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Original Article

Expression and Biological Functions of EPC1 in Nasopharyngeal Carcinoma

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Abstract

Background: Comb homolog enhancer 1 (EPC1) gene is one of the important members of epigenetic inhibitor PCG family. It shows carcinogenic potential in a variety of malignant tumors, but the expression and role of EPC1 in nasopharyngeal carcinoma are unclear. The aim of this study was to explore the expression and function of enhancer of polycomb homolog 1 (EPC1) in nasopharyngeal carcinoma (NPC).

Methods: The differential expression of *EPC1* in the cancer tissues and cell lines of NPC was examined by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). *EPC1* expression, cell proliferation, and apoptosis were detected in NPC cell lines after *EPC1* silencing, and the levels of the epithelial-mesenchymal transition (EMT)-related proteins E-cadherin and vimentin were detected in NPC cells after *EPC1* silencing. The study was performed at Fujian Provincial Hospital, Fujian, China, from 2018 to 2019.

Results: We found that EPC1 was significantly upregulated in the cancer tissues and cell lines of NPC ($P<0.001$). Furthermore, knockdown of EPC1 inhibited the growth and metastasis of NPC cells. E-cadherin and vimentin were detected in NPC cells after EPC1 was knocked out. It was confirmed that inhibition of EPC1 resulted in increased E-cadherin expression ($P<0.001$) and decreased vimentin expression ($P<0.001$), suggesting that inhibition of EPC1 could inhibit the EMT in NPC cells.

Conclusion: EPC1 expression was upregulated in NPC tissues and cell lines. Knockout of EPC1 effectively inhibited the growth of NPC cells, induced apoptosis, and inhibited invasion and metastasis. Inhibition of EPC1 could inhibit the EMT in NPC cells. All of the above findings support the viewpoint that EPC1 plays a pro-cancer role in NPC.

Keywords: EMT, *EPC1*, Invasion, Nasopharyngeal carcinoma, Proliferation

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Introduction

Nasopharyngeal carcinoma (NPC; OMIM: 607107) is one of the most common head and neck carcinomas in China. According to global cancer statistics, approximately 129 000 patients were diagnosed with NPC in 2018, accounting for 0.7% of all cancers, and the mortality rate was 0.8%.[1] The overall treatment strategy for NPC is completely based on the disease stage. Radical radiotherapy is used in patients with early-stage disease, with notably good clinical and survival results after intensity-modulated radiation therapy.[2,3] The 5-year local regional control rates for T3 and T4 NPC are estimated to be 90% and 75–80%, respectively.[4,5] However, about 5–30% of patients develop local recurrence or distant metastasis.[6] Approximately 50% of patients show local recurrence with distant metastases.[7] Treatment of recurrent and metastatic NPC is challenging and has yielded disappointing results.[8,9]

Novel functions of *EPC1* (enhancer of polycomb homolog 1; OMIM:610999) in DNA damage protection have been reported in the literature.[10] In genotoxic therapy, *EPC1* deletion enhanced *E2F1* mediated apoptosis and eliminated the motility of tumor cells. *E2F1* (OMIM: 189971) binds directly to the *EPC1* promoter, and *EPC1* physically interacts with bifunctional *E2F1* to regulate its transcriptional activity in a target gene specific manner. Cooperation between *EPC1* and *E2F1* has been revealed to trigger a metastasis related gene signal in advanced cancer and predict poor survival. These findings reveal a novel carcinogenic function of *EPC1* in inducing the expression of tumor progression related genes, which may contribute to new therapeutic methods. However, there are no relevant reports on the expression of *EPC1* in NPC and its role in the occurrence and progression of NPC.

In this study, nasopharyngeal tissues were collected from NPC patients and healthy subjects, and qRT-PCR was used to detect *EPC1* expression in nasopharyngeal biopsy specimens from the NPC patients at the initial diagnosis. The differential expression of *EPC1* in NPC and normal nasopharyngeal tissues was detected by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). Differential expression of *EPC1* in different cell lines was examined by qRT-PCR after normal

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nasopharyngeal epithelial cells and NPC cell lines were cultured. In NPC cells, *EPC1* was silenced and transfected into NPC cell lines. *EPC1* expression and cell proliferation and apoptosis after *EPC1* silencing in the NPC cell lines were detected. The levels of the epithelial-mesenchymal transition (EMT)-related proteins E-cadherin and vimentin in NPC cells after *EPC1* silencing were detected to explore the expression and biological functions of *EPC1* in NPC.

Materials and methods

Tissues Samples

We recruited a total of 35 NPC patients enrolled at Fujian Provincial Hospital during 2018 to 2019 and 35 healthy subjects matched for age, sex, and oral habits. Among them, all NPC patients had given their written informed consent. The clinical stages of NPC patients were classified according to the TNM system of the American Joint Committee on Cancer, combined with other malignancy and incomplete clinical or prognostic information as exclusion criteria. The tissues samples were collected within 15 min after removal from the body. Meanwhile, 35 pairs of NPC tissues and matched control samples were used to compare the expression level of genes of interest.

Cell Culture

NPC cell lines (5-8F, C666-1, CNE-2, 6-10B, HONE-1 and CNE-1) and nasopharyngeal epithelial cell line (NP69) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sangon Biotech, Shanghai, China) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 100 μg/mL penicillin/streptomycin (Gibco, Gaithersburg, MD, USA) at 37˚C with 5% CO_2.

Cell Transfection

The transfection reagent si-*EPC1* was purchased from Santa Cruz Biotechnology Inc., USA. si-*EPC* and the control (si-NC) were transfected into CNE1 and HONE-1 cells using a Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) according to the instructions.

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction

Total RNA from tissues and cells was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Approximately 1.2 μg of RNA was reverse transcribed to cDNA using a Fast Quant RT Kit (TaKaRa, Dalian, China), and the qRT-PCR was carried out using a SYBR Premix Ex Taq II kit (TaKaRa) and Roche LightCycler® 480 System (Roche, Chicago, USA). The relative expression changes of the targets were analyzed by the $2^{-\Delta\Delta Ct}$ method,[11] and GAPDH served as the internal reference. The following primers were used: *EPC1*: forward primer: 5'-AGCCGATCTTATCCGACCGAA-3', reverse primer: 5'-TCCAAGGCCAGTTGCCAGTTT -3'; GAPDH: forward primer: 5'-CGAGAGAATCCGCGGACAT-3', reverse primer: 5'-TTGTGCAATACAGCGTGGAC-3'.

CCK-8 Assay

Cell proliferation was detected by Cell Counting Kit-8 (Dojindo Laboratories, Kumamo-to, Japan). Approximately 2000 cells per 100 μL of medium were seeded into 96-well plates and cultured for different times, including twenty-four, forty-eight, seventy-two and ninety-six hours. Absorbance was detected at 450 nm with the CCK-8 kit.

Colony Formation

Cells were collected and seeded into 6-well plates with approximately 1000 cells per well. After two weeks, the cells were fixed with methanol for 30 minutes and subsequently stained with 1% crystal violet dye. Finally, the numbers of colonies were counted.

Transwell Assay

The invasion and migration of NPC cells was evaluated by transwell assay as previously described.[12] Briefly, approximately 1×10^5 cells with different treatments were added in the upper uncoated (migration) or 8.0-μm-pore Matrigel™-coated membranes (for invasion) with serum-free medium. RPMI 1640 medium with 10% FBS was added into the lower wells. After twenty-four hours, cells were fixed by 4% paraformaldehyde and stained with crystal violet. The cells that had invaded the lower wells were photographed and counted under a microscope in 10 randomly selected fields.

Apoptosis Analysis

Cell apoptosis was detected by an Annexin V-FITC/PI apoptosis detection kit (Becton, Dickinson and Company). Briefly, cells were seeded into 6-well plates at a density of 1×10^5 cells/mL. After forty-eight hours, cells were harvested by trypsinization and cell pellet were collected by centrifugation. Cells were resuspended with 200 μL of binding buffer for 1×10^6 cell/mL and stained with Annexin V-FITC for 15 minutes. Then, PI was added, followed by incubation for another 5 minutes. Subsequently, flow cytometric analysis was performed with a ACSCalibur™ Flow Cytometer (Becton, Dickinson and Company).

Western Blot

Total proteins were isolated from tissues or cultured cells with RIPA Lysis Buffer (Beyotime Institute of Biotechnology). Approximately the same amount of protein was separated by 8–12% SDS-PAGE and then transferred to PVDF membrane. After sealing the membrane with 5% skim milk, the first antibody (including E-cadherin and vimentin) was incubated at the ratio of 1:500 at 4˚C for one night, and GAPDH was used as the internal reference. Subsequently, the membranes were incubated with horseradish peroxidase-labeled IgG

for one hour. All primary antibodies were purchased from Abcam. Finally, the bands of interest were visualized and observed under a Bio-Rad imaging system.

Statistical Analysis

Data were expressed as mean ± SD, and statistical analysis was performed using GraphPad Prism 6. All experiments were repeated three times or more. The data between groups were compared by one-way analysis of variance (ANOVA). The differences between the two groups were compared by *t* test. The difference was considered statistically significant at $P<0.05$.

Results

Clinical Data of NPC Patients

The clinical data for 35 NPC patients are shown in Table 1. The median age was 46 years, and the sample consisted of 23 males (65.7%) and 12 females (34.29%). Four patients had distant metastasis.

EPC1 Expression in Nasopharyngeal Tissues from NPC Patients and Matched Healthy Individuals

EPC1 expression in nasopharyngeal tissues from NPC patients and matched healthy subjects was detected by qRT-PCR. The results showed that the relative expression level of *EPC1* was elevated in most NPC tissues compared to nasopharyngeal tissues from healthy subjects. The difference was statistically significant ($P<0.001$) (Figure 1).

Expression of EPC1 in NPC Cells and Nasopharyngeal Epithelial Cells

EPC1 expression in the NPC cell lines 5-8F, C666-1, CNE-1, CNE-2, HONE-1, and 6-10B and the normal nasopharyngeal epithelial cell line NP69 was detected by qRT-PCR. Data were analyzed with one-way ANOVA. We investigated the satisfaction of assumptions of normality and homogeneity of error variance, prior to application of ANOVA. The results showed that the relative expression levels of *EPC1* mRNA in NPC cells were significantly higher than NP69 cells ($P<0.001$) (Figure 2), suggesting that EPC1 was highly expressed in NPC cells.

Table 1. Clinical Data for 35 NPC Patients

Variable	Value
Age (Median, 25%,75%) (years)	46, 38, 52.5
Gender	
Male	23 (65.71%)
Female	12 (34.29%)
Distant metastasis	
Yes	4 (11.43%)
No	31 (88.57%)
T stage	
T1-2	7 (20%)
T3	17 (48.57%)
T4	11 (31.43%)
N stage	
N0	2 (5.71%)
N1	6 (17.14%)
N2	26 (74.29%)
N3	1 (2.86%)

Silencing of EPC1 Inhibited the Growth of NPC Cells in vitro

The expression of mRNAs is closely associated with the occurrence and development of NPC, and plays an important role in the proliferation, invasion, and migration of NPC cells. We designed si-*EPC1* (gene knockout of *EPC1*) and transfected it into NPC cell lines（CNE-1 and HONE-1 cells). We observed whether gene knockout of *EPC1* affects the proliferation, invasion, and metastasis of NPC cell lines.

qRT-PCR Detection of EPC1 Expression in NPC Cell Lines after EPC1 Silencing

EPC1 expression in NPC cell lines after *EPC1* silencing was detected by qRT-PCR. The results showed that the relative expression levels of *EPC1* in NPC cells were decreased after si-*EPC1* transfection compared with the control group (si-NC). The difference was statistically

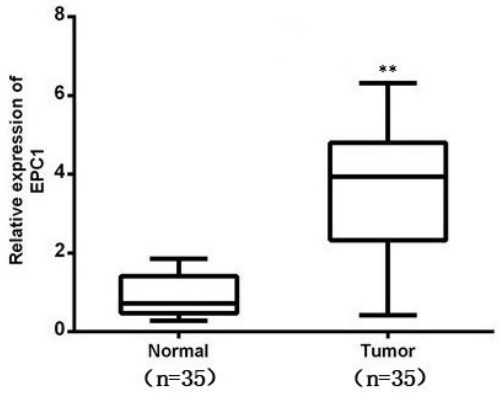

Figure 1. Relative Expression of EPC1 in NPC Tissues and Matched Normal Tissues (n=35) (**$P<0.001$).

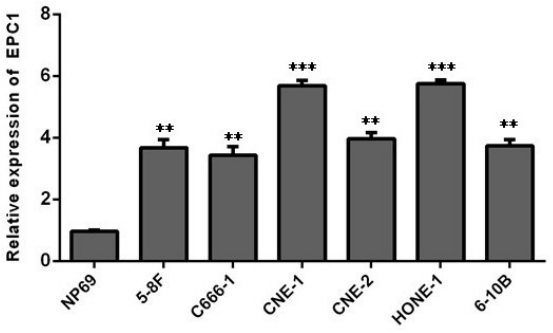

Figure 2. EPC1 expression in the NPC cell lines and the normal nasopharyngeal epithelial cell line was evaluated by qRT-PCR (n=3). Data were analyzed using one-way ANOVA. ***$P<0.001$, NP69 vs. 5-8F, -2.72 (95% CI, -3.22 to -2.22); ***$P<0.001$, NP69 vs. C666-1, -2.47 (95% CI, -2.97 to -1.97); ***$P<0.001$, NP69 vs. CNE-1, -4.73 (95% CI, -5.23 to -4.23); ***$P<0.001$, NP69 vs. CNE-2, -3.01 (95% CI, -3.51 to -2.51); ***$P<0.001$, NP69 vs. HONE-1, -4.79 (95% CI, -5.29 to -4.29); ***$P<0.001$, NP69 vs. 6-10B, -2.78 (95% CI, -3.28 to -2.28).

significant ($P<0.01$) (Figure 3).

Proliferation of NPC Cells after EPC1 Silencing

We used CCK-8 and colony formation assays to evaluate the effect of *EPC1* silencing on the proliferation of NPC cells. The absorbance values (optical density values) of cells in each group were measured at 0, 24, 48, 72, and 96 hours and the growth curves of cell proliferation were plotted. The results showed that compared with si-NC, si-*EPC1* significantly inhibited the growth of NPC cells ($P<0.01$) (Figure 4). The colony formation assays showed that the number of NPC cells colonies was significantly higher in the si-NC group than the si-*EPC1* group ($P<0.01$) (Figure 5), indicating that *EPC1* silencing inhibited the proliferation of NPC cell lines.

EPC1 silencing affected the invasion and migration of NPC cells

Transwell invasion assays were performed to evaluate the invasion of NPC cells after *EPC1* silencing. Compared with si-NC, si-*EPC1* significantly inhibited the invasion of NPC cells ($P<0.01$) (Figure 6).

Transwell migration assays were performed to evaluate the effect of *EPC1* silencing on the metastasis of NPC cells. Compared with si-NC, si-*EPC1* significantly inhibited the metastasis of NPC cells ($P<0.01$) (Figure 7).

EPC1 Silencing Promoted NPC Cell Apoptosis

Flow cytometry was used to detect the effect of *EPC1* silencing on the apoptosis of NPC cells. The results showed that si-*EPC1* could effectively induce NPC cell apoptosis, and the difference was statistically significant ($P<0.016$) (Figure 8).

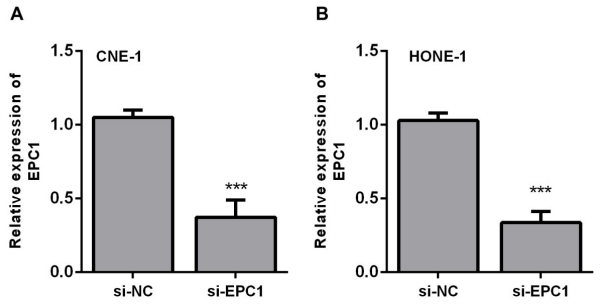

Figure 3. NPC cell lines CNE-1 **(A)** and HONE-1 **(B)** were transfected with si-EPC1 or si-NC. Transfection efficiency was evaluated by qRT-PCR (n=3) (***$P<0.001$).

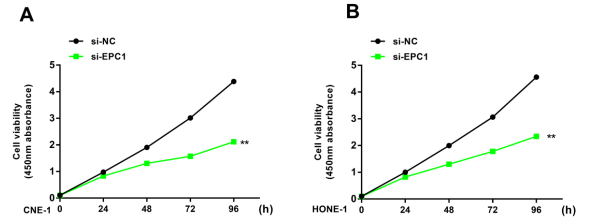

Figure 4. Cell proliferation was evaluated by a CCK-8 kit. **(A)** CNE-1 and **(B)** HONE-1 cells (n=3) (***$P<0.001$).

EPC1 Silencing Inhibited EMT of NPC Cells

To investigate the factors underlying the inhibition of invasion and metastasis of NPC cells by *EPC1*, si-*EPC1* and si-NC cells were transfected into the cultured NPC cell lines. Western blotting was used to detect the EMT-related proteins E-cadherin and vimentin in cells from both groups. The results showed that si-*EPC1* significantly increased E-cadherin protein levels and decreased vimentin expression ($P<0.01$) (Figure 9). These results confirmed that inhibition of *EPC1* resulted in increased expression of E-cadherin and decreased expression of vimentin, confirming that *EPC1* silencing inhibited the EMT process.

Discussion

NPC is a multifactorial malignancy closely related with genetic factors and Epstein-Barr virus infection.[13] Currently, treatment of malignant tumors tends to be precise and individualized. Molecular targeting of tumors is based on specific binding of antibodies/ligands to the target molecules of tumor cells, thereby blocking the downstream signaling pathways associated with the promotion of tumor occurrence and development. Targeted treatment is dependent on the genetic and epigenetic states of tumor cells and is currently considered the most

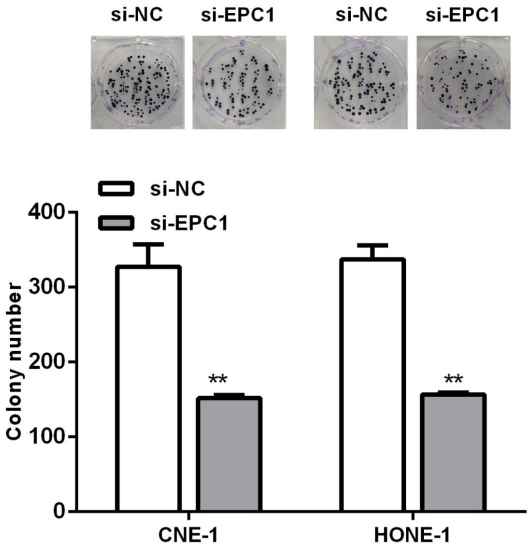

Figure 5. Cell proliferation was evaluated by colony formation assay (n=3) (***$P<0.001$).

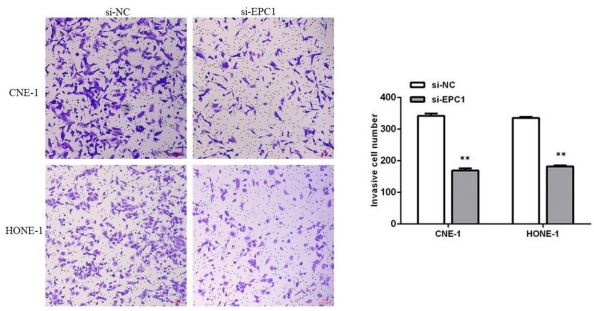

Figure 6. Transwell Invasion Assay. Scale bar=100 μm (n=3) (***$P<0.001$).

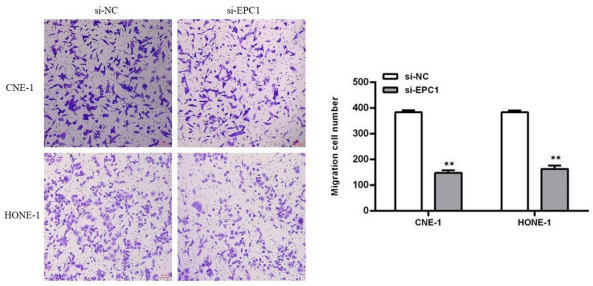

Figure 7. Transwell Migration Assay. Scale bar = 100 μm (n = 3) (***P < 0.001).

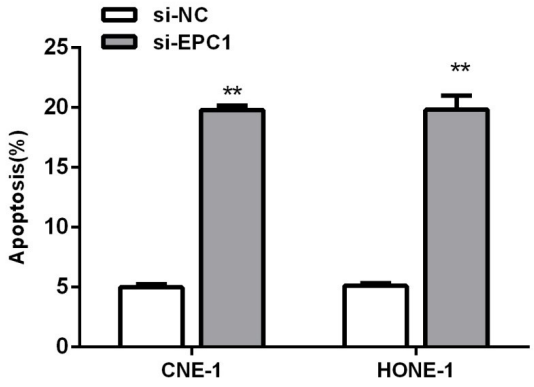

Figure 8. Apoptosis rate was measured by flow cytometry (n = 3) (**P < 0.01).

promising method for treating cancers.[14] Currently, the main molecularly targeted therapeutic drugs with proven efficacy in the treatment of NPC are epidermal growth factor receptor (OMIM: 131550) inhibitors and vascular endothelial growth factor inhibitors.[15,16] Previous studies have demonstrated the clinical efficacy and good safety of molecular targeted therapy in NPC. However, many studies are only in the preclinical or early research stage,[17] and more phase III clinical trials are needed. As cancer treatment becomes more precise and individualized, biomarkers will become signposts for the development of targeted therapies and immunotherapeutic pathways. Building predictive models and comprehensive assessment using predictive factors can be used more accurately to select beneficiary populations.

Therefore, investigating the molecular mechanisms of NPC underlying the occurrence and development, identifying specific biomarkers that contribute to early diagnosis and prognosis prediction of NPC, understanding the mechanisms underlying the recurrence and metastasis of NPC, and seeking molecule therapeutic targets for drugs are key tasks for improving the early diagnosis rate and survival rate of NPC patients. Tumors have complex pathogeneses involving changes in multiple genes and multiple signaling pathways, including maintenance of growth signal transduction, evading growth inhibitors, resistance to cell death, achieving unlimited proliferation, inducing angiogenesis, and activation of invasion and metastasis. With the in-depth study of epigenetics

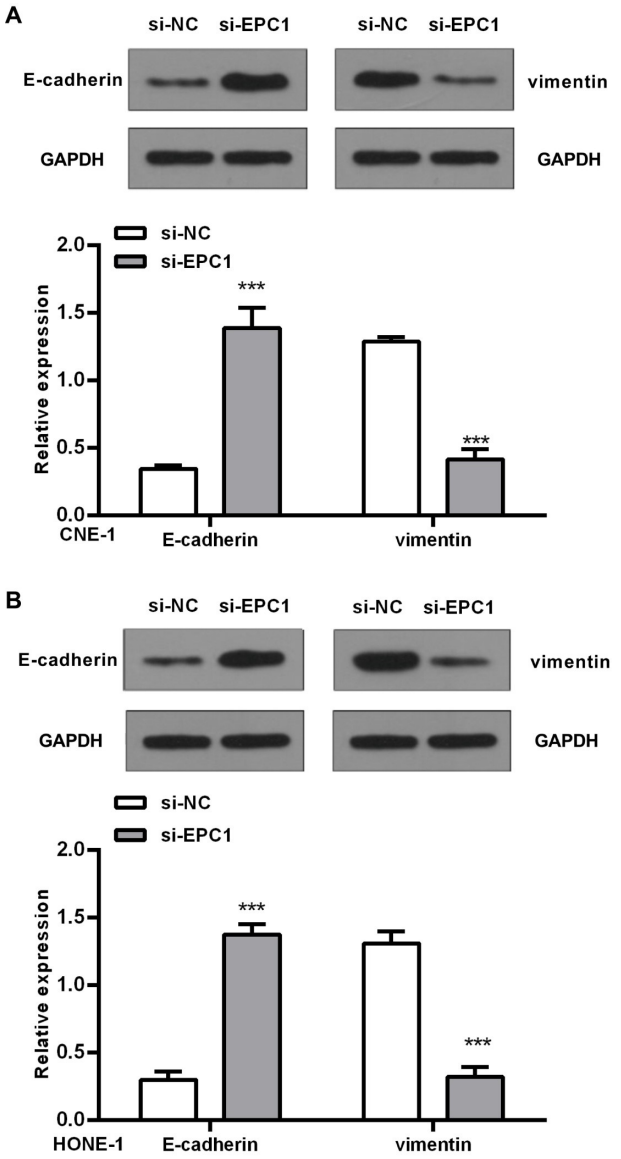

Figure 9. Protein expression of E-cadherin and vimentin was detected by western blot. (A) CNE-1 and (B) HONE-1 cells (n = 3) (***P < 0.001).

and proteomics, the normal expression of genes can be disturbed through DNA methylation, histone modification, chromatin remodeling, and noncoding RNA regulation, thereby affecting the transcription and expression of related genes. Oncogene activation and tumor suppressor gene deletion[18,19] have received extensive attention in cancer research in recent years. In most cases, oncogenes are considered important factors in the occurrence and development of NPC.

The *EPC1* gene was first identified in *Drosophila* and belongs to the *PcG* gene family. The gene mainly functions through epigenetic silencing and maintenance of cell-type specificity.[20] The *EPC1* gene can increase homozygous mutant phenotypes of other *PcG* genes.[21] To the best of our knowledge, several studies have reported the role and association of the *EPC1* gene in malignant tumors. For example, *EPC1* and *EPC2* (OMIM: 611000) are components of a complex that inhibits *MYC* (OMIM: 190080) accumulation and apoptosis, thereby maintaining

carcinogenic potential.[22] *EPC1* is a major regulator of the DNA damage response. *EPC1* silences death and activates metastasis-associated gene signals by interacting with *E2F1*. The synergy between *EPC1* and *E2F1* triggers metastasis-associated gene signatures in advanced tumors, which predicts a poor survival prognosis.[10] Genomic studies in pancreatic adenocarcinoma (OMIM: 260350) discovered abnormalities in genes of axon-oriented pathways, including *EPC1* and *ARID2*(OMIM: 609539) genes.[23] RNA interference inhibition or silencing of *EPC1* inhibited cell proliferation and tumor growth in lung cancer (OMIM: 211980) patients.[24] These results suggest that the *EPC1* gene plays an important role in the pathogenesis of malignant tumors.

We used qRT-PCR to detect *EPC1* expression in the tissues of 35 NPC patients and paired normal nasopharyngeal tissues. The results showed that *EPC1* was highly expressed in the tumor tissues, suggesting that *EPC1* may be associated with NPC. However, the mechanism of *EPC1* in the occurrence and development of NPC has not been studied and deserves further investigation.

Invasion and metastasis are the main biological features of malignant tumors and are the main factors leading to the death of patients with malignant tumor. Tumor invasion and metastasis are complex multistep processes regulated by the microenvironment of tumor cells and a series of signaling pathways.[25] Cell migration is a key step in the process of malignant tumor invasion and metastasis when cells leave the original position to a new position after sensing a substance concentration gradient or receiving a certain signal. In this study, qRT-PCR was used to detect the expression of *EPC1* in NPC cell lines and normal nasopharyngeal epithelial cells (NP69). The results showed that *EPC1* expression was significantly upregulated in these NPC cell lines compared to NP69. To determine the carcinogenic role of *EPC1* in NPC, we designed an *EPC1*-knockout NPC cell line. The results showed that after *EPC1* was knocked out, the relative expression levels of *EPC1* in NPC cells decreased, and the growth, invasion, and migration of NPC cells were inhibited. These results suggest that downregulation of *EPC1* can effectively inhibit the growth of NPC cells and induce cell apoptosis, thus suppressing the invasion and metastasis of NPC cells.

EMT refers to the transition of epithelial cell from polarized epithelial cells to motile mesenchymal cells mediated by a series of activation signals.[26] Epithelial cells acquire the basic morphogenetic process of mesenchymal phenotypes, which modify the adhesion molecules expressed by the cells, resulting in migration and invasion behaviors of epithelial cells. Multiple data indicate that EMT plays a key role in tumor development. In tumors, EMT can be activated and promoted by a variety of carcinogenic signaling pathways, tumor microenvironment signals and hypoxia, resulting in loss of cell polarity and intercellular adhesion of epithelial cells and thus, yielding migratory and invasive freatures.[27] EMT is activated and regulated by specific microenvironmental factors, endogenous triggering factors, and a complex network of signaling pathway, which mainly include epigenetic events that affect protein translational control factors and proteases.[28] Down regulation of E-cadherin is considered an indicator of poor prognosis of cancer. Many tumors are characterized by incomplete EMT. Tumor cells have mesenchymal characteristics but retain epithelial markers, especially E-cadherin. In cells with a hybrid epithelial-mesenchymal phenotype, E-cadherin accumulates in adhesion junctions, whereas in normal epithelial cells, the adhesion junctions of E-cadherin are less stable than adhesion junctions of cells with a hybrid phenotype. Adhesion junction based on E-cadherin is crucial for the migration, invasion and survival of cancer cells. The plasticity of hybrid epithelial mesenchymal phenotype improves the adaptability of tumor cells. By undergoing EMT, cancer cells become resistance to chemotherapy[29] and obtain the ability to suppress immune responses. To investigate the factors underlying the inhibition of NPC cell invasion and metastasis by *EPC1*, *EPC1* was knocked down in NPC cells, and the EMT-related proteins E-cadherin and vimentin were detected. Inhibition of *EPC1* was confirmed to result in increased expression of E-cadherin and decreased expression of vimentin, suggesting that inhibition of *EPC1* can inhibit EMT in NPC cells.

In conclusion, *EPC1* expression is upregulated in NPC tissues and NPC cell lines, and low *EPC1* expression is associated with a poor prognosis. Gene knockout of *EPC1* can effectively inhibit the growth of NPC cells and induce apoptosis, thereby inhibiting the invasion and metastasis of NPC cells. Inhibition of *EPC1* resulted in increased expression of E-cadherin and decreased expression of vimentin, suggesting that *EPC1* promoted invasion and metastasis of NPC cells. The above findings provide a basis for the role of *EPC1* in promoting NPC, but how does *EPC1* play a role in the occurrence and development of NPC? Does *EPC1* participate in the radiotherapy resistance of NPC and what are the possible mechanisms? The above questions deserve further studies.

Authors' Contribution

LC and TC designed this study. YD, WC, CH, SL and LX were involved in the treatment and management of the patients. YD, JH, JX and QC were involved in the conception of the manuscript. YD, WC and GJ were involved in the drafting of the manuscript or critical revision of the manuscript for important intellectual content. All authors reviewed the manuscript and approved the final version.

Conflict of Interest Disclosures

None.

EthicalStatement

The study protocol was approved by the local medical ethics committee of Fujian Provincial Hospital, Fujian, China. The study was conducted at Fujian Provincial Hospital, Fujian, China between 2018 and 2019.

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