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NEAT1 promotes cell proliferation in multiple myeloma by activating PI3K/AKT pathway

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Abstract. – OBJECTIVE: The role of long-chain non-coding NEAT1 (Nuclear enriched abundant transcript1) in multiple myeloma (MM) and its underlying mechanisms were investigated.

PATIENTS AND METHODS: The expression of NEAT1 and SOX13 was detected in CD138+ positive cells collected from MM and healthy subjects. Meanwhile, the relationship between NEAT1 level and the prognosis as well as clinical staging was analyzed. Virus transfection method was used to change the expression of NEAT1 and SOX13 in tumor cells, and then the effect of NEAT1 on cell proliferation, apoptosis and cell cycle was examined. The influence of NEAT1 on the regulation of SOX13 was explored through recovery experiments and Western blot. In addition, the regulation effect of NEAT1 on tumor formation was explored *in vivo*.

RESULTS: NEAT1 and SOX13 were highly expressed in MM patients and MM cell lines, and the patient survival rate and platelet count were significantly decreased in the highly expressed NEAT1 group. Low expression of NEAT1 could inhibit the PI3K/AKT pathway to suppress cell proliferation, promote apoptosis, and inhibit cell cycle. Overexpression of SOX13 was able to partially restore the inhibitory effect of NEAT1 on cell proliferation. Meanwhile, it was found that low expression of NEAT1 significantly inhibited tumor formation *in vivo*.

CONCLUSIONS: Highly expressed NEAT1 promoted cell proliferation through activation of PI3K/AKT pathway, thus participating in the development of MM.

Key Words:
NEAT1, PI3K/AKT pathway, Cell proliferation, Multiple myeloma.

Introduction

Multiple myeloma (MM) is a malignant plasma cell disease derived from terminally differentiated B lymphocytes[1]. Most of the clinical manifestations were bone pain, hypercalcemia, anemia, renal impairment, infection and so on. Statistical result of American Cancer Society showed that about 20,000 new cases are diagnosed with MM each year in USA[2]. The prevalence of MM in black people is about twice that in white men, and men's is slightly higher than women's[3]. The age of onset of MM is mostly over 60 years old, and only 2% of patients have an onset age of less than 40 years[4]. Recently, new treatment options, such as high-dose chemotherapy, hematopoietic stem cell transplantation and the application of new drugs, have increased the complete remission rate of MM, improve the prognosis of MM patients, and significantly increase the survival of patients. However, almost all remission patients are at risk of recurrence, which make MM still an incurable malignant disease[5]. The unclear etiology of molecular biology of MM restricts the further development of MM treatment.

Researches show that epigenetic inheritance is closely related to tumorigenesis and development, which is regulated by non-coding RNA (ncRNA). With the deepening of understanding of ncRNAs, more and more attention has been paid to the important regulatory functions of long-chain non-coding RNAs that belong to ncRNAs[6]. Long non-coding RNA (lncRNA) is a type of RNA molecule with a length of more than 200 nt. It does not have the ability to encode proteins or only encode short polypeptides, but it can play an extremely important regulatory function at multiple levels by interacting with DNA, RNA, proteins and other molecules[7,8]. Numerous studies have demonstrated that IncRNA is widely involved in the regulation of human growth and development, cell proliferation, apoptosis, and the cycle of normal physiological activities. Additionally, it is also found to be associated with the occurrence of cardiovascular diseases, neurodegenerative diseases, and cancer[9].

NEAT1 (Nuclear enriched abundant transcript1) is a kind of IncRNA transcribed from

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chromosome 11 in human body[10]. Its transcription is crucial for the assembly of subspeckles, which are newborn nuclear substructure[11]. NEAT1 influences the assembly of paraspeckles by changing the intracellular distribution of the marker proteins including p54nrb and PSF[12]. In recent years, a series of studies have shown that the upregulated NEAT1 is associated with the poor prognosis of lung cancer, liver cancer, and breast cancer[13-15], and promote malignant invasion as well as distant metastasis of tumors, suggesting that NEAT1 is closely related to occurrence and metastasis of many other malignancies. Other studies have shown that NEAT1 is involved in p53-mediated non-coding RNA networks in leukemia[16]. However, the functional role and underlying mechanism of NEATl in osteosarcoma remains unknown.

Patients and Methods

Patients

We selected 40 hospitalized patients with multiple myeloma from June 2015 to June 2017 in the Department of Hematology of The Affiliated Yixing Hospital of Jiangsu University and 18 normal subjects as controls. The primary CD138+ cells in the bone marrow were isolated and collected, and the clinical data and laboratory examination data of patients with multiple myeloma and healthy controls were recorded. The above date included age, gender, hemoglobin concentration, platelet levels, DS (Durie-Salmon) staging and ISS (international staging system) staging and other relevant information. This study was approved by The Ethics Committee of the Affiliated Yixing Hospital of Jiangsu University, and all participants signed informed consent.

Cell Culture

Human myeloma cell lines including RPMI-8226, U266, MM.1S, KM3, and H929 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 37°C in an incubator with 5% CO_2. The culture medium was Roswell Park Memorial Institute-1640 (RPMI-1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin 100 U/mL and streptomycin 0.1 mg/mL. Normal plasma cells (nPCs) were cultured in the same environment.

Transfection of Cells

10^5 of KM3 and H929 cells in logarithmic phase were collected and centrifuged at 1000 rpm. After the supernatant was removed, the cells were washed twice with RPMI-1640 serum-free medium, and different volumes of virus were added at a multiplicity of infection (MOI) of 80 in cell culture plate, which was then incubated at 37°C incubator for 2 hours and gently shaken every 15 minutes. Subsequently, after the same volume of RPMI-1640 medium containing 30% fetal bovine serum (FBS) was added, the cells were cultured and incubated for another 72 hours. At last, the expression of green fluorescent protein was observed under a fluorescence microscope. The LV-Vector, LV-shNEAT1, and LV-SOX13 required for the experiment were all purchased from Shanghai Zima.

RNA Extraction and Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added to samples to extract the total RNA according to the TRIzol instructions. The concentration of the extracted RNA was determined using UV analysis. Reverse transcription synthesis of complementary Deoxyribose Nucleic Acid (cDNA) and Real-time quantitative PCR reactions were performed. Reaction conditions were as follows: pre-denaturation at 95°C for 30 s, 95°C for 5 s, 60°C for 31 s, with 40 cycles. The primer sequence was as follows: NEAT1 F: GCTGGAGTCTTGGGCACGGC; R: TCAACCGAGGCCGCTGTCTC; SOX13 F: ATTGGTTGAGGACCATGTGC; R: GCGAGCTGTCTCTCTCCAAA; GAPDH F: TGCACCACCAACTGCTTAGC; R: GGCATGGACTGTGGTCATGAG

CDl38+ Plasma Cell Sorting

Bone marrow was withdrawn and diluted with phosphate-buffered saline (PBS) or saline. Mononuclear cells were isolated using a lymphatic fluid and washed twice with PBS saline. After red blood cells were removed, the mononuclear cells are thoroughly mixed with magnetic beads and incubated at 4°C for 15-20 minutes. Unbound magnetic beads were washed away and CDl38+ cells were collected by LS-type sorting columns. The consumables needed for the experiment were all purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

Cell Counting kit-8 (CCK-8) Assay

The transfected cells were gently resuspended and seeded into 96-well plates at 1000 cells/well. 10 μL of CCK8 (Dojindo, Kumamoto, Japan) was added in each well after 24 hours, 48 hours, 72 hours, and 96 hours, respectively, and then the plate was gently shaken to mix. After incubation for 3.5 hours, the absorbance of each well was determined at 450 am with a microplate reader.

Plate Clone Formation Experiment

After a single cell suspension of the transfected cells and the corresponding control cells were make, the cells were seeded at a density of 200 cells/well and continued to be cultured in the incubator for 2 weeks. When cell cloning in the culture dish was observed, 5 mL of 100% methanol was added and fixed for 30 minutes. After the methanol fixative was discarded, 2 mL of crystal violet staining solution was added to each well and stained for 20 minutes. After naturally dried in the air, the number of colonies was counted.

Western Blot

After the medium in the culture dish was aspirated, the cells were washed twice with PBS. 100 μL of radioimmunoprecipitation assay (RIPA) lysate with 1 μL phenylmethylsulfonyl fluoride (PMSF) was added to lyse the cells (Beyotime, Shanghai, China), which were then transferred to an EP tube. After centrifugation, the supernatant was collected, and the protein concentration was determined by bicinchoninic acid (BCA) colorimetry (Pierce, Rockford, IL, USA). Next, the protein samples were separated in electrophoresis with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), which was blocked with 5% skim milk for 1 h and then incubated with specific primary antibodies overnight at 4°C. The membrane was washed with Tris-buffered saline and Tween (TBST) for 3 times the next day and incubated with secondary antibody. Then the membrane was washed 3 times with TBST again. Electrochemiluminescence (ECL) (Thermo Fisher Scientific, Waltham, MA, USA) was used for the detection of protein bands.

Apoptosis

The transfected cells were collected after centrifugation. 50 μL of Binding Buffer was added in each set of cell pellets to gently resuspend the cells, and 5 μL of 7-AAD staining solution was added to each tube. 5 μL of Annexin V-PE were added to each tube at room temperature in the dark and allowed to react for 15 minutes. Lastly, cell apoptosis was analyzed by flow cytometry.

Cell Cycle

The transfected cells and the corresponding control cells were washed with cold PBS solution, and then fixed with 1 mL of 70% pre-cooled ethanol and left overnight at 4°C. On the next day, 150 μL of propidium iodide (PI, 50 μg/mL), 5 μL of Rnase and 145 μL of PBS solution were successively added and then allowed to stand in the dark for 30 minutes at room temperature. Flow cytometry was performed to detect cell cycle of each group.

Establishment of Transplantation Tumor Model in Nude Mice

The nude mice were pretreated with cyclophosphamide for 3 days before inoculation with cells. Cells in logarithmic growth phase were collected, and the concentration was adjusted to 1×10^7 cells/100 μL. After mixed with an equal volume of Matrigel, 200 μL of cell suspension was injected subcutaneously. The nude mice were observed every other day during the feeding process to record their body weight and length as well as width of the tumors. The observation ended when the nude mice showed wilting, hair loss, blindness, hemiplegia, or the body weight decreased by more than 20% on one day, and the rest of the nude mice were raised to 15 days after inoculation. After the nude mice sacrificed by cervical dislocation, the tumors were bluntly dissected and weighed. The specimens were then fixed in 4% paraformaldehyde. The mice needed for the experiment were purchased at the animal model center of Nanjing University and reviewed and approved by Animal Ethics Committee of Jiangsu University.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (La Jolla, CA, USA). Measurement data were expressed as mean±standard deviation (`x ± s). The *t*-test was used for comparison between two groups. Kaplan-Meier survival curves were used when analyzing clinical data. When $p < 0.05$, the difference was considered statistically significant.

Results

NEAT1 was Highly Expressed in MM

A total of 40 MM patients and 18 healthy controls were collected in this experiment. Analysis of the collected tumors and corresponding healthy control samples revealed that NEAT1 level was significantly higher in tumor tissues (Figure 1A). And as the stage of MM ISS increased, the expression gradually increased (Figure 1B). According to the expression of NEAT1, the patients were divided into high expression group and low expression group. The prognosis of the high expression group was significantly lower than that of the low expression group (Figure 1C). And NEAT1 expression was significantly negatively correlated with platelet count but not with age, gender, hemoglobin concentration, and DS staging (Table I). At the same time, it was found that the expression of NEAT1 was significantly enhanced in MM cell lines compared with nPCs, especially in KM3 and H929 cells, which were therefore selected for subsequent experiments (Figure 1D).

NEAT1 Activated PI3K/AKT Pathway to Promote Cell Proliferation

Further, we reduced intracellular NEAT1 expression by viral transfection and found that low expression of NEAT1 significantly inhibited cell proliferation (Figure 2A, 2B). Clone formation experiments also demonstrated the inhibition ef-

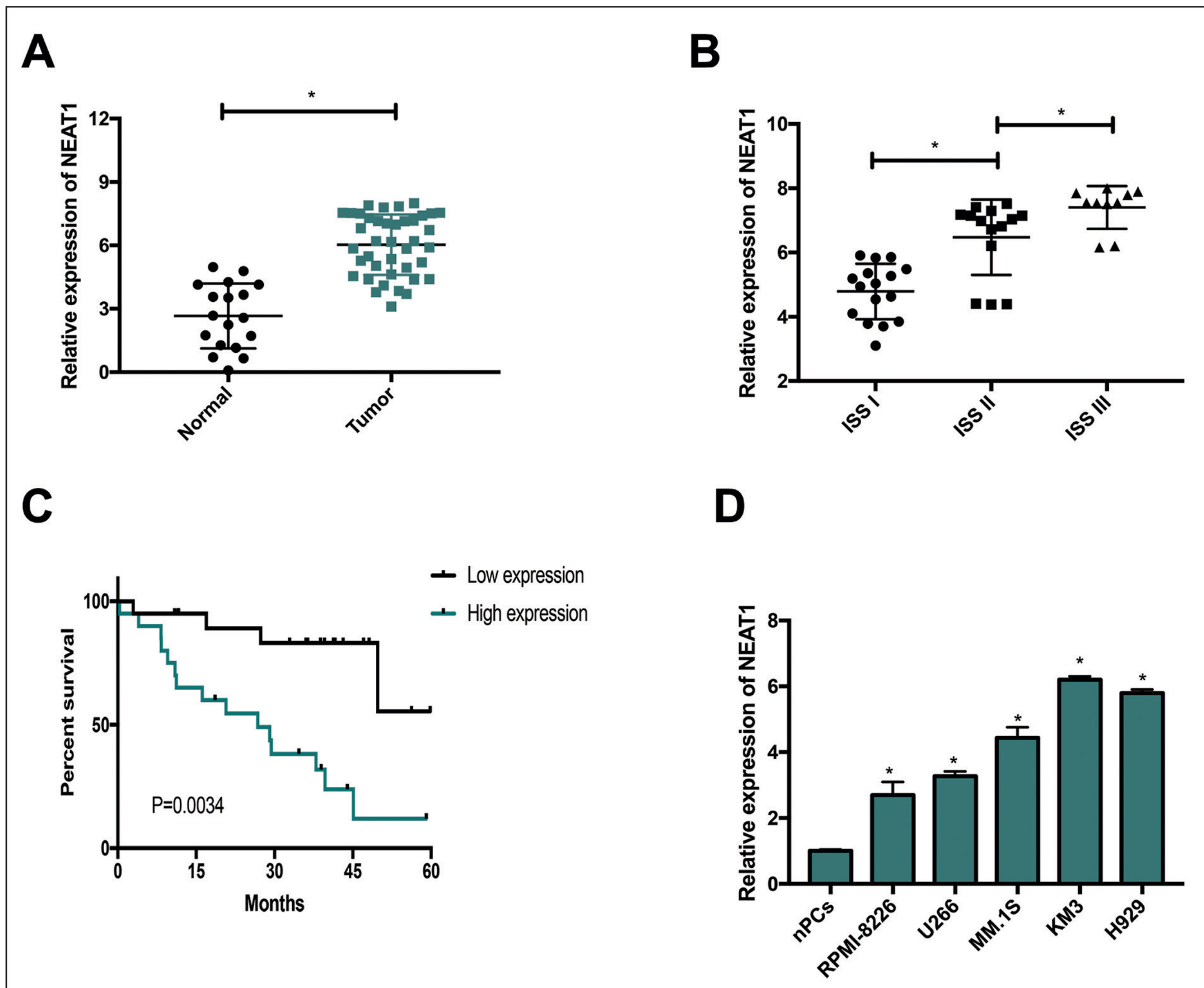

Figure 1. High expression of NEAT1 in MM. ***A,*** PCR detection of NEAT1 mRNA expression in MM and healthy people. ***B,*** PCR detection of NEAT1 mRNA expression in different ISS stages of MM. ***C,*** The survival time of patients in NEAT1 high expression group was lower than that in low expression group. ***D,*** PCR detection of NEAT1 expression in normal plasma cells and MM cell lines.

Table I. NEAT1 expression in clinicopathological features.

Clinicopathological features	Number of cases	LncRNA NEAT1 expression		*p*-value
		Low (n = 20)	High (n = 20)	
Age (years)		20	20	0.5073
≤ 65	26	12	14	
> 65	14	8	6	
Gender				0.7440
Male	25	13	12	
Female	15	7	8	
ISS stage				
0.0267*				
I-II	21	14	7	
III	19	6	13	
DS stage				0.0578
I-II	20	13	7	
III	20	7	13	
Hemoglobin (g/L)				0.2036
≤ 85	18	11	7	
> 85	22	9	13	
Platelets ($\times 10^9$)				0.0110*
≤ 100	28	7	15	
> 100	12	13	5	

fect of NEAT1 on cell proliferation (Figure 2C). Further exploration of the mechanism revealed that low expression of NEAT1 significantly inhibited the PI3K/AKT pathway (Figure 2D). At the same time, low expression of NEAT1 promoted cell apoptosis (Figure 2E) and resulted in cell cycle arrest in the GO/G1 phase (Figure 2F).

NEAT1 Promoted the Expression of SOX13

Through starBase, the bioinformatics prediction site (http://starbase.sysu.edu.cn/mrnaCeRNA.php), we found that NEAT1 can regulate the expression of SOX13. In addition, SOX13 mRNA and protein levels (Figure 3A and Figure 3B) were significantly higher in tumor tissues, and decreasing NEAT1 expression reduced SOX13 mRNA (Figure 3C) and protein (Figure 3D) levels. Through recovery experiments, we found that upregulation of SOX13 could partially reverse the increased apoptosis (Figure 3E), decreased proliferation (Figure 3F, 3G), and cell cycle arrest (Figure 3H) caused by NEAT1.

NEAT1 Promoted Tumor Formation in Vivo

We further explored the role of NETA1 *in vivo*. The results showed that tumor formation was significantly inhibited in low expressed NEAT1 group, and the weight (Figure 4A) and volume of the tumor (Figure 4B) were significantly lower than those in the control group. And analysis of gene expression in tumor tissue revealed that both the levels of NEAT1 and SOX13 in the LV-shNEAT1 group were significantly reduced (Figure 4C).

Discussion

The pathogenesis of multiple myeloma is so complex that genetic mutations cannot fully explain and clarify the complexity of the development of multiple myeloma. Researches have shown that epigenetic regulation, such as lncRNAs that belong to non-coding RNAs, play an important role in the development of multiple myeloma. Studies have found that rearrangements on chromosome 8q24 occur occasionally in multiple myeloma, which is associated with the disease progression. By FISH, 8q24 rearrangements were detected in 12 of 54 patients (22.2%) with multiple myeloma and in 8 out of 11 multiple myeloma cell lines (72.7%). A 360-kb fragment, PVT1, was found in 10 patients with multiple myeloma and in all multiple myeloma cell lines, and was relatively close to the transcription factor c-Myc. The translocation of 8q24 leads to the overexpression of oncogenes, which correlates with the occurrence of multiple myeloma. What's more, PVTl

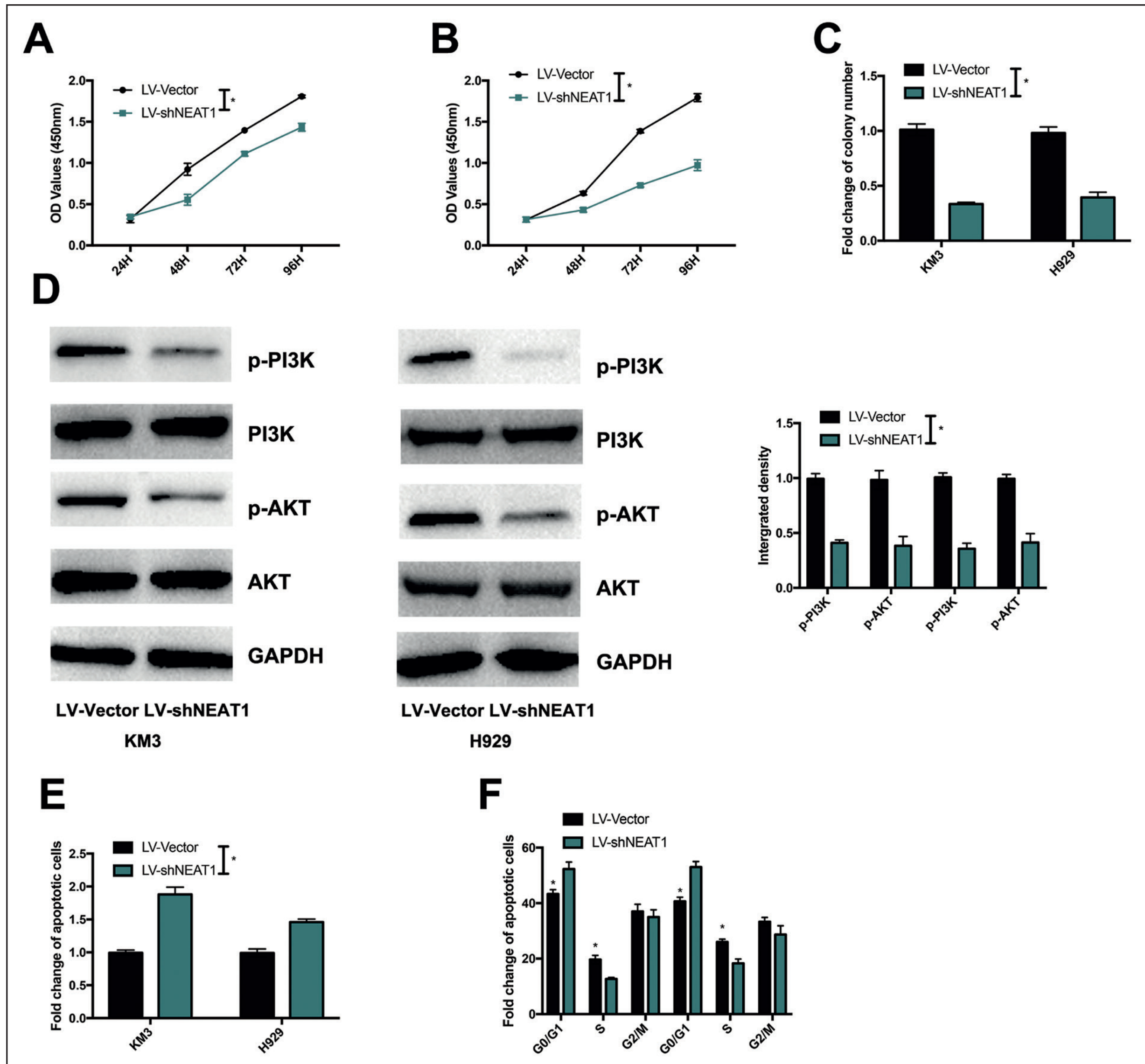

Figure 2. High expression of NEAT1 promoted cell proliferation. ***A,*** CCK8 was used to detect proliferation of KM3 cells with low expression of NEAT1. ***B,*** CCK8 was used to detect proliferation of H929 cells with low expression of NEAT1. ***C,*** Colony formation assay was performed to detect proliferation of KM3 and H929 cells with low expression of NEAT1. ***D,*** PI3K/AKT pathway was detected in KM3 and H929 cells after low expression of NEAT1. ***E,*** Apoptosis of KM3 and H929 cells was analyzed after low expression of NEAT1. ***F,*** Cell cycle of KM3 and H929 was detected after low expression of NEAT1.

was found as an oncogene[17]. In fact, upregulation of PVT1 contributes to tumor survival and drug resistance, while its down-regulation inhibits cell proliferation and induces a strong apoptotic response[18]. In this study, we found that NEAT1 was highly expressed in MM patients, and its expression level was related to the patient's ISS stage and platelet count. In addition, high expression of NEAT1 could promote cell proliferation and inhibit apoptosis *in vitro* while promote the formation of tumors *in vivo*. The underlying mechanism might be related to the positive regulation of SOX13 expression and activation of the PI3K/AKT pathway.

A large number of studies[19,20] have demonstrated that abnormal activation of the PI3K/Akt signaling pathway exists in many malignant solid tumors and hematological tumors, and Akt hyperphosphorylation activation is thought to be closely associated with tumor cell growth, escape, invasion, and tumor resistance. Throughout all current findings about Akt and cancer,

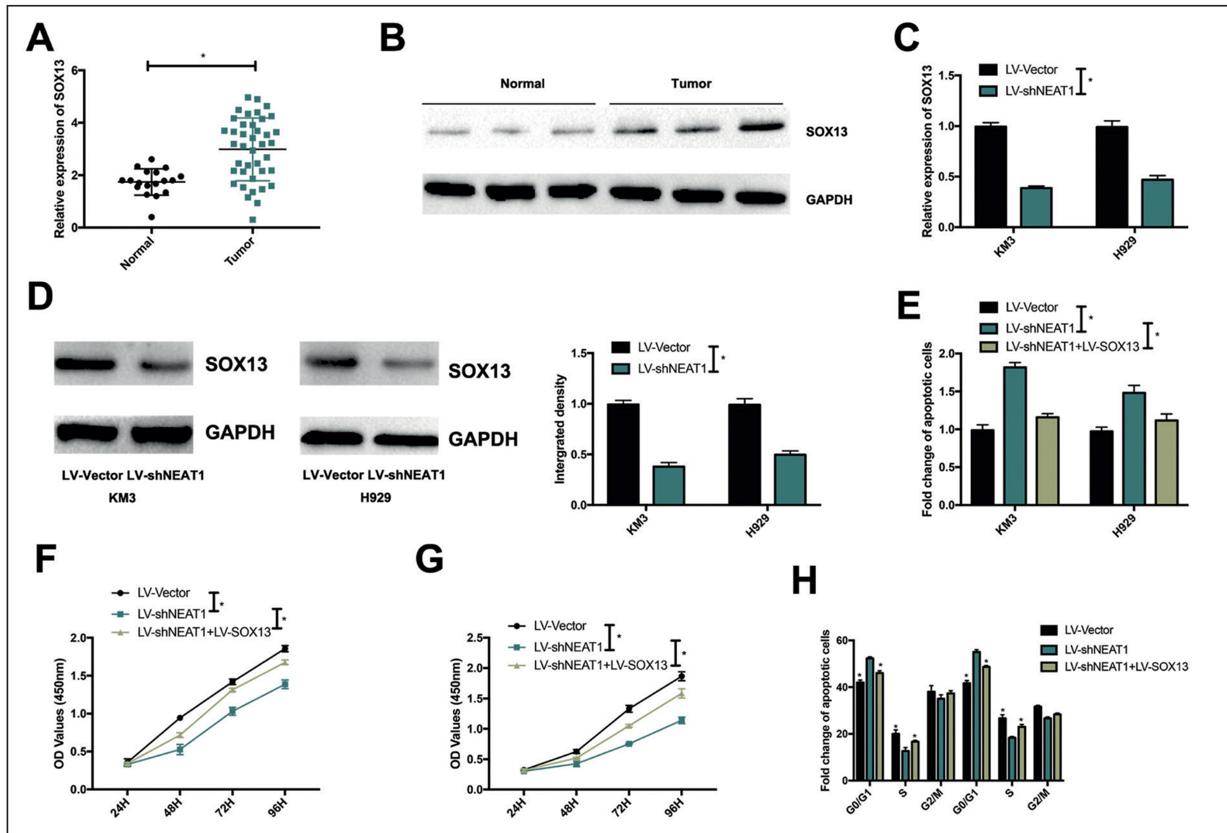

Figure 3. High expression of SOX13 in MM. ***A,*** PCR detection of SOX13 expression in MM and healthy people. ***B,*** Detection of SOX13 protein expression in MM and healthy people. ***C,*** RNA expression of SOX13 in KM3 and H929 cells after low expression of NEAT1. ***D,*** Detection of SOX13 protein expression in KM3 and H929 cells after low expression of NEAT1. ***E,*** Apoptosis of KM3 and H929 cells after low-expression of NEAT1 and over-expression of SOX13 was detected. ***F,*** KM3 cell proliferation after simultaneous low expression of NEAT1 and overexpression of SOX13 was detected. ***G,*** H929 cell proliferation after simultaneous low expression of NEAT1 and overexpression of SOX13 was detected. ***H,*** Cell cycles of KM3 and H929 after simultaneous low expression of NEAT1 and overexpression of SOX13 were detected.

we found that increased phosphorylation of Akt seems to play a more important role in tumor development compared to the enhanced amount of Akt expression, as Akt is not highly expressed in all solid tumors or blood system tumors, but its phosphorylation level is significantly

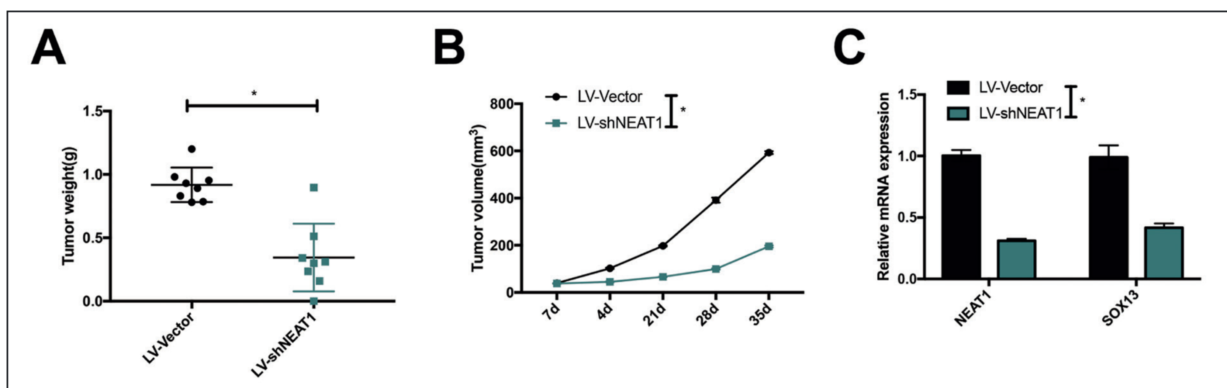

Figure 4. NEAT1 promoted tumor formation *in vivo*. ***A,*** Tumor weight in low expression group and control group was detected. ***B,*** Tumor size in low-expression and control groups was detected. ***C,*** NEAT1 and SOX13 expression was detected in tumor tissues of low expression and control groups.

increased. Therefore, we suggested that the hyperphosphorylation of Akt and mTOR levels is closely related to tumor recurrence and drug resistance[21]. Activated PI3K is able to promote phosphorylation of PIP2 to PIP3, which further activating downstream proteins such as Akt and other related proteins to adjust a series of cytokines, including the platelet-derived growth factor (PDGF), nerve growth factor, insulin-like growth factor I (IGF-I) and interleukin-3, thereby promoting resistance, relapse, immune escape of multiple myeloma[22].

Conclusions

We found that NEAT1 was highly expressed in MM, which was significantly negatively correlated with patient prognosis. Highly expressed NEAT1 could enhance cell proliferation and inhibit cell apoptosis so as to promote the tumor formation *in vivo*. The mechanism might be related to regulating SOX13 and activating the PI3K/AKT pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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