

UNC119 promotes the malignant progression of nasopharyngeal carcinoma cells by regulating Wnt/ β -catenin pathway

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ABSTRACT: UNC119, also known as human retinal gene 4 (HRG4), has been found to contribute to the tumorigenesis of hepatocellular carcinoma. However, the role and mechanism of UNC119 in nasopharyngeal carcinoma has not been systematically investigated yet. Data from western blot and RT-qPCR assays showed that UNC119 was elevated in nasopharyngeal carcinoma cells and tissues. Functional assays demonstrated that transfection with siRNA targeting UNC119 reduced cell viability and suppressed proliferation, invasion, and migration of nasopharyngeal carcinoma cells. Moreover, silence of UNC119 decreased the protein expressions of N-cadherin and vimentin while, on the other hand, increased the protein expressions of E-cadherin and zonula occludens-1 (ZO-1) to suppress epithelial-mesenchymal transition in nasopharyngeal carcinoma. The protein expressions of Axin2 and adenomatous polyposis coli (APC) were up-regulated, while β -catenin and matrix metalloproteinase-7 (MMP-7) were down-regulated by silence of UNC119 in nasopharyngeal carcinoma cells. In conclusion, knockdown of UNC119 suppressed cell growth and metastasis, and repressed epithelial-mesenchymal transition in nasopharyngeal carcinoma through inactivation of Wnt/ β -catenin pathway.

KEYWORDS: UNC119, metastasis, epithelial-mesenchymal transition, nasopharyngeal carcinoma, Wnt/ β -catenin

INTRODUCTION

Nasopharyngeal carcinoma [1] and laryngeal or hypopharyngeal cancer [2] are throat cancers with highly prevalence in Southeast Asia and Southern China. Although nasopharyngeal carcinoma cells are sensitive to radiation, chemotherapy and radiotherapy are the preferred strategy for its treatment, and the 5-year survival rate of patients with this cancer at early stage is up to 80% [3]. However, radiotherapy often leads to serious adverse effects, and patients with distant metastasis and local recurrence are resistant to chemotherapy and radiotherapy [4]. Therefore, the strategies to suppress nasopharyngeal carcinoma metastasis are essential for the individualized clinical treatment of patients with metastatic nasopharyngeal carcinoma and the improvement of poor prognosis.

UNC119, also known as human retinal gene 4 (HRG4), was firstly identified as a photoreceptor synaptic protein [5]. UNC119 was implicated in the pathogenesis of retinal degeneration [6]. Recently, UNC119 was found to be up-regulated in hepatocellular carcinoma tissues, and the knockdown of UNC119 suppressed cell proliferation and induced cell cycle arrest at G0/G1 stage in hepatocellular carcinoma [7]. Over-expression of UNC119 enhanced cell proliferation and metastasis in hepatocellular carcinoma through the Wnt/ β -catenin and TGF- β /EMT signaling pathways [8]. Interestingly, low UNC119 expression was associated with poor prognosis of patients,

and UNC119 interacted with Ras-association domain family 6 to promote cell apoptosis [9]. UNC119 functioned as a tumor suppressor through antagonism of Ras-association domain family 6/MDM2/p53-mediated malignant transformation [10]. Nonetheless, the role of UNC119 in nasopharyngeal carcinoma progression remains unknown.

The Wnt/ β -catenin pathway was activated in nasopharyngeal carcinoma to promote cell proliferation and metastasis [11]. Suppression of Wnt/ β -catenin pathway could ameliorate tumorigenesis in nasopharyngeal carcinoma [12]. Considering that UNC119 promoted the activation of Wnt/ β -catenin during the progression of hepatocellular carcinoma [7, 8], UNC119 was hypothesized to be an oncogene in nasopharyngeal carcinoma through activation of Wnt/ β -catenin.

In this study, the expression levels of UNC119 in nasopharyngeal carcinoma tissues and cell lines were firstly determined, and loss-of functional assays were then performed to clarify its functional role in nasopharyngeal carcinoma.

MATERIALS AND METHODS

Tissue specimens

This study was in accordance with the standards upheld by the Ethics Committee of Hangzhou Hospital of Zhejiang Medical and Health Group (Approval no. 20210009) and with those of the 1964 Helsinki Dec-

laration and its later amendments for ethical research involving human subjects. Tumor and paracancerous tissues were collected from nasopharyngeal carcinoma patients ($N = 40$), with written informed consents, recruited at the Hangzhou Hospital of Zhejiang Medical and Health Group, whose.

Cell culture and transfection

Three common and widely studied nasopharyngeal carcinoma cell lines (C666-1, SUNE-1, and 5-8F) and nasopharyngeal epithelial cell line (NP69) were purchased from Chinese Academy of Sciences (Shanghai, China), and identified via PCR analysis of HPV18 and STR analysis. Following the test of mycoplasma contamination, cells were cultured in RPMI-1640 medium with 100 U/ml streptomycin-penicillin and 10% fetal bovine serum (Gibco, Carlsbad, CA, USA). siRNA targeting UNC119 (si-UNC119) and the negative control (si-NC) were synthesized by GenePharma (Suzhou, China). C666-1 cell line was transfected with siRNAs by Lipofectamine 2000 (Gibco) for 48 hours before functional assays. Cells treated with Lipofectamine 2000 were regarded as control group.

Cell viability and colony formation assays

After transfection, C666-1 cell line was seeded in 96-well plates and incubated for 24, 48, 72, or 96 h. Cells were incubated with CCK 8 solution (Dojindo, Tokyo, Japan) for 2 h. Absorbance at 450 nm was measured by Microplate Autoreader (Thermo Fisher, Waltham, MA, USA). For cell colony formation assay, C666-1 cell line was seeded in 6-well plates and cultured in the medium for 10 days. Cells were fixed and then stained with crystal violet before measurement under light microscope (Olympus, Tokyo, Japan).

Wound healing and transwell assays

After transfection, C666-1 cell line was seeded in 96-well plates and, then, scratched by a pipette tip for wound healing assay. 24 h later, the wound gaps were photographed under the microscope. For cell invasion assay, C666-1 cells in serum-free medium were added into the upper chamber of Matrigel-coated well (Corning, Tewksbury, MA, USA). Medium containing 10% fetal bovine serum was added to the lower chamber. 24 h later, the methanol-fixed and crystal violet-stained cells were photographed under the microscope.

RT-qPCR

RNAs isolated from nasopharyngeal carcinoma tissues and cells were reverse-transcribed into cDNAs, and then performed with SYBR Green Master (Roche, Mannheim, Germany) to determine the mRNA levels of UNC119. Primers: UNC119 (forward: 5'-GAGGACGTGCTGGGGCT-3' and reverse: 5'-CTGAGTCCATGTCCCGAATC-3') and GAPDH (endogenous control; forward: 5'-ACCACAGTCCATGCCA

TCAC-3' and reverse: 5'-TCCACCACCCTGTTGCTGTA-3'), were used in this study.

Western blot

Protein extracted from nasopharyngeal carcinoma tissues and cells were segregated by SDS-PAGE and, then, transferred onto nitrocellulose membranes. The membranes were blocked by 5% bovine serum albumin, and then incubated with primary antibodies: anti-UNC119 and anti-GAPDH (1:2000); anti-N-cadherin and anti-E-cadherin (1:2500); anti-Vimentin and anti-ZO-1 (1:3000); anti- β -catenin and anti-Axin2 (1:3500); and anti-AP and anti-MMP-7 (1:4000); then, incubated with the corresponding secondary antibodies (1:5000). Chemiluminescence reagent kit (Beoytime Biotechnology, Beijing, China) was used to visualize the protein strips. All the antibodies were purchased from Abcam (Cambridge, MA, USA).

Statistical analysis

All data were expressed as mean \pm SEM, and analyzed by student's *t*-test or one-way analysis of variance. A *p*-value of < 0.05 was considered as statistically significant.

RESULTS

UNC119 was up-regulated in nasopharyngeal carcinoma tissues and cell lines

The mRNA (Fig. 1A) and protein (Fig. 1B) expression levels of UNC119 were firstly found to be elevated in the nasopharyngeal carcinoma tissues compared with the normal tissues. Moreover, nasopharyngeal carcinoma cell lines, C666-1, SUNE-1, and 5-8F also expressed higher levels of UNC119 than nasopharyngeal epithelial cell line NP69 (Fig. 1C,D). C666-1 cell line with the highest expression of UNC119 was used for the functional assays. These results demonstrated that UNC119 might be involved in the progression of nasopharyngeal carcinoma.

Silence of UNC119 repressed proliferation of nasopharyngeal carcinoma cells

C666-1 cell line was transfected with si-UNC119, and the protein expression of UNC119 was significantly reduced compared with the control or the negative control (si-NC) (Fig. 2A). The viability and colonies numbers of C666-1 cells were decreased by si-UNC119 transfection as compared with the si-NC (Fig. 2B,C), suggesting the proliferative effects of UNC119 on nasopharyngeal carcinoma cells.

Silence of UNC119 repressed invasion and migration of nasopharyngeal carcinoma cells

In addition to its anti-proliferative effect, silence of UNC119 also repressed the migratory number of C666-1 cells as compared with the si-NC (Fig. 3A). Moreover, the invasive number of C666-1 cells was also

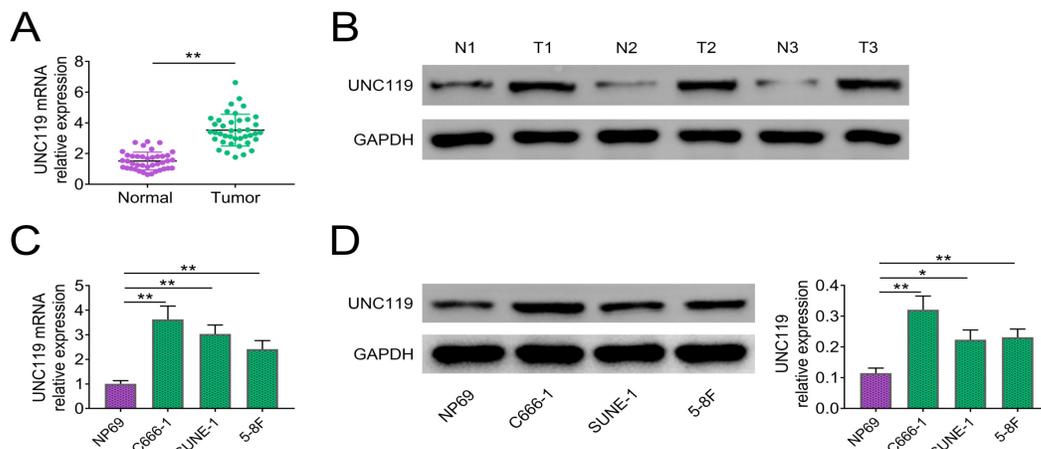


Fig. 1 UNC119 was up-regulated in the nasopharyngeal carcinoma tissues compared with the normal tissues: (A) UNC119 mRNA and (B) UNC119 protein; and in the nasopharyngeal carcinoma cell lines (C666-1, SUNE-1, 5-8F) compared with the nasopharyngeal epithelial cell line (NP69): (C) UNC119 mRNA and (D) UNC119 protein. * $p < 0.05$, ** $p < 0.01$.

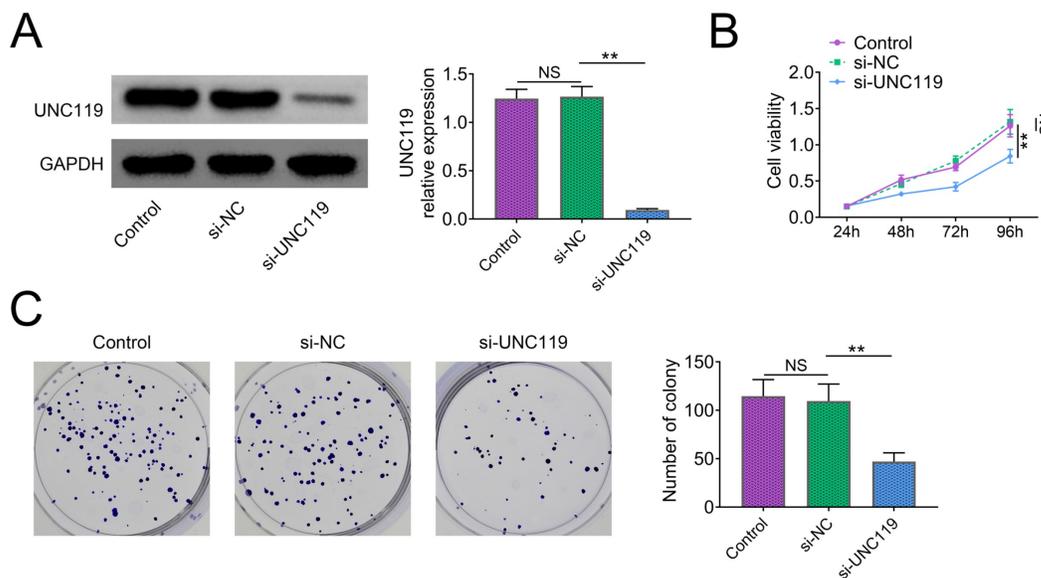


Fig. 2 Silence of UNC119 repressed cell proliferation of nasopharyngeal carcinoma. Transfection with si-UNC119: (A) decreased protein expression of UNC119; (B) reduced C666-1 cell viability; and (C) suppressed C666-1 cell proliferation. ** $p < 0.01$.

suppressed by knockdown of UNC119 as compared with the si-NC (Fig. 3B), indicating the metastatic role of UNC119 in nasopharyngeal carcinoma.

Silence of UNC119 repressed epithelial-mesenchymal transition of nasopharyngeal carcinoma

Knockdown of UNC119 decreased the protein expressions of N-cadherin and Vimentin but enhanced the protein expressions of E-cadherin and ZO-1 in C666-1 cell line (Fig. 4). These results suggested that UNC119

contributed to epithelial-mesenchymal transition of nasopharyngeal carcinoma.

Silence of UNC119 suppressed the activation of Wnt/ β -catenin pathway

The protein expressions of β -catenin and MMP-7 were reduced by the knockdown of UNC119 in C666-1 cell line (Fig. 5). However, Axin2 and APC were up-regulated by the knockdown (Fig. 5), revealing that UNC119 contributed to the activation of Wnt/ β -

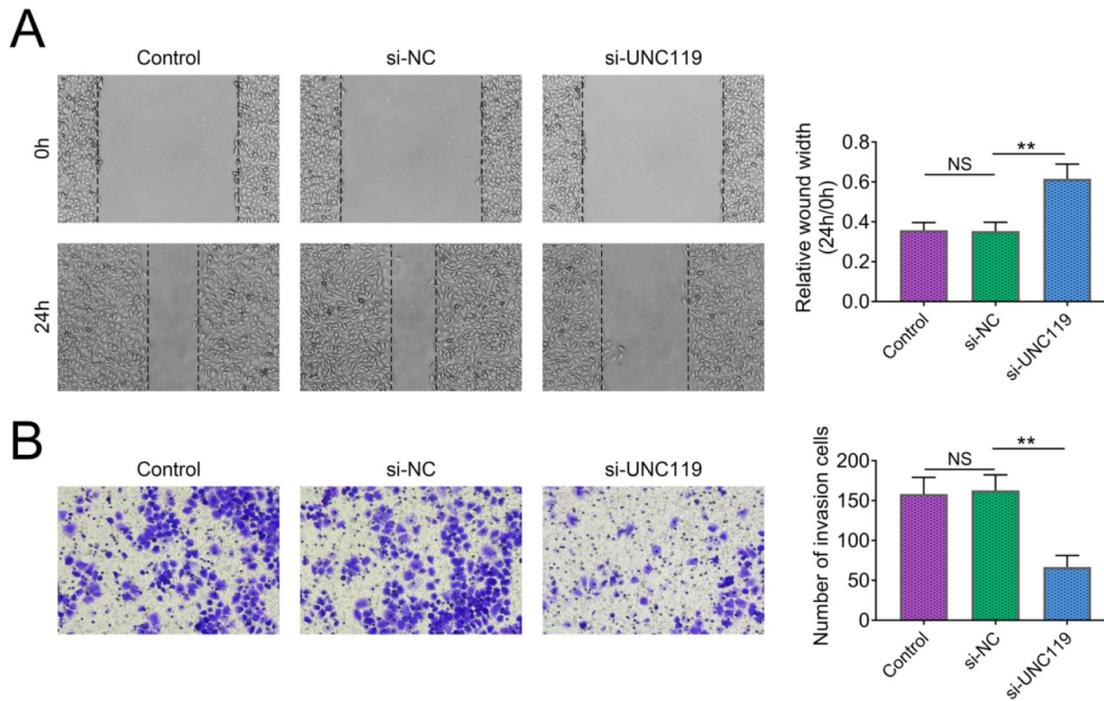


Fig. 3 Silence of UNC119 repressed invasion and migration of nasopharyngeal carcinoma cells. Transfection with si-UNC119 suppressed C666-1 cell: (A) migration and (B) invasion. ** $p < 0.01$.

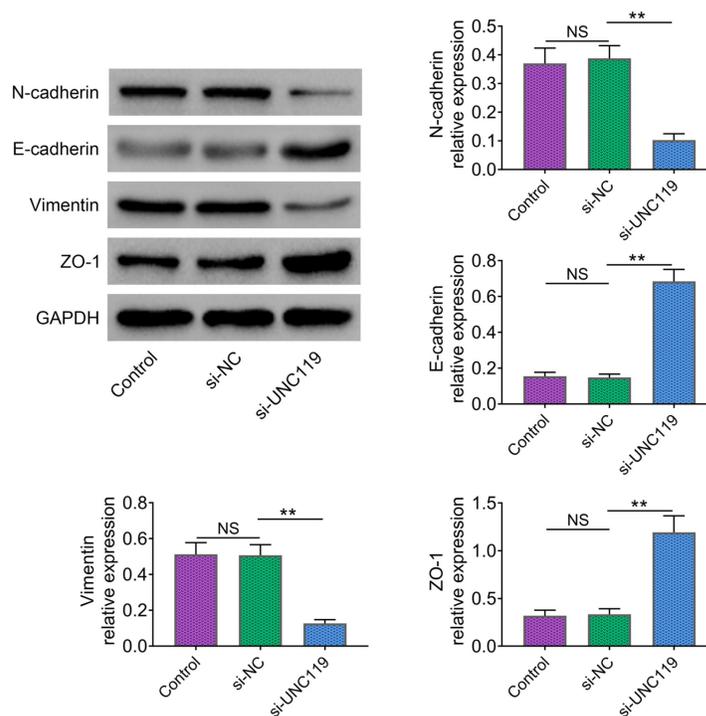


Fig. 4 Silence of UNC119 repressed epithelial-mesenchymal transition of nasopharyngeal carcinoma. Transfection with si-UNC119 enhanced the protein expressions of E-cadherin and ZO-1, and reduced the protein expressions of N-cadherin and Vimentin in C666-1 cell line. ** $p < 0.01$.

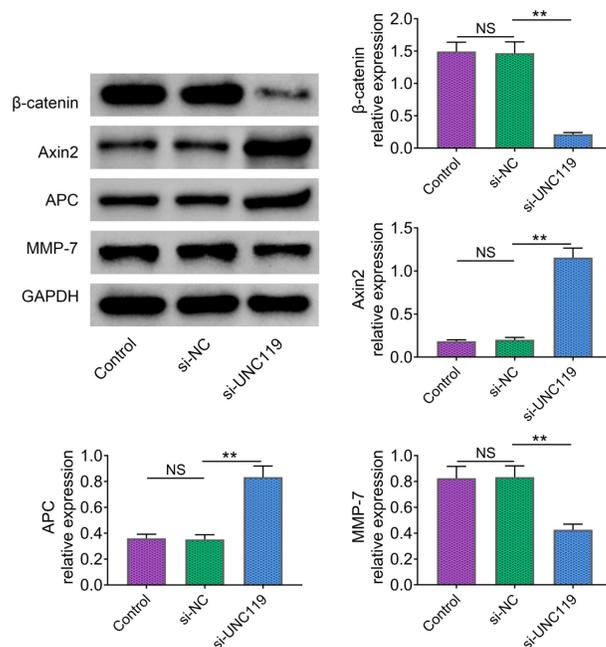


Fig. 5 Silence of UNC119 suppressed the activation of Wnt/ β -catenin pathway. Transfection with si-UNC119 enhanced the protein expressions of Axin2 and APC, and reduced the protein expressions of β -catenin and MMP-7 in C666-1 cell line. ** $p < 0.01$.

catenin pathway in nasopharyngeal carcinoma cells.

DISCUSSION

Nasopharyngeal carcinoma tends to metastasize at the advanced stage, and the distant metastasis increases the mortality rate of patients [13]. Targeted therapies, such as EGFR1 and C-kit, have been clinically investigated to prevent metastatic nasopharyngeal carcinoma [14]. UNC119 was found to act as either an oncogene [7, 8] or tumor suppressor in various tumors [9, 10]. The exact role of UNC119 in the metastasis of nasopharyngeal carcinoma was, therefore, investigated in this study.

Here, UNC119 was elevated in nasopharyngeal carcinoma tissues and cell lines. Although high UNC119 expression was not significantly associated with TNM stage, tumor size, and liver cirrhosis in patients with hepatocellular carcinoma; it predicted poor prognosis in patients with hepatocellular carcinoma [7]. Correlation between UNC119 expression and clinicopathological parameters of patients with nasopharyngeal carcinoma should be further investigated to verify whether UNC119 was a metastasis-associated biomarker or prognostic biomarker of nasopharyngeal carcinoma.

Loss-of functional assays showed that silence of UNC119 reduced the cell viability and suppressed the proliferation, the migration, and the invasion of nasopharyngeal carcinoma cells, suggesting that

UNC119 might be a therapeutic target for its treatment. Epithelial-mesenchymal transition is considered to be a key process of metastasis in nasopharyngeal carcinoma [15], of which the epithelial markers (including E-cadherin and ZO-1) were down-regulated and the mesenchymal markers (including N-cadherin and Vimentin) were up-regulated [16]. As a result, the epithelial-to-mesenchymal transition-induced metastasis in nasopharyngeal carcinoma was attenuated [17]. UNC119 has been shown to induce epithelial-to-mesenchymal transition and promote the migration of hepatocellular carcinoma [8]. Knock-down of UNC119 in this study reduced the protein expressions of N-cadherin and Vimentin, and enhanced the protein expressions of E-cadherin and ZO-1 to suppress epithelial-to-mesenchymal transition in nasopharyngeal carcinoma.

Upon binding with Wnt ligands, the Wnt/ β -catenin pathway was initiated, and the downstream targets were then activated by T-cell factor/lymphoid enhancer factor, thus involving in tumor metastasis [18]. β -catenin pathway also interacts with TGF- β , epithelial-mesenchymal transition, and other pathways to promote self-renewal networks and inhibit tumor suppressive pathways during the development of nasopharyngeal carcinoma [19]. Inhibition of Wnt/ β -catenin pathway in nasopharyngeal carcinoma also suppressed tumor cell growth and arrested tumor cell stemness [20]. In particular, Wnt/ β -catenin pathway

was activated by UNC119 in hepatocellular carcinoma [7,8]. Here, results showed that β -catenin was decreased by the knockdown of UNC119 in nasopharyngeal carcinoma cells. Moreover, MMP-7, a transcriptional target of Wnt/ β -catenin pathway, was also reduced by the knockdown. However, the knockdown of UNC119 increased the expression of Wnt suppressor, Axin2 and promoted the destruction of β -catenin APC complex components in nasopharyngeal carcinoma cells, thereby inhibiting tumor metastasis. However, since MDM2/p53-mediated tumor cell apoptosis was promoted by interaction between UNC119 and tumor suppressor Ras-association domain family 6 [9,10], the UNC119 effect on Ras-association domain family 6/MDM2/p53 axis during nasopharyngeal carcinoma progression should be further investigated.

Taken together, UNC119 functioned as an oncogene in nasopharyngeal carcinoma, and knockdown of UNC119 suppressed viability, colony formation, invasion, and migration of nasopharyngeal carcinoma cells through inactivation of Wnt/ β -catenin pathway. These results implied that UNC119 might be a crucial therapeutic target for the treatment of nasopharyngeal carcinoma.

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