Long non-coding RNA NEAT1 promotes malignant progression of thyroid carcinoma by regulating miRNA-214

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Abstract. The long non-coding RNA nuclear enrich abundant transcript 1 (NEAT1) has been identified to be carcinogenic in various cancers and elevated NEAT1 expression was tightly linked to tumorigenesis and progression. However, the mechanism has not been revealed in progression of thyroid cancer. Tumor xenograft mouse model was established and tumor size was evaluated. Arg-1, NEAT1 and miR-214 expression in CBMs, TAMs, BMDMs and RAW 264.7 cell lines were detected. TPC-1 cells were subjected to si-NEAT1 transfection in vitro for cell viability study. A direct target of miRNA-214 (β-catenin) was assessed, cell survival and invasion in TAMs were investigated. NEAT1, Arg-1 was highly expressed and miRNA-214 had lower expression in patients with thyroid cancer. NEAT1 knockout inhibited thyroid cancer cell survival, migration and invasion, along with reduced β-catenin (a direct target of miRNA-214) protein expression. Furthermore, NEAT1 significantly accelerated thyroid cancer cell growth and metastasis in vitro and increased tumor size in vivo. Upregulation of NEAT1 decreased the expression of miRNA-214, presenting a reciprocal repression correlation. In conclusion, these results suggest that high expression of NEAT1 promoted the onset of thyroid carcinoma. In addition, NEAT1 promoted the malignant progression of thyroid cancer through regulating miRNA-214 expression, which adds to our understanding of the molecular mechanisms in thyroid carcinoma.

Introduction

With the development of modern industry, thyroid cancer is becoming the most common endocrine malignancy, and its incidence has increased by nearly 3-fold during the past several years (1-4). Papillary thyroid cancer (PTC) accounts for 85-90% of the predominant histological subtypes, and >90% of patients have a 10 year survival rate (5,6). Although the overall prognosis is good, 15-30% PTC patients will have persistence or recurrence and 5-10% will suffer progressive, distant metastasis, for whom 5 years of survival is only less than half (7). As for these patients, the available adjunctive therapies currently are often of limited benefit (5). Therefore, it is urgent to discover the molecular mechanisms by which PTC is maintained and provide a new focus for the development of PTC treatments.

The mechanisms underlying aggressive PTC continue to be elucidated and may include tumor-mediated immune suppression (8). Nowadays, immunotherapy has gradually become a new therapeutic approach for patients with recurrent or progressive PTC. There are studies showing that the level of immunosuppressive molecules and specific patterns of tumor infiltrating leukocytes (TIL) have predicted tumorigenesis and recurrence in some solid malignancies (9-11). In addition, immunotherapy regimens such as programmed cell death 1 ligand 1 (PD-L1) and IL-2 have induced remarkable durable cancer regressions in patients with metastatic diseases, including lung, prostate, and renal cell cancers (12,13). It is now established that host immune cells can recognize and eliminate malignant cells, but that abnormal antigens present in tumors, neoplastic growths frequently evolve mechanisms to escape immune destruction, including downregulation of antigen recognition, the expression of immune-inhibitory ligands, and the recruitment of suppressor cell populations (11,14,15). In order to effectively treat cancer, immunotherapy could reverse the tumor-driven immune dysfunction, further restore immune responses and bring antigen specific reaction.

Long non-coding RNAs (lncRNAs) are a class of RNAs that do not encode proteins, with length of >200 nt (16-18). They are widely distributed in a variety of human tissues and play an important role in physical processes, with increasing evidence revealing that they are involved in cell-type feature and diverse cellular events, including epigenetic regulation, gene transcription and mRNA processing (19). LncRNAs are ubiquitous disorders in cancers, especially resulting in aberrant proliferation, migration and invasion, and apoptosis (20), which contribute to the advance of human tumors.
and tumor outcomes (21-23). As a kind of lncRNA, IncRNA nuclear enrich abundant transcript 1 (NEAT1), a 4-kb lncRNA localized to the nucleus (24), serves as a crucial architectural component of a paraspeckle structure (25-27). NEAT1 has been demonstrated to act as a key role in various cancers, including acute promyelocytic leukemia cells (28), prostate cancer (29), breast cancer (30), hepatocellular carcinoma (31), ovarian carcinoma (32) and glioma (33). However, whether NEAT1 is associated with the malignant progression of PTC remains unclear.

MicroRNAs (miRNAs, ~22 nt) are a group of small non-coding RNAs with aberrant expression in various tumors (34), miRNAs, as a major class of well characterized, conserved and endogenous small interfering RNA that regulate gene expression (35). It has been indicated that miRNAs are involved in diverse biological processes, such as cell growth, migration, apoptosis and differentiation through mainly binding to the 3'-untranslated region (3'-UTR) of their target genes (34,36). In addition, miRNAs can function as either oncogenes or tumor suppressor genes via regulation of cell proliferation or cell death (37). miRNA-214 also plays a vital role as a tumor inhibitor by downregulating oncogenes, such as GALNT7, Bcl2l2 and TFAM in cervical cancer (38-40) and the NEAT1 overexpression (Ad-NEAT1) plasmid, as well as the respective non-targeting sequence (negative control, si-control and Ad-GFP) were synthesized by GenePharma Co. (Shanghai, China). Cells were transient transfected through the use of Lipofectamine 2000 transfection reagent (Life Technologies Corp., Shanghai, China) according to the manufacturer's instructions when cells were at 50-70% confluence. The transfection efficiency was verified by qRT-PCR analysis.

Cell transfection. The NEAT1 knockdown (si-NEAT1) plasmid and the NEAT1 overexpression (Ad-NEAT1) plasmid, as well as the respective non-targeting sequence (negative control, si-control and Ad-GFP) were synthesized by GenePharma Co. (Shanghai, China). Cells were transient transfected through the use of Lipofectamine 2000 transfection reagent (Life Technologies Corp., Shanghai, China) according to the manufacturer's instructions when cells were at 50-70% confluence. The transfection efficiency was verified by qRT-PCR analysis.

RNA extraction and qRT-PCR. Total RNA was extracted from clinical specimens and cells with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A One-Step SYBR Prime Script RT-PCR Kit was used for qRT-PCR. cDNA was generated from miRNA with a TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). TaqMan Universal Master Mix II was used to perform TaqMan miRNA assays for target genes on the ABI 7500 Fast Real-Time PCR system (Applied Biosystems). GAPDH and U6 were used as endogenous controls, respectively. The relative expression level was calculated using the 2-ΔΔCt method. The formula and its derivations were obtained from the ABI Prism 7300 sequence detection system user guide. Statistical analysis was performed on the fold-change.

Cell survival and viability assay. After transfection efficacy was confirmed, cells were dissociated with Accutase® Cell Dissociation buffer (Life Technologies Corp.) resuspended and seeded in 96-well micro-hole plates at 1.0×10³ cells/well. Volume of 10 μl CCK-8 was added to each well at the time of harvest, according to the manufacturer's instructions. The plate was incubated for 2 h at 37°C and then recorded cell survival and viability by measuring the absorbance of the converted dye at 450 nm on a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Cell migration and invasion assay. To measure cell migration, 8-mm pore size culture inserts (Transwell; Costar, Cambridge, UK) were placed into the 24-well culture plates, separating
the upper and the lower chambers. In the lower chamber, DMEM containing 10% FBS was added. Serum-free medium containing 5x10^4 cells were placed to the upper chamber for migration and invasion assay. After incubation at 37˚C for 48 h, the cells on the upper membrane surface were scraped off. The cells on the lower side of the member were fixed and then stained with 0.1% crystal violet. The number of cells that had migrated through the pores was quantified by counting 10 independent visual fields under the microscope for statistics. At magnification x20. Each experiment was performed at least 3 times.

RNA pull-down assay. NEAT1 and its antisense RNA were in vitro transcribed and biotin-labeled using a biotin RNA labeling mix and T7/SP6 RNA polymerase, treated with RNase-free DNase I and purified using an RNeasy mini kit (Qiagen, Valencia, CA, USA) to detect Argonaute 2 (Ago2) expression by western blotting. All these procedures were according to the manufacturer's protocol. One milligram of protein from cell extracts was mixed with 50 pmol biotin labeled RNA, incubated with streptavidin-agarose beads, and washed 3 times with NaCl/Pi at room temperature. The retrieved proteins were detected using a standard western blotting technique.

Tumor xenografts. Four-week-old nude C57BL/6 mice were purchased from Henan Research Center of Laboratory Animals (Zhengzhou, China). These mice were given free access to sterile food and water during the experiment process. All animal experiments with nude mice were performed strictly in accordance with a protocol approved by Henan Research Center of Laboratory Animal. To establish thyroid cancer xenograft model. The mice were subcutaneously injected with 5x10^4 TPC-1 cells (n=6 in each group). After 8 days of transplantation, the transplanted nude mice were divided into two groups: si-control, and si-NEAT1. Si-control or si-NEAT1 was directly injected into the implanted tumors per mouse every 4 days for 4 times. The subcutaneous tumor-bearing mice were sacrificed 2 weeks after injection. The tumor volume was calculated by the formula: volume (mm^3) = length x width^2/2.

Statistical analysis. Data are presented as the mean ± standard deviation (SD). All experimental results were statistically analyzed with Student's t-test or one-way analysis of variance (ANOVA). All statistical analyses were performed with SPSS 18.0 statistical software. A value of P<0.05 was considered to indicate statistically significant difference.

Results

NEAT1 is upregulated while miR-214 is downregulated in thyroid cancer cell TAMs. As previously reported, NEAT1 was upregulated in glioblastoma tissues (46). Similarly, we found NEAT1 was upregulated in TAM thyroid cancer cell line TAMs compared with CBMs (Fig. 1B). In addition, quantitative real-time PCR (qRT-PCR) was conducted to determine the expression of Arg-1 and miR-214 in the two cell types TAM and CBM. Arg-1 expression was significantly upregulated in TAM cells, however, miR-214 expression was significantly lower in TAM cell lines as compared with those of CBMs (Fig. 1A and C). These results suggested that NEAT1 promotes the occurrence of tumor in TAMs, while miR-214 functions as a tumor suppressor.

NEAT1 is upregulated while miR-214 is downregulated in BMDMs treated with IL-4. To further explore the oncogenic properties and roles of NEAT1 on thyroid cancer in vitro, we established thyroid cancer cell lines (BMDMs treated with 20 ng/ml IL-4), and detected the mRNA expression of Arg-1, NEAT1 and miR-214 genes at 3, 6, and 12 h, respectively. In this study, we found that the relative Arg-1 mRNA level was significantly increased after 12 h of treatment in thyroid cancer cell lines (Fig. 2A). Moreover, qRT-PCR analysis for the expression of NEAT1 showed that NEAT1 was obviously increased in BMDM cells at 3, 6, and 12 h (Fig. 2B). On the contrary, miR-214 gene expression was significantly decreased with 6 and 12 h treatment (Fig. 2C). The change of the above three parameters all showed a time-dependent manner. These data demonstrate that the upregulation of NEAT1 may play important roles on thyroid cancer development and progression.

Transfection with si-NEAT1/Ad-NEAT1 attenuates/increases IL-4 induced Arg-1 expression in BMDMs and RAW 264.7 cells. The expression of Arg-1 was assessed by qRT-PCR. As mentioned above, NEAT1 expression was upregulated under
IL-4 treatment, enforced NEAT1 silence was firstly performed and analyzed by transfecting si-NEAT1 that targets NEAT1 transcript into BMDMs and RAW 264.7 cells. Analysis for Arg-1 gene level demonstrated to be strongly attenuated in NEAT1 silencing thyroid cancer cell lines close to a basal expression level (Fig. 3A). Moreover, thyroid cancer cell lines were transfected with Ad-NEAT1 to overexpress NEAT1. The expression of Arg-1 was markedly promoted in BMDMs and RAW 264.7 cells transfected with Ad-NEAT1 compared to that in the control group (Fig. 3B).
Transfection with si-NEAT1/Ad-NEAT1 reverses/decreases IL-4 induced miR-214 and β-catenin expression in BMDMs and RAW 264.7 cells. Similarly, in order to determine the effect of si-NEAT1/Ad-NEAT1 on the expression of miR-214 and β-catenin, we divided cells into three groups: si-control group (transfected with si-control plasmid), si-NEAT1 group (transfected with si-control plasmid and the si-NEAT1 plasmid) and the IL-4 group (transfected with si-control plasmid and 20 ng/ml IL-4). The qRT-PCR assay indicated that miR-214 gene level was reduced in IL-4 group, while it was obviously reversed in NEAT1 silencing BMDMs and RAW 264.7 cells. β-catenin, as the direct target molecule of miR-214, was also determined by western blotting. The protein level of β-catenin was remarkably downregulated in BMDMs and RAW 264.7 cells transfected with si-NEAT1 when compared with IL-4 group (Fig. 4A). BMDMs and RAW 264.7 were simultaneously transfected with the Ad-NEAT1 plasmid, the change of miR-214 level and β-catenin expression was analyzed accordingly, opposite results were observed in treated groups (Fig. 4B).

NEAT1 is a direct target of miR-214 in thyroid cancer cell lines. Increasing evidence has demonstrated that lncRNAs could act as a molecular sponge or a competition endogenous RNA (ceRNA) in regulating the accumulation of miRNA and in turn affecting its biological functions. Using a bioinformatics database (StarBase), we determined that NEAT1 harbors seven putative binding sites for miR-214 (Fig. 5A). Furthermore, an RNA pull-down experiment was conducted to determine whether NEAT1 and Ago2 were in the expected RNA-induced silencing complex (RISC). A significant enrichment was found of Ago2 in the presence of NEAT1 compared with antisense RNA (negative control) (Fig. 5B). In addition, the miR-214 gene expression was remarkably promoted in NEAT1 interfered group compared with antisense RNA group (Fig. 5C). These results revealed miR-214 could directly bind to NEAT1 at the miRNA recognition site.

Knockdown of NEAT1 impairs the malignant progression of thyroid papillary carcinoma-1. To investigate whether the NEAT1 silencing can block cell survival and invasion, we used two different approaches to evaluate the role of NEAT1 on TPC-1 cell malignant progression. The CCK-8 assay indicated that TPC-1 survival was lower in the si-NEAT1 group than in the si-control group (Fig. 6A). We also evaluated cancer cell migration and invasion through Transwell assays. The migration and invasion of TPC-1 were also significantly impeded in the NEAT1-knockdown group compared to the si-control group (Fig. 6B). Moreover, the inhibition of NEAT1 had no significant effect on the cell viability of TPC-1 cells between si-NEAT1 and si-control groups (Fig. 7). These results indicated that NEAT1 may act as a oncogene in TPC-1 cells.

Knockdown of NEAT1 inhibits thyroid tumor growth in vivo. To verify the effects of NEAT1 on tumorigenesis in vivo, TPC-1 cells or appropriate control cells were subcutaneously
injected into nude mice. Si-NEAT1 was directly injected into the implanted tumor at the 8th day after injection every 4 days. Knockout of NEAT1 significantly decreased tumor size and tumor growth in vivo compared with si-control group (Fig. 8A). Using qRT-PCR analysis, we confirmed the NEAT1 knockout in the xenograft tumors generated from thyroid cancer TAM cells (Fig. 8B). Taken together, these results demonstrated that NEAT1 plays a crucial role in thyroid cancer TAM cell progression.

**Discussion**

Previous studies have explored the functional roles of lncRNAs (18,47,48), and indicated new insights into the underlying molecular mechanisms by which lncRNAs take effect in various human cancers (49-51). However, the mechanisms of NEAT1 in thyroid cancer have not been thoroughly revealed. In the present study, we provided new evidence that highly expressed NEAT1 in thyroid cancer cells acted as an oncogenic
biomarker. NEAT1 was found to be a direct target of miR-214 and there was a negative correlation between them. NEAT1 served as an oncogene to promote tumor process partially due to its ability to depress the expression of miR-214. Hence, our findings may help to improve the literature supporting the importance of lncRNA species in cancer treatment.

In this study, we demonstrated that NEAT1 was upregulated in thyroid cancer cells. NEAT1 inhibition impaired the malignant process of thyroid papillary carcinoma-1 and attenuated β-catenin expression. On the contrary, miR-214 expression was downregulated in thyroid cancer TAMs, BMDMs and RAW 264.7 cells. Moreover, miR-214 was found to bind to NEAT1 in a specific manner, and showed a reciprocal repression correlation. β-catenin was considered to be a direct target of miR-214 and was involved in the NEAT1-induced malignant behavior of thyroid cancer. Collectively, our observations indicated NEAT1 may serve as an oncogene and play an important role in thyroid cancer initiation, development and progression.

Emerging evidence presented that lncRNAs are abnormally expressed in various cancers (46). Because they are involved in the occurrence and progression of cancer, lncRNAs could be used as diagnostic or prognostic markers and potential therapeutic targets. Previous study indicated that NEAT1 was upregulated in glioma, and promoted cell proliferation, migration and invasion while inhibiting apoptosis in glioma cell lines (33). Similar findings were observed in this study, knockout NEAT1 suppressed the malignant progression of thyroid papillary carcinoma-1 cells. In addition, we also further determined the expression of β-catenin and found that NEAT1 knockout significantly reduced β-catenin level in thyroid cancer cells. However, whether β-catenin is also involved in the NEAT1-induced enhancement of thyroid cancer progression needs to be further investigated.

So far, NEAT1 has been considered to act as a cancer inducing gene, but the deep mechanism by which NEAT1-regulated gene expression remains to be clarified (52). It has been demonstrated that NEAT1 played an important role in numerous biological processes, including cellular differentiation and stress response through paraspeckles pathway (53). Evaluation of the effect of NEAT1 on transcriptional regulation by sequestering SFPQ from the RNA-specific adenosine deaminase, RNA specific B2 (ADARB2) gene in response to proteasome inhibition effect has been reported (54). In the present study, we intended to discover another underlying molecular mechanism of NEAT1 on thyroid cancer progression, that is, functioning as ‘molecular sponges’ to regulate miRNAs. A previous study proved that in a variety of cell processes, lncRNAs played a vital role through acting as ceRNAs to mediate the miRNAs (17). Various lncRNAs have been investigated in cancer research, including lncRNA GAS5 (54) and CCAT1 (55). In this study, we evaluated the effect of NEAT1 on thyroid cancer cells and discovered that NEAT1 participated in the development of ceRNA regulatory networks and acted as an endogenous miRNA sponge, binding to miR-214 and regulating its function. There are studies indicating miR-214 tumor inhibitory effect in various acute or chronic diseases. Study of Xiong et al found that down-regulation of miR-214 induced G1 cell cycle arrest in gastric cancer cells by upregulating PTEN (56). Yang et al reported that miR-214 induces cell survival and cisplatin resistance through targeting the 3′-UTR of PTEN (45). miR-214 was downregulated in cervical cancer tissue and could negatively regulate HeLa cell growth (38,57), while its role on thyroid cancer cells have not been investigated. In this experiment, we found that miR-214 expression was low in thyroid cancer cells.

Figure 8. NEAT1 silencing impairs the malignant progression of thyroid carcinoma in vivo. (A) Tumor size of subcutaneous implantation models of TPC-1 cells. (B) Relative NEAT1 expression in tumors isolated from si-control and si-NEAT1 groups. *P<0.01. Means ± SD are shown. Statistical analysis was conducted using Student’s t-test.
In addition, the expression of NEAT1 and miR-214 showed a significantly negative correlation in thyroid cancer cell lines. Knockout of NEAT1 remarkably increased miR-214 expression, while overexpression of NEAT1 decreased its expression. Moreover, a pull-down assay showed NEAT1 could pull-down miR-214.

In conclusion, our results indicate that highly expressed NEAT1 is an oncogenic IncRNA that promotes tumorigenesis and progression of thyroid cancer through regulating the expression of miR-214. The significance of the correlation among NEAT1, miR-214 and thyroid cancer was highlighted for the first time. Thus, NEAT1 could be a useful marker and the combination of NEAT1/miR-214 may be a promising option for the treatment of human thyroid cancer.

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References


