European Review for Medical and Pharmacological Sciences 2021; 25: 770-778

Long non-coding RNA HEIH modulates CDK8 expression by inhibiting miR-193a-5p to accelerate nasopharyngeal carcinoma progression

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Abstract. – OBJECTIVE: Nasopharyngeal carcinoma (NPC) is the commonest malignant tumor. In this article, we aimed to examine the molecular role of lncRNA HEIH in the progression of NPC.

PATIENTS AND METHODS: We assessed the expression of HEIH, miR-193a-5p and CDK8 in NPC tissues and cells by real-time PCR. The cell proliferation, invasion and migration of SUNE-1 cells were examined by CCK-8 and transwell assay. Western blot assay was adopted to measure the protein expression level of CDK8. Dual-Luciferase reporter assay was adopted to evaluate the correlation between HEIH, miR-193a-5p and CDK8.

RESULTS: We discovered that HEIH was high expressed and miR-193a-5p was reduced in both NPC tissues and cells. The upregulation of HEIH facilitated cell proliferation, migration and invasion of SUNE-1 cells. In addition, overexpression of miR-193a-5p restrained cell progression of SUNE-1 cells. Moreover, HEIH was proved to be a molecular sponge of miR-193a-5p in NPC. Besides that, CDK8 was found to be a direct target gene of miR-193a-5p in NPC. Furthermore, CDK8 knockdown suppressed cell progression of SUNE-1 cells.

CONCLUSIONS: Our data demonstrated that HEIH overexpression promoted cell progression by sponging miR-193a-5p and upregulating CDK8.

Key Words:
Nasopharyngeal carcinoma, HEIH, MiR-193a-5p, CDK8.

Introduction

Nasopharyngeal carcinoma (NPC) is a kind of malignant tumor which derived from the nasopharyngeal epithelial cells. It is one of the most frequently occurring malignant tumors. Radiotherapy is one of the main methods for the treatment of nasopharyngeal carcinoma[1]. Despite the local control rate of NPC has been greatly improved, poor prognosis is still a huge challenge for the NPC treatment[2]. Consequently, it is important to explore the pathogenesis and development of NPC.

Long non-coding RNAs (lncRNAs) have been informed to serve as essential regulatory factors in the progression of multiple tumors, such as colorectal cancer, lung cancer and gastric cancer[3-4]. Lnc-PVT1 was found to promote cell proliferation and invasion in colon cancer by modulating RUNX2[5]. In osteosarcoma, LINC00963 was found to accelerate cell viability, and significantly correlated with poor prognosis[6]. LncRNAs have been observed to inhibit the development of cancers. Consistently, in breast cancer, lncRNA-TSLNC8 suppressed cell proliferation and the G1/S phase transition by regulating miR-214-3p/FOXP2 axis[7]. LncRNA-HEIH (high expression in hepatocellular carcinoma) has been found to be a cancer promoting RNA in hepatocellular carcinoma[8]. Additionally, HEIH was reported to act as an oncogenic in cell proliferation and apoptosis via regulating miR-4458/SOCS1 in triple-negative breast cancer[9]. However, the role of HEIH is still unknown in NPC.

MicroRNAs (miRNAs) have been reported to regulated by lncRNAs in the process of NPC cells. LncRNA XIST was found to regulated cell metastasis and hypoxia-induced glycolysis by inhibiting miR-381-3p in NPC[10]. Depletion of TUG1 was found to retain cell growth by regulat-

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ing miR-384 in NPC[11]. Furthermore, miR-193a-5p was confirmed to participate in the cell progression of pediatric chordomas[12] and pancreatic cancer[13]. Nevertheless, the expression level and function of miR-193a-5p in NPC is still unclear.

This research is designed to explore the expression level of HEIH in NPC and investigate the role of HEIH on the tumorigenesis and progression of NPC cells. In addition, we also revealed the role of miR-193a-5p/CDK8 on the tumorigenesis and progression of NPC cells. The correlation between HEIH, miR193a-5p and CDK8 expressions in NPC was also illustrated in this work.

Patients and Methods

Patients and Specimens

46 NPC tissues and the corresponding normal tissues were collected from Qilu Hospital (Qingdao), Cheeloo College of Medicine, Shandong University (China). After surgical resection, the clinical samples were frozen in liquid nitrogen immediately and then stored at a -80°C refrigerator. All patients have not been received any anti-tumor therapy. Before the investigation, all NPC patients have written the informed consents. In addition, our experiment was executed according to the Declaration of Helsinki Principles and authorized by the Ethics Committee of Qilu Hospital (Qingdao), Cheeloo College of Medicine, Shandong University (China).

Cell Lines and Cell Transfection

NPC cell line 6-10B, NP69, SUNE-1C666-1 and 5-8F were obtained from BeNa Culture collection. NPC cells were planted in Roswell Park Memorial Institute-1640 (RPMI-1640; Thermo Fisher Scientific, Waltham, MA, USA) medium with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) at 37°C and 5% CO_2. When the cell fusion rate was 80%, cell passage was carried out with 0.25% trypsin.

Si-HEIH, si-CDK8, miR-193a-5p mimics, miR-193a-5p inhibitor, pcDNA3.1-HEIH and pcDNA3.1-CDK8 were obtained from GenePharma (Shanghai, China). Then, we transfected them into SUNE-1 cells by Lipofectamine 2000 (Thermo Fisher Scientific Waltham, MA, USA).

Quantitative Real Time-PCR

Total RNA samples were isolated from NPC tissues and cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). GoScript Reverse Transcription System was used to reverse RNA transcription into cDNA. Then, LightCycle 96 thermocycle (Roche, Basel, Switzerland) was adopted to quantify the expression level of HEIH, miR-193a-5p and CDK8. HEIH and miR-193a-5p were standardized to GAPDH, while β-actin was normalized to CDK8. The primer sequences are shown in Table I.

Cell Counting Kit-8 (CCK-8) Assay

SUNE-1 cells were cultured in 96-well plates (1,000 cells/well). After cultivation for 24, 48, 72 or 96 h, CCK-8 reagents (Dojindo Molecular Technologies, Kumamoto, Japan) were added in each well. Then, SUNE-1 cells were subsequently incubated at 37°C for another 2 h. Finally, the optical density was measured at 450 nm.

Transwell Assay

Transwell chambers (8 μm; Millipore, Billerica, MA, USA) were performed to examine migration and invasion abilities of NPC cells. First, cells (2×10^3) were inoculated in the upper transwell compartment. Unlike cell migration, cell invasion capability was tested in the upper chamber with Matrigel. Then, 10% FBS was add-

Table I. Primer sequences in qRT-PCR.

Gene		Primers5′-3′
HEIH	Forward	5’-AAGAACTCTTCGCTCCAGCC-3’
	Reverse	5’-ACAAAAGCAGACTAGGGCGG-3’
miR-193a-5p	Forward	5’-CGAGGATGGGAGCTGAGGG 3’
	Reverse	5’ GCCGAGAACTGGGACTTTGT 3’
CDK8	Forward	5’-ACCGTTGGCCGAGGCACTTA-3’
	Reverse	5’-TGCCGACATAGAGATCCCAG-3’
β-actin	Forward	5’-CCACTGGCATCGTGATGGA-3’
	Reverse	5’-CGCTCGGTGAGGATCTTCAT-3’
GAPDH	Forward	5’-CCTGACCTGCGTGTGGACT-3’
	Reverse	5’-GCTGTGGATGGGGAGGTGTC-3’

ed to the lower compartment. After incubation for 24 h, SUNE-1 cells were dyed with 0.5% crystal violet. Finally, counted the migrated and invaded cells numbers under an optical microscope.

Western Blot Assay

Total proteins samples were split by 10% sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto the NC membrane. After incubating with 5% skimmed milk, membranes were incubated with specific antibodies. Enhanced chemiluminescence (ECL) Western blotting substrate was used to detect the protein signals.

Dual-Luciferase Reporter Assay

HEIH-mut or HEIH-wt, miR-193a-5p mimics or miR-NC, and CDK8-mut or CDK8-wt was transfected into SUNE-1 cells by Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). SUNE-1 cells were cultured in the incubator at 37°C, 5% CO_2, and 95% humidity. After 48 hours, the Luciferase activity was tested by the Dual-Luciferase reporter assay kit.

Statistical Analysis

All data were presented as mean±SD. GraphPad Prism 6.0 (La Jolla, CA, USA) was carried out for statistical analysis. Differences between two groups was tested by Student's *t*-test Moreover, one-way ANOVA was adopted to analyze the differences of multiple groups. Pearson's correlation analysis was used to explore the correlation between HEIH, miR-193a-5p and CDK8. Statistical significance was set as $p<0.05$.

Results

The Ectopic Expressions of HEIH and MiR-193a-5p Were Found in NPC

First, we explored the expression pattern of HEIH and miR-193a-5p in NPC tissues and cell lines. We found that HEIH was higher expressed in NPC tissues than in normal NPC tissues (Figure 1A). Moreover, HEIH was up-regulated in NPC cell lines (6-10B, SUNE-1, 5-8F and C666-1) compared to the normal cell line (NP69) (Figure 1B). Inversely, miR-193a-5p was found to be down regulated in NPC tissues contrast to normal NPC tissues (Figure 1C). Similarly, the expression of miR-193a-5p was lower in NPC cell lines than in NP69 cells (Figure 1D). Our results verified that ectopic expression of HEIH and miR-193a-5p existed in the tumorigenesis of NPC.

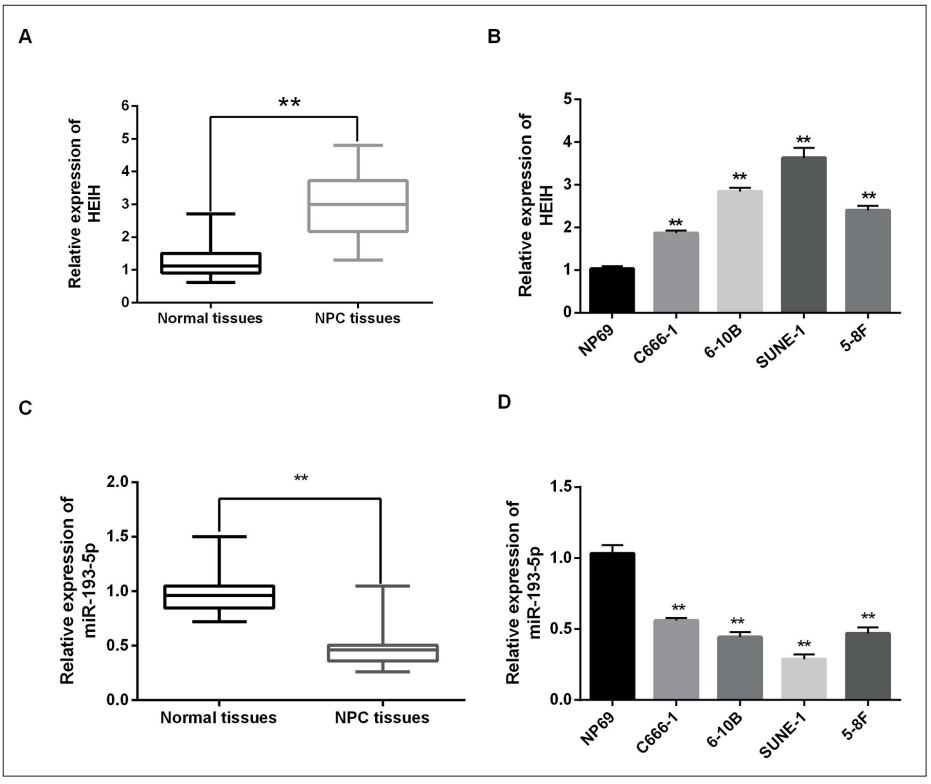

Figure 1. The expression of HEIH and miR-193a-5p were found in NPC. **A,** The expression of HEIH in NPC tissues (n=46). **B,** HEIH expression in NPC cell lines (SUNE-1, 6-10B, 5-8F and C666-1) and NP69 cells. **C,** MiR-193a-5p expression in NPC tissues. **D,** MiR-193a-5p expression in NPC cell lines (SUNE-1, 6-10B, 5-8F and C666-1) and NP69 cells. $^{**}p<0.01$.

HEIH Served as a Sponge of MiR-193a-5p in NPC

In our paper, StarBase predicted that HEIH had special binding sites with miR-193a-5p (Figure 2A). Dual-Luciferase reporter assay was applied to further observe this prediction. These results demonstrated that miR-193a-5p mimics significantly reduced the Luciferase activity of HEIH-wt, while had no effect on HEIH-mut (Figure 2B). Next, qRT-PCR was adopted to explore the relationship between HEIH and miR-193a-5p expression in SUNE-1 cells. Our results displayed that the expression level of miR-193a-5p was reduced by HEIH vector and increased by si-HEIH (Figure 2C). Moreover, miR-193a-5p mimics reduced HEIH expression, while miR-193a-5p inhibitor enhanced HEIH expression (Figure 2D). Moreover, Pearson's correlation analysis showed that HEIH was inversely correlated with miR-193a-5p in NPC tissues (Figure 2E). These findings demonstrated that HEIH could be a molecular sponge of miR-193a-5p in NPC.

HEIH Promoted the Cell Progression by Modulating MiR-193a-5p in NPC

To further probe the function of HEIH/miR-193a-5p in the progression of NPC, miR-193a-5p mimics, miR-193a-5p inhibitor, si-HEIH or HEIH vector was transfected into SUNE-1 cells respectively. As shown in Figure 3A, the reduction of HEIH caused by si-HEIH was restored by miR-193a-5p inhibitor. Moreover, CCK-8 assay and transwell assay were used to verify cell proliferation, migration and invasion of SUNE-1 cells. These results demonstrated that si-HEIH restrained cell proliferation of SUNE-1 cells, while miR-193a-5p downregulation abolished the inhibition effect of cell proliferation induced by si-HEIH (Figure 3B). Furthermore, the suppression of cell migration and invasion stimulated by HEIH silencing was also recovered by miR-193a-5p inhibitor (Figure 3C, D). Next, SUNE-1 cells were co-transfected with HEIH vector and miR-193a-5p mimics. We found that miR-193a-5p expression was advanced by miR-193a-5p mimics, while decreased by HEIH vector (Figure 3E). Functionally, cell proliferation was restrained by miR-193a-5p overexpression. Moreover, the reverse effect of HEIH vector on miR-193a-5p mimics was also identified in SUNE-1 cells (Figure 3F). Besides that, similar patterns were found in SUNE-1 cell migration and invasion (Figure 3G, H). The results implied that HEIH overexpression enhanced NPC progression by competing binding with miR-193a-5p.

CDK8 Was a Direct Target Gene of MiR-193a-5p in NPC

Furthermore, TargetScan predicted that CDK8 was a potential target gene of miR-193a-5p (Figure 4A). Luciferase reporter assay indicated that miR-193a-5p mimics reduced the Luciferase activity

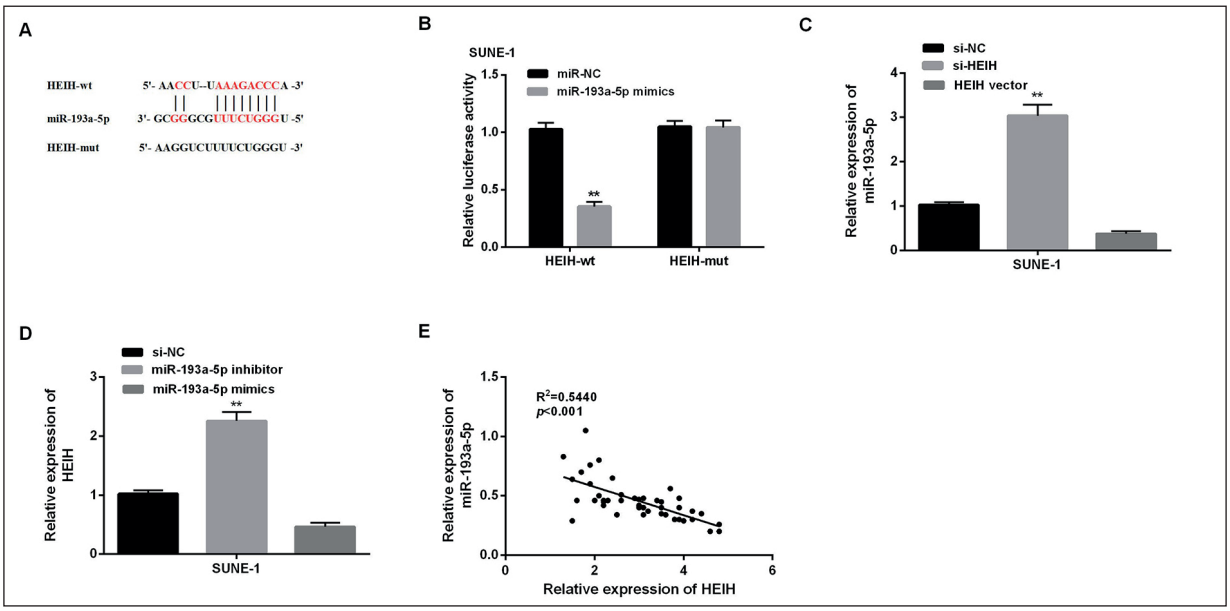

Figure 2. HEIH played as a sponge of miR-193a-5p in NPC. **A,** The binding sites between HEIH and miR-193a-5p. **B,** Dual-Luciferase assay. **C,** The expression of miR-193a-5p with si-HEIH and HEIH vector in SUNE-1 cells. **D,** HEIH expression regulated by miR-193a-5p mimic or inhibitor. **E,** HEIH negatively regulated miR-193a-5p expression in NPC tissues. $^{**}p<0.01$.

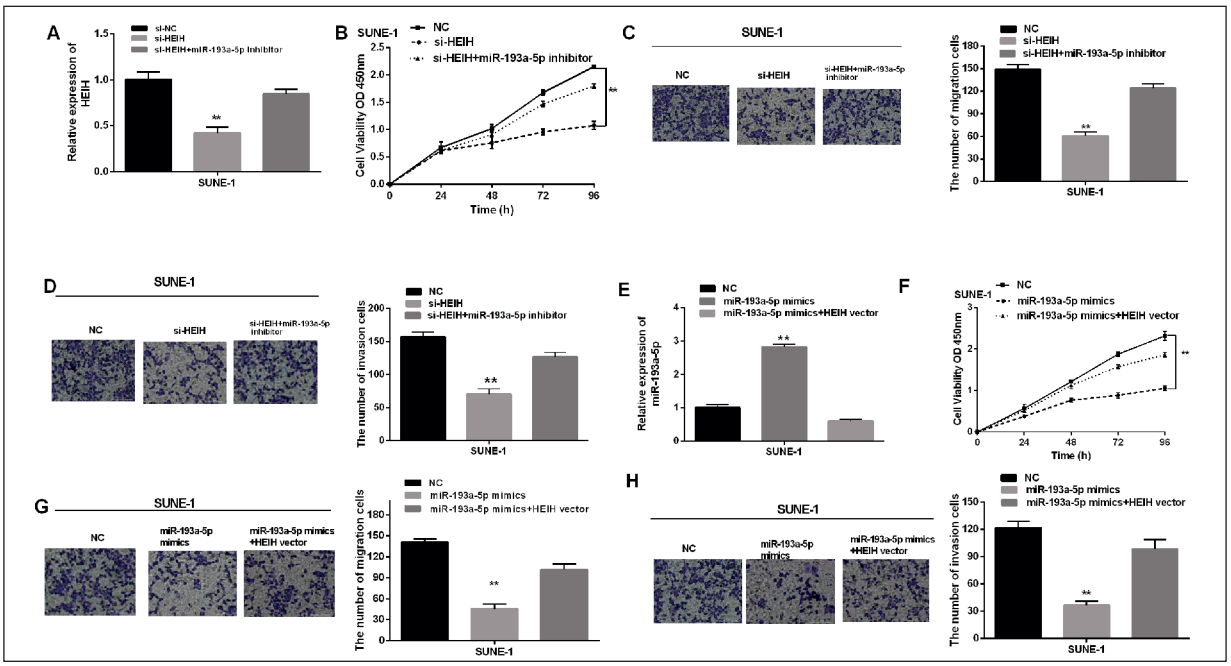

Figure 3. HEIH accelerated the NPC progression by regulating miR-193a-5p. **A,** The expression of HEIH with si-HEIH or si-HEIH+miR-193a-5p inhibitor in SUNE-1 cells. **B-D,** Cell proliferation, migration and invasion in SUNE-1 cells with si-HEIH or si-HEIH +miR-193a-5p inhibitor (magnification: 20×). **E,** The expression of miR-193a-5p in SUNE-1 cells with miR-193a-5p mimic or HEIH vector+miR-193a-5p mimic. **F-H,** Cell proliferation, migration and invasion in SUNE-1 cells with miR-193a-5p mimic or HEIH vectoe+miR-193a-5p mimic (magnification: 20×). **$p<0.01$.

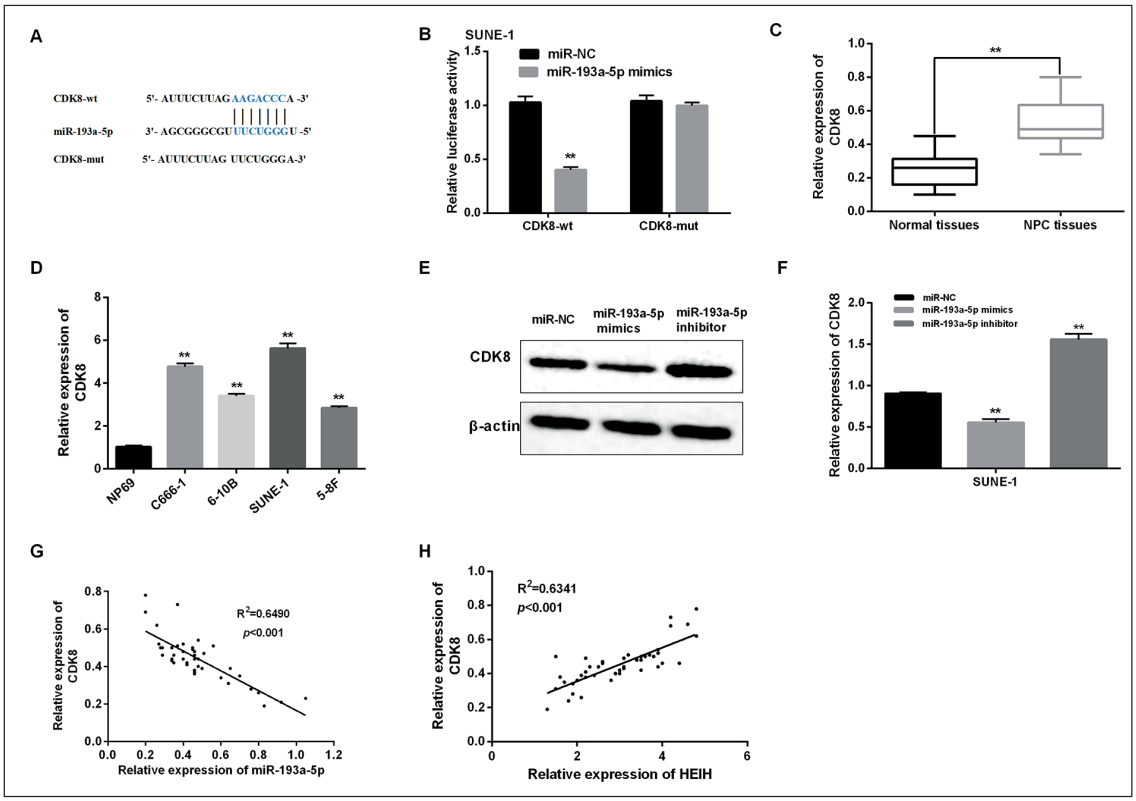

Figure 4. CDK8 was a target gene of miR-193a-5p. **A,** The binding sites between miR-193a-5p and CDK8. **B,** Dual-Luciferase reporter assay. **C,** The expression of CDK8 in NPC tissues. **D,** CDK8 expression in NPC cell lines and NP69 cells. **E-F,** CDK8 mRNA expression and protein expression in SUNE-1 cells with miR-193a-5p mimic or inhibitor. **G,** CDK8 was negatively correlated with miR-193a-5p in NPC tissues. **H,** CDK8 was positively correlated with HEIH in NPC tissues. **$p<0.01$.

of CDK8-wt, while not CDK8-mut (Figure 4B). In addition, CDK8 was discovered to be upward in NPC tissues and cell lines contrast to normal groups (Figure 4C, D). Furthermore, Western blot assay demonstrated that miR-193a-5p mimics suppressed the protein expression level of CDK8, while miR-193a-5p inhibitor accelerated CDK8 expression (Figure 4E). Similarly, we detected that miR-193a-5p overexpression reduced CDK8 mRNA expression, while miR-193a-5p downregulation promoted the expression of CDK8 (Figure 4F). Pearson's correlation analysis displayed that CDK8 was negatively correlated with miR-193a-5p in NPC tissues (R^2=0.6490, Figure 4G). However, CDK8 and HEIH expression presented a positive correlation in NPC tissues (R^2=0.6341, Figure 4H). Based on these findings, we thought that CDK8 was a target gene of miR-193a-5p in NPC.

HEIH Regulated NPC Progression by Mediating MiR-193a-5p /CDK8 Axis

To detect the mechanism of HEIH/miR-193a-5p/CDK8 in progression of NPC, HEIH vector or miR-193a-5p inhibitor was transfected into SUNE-1 cells with si-CDK8. Our results showed that HEIH vector or miR-193a-5p inhibitor restored this reduction of CDK8 mRNA expression by si-CDK8 (Figure 5A). Moreover, HEIH vector or miR-193a-5p inhibitor had a similar effect on CDK8 protein expression (Figure 5B). Next, we investigated the effect of HEIH vector or miR-193a-5p inhibitor on SUNE-1 cells activity. Primarily, si-CDK8 was found to suppress cell proliferation, whereas HEIH vector or miR-193a-5p inhibitor impaired the inhibitory of cell proliferation induced by si-CDK8 (Figure 5C). Similarly, upregulation of HEIH or reduction of miR-193a-5p abolished the inhibition effect of si-CDK8 on cell migration and invasion of SUNE-1 cells (Figure 5D, 5E). Taken together, HEIH promoted the cell progression of NPC by sponging miR-193a-5p expression and upregulating CDK8 expression.

Discussion

In recent years, many lncRNAs have been observed to promote tumorigenesis and pro-

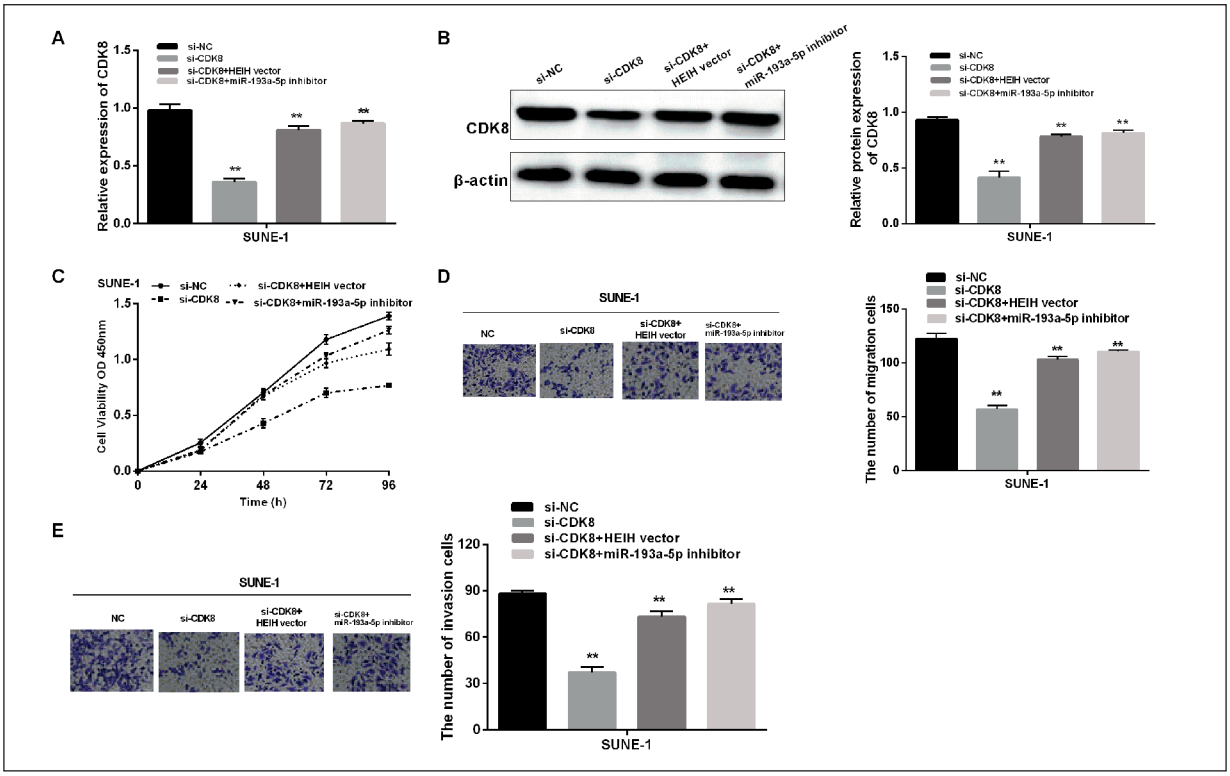

Figure 5. HEIH regulated NPC progression by mediating miR-193a-5p/CDK8 axis. **A, B,** The mRNA expression and protein expression of CDK8 in SUNE-1 cells with si-CDK8, si-CDK8+HEIH vector or si-HEIH+miR-193a-5p inhibitor. **C,** Cell proliferation in SUNE-1 cells with si-CDK8, si-CDK8+HEIH vector or si-HEIH+miR-193a-5p inhibitor. **D, E,** Cell migration and invasion in SUNE-1 cells with si-CDK8, si-CDK8+HEIH vector or si-HEIH+miR-193a-5p inhibitor (magnification: 20×). **p<0.01.

gression in NPC, such as LncRNA XIST, LINC00958, HCG18 and TUG1[11,14-16]. Yang et al[17] were the first to report that LncRNA-HEIH promoted cell invasion in hepatocellular carcinoma. Next, HEIH was detected to be upregulated in various cancers and promote tumor progression. Moreover, Guo et al[18] claimed that HEIH accelerated cell proliferation and enhanced chemoresistance of endometrial cancer cells by activating MAPK pathway. However, the mechanism of HEIH in NPC has not been reported. In this study, we noticed that the expression of HEIH was markedly high regulated in both NPC tissues and cells. Moreover, functional experiments showed that HEIH knockdown restrained cell proliferation, migration and invasion capability, which consistent with the effect of HEIH in colorectal cancer[19]. In addition, Jia et al[20] discovered that HEIH was upregulated and enhanced cell proliferation and metastasis in non-small cell lung cancer.

LncRNAs acted as ceRNAs of miRNAs, and affected miRNAs to regulate the expression of relevant target gene. HEIH was remarkably upregulated, and enhanced development by modulating miR-200b in breast cancer[21]. In addition, miR-199a-3p was also found to have binding sites with HEIH[22]. In prostatic cancer, miR-193a-5p emerged as a tumor inhibitor regulated by TTN-AS1[23]. In addition, miR-193a-5p was recovered to play similar effect in colon cancer and glioblastoma[24,25]. In this investigation, we predicted that HEIH had binding sites with miR-193a-5p and verified the hypothesis by Dual-Luciferase assay and qRT-PCR. Consistent with previous reports[26,27], miR-193a-5p was down expressed in NPC tissues and cells and had an inhibitory effect on the pathogenesis and development of SUNE-1 cells. Furthermore, we verified that HEIH overexpression accelerated NPC progression by mediating miR-193a-5p. In addition, we found an inverse correlation between HEIH and miR-193a-5p.

CDK8, a member of the cyclin-dependent kinases (CDKs) family, has conserved function as a part of mediator complex in transcription[28]. Recently, CDK8 has been reported to be involved in the EMT progress and act as a crucial regulator of cell growth, cell differentiation and angiogenesis[29,30]. In breast cancer, CDK8 was found to play a critical role in cell proliferation and migration[31]. Despite CDK8 has been reported in different types of human cancers, the role of CDK8 in NPC is still unknown. In our research, we demonstrated that CDK8 was a target gene of miR-193a-5p. In addition, upregulation of CDK8 facilitated cell proliferation and metastasis which was similar in colon cancer[32]. Furthermore, we also found that the expression of HEIH and CDK8 existed a positive correlation. Additionally, HEIH was proved to be a ceRNA of miR-193a-5p to modulate CDK8 expression in NPC.

Conclusions

To sum up, our study first revealed that HEIH was up expressed in NPC tissues and cells. We also indicated that HEIH enhanced the NPC development by modulating miR-193a-5p/CDK8. Therefore, we suggested that HEIH could be a possible therapeutic target for the treatment of NPC in the further. However, further studies are needed to verify the findings of this study.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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