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Long non-coding RNA NEAT1 promotes tumor development and metastasis through targeting miR-224-5p in malignant melanoma

J.-X. ZOU[1], T.-W. GE[2]

[1]Department of Dermatology, The People's Hospital of Danyang, Danyang, [illegible]ina
[2]Department of Dermatology, Affiliated Jiangyin Hospital of Southeast U[illegible]rsity M[illegible]ical Colle[illegible] Jiangyin, China

Abstract. – OBJECTIVE: Melanoma is one of the most ordinary malignant tumors. Recent studies have revealed that long noncoding RNAs (lncRNAs) play an important role in the progression of tumorigenesis. This work aims to identify how lncRNA NEAT1 functions in the progression of melanoma.

PATIENTS AND METHODS: NEAT1 expression of both melanoma patients' tissue samples and cell lines was detected by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). Moreover, the function of NEAT1 was ide[illegible] by performing the proliferation and trans[illegible] assay in vitro. Besides, the underlying mech[illegible]sm was explored through the Luciferase assay [illegible]d RNA immunoprecipitation (RIP) assay. In [illegible]dition, tumor formation and met[illegible]asis assa[illegible] were also conducted *in vivo*.

RESULTS: In this researc[illegible]EAT[illegible]pression was significantly higher [illegible] melano[illegible] tissues compared with that in sk[illegible]es w[illegible] melanocytic nevus. Cell p[illegible]lifera[illegible] of melanoma were inh[illegible]ed after [illegible]ockdown of NEAT1 in vitro. M[illegible]over, the re[illegible] of further experiments [illegible] that micr[illegible]-224-5p (miR-224-5p) [illegible]as u[illegible]lated via the knockdown of N[illegible]AT1 and wa[illegible]o a direct target of NEAT1 i[illegible]elanoma. Furt[illegible]re, tumor formation a[illegible]etastasis of mela[illegible]a were inhibited via t[illegible]knockdown of NEAT1 in nude mice.

[illegible]LUSI[illegible]S: Our study suggests that NEA[illegible]ces mel[illegible]oma cell proliferation and me[illegible]is via s[illegible]nging miR-224-5p *in vi*[illegible]nd *in* [illegible]

Key[illegible]ords:
[illegible]ncoding RNA, NEAT1, Melanoma, MiR-224-5p.

Introduction

Melanoma is a major public health problem in many countries and it is the most aggressive and life-thr[illegible] skin cancer [illegible] world, accounting [illegible] mor[illegible] 80% of sk[illegible]-cancer related deaths[1]. The mor[illegible] of melanoma is increasing [illegible]y by approx[illegible]ly 2.8% every year si[illegible]198[illegible][2,3]. Accurate dia[illegible]osis and appropriate t[illegible]tment at an early clinical stage are responsible [illegible]a better surv[illegible][4]. However, the prognosis for m[illegible]oma patie[illegible] who develop local or distant me[illegible]is rema[illegible] very poor[5]. Thus, it is important to [illegible]e mechanisms underlying tumorigenesis and metastasis of melanoma and explore [illegible]ve intervention.

[illegible]ding RNAs (ncRNAs) are classified as long or small, respectively according to a nucleotide length. As one subtype of noncoding RNA (ncRNA), long noncoding RNAs (lncRNAs) regulate a variety of cellular processes and pathways in the development of cancers. The downregulation of lncRNA linc-ITGB1 inhibits cell invasion, cell migration, and epithelial-mesenchymal transition in non-small cell lung cancer by decreasing the Snail expression[6]. Upregulation of CASC15 promotes cell proliferation in gastric cancer which may function as a risk factor for the prognosis of gastric cancer patients[7]. The expression level of lncRNA-CCHE1 is positively related to the malignancy of colorectal carcinoma and it regulates the ERK/COX-2 pathway[8]. LncRNA HCCL5 accelerates cell viability, cell migration, epithelial-mesenchymal transition, and the malignancy of hepatocellular carcinoma[9]. Moreover, lncRNAs also function in the melanoma progression. Through the modulation of microRNA-200b (miR-200b)/a/429 pathway, knockdown of lncRNA ILF3-AS1 depresses the proliferation and migration of melanoma cell[10].

LncRNA NEAT1 is a novel lncRNA which has been reported to function in many tumors. However, the clinical role and underlying mechanisms of NEAT1 in the development of melanoma re-

Corresponding Author: Tianwei Ge, MM; e-mail: 763824460@qq.com

mains unexplored. Our work aims to explore the role of NEAT1 in melanoma development and metastasis.

Patients and Methods

Tissue Specimens

Melanoma patients who received surgery at The People's Hospital of Danyang were enrolled for 53 malignant melanoma tissues and 36 skin tissues with melanocytic *nevus* and received surgery at The People's Hospital of Danyang. All tissues were kept at –80°C. This investigation was approved by the Ethics Committee of The People's Hospital of Danyang. Signed written informed consents were obtained from all participants before the study.

Cell Culture

The Chinese Academy of Science (Shanghai, China) offered four melanoma cancer cell lines (SK-MEL-28, A375, WM266-4, and SK-MEL-2), and one human epidermal melanocyte (HEMa-LP), which were then cultured in [illegible] fetal bovine serum (FBS; Gibco, Rockvil[illegible] USA) and Roswell Park Memorial Institut[illegible]40 (RPMI-1640; HyClone, South Logan, UT, [illegible]) supplemented with 1% penicillin/streptom[illegible] (Sigma-Aldrich, St. Louis, MO, [illegible] in an inc[illegible]bator containing 5% CO_2 at 3[illegible].

Cell Transfection

We purchased shor[illegible]airp[illegible] [illegible]rectly against NEA[illegible] from Ge[illegible]rma (Shanghai, China). Neg[illegible] control sh[illegible] was also synthesized. T[illegible] co[illegible]mentary D[illegible] encoding NEAT1 was [illegible]CR-am[illegible]d and then inserted into pcDN[illegible].1 (Invitroge[illegible]rlsbad, CA, USA), which [illegible] subsequently tra[illegible]cted into melanom[illegible]lls through Lipofectamine 2000 reagent (In[illegible]en, C[illegible]sbad, CA, USA) according to the manu[illegible] protoco[illegible]

[illegible] Extra[illegible]d Real Time-Quantitative Po[illegible]merase [illegible]in Reaction (RT-qPCR)

[illegible]al RNA was extracted from cultured melano[illegible] [illegible]ents' tumor tissues using TRIzol re[illegible]ent (Invitrogen, Carlsbad, CA, USA). First-strand [illegible]plementary deoxyribose nucleic acid (cDNA) w[illegible]nthesized using the Transcriptor first strand cDNA synthesis kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. The thermocycling conditions were as follows: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 10 s, annealing at 60°C for 30 s, for a total of 35 cycles. The primer sequences used for RT-qPCR were as follows: NEAT1 primers fo[illegible]rd 5′-GCTCTGGGACCTTCGTGACTCT-3[illegible] 5′-CTGCCTTGGCTTGGAAATGTA[illegible]; Glyceraldehyde 3-phosphate dehydroge[illegible] (GAPDH), forward: 5′-GCACCGTCAAGG[illegible]AGAAC-3′ and reverse: 5′-TGGTGAAG[illegible]CGCC[illegible]GGA-3′. The $2^{-\Delta\Delta Ct}$ method was utili[illegible] to calcula[illegible] relative expression.

Cell Proliferation A[illegible]

Cell viabilit[illegible] was [illegible]red w[illegible] MTT (3-(4,5-dimet[illegible]iazol-2-yl)-[illegible]yl tetrazolium bro[illegible]ay (Sigma-A[illegible], St. Louis, MO, USA[illegible] Brie[illegible]elanoma cells (1000 cells/well) were seeded i[illegible] 96-well plate for 12 h. Aft[illegible] were cultu[illegible] different times (0, 2[illegible]8, and 72 h), 15 μL M[illegible]T was added to each [illegible]l and incubated for 4 h. To stop the reaction, t[illegible] were adde[illegible]th 100 μL dimethyl sulfoxide ([illegible]O). Absor[illegible]ce at 490 nm was assessed usi[illegible] enzy[illegible]linked immuno sorbent assay (ELIS[illegible] system (Multiskan Ascent, LabSystems, Helsinki, Finland).

[illegible]ell Assay

24 h after transfection, 2×10^5 cells in 100 μL serum-free RPMI-1640 were transformed to top chamber of an 8-μm culture insert (Corning, Corning, NY, USA) coated with 50 μg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). 20% FBS-RPMI-1640 was added to the lower chamber of the culture inserts. 24 h later, these inserts were treated by methanol for 30 min and stained by hematoxylin for 20 min. An inverted microscope (×40) was utilized for counting invaded cells in three random fields.

Luciferase Assay and Bio-Informative Analysis

The miRNAs that contained complementary base with NEAT1 are found by conducting Starbase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php). The NEAT1 3′-untranslated region (3′-UTR) wild-type (WT) sequence was named NEAT1-WT and the mutant sequence of NEAT1 3′-UTR missing the binding site with miR-224-5p was named NEAT1-MUT. Luciferase reporter gene assay kits (Promega, Madison, WI, USA) were used to detect the Luciferase activity of cells. The Luciferase reporter gene vector was constructed, and cells were transfected.

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RNA Immunoprecipitation (RIP) Assay

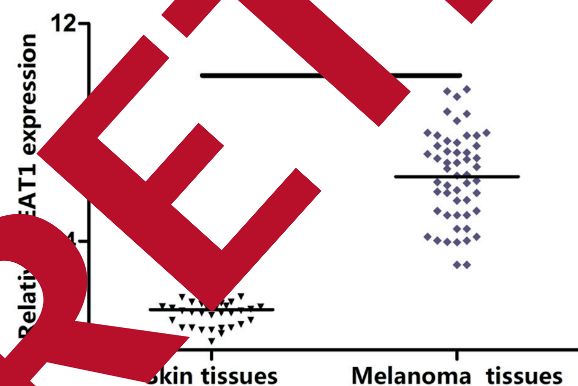
For the RIP assay, Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was performed according to the protocol. Then, RT-qPCR was used to detect co-precipitated RNAs. Treated cells were collected and lysed using RIP lysis buffer containing protease inhibitor and RNase inhibitor. Cells were incubated with the RIP buffer containing magnetic beads coated with Ago2 antibodies (Millipore, Billerica, MA, USA). IgG acted as a negative control (input group). After incubation for 2 h at 4°C, co-precipitated RNAs were isolated and measured by RT-qPCR analysis.

Xenograft Model

For the tumor formation assay, transfected WM266-4 cells were subcutaneously injected into NOD/SCID mice (4-5 weeks old). Tumor diameters were detected every 5 days after inoculation. Tumor volume was calculated as the formula (volume = length × width^2 × 1/2). The mice were sacrificed and tumors were extracted after 4 weeks. For the tumor metastasis assay, transfected WM266-4 cells were injected into the tai[illegible] of NOD/SCID mice (4-5 weeks old). Th[illegible] were sacrificed and the lung was extracte[illegible] after 4 weeks. Next, the number of metastatic no[illegible]s in the lung was counted. The animal experim[illegible] were approved by the Animal E[illegible] Committ[illegible] of The People's Hospital of D[illegible]ang[illegible]

Statistical Analysis

GraphPad Prism 5[illegible] (Gr[illegible] d[illegible] a Jolla, CA, USA) w[illegible] adopted [illegible] duct the statistical analysis. Data were expressed as mean ± SD (standard deviation). The Student's *t*-test method was utilized for the analysis. It was considered statistically significant when the *p*-[illegible] was less than 0.05.



[illegible] 1. Expression levels of NEAT1 were increased in me[illegible]ma tissues and cell lines. NEAT1 expression was significantly increased in the melanoma tissues compared with adjacent tissues. Data are presented as the mean ± standard error of the mean. $^*p<0.05$.

Results

Expression Level of N[illegible]1 in Tiss[illegible] and Cells of Melano[illegible]

To determine the bi[illegible]ical fu[illegible]on of NEA[illegible]1 in the tumorigenesis o[illegible]a, we d[illegible]tected NEAT1 expressi[illegible] levels i[illegible]ed mel[illegible]ma tissues and skin [illegible]ues with m[illegible] *nevus* by RT-qPCR. [illegible]howed that [illegible]1 was significantly [illegible]pregu[illegible] in melanoma tissue samples than in skin tiss[illegible] Figure 1).

K[illegible]ckdown of NEAT[illegible]epressed Cell [illegible]oliferation and Invasion in Melanoma

[illegible]EAT1 expre[illegible]n was also detected *via* RT-qP-C[illegible] four melan[illegible]a cell lines. NEAT1 level was sig[illegible]tly hi[illegible] in melanoma cells than that of HEMa-[illegible]e 2A). To further investigate wheth-[illegible] NEAT1 is connected to the development and me-[illegible]f melanoma, we researched the function of [illegible]n vitro*. In our work, we chose WM266-4 cell line for the knockdown of NEAT1. Then, NEAT1 expression was detected by RT-qPCR (Figure 2B). In this research, the ability of cell proliferation was examined *via* the MTT assay after the knockdown of NEAT1 in the WM266-4 cells. The MTT assay showed that the cell growth ability of WM266-4 cells was significantly repressed after NEAT1 was knocked down (Figure 2C). Transwell assay showed that the number of invaded cells was significantly reduced after NEAT1 was knocked down (Figure 2D).

The Interaction Between MiR-224-5p and NEAT1 in Melanoma

Starbase v2.0 (http://starbase.sysu.edu.cn/starbase2/rbpLncRNA.php) was used to find the target miRNAs of NEAT1. MiR-224-5p was selected from these miRNAs which were interacted with NEAT1 (Figure 3A). The RT-qPCR assay showed that the expression of miR-224-5p was higher in sh-NEAT1 cells than in negative control shRNA cells (Figure 3B). The Luciferase assay revealed that the Luciferase activity was significantly inhibited *via* co-transfection of NEAT1-WT and miR-224-5p (Figure 3C). RIP assay results showed that miR-224-5p was enriched in NEAT1 group when compared to control group (Figure 3D).

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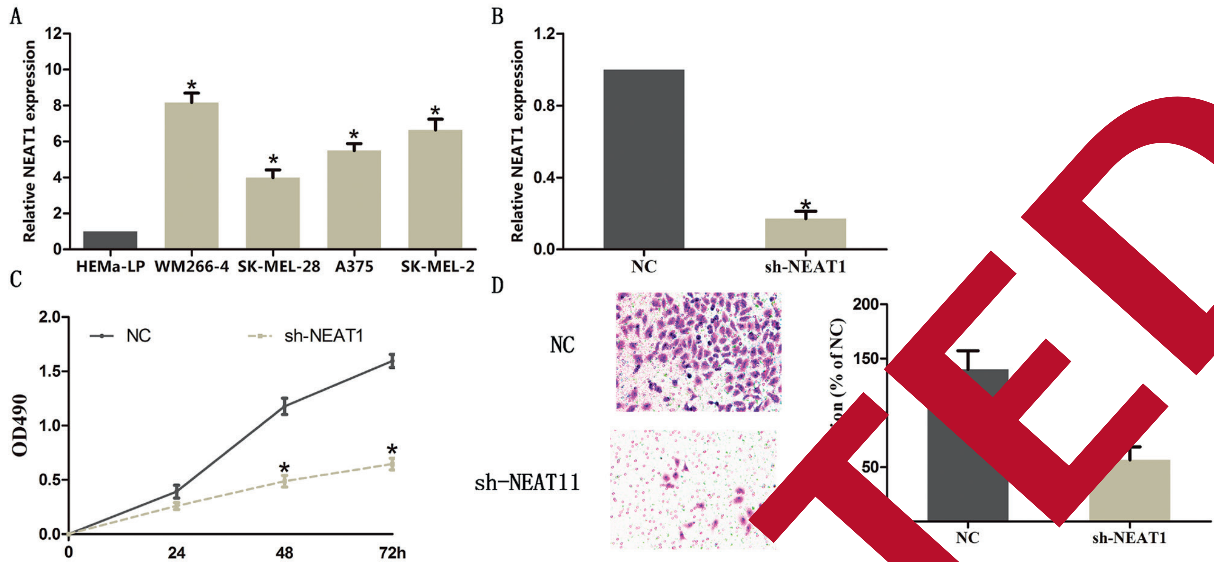
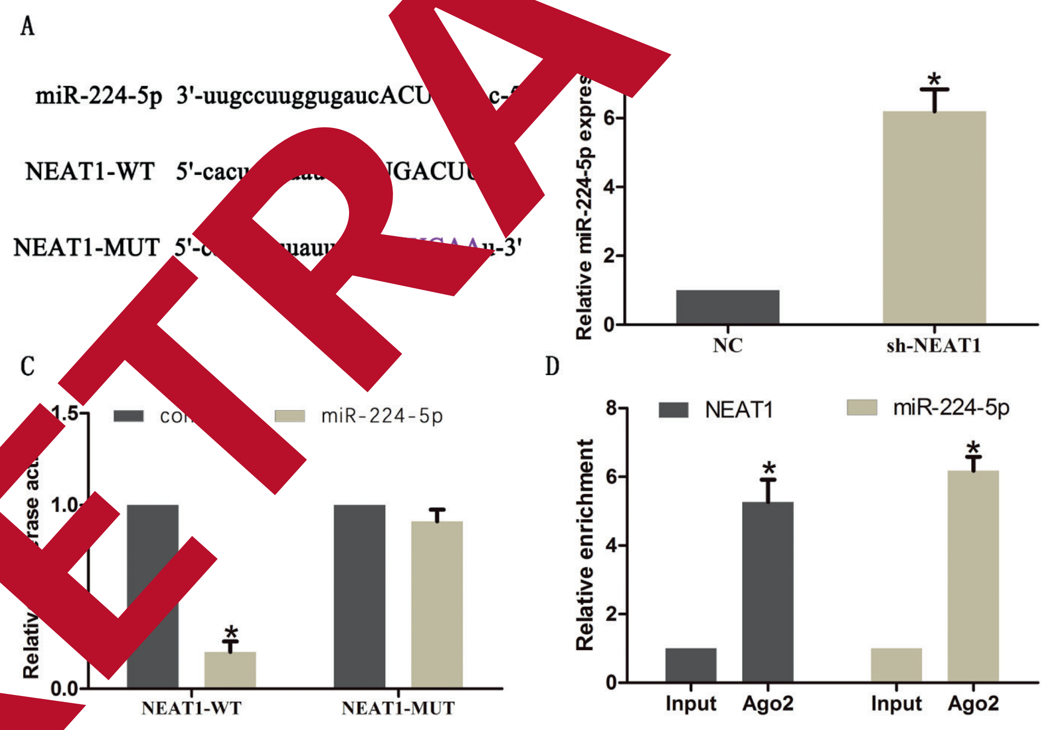


Figure 2. Knockdown of NEAT1 inhibited melanoma proliferati[illegible]pression levels o[illegible]T1 relative to GAPDH were determined in the human melanoma cell lines and a human e[illegible]rmal melanocyte (HEMa-L[illegible]) by RT-qPCR. **B,** NEAT1 expression in melanoma cells transfected with negative control sh[illegible]A (NC) or NEAT1 shRNA (sh-NEAT1) was detected by RT-qPCR. GAPDH was used as an internal control. **C,** MTT assay [illegible]wed that knoc[illegible]n of NEAT1 significantly repressed cell proliferation in melanoma cells. **D,** Transwell assay showed th[illegible]ockdown of N[illegible]T1 significantly repressed cell invasion in melanoma cells (magnification: 40×). *$p<0.05$, as compared t[illegible]ontrol cell[illegible]



[illegible]re 3. The association between NEAT1 and miR-224-5p in melanoma. **A,** Binding area of miR-224-5p in NEAT1. **B,** [illegible]R results showed that the miR-224-5p expression was increased in sh-NEAT1 group compared with NC group. **C,** Co-[illegible]sfection of miR-224-5p and NEAT1-WT strongly decreased the Luciferase activity, while co-transfection of miR-224-5p and NEAT1-MUT did not change the Luciferase activity either. **D,** RIP assay results demonstrated that the enrichment of NEAT1 and miR-224-5p Ago2-containing beads. The results represent the average of three independent experiments. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

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Knockdown of NEAT1 Repressed Tumor Formation and Metastasis In Vivo

The ability of NEAT1 in tumor formation and metastasis was detected *in vivo*. The tumor size in sh-NEAT1 group was smaller compared to negative control shRNA group (Figure 4A). The weight of dissected tumors in sh-NEAT1 group was smaller compared to negative control shRNA group (Figure 4B). Furthermore, the number of metastatic nodules in the lung from the sh-NEAT1 group was significantly reduced compared to negative control shRNA group (Figure 4C).

Discussion

Some studies in molecular technologies have suggested the important role of lncRNAs in melanoma progression, immune response, proliferation, oncogenesis and so on. For instance, PVT1 promotes cell proliferation and cell migration in melanoma[11] which may offer a potential diagnostic biomarker and target for intervention. LncRNA FALEC facilitates cell proliferation in melanoma by silencing p21 which is associated with poor prognosis for patients with melanoma[12]. By inhibiting miR-200b/a/429, lncRNA HEIH serves as an oncogene in melanoma and enhances cell proliferation and invasion[13]. Nuclear Enriched Abundant Transcript 1(NEAT1) encodes two isoforms of lncRNAs, 3.7-kb NEAT1-1 and -23-kb NEAT1-2. It has been reported that NEAT1 plays an important role in nuclear paraspeckles and serves as a crucial modulator in RNA splicing and transcription. Li et al[14] have indicated that NEAT1 acts as an oncogene in multiple types of cancers. For example, through the regulation of miR-211/HMGA2 axis, NEAT1 facilitates cell proliferation and cell invasion in breast cancer. Regulated by [illegible], NEAT1 promotes progression and tumorigenesis in ovarian cancer[15]. NEAT1 facilitates the malignancy of biological behaviors in gastric cancer and induces chemotherapy resistance[16]. The knockdown of NEAT1 inhibits cell proliferation and promotes cell apoptosis in colorectal cancer through regulating Akt Signaling[17]. In the present study, NEAT1 was found to be up-regulated in both melanoma tissue and cells. Fur-

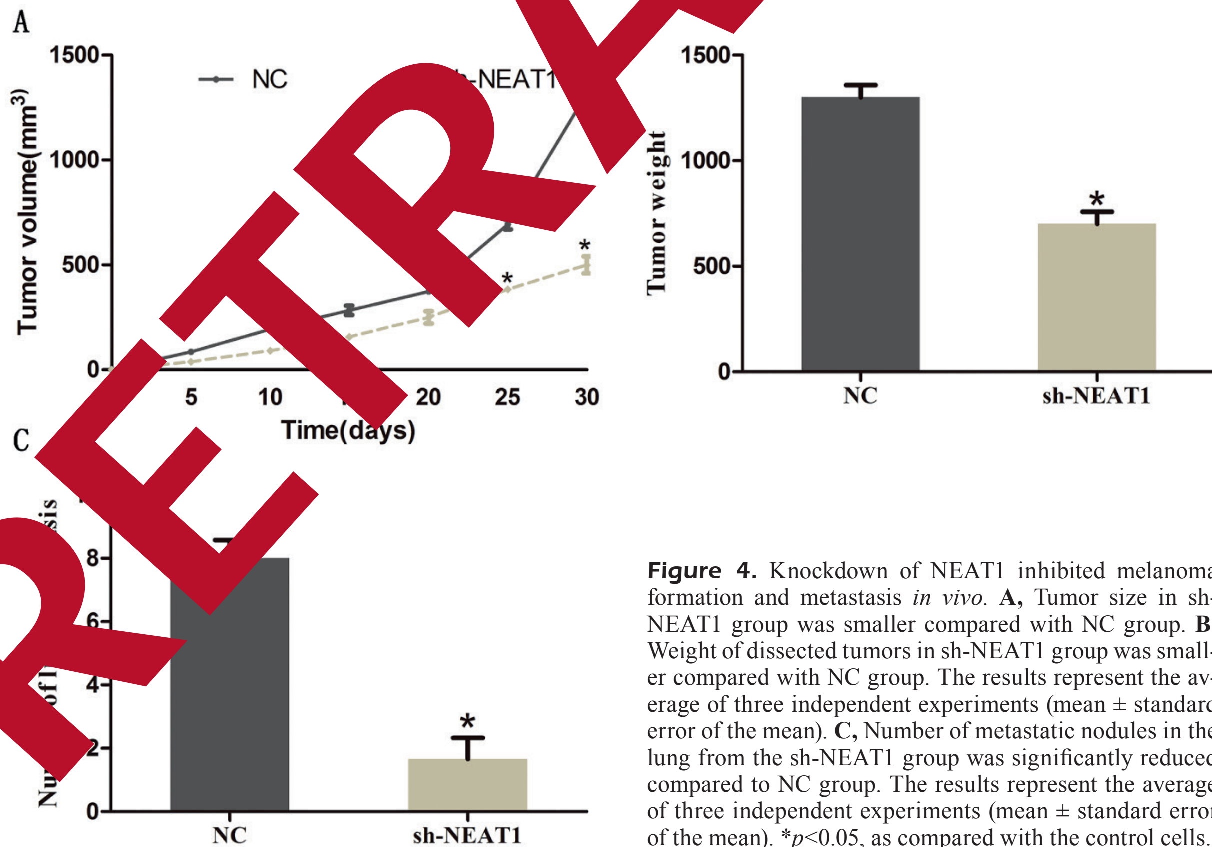

Figure 4. Knockdown of NEAT1 inhibited melanoma formation and metastasis *in vivo*. **A,** Tumor size in sh-NEAT1 group was smaller compared with NC group. **B,** Weight of dissected tumors in sh-NEAT1 group was smaller compared with NC group. The results represent the average of three independent experiments (mean ± standard error of the mean). **C,** Number of metastatic nodules in the lung from the sh-NEAT1 group was significantly reduced compared to NC group. The results represent the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with the control cells.

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thermore, after NEAT1 was knocked down, the ability of melanoma cell growth and invasion was suppressed. These results indicated that NEAT1 functioned as an oncogene and promoted the tumorigenesis of melanoma.

In our work, bioinformatics software predicted miR-224-5p as a possible target miRNA of NEAT1. The mature miRNA miR-224-5p participates in a series of biological processes, including cell proliferation, migration, and invasion in various malignancies. MiR-224-5p is up-regulated and has the potential to become a diagnostic and prognostic biomarker in digestive system cancers[18]. By serving as a sponge of miR-224-5p, lncRNA FTH1P3 promotes the progression of oral squamous cell carcinoma *via* modulating the expression of fizzled 5[19]. In our work, the miR-224-5p expression could be upregulated through the knockdown of NEAT1. The results of Luciferase assay and RIP assay showed that miR-224-5p could be directly targeted by NEAT1. Furthermore, the knockdown of NEAT1 also inhibited tumor formation and metastasis *in vivo*. All these results showed that miR-224-5p was directly targeted by NEAT1 in melanoma.

Conclusions

We identified that NEAT1 [illegible] facilita[illegible] melanoma cell proliferation [illegible] invasio[illegible] by sponging miR-224-5p, [illegible]ich sug[illegible]ted that NEAT1/miR-224-5p axis [illegible] serve [illegible] promising marker for melan[illegible]a.

Conflict of Int[illegible]

The Authors declare that th[illegible] no conflict of interests.

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