-AS1 inhibited the migration of Eca-109 and Kyse-30 cells.

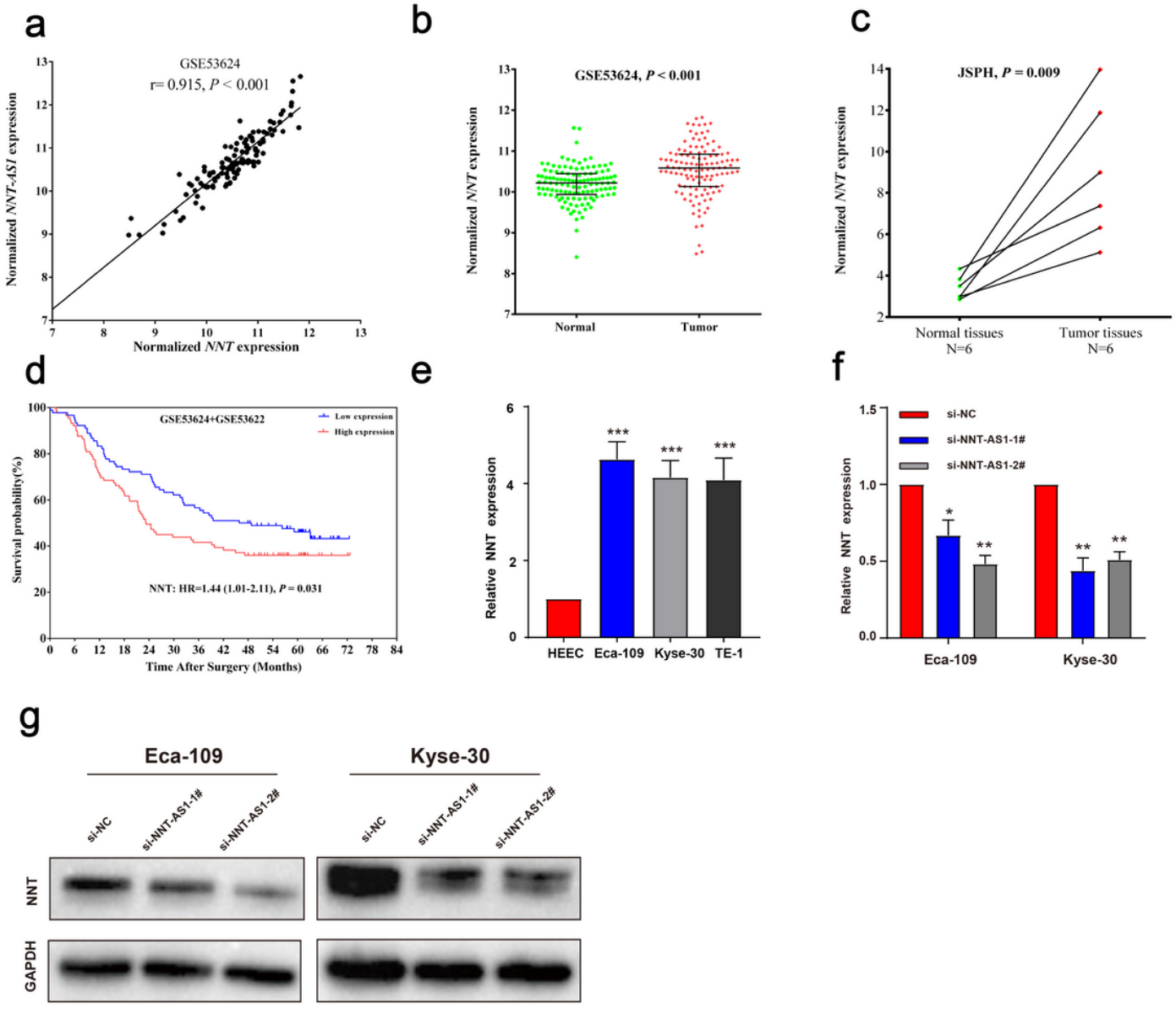

Figure 3

NNT was upregulated in ESCC and positively regulated by NNT-AS1. a: NNT was positively correlated with NNT-AS1 in GSE53624 (r = 0.915, P < 0.001); b-c: NNT was upregulated in ESCC tissues (b, GSE53624, P < 0.001; c, JSPH, P = 0.009); d: High expression of NNT was significantly associated with a poorer ESCC prognosis; e: The expression of NNT in three ESCC cells and HEEC cells; f-g: Knockdown of NNT-AS1 suppressed NNT in mRNA and protein levels.

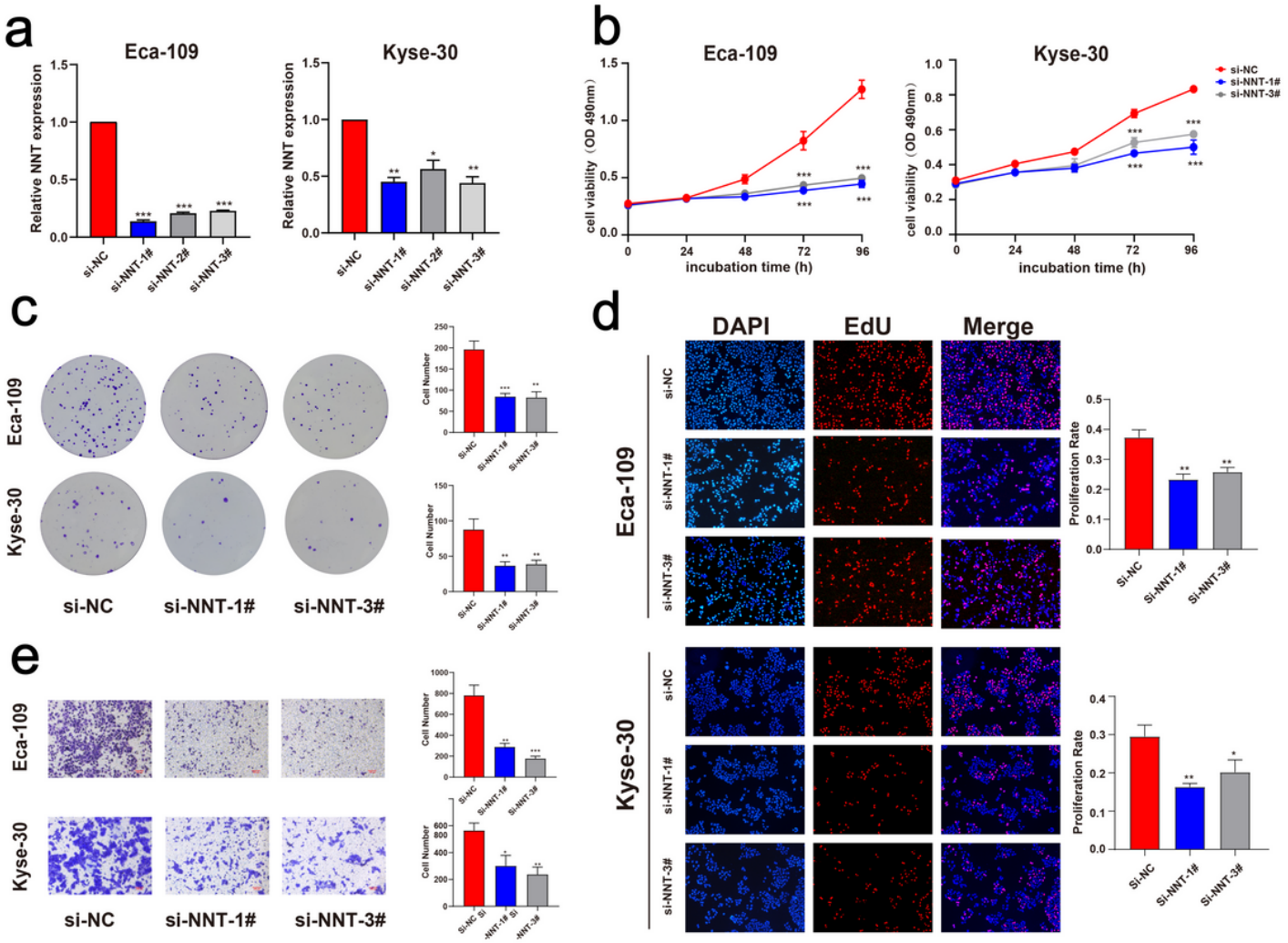

Figure 4

Knockdown of NNT suppressed ESCC cells proliferation and migration. a: The knockdown efficiency of short interfering RNAs for NNT was measured in Eca-109 and Kyse-30; b-d: Knockdown of NNT inhibited proliferation of Eca-109 and Kyse-30 cells using colony forming assays, MTT assays, and EdU assays; e: Knockdown of NNT inhibited the migration of Eca-109 and Kyse-30 cells.

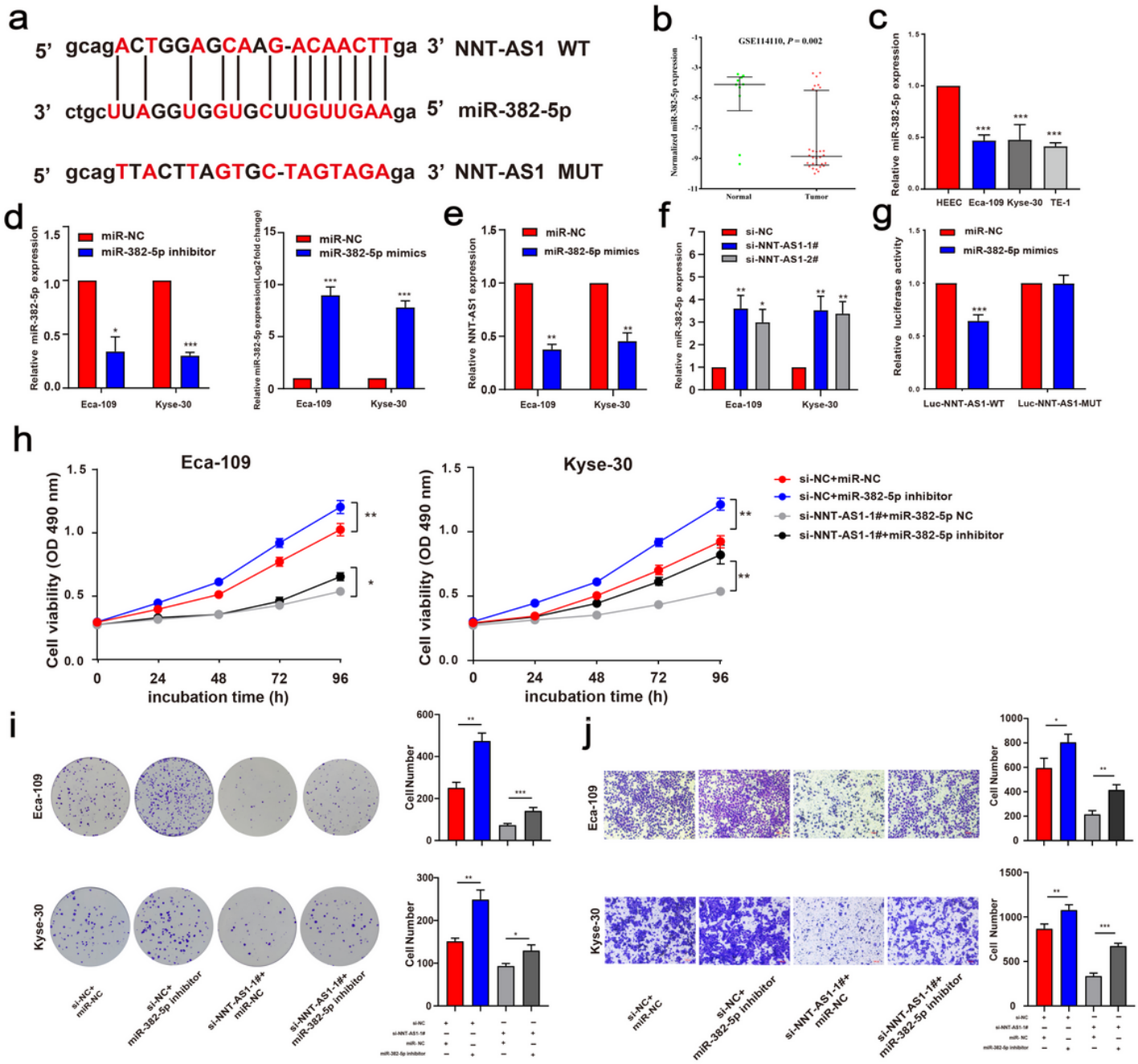

Figure 5

NNT-AS1 could sponge miR-382-5p and knockdown of miR-382-5p could rescue the decreased ESCC proliferation and migration mediated by NNT-AS1 inhibition a: MiR-382-5p had a binding site for NNT-AS1; b: MiR-382-5p was downregulated in ESCC tissues based on GSE114110 (P = 0.002); c: The expression of miR-382-5p in three ESCC cells and HEEC cells; d: The transfection efficiency of miR-382-5p mimics and inhibitor were verified; e: Overexpression of miR-382-5p inhibited the NNT-AS1 expression; f: Knockdown of NNT-AS1 increased miR-382-5p expression; g: Dual-Luciferase assays detected the luciferase activities in Eca-109 cells after being co-transfected with miRNA (miR-NC or miR-mimics) and luciferase reporter vectors (NNT-AS1-WT or NNT-AS1-MUT); h-j: Mi-382-5p partly retrieved the suppressed

abilities of proliferation (h: MTT; i: colony formation) and migration (j) that NNT-AS1 knockdown mediated in ESCC cell lines.

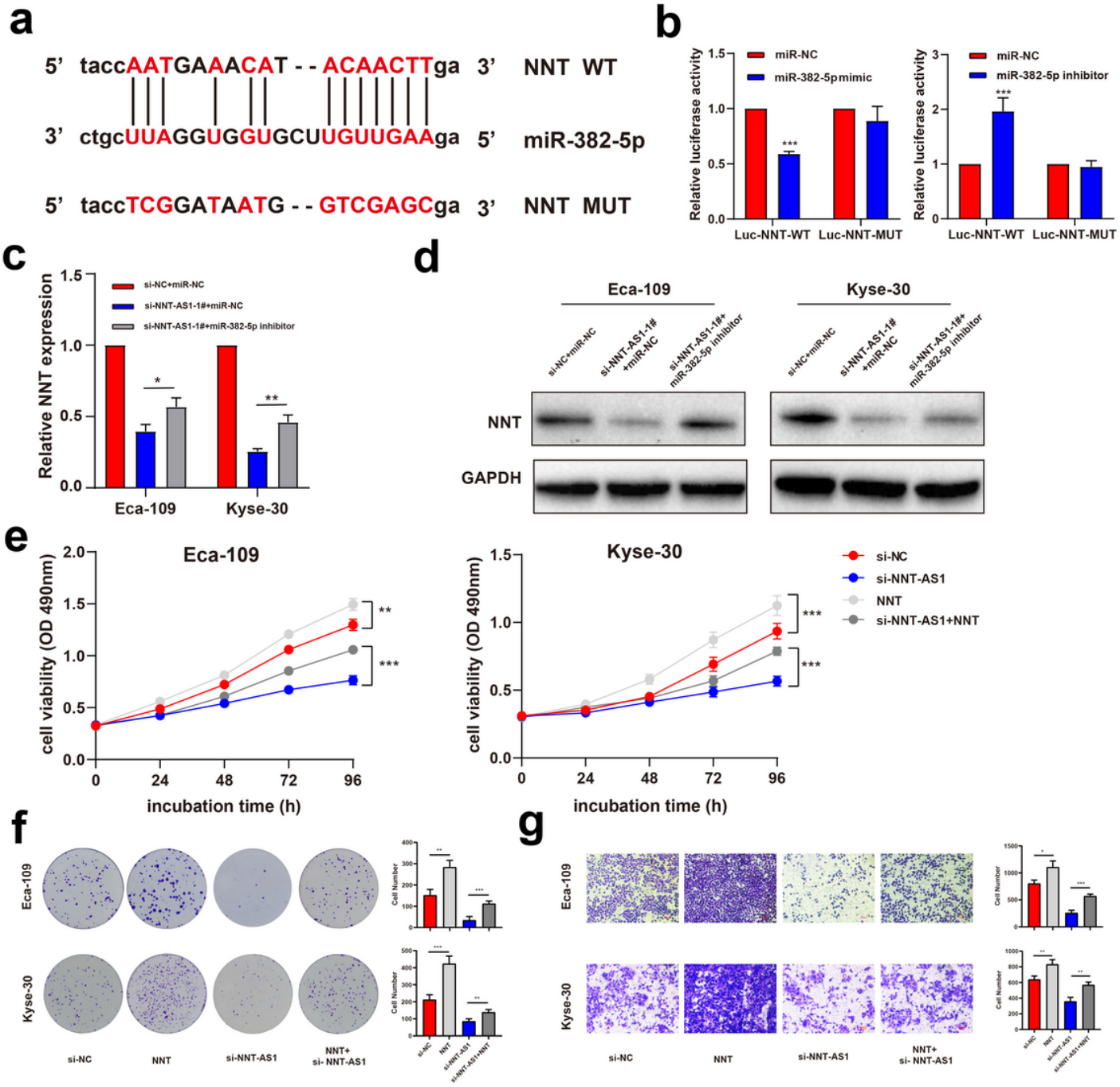

Figure 6

MiR-382-5p negatively regulated NNT and overexpression of NNT rescued the decline of cellular function that NNT-AS1 knockdown mediated in ESCC. a: MiR-382-5p had a binding site for NNT; b: Dual-Luciferase assays were utilized to detect the luciferase activities in Eca-109 cells after being co-transfected with miRNAs (miR-NC, miR-382-5p mimics, miR-382-5p inhibitor) and luciferase reporter vectors (NNT- WT and NNT- MUT) respectively; c-d: Downregulation of miR-382-5p rescued the decreased NNT expression mediated by NNT-AS1 inhibition in Eca-109 and Kyse-30 cells; e-g: Rescue assays were conducted to

measure the cellular functions of NNT-AS1 knockdown cells after overexpressing NNT in Eca-109 and Kyse-30 cells.

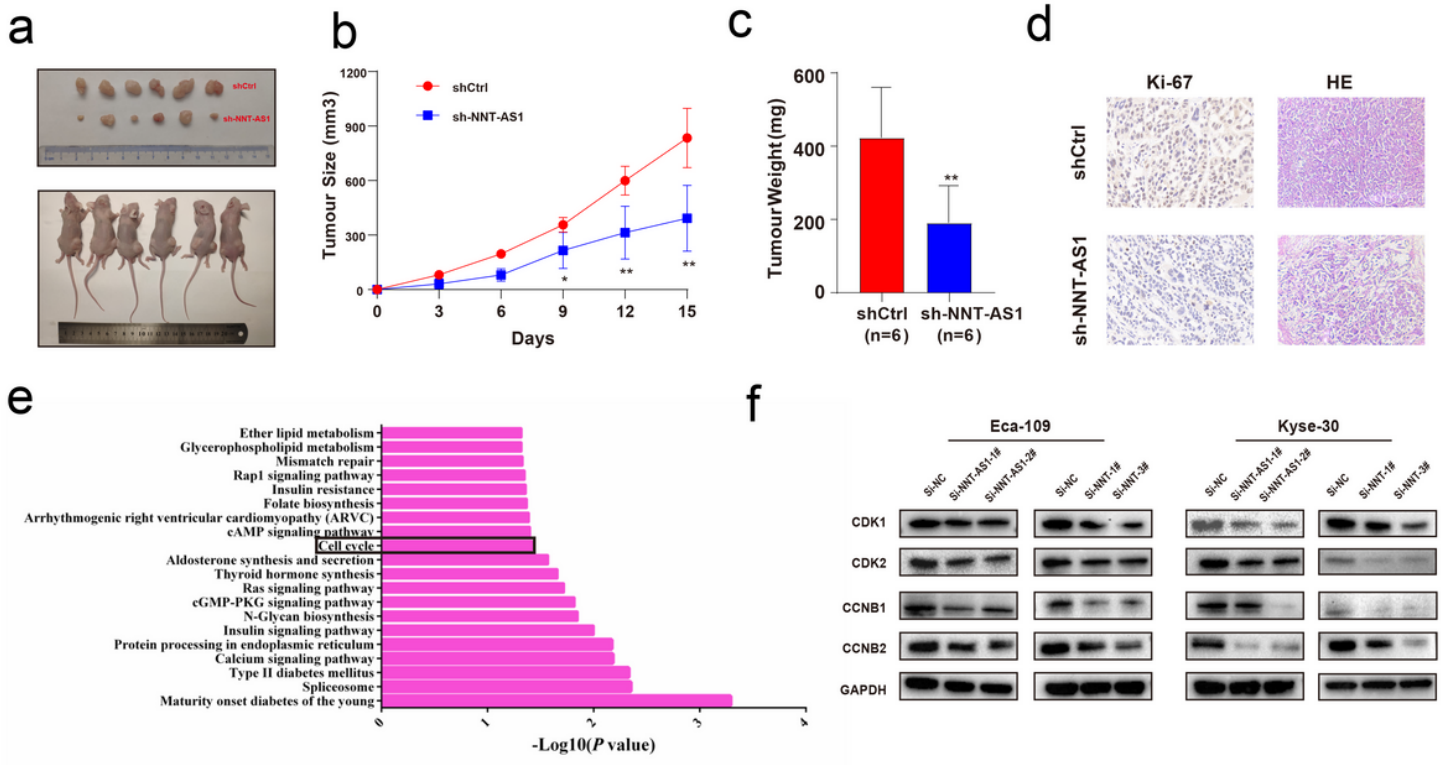

Figure 7

NNT-AS1 promoted cell growth in vivo and could regulate cell cycle signaling pathway. a: ShCtrl or shCCAT1 was stably transfected into Eca-109 cells, which were respectively injected into the back of nude mice; b: Tumor volumes were measured every 3 days and the growth curve of tumors was plotted; c: The tumor weight in shCtrl group was higher than that in shNNT-AS1 group; d: The tumor sections were undergone ki-67 staining and HE staining; e: Pathway enrichment analysis was conducted to predict the potential pathways that NNT participated in ESCC; f: Several checkpoints of cell cycle pathway were measured using western blot assays after silencing NNT and NNT-AS1.

Supplementary Files

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- SupplementaryInformation.docx