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LINC00662 promotes cell proliferation, migration and invasion of melanoma by sponging miR-890 to upregulate ELK3

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Abstract. – OBJECTIVE: Melanoma is one of the most malignant types of skin tumors and accounts for the majority of skin cancer-related deaths. LINC00662 is a tumor promoter in multiple types of cancer, but the role of LINC00662 in melanoma has not been fully elucidated.

MATERIALS AND METHODS: The expression levels of LINC00662, miR-890, and ELK3 were detected by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). MTT assay was performed to measure the cell proliferation ability in A375 and SK-MEL-1 cells. Cell migration and invasion abilities were measured by wound healing assay and transwell assay, respectively. Besides, Luciferase reporter assay was employed to examine the interaction between miR-890 and LINC00662 or ELK3.

RESULTS: In the present study, it was demonstrated that melanoma patients with high expression levels of LINC00662 had a shorter survival time than those with low expression levels of LINC00662. LINC00662 exhibited higher expression levels in melanoma tissues and cell lines. Additionally, suppression of LINC00662 impaired cell proliferation, migration, and invasion. Furthermore, animal experiments demonstrated that LINC00662 facilitated tumor growth *in vivo*. LINC00662 was confirmed to bind with miR-890, and ELK3 was identified as a downstream target gene of miR-890. Furthermore, miR-890 was found to negatively regulate ELK3 expression. Through rescue assays, overexpression of ELK3 reversed the inhibitive effects of LINC00662 knockdown or miR-890 mimics on the cell proliferative, migratory, and invasive abilities.

CONCLUSIONS: Our results demonstrated that LINC00662 facilitated the occurrence and development of melanoma by sponging miR-890 to upregulate ELK3. This discovery implied that LINC00662 may be a promising prognostic and therapeutic biomarker for patients with melanoma.

Key Words:
LINC00662, MiR-890, ELK3, Melanoma.

Introduction

Melanoma is a malignant tumor which originated from melanocytes[1,2]. Despite recent major advances in tumor therapy, including surgery, radiotherapy and chemotherapy, the survival rate for patients with melanoma is still unsatisfactory[3,4]. Accordingly, the study of novel molecular targets in melanoma is an important challenge.

Long non-coding RNAs (LncRNAs) are a class of transcribed RNAs with a length of more than 200 nucleotides[5-7] and they participate in the regulation of various types of cancer by acting as a tumor promoter or suppressor[8,9]. In fact, lncRNA PVT1 accelerates cell proliferation and invasion in gliomas by sponging miR-200a[10]. The upregulation of lncRNA LINC00163 inhibits the development of lung cancer[11]. LncRNA PICART1 represses cell proliferation and invasion in non-small cell lung cancer through affecting the AKT1 signaling pathway[12]. Many lncRNAs are also involved in the progression of melanomas. LncRNA CPS1-IT1 restrains melanoma cell metastasis by competitively binding to BRG1 to suppress Cyr61[13]. LncRNA RMEL3 enhances cell proliferation and tumor growth in melanoma[14]. LINC00662 is a novel lncRNA and acts as an oncogene in human cancers. For example, lncRNA LINC00662 modulates the Hippo-YAP1 pathway to stimulate the progression of gastric cancer[15]. LINC00662 acts as a competitive endogenous RNA (ceRNA) to promote prostate cancer tum-

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origenesis by regulating miR-34a expression[16]. However, the underlying molecular mechanisms of LINC00662 in melanoma are unknown.

Another important set of non-coding RNAs are microRNAs (miRNAs), whose length is typically 18-24 nucleotides[17,18]. MiRNAs play important roles in the occurrence and development of various types of cancer, including melanoma. MiR-375 attenuates cell proliferation in esophageal squamous cell carcinoma by directly targeting SP1[19]. miR-485-5p suppresses cell proliferation in gliomas by directly targeting paired box 3[20]. MiR-338-5p targets CD82 to enhance the growth and metastasis of malignant melanomas[21]. The miR-425/IGF-1 axis weakens melanoma metastasis by blocking the PI3K-Akt pathway[22]. Recently, lncRNAs have been reported to act as 'sponges' to bind with miRNAs, which can regulate cancer. LncRNA small nucleolar RNA host gene 1 (SNHG1) accelerates osteosarcoma progression by sequestrating miR-577 and activating the WNT2B/Wnt/β-catenin signaling pathway[23]. LncRNA LINC00511 aggravates breast cancer tumorigenesis by targeting the miR-185-3p/E2F1/Nanog axis[24]. LncRNA DSCAM-AS1 contributes to the development of melanomas by sponging miR-136[25]. In the present study, bioinformatics tools predicted that LINC00662 had the binding sequences for miR-890. miR-890 has been reported to attenuate triple-negative breast cancer progression[26], but its role in melanomas has not been investigated. As such, the present investigation further investigated the interaction between LINC00662 and miR-890 in melanoma.

In the present study, the role of LINC00662 in melanoma was explored. It was confirmed that LINC00662 accelerated the progression of melanoma by targeting the miR-890/ELK3 axis.

Materials and Methods

Cell Culture and Transfection

The normal human epidermal melanin cell line, HEMa-LP, was acquired from Invitrogen (Carlsbad, CA, USA), and melanoma cell lines including A375, SK-MEL-1, and SK-MEL-2 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Melanoma cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM; Corning Life Sciences, MA, USA) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and penicillin-streptomycin (penicillin: 100 UI/mL and streptomycin: 100 μg/mL; Thermo Fisher Scientific, Waltham, MA, USA). HEMa-LP cells were cultured in medium 254 (Thermo Fisher Scientific, Waltham, MA, USA) and Human Melanocyte Growth Supplement-2 (HMGS-2; Thermo Fisher Scientific, Waltham, MA, USA). The cells were cultured at 37°C in an atmosphere with 5% CO_2.

The short-hairpin RNA against LINC00662 (sh-LINC00662; 5'-GCUGCUGCCACUGUAAUAAUU-3') with negative control (sh-NC; 5'-AAUUCUCGGAACGUCUGACGU-3'), miR-890 mimics (5'- UACUUGGAAAGGCAUCAGUUGtt-3'), miR-890 inhibitor (5'-CAACUGAUGCCUUUCCAAGUAtt-3') and negative control (NC, 5'- AGACGUGUAUCGUAACUGAUGtt-3') were synthesized by GenePharma (Shanghai, China). The full length of ELK3 was subcloned into pcDNA3.1 to overexpress ELK3 levels with empty pcDNA3.1 serving as the control. The pcDNA3.1 vector was bought from GenePharma (Shanghai, China). The aforementioned plasmids were transfected into A375 or SK-MEL-1 cells using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA).

Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's protocol to isolate total RNA from melanoma cells. Total RNA was reverse transcribed into cDNA using a PrimeScript RT reagent kit (Takara, Dalian, China). PCR amplification reaction was prepared using SYBR Green Real-Time PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method. The expression levels of LINC00662 or ELK3 were normalized to GAPDH. The expression levels of miR-890 were normalized to U6. The sequences of the primers were: LINC00662 forward, 5'-CACGCTTCTGAAACTGGTGT-3' and reverse, 5'-TGTACAGCCTGGTGACAGAG-3'; miR-890 forward, 5'-GCTTGGAAAGGCATCAGTTG-3' and reverse, 5'-CTCAACTGGTGTCGTGGAGTC-3'; ELK3 forward, 5'-ACCCAAAGGCTTGGAAATCT-3' and reverse, 5'-TGTATGCTGGAGAGCAGTGG-3'; GAPDH forward, 5'-ACCACAGTCCAT GCCATCAC-3' and reverse 5'-TCCACCACCCTGTTGCTGTA-3'; U6 forward, 5'-CTCGCTTCGGCAGCACAT-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

MTT Assay

Cell proliferation was measured using MTT assays. Melanoma cells, at a density of $1x10^4$ cells/well, were cultured on 96-well plates. 10 μL MTT (5 mg/ml; Bioswamp, Wuhan, China) was added into each well at the time of determination (0, 24, 48, and 72 h). Subsequently, dimethyl sulfoxide (~100 μl; Bio-Swamp, Wuhan, China) was then added after incubation for 4 h. The optical density was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Wound Healing Assay

The transfected melanoma cells were seeded onto 6-well plates and cultured in standard conditions until 80-90% confluent. A straight wound was made using a sterile pipette tip. The size of the wound was captured at 0 and 24 h using an optical microscope (Leica DMI4000B, Milton Keynes, Bucks, UK) and the migration ratio was calculated.

Transwell Assay

Cell invasive capabilities were evaluated using transwell chambers (Corning Inc., Corning, NY, USA) coated with Matrigel (BD Biosciences, Bedford, MA, USA). The transfected melanoma cells in 200 μl serum-free Dulbecco's Modified Eagle Medium (DMEM; Corning Life Sciences, MA, USA) were placed into the upper chamber. A total of 600 μl culture medium containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) was put into the lower chamber. After cultivating for 48 h, the cells were fixed with methanol and stained with crystal violet. After washing with PBS buffer, invaded cells were imaged and counted using an inverted microscope.

Bioinformatics Prediction and Luciferase Reporter Assay

StarBase 2.0 (http://starbase.sysu.edu.cn/) was utilized for the analysis of the lncRNA-miRNA-mRNA interaction. The pmirGLO-LINC00662-wild type (Wt) and pmirGLO-LINC00662-mutant (Mut) vectors were co-transfected with miR-NC or miR-890 mimics into 293T cells, respectively. The pmirGLO-ELK3-Wt and pmirGLO-ELK3-Mut vectors were also co-transfected with miR-NC or miR-890 mimics into 293T cells, respectively. After 48 h of transfection, the relative Luciferase activity was determined using the dual-luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Animal Experiments

Animal experiments were performed to determine the impact of LINC00662 on tumor growth *in vivo*. The experiments were carried out on male BALB/C nude mice. Briefly, cells transfected with sh-NC or sh-LINC00662 were injected into nude mice. Every 7 days, the volume and weight of tumors were calculated. 4 weeks later, mice were sacrificed. The tumors were dissected and detected. The animal experiments were approved by the Ethics Committee of Shanghai Ninth People's Hospital.

Statistical Analysis

Data are expressed as the mean ± SD and all experiments were repeated 3 times. Statistical analysis was conducted using the SPSS 22.0 software (IBM Inc., Armonk, NY, USA). Comparisons among the groups were tested using Student's *t*-tests (for two groups) or one-way ANOVAs (for more than two groups) followed by Tukey's test. Kaplan-Meier analysis and the log-rank test were used to estimate survival curves. Cut-off values were determined using the Youden's index. $p<0.05$ was considered to indicate a statistically significant difference.

Results

LINC00662 Facilitates Cell Proliferation, Migration, and Invasion in Melanoma

Firstly, the role of LINC00662 was investigated in melanomas. Using the TCGA database, it was found that melanoma patients with high expression levels of LINC00662 had a shorter survival time than those with low expression levels of LINC00662 (Figure 1A) and the expression levels of LINC00662 were significantly higher in skin cutaneous melanoma (SKCM) tissues than that in normal tissues (Figure 1B). RT-qPCR assays were performed to detect the expression levels of LINC00662 in the melanoma cell lines (A375, SK-MEL-1, and SK-MEL-2) and normal human epidermal melanin cell line (HEMa-LP). The results showed that LINC00662 presented higher expression levels in the melanoma cells than that of the HEMa-LP cells (Figure 1C). The knockdown efficiency of LINC00662 was measured. The expression of LINC00662 was markedly reduced by LINC00662 silencing (Figure 1D). Additionally, the proliferative ability of A375 and SK-MEL-1 cells was weakened following suppression of LINC00662 (Figure 1E).

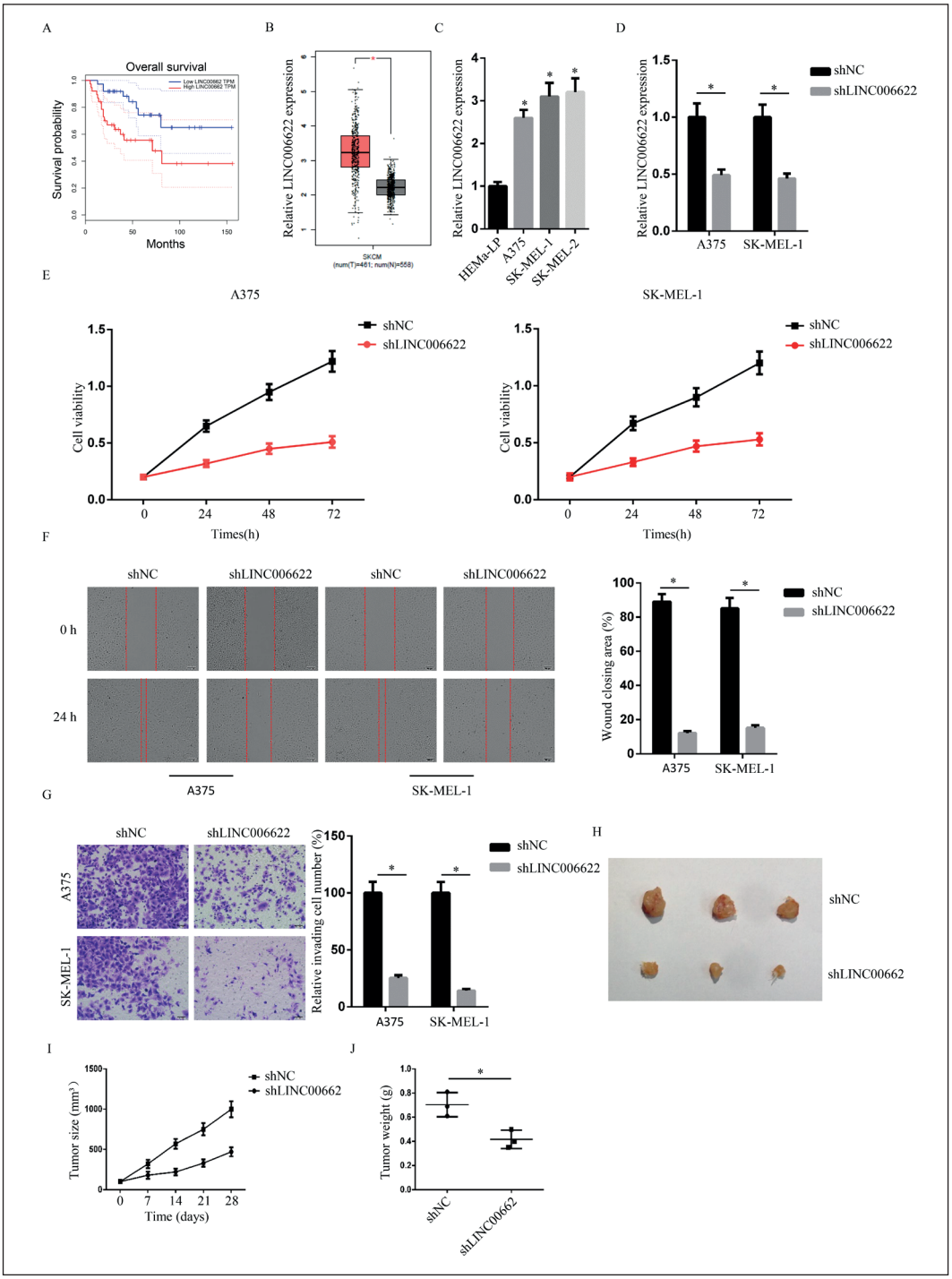

Figure 1. High expression of LINC00662 facilitated cell proliferation, migration and invasion in melanoma. **A**, The prognosis of melanoma patients with high or low LINC00662 expression was analyzed through TCGA database. **B**, Bioinformatic analysis of TCGA database showed the levels of LINC00662 in melanoma tissues (red; n=461) and normal tissues (gray; n=558). **C**, RT-qPCR analysis showed the relative expression level of LINC00662 in normal human epidermal melanin cell line (HEMa-LP) and melanoma cell lines (A375, SK-MEL-1 and SK-MEL-2). **D**, The knockdown efficiency of LINC00662 in A375 and SK-MEL-1 cells was detected by RT-qPCR assay. **E**, Cell proliferation after suppressing LINC00662 in A375 and SK-MEL-1 cells was tested via MTT assay. **F**, The migration ability of A375 and SK-MEL-1 cells by knocking down LINC00662 was evaluated through wound healing assay (magnification ×40). **G**, The invasion ability of A375 and SK-MEL-1 cells by repressing LINC00662 was estimated by transwell assay (magnification ×40). **H**, Xenograft tumor assay shows the tumor growth of cells transfected with sh-NC and sh-LINC00662. I, The volume of tumors throughout the experiment. **J**, Statistical analysis of tumor weights. *p<0.05.

Through wound healing and transwell assays, it was discovered that the migratory and invasive abilities of melanoma cells were impaired by silencing LINC00662 (Figure 1F and G). Animal experiments were performed to examine the impact of LINC00662 on tumor growth *in vivo*. The tumors collected from mice in the shLINC00662 group were significantly smaller than that in the shNC group (Figure 1H and I). In Figure 1J, a statistically significant difference in tumor weight was observed between the two groups. Taken together, these data demonstrated that LINC00662 promoted melanoma progression *in vitro* and *in vivo*.

LINC00662 Sponges MiR-890 to Regulate the Progression of Melanoma

It was predicted that LINC00662 had binding sites for miR-890 using the starBase website (Figure 2A). RT-qPCR analysis showed that the expression levels of miR-890 were reduced in melanoma cells compared with that in the normal cells (Figure 2B). The expression of miR-890 was notably enhanced in the miR-890 mimics group (Figure 2C). In order to study the interactions between LINC00662 and miR-890, Luciferase reporter assays were carried out. The Luciferase activity of pmirGLO-LINC00662-Wt vectors was weakened by the overexpression of miR-890, but no change was discovered in the Luciferase activity of pmirGLO-LINC00662-Mut vectors in 293T cells (Figure 2D). The expression of miR-890 was elevated by knocking down LINC00662, but this effect was abolished by treatment with miR-890 inhibitor (Figure 2E). Suppressing miR-890 could reverse the inhibitive effect of LINC00662 knockdown on cell proliferation (Figure 2F). Furthermore, the repression of LINC00662 significantly damaged the migratory and invasive abilities of melanoma cells, which was restored by miR-890 inhibitor (Figure 2G and H). Hence, it was revealed that LINC00662 sponged miR-890 to regulate the progression of melanomas.

ELK3 is a downstream target gene of miR-890

The starBase v3.0 website was utilized to identify potential target genes of miR-890, which indicated that there were binding sites between miR-890 and ELK3 (Figure 3A). Kaplan-Meier analysis showed that patients with high ELK3 expression levels exhibited a worse prognosis compared with patients with low ELK3 expression levels (Figure 3B). Moreover, the expression levels of ELK3 were significantly higher in SKCM tissues than that in normal tissues (Figure 3C). RT-qPCR results showed that the expression levels of ELK3 were increased in melanoma cells compared with that in normal cells (Figure 3D). To validate the interaction between miR-890 and ELK3, luciferase reporter assays were performed. The results showed that the luciferase activity of pmirGLO-ELK3-Wt vectors was weakened by overexpression of miR-890. There was no change in the luciferase activity of pmirGLO-ELK3-Mut vectors in 293T cells (Figure 3E). These results were further verified by testing the overexpression efficiency of miR-890 mimics and it was found that miR-890 overexpression decreased the expression levels of ELK3 (Figure 3F). These results revealed that ELK3 was a downstream target gene of miR-890.

The LINC00662/miR-890/ELK3 Axis Promotes Melanoma Progression

Rescue assays were performed to verify whether LINC00662 promoted the progression of melanoma through the miR-890/ELK3 axis. The upregulation of ELK3 reversed the inhibitive effect of LINC00662 knockdown or miR-890 overexpression on ELK3 expression levels (Figure 4A). Cell proliferation was reduced following knocking down of LINC00662 or overexpression of miR-890, but this effect was reversed by overexpressing ELK3 (Figure 4B). Overexpression of ELK3 counteracted the inhibitive effect of LINC00662 suppression or miR-890 mimics on cell migration and invasion (Figure 4C and D). Collectively, these findings revealed that the LINC00662/miR-890/ELK3 axis promoted the development and progression of melanoma.

Discussion

LncRNAs play vital roles in the progression of various types of tumors and function as either pro-tumor or anti-tumor factors[10-13]. LINC00662 has been reported to be an oncogene in several types of cancer, including gastric cancer[15], oral squamous cell carcinoma[27], and lung cancer[28]. However, the regulatory mechanisms of LINC00662 in melanoma are still unidentified. In the present study, high expression levels of LINC00662 were found in melanoma tissues and cell lines, and were related to a poor prognosis for patients with melanoma. Suppression of

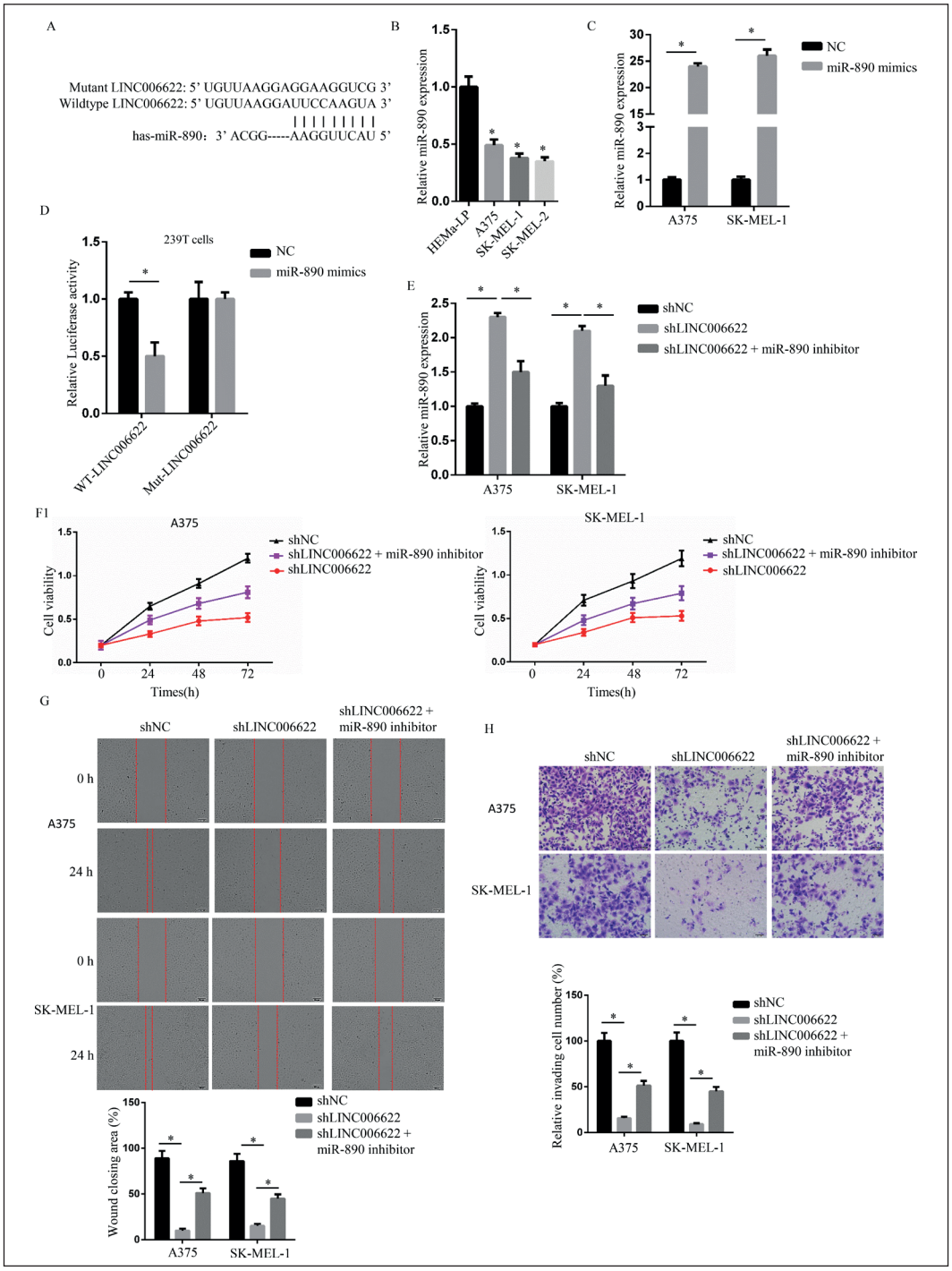

Figure 2. LINC00662 sponged miR-890 to regulate the progression of melanoma. **A**, The binding sequences between LINC00662 and miR-890 were predicted through starBase website. **B**, RT-qPCR analysis showed the relative expression level of miR-890 in normal human epidermal melanin cell line (HEMa-LP) and melanoma cell lines (A375, SK-MEL-1 and SK-MEL-2). **C**, The overexpression efficiency of miR-890 in A375 and SK-MEL-1 cells was detected by RT-qPCR. **D**, The binding ability between LINC00662 and miR-890 was confirmed by luciferase reporter assay. **E**, The expression of miR-890 was verified in A375 and SK-MEL-1 cells transfected with sh-NC, sh-LINC00662 and sh-LINC00662+miR-890 inhibitor via RT-qPCR assay. **F**, Cell proliferation was detected in A375 and SK-MEL-1 cells transfected with sh-NC, sh-LINC00662 and sh-LINC00662+miR-890 inhibitor through MTT assay. **G**, Cell migration was evaluated in A375 and SK-MEL-1 cells transfected with sh-NC, sh-LINC00662 and sh-LINC00662+miR-890 inhibitor via wound healing assay (magnification ×40). **H**, Cell invasion was examined in A375 and SK-MEL-1 cells transfected with sh-NC, sh-LINC00662 and sh-LINC00662+miR-890 inhibitor by transwell assay (magnification ×40). *$p<0.05$.

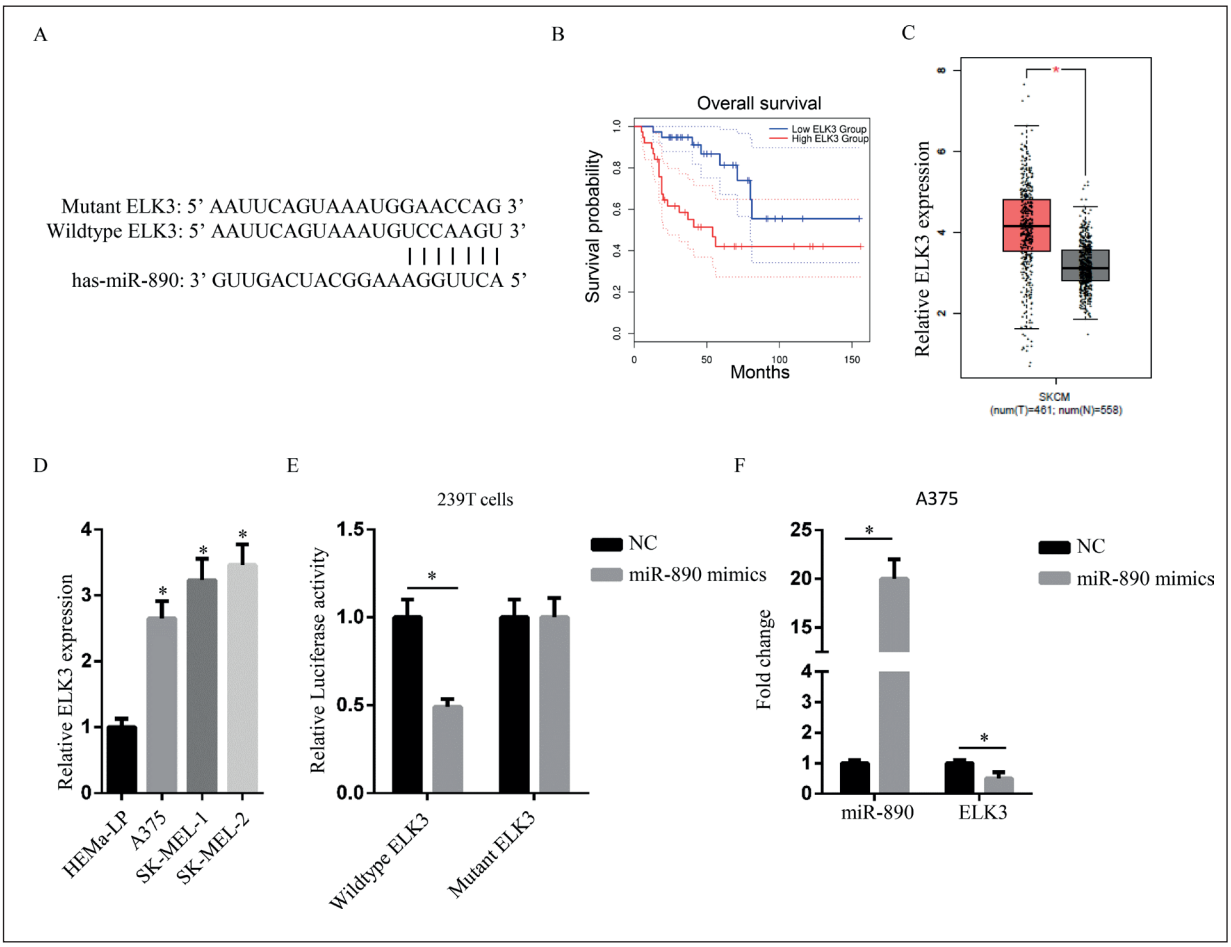

Figure 3. ELK3 was a downstream target gene of miR-890. **A**, The binding sites between miR-890 and ELK3 were predicted by starBase website. **B**, The prognosis of melanoma patients with high or low ELK3 expression was analyzed through TCGA database. **C**, Bioinformatic analysis of TCGA database showed the levels of ELK3 in melanoma tissues (red; n=461) and normal tissues (gray; n=558). **D**, RT-qPCR analysis showed the relative expression level of ELK3 in normal human epidermal melanin cell line (HEMa-LP) and melanoma cell lines (A375, SK-MEL-1 and SK-MEL-2). **E**, The binding ability between miR-890 and ELK3 in 293T cells was verified via luciferase reporter assay. **F**, The expression of miR-890 and ELK3 was detected in A375 cells transfected with miR-NC and miR-890 mimics through RT-qPCR assay. *p<0.05.

LINC00662 impaired cell proliferation, migration, and invasion. These findings demonstrated that LINC00662 exhibited a pro-oncogenic role in melanomas.

Accumulating evidence suggests that lncRNAs can act as molecular sponges to absorb miRNA and regulate the progression of human cancers. For example, lncRNA RP4 acts as a ceRNA by sponging miR-7-5p in colorectal cancer[29]. LncRNA myocardial infarction association transcript (MIAT) enhances cell proliferation and invasion in hepatocellular carcinoma (HCC) by sponging miR-214[30]. LncRNA ILF3 antisense RNA 1 (ILF3-AS1) negatively regulates miR-200b/a/429 to strengthen cell proliferation, migration, and invasion in melanomas[31]. miR-890 has only currently been found to be involved in triple-negative breast cancer progression[26], but the mechanism of action related to LINC00662/miR-890 in melanomas is unknown. In the present study, it was found that the expression levels of miR-890 were reduced in melanoma cells. Functional assays revealed that LINC00662 promoted the progression of melanoma by directly interacting with miR-890.

Growing evidence has demonstrated that lncRNAs function as ceRNAs by sponging miRNAs, thus blocking the target binding ability of miRNAs to release mRNAs. LncRNA H1 acts as a ceRNA of miR-138 and releases the enhancer of zeste homolog 2 (EZH2) to heighten cell proliferation and invasion in oral squamous cell carcinoma[32]. LncRNA SNHG1 facilitates the progression

of non-small cell lung cancer by sponging miR-145-5p to upregulate MTDH[33]. ELK3 is a transcription factor of Ets family. Different signaling pathways, such as the PI3K/Akt/mTOR and ERK signaling pathways activate ELK3. Several studies have identified that ELK3 participates in the tumorigenesis of various types of cancers. ELK3 expressed in lymphatic endothelial cells enhances breast cancer progression through exosomal miRNAs[34]. ELK3 targets HIF-1α to strengthen the migratory and invasive abilities of liver cancer stem cells[35]. In addition, ELK3 has been reported to bind with miRNAs to regulate cancer progression. For example, lncRNA LINC00525/miR-507/ELK3 axis accelerates the stemness and chemoresistance of colorectal cancer[36]. Tumor-suppressive miRNA-135a targets ELK1 and ELK3 oncogenes to repress breast cancer cell proliferation[37].

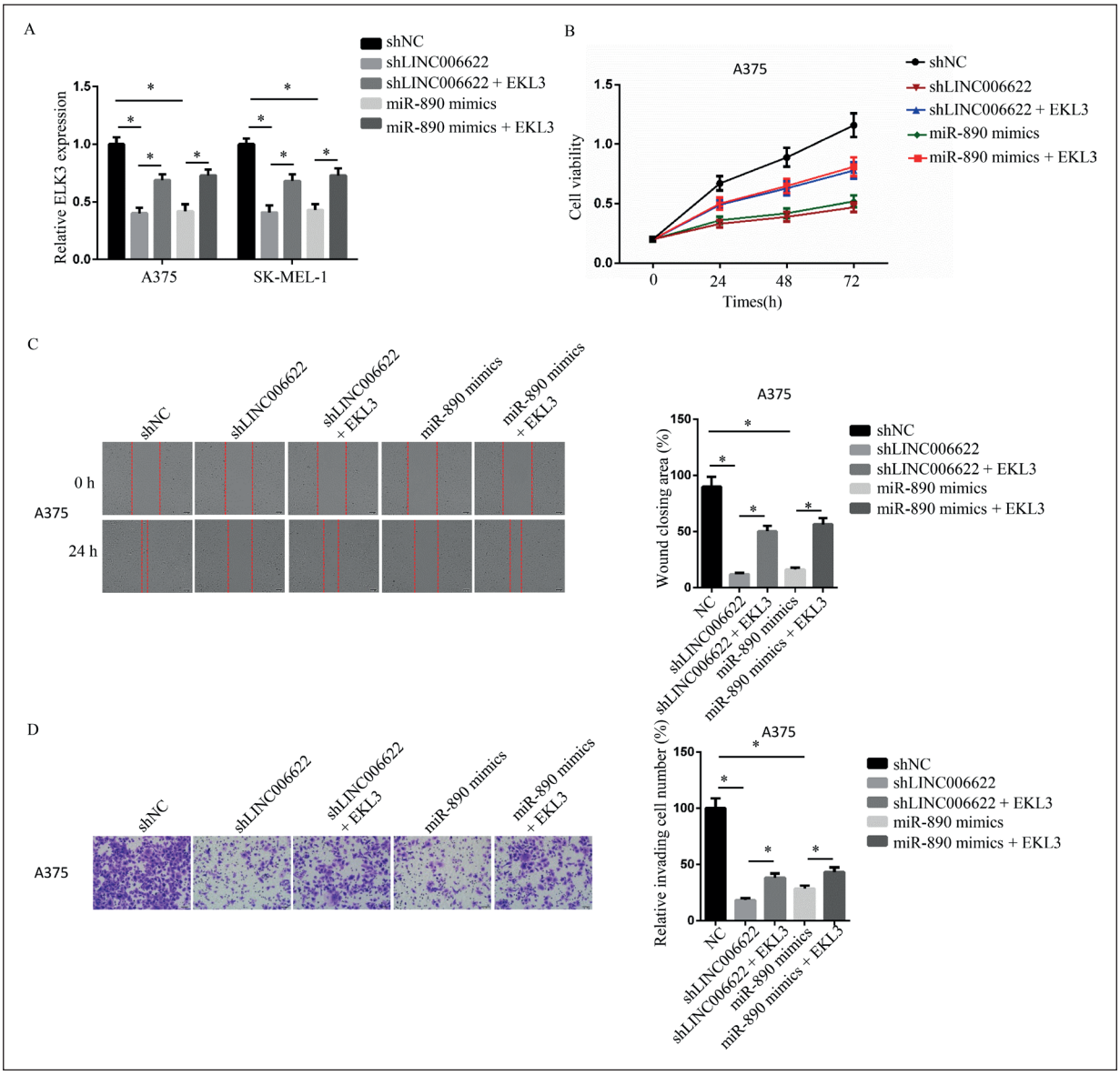

Figure 4. The LINC00662/miR-890/ELK3 axis promoted the progression of melanoma. **A**, The expression of ELK3 was measured in A375 and SK-MEL-1 cells transfected with NC, sh-LINC00662, sh-LINC00662+ELK3, miR-890 mimics and miR-890 mimics+ELK3 via RT-qPCR assay. **B**, Cell proliferation was detected in A375 cells transfected with NC, sh-LINC00662, sh-LINC00662+ELK3, miR-890 mimics and miR-890 mimics+ELK3 through MTT assay. **C**, Cell migration was estimated in A375 cells transfected with NC, sh-LINC00662, sh-LINC00662+ELK3, miR-890 mimics and miR-890 mimics+ELK3 via wound healing assay (magnification ×40). **D**, Cell invasion was tested in A375 cells transfected with NC, sh-LINC00662, sh-LINC00662+ELK3, miR-890 mimics and miR-890 mimics+ELK3 by transwell assay (magnification ×40). *p<0.05.

In the present study, ELK3 was found to be a downstream target gene of miR-890. Furthermore, miR-890 negatively regulated ELK3 expression levels. Through rescue assays, overexpression of ELK3 abrogated the inhibitive effects of LINC00662 knockdown or miR-890 mimics on cell proliferation, migration, and invasion.

Conclusions

Our results demonstrated for the first time that the LINC00662 facilitated the occurrence and development of melanoma through the miR-890/ELK3 axis, suggesting that this work may provide clues for the development of new therapeutic targets for patients with melanoma.

Conflict of Interests

The Authors declare that they have no conflict of interests..

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