LncRNA NEAT1 exacerbates non-small cell lung cancer by upregulating EIF4G2 via miR-582-5p sponging

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Abstract: In this study, we aimed to elucidate the role of long non-coding RNA nuclear enriched abundant transcript 1 (lncRNA NEAT1) in non-small cell lung cancer (NSCLC). Quantitative real-time polymerase chain reaction (qRT-PCR) was employed to detect the abundance of NEAT1, microRNA-582-5p (miR-582-5p) and eukaryotic translation initiation factor 4 gamma 2 (EIF4G2). Proliferation, apoptosis, metastasis and glycolytic metabolism were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) flow cytometry, transwell assays and fluorescence-based glucose and lactate assay kits. The targets of NEAT1 and miR-582-5p were predicted by the starBase website, and dual-luciferase reporter and RNA immunoprecipitation (RIP) assays were performed to verify these predictions. Western blot analysis was conducted to detect the protein expression of EIF4G2. A xenograft tumor model was built to clarify the role of NEAT1 *in vivo*. Results showed that NEAT1 interference inhibited proliferation, metastasis and glycolysis, and facilitated the apoptosis of NSCLC cells. MiR-582-5p was a functional target of NEAT1, and the biological influence of NEAT1 intervention on NSCLC cells was alleviated by transfection with anti-miR-582-5p. MiR-582-5p could bind to EIF4G2 messenger RNA (mRNA); it exerted its antitumor role in NSCLC cells by inhibiting EIF4G2. EIF4G2 was regulated by NEAT1/miR-582-5p signaling. NEAT1 accelerated NSCLC tumor growth via the miR-582-5p/EIF4G2 axis *in vivo*. In conclusion, NEAT1 affected NSCLC by elevating their malignant potential via the miR-582-5p/EIF4G2 axis.

Keywords: NEAT1; miR-582-5p; EIF4G2; metastasis; glycolysis

Abbreviations: long non-coding RNA (lncRNA); nuclear enriched abundant transcript 1 (NEAT1); non-small cell lung cancer (NSCLC); eukaryotic translation initiation factor 4 gamma (EIF4G); eukaryotic initiation factor 4F (EIF4F)

INTRODUCTION

As a major histopathological type of lung cancer, non-small cell lung cancer (NSCLC) accounts for 70%-80% of lung cancer cases [1,2]. Tumor cells consume glucose and metabolize it into lactate even in the presence of oxygen, and this phenomenon is termed the Warburg effect [3]. The suppression of glycolysis could impede the development of cancers [4]. Although the treatment strategy has been improved, the prognosis of NSCLC patients remains dismal owing to metastasis and relapse [5]. Therefore, it is urgent to uncover the potential mechanism behind the proliferation, motility and glycolysis of NSCLC cells.

Long non-coding RNAs (lncRNAs) are a group of non-coding RNAs (ncRNAs) with at least 200 nucleotides [6]. There is accumulating evidence that lncRNAs are involved in the initiation and progression of multiple cancers including NSCLC [7-9]. LncRNA nuclear enriched abundant transcript 1 (NEAT1) has been reported as a prognostic marker for patients with breast cancer [10]. It was claimed that NEAT1 elevated paclitaxel resistance of ovarian cancer cells [11]. The protumor role of NEAT1 in NSCLC has also been reported in previous studies [12-14]. Nevertheless, the regulatory network behind NEAT1 in NSCLC is still not fully understood.

MicroRNAs (miRNAs) are another class of ncRNAs that contain 19-24 nucleotides. MiRNAs could regulate cellular biological functions by inducing degradation or by repressing the translation of messenger RNAs (mRNAs) by direct combination [15,16]. The crucial role of miRNAs in diverse cancers has been reported [17-19]. Herein, we focused on the role of miR-582-5p in NSCLC. It was shown that miR-582-5p suppressed the progression of NSCLC via mitogen-activated protein kinase kinase kinase 2 (MAP3K2) [20], and that miR-582-5p restrains the proliferation and invasion of NSCLC cells via Notch homolog 1, translocation-associated (NOTCH1) [21]. In the present study, we found a new regulatory mechanism involving miR-582-5p in NSCLC.

Eukaryotic translation initiation factor 4 gamma 2 (EIF4G2) is a component of eukaryotic initiation factor 4F (EIF4F). The protumor role of EIF4G2 in multiple cancers has been described in previous studies [22,23]. It was demonstrated that miR-379 could inhibit cisplatin resistance of NSCLC cells through downregulation of EIF4G2, suggesting a protumor role of EIF4G2 in NSCLC [24]. However, the molecular mechanism involving EIF4G2 in NSCLC remains largely unexplored.

In the current study, we examined the expression profile of NEAT1 in NSCLC, and loss-of-function experiments were employed to investigate the role of NEAT1 in NSCLC. The lncRNA-miRNA-mRNA signal network was then examined in order to study the working mechanism of NEAT1 in NSCLC.

MATERIALS AND METHODS

Tissue samples

Paired NSCLC tissues (n=40) and adjacent non-tumor tissues (n=40) were obtained from NSCLC patients in the Affiliated Hospital of Shandong University of Traditional Chinese Medicine who underwent surgical resection. The tissues were snap-frozen in liquid nitrogen to detect the expression of NEAT1, miR-582-5p and EIF4G2. The procedures in this study were approved by the Ethics Committees of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine. Written informed consent was provided from all subjects before radical resection.

Cell culture

NSCLC cell lines (A549 and H1299) and the normal human lung epithelial cell line BEAS-2B were purchased from BeNa Culture Collection (Beijing, China). All cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 10% penicillin (100 U/mL) and 10% streptomycin (100 μg/mL) at 37°C in a humidified incubator with 5% CO_2.

Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used for RNA extraction from tissues and cells. RNA concentration was measured using NanoDrop 2000 (NanoDrop Technologies, Wilmington, DE, USA). Complementary DNA (cDNA) was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and an All-in-One miRNA First stand cDNA Synthesis Kit (GeneCopoeia, Rockville, MD, USA). The amplification reaction was conducted using SYBR® Premix Ex Taq™ II (Takara, Osaka, Japan). The PCR reaction system and conditions were as follows: PCR reaction system (25 μL): SYBR® Premix Ex Taq™ (2×; 12.5 μL), forward primer (10 μM; 0.5 μL), reverse primer (10 μM; 0.5 μL), DNA template (2 μL), dH_2O (9.5 μL). PCR condition: 95°C for 30 s followed by 35 cycles of 95°C for 5 s and 60°C for 30 s. The abundance of NEAT1, miR-582-5p and EIF4G2 was calculated using the $2^{-\Delta\Delta Ct}$ method. The level of miR-582-5p was normalized to U6 small nuclear RNA (snRNA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal control for NEAT1 and EIF4G2. The primers used in this study were designed as follows: NEAT1 (forward, 5'-ATGCCACAACGCAGATTGAT-3'; reverse, 5'-CGAGAAACGCACAAGAAGG-3'), miR-582-5p (forward, 5'-GCGGTTACAGTTGTTCAACC-3'; reverse, 5'-CTCAACTGGTGTCGTGGA-3'), EIF4G2 (forward, 5'-CCCTTCAAGATGCACCTCAT-3'; reverse, 5'-GTGGTCCACAGCATTTCCTT-3'), U6 (forward, 5'-CTCGCTTCGGCAGCACA-3'; reverse, 5'-AACGCTTCACGAATTTGCGT-3') and GAPDH (forward, 5'-GGAGCGAGATCCCTCCAAAAT-3'; reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3').

Cell transfection

NEAT1 specific small interfering RNA (si-NEAT1 #1 and si-NEAT1 #2) and siRNA negative control (si-NC), NEAT1 specific short hairpin RNA (sh-NEAT1) and control (sh-NC), NEAT1 ectopic expression plasmid (NEAT1), EIF4G2 ectopic expression plasmid (EIF4G2) and control (vector), miR-582-5p mimic (miR-582-5p) and miR-NC, miR-582-5p inhibitor (anti-miR-582-5p) and control (anti-miR-NC) were obtained from Genepharma (Shanghai, China). Transfection was carried out using Lipofectamine 3000 (Invitrogen, USA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

After transfection for 0 h, 24 h, 48 h or 72 h, NSCLC cells were incubated with 10 μL MTT (Invitrogen, USA) for 4 h. The absorbance at 490 nm was detected by a microplate reader.

Flow cytometry for apoptosis analysis

The apoptotic rate of NSCLC cells with specific treatment was analyzed by flow cytometry using an Annexin V apoptosis detection kit (BD Pharmingen™, San Diego, CA, USA). The apoptotic NSCLC cells were identified by a FC-500 flow cytometer (Beckman Coulter, Pasadena, CA, USA).

Transwell assays

To measure the invasive ability of NSCLC cells, transwell upper chambers (Costar, Corning, NY, USA) were pre-coated with Matrigel (BD Pharmingen™). NSCLC cells (2×10^4 cells per well) suspended in 100 μL serum-free medium were added into the pre-coated upper chambers; 500 μL medium supplemented with 10% fetal bovine serum (FBS) was added to the lower chambers. After incubation for 24 h, invaded NSCLC cells were stained with 0.5% crystal violet (0.5%; Sigma, USA). The number of invaded NSCLC cells in five random fields was counted using an optical microscope. To detect the migration capacity of NSCLC cells, a cell suspension was added to un-coated upper chambers following the same procedures.

Glucose uptake and lactate production analysis

Glycolysis of NSCLC cells was analyzed by measuring glucose uptake and lactate production using fluorescence-based glucose and lactate assay kits (BioVision, Milpitas, California, USA).

Dual-luciferase reporter assay

The candidate targets of NEAT1 and miR-582-5p were predicted by the starBase database. The sequence of NEAT1 and the 3' untranslated region (3'UTR) of EIF4G2 mRNA, including the binding sites with miR-582-5p, was cloned into pGL3 vector (Promega, Madison, WI, USA), generating NEAT1-WT and EIF4G2 3'UTR-WT. The corresponding mutant-type reporter vectors were termed NEAT1-MUT and EIF4G2 3'UTR-MUT. Luciferase activity was measured in A549 and H1299 cells cotransfected with miR-NC or miR-582-5p and the above reporter plasmid through the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

RNA immunoprecipitation (RIP) assay

The RIP assay was performed in order to extract RNA-RNA/protein complexes with the Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA).

Western blot assay

Equal amounts of protein samples (30 μg) were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane, followed by blocking with 5% non-fat milk for 1 h at room temperature. The specific primary antibodies, including anti-EIF4G2 (HPA006773; Sigma, USA) and anti-actin beta (ACTB) (ab8224; Abcam, Cambridge, MA, USA), were used to probe the membrane; the membrane was probed with horseradish peroxidase (HRP)-combined secondary antibodies (ab97080, Abcam and ab97040, Abcam, UK). The protein signal was determined using enhanced chemiluminescence (ECL) chromogenic substrate (GE Healthcare, Amersham, UK).

Xenograft tumor model

This study was approved by the Animal Research Committee of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine. BALB/c-nu mice (5 weeks old) were purchased from the Chinese Academy of Medical Sciences (Beijing, China). A549 cells (2×10^6) stably transfected with sh-NC or sh-NEAT1 were suspended in 100 µL phosphate buffered saline (PBS). The back region of nude mice (n=5) was subcutaneously inoculated with A549 cells. The volume of NSCLC tumors was calculated using the following formula of volume=$\pi\times$(length$\times$width^2)/6. Mice were killed after 35 days inoculation and the tumors were resected and weighed.

Statistical analysis

The data are presented as the mean±standard deviation (SD). Student's t-test was utilized to compare the differences between two groups, while the differences among multiple groups were assessed using one-way or two-way analysis of variance (ANOVA) followed by Tukey's test. The survival curve was generated using the Kaplan-Meier plot and log-rank test. The linear relationship was evaluated by Spearman's correlation coefficient. A P value less than 0.05 was considered statistically significant.

RESULTS

NEAT1 is aberrantly upregulated in NSCLC tissues and cells

Abnormal upregulation in the expression of NEAT1 was observed in NSCLC tumor samples when compared with that in adjacent normal tissues (Fig. 1A). As shown in Fig. 1B, high expression of NEAT1 was associated with the poor survival rate of NSCLC patients. To uncover the molecular mechanism of NEAT1 in NSCLC, two
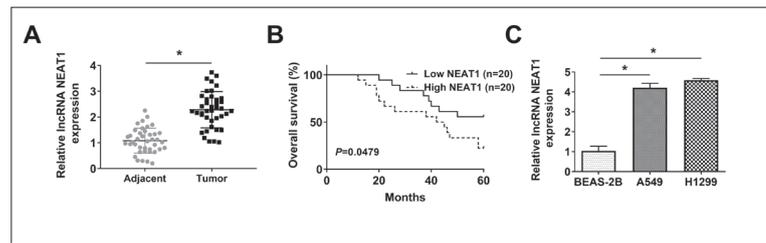

Fig. 1. NEAT1 is aberrantly upregulated in NSCLC tissues and cells. **A** – The expression of NEAT1 measured in NSCLC tissue specimens (n=40) and adjacent normal tissue specimens (n=40) by qRT-PCR. **B** – The survival curve of NSCLC patients generated using the Kaplan-Meier plot and log-rank test. **C** – qRT-PCR employed to determine the abundance of NEAT1 in NSCLC cell lines, including A549 and H1299 and the normal human lung epithelial cell line BEAS-2B. *$P<0.05$.
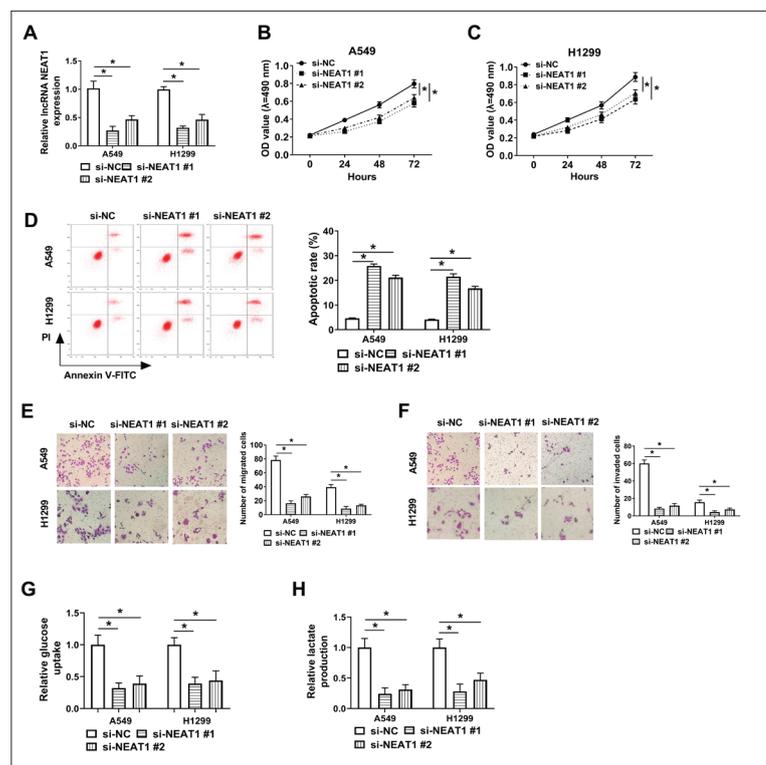

Fig. 2. NEAT1 potentiates the malignancy of NSCLC cells. A549 and H1299 cells were transfected with si-NC, si-NEAT1 #1 or si-NEAT1 #2. **A** – The enrichment of NEAT1 examined in A549 and H1299 by qRT-PCR. **B, C** – MTT assay employed to assess the proliferation of NSCLC cells. **D** – The apoptotic rate of NSCLC cells evaluated by flow cytometry. **E, F** – Transwell assays conducted to detect the capacities of migration and invasion of NSCLC cells. **G, H** – Aerobic glycolysis of NSCLC cells analyzed using fluorescence-based glucose and lactate assay kits. *$P<0.05$.

NSCLC cell lines (A549 and H1299) and normal human lung epithelial cell line BEAS-2B were used in this study. The level of NEAT1 was markedly enhanced in NSCLC cells in comparison with that in BEAS-2B cells (Fig. 1C). In summary, NEAT1 was highly expressed in NSCLC tissues and cells.
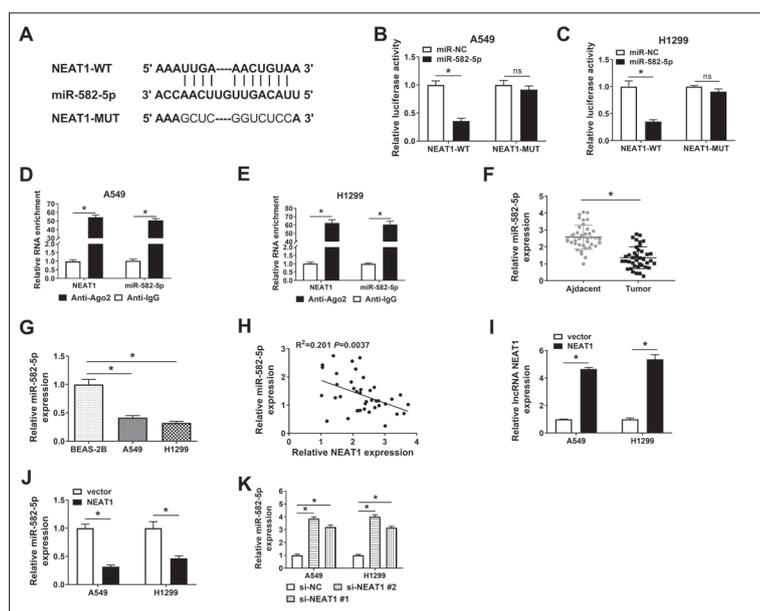
Fig. 3. NEAT1 can sponge miR-582-5p. **A** – The binding sites between NEAT1 and miR-582-5p predicted by starBase database; mutant binding sites in NEAT1 are shown in red. **B**, **C** – The target relationship between NEAT1 and miR-582-5p confirmed by the dual-luciferase reporter assay. **D**, **E** – RNA immunoprecipitation (RIP) assay applied to validate the combination between NEAT1 and miR-582-5p. **F**, **G** – The expression of miR-582-5p detected in NSCLC tissues (n=40) and cells and corresponding normal tissues (n=40) and BEAS-2B cells by qRT-PCR. **H** – The linear relationship between miR-582-5p and NEAT1 analyzed by Spearman's correlation coefficient. **I** – The overexpression efficiency of NEAT1 overexpression plasmid was evaluated by qRT-PCR. **J** – The abundance of miR-582-5p measured in A549 and H1299 cells transfected with vector or NEAT1 by qRT-PCR. **K** – qRT-PCR employed to detect the level of miR-582-5p in A549 and H1299 cells transfected with si-NC, si-NEAT1 #1 or si-NEAT1 #2, respectively. *$P<0.05$.

NEAT1 potentiates the malignancy of NSCLC cells

qRT-PCR revealed that the knockdown efficiencies of two NEAT1 small interfering RNAs (si-NEAT1 #1 and si-NEAT1 #2) in NSCLC cells were high (Fig. 2A). Loss-of-function experiments were conducted to explore the biological functions of NEAT1 in NSCLC. NEAT1 intervention significantly restrained the proliferation of NSCLC cells (Fig. 2B and C). The apoptotic rate of NSCLC cells was increased with the transfection of si-NEAT1 #1 or si-NEAT1 #2 (Fig. 2D), suggested that NEAT1 suppressed the apoptosis of NSCLC cells. The motility of NSCLC cells with NEAT1 interference was also assessed by performing a transwell migration assay and a transwell invasion assay. As mentioned in Fig. 2E and F, NEAT1 interference notably inhibited the migration and invasion of NSCLC cells. Cancer cells

obtained energy mainly through glycolysis instead of oxidative phosphorylation [3]. The influence of NEAT1 silencing on the glycolysis of NSCLC cells was also evaluated by measuring glucose uptake and lactate production. NEAT1 intervention caused decreased consumption of glucose and production of lactate (Fig. 2G and H), demonstrated that NEAT1 could enhance the glycolysis of NSCLC cells. Taken together, we can conclude that NEAT1 promoted the proliferation, metastasis and glycolysis and suppressed the apoptosis of NSCLC cells. Si-NEAT1 #1 was chosen for the subsequent experiments owing to its higher interference efficiency in NSCLC cells.

NEAT1 can sponge miR-582-5p

Studies have reported that lncRNAs could serve as miRNAs sponges to modulate the levels of target miRNAs and matching downstream genes [25]. LncRNA NEAT1-miRNAs interactions were investigated using starBase online software, and miR-582-5p was chosen for the following experiments due to its tumor suppressor role in NSCLC [20, 21]. The binding sequence between NEAT1 and miR-582-5p predicted by starBase database is shown in Fig. 3A. A dual-luciferase reporter assay revealed that luciferase activity was prominently decreased in miR-582-5p and the NEAT1-WT cotransfected group as compared with that in the miR-NC and NEAT1-WT cotransfected group; cotransfection of miR-NC or miR-582-5p made no difference in the luciferase activity of NEAT1-MUT group (Fig. 3B and C), suggesting that miR-582-5p could directly interact with NEAT1 through putative binding sites. Besides, NEAT1 and miR-582-5p were both enriched in the Anti-Ago2 group (Fig. 3D and E), which indicated that NEAT1 could bind to the RNA-induced silencing complex (RISC), likely through a target relationship with miR-582-5p. Subsequently, we detected the expression of miR-582-5p in NSCLC tissues and cells. As indicated in Fig. 3F and G, the level of miR-582-5p was conspicuously decreased in NSCLC tissues and cells. A prominent
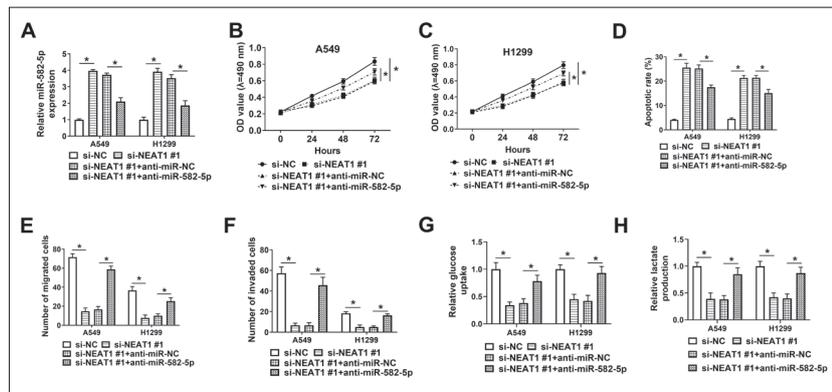
Fig. 4. NEAT1 promotes the progression of NSCLC through sponging of miR-582-5p. A549 and H1299 cells were transfected with si-NC, si-NEAT1 #1, si-NEAT1 #1+anti-miR-NC or si-NEAT1 #1+anti-miR-582-5p. **A** – The level of miR-582-5p examined in NSCLC cells by qRT-PCR. **B, C** – The proliferation of NSCLC cells analyzed by the MTT assay. **D** – Apoptosis of NSCLC cells measured by flow cytometry. **E, F** – The metastasis ability of NSCLC cells, including the migration and invasion abilities, assessed by transwell assays. **G, H** – Glucose uptake and lactate production of NSCLC cells determined using fluorescence-based glucose and lactate assay kits. *$P<0.05$.
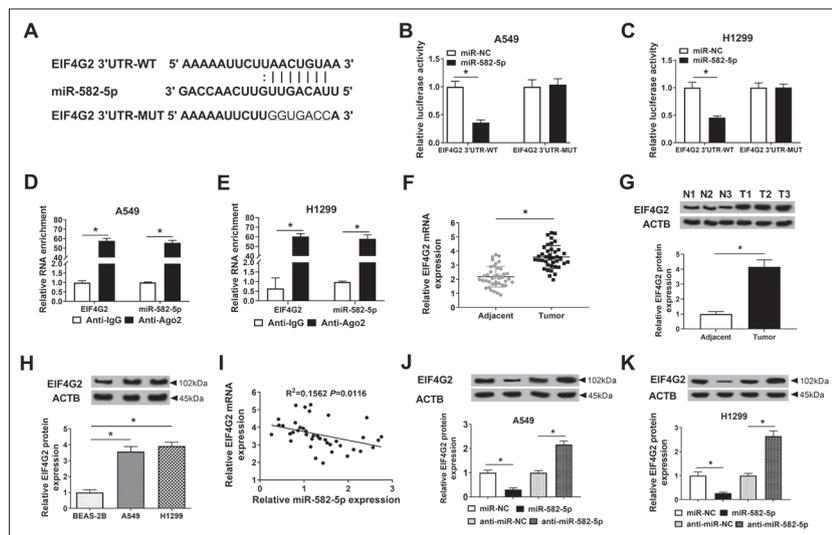

Fig. 5. EIF4G2 is a direct target of miR-582-5p in NSCLC cells. **A** – Complementary sites between miR-582-5p and EIF4G2 predicted by starBase bioinformatic software. **B, C** – Luciferase activity measured in NSCLC cells transfected with miR-NC or miR-582-5p and EIF4G2 3'UTR-WT or EIF4G2 3'UTR-MUT. **D, E** – The target relationship between miR-582-5p and EIF4G2 verified by the RIP assay. **F** – mRNA expression of EIF4G2 in NSCLC tissues (n=40) and adjacent normal tissues (n=40) determined by qRT-PCR. **G** – Western blotting carried out to detect the protein level of EIF4G2 in NSCLC tissues (n=3) and matching non-tumor tissues (n=3). **H** – Protein expression of EIF4G2 was examined in NSCLC and BEAS-2B cell lines by Western blotting. **I** – The correlation between miR-582-5p and EIF4G2 mRNA analyzed using Spearman's correlation coefficient. **J, K** – The protein level of EIF4G2 in NSCLC cells transfected with miR-NC, miR-582-5p, anti-miR-NC or anti-miR-582-5p determined by Western blot analysis. *$P<0.05$.

negative correlation between the expression of miR-582-5p and NEAT1 in NSCLC tissues was observed (Fig. 3H). The expression of NEAT1 was elevated with the transfection of NEAT1 ectopic expression plasmid, as compared with that in the vector group (Fig. 3I). The accumulation of NEAT1 caused a significant reduction in the expression of miR-582-5p in NSCLC cells (Fig. 3J), and the intervention of NEAT1 elevated the enrichment of miR-582-5p in NSCLC cells (Fig. 3K). In summary, lncRNA NEAT1 suppressed the expression of miR-582-5p through direct interaction.

NEAT1 promotes the progression of NSCLC through sponging miR-582-5p

Rescue experiments were conducted to explore whether NEAT1 exerted its protumor role through sponging miR-582-5p in NSCLC cells. The upregulation of miR-582-5p expression caused by NEAT1 interference was counteracted by the introduction of anti-miR-582-5p (Fig. 4A). The proliferation of NSCLC cells was impeded by the silencing of NEAT1, and the cotransfection of anti-miR-582-5p partly recovered the proliferation of NSCLC cells (Fig. 4B and 4C). The promoting effect of si-NEAT1 #1 transfection on the apoptosis of NSCLC cells was attenuated by the addition of anti-miR-582-5p (Fig. 4D). The number of migrated or invaded NSCLC cells was greatly decreased in the si-NEAT1 #1 group, while the metastasis ability was regained in the si-NEAT1 #1 and the anti-miR-582-5p cotransfected groups (Fig. 4E and F). The addition of anti-miR-582-5p also recovered

glucose uptake and lactate production in NSCLC cells, which were held back by the intervention of NEAT1 (Fig. 4G and H). These results indicated that NEAT1 facilitated the malignant potential of NSCLC cells through suppression of miR-582-5p.

EIF4G2 is a direct target of miR-582-5p in NSCLC cells

The targets of miR-582-5p were also predicted by the starBase online website. Among the candidate targets of miR-582-5p, EIF4G2 was selected due to its promoting role in proliferation and metastasis of cancer cells [22,23]. The complementary sequence in miR-582-5p and the 3'UTR of EIF4G2 mRNA is shown in Fig. 5A. As can be seen in Fig. 5B and C, the accumulation of miR-582-5p markedly reduced luciferase activity in the EIF4G2

3'UTR-WT group when compared to the EIF4G2 3'UTR-MUT group, suggesting direct interaction between miR-582-5p and EIF4G2. To further confirm this combination, the RIP assay was employed. MiR-582-5p and EIF4G2 were substantially enriched when using Ago2 antibody (Fig. 5D and E), demonstrating that miR-582-5p could bind to EIF4G2. The mRNA and protein expression of EIF4G2 was aberrantly upregulated in NSCLC tissues relative to adjacent normal tissues (Fig. 5F and G). Also, striking upregulation in the abundance of EIF4G2 protein was observed in NSCLC cells in comparison to BEAS-2B cells (Fig. 5H). There was a significant inverse correlation between the levels of miR-582-5p and mRNA expression of EIF4G2 in NSCLC tissues (Fig. 5I). To further test the regulatory relationship between EIF4G2 and miR-582-5p, miR-582-5p mimic (miR-582-5p) and miR-582-5p inhibitor (anti-miR-582-5p) were transfected into NSCLC cells. As mentioned above (for Fig. 5J and K), miR-582-5p transfection significantly decreased the protein level of EIF4G2, with the protein level of EIF4G2 being markedly elevated after transfection with anti-miR-582-5p in the two NSCLC cell lines. These findings
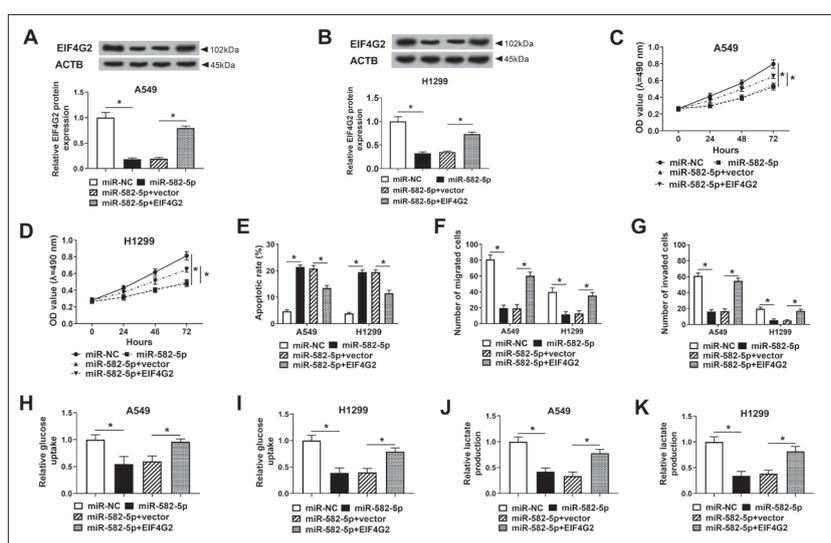

Fig. 6. EIF4G2 accumulation overturns the biological functions of miR-582-5p in NSCLC cells. A549 and H1299 cells were transfected with miR-NC, miR-582-5p, miR-582-5p+vector or miR-582-5p+EIF4G2. **A, B** – The protein level of EIF4G2 in NSCLC cells measured by Western blotting. **C, D** – The optical density of NSCLC cells after transfection for 0 h, 24 h, 48 h or 72 h analyzed by the MTT assay. **E** – The influence of miR-582-5p and EIF4G2 on apoptosis of NSCLC cells assessed by flow cytometry. **F, G** – The number of migrated and invaded NSCLC cells determined by transwell assays. **H-K** – Glucose consumption and production of lactate in NSCLC cells analyzed using fluorescence-based glucose and lactate assay kits. *P<0.05.

revealed that miR-582-5p could negatively regulate the expression of EIF4G2 through direct interaction.

EIF4G2 accumulation reverses the biological functions of miR-582-5p on NSCLC cells

MiR-582-5p and EIF4G2 were cotransfected into NSCLC cells to address the question of the biological role of EIF4G2 in the miR-582-5p-mediated influence of NSCLC cells. As shown in Fig. 6A and B, the downregulation of EIF4G2 protein expression caused by transfection with miR-582-5p was alleviated by the addition of EIF4G2. MiR-582-5p overexpression suppressed the proliferation, while accelerating apoptosis of NSCLC cells, and these effects were partly counteracted by the introduction of EIF4G2 (Fig. 6C-E). The overexpression of EIF4G2 also ameliorated the inhibitory effects of miR-582-5p accumulation on migration, invasion and glycolysis of NSCLC cells (Fig. 6F-K). Taken together, miR-582-5p inhibited the proliferation, metastasis and glycolysis while promoting apoptosis of NSCLC cells through a decrease in the level of EIF4G2.
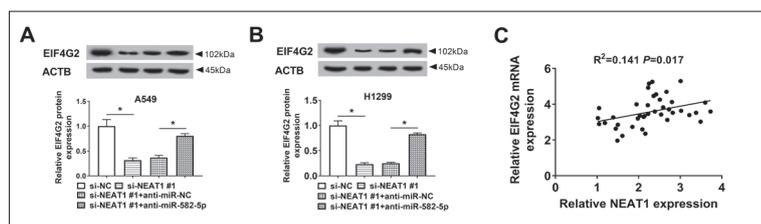
Fig. 7. NEAT1 elevates the expression of EIF4G2 through sponging of miR-582-5p in NSCLC cells. **A**, **B** – The protein level of EIF4G2 detected in NSCLC cells transfected with si-NC, si-NEAT1 #1, si-NEAT1 #1+anti-miR-NC or si-NEAT1 #1+anti-miR-582-5p by Western blotting. **C** – The linear relationship between the expression of NEAT1 and EIF4G2 mRNA analyzed through Spearman's correlation coefficient. *$P<0.05$.
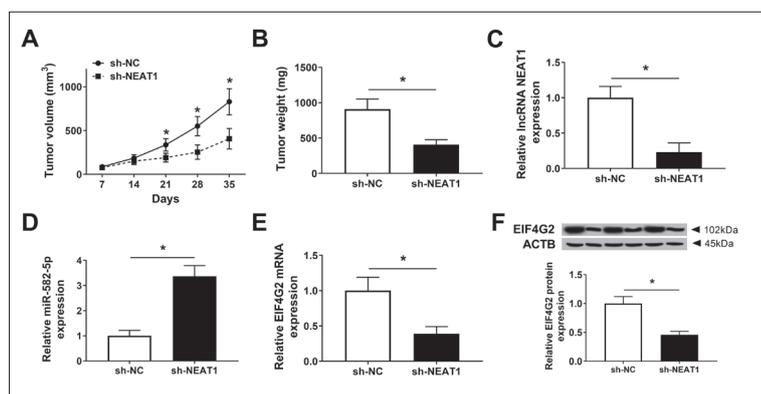

Fig. 8. NEAT1 accelerates tumor growth of NSCLC via miR-582-5p/EIF4G2 axis *in vivo*. **A** – The size of the tumor was recorded every 7 days using the following method: volume $=\pi\times(length\times width^2)/6$. **B** – Tumors were resected from nude mice after injection for 35 days, and the weights of the tumors in the sh-NC (n=5) and the sh-NEAT1 groups (n=5) were measured using an analytical balance. **C-F** – The expression of NEAT1, miR-582-5p and EIF4G2 mRNA and protein in tumor tissues determined by qRT-PCR and Western blot analysis. *$P<0.05$.

NEAT1 elevates the expression of EIF4G2 by the sponging miR-582-5p in NSCLC cells

The interference with NEAT1 downregulated the expression of EIF4G2, and the level of EIF4G2 was partly recovered in the si-NEAT1 #1 and the anti-miR-582-5p cotransfected group (Fig. 7A and B). The expression of EIF4G2 mRNA was positively correlated with the expression of NEAT1 (Fig. 7C). Collectively, lncRNA NEAT1 could sequester miR-582-5p from EIF4G2 mRNA, thus upregulating the expression of EIF4G2 at the post-transcriptional level in NSCLC cells.

NEAT1 accelerates tumor growth of NSCLC via the miR-582-5p/EIF4G2 axis *in vivo*

We validated the role of NEAT1 using a murine xenograft model. A549 cells stably expressing sh-NC or sh-NEAT1 were subcutaneously injected into the back region of nude mice. As can be seen in Fig. 8A and B, NSCLC tumors were smaller in the sh-NEAT1 group relative to the sh-NC group. The resected tumor tissues were used to measure the expression of NEAT1, miR-582-5p and EIF4G2. The abundance of NEAT1 was declined significantly in the sh-NEAT1 group in comparison to the sh-NC group (Fig. 8C). The level of miR-582-5p exhibited a reverse trend with respect to NEAT1 (Fig. 8D). Moreover, the mRNA and protein expression of EIF4G2 were decreased in the sh-NEAT1 group relative to the sh-NC group (Fig. 8E and F). In summary, NEAT1 contributed to the malignancy of NSCLC through the miR-582-5p/EIF4G2 axis *in vivo*.

DISCUSSION

The identification of crucial molecules that are involved in the occurrence and development of NSCLC is urgently need to improve the prognosis of NSCLC patients. We found that NEAT1 was abnormally upregulated in NSCLC tissues and cells, which is consistent with previous findings [12,26-29]. High expression of NEAT1 is related to the poor survival rate of NSCLC patients. Knockdown experiments revealed that NEAT1 accelerated the proliferation, metastasis and glycolysis, while restricting apoptosis of NSCLC cells. The protumor role of NEAT1 is in agreement with published articles, thus, NEAT1 facilitates the development of breast cancer via miR-448 and zinc finger E-box-binding homeobox 1 protein (ZEB1) [30], and NEAT1 contributes to the progression of colorectal cancer via the enzyme probable ATP-dependent RNA helicase DDX5 also known as DEAD box protein 5 or RNA helicase p68 (DDX5) [31].

LncRNAs regulate cellular physiological and pathological processes mainly by functioning as miRNAs sponges [32]. It was shown that LncRNA-00319 aggravated ovarian cancer by upregulating the nucleus accumbens associated protein (NACC1) via miR-423-5p sponging [33]. LncRNA XLOC_006390 potentiates the malignancy of cervical cancer cells through miR-331-3p and miR-338-3p sponging [34]. MiR-582-5p serves as a tumor suppressor in many cancers, including hepatocellular carcinoma, colorectal carcinoma, gastric cancer and NSCLC [20,21,35-37]. MiR-582-5p suppresses the proliferation and metastasis of colorectal cancer cells via Ras-related protein Rab27a [36], and impedes the proliferation and motility of NSCLC cells [21]. MiR-582-5p was found to be a direct target of NEAT1, and functional experiments showed that NEAT1 undermined NSCLC by sponging miR-582-5p.

EIF4G contains two forms, including EIF4G1 and EIF4G2 [38]. EIF4G is a member of EIF4F, which regulates the initiation process of mRNA translation [39]. The dysregulation in mRNA translation causes initiation and progression of multiple cancers [40,41]. MiR-379 hinders the malignant potential of osteosarcoma cells by inhibiting EIF4G2 [22]. MiR-379 contributes to cisplatin chemosensitivity of NSCLC cells by suppressing EIF4G2 [24]. The dual-luciferase reporter and RIP assays confirmed that EIF4G2 was a direct target of miR-582-5p in NSCLC cells. Moreover, the accumulation of EIF4G2 counteracted the inhibitory effect of miR-582-5p overexpression on the malignancy of NSCLC cells. Subsequently, we found that NEAT1 could enhance the expression of EIF4G2 through the sponging of miR-582-5p in NSCLC cells. The function of the NEAT1/miR-582-5p/EIF4G2 axis was also verified in a xenograft tumor model *in vivo*, and the depletion of NEAT1 in tumor cells blocked the growth of NSCLC tumors, at least in part, through miR-582-5p/EIF4G2 signaling.

In summary, NEAT1 potentiated the malignant potential of NSCLC cells through the miR-582-5p/EIF4G2 axis *in vitro* and *in vivo*. The NEAT1/miR-582-5p/EIF4G2 axis could provide a new diagnostic and prognostic marker in NSCLC treatment.

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