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LncRNA NEAT1 regulates the growth, migration, and invasion of the human esophageal cancer cells *via* the miR-377/E2F3 axis

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Esophageal cancer is a highly aggressive and lethal human malignancy. The current study was established with the aim of studying the role of the lncRNA NEAT1 in regulating the growth and progression of esophageal cancer. Esophageal cancer tissues and cell lines showed significantly ($P<0.05$) upregulated transcript levels of lncRNA NEAT1. The expression of NEAT1 was also upregulated in metastatic tissues compared to nonmetastatic. The elimination of lncRNA NEAT1 led to a significant decrease ($P<0.05$) decrease in the viabilities of cancer cells due to the induction of apoptosis. Cancer cell migration and invasion were also significantly reduced ($P<0.05$) upon lncRNA NEAT1. *In silico* analysis indicated that miR-377 targets lncRNA NEAT1 at the post-transcriptional level, whose overexpression in cancer cells was found to mimic the tumor-suppressive regulatory effects of lncRNA-NEAT1. At the molecular level, the regulatory effects of lncRNA NEAT1 were shown to be modulated by the miR-377/E2F3 signaling axis. The results suggest that the molecular targeting of the lncRNA NEAT1 and miR-377/E2F3 axis could prove beneficial in the management of esophageal cancer.

Keywords: Esophageal cancer, long noncoding RNA, proliferation, apoptosis migration, invasion.

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Abbreviations: lncRNAs, long noncoding RNAs; NEAT1, Nuclear Enriched Abundant Transcript 1; NCE, Non-cancerous esophageal cells; cDNA complementary DNA

INTRODUCTION

The gene regulatory machinery is highly complex in higher organisms (Britten & Davidson, 1969). Although the transcriptional level of gene regulation is the primary way of eukaryotic gene regulation, there are different players operating at post-transcriptional and translational stages which fine-tune the regulation of eukaryotic genes (Vogel & Marcotte, 2012; Barrett *et al.*, 2012). Among post-transcriptional regulatory molecules, non-coding RNAs form an important class of gene regulators and are mainly grouped into two broad classes-microRNAs (miRs) and long noncoding RNAs (lncRNAs) (Di Gesualdo *et al.*, 2014). lncRNAs are the transcriptional pervasive products of RNA polymerase II that are not translated to proteins, but aid in gene regulation at the post-transcriptional or translational level (Jensen *et al.*, 2013; Krishnan *et al.*, 2014). The production of lncRNAs is developmentally regulated in mammals and higher organisms (Morris & Mattick, 2014). LncRNAs regulate the development and differentiation of human cells (Fatica & Bozzoni, 2014). They have been shown to regulate crucial aspects of growth and development in humans (Ponting *et al.*, 2009). The impairment in human lncRNA transcript levels has unfavorable effects and results in the onset of various diseases (Taft *et al.*, 2010). Recent studies have found that lncRNAs also regulate the development of human cancers (Prensner & Chinnaiyan, 2011). Dysregulation of lncRNAs has been shown to be responsible for the growth and progression of a number of human cancers such as gastric cancer, pancreatic cancer, and lung cancer (Li *et al.*, 2014; Haung *et al.*, 2016; Lowen *et al.*, 2014). Therefore, lncRNAs have been reported to aid in the diagnosis and therapeutic interventions against human cancers (Qi & Du, 2013). Esophageal cancer is one of the most aggressive cancers of humans and accounts for a significant number of cancer-related deaths (Crew & Neugut, 2004). It is ranked as sixth most death-causing human cancer and the overall 5-year survival rate is still less than 10% (Jemal *et al.*, 2011). Looking at the regulatory role of lncRNAs in human cancers, the study aimed to explore the role of Nuclear Enriched Abundant Transcript 1 (NEAT1) of lncRNA in regulating the growth and proliferation of human esophageal cancer. Additionally, the interactional analysis of NEAT1 was studied to identify its molecular regulatory interaction with the specific targeting of miR at the post-transcriptional level, as is true for most lncRNAs. Together, the study pointed out the importance of lncRNA NEAT1 for acting as a molecular target for the treatment of human esophageal cancer.

MATERIALS AND METHODS

Culture and maintenance of cell lines

Non-cancerous esophageal cells (NCE) and esophageal cancer cell lines (CE48T, CE146T, KYSE150, and KYSE510) were obtained from ATCC, USA. For cell line culture, the temperature of 37°C with a CO_2 concentration of 5% was maintained using a humidified incubator. The growth medium used for cell culture was Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientifc), 100 μg/ml streptomycin and 100 U/ml ampicillin (both from Himedia).

Clinical specimens and ethical statement

The esophageal clinical specimens (cancer and normal surrounding part) were obtained from the Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China, after informed written consent of the patients. The collection, maintenance, and laboratory usage of specimens were carried out following the standard ethical guidelines and as approved by the institutional ethics committee. The tissues were frozen in liquid nitrogen and stored in a –80°C refrigerator until investigation.

Gene expression analysis using the qRT-PCR method

The Pure-Link RNA Mini Kit was used to isolate total RNA from different esophageal clinical tissues and cell lines (normal and cancerous). The RNA was treated with DNase I (Thermo Fisher Scientific) to remove any DNA contamination and then reverse transcribed to complementary DNA (cDNA) using the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific). The SYBR Green method was used to perform quantitative real-time PCR using cDNA as a template. The human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control in gene expression studies. The PCR reaction conditions were: 3 min at 95°C, 15 s at 95°C, 15 s at 58°C, 15 s at 68°C, 1 min at 95°C, 1 min at 55°C and 6 s at 70°C: 40 cycles. The primer sequences were GADPH-F 5'-CCAGAAGACTGTGGATG-3', GADPH-R 5'-CTAGACGGCAGGTCAGG-3', NEAT1-F 5'-3', NEAT1-R 5'-3', miR-377-F 5'-3' and miR-377-R 5'-3'.

Cancer cell transfection and transfection constructs

At 50% confluence of cell culture in DMEM, CE146T cancer cells were transfected using the Lipofectamine 2000 method (Thermo Fisher Scientific) method. The NEAT1 RNAi construct (si-NEAT1) and the silencing control (si-Ctrl), the microRNA down-regulation construct, the miR-377 inhibitor and its silencing control (miR-NC) were purchased from the RiboBio company, China.

Determination of cell proliferation

The proliferative rates of CE146T cancer cells and noncancerous esophageal cells (NCE) were calculated with the help of 3-[4,5-dimethylthiazolyl-2]-2, 5-diphenyltetrazolium bromide (MTT, Thermo Fisher Scientific) reagent. Briefly, after 0, 12, 24, 48 or 96 hours of cell transfection, CE146T or NCE cells were seeded in the 96-well plate at a cellular density of 0.6×10^6 cells/well. The cells were then cultured for 24 h at 37°C A total of 10 μL MTT solution (0.5%) was then added to each well followed by an additional 4 h incubation at 37°C Subsequently, each well was supplied with 100 μL of DMSO (Sigma-Aldrich) to dissolve the formazan crystals. Finally, cell proliferation was determined by measuring the absorbance at 570 nm with the help of a spectrophotometer and comparisons were made using percent values.

DAPI staining to analyze cell viability

The DAPI staining method was used to analyze the viability and apoptosis of CE146T cancer cells. Briefly, CE146T cancer cells were transfected with si-NEAT1 or si-Ctrl for 24 h using Lipofectamine 2000 and then seeded in a 12- well plate at cellular densities of 2×10^5 cells/well Cells were allowed to grow for 24 h in a humidified incubator at 37°C with 5% CO_2. Subsequently, cells were harvested and fixed with 70% ethanol and then stained with DAPI solution. Finally, the fluorescent microscope was used to examine the nuclear morphology of CE146T cells. The Annexin V/PI assay was performed as previously described (Huang *et al.*, 2016).

Wound healing assay

The migratory potential of CE146T cells, transfected with si-NEAT1 or si-Ctrl, was estimated by the wound healing method. CE146T cancer cells (2×10^5 cells/well) were seeded in a 6-well plate, after 24 h of transfection and cultured for 24 h. Using a sterile pipette tip, a scratch line was drawn on the cell surface. The width of the scratch was recorded at 0 and 24 h. Migration was estimated similarly for miR-377 inhibitor or miR-NC transfected CE146T esophageal cancer cells.

Transwell assay

The invasion of CE146T cells was estimated using the transwell chamber method. Here, the culture medium containing 1×10^5 CE146T cells transfected with si-NEAT1, or si-Ctrl and the miR-377 inhibitor or miR-NC was placed in the upper chamber of the transwell coated with Matrigel (BD Biosciences, USA). The pore size of the transwell used was 8 μm. 600 μL DMEM supplemented with 10% FBS was placed in the lower chamber of the transwell. After 24 h of incubation at 37°C with 5% CO_2 cells invading the lower chamber were fixed with methanol and then stained with crystal violet (0.1%). A high-magnification light microscope was used to examine the stained cells.

In silico analysis and luciferase reporter assay

StarBase v2.0 (http://starbase.sysu.edu.cn/starbase2/index.php) was used to predict the microRNA targeting NEAT1, post-transcriptionally. The Dual Luciferase Reporter System (Promega) was used to perform the luciferase assay to determine the interaction between miR-377 and NEAT1, following the manufacturer's protocol. The reporter construct pGL3-NEAT1wt (wild type) or pGL3-NEAT1mut (mutant) was cotransfected with miR-377 mimics in CE146T esophageal cancer cells. The luciferase activity of Renilla was used as an internal control in the luciferase reporter assay.

Western blot

Cell lysis and protein extraction buffer (RIPA) were used to achieve total protein extraction. Protein concentration was estimated using the Bradford method. About 50 μg proteins, from each sample, were separated on 12% SDS PAGE gel. The gel blotting was made on a PVDF membrane. Exposure of primary antibodies (ant-E2F3) was administered to blotted membranes. Using specific secondary metabolites, protein bands were visualized with the help of efficient chemiluminescent reagent. The human β-actin protein acted as an internal control in protein expression studies.

Statistical analysis

Each experiment was carried out with at least three independent replicates and quantitative data were presented as means ± standard deviation (S.D.). Statistical analysis was performed with SPSS 19.0 software. The two-tailed *t*-test was performed to execute pairwise com-

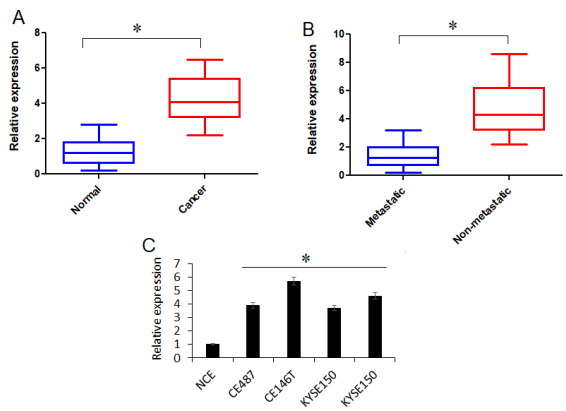

Figure 1. NEAT1 is upregulated in esophageal cancer.
The qRT-PCR analysis of NEAT1 in (**A**) normal and cancerous esophageal tissues (**B**) nonmetastatic and metastatic esophageal cancer tissues (**C**) non-cancerous (NCE) and cancerous (CE48T, CE146T, KYSE150 and KYSE510) esophageal cell lines. The experiments were carried out in triplicate and expressed as means ± S.D. (**P*<0.05)

parisons. The *P*-value <0.05 was taken as the measure of the statistical significancy of the differences, which was symbolized as *.

RESULTS

NEAT1 is up-regulated in esophageal cancer

The qRT-PCR analysis of NEAT1 was performed to understand the pattern of its gene expression in esophageal cancer. The results were indicative that the transcript level of NEAT1 is significantly higher (*P*<0.05) higher in cancerous tissues compared to normal tissues (Fig. 1A). It suggests the probable regulatory role of NEAT1 in esophageal cancer. The regulatory role of NEAT1 in esophageal cancer was further evident from the study of its expression in metastatic versus nonmetastatic cancerous tissue samples. The expression was significantly (*P*<0.05) high in metastatic tissues than in non-metastatic tissues (Fig. 1B). The expression of NEAT1 was also studied in four different esophageal cancer cell lines (CE48T, CE146T, KYSE150 and KYSE510) and non-cancerous esophageal cells (NCE). The relative transcript level of NEAT1 was significantly (*P*<0.05) higher in all cancer cell lines (Fig. 1C). NEAT1 expression was highest in the CE146T cancer cell line and thus was chosen for further investigation. Taken together, the results suggest the negative regulatory role of NEAT1 in human esophageal cancer.

NEAT1 repression reduces the viability of cancer cells to suppress their proliferation

To gain insight into the molecular role of lncRNA NEAT1 in esophageal cancer, CE146T cancer cells were transfected with si-NEAT1 to achieve down-regulation of NEAT1, which was confirmed by qRT-PCR (Fig. 2A). CE146T cancer cells transfected with the si-Ctrl vector were used as control. The MTT assay-based proliferation assessment of cancer cells transfected with si-NEAT1 or si-Ctrl showed that cancer cell proliferation rates were significantly lower under NEAT1 downregulation (Fig. 2B). Transfection of CE146T cancer cells with si-NEAT1 for longer duration resulted in a more severe decline in proliferation levels due to the fact that the

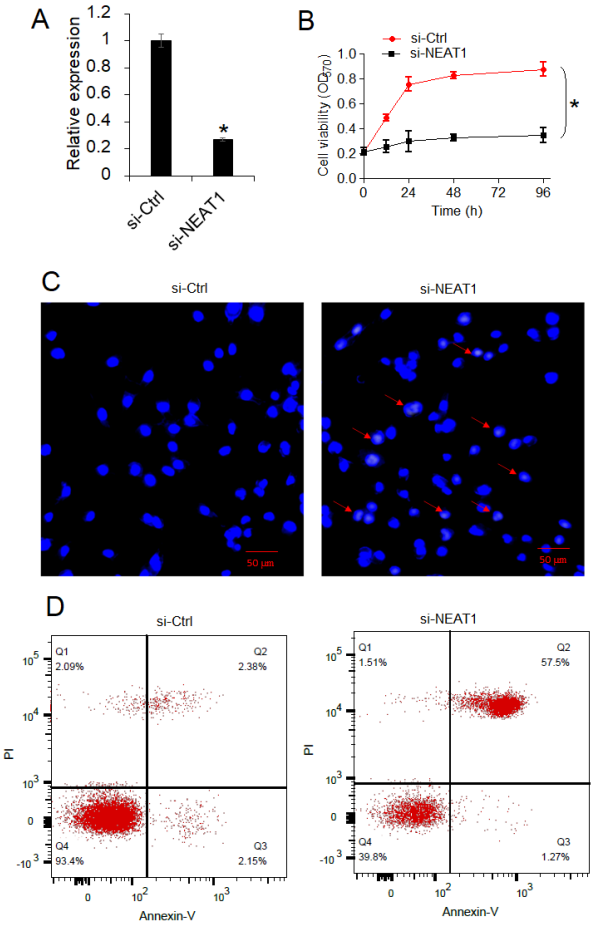

Figure 2. Down-regulation of NEAT1 inhibits esophageal cancer cell proliferation through apoptosis.
(**A**) NEAT1 expression in CE146T cells transfected with si-Ctrl or si-NEAT1 (**B**) relative percentage of proliferation of CE146T cells transfected with si-Ctrl or si-NEAT1 (**C**) DAPI staining of CE146T cells transfected with si-Ctrl or si-NEAT1. (**D**) Annexin V/PI staining of CE146T cells transfected with si-Ctrl or si-NEAT1. The experiments were carried out in triplicate and expressed as mean ± S.D. (**P*<0.05)

longer duration of transfection results in a higher level of down-regulation of NEAT1. The study of the nuclear morphology of cancer cells revealed that the repression of NEAT1 transcript levels decreases cell viability due to the induction of cell apoptosis, as observed by DAPI and Annexin V/PI staining (Fig. 2C and 2D). The percentage of apoptosis increased from 4.41% in si-NC to 58.77% in CE1467 cells transfected with si-NEAT1 (Fig. 2D). Collectively, the results show that the reduction in esophageal cancer cell proliferation was due to induction of apoptosis in cancer cells.

Down-regulation of NEAT1 reduces the migration and invasion of cancer cells

The wound healing assay was performed to assess the effect of NEAT1 down-regulation on CE146T cancer cell migration. CE146T cancer cells that negatively regulate NEAT1 were observed to show very low levels of migration (Fig. 3A). NEAT1 repression also had an inhibitory effect on CE146T esophageal cancer cell invasion, as revealed by a transwell assay (Fig. 3B). Together, these results suggest that down-regulation of NEAT1 might be valuable in limiting esophageal cancer metasta-

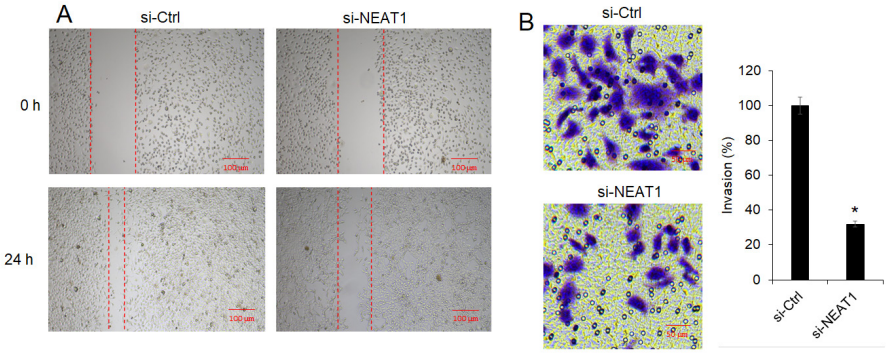

Figure 3. NEAT1 repression restricts the migration and invasion of CE146T cancer cells.
(**A**) wound healing assay; (**B**) trans-well assay. The experiments were carried out in triplicate and expressed as mean ± S.D. (**P*<0.05)

sis and point out the therapeutic potential of NEAT1 in the treatment of human esophageal cancer.

NEAT1 is targeted by miR-377

In silico analysis inferred that NEAT1 acts as the post-transcriptional target of miR-377 and showed that miR-377 interacts and binds in a specific complementary region of NEAT1 (Fig. 4A). The expression of miR-377 was also found to co-relate with the expression of NEAT1 in cancer cell lines (Fig. 4B). This further supports the interaction of miR-377 and NEAT1. Finally, the interaction was confirmed by a dual luciferase assay, in which it was seen that when the reporter construct pGL3-NEAT1wt is cotransfected with miR-377 mimics, cancer cells show significantly (P<0.05) higher luciferase activity compared to cancer cells cotransfected with pGL3-NEAT1mut and miR-377 mimics (Fig. 4C). Together, the results show that miR-377 interacts and targets the lncRNA NEAT1 post-transcriptionally.

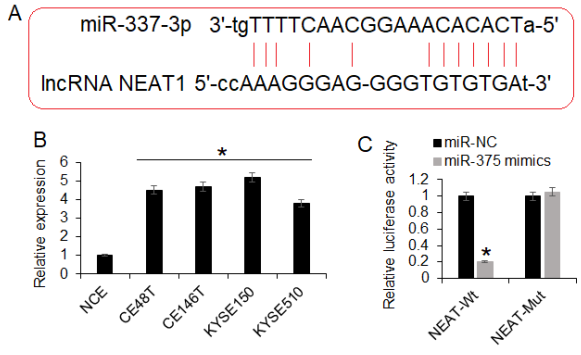

Figure 4. NEAT1 acts as the post-transcriptional target of miR-377.
(**A**) In silico analysis of the miR-377-3p and NEAT1 interaction (**B**) qRT-PCR analysis of miR-377 in non-cancerous (NCE) and cancerous (CE48T, CE146T, KYSE150 and KYSE510) esophageal cell lines (**C**) dual luciferase reporter assay. The experiments were carried out in triplicate and expressed as mean ± S.D. (**P*<0.05)

Exertion of NEAT1 regulatory effects through the miR-377/E2F3 axis

Whether the regulatory effects of NEAT1 in esophageal cancer are passed on through miR-377, the silencing of miR-377 was studied. CE146T cancer cells were transfected with miR-377 inhibitor construct to cause silencing of miR-377, which was confirmed by qRT-PCR analysis (Fig. 5A). The down-regulation of miR-377 resulted in the decline in proliferation, migration, and invasion of CE146T cancer cells in a similar manner as the NEAT1 repression (Fig. 5B–D). The decline in miR-377 transcripts further resulted in the enhancement of E2F3 transcription factor protein levels (Fig. 6A). Cyclin A protein concentration was also found to decrease in miR-377 inhibitor-transfected CE146T cancer cells (Fig. 6B). Thus, the results show that the regulatory effects of NEAT1 on human esophageal cancer are exerted through the miR-377/E2F3 axis.

DISCUSSION

Advancement in anticancer approaches in recent era has improved the overall survival rates of cancer patients (Sharma *et al.*, 2011). However, human esophageal cancer is still a highly lethal malignancy. It is a very aggressive type of human cancer and ranks eighth in terms of total cancer occurrence (Rahib *et al.*, 2014). In terms of annual deaths, it is ranked as the sixth most lethal cancer worldwide (Jemal *et al.*, 2011). More than 70% of patients die within the first year after the diagnosis of esophageal cancer (working, 2002). Hence, understanding the basic mechanics of human esophageal cancer is of immense importance for the development of the more promising anticancer approaches for esophageal cancer. Noncoding RNAs have gained tremendous attention for their role in human cancer regulation (Gibb *et al.*, 2011). These noncoding RNAs are greater than 200 nucleotides in length termed long noncoding RNAs (lncRNAs) and regulate important aspects of cellular development such as growth and differentiation (Fatica & Bozzoni, 2014) Studies have found that there is a substantial level of expressional dysregulation of lncRNA in different human cancers (Yan *et al.*, 2015). Our results also indicated a similar correlation between the lncRNA NEAT1 and esophageal cancer, further strengthened by relatively higher levels of NEAT1 expression in all cancer cell lines investigated. lncRNAs have been reported to be active in influencing cancer cell proliferation through various research studies (Gutschner & Diederichs, 2012). Confirming this, downregulation of NEAT1 resulted in a decrease in cellular proliferation of esophageal cancer cells due to the induction of cancer cell apoptosis. lncRNAs have been reported to regulate metastasis of various human cancers (Qiao *et al.*, 2013; Hajjari *et al.*, 2013; Hajjari & Salavaty, 2015). Here, when the analysis of the expression of NEAT1 was carried out in metastatic and non-metastatic esophageal cancer tissue specimens, NEAT1 was found to be upregulated in metastatic tissue. Repres-

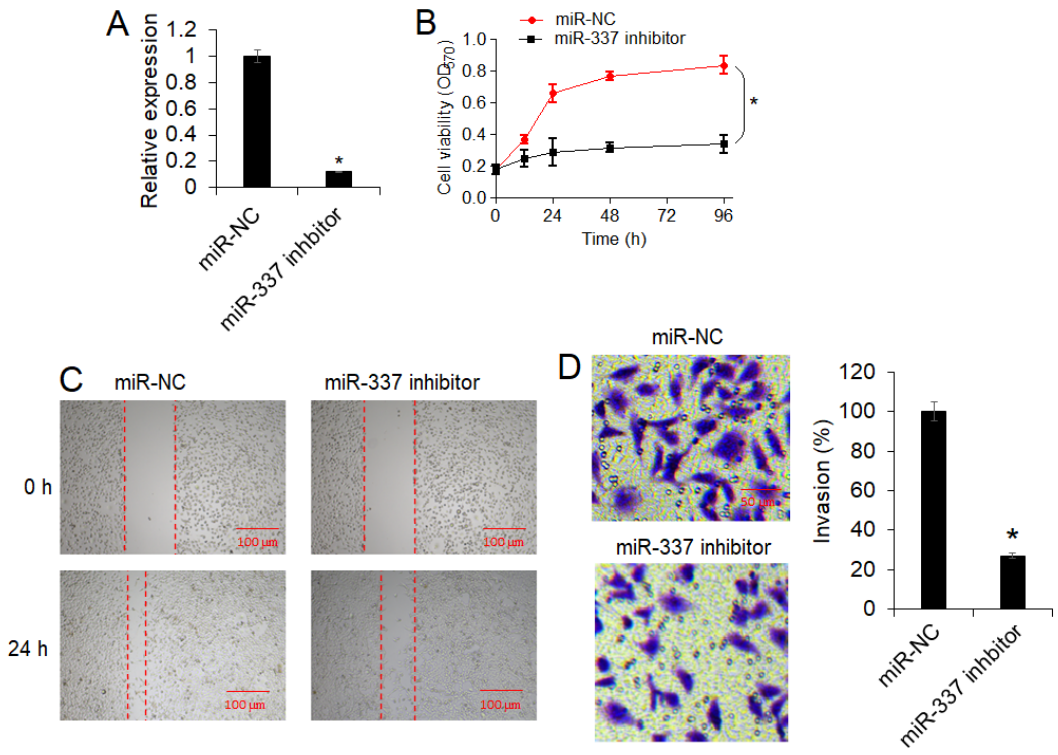

Figure 5. miR-377 mimics the regulatory effects of NEAT1 in esophageal cancer.
(**A**) relative expression of miR-377 in CE146T cancer cells transfected with miR-NC or miR-377 inhibitor; (**B**) relative percentage of proliferation of CE146T cells transfected with miR-NC or miR-377 inhibitor; (**C**) wound healing assay; (D) transwell assay. The experiments were carried out in triplicate and expressed as means ± S.D. (**P*<0.05)

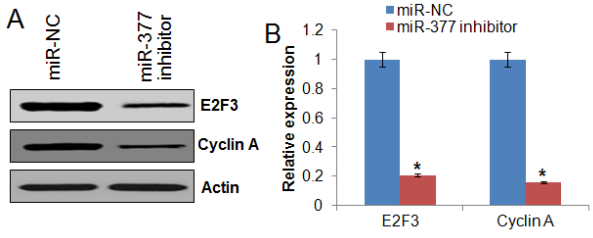

Figure 6. NEAT1 regulatory effects are modulated through the miR-377/E2F3 axis.
Western blotting of E2F3 and Cyclin A with the human β-actin gene as an internal control. The experiments were carried out in triplicate and expressed as mean ± S.D. (**P*<0.05)

sion of NEAT1 was also found to restrict the migration and invasion of esophageal cancer cells, highlighting the role of NEAT1 in esophageal cancer metastasis. Research investigations have shown that lncRNAs have microRNA (miR) sponging functions and their regulatory effects are modulated *via* miRs specifically targeting them at the post-transcriptional level (Liang *et al.*, 2017; Su *et al.*, 2016). Bioinformatic analysis predicted NEAT1 as the molecular target of miR-377. The results were confirmed by interaction assay and qRT-PCR expression study, which showed up-regulation of miR-377 in esophageal cancer cell lines. Interestingly, the knockdown of miR-377 in esophageal cancer cells reduced their proliferation, migration, and invasion considerably. The regulatory effects of NEAT1 were found to be modulated through the miR/E2F3 signaling axis. The E2F3 transcription factor plays a prominent role in different cellular aspects, including cell division (Humbert *et al.*, 2000). Reports have shown that it regulates cyclin proteins to control the entry of dividing cells into the mitotic phase of the cell cycle (Jensen *et al.*, 2013). Repression of miR-377 transcripts decreased E2F3 protein levels, which may occur through some regulatory intermediate that operates between miR-377 and E2F3 whose molecular targeting results in the expression of the E2F3 protein. The decrease in the concentration of E2F3 protein led to the decrease in the cyclin A protein in CE146T cancer cells. In summary, the results designate that lncRNA NEAT1 regulates esophageal cancer growth and proliferation through its molecular interaction with miR-377 and regulatory effects are fine-tuned through the miR-377/E2F3 molecular signaling axis. However, studies that involve more cell lines and *in vivo* systems should be carried out to establish NEAT1 as a therapeutic target. The effects of already established drugs on the expression of NEAT1 need to be determined. Furthermore, chemotherapeutic drugs that can alter the expression of NEAT1 in esophageal cancer cells need to be developed.

CONCLUSION

Taken together, the results of the present study showed that NEAT1 is up-regulated in human esophageal cancer. Silencing of NEAT1 inhibits the proliferation, migration, and invasion of esophageal cancer cells *via* the miR-377/E2F3 axis. The results highlight the potential of NEAT1 in the management of esophageal cancer.

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