OncoTargets and Therapy

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ORIGINAL RESEARCH

RETRACTED ARTICLE: LINC00958 Promotes The Malignancy Of Nasopharyngeal Carcinoma By Sponging microRNA-625 And Thus Upregulating NUAK1

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Purpose: The aberrant expression of long noncoding RNAs (lncRNAs) indicates progression of various diseases. LINC00958 has been well studied in several types of human cancer; however, the expression profile, functions, and potential mechanisms of action of this lncRNA in nasopharyngeal carcinoma (NPC) remain largely unclear and still need to be elucidated. In the present study, we aimed to measure LINC00958 expression in NPC, determine its clinical value, and explore its roles in NPC progression as well as the mechanisms behind these processes.

Methods: The expression profile of LINC00958 in NPC was evaluated by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). A series of functional assays, including the Cell Counting Kit-8 assay, flow cytometry, a Transwell assay, and an in vivo nude mouse model, were utilized to determine the participation of LINC00958 in the malignancy of NPC.

Results: LINC00958 was found to be upregulated in NPC tissue specimens and cell lines. The LINC00958 overexpression was significantly correlated with tumor size, lymph node status, TNM stage, and worse overall survival among NPC patients. Downregulation of LINC00958 suppressed NPC cell proliferation, migration, and invasion and induced apoptosis in vitro. Additionally, the LINC00958 knockdown impaired tumor growth in vivo. Mechanistically, LINC00958 was found to serve as a molecular sponge of microRNA-625 (miR-625), thereby upregulating NUAK family SNF1-like kinase 1 (NUAK1) in NPC cells. Lastly, rescue experiments validated the involvement of the miR-625–NUAK1 axis in LINC00958-mediated biological functions in NPC.

Conclusion: Our results demonstrated that LINC00958 works as an oncogene in NPC and plays a key role in the malignant phenotype of NPC cells by sponging miR-625 and increasing NUAK1 expression. The LINC00958–miR-625–NUAK1 pathway might be a target for anticancer therapy in patients with NPC.

Keywords: NUAK family SNF1-like kinase 1, LINC00958, nasopharyngeal carcinoma, microRNA-625

Introduction

Nasopharyngeal carcinoma (NPC), derived from epithelial cells located in the nasopharynx, is a type of human cancer that occurs frequently in Southeast Asia populations.[1] It is estimated that there will be approximately 60 000 new cases and 34 000 deaths resulting from NPC every year in China.[2] Multiple factors, including Epstein–Barr virus infection, environmental factors, diet, and genetic factors, are involved in the NPC pathogenesis;[3–5] however, the detailed mechanisms governing NPC initiation and progression have not yet been elucidated. In spite of the

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considerable progress in the diagnostic and treatment strategies, the clinical outcomes of NPC diagnosed at an advanced stage are still not satisfactory, owing to local recurrence, distant metastasis, and chemoresistance.[6,7] Hence, a complete exploration of the mechanisms underlying NPC formation and progression is necessary for the identification of novel and promising diagnostic biomarkers and targets for anticancer therapies.

Long noncoding RNAs (lncRNAs) belong to a group of RNAs with a length of >200 nucleotides and do not encode proteins.[8] LncRNAs are reported to perform important functions in distinct biological phenomena, such as differentiation, metabolism, immunity, and especially carcinogenesis including cancer progression.[9–11] A vast number of lncRNAs are aberrantly expressed in NPC. For instance, NEAT1,[12] DANCR,[13] and HOTTIP[14] are overexpressed in NPC and function as oncogenes, whereas ZNF674-1,[15] LET,[16] and LINC0086[17] are underexpressed in NPC and exert tumor-suppressive actions. Therefore, lncRNAs may represent useful therapeutic targets in NPC.

MicroRNAs (miRNAs) are defined as a group of noncoding 19–25 nucleotide-long RNA molecules that take part in the regulation of gene expression.[18] MiRNAs negatively modulate gene expression through base pairing with partially complementary sites in the 3′ untranslated regions (3′-UTRs) of their target mRNAs, thus affecting a wide range of basic biological processes.[19] A number of studies have revealed aberrant expression of miRNAs in a variety of human diseases, including cancer.[20–22] To date, numerous miRNAs have been demonstrated to be dysregulated in NPC.[23–25] The dysregulation of miRNAs is implicated in diverse tumor processes and exerts tumor-suppressive or oncogenic actions.[24] Therefore, the investigation of cancer-related miRNAs in NPC may help to identify effective novel targets for NPC therapy.

Although LINC00958 has been well studied in several types of human cancer, the expression profile, functions, and potential mechanisms of action of this lncRNA in NPC remain largely unclear and still need to be elucidated. Hence, this study was aimed at measuring LINC00958 expression in NPC, determining its clinical value, and exploring its roles in NPC progression as well as elucidating the relevant molecular mechanisms of action.

Materials And Methods

Clinical Specimens

A total of 59 freshly frozen NPC tissue samples and matched adjacent non-tumor nasopharyngeal epithelial tissue samples were collected at Yidu Central Hospital of Weifang. None of the patients had been treated with any type of antitumor therapy before surgical resection. All specimens were quickly frozen in liquid nitrogen after tissue resection and then preserved at –80°C. The research protocols were approved by the Ethics Committee of Yidu Central Hospital of Weifang and were carried out according to the Declaration of Helsinki. Written informed consent for the use of tissue specimens was provided by all the participants.

Cell Culture

Four human NPC cell lines—CNE-1, CNE-2, HONE-1, and SUNE-1—were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% (v/v) of fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). A normal nasopharyngeal epithelial cell line, NP69, was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA).

Keratinocyte serum-free medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 30 μg/mL bovine pituitary extract (BD Biosciences, San Diego, CA, USA) was utilized for the cultivation of the NP69 cell line. All the above cell lines were grown at 37°C in a humidified atmosphere containing 5% of CO_2.

Oligonucleotide And Plasmid Transfection

The small interfering RNA (siRNA) that targeted LINC00958 (si-LINC00958) and negative control siRNA (si-NC) were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Agomir-625, agomir-NC, antagomir-625, and antagomir-NC were constructed at Shanghai GenePharma Co., Ltd. (Shanghai, China). The empty pcDNA3.1 plasmid and NUAK1-overexpressing plasmid pcDNA3.1-NUAK1 (pc-NUAK1) were chemically synthesized by the Chinese Academy of Sciences (Changchun, China).

Cells were seeded in 6-well plates with the culture medium without antibiotics one night before transfection. To obtain cell models with high or low expression of a target gene, the si-LINC00958 (100 pmol), si-NC (100 pmol), agomir-625 (50 nM), agomir-NC (50 nM), antagomir-625 (100 nM), antagomir-NC (100 nM) and plasmids (4 μg) were transfected into cells using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). After different periods of

incubation, the transfected cells were collected for the subsequent functional assays.

Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

The TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was employed for the total RNA isolation from tissue specimens and cells. To detect LINC00958 and *NUAK1* mRNA expression, reverse transcription was conducted to convert total RNA to cDNA with the PrimeScript RT-Reagent Kit (Takara Bio, Kusatsu, Japan). Subsequently, the amplification reaction was carried out using the SYBR Premix Ex *Taq*™ Kit (Takara Bio) on an ABI Prism 7900 sequence detection system (Applied Biosystems Inc.). LINC00958 and *NUAK1* mRNA levels were normalized to *GAPDH*. To quantify miR-625 expression, the synthesis of cDNA was performed with the miScript Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany), and the cDNA was then subjected to qPCR by means of the miScript SYBR Green PCR Kit (Qiagen GmbH). U6 small nuclear RNA served as an internal control of miR-625 expression. The $2^{-\Delta\Delta Cq}$ method was employed to analyze relative gene expression.

Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were collected after 24 h of incubation and were inoculated into 96-well plates at an initial density of 1×10^3 cells/well. The cells were then incubated at 37°C in a humidified atmosphere containing 5% of CO_2 for 0, 1, 2, or 3 days. At various time points, the CCK-8 assay was performed to evaluate cellular proliferation. Briefly, the culture medium was discarded, and 90 µL of a fresh culture medium supplemented with 10 µL of the CCK-8 solution (Dojindo Molecular Technologies, Tokyo, Japan) was added into each well. After 2 h of incubation, the absorbance was read at 450 nm wavelength on a microplate absorbance reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Flow-Cytometric Analysis

After cultivation for 48 h, transfected cells were collected for the determination of cell apoptosis using the Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit (Biolegend, San Diego, CA, USA). In particular, the transfected cells were harvested with centrifugation and washed twice with ice-cold phosphate-buffered saline (Gibco; Thermo Fisher Scientific, Inc.), followed by resuspension in 100 µl of binding buffer, which was then supplemented with 5 µl of Annexin V-FITC and 5 µl of the kit propidium iodide solution. The proportion of apoptotic cells was determined on a flow cytometer (FACScan™; BD Biosciences, Franklin Lakes, NJ, USA) within 15 min of incubation in darkness.

Transwell Assays

The migratory and invasive abilities were evaluated using 8-µm Transwell inserts without and with Matrigel precoating (BD Biosciences, San Jose, CA, USA), respectively. In total, 5×10^4 cells resuspended in 200 µL of DMEM were plated into the upper compartments of the Transwell inserts, and 500 µL of DMEM containing 20% of fetal bovine serum was added into the lower compartments as a chemoattractant. Following 24-h incubation, the cells remaining on the upper side of the polycarbonate membrane were gently removed with a cotton swab. Cells that moved to the bottom of the membranes were fixed with 70% ethanol, stained with 0.1% crystal violet, and rinsed thrice with double-distilled water. Finally, the migratory and invading cells were photographed and counted in at least five randomly selected visual fields under an Olympus microscope (Olympus Corporation, Tokyo, Japan), and the average was calculated.

Establishment Of In Vivo Nude Mouse Model

Female BALB/C nude mice (5–6 weeks of age, 16–18 g) were purchased from Shanghai Laboratory Animals Center of the Chinese Academy of Sciences (Shanghai, China). CNE-1 cells transfected with either si-LINC00958 or si-NC were subcutaneously inoculated into the flanks of the nude mice (n=4 for each group). Two weeks later, the tumor volume was measured every 2 days via the following formula: 1/2 × tumor length × tumor width^2. All the nude mice were killed 4 weeks after the injection, and all the tumor xenografts were surgically removed and stored for subsequent analyses. The Animal Care and Use Committee of Yidu Central Hospital of Weifang approved this animal experiment, and the experimental procedures were in accordance with the Animal Protection Law of the People's Republic of China-2009 for experimental animals.

Bioinformatic Prediction Of Targets

The binding site in LINC00958 for miRNAs was predicted using starBase 3.0 (http://starbase.sysu.edu.cn/). miRDB database (http://mirdb.org), and TargetScan Human 6.2

(http://www.targetscan.org/vert_72/) were used to search for the potential targets of miR-625.

Luciferase Reporter Assay

A wild-type (Wt) LINC00958 fragment containing the putative binding site for miR-625 was chemically amplified by Shanghai GenePharma Co., Ltd., and inserted into the pmirGLO plasmid (Promega Corporation, Madison, WI, USA), and the resultant vector was designated as LINC00958-Wt. The corresponding mutant (Mut) plasmid (LINC00958-Mut) was created by mutating the seed regions of the miR-625–binding site in LINC00958-Wt. The luciferase reporter plasmids, NUAK1-Wt and NUAK1-Mut, were chemically produced in the same manner. For a reporter assay, cells were seeded in 24-well plates and were transiently cotransfected with the Wt or Mut reporter plasmid in the presence of either agomir-625 or agomir-NC using the Lipofectamine 2000 reagent. After 48-h incubation, the transected cells were harvested and subjected to the measurement of luciferase activity using a Dual-Luciferase Reporter Assay System (Promega Corporation). *Renilla* luciferase activity served for normalization.

RNA-Binding Protein Immunoprecipitation (RIP) Assay

The RIP assay was performed with the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (EMD Millipore, Billerica, MA, USA). In brief, a whole-cell lysate was prepared and incubated with RIP buffer containing magnetic beads, which had been conjugated with a human anti-Ago2 antibody (Abcam, Cambridge, UK) or normal Immunoglobulin G (IgG). Then, proteinase K was applied to the cell lysate to remove the protein. Finally, total RNA was isolated and analyzed by RT-qPCR.

Western Blot Analysis

Radioimmunoprecipitation assay buffer (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) was employed to extract total protein from tissues or cells. The concentration was determined using the Bicinchoninic Acid Assay Kit (Pierce Biotechnology Inc., Rockford, IL, USA). Equal amounts of protein samples were loaded on a gel and were separated by SDS polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes. Next, the membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% of Tween 20 at 37°C for 2 h. The membranes were probed with primary antibodies against NUAK1 (cat. No. sc-271827; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and GAPDH (cat. No. sc-66163; Santa Cruz Biotechnology, Inc.) at 4 °C overnight. After that, the membranes were incubated with a horseradish peroxidase–conjugated goat anti-mouse IgG secondary antibody (cat. No. sc-516102; Santa Cruz Biotechnology, Inc.), and protein bands were visualized with the Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore). GAPDH served as a loading control.

Statistical Analysis

All the experiments were repeated at least three times, and all data were presented as mean ± standard deviation. Differences between two groups were analyzed by Student's *t*-test, and differences among multiple groups by one-way ANOVA followed by the Student–Newman–Keuls multiple-comparison *post hoc* test. The χ^2 test was performed to investigate the association between LINC00958 and clinicopathological characteristics in patients with NPC. The expression correlation between LINC00958 and miR-625 was determined via Spearman correlation analysis. Survival analysis was carried out by the Kaplan–Meier method and log rank test. Data with a P value less than 0.05 were considered statistically significant.

Results

Elevated levels of LINC00958 are associated with adverse clinical parameters and poor prognosis in patients with NPC

To determine the importance of LINC00958 in NPC, its expression in 59 pairs of NPC tissue samples and matched adjacent non-tumor nasopharyngeal epithelial tissues was analyzed by RT-qPCR. The data indicated that LINC00958 expression was significantly higher in NPC tissues than in adjacent non-tumor nasopharyngeal epithelial tissue samples (Figure 1A, $P < 0.05$). LINC00958 expression was then measured in four NPC cell lines: CNE-1, CNE-2, HONE-1, and SUNE-1. Normal nasopharyngeal epithelial cell line NP69 served as the control. The results showed that expression of LINC00958 was higher in all four tested NPC cell lines than in NP69 cells (Figure 1B, $P < 0.05$).

Next, we assessed the clinical value of the overexpression of LINC00958 in patients with NPC. The median value of LINC00958 in NPC tissues (2.31) was defined as the cutoff, and all patients were divided into two groups: LINC00958 low (n = 29) and high (n = 30) expression

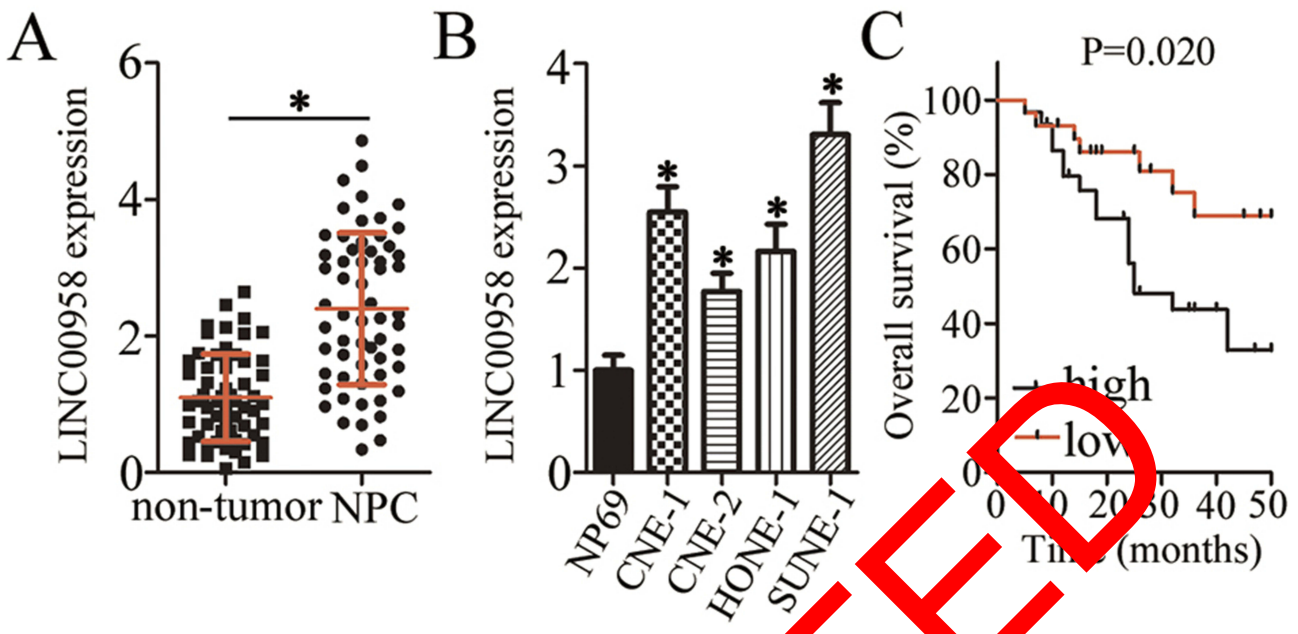

Figure 1 LINC00958 is upregulated in NPC and predicts poor prognosis. (**A**) The expression profile of LINC00958 in 59 pairs of NPC tissue samples and matched adjacent non-tumor nasopharyngeal epithelial tissue samples was determined via RT-qPCR. *P < 0.05 vs non-tumor nasopharyngeal epithelial tissues. (**B**) Quantitation of LINC00958 in four NPC cell lines (CNE-1, CNE-2, HONE-1, and SUNE-1) and the normal nasopharyngeal epithelial cell line NP69 was conducted by RT-qPCR. *P < 0.05 vs group NP69. (**C**) Kaplan–Meier curves of overall survival among NPC patients with LINC00958 low (n = 29) and high (n = 30) expression. P = 0.020.

groups. As shown in Table 1, high expression of LINC00958 manifested an obvious association with tumor size (P = 0.021), lymph node status (P = 0.037) and TNM stage (P = 0.009). In addition, NPC patients with high LINC00958 expression in the tumor had a remarkably lower overall survival rate (Figure 1C, P = 0.020) than did the patients with low LINC00958 expression. These results suggested that increased expression of LINC00958 may be related to NPC progression.

Downregulation Of LINC00958 Inhibits NPC Cell Proliferation, Migration, And Invasion And Induces Cell Apoptosis

Because the expression of LINC00958 was much higher in CNE-1 and SUNE-1 cell lines among the four tested NPC cell lines, these two cell lines were chosen for subsequent assays. To explore the roles of LINC00958 in NPC progression, loss-of-function assays were performed by transfection of si-LINC00958 into CNE-1 and SUNE-1 cells. RT-qPCR analysis confirmed that LINC00958 was efficiently silenced in CNE-1 and SUNE-1 cells after si-LINC00958 transfection (Figure 2A, P < 0.05). The results of the CCK-8 assay meant that downregulation of LINC00958 remarkably decreased the proliferative ability of CNE-1 and SUNE-1 cells (Figure 2B, P < 0.05). After that, we conducted flow cytometric analysis to test whether silencing of LINC00958 affected apoptosis. As presented in Figure 2C, transfection of si-LINC00958

Table 1 The Relationship Between The Expression Of LINC00958 And Clinicopathological Characteristics Among Patients With NPC

Characteristics	LINC00958 Expression		P
	High (n=30)	Low (n=29)	
Age (years)			0.785
< 60	12	9	
≥ 60	18	20	
Gender			0.387
Male	15	17	
Female	15	12	
Tumor size (cm)			0.021
< 5	20	27	
≥ 5	10	2	
Lymph node status			0.037
N0	12	20	
N1-3	18	9	
Distant metastasis			0.612
No	27	28	
Yes	3	1	
TNM stage			0.009
I-II	9	19	
III-IV	21	10	

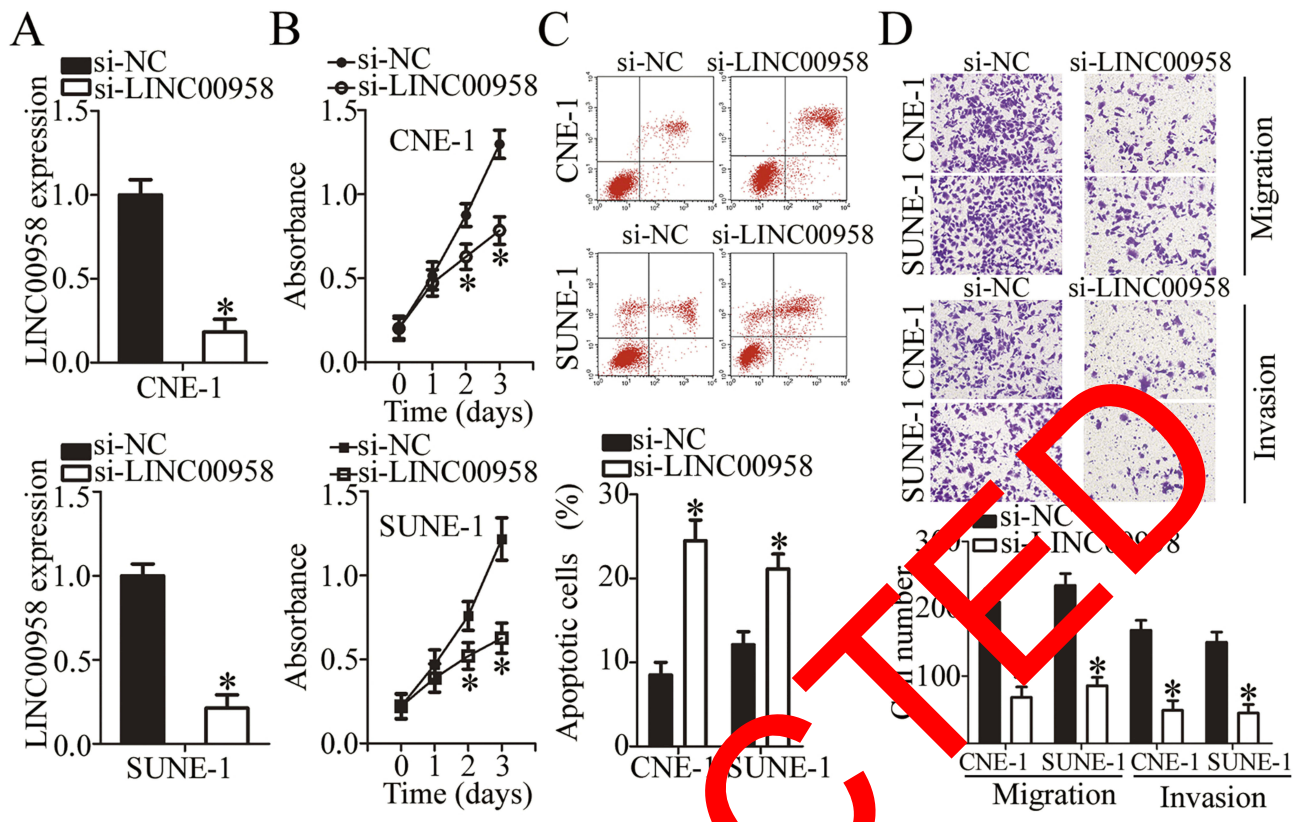

Figure 2 LINC00958 silencing restrains the growth and metastasis of NPC cells in vitro. (**A**) CNE-1 and SUNE-1 cells were transfected with either si-LINC00958 or si-NC. The transfected cells were collected after 48-h incubation and used for the determination of transfection efficiency. *P < 0.05 vs the si-NC group. (**B**) CCK-8 assays revealed a reduction in the cellular proliferative abilities of LINC00958-deficient CNE-1 and SUNE-1 cells at 0, 24, 48, 72, and 96 h after inoculation. *P < 0.05 vs group si-NC. (**C**) The apoptosis of CNE-1 and SUNE-1 cells treated with si-LINC00958 or si-NC was assessed by flow cytometry. *P < 0.05 vs the si-NC group. (**D**) Transwell assays were conducted to evaluate the impact of the LINC00958 knockdown on the migration and invasiveness of CNE-1 and SUNE-1 cells. Representative images are presented. *P < 0.05 vs group si-NC.

notably promoted the apoptosis of CNE-1 and SUNE-1 cells ($P < 0.05$). Furthermore, the migration or invasion alterations of CNE-1 and SUNE-1 cells (when LINC00958 was silenced) were investigated in Transwell assays. Silencing of LINC00958 expression resulted in obvious suppression of CNE-1 and SUNE-1 cell migration and invasion (Figure 2D, $P < 0.05$). Taken together, these results implied that LINC00958 may exert oncogenic actions on the growth and metastasis of NPC cells in vitro.

LINC00958 Acts As A Molecular Sponge For miR-625 In NPC

To uncover the mechanisms through which LINC00958 is implicated in the progression of NPC, the potential target miRNAs of LINC00958 were predicted by bioinformatic analysis. Among these miRNAs, miR-625 (Figure 3A), which has been fully studied in human cancers,[26–32] was chosen for further investigation. To determine whether miR-625 can interact with LINC00958 directly, a luciferase reporter assay was performed on CNE-1 and SUNE-1 cells after cotransfection with either LINC00958-Wt or LINC00958-Mut in the presence of either agomir-625 or agomir-NC. The efficiency of agomir-625 is illustrated in Figure 3B ($P < 0.05$). The luciferase activity of LINC00958-Wt significantly decreased in CNE-1 and SUNE-1 cells after miR-625 upregulation (Figure 3C, $P < 0.05$); however, the luciferase activity of LINC00958-Mut was not affected by agomir-625 transfection. Furthermore, LINC00958 and miR-625 were successfully coimmunoprecipitated in CNE-1 and SUNE-1 cells by the anti-Ago2 antibody but not by the IgG antibody (Figure 3D, $P < 0.05$), as revealed by the RIP assay.

Then, miR-625 expression was confirmed to be significantly lower in NPC tissue samples when compared with matched adjacent non-tumor nasopharyngeal epithelial tissues (Figure 3E, $P < 0.05$). The expression levels of LINC00958 and miR-625 showed a negative correlation among NPC tissue samples (Figure 3F; $R^2 = 0.2925$, $P < 0.0001$). Moreover, LINC00958 downregulation obviously increased miR-625 expression in CNE-1 and SUNE-1 cells, as revealed by

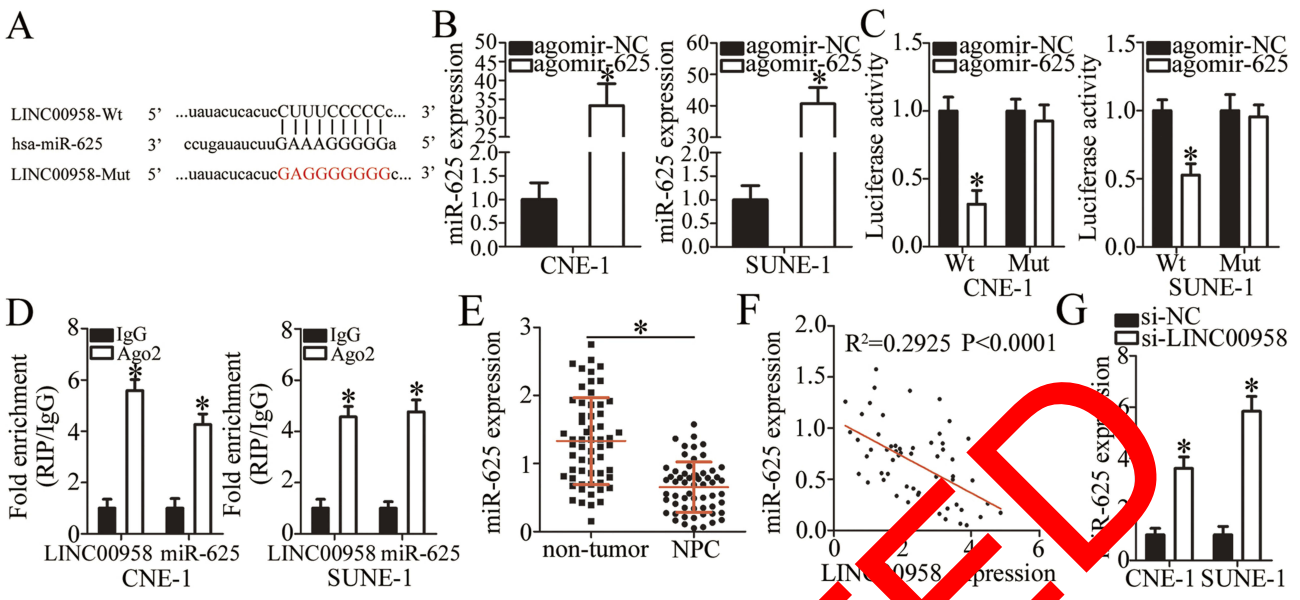

Figure 3 LINC00958 acts as a molecular sponge of miR-625 in NPC. (**A**) The wild-type and mutant binding sites in LINC00958 for miR-625 were found by means of StarBase 3.0. (**B**) Assessment of miR-625 expression by RT-qPCR analysis in CNE-1 and SUNE-1 cells after agomir-625 or agomir-NC transfection. *P < 0.05 vs the agomir-NC group. (**C**) The luciferase reporter assay was carried out to confirm the binding of LINC00958 to miR-625 in NPC cells. *P < 0.05 vs group agomir-NC. (**D**) The RIP assay was employed to reveal the enrichment of LINC00958 and miR-625 in an Ago2 immunoprecipitate. *P < 0.05 vs group IgG. (**E**) Relative miR-625 expression in 59 pairs of NPC tissue samples and matched adjacent non-tumor nasopharyngeal epithelial tissues was determined through RT-qPCR analysis. *P < 0.05 vs non-tumor nasopharyngeal epithelial tissues. (**F**) The expression correlation between LINC00958 and miR-625 among the NPC tissue samples was studied by Spearman correlation analysis. $R^2 = 0.2925$, P < 0.0001. (G) RT-qPCR assay was performed to measure miR-625 expression when LINC00958 was silenced in CNE-1 and SUNE-1 cells. *P < 0.05 vs the si-NC group.

RT-qPCR analysis (Figure 3G, P < 0.05). These results provided evidence that LINC00958 serves as a molecular sponge for miR-625 in NPC.

MiR-625 Suppresses The Growth And Metastasis Of NPC Cells In Vitro

Because miR-625 was revealed to be sponged by LINC00958, the physiological effects of miR-625 on the aggressive phenotypes of NPC cells were investigated in detail. As determined by the CCK-8 assay and flow cytometric analysis, exogenous miR-625 expression significantly suppressed proliferation (Figure 4A, P < 0.05) and induced apoptosis (Figure 4B, P<0.05) of CNE-1 and SUNE-1 cells. In addition, the numbers of migratory (Figure 4C, P < 0.05) and invading (Figure 4D, P < 0.05) cells were lower among miR-625–overexpressing CNE-1 and SUNE-1 cells as compared with the cells transfected with agomir-NC. These observations meant that miR-625 may exert an inhibitory action on the growth and metastasis of NPC cells in vitro.

NUAK1 Is A Direct Target Gene Of miR-625 In NPC Cells

To understand the mechanism of action of miR-625 in NPC progression, two algorithms were applied to search for the putative potential target of miR-625. The 3′-UTR of *NUAK1* was found to contain a complementary site for the seed region of miR-625 (Figure 5A) and was chosen for further analysis because this gene is also closely related to NPC tumorigenesis.[33,34] Then, the luciferase reporter assay was carried out to determine whether the 3′-UTR of *NUAK1* could be directly targeted by miR-625. It was observed that the luciferase activity of NUAK1-Wt was notably lowered by miR-625 overexpression in CNE-1 and SUNE-1 cells (P < 0.05); by contrast, no difference in luciferase activity between agomir-625 and agomir-NC groups was noted when the cells were cotransfected with the NUAK1-Mut plasmid (Figure 5B).

In addition, the data obtained from RT-qPCR and Western blotting confirmed that transfection of agomir-625 led to an obvious reduction in the mRNA (Figure 5C, P < 0.05) and protein levels (Figure 5D, P < 0.05) of NUAK1 in CNE-1 and SUNE-1 cells. NUAK1 expression was subsequently measured in NPC tissue samples and in matched adjacent non-tumor nasopharyngeal epithelial tissues via RT-qPCR. The results revealed that NPC tissues remarkedly overexpressed NUAK1 at the mRNA level in comparison with the matched adjacent non-tumor nasopharyngeal epithelial tissues (Figure 5E, P < 0.05). Then, an inverse correlation

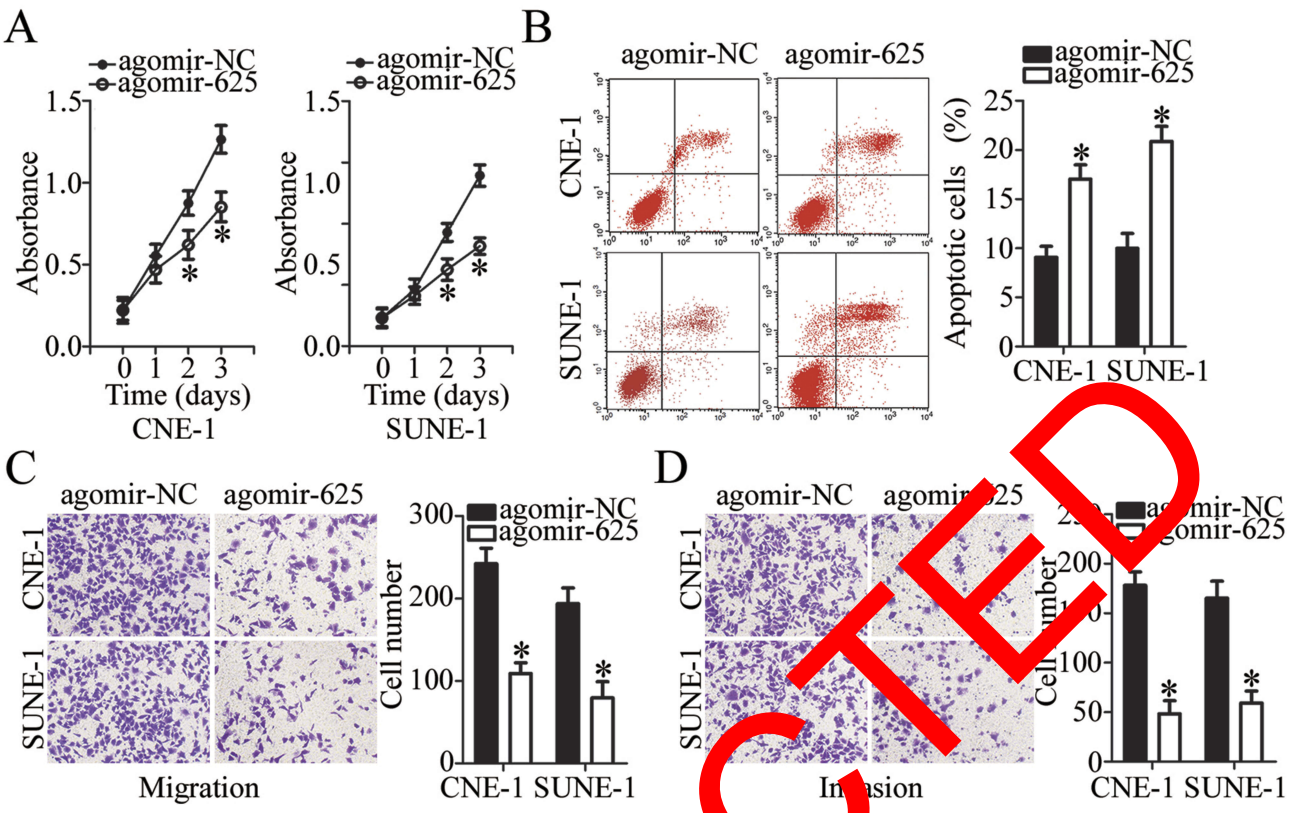

Figure 4 MiR-625 exerts an inhibitory action on the growth and metastasis of CNE-1 and SUNE-1 cells. (**A**–) Quantitation of proliferation by the CCK-8 assay and of apoptosis by flow cytometric analysis of CNE-1 and SUNE-1 cells after transfect omir-625 or C. *P < 0.05 vs the agomir-NC group. (**C, D**) Transwell assays showed alteration of migratory and invasive abilities of CNE-1 and SUNE-1 cells at we ted with agomir-625. *P < 0.05 vs group agomir-NC.

between miR-625 and NUAK1 mRNA leve among the NPC tissue samples was observed in Spea an cor ation analysis (Figure 5F; $R^2 = 0.3739$, P < 0.0001). ummary, these experiments identified NUA as a direct rget of miR-625 in NPC cells.

Restoration Of UAK Expression Rescues NPC Ce rom iR-625–Induced G owt An Metastasis Inhibitio In Vi o

Having demon ed that NUAK1 is a direct target of miR-625, we next test whether the tumor-suppressive role of miR-625 in NPC cells was mediated by the decrease in NUAK1 expression. MiR-625–overexpressing CNE-1 and SUNE-1 cells were cotransfected with NUAK1-overexpressing plasmid (pc-NUAK1) or the empty pcDNA3.1 plasmid. The downregulation of NUAK1 in CNE-1 and SUNE-1 cells by agomir-625 transfection was reversed after cotransfection with pc-NUAK1, as evidenced by Western blotting (Figure 6A, P < 0.05). The results of the CCK-8 assay suggested that the growth-inhibitory effect of miR-625 overexpression in CNE-1 and SUNE-1 cells was attenuated by NUAK1 restoration (Figure 6B, P < 0.05). In addition, reintroduction of NUAK1 hampered the influence of miR-625 overexpression on the apoptosis (Figure 6C, P < 0.05), migration, and invasiveness (Figure 6D, P < 0.05) of CNE-1 and SUNE-1 cells. Collectively, these findings suggested that NUAK1 downregulation mediates the tumor-suppressive effects of miR-625 on NPC growth and metastasis in vitro.

MiR-625 Downregulation Neutralizes The Effects Of LINC00958 Knockdown On NPC Cell Growth And Metastasis In Vitro

We next tested whether the oncogenic roles of LINC00958 in NPC cells were dependent on the sponging of miR-625. To this end, rescue experiments were conducted with LINC00958-deficient CNE-1 and SUNE-1 cells by cotransfection with either antagomir-625 or antagomir-NC. The efficiency of antagomir-625 was confirmed by RT-qPCR analysis (Figure 7A, P < 0.05). The LINC00958 knockdown significantly increased miR-625 amounts (Figure 7B, P < 0.05) and reduced NUAK1 protein levels (Figure 7C, P < 0.05) in CNE-1

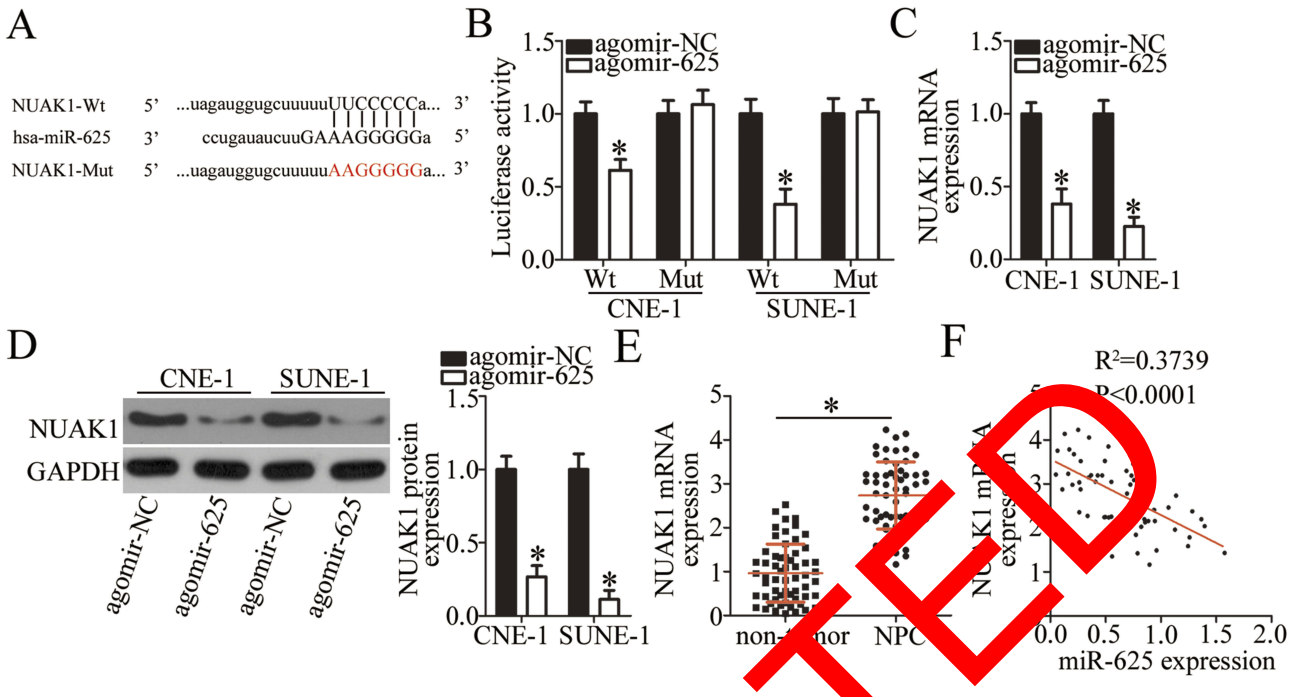

Figure 5 *NUAK1* is a direct target gene of miR-625 in NPC. (**A**) The binding sequences of miR-625 in the 3′-UTR of *NUAK1* mRNA predicted by miRDB and TargetScan. The positions of mutated nucleotides (red) in the 3′-UTR of *NUAK1* mRNA are also shown. (**B**) CNE-1 and SUNE-1 cells that were cotransfected with either agomir-625 or agomir-NC and either NUAK1-Wt or NUAK1-Mut were harvested at 48 h post-transfection and subjected to the detection of luciferase activity. *P < 0.05 vs group agomir-NC. (**C, D**) RT-qPCR and Western blotting were carried out to assess the expression of NUAK1 mRNA and protein in CNE-1 and SUNE-1 cells transfected with either agomir-625 or agomir-NC. *P < 0.05 vs the agomir-NC group. (**E**) Quantification of NUAK1 expression in 15 pairs of NPC tissue samples and matched adjacent non-tumor nasopharyngeal epithelial tissue samples. *P < 0.05 vs non-tumor nasopharyngeal epithelial tissue samples. (**F**) Spearman correlation analysis was conducted to determine the correlation of miR-625 with NUAK1 expression among the NPC tissue samples. $R^2 = 0.373$ $P < 0.0001$.

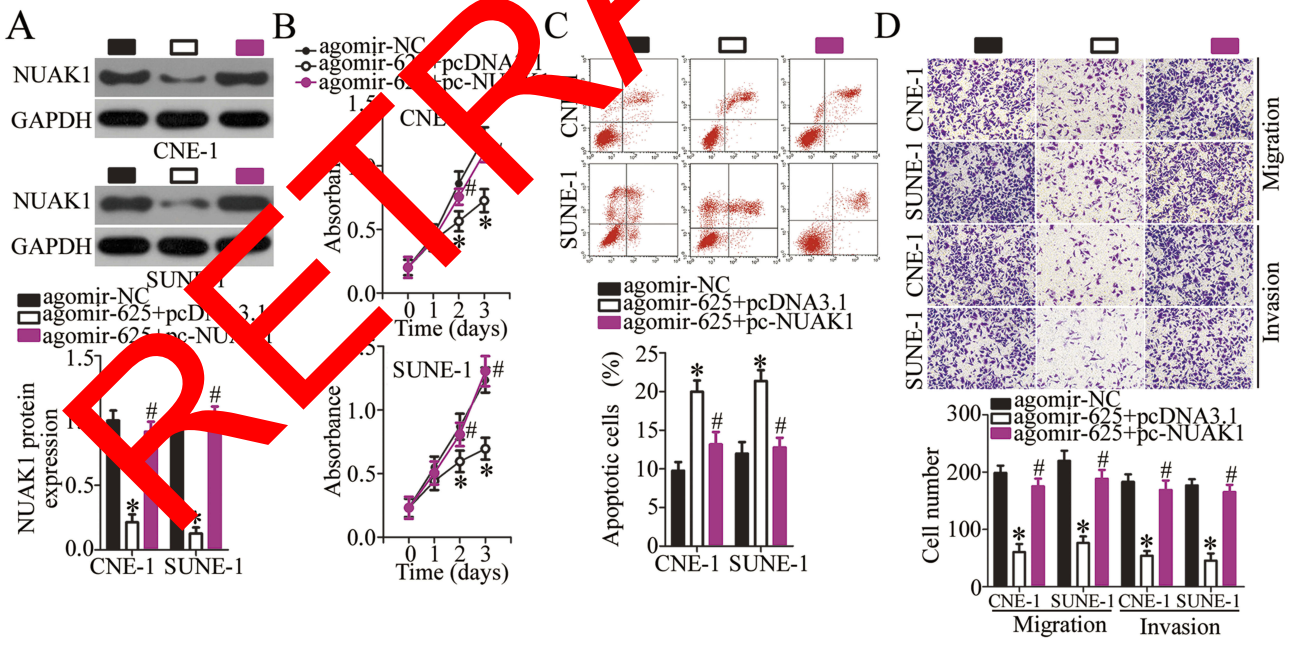

Figure 6 NUAK1 restoration rescues CNE-1 and SUNE-1 cells from miR-625 overexpression–induced inhibition of growth and metastasis in vitro. (**A**) MiR-625–overexpressing CNE-1 and SUNE-1 cells that were next cotransfected with either pc-NUAK1 or pcDNA3.1 were subjected to Western blotting for the measurement of NUAK1 protein expression. *P < 0.05 vs group agomir-NC. #P < 0.05 vs the agomir-625+pcDNA3.1 group. (**B**–**D**) The proliferation, apoptosis, migration, and invasiveness of CNE-1 and SUNE-1 cells treated as described above were investigated by the CCK-8 assay, flow cytometry, and Transwell assays, respectively. *P < 0.05 vs group agomir-NC. #P < 0.05 vs the agomir-625+pcDNA3.1 group.

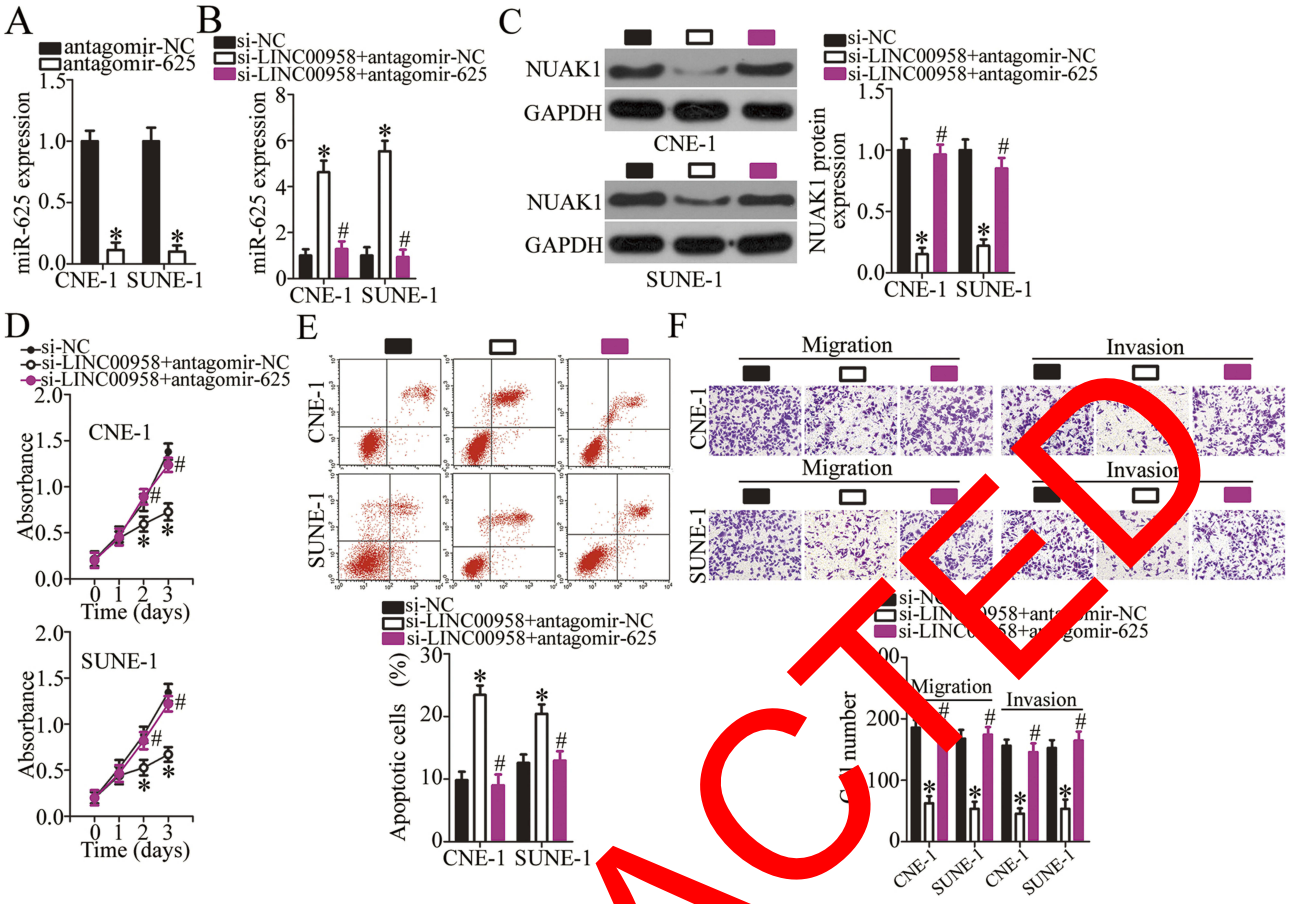

Figure 7 The influence of LINC00958 downregulation on the malignant characteristics of CNE-1 and SUNE-1 cells was partially reversed by the miR-625 knockdown. (**A**) The efficiency of antagomir-625 in CNE-1 and SUNE-1 cells was validated by RT-qPCR. *P < 0.05 vs the antagomir-NC group. (**B, C**) CNE-1 and SUNE-1 cells were cotransfected with si-LINC00958 and either antagomir-625 or antagomir-NC. The miR-625 and NUAK1 protein levels were analyzed by RT-qPCR and Western blotting, respectively. *P < 0.05 vs group si-NC. #P < 0.05 vs the si-LINC00958+antagomir-NC group. (**D–F**) The proliferation, apoptosis, migration, and invasiveness of the aforementioned cells were assessed by the CCK-8 assay, flow cytometry, and cell assays, respectively. *P < 0.05 vs group si-NC. #P < 0.05 vs the si-LINC00958 +antagomir-NC group.

and SUNE-1 cells, and these outcomes were abrogated following cotransfection with antagomir-625. The functional experiments were performed, and revealed that the effects of the LINC00958 knockdown on proliferation (Figure 7D, P < 0.05), apoptosis (Figure 7E, P < 0.05), migration, and invasiveness (Figure 7F, P < 0.05) of CNE-1 and SUNE-1 cells were attenuated by the transfection of antagomir-625. These observations indicated that LINC00958 exerts oncogenic effects on NPC progression by functioning as a competitive endogenous RNA for miR-625.

LINC00958 Silencing Restrains The Tumor Growth Of NPC Cells In Vivo Through Alleviation Of Sponging Of miR-625

To test whether downregulation of LINC00958 impairs the tumor growth of NPC cells in vivo, an in vivo nude mouse model was implemented by injection of LINC00958-deficient CNE-1 cells into the flanks of nude mice. After an observation period of 4 weeks, the tumor growth (Figure 8A and B, P < 0.05) and weight (Figure 8C, P < 0.05) of the si-LINC00958 group was found to be significantly lower than that in the si-NC group. Total-RNA and total-protein samples were isolated from tumor xenografts and subjected to RT-qPCR and Western blot assays, respectively. The results indicated that the tumor xenografts derived from si-LINC00958–transfected CNE-1 cells featured significant upregulation of miR-625 (Figure 8D, P < 0.05) and downregulation of NUAK1 protein (Figure 8E) levels. To confirm that these effects were due to the LINC00958 knockdown, RT-qPCR analysis was performed to evaluate LINC00958 expression in the tumor xenografts. The expression levels of LINC00958 were still lower in the tumor xenografts obtained from the si-LINC00958 group relative to the si-NC group (Figure 8F, P < 0.05). These data confirmed the effects of the

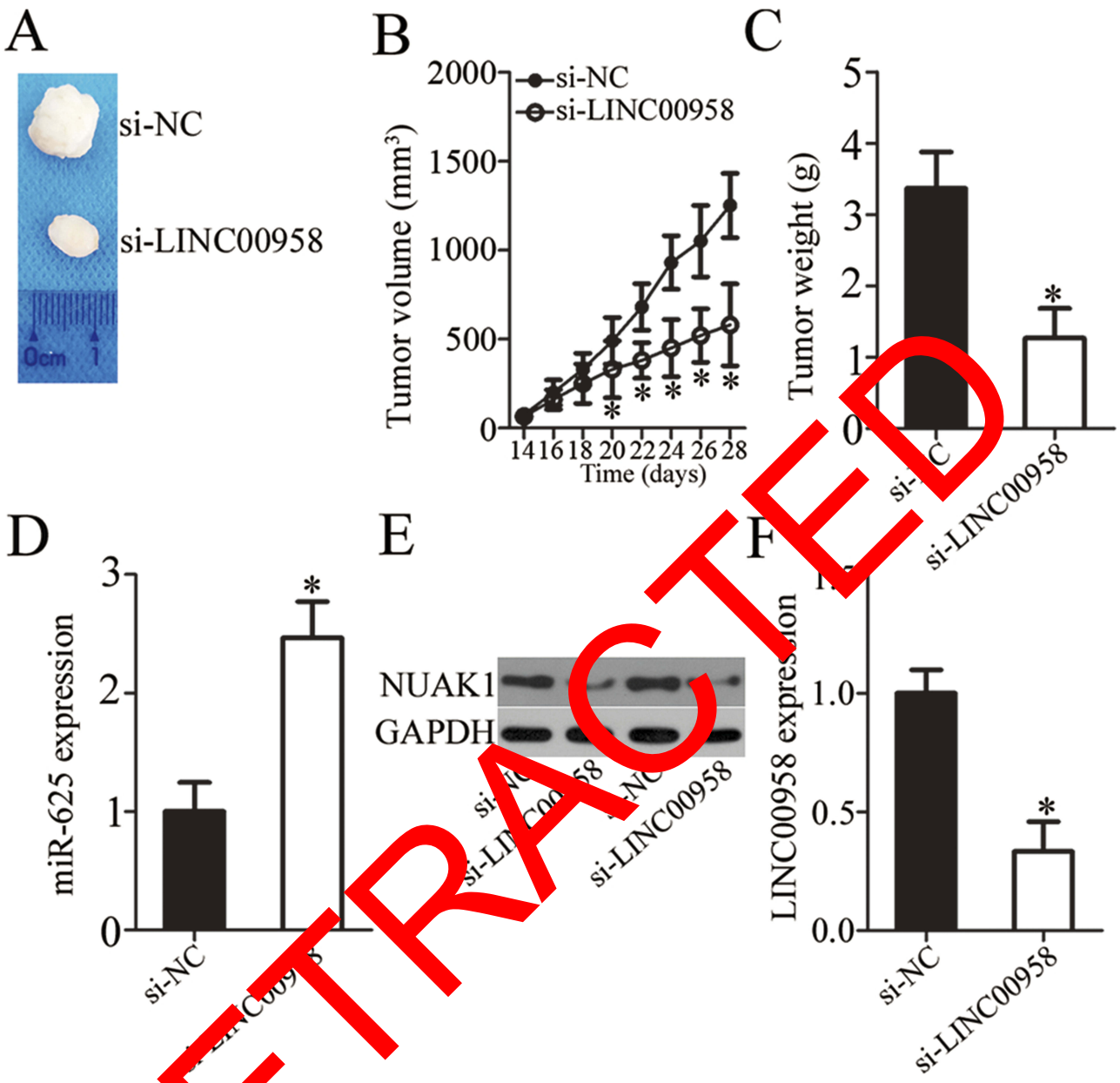

Figure 8 LINC00958 knockdown impairs the tumor growth of NPC cells in vivo. (**A**) A representative image of tumor xenografts derived from si-LINC00958–transfected or si-NC–transfected CNE-1 cells. (**B**) Either si-LINC00958–transfected or si-NC–transfected CNE-1 cells were subcutaneously injected into nude mice. Tumor volumes were monitored every 2 days starting 2 weeks after the injection. *P < 0.05 vs group si-NC. (**C**) The weight of tumor xenografts was determined at 4 weeks after the cancer cell inoculation. *P < 0.05 vs group si-NC. (**D, E**) The expression levels of miR-625 and of the NUAK1 protein were measured in tumor xenografts by RT-qPCR and Western blotting, respectively. *P < 0.05 vs the si-NC group. (**F**) RT-qPCR analysis of LINC00958 expression in tumor xenografts formed by si-LINC00958–transfected or si-NC–transfected CNE-1 cells. *P < 0.05 vs the si-NC group.

LINC00958–miR-625–NUAK1 pathway on NPC tumor growth in vivo.

Discussion

Increasing numbers of studies show that the dysregulation of lncRNA is an indicator of different types of human cancer, including NPC.[35–37] LncRNAs can play either tumor-suppressive or oncogenic roles in NPC and affect numerous molecular processes associated with carcinogenesis including cancer progression.[38,39] Therefore, a thorough understanding of the important functions of cancer-specific lncRNAs in the malignant characteristics of NPC is critical for identification of promising targets for anticancer therapies. In this study, we evaluated LINC00958 expression in NPC and investigated its clinical significance in patients with NPC. The potential roles of LINC00958 in the malignancy of NPC and the molecular

mechanisms governing these processes were explored at a molecular level. Our findings provide a novel insight into a potential therapeutic approach to NPC via targeting of the LINC00958–miR-625–NUAK1 pathway.

LINC00958 has been well studied in several types of human cancer. For instance, LINC00958 is upregulated in gastric cancer; upregulation of LINC00958 manifests an obvious correlation with adverse clinical parameters.[40] Patients with gastric cancer exhibiting high LINC00958 expression show worse overall survival than do patients with low LINC00958 expression.[40] Notably, LINC00958 has been validated as an independent prognostic factor of gastric cancer.[40] The upregulation of LINC00958 in pancreatic cancer,[41] glioma,[42] and bladder cancer[43] has also been reported. However, little is known about the expression profile of LINC00958 in NPC. In this study, we demonstrated that LINC00958 is overexpressed in both NPC tissues and cell lines. The high LINC00958 expression was significantly associated with tumor size, lymph node status and TNM stage among patients with NPC. An obvious connection was identified between the worse overall survival and high LINC00958 expression among the NPC patients. Accordingly, LINC00958 may be developed as a biomarker for the diagnosis and prognosis of NPC.

Some studies suggest that LINC00958 acts as an oncogene in multiple human cancer types. For example, downregulation of LINC00958 inhibits epithelial–mesenchymal transition, invasiveness, and metastasis of pancreatic cancer cells.[41] Silencing of LINC00958 expression restricts glioma cell proliferation and invasion, promotes cell cycle arrest at the G0–G1 transition in vitro, and impairs tumor growth in vivo.[42] In bladder cancer, knockdown of LINC00958 decreases cell viability, migration, invasion and resistance to anoikis.[43] In contrast, the regulatory roles of LINC00958 in NPC progression have not been investigated to date. Herein, functional assays revealed that the LINC00958 knockdown decreased NPC cell growth and metastasis in vitro and hindered tumor growth in vivo. These data suggest that LINC00958 may be considered a potential target for the treatment of patients with NPC.

Identification of the specific mechanisms of LINC00958 action is crucial for the early diagnosis and improvement of clinical outcomes among patients with NPC. In this study, we demonstrated that the LINC00958 knockdown alleviates miR-625 sponging thereby reducing NUAK1 expression in NPC and suppressing the malignant characteristics in vitro and in vivo. MiR-625 expression is low in a variety of human cancers, including colorectal cancer,[26] hepatocellular carcinoma,[27] esophageal cancer,[28] breast cancer,[29] laryngeal squamous cell carcinoma,[30] gastric cancer,[31] and glioma.[32] In terms of the function, miR-625 acts as a tumor-suppressor in the aforementioned human cancer types. In this study, to the best of our knowledge, the expression status, roles, and mechanisms of action of miR-625 in NPC were investigated for the first time. Our results revealed that miR-625 is underexpressed in NPC. MiR-625 overexpression downregulates NUAK1 thereby inhibiting NPC cell proliferation, migration, and invasion but promoting apoptosis.

NUAK1, a member of the AMP-activated protein kinase (AMPK) catalytic subunit family,[44] is overexpressed in NPC.[33] Clinically, elevated NUAK1 expression significantly correlates with the maximum neck lymph node diameter and World Health Organization histological type among patients with NPC.[33] In addition, an increased NUAK1 level strongly inversely correlates with overall survival and disease-free survival of NPC patients.[33] Functionally, downregulation of NUAK1 restrains NPC cell migration and invasion in vitro and tumor metastasis in vivo.[34] In the present study, our results make it clear that the regulatory participation of LINC00958 in the aggressive behaviors of NPC cells in vitro and in vivo is partly mediated by its role as a competitive endogenous RNA for miR-625 and consequent upregulation of NUAK1.

Conclusion

In summary, we show for the first time, to our knowledge, that LINC00958 works as a novel oncogene promoting the malignancy of NPC by sponging miR-625 and thereby disinhibiting the expression of the downstream target NUAK1. This study expands the understanding of NPC pathogenesis and will facilitate identification of lncRNA-directed diagnostic biomarkers and therapeutic targets in NPC.

Abbreviations

3′-UTR, 3′ untranslated region; CCK-8, Cell Counting Kit-8; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; lncRNA, long noncoding RNA; miRNA, miR, microRNA; NPC, nasopharyngeal carcinoma; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; siRNA, small interfering RNA.

Ethics Approval And Informed Consent

The research protocols were approved by the Ethics Committee of Yidu Central Hospital of Weifang and were carried out according to the Declaration of Helsinki. Written informed consent for the use of tissue specimens was provided by all the participants. The Animal Care and Use Committee of Yidu Central Hospital of Weifang approved this animal experiment, and the experimental procedures were in accordance with the Animal Protection Law of the People's Republic of China-2009 for experimental animals.

Disclosure

The authors report no conflicts of interest in this work.

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