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Long non-coding RNA NEAT1 regulates Hodgkin's lymphoma cell proliferation and invasion *via* miR-448 mediated regulation of DCLK1

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Abstract. – OBJECTIVE: To explore whether long non-coding RNA nuclear enriched abundant transcript 1 (lncRNA NEAT1) could regulate Hodgkin's lymphoma (HL) cell proliferation and invasion through miR-448, which could target doublecortin like kinase 1 (DCLK1) and mediate DCLK1 expression.

PATIENTS AND METHODS: Expressions of NEAT1, miR-448 and DCLK1 were evaluated by qRT-PCR or Western blot assay. Cell Counting Kit-8 (CCK-8) and transwell assay were utilized to detect cell proliferation and invasion capability in L428 cells respectively. The target relationship between NEAT1, miR-448 and DCLK1 was confirmed by Luciferase reporter assay.

RESULTS: QRT-PCR results showed that NEAT1 expressed higher in HL tissues and cell lines than that in controls. *In vitro* experiments, NEAT1 downregulation could decrease cell proliferation and invasion capability in L428 cells. NEAT1 directly interacted with miR-448 and negatively regulated it. Moreover, DCLK1 was confirmed as a target of miR-448. DCLK1 expression was increased in L428 cells and positively regulated by NEAT1. NEAT1 overexpression upregulated the protein level of DCLK1 in L428 cells according to Western blot analysis. Additionally, DCLK1 overexpression could reverse the suppression on cell proliferation and invasion capability induced by NEAT1 knockdown or miR-448 overexpression.

CONCLUSIONS: NEAT1 might be contributed to HL progression by promoting cell proliferation and invasion capability *via* miR-448 mediated DCLK1 expression.

Key Words:
NEAT1, MiR-448, DCLK1, Hodgkin's lymphoma.

Introduction

Hodgkin's lymphoma (HL) is the most common malignant lymphoma originating in the lymphoid hematopoietic system, especially in young adults. HL is divided into nodular lymphocyte-predominant Hodgkin's lymphoma (NLPHL) and classical Hodgkin's lymphoma (cHL) according to World Health Organisation (WHO) classification[1,2]. The current treatment methods of HL patients mainly include radiotherapy, surgical excision, chemotherapy and bone marrow transplant[2]. Most HL patients have a good prognosis through those current treatments, especially the young and middle-aged people[2]. But the underlying mechanisms of HL still need to further explore to find new targets for better prediction and treatment of HL patients.

Long non-coding RNAs (lncRNAs) are a class of RNA molecules with a length of more than 200 nucleotides which have no protein coding ability and involved in the regulation of various intracellular processes[3]. LncRNAs are dysregulated in cancers as oncogenes or tumor suppressors and associated with the progression and pathogenesis of cancer[4]. There is no doubt that lncRNAs have

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become novel research focus in tumorigenesis and therapeutic approaches after miRNAs although the specific regulatory mechanisms of most lncRNAs have not been confirmed.

Among cancer-related lncRNAs, Nuclear Enriched Abundant Transcript 1 (NEAT1) generally serves oncogenic role in most human malignancies[5]. NEAT1 has been demonstrated to be involved in the carcinogenesis by regulating cancer-related pathways (Akt or Wnt signaling pathways) in several cancers[6]. Besides, NEAT1 could function as a competing endogenous RNA (ceRNA) to inhibit specific tumor suppressor miRNAs and further positively regulated miRNAs target genes[6]. The upregulation of NEAT1 was bound up with poor prognosis, thus it was considered to be a convinced adverse prognostic factor in several cancers[7]. However, the regulatory mechanism of NEAT1 related miRNAs and target genes in HL cells remain unclear and deserve further research. Therefore, the present study was designed to probe into the detailed molecular mechanism for NEAT1 in HL cells.

Patients and Methods

Tissue Samples and Cell Lines

20 cases HL tissues and reactive lymphadenopathy (RL) samples were obtained from the Qingdao Hospital of Traditional Chinese Medicine between March 2015 and April 2018 respectively. Germinal center (GC)-B cells were sorted from tonsil tissue samples of three HL donors aged between 2 and 9 years. GC-B cells were purified from human tonsil tissues based on the expression of $CD20^{+}lgD^{-}CD38^{+}$ or $IgD^{-}CD138^{-}CD3^{-}CD10^{+}$ as previously described[8,9]. Inclusion criteria: (1) all pathological diagnoses were HL; (2) the clinical data completed and could cooperate with the completion of this study. Exclusion criteria: (1) people with severe heart, liver or kidney dysfunction; (2) patients undergoing related treatment before diagnosis; (3) patients with other malignant tumors; (4) people with mental or mental disorders who could not cooperate with treatment. This investigation was approved by the Ethics Committee of Qingdao Hospital of Traditional Chinese Medicine. Signed written informed consents were obtained from all participants before the study.

L1236 (CSC-C0538), KM-H2 (CSC-C0104), L428 (CSC-C0322) and L540 (CSC-C0233) HL cell lines were obtained from Creative Bioarray (Shirley, NY, USA). Then, all cells were cultured in the Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) which contained 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, and incubated at 37°C with 5% CO_2 humidified atmosphere.

Cell Transfection

siNEAT1 (small interfering RNA targeting NEAT1), miR-448 mimics and their corresponding controls were designed and synthesized from GenePharma company (Shanghai, China). The pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) was devoted to construct NEAT1 or DCLK1 overexpression plasmid (opNEAT1 or opDCLK1). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was applied to L428 cells transfection according to the requirements.

Cell Counting Kit-8 (CCK-8) Assays

Cell proliferation was detected by CCK8 assays. Cells were cultured in the 96well plate. At 0, 24, 48, 72 h, each well was added 10 μL CCK-8 reagents (Dojindo, Molecular Technologies, Kumamoto, Japan) followed by manufacturer's protocol and being cultured 2 h at 37°C. Then, the absorbance value at 450 nm was recorded by a microplate reader.

Transwell Assay

Transwell assay (Corning, Corning, NY, USA) was performed to assess the invasion capability of L428 cells transfected with plasmids and/or oligonucleotides. Briefly, cell density of each group was adjusted with serum-free RPMI-1640 culture medium, and the upper chamber inserted with 200 μL cell suspension, while 400 μL RPMI-1640 culture medium containing 20% FBS medium was added to the lower chamber. After incubation for 48 h at 37°C, cells migrated into the lower chamber were fixed (4% paraformaldehyde), and stained (0.1% crystal violet). Cells invasion capability was represented by the mean value of 5 random selected different fields.

Dual-Luciferase Activity Assay

The potential NEAT1-miRNA interactions and the potential miR-448 targets were searched by the online prediction databases StarBase 3.0 (http://starbase.sysu.edu.cn/) and TargetScan (www.targetscan.org/) respectively. Luciferase reporter vector of NEAT1 and DCLK1 [wildtype (wt) or mutant-type (mut)] containing the miR-

448 binding sites were amplified and ligated into the pGL3 vector (Promega, Madison, WI, USA). Reporter plasmids (NEAT1-wt, NEAT1-mut, DCLK1-wt or DCLK1-mut) along with miR-448 mimic or NC were cotransfected into L428 cells. Luciferase activity was examined by the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA) after transfection 48 h.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribe RNA was performed by reverse transcription kit (TaKaRa, Dalian, China). QRT-PCR was performed on ABI 7500 fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using the SYBR-Green RT-PCR kit (TaKaRa, Dalian, China). The expression levels of NEAT1, miR-448 and DCLK1 were analyzed using the $2^{-\Delta\Delta CT}$ method and glyceraldehyde phosphate dehydrogenase (GAPDH) or U6 was used as an endogenous control. All primers were listed in Table I.

Western Blot

Cell total proteins were extracted by radioimmunoprecipitation assay (RIPA) lysis buffer with fresh protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO, USA). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate proteins. Polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) which transferred with the protein bands were incubated with primary antibodies (DCLK1 and GAPDH antibodies, 1:1,000; Cell Signaling, Danvers, MA, USA) after blocked with 5% non-fat skim milk, and then, probed with horse radish peroxidase (HRP)conjugated secondary antibody (1:5,000; Abcam, Cambridge, MA, USA). The protein bands were visualized by the enhanced chemiluminescence (ECL) prime kit (Thermo Fisher Scientific, Waltham, MA, USA) with Image Lab analysis software (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA). Data were represented as mean ± Standard Deviation (SD). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ indicated statistical significance.

Results

NEAT1 Expression Was Upregulated In HL

The expression of NEAT1 in HL tissues and cells was investigated. The analysis of qRT-PCR revealed that NEAT1 expression was upregulated in HL tissues versus RL tissue (Figure 1A). Consistently, we found that NEAT1 expression was also increased in HL cells (L1236, L540, L428 and KM-H2) compared with GC-B cells (Figure 1B). Data proposed that NEAT1 was upregulated in HL.

NEAT1 Knockdown Suppressed L428 Cell Proliferation and Invasion

To explore the function of NEAT1 on cell proliferation and invasion, L428 cells with NEAT1 highest expression was selected to transfect with

Table I. Primer sequences for real-time fluorescence quantification PCR.

Gene	Primer sequences
GAPDH	Forward primer: 5′-ACGCTGCATGTGTCCTTAG-3′ Reverse primer: 5′-GAGCCTCTTATAGCTGTTTG-3′
U6	Forward primer: 5′-CTCGCTTCGGCAGCACA-3′ Reverse primer: 5′-AACGCTTCACGAATTTGCGT-3′
lncRNA NEAT1	Forward primer: 5′-TGGCTAGCTCAGGGCTTCAG-3′ Reverse primer: 5′-TCTCCTTGCCAAGCTTCCTTC-3′
miR-448	Forward primer: 5′-TTATTGCGATGTGTTCCTTATG-3′ Reverse primer: 5′-ATGCATGCCACGGGCATATACACT-3′
DCLK1	Forward primer: 5′-TGAAGGGTACGCTCCTCAGT-3′ Reverse primer: 5′-GCTACACTCTGACCGCATGA-3′

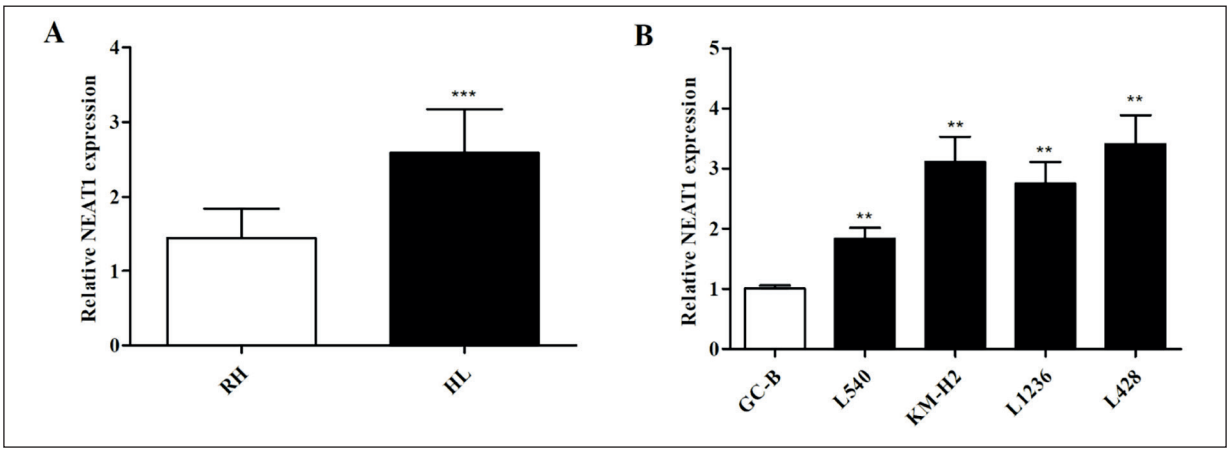

Figure 1. Expressions of NEAT1 was examined in HL tissues and cell lines. **A,** NEAT1 expression was determined by qRT-PCR in HL tissues. **B,** The expression of NEAT1 was examined by qRT-PCR in HL cell lines. $^{**}p<0.01$, $^{***}p<0.001$, compared with RL tissues or GC-B cells.

siNEAT1. The results found that NEAT1 expression was conspicuously lower in cells transfected with siNEAT1 than siNC (Figure 2A). In CCK-8 assay, compared with siNC group, NEAT1 knockdown caused a significant suppression of L428 cell proliferation (Figure 2B). Besides, cell invasion was significantly suppressed in L428 cells transfected with siNEAT1 according to the transwell assay results (Figure 2C). Together, we proposed that NEAT1 has a great relationship to cell proliferation and invasion capability of L428 cells.

NEAT1 Directly Interacted With miR-448 In L428 Cells

To explore the ceRNA regulator mechanisms of NEAT1, StarBase v3.0 database was used to predict potential NEAT1-miRNA interactions. It turns out that miR-448 could bind to NEAT1 and the binding sites for NEAT1 were shown in Figure 3A. To confirm the prediction, NEAT1-wt or NEAT1-mut plasmid were transfected into L428 along with mimics NC or miR-448 mimics. The results revealed that miR-448 mimics could significantly reduce the Luciferase activity of the NEAT1-wt while there was no change in the NEAT1-mut (Figure 3B). Furthermore, expression of miR-448 in L428 cells was detected after NEAT1 knockdown, and we found that NEAT1 knockdown led to a significant increasing of miR-448 expression (Figure 3C). Finally, compared with the expression in GC-B cells, miR-448 exhibited a decreasing trend in L428 cells (Figure 3D). We proposed that NEAT1 could interact with miR-448 and negatively regulate it in L428 cells.

DCLK1 Directly Targets MiR-448 and Regulated By NEAT1 In L428 Cells

To investigate whether the regulatory mechanism of NEAT1 in L428 cells was associated with its regulating of miR-448 target genes, we explored the TargetScan database to predict miR-448 targets. DCLK1 was selected and the target sites with miR-448 was shown in Figure 4A. After bioinformatics analysis, DCLK1-wt and DCLK1-mut plasmids containing binding cites with miR-448 were constructed, and transfected into L428 with miR-448 mimics or mimics NC. Luciferase activity was significantly inhibited in the cells co-transfected with DCLK1-wt and miR-448 mimics compared with mimics NC and DCLK1-wt co-transfection, while there was no effect in cells co-transfected with DCLK1-mut and miR-448 mimics or mimics NC (Figure 4B). In addition, it was demonstrated that DCLK1 expression was higher in L428 cells than that in GC-B cells (Figure 4C and 4D). We also found that overexpression of miR-448 caused a significant reduction of DCLK1 protein level (Figure 4E). Therefore, data indicated that DCLK1 was a target of miR-448 and negatively regulated by miR-448.

Meanwhile, the protein level of DCLK1 was analyzed by Western blot in L428 cells when miR-448 and NEAT1 overexpressed. We found that NEAT1 overexpression significantly abrogated the decrease expression of DCLK1 induced by miR-448 mimics (Figure 4E). These findings suggested that NEAT1 may regulate the expression of DCLK1 by sponging miR-448.

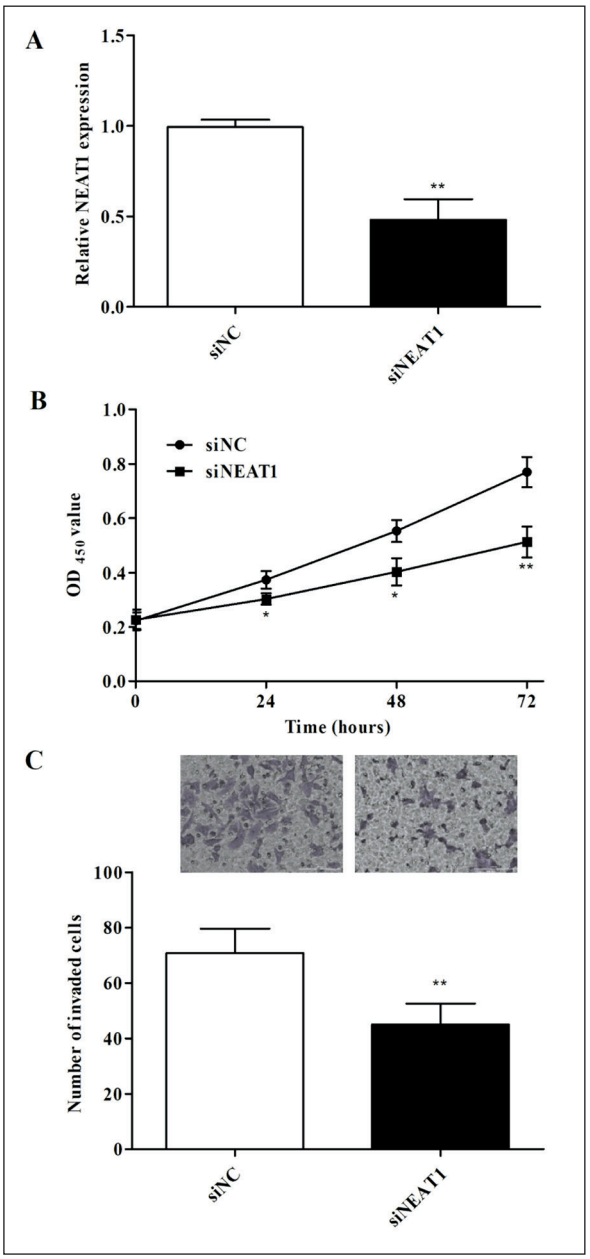

Figure 2. NEAT1 knockdown inhibited L428 cell proliferation and invasion. **A,** NEAT1 expression after cells transfected with siNEAT1 or siNC. **B,** Cell proliferation of L428 cells after NEAT1 knockdown. **C,** Cell invasion capability of L428 cells after NEAT1 knockdown (200×). *p<0.05, **p<0.01, compared with siNC group.

NEAT1/MiR-448/DCLK1 Axis Regulated L428 Cell Proliferation and Invasion

On the basis of above experiment results, we speculated that NEAT1/miR-448/DCLK1 regulatory network may be associated to HL development. To validate our hypothesis, CCK-8 and transwell assays were carried out to determine cell proliferation and invasion capability in L428 cells transfected siNEAT1 or miR-448 mimic in combination with opDCLK1. As expected, suppressed cell proliferation (Figure 5A and 5B) and invasiveness (Figure 5C and 5D) in L428 cells were observed after NEAT1 knockdown or miR-448 overexpression, while the inhibition was notably abrogated by DCLK1 overexpression (Figure 5). As aforementioned, it suggested that NEAT1 may contribute to HL progression by promoting cell proliferation and invasion capability through miR-448 mediated regulation of DCLK1.

Discussion

LncRNAs served as important regulators and biomarkers in most type human cancers. Although NEAT1 is reported to be a promoting role in several cancers progression[5,10], the specific potential mechanism of NEAT1 in HL cell proliferation and invasion capability remains unclear. Our study validated that NEAT1 was observably upregulated in HL tissues and cells compared to control group. In previous studies, NEAT1 expression was significantly increased in some cancers. In breast cancer (BC), NEAT1 was notably overexpressed in BC cell lines and tissues, and NEAT1 knockdown inhibited cell growth and invasion capability[11]. Expression of NEAT1 was signally enhanced and downregulation of NEAT1 suppressed cells migration and invasion in glioma[12]. NEAT1 expression was distinctly increased in colorectal cancer, and promoted cell proliferation by activating Akt signaling[13]. Our results were consistent with these previous studies. In the meanwhile, reducing NEAT1 expression was significantly decreased the proliferation and invasion capability in L428 cells.

NEAT1 could function as a ceRNA to directly interact with some miRNAs, thereby regulating target genes of miRNA. NEAT1 participated in the non-small cell lung cancer progression by acting as a ceRNA of miR-377-3p and further positively modulating E2F3 expression[14]. NEAT1 functioned as a ceRNA to competitively bind to miR-133a and positively regulate SOX4 expression in promoting cervical cancer progression[15]. In ovarian cancer, NEAT1 overexpression could promote cell proliferation and invasion by sponging miR-34a-5p to modulate BCL2 expression[16]. Moreover, NEAT1 promoted glioma development by regulating CDK14

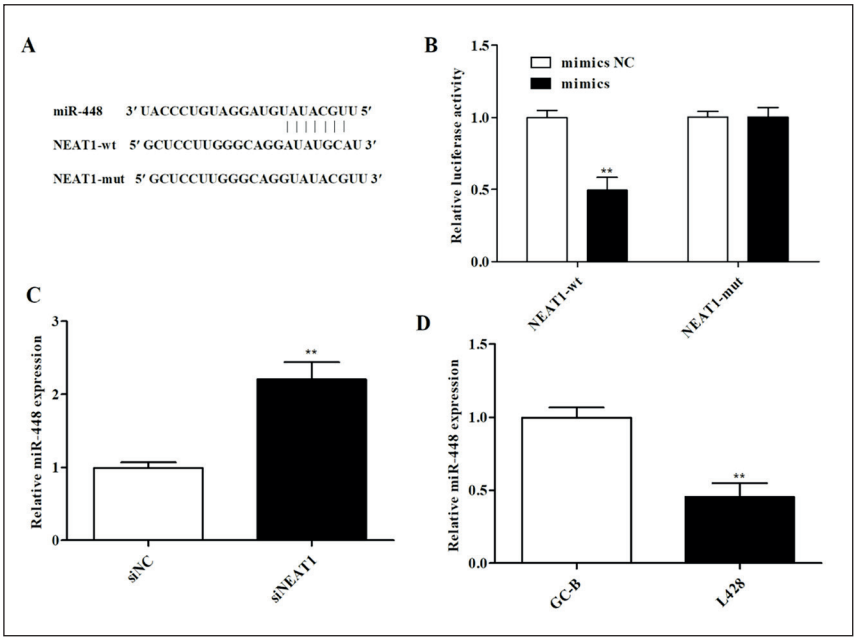

Figure 3. NEAT1 directly interacted with miR-448. **A,** Sequence alignment of miR-448 with the putative binding sites with NEAT1. **B,** Targeting relationship between NEAT1 and miR-448 in L428 cells was confirmed by dual-luciferase reporter assay. **C,** MiR-448 expression in L428 cells transfected with siNEAT1 or siNC. **D,** MiR-448 expression in L428 cells. **$p<0.01$, compared with mimics NC, siNC or GC-B group.

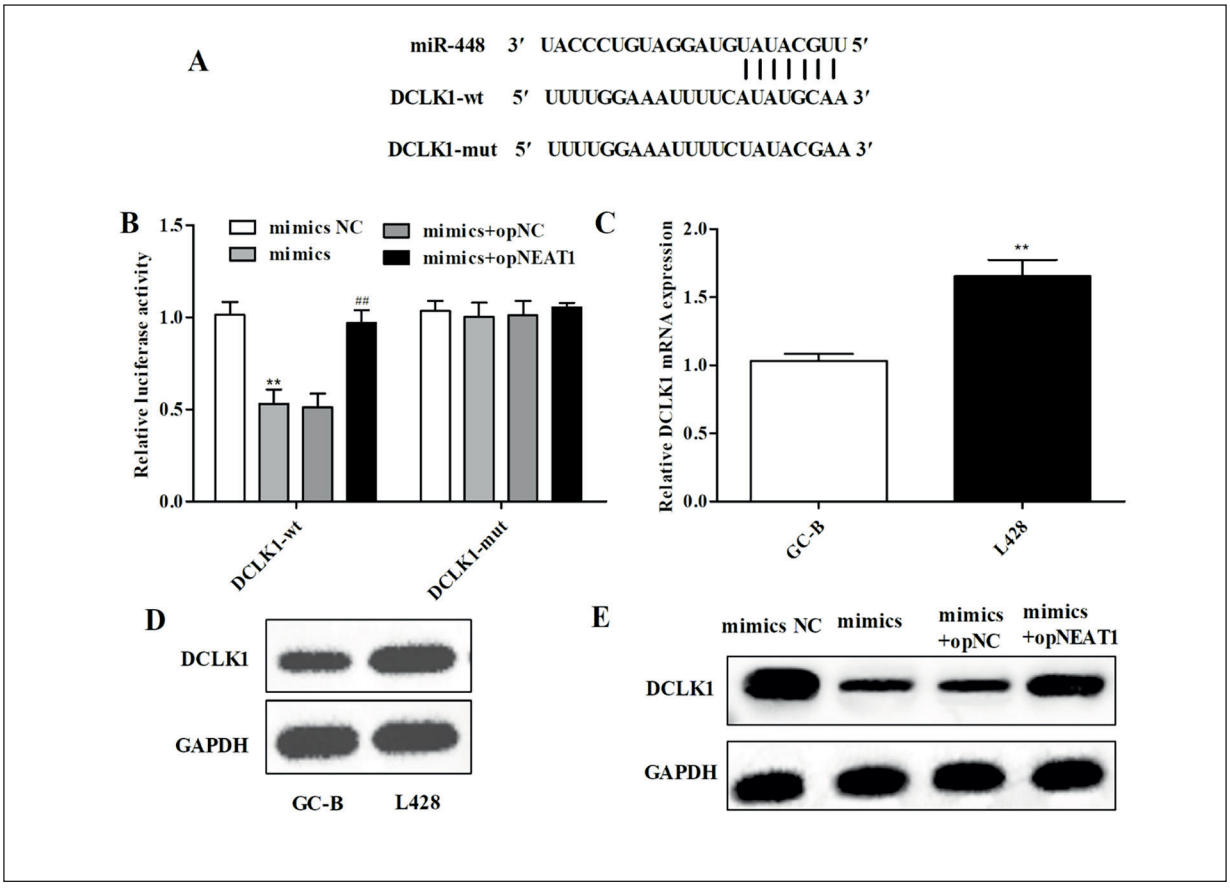

Figure 4. DCLK1 directly targets miR-448 and regulated by NEAT1. **A,** Sequence alignment of miR-448 with the putative binding sites with DCLK1. **B,** The relative luciferase activity in L428 cells co-transfected with DCLK1-wt and miR-448 mimics or NC, DCLK1-mut and miR-448 mimics or NC, or in combination with opNC or opNEAT1. **C-D,** DCLK1 expression in L428 cells. **E,** DCLK1 protein level in L428 cells transfected with mimics NC, mimics, mimics NC and opNC, or mimics NC and opNEAT1. **$p<0.01$, ##$p<0.01$, compared with mimics NC, GC-B cells or mimics+opNEAT1 group.

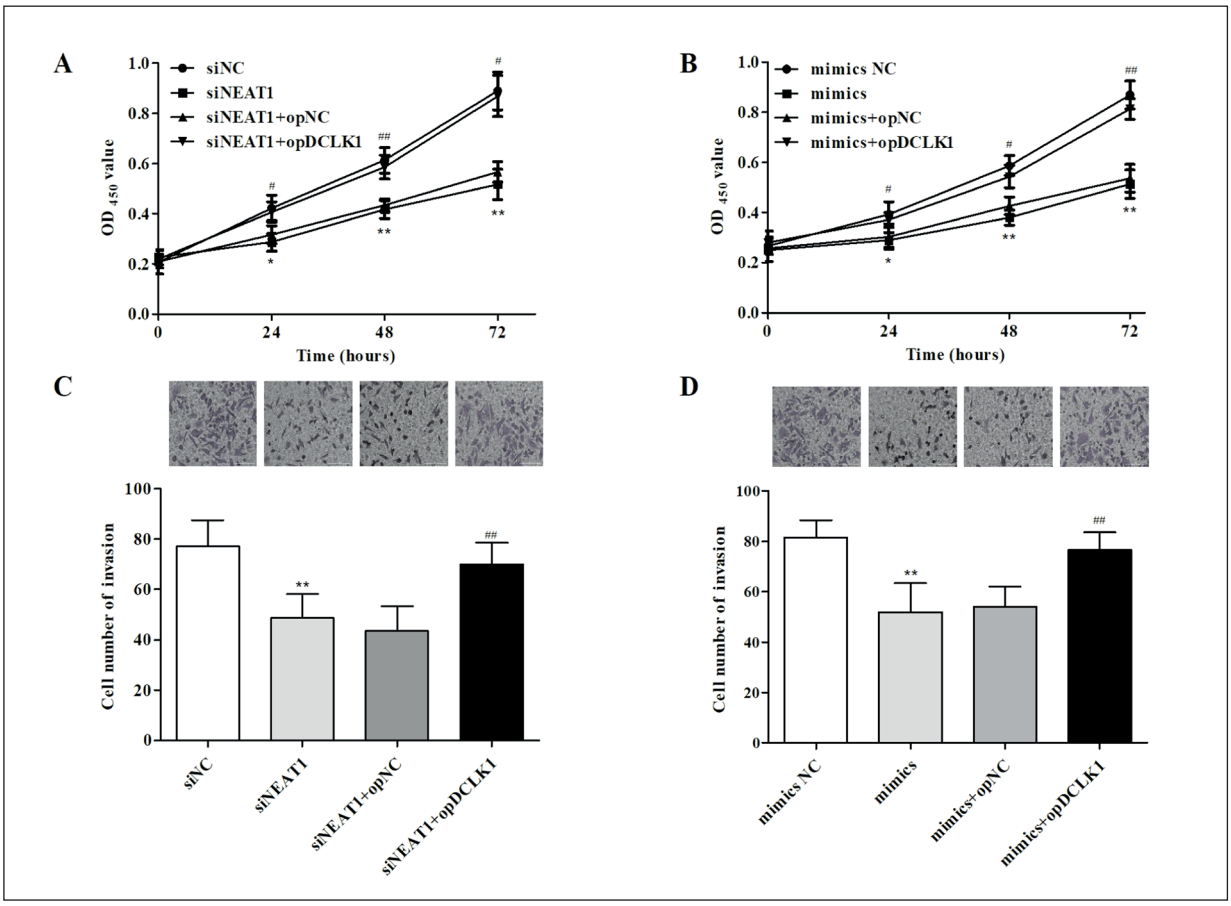

Figure 5. DCLK1 overexpression overturned suppression on cell proliferation and invasion capability of L428 cells mediated by NEAT1 knockdown or miR-448 overexpression. **A-B,** L428 cells proliferation and invasion capability after transfected with siNC, siNEAT1, siNEAT1 and opNC, or siNEAT1 and opDCLK1 were determine by CCK-8 and transwell assay (200×) respectively. **C-D,** Cell proliferation and invasion capability of L428 cells transfected with mimics NC, miR-448 mimics, miR-448 mimics and opNC, or miR-448 mimics and op opDCLK1 were measured by CCK-8 and transwell assay (200×) respectively. *p<0.05, **p<0.01, #p<0.05, ##p<0.01, compared with mimics NC or mimics+opNC group.

expression as a ceRNA of miR-107[17]. In present study, we revealed that NEAT1 directly interacted with miR-448 in L428 cells, and also negatively regulated miR-448, thus we proposed that NEAT1 may promote HL cell proliferation and invasion capability by regulating miR-448 to mediate miR-448 target genes.

Later, miR-448 potential target genes were explored and DCLK1 was selected for following experiments. DCLK1 is involved in microtubule and has been confirmed to be related to cancer tumorigenesis in previous studies[18]. DCLK1 expression was strictly related to lymph node invasion, tumor differentiation and clinical stage[19]. In colorectal cancer (CC), upregulation of DCLK1 was accompanied with metastasis and poor prognosis of CC patients[20]. DCLK1 knockdown inhibited epithelial-mesenchymal transition (EMT) and reduced cell invasion of renal clear cell carcinoma (RCC)[21]. In lung squamous cell carcinoma, miR-448 could negatively regulate DCLK1 expression to suppress cells growth and metastasis[22]. However, the relationship between miR-448 and DCLK1 in HL was still unknown. In our study, DCLK1 expression was increased in L428 cells versus GC-B cells. NEAT1 regulated the expression of DCLK1 as a ceRNA of miR-448. To our surprise, DCLK1 overexpression could abrogate the suppression on L428 cell proliferation and invasion capability mediated by NEAT1 knockdown or miR-448 overexpression, suggesting that NEAT1 promoted L428 cell proliferation and invasion capability by regulating miR-448 and NEAT1 expression.

In summary, we first reported that the NEAT1/miR448/DCLK1 axis could regulate cell prolifer-

ation and invasion in L428 cells. This is helpful to our further understanding of lncRNA-miRNA-mRNA regulatory networks in HL. However, the present study has many limitations, such as lacking of animal and other HL cell lines experiments. Thus, further studies were deserved to confirm the function of this regulatory network *in vivo* and *in vitro*.

Conclusions

As far as we know, we first studied the impact of MALAT1 on cell proliferation and invasion in HL cells. The results first put forward that NEAT1 was upregulated in HL tissues and cells, and could acted as a ceRNA of miR-448 to modulate DCLK1 expression. Moreover, we also were the first to identify NEAT1 facilitated to HL progression by promoting cell proliferation and invasion through miR-448/DCLK1 axis. This newly identified MALAT1/miR-448/DCLK1 axis may provide novel therapeutic strategy for HL patients in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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