FEBS Letters 588 (2014) 3732–3738

journal homepage: www.FEBSLetters.org

# MicroRNA-320a inhibits cell proliferation, migration and invasion by targeting BMI-1 in nasopharyngeal carcinoma

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ARTICLE INFO

*Article history:*
Received 2 August 2014
Revised 18 August 2014
Accepted 18 August 2014
Available online 27 August 2014

Edited by Tamas Dalmay

*Keywords:*
miR-320a
BMI-1
Proliferation
Invasion
Nasopharyngeal carcinoma

ABSTRACT

**In the present study, we investigated the roles and molecular mechanisms of miR-320a in human nasopharyngeal carcinoma (NPC). miR-320a expression was strongly reduced in NPC tissues and cell lines. Overexpression of miR-320a significantly suppressed NPC cell growth, migration, invasion and tumor growth in a xenograft mouse model. A luciferase reporter assay revealed that miR-320a could directly bind to the 3′ UTR of BMI-1. Overexpression of BMI-1 rescued miR-320a-mediated biological function. BMI-1 expression was found to be up-regulated and inversely correlated with miR-320a expression in NPC. Collectively, our data indicate that miR-320a plays a tumor suppressor role in the development and progression of NPC and may be a novel therapeutic target against NPC.**


## 1. Introduction

Nasopharyngeal carcinoma (NPC) is a malignant squamous cell carcinoma arising from epithelial cells located in the nasopharynx, which is a leading form of cancer in Southeast Asia and Middle East/North Africa [1]. Moreover, the incidence of NPC in such regions remains quite high. When treated with radiation therapy and adjuvant chemotherapy, NPC responds well and the 5-year overall survival rate is approximately 70% [2,3]. However, NPC can invade local tissues easily and within 4 years, 30–40% of patients will develop distant metastases with poor prognosis [4]. Therefore, an improved understanding of the molecular basis of pathogenesis is needed for the development of more effective therapies for NPC.

MicroRNAs (miRNAs) are evolutionarily conserved, small non-coding RNAs that play regulatory roles by binding to the 3′-untranslated region (3′-UTR) of target mRNAs in a sequence-specific manner [5,6]. miRNAs have the potential to influence almost every cellular process, such as proliferation, apoptosis, differentiation, invasion and metabolism [7,8]. Moreover, accumulating evidence has shown that miRNAs play critical roles in the regulation of cancer initiation and progression [9]. A variety of miRNAs have been found to be dysregulated in NPC, such as miR-663, miR-144, miR-26a, miR-451 and miR-9 [10–14], which function as oncogenes or tumor suppressors depending on their targets. Human miR-320a has been reported to be involved in progression of several cancers [15–17], but its specific roles and mechanisms in NPC have not been well established.

In the present study, the expression levels of miR-320a were found to be significantly down-regulated in NPC tissues and cell lines. NPC cell proliferation, migration, invasion and tumor growth in the xenograft mouse model were all strongly suppressed when miR-320a was overexpressed. Furthermore, BMI-1 was found to be a direct target gene of miR-320a, and overexpression of BMI-1 could rescue miR-320a-induced biological functions. In addition, a negative correlation between the expression of miR-320a and BMI-1 was found in NPC specimens. Collectively, our data indicate that miR-320a plays a tumor suppressor role in the development and progression of NPC and may be a novel molecular therapeutic target against NPC.

## 2. Materials and methods

### 2.1. Clinical specimens and cell culture

Sixteen primary NPC biopsy specimens and ten normal nasopharyngeal epithelial specimens were collected from patients at the 88th Hospital of PLA at the time of surgery and immediately

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http://dx.doi.org/10.1016/j.febslet.2014.08.021
0014-5793/

stored in liquid nitrogen until use. All patients provided written informed consent for the use of these clinical materials in research, and the project was approved by the Institutional Ethics Committee. The human NPC cell lines CNE1, CNE2, C666-1, HONE1 and SUNE1 were cultured in RPMI-1640 medium supplemented with 10% FBS. The nasopharyngeal epithelial cell line NP69 was cultured in keratinocyte-SFM (Life Technologies, Inc., Grand Island, NY) supplemented with bovine pituitary extract (BD Biosciences, San Diego, CA) as described previously [12]. All cell lines were cultured in a humidified incubator in an atmosphere of 5% (v/v) $CO_2$ at 37 °C.

### 2.2. RNA isolation and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated from cell lines and tumor samples using the miRVANA Kit (Ambion, Carlsbad, MA) according to the manufacturer's protocols. Complementary DNA synthesis was performed using the PrimeScript RT Reagent Kit (TaKaRa, Osaka, Japan). miRNA was converted to cDNA using the TaqMan microRNA reverse transcription kit (Applied Biosystems, Foster City, CA). The expression levels of miR-320a were determined using the Taqman miRNA assay kit (Applied Biosystems) according to the manufacturer's instructions, and calculated by normalization to the signal for U6 expression using the $2^{-\triangle\triangle Ct}$ method. The expression levels of BMI-1 (forward: 5′-ACTGGAAAGTGACTCTGGGA-3′; reverse 5′-TACTGGGGCTAGGCAAACAA-3′) were evaluated using SYBR green PCR master mix (Applied Biosystems) and normalized to β-actin. All of the reactions were run in triplicate.

### 2.3. Western blotting

Cells were lysed in RIPA buffer containing protease inhibitor cocktail (Calbiochem, La Jolla, CA). After electrophoresis on a 10% SDS–PAGE gel, proteins were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% milk and incubated with primary antibodies against BMI-1 and β-actin (Abgent, San Diego, CA) at 4 °C overnight. The corresponding horseradish peroxidase (HRP)-conjugated secondary antibody was added and incubated at room temperature for 1 h. Signals were visualized after chemiluminescence reaction with HRP substrate.

### 2.4. Lentivirus transduction

The lenti-viral system with EGFP expressing miR-320a (lenti-miR-320a) and the negative control lenti-vector (lenti-NC) were purchased from Genechem (Shanghai, China). The NPC cell lines C666-1 and SUNE1 were infected with lenti-miR-320a or lenti-NC according to the manufacturer's protocol.

### 2.5. Plasmid construction

The plasmid pcDNA-BMI-1 was constructed by inserting the BMI-1 CDS (Proteintech Group, Inc., Chicago, IL) into the pcDNA3.1(+) vector (Life Technologies, Inc.) at *Bam*H I and *Xba* I sites. A 370 bp fragment of the BMI-1 3′-UTR (position 1-370) containing the predicted binding site of miR-320a was amplified by PCR and cloned into pGL3 Basic vector (Promega, Madison, WI). The corresponding mutant constructs were created by mutating the seed region of the miR-320a-binding site using a site-directed mutagenesis kit (SBS Genetech, Beijing, China). The constructs were confirmed by sequencing.

### 2.6. Cell proliferation and colony formation assays

Cell proliferation was determined by MTT assay according to a standard method as described previously [18]. In brief, infected or transfected cells seeded into 96-well plates ($2 \times 10^3$/well) were stained at the indicated time points with 100 μL sterile MTT dye for 4 h. The absorbance at 570 nm of each well was measured using a microplate reader, with 655 nm as the reference wavelength. For colony formation assay, 500 cells were seeded into each well of 6-well plates. After 10 days in culture, the cells were fixed using 70% ethanol and stained with 1% crystal violet solution for 20 min to visualize colonies for counting.

### 2.7. In vitro migration and invasion assays

For the invasion assay, the transwell system (24 wells, 8 μm pore size with poly-carbonate membrane) and Matrigel (BD Biosciences) were used according to the manufacturer's protocols. Aliquots of $1 \times 10^5$ cells were seeded into the upper chambers precoated with Matrigel and cultured in serum-free RPMI-1640 medium. The lower compartment was filled with RPMI-1640 with 10% FBS as a chemoattractant. After incubation for 24 h, the cells remaining in the upper chamber were removed, and the cells at the bottom of the insert were fixed, stained in 0.5% crystal violet and counted under a microscope (Olympus Corp., Tokyo, Japan). The results were averaged over three independent experiments. For the migration assay, cells were seeded into the upper chambers without a Matrigel coating. The rest of the assay was carried out in the same way as the invasion assay.

### 2.8. Luciferase reporter assay

The luciferase reporter assay was performed as described previously [19]. In brief, the NPC cell lines C666-1 and SUNE1 were transfected with 100 ng pGL3-BMI-1-3′ UTR (WT/MUT) plasmid and 1 ng pRL-TK vector (Promega) containing Renilla luciferase in combination with miRNA (100 nM) using Lipofectamine 2000 (Life Technologies, Inc.). Luciferase activity was measured 48 h after transfection using the dual luciferase reporter assay kit (Promega) according to the manufacturer's protocol. All transfection experiments were conducted in triplicate and repeated three times independently.

### 2.9. In vivo tumor growth assay

The established lenti-NC and lenti-miR-320a cells ($1 \times 10^6$) were collected and injected subcutaneously into the right lateral axilla region of BALB/c nude mice (6 mice per group). Tumor size was measured every 3 days using vernier calipers. After 26 days, mice were killed and tumors were dissected and weighed. Tumor volumes were calculated using the formula: length $\times$ width$^2$ $\times$ 0.5. Animal handling and experimental procedures were approved by the Animal Experimental Ethics Committee of the 88th Hospital of PLA.

### 2.10. Statistical analysis

All data are expressed as the means ± S.D. from at least 3 independent experiments. The relationship between miR-320a expression and BMI-1 mRNA expression level was analyzed using Spearman's correlation. The statistical significance of differences between groups was evaluated using Student's *t*-test and a *P*-value less than 0.05 was considered statistically significant.

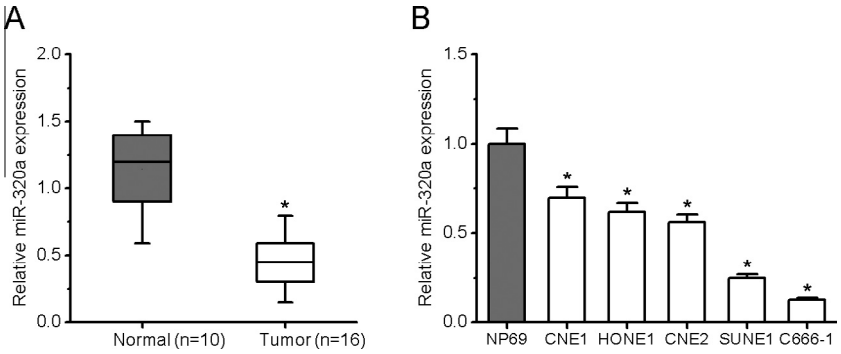


**Fig. 1.** The levels of miR-320a in NPC tissues and cell lines. (A) Relative miR-320a expression was examined by qPCR in NPC specimens and normal nasopharyngeal epithelial tissues. (B) Relative miR-320a expression in NPC cell lines. U6 was used as the endogenous control for miR-320a. Data are expressed as mean ± S.D. of three independent experiments ($^*P < 0.05$).

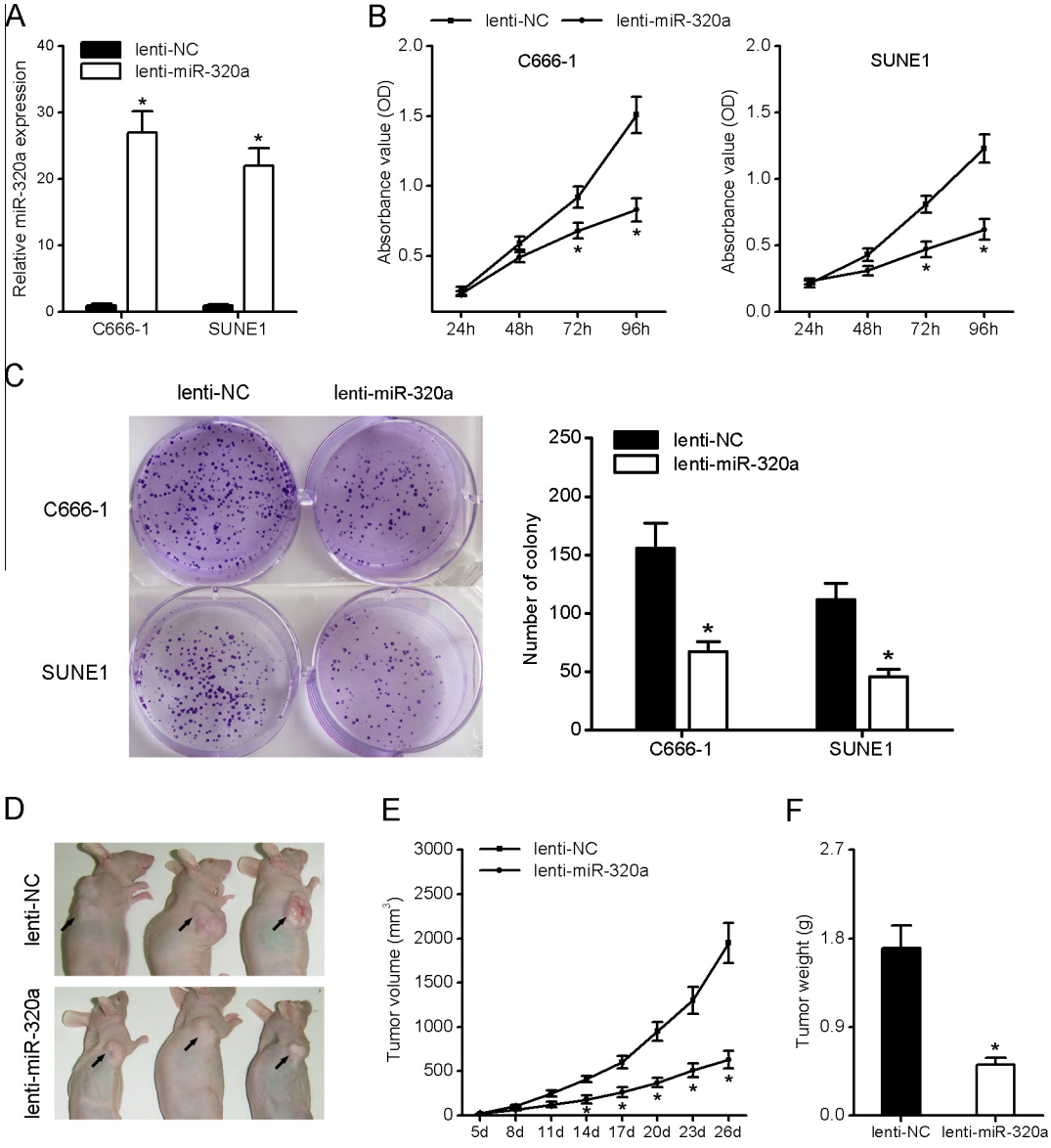


**Fig. 2.** Overexpression of miR-320a inhibits NPC cell proliferation in vitro and in vivo. C666-1 and SUNE1 cells infected with lentivirus encoding pre-miR-320a were used in these studies. (A) Relative miR-320a expression was examined by qPCR. (B) Cell viability was examined by MTT assay. (C) Cell growth capacity was evaluated using the colony formation assay. (D) Flank tumors were established in nude mice (6 mice per group) as described in materials and methods. Representative images of tumors are shown. (E) Tumor volume was measured at the indicated days. (F) After 26 days, mice were killed, and tumor weight was calculated. Data are expressed as mean ± S.D. of three independent experiments ($^*P < 0.05$).

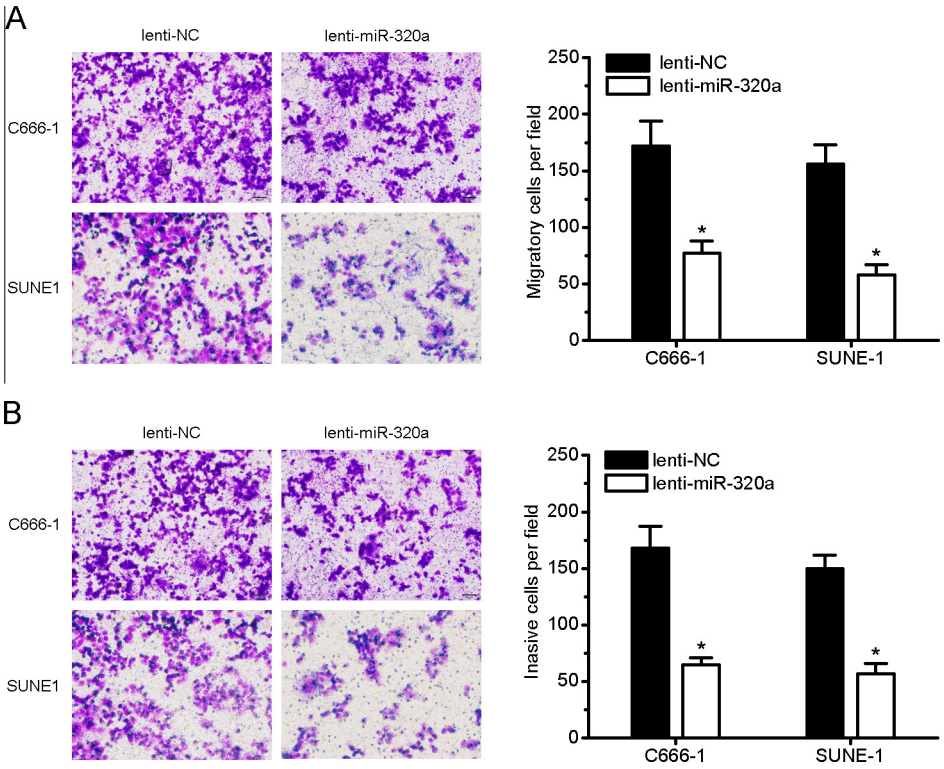


**Fig. 3.** Overexpression of miR-320a suppresses NPC cell migration and invasion. (A) Migration and (B) invasion of C666-1 and SUNE1 cells stably overexpressing miR-320a were evaluated using the Transwell migration and Matrigel invasion assay. Data are expressed as mean ± S.D. of three independent experiments (*$P < 0.05$).

## 3. Results

### 3.1. Levels of miR-320a are frequently lower in NPC cell lines and tissues

First, we analyzed the expression patterns of miR-320a by qRT-PCR in 16 NPC and 10 normal nasopharyngeal epithelial specimens. The results showed that miR-320a was frequently down-regulated in NPC tissues compared with normal nasopharyngeal epithelial specimens (Fig. 1A). Moreover, the expression levels of miR-320a were also decreased in all 5 NPC cell lines examined, particularly in SUNE1 and C666-1, when compared with the nasopharyngeal epithelial cell line NP69 (Fig. 1B).

### 3.2. Overexpression of miR-320a suppresses NPC cell proliferation in vitro and in vivo

To explore whether miR-320a has functional effects on NPC cell proliferation, we stably infected C666-1 and SUNE1 cells with lentivirus containing pre-miR-320a. The highly up-regulated expression of miR-320a was confirmed by qPCR (Fig. 2A). MTT assay showed that the proliferation rates of C666-1 and SUNE1 stably overexpressing lenti-miR-320a were significantly decreased when compared with those of lenti-NC infected cells (Fig. 2B). Similar results were also observed in the colony formation assay, suggesting a proliferation-inhibiting role of miR-320a in NPC cells (Fig. 2C). To further determine whether miR-320a was associated with tumorigenesis, C666-1 cells stably overexpressing miR-320a were injected into nude mice. As indicated in Fig. 2D and E, the tumors in mice bearing lenti-miR-320a grew more slowly than those from lenti-NC cells within 14 days after inoculation, and this difference continued to increase until the endpoint of the test. When the tumors were harvested, the average weight of the tumors from the lenti-miR-320a group was also significantly reduced compared with that of the lenti-NC group (Fig. 2F). Taken together, these results indicate that miR-320a inhibits NPC cell proliferation in vitro, and suppresses tumorigenesis in vivo.

### 3.3. Overexpression of miR-320a reduces migration and invasion of NPC cells

To study the contribution of miR-320a to NPC cell migration and invasion, transwell migration and Matrigel invasion assays were performed. As shown in Fig. 3A, when infected with lenti-miR-320a, the cell migration capability of both C666-1 and SUNE1 cell lines was reduced significantly. Their capacity for invasion was also obviously reduced in both cell lines infected with lenti-miR-320a compared with those infected with lenti-NC (Fig. 3B). These results indicate that miR-320a reduces NPC cell migration and invasion.

### 3.4. miR-320a directly targets BMI-1

To identify direct targets of miR-320a for regulating NPC cell proliferation and invasion, the two most-used public bioinformatic algorithms, TargetScan and miRanda, were used in combination. As shown in Fig. 4A, BMI-1 (B lymphoma mouse Moloney leukemia virus insertion region 1), a polycomb gene family member, is theoretically a potential target gene of miR-320a, and the predicted binding site between miR-320a and BMI-1 3′-UTR is also illustrated. Western blot analysis showed that the expression level of BMI-1 was significantly decreased in both C666-1 and SUNE1 cell lines infected with lenti-miR-320a compared with those infected with lenti-miR-NC (Fig. 4B). Consistently, qPCR identified reduced expression of BMI-1 targeted by miR-320a (Fig. 4C). To further test whether BMI-1 is a direct target of miR-320a, the 3′-UTR fragments of BMI-1 which contain a wild-type or mutant binding site of miR-320a were subcloned into the luciferase reporter vector. As shown in Fig. 4D, overexpression of miR-320a in both C666-1 and SUNE1 cell lines significantly reduced the luciferase activity of BMI-1

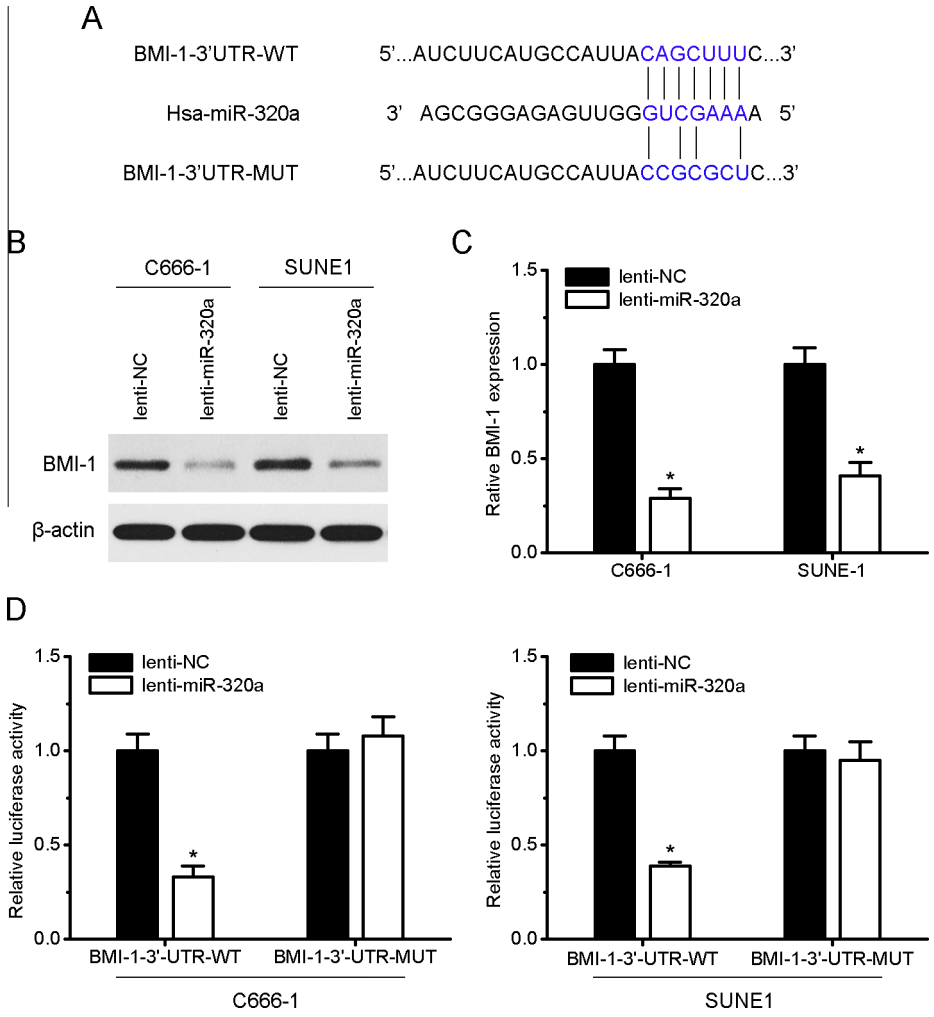


**Fig. 4.** BMI-1 is a downstream target of miR-320a. (A) Sequence alignment of a putative miR-320a binding site in the 3′-UTR of BMI-1 mRNA. BMI-1 protein (B) and mRNA expression levels (C) were detected by Western blotting and qPCR, respectively. (D) Luciferase activity of the wild-type (WT) and the mutant (MUT) BMI-1 3′-UTR reporter constructs in the presence of miR-320a. Data are expressed as mean ± S.D. of three independent experiments (*$P < 0.05$).

containing a wild-type 3′-UTR, but did not suppress activity of BMI-1 with a mutant 3′-UTR. These results suggest that miR-320a downregulates BMI-1 expression by directly targeting its 3′-UTR.

### *3.5. The tumor suppressor role of miR-320a is mediated by downregulating BMI-1*

To confirm that the tumor suppressor role of miR-320a in NPC is mediated by repressing the expression of BMI-1, we performed a gain-of-function assay. C666-1 and SUNE1 cell lines stably overexpressing miR-320a were transfected with pcDNA3.1-BMI-1, which encoded the full-length coding sequence without the 3′-UTR region (Fig. 5A). As shown in Fig. 5B, the inhibitory role of miR-320a in proliferation was rescued under the condition of BMI-1 overexpression. Accordingly, similar results could be observed when cell migration and invasion assays were carried out (Fig. 5C and D). These results suggest that a tumor suppressor role of miR-320a in NPC cells is mediated by downregulation of BMI-1.

### *3.6. BMI-1 is up-regulated in NPC specimens and inversely correlated with miR-320a levels*

We further examined the expression levels of BMI-1 in clinical NPC specimens by qPCR. As shown in Fig. 6A, the average expression of BMI-1 was significantly higher in NPC tissues ($n = 16$) than in normal nasopharyngeal tissues ($n = 10$). In addition, correlation analysis revealed that miR-320a levels negatively correlated with levels of BMI-1 in NPC tissues ($r = -0.603$, $P = 0.013$; Fig. 6B).

## 4. Discussion

Previous studies have shown that miR-320a is dysregulated in several cancers and its potential function has also been partly explored in several studies. For example, Zhang et al. found that miR-320a is associated with liver metastasis in colorectal cancer (CRC) and inhibits tumor invasion by targeting neuropilin 1 [16]. Zhao et al. showed similar results, in that miR-320a significantly suppressed CRC cell migration/invasion and induced G0/G1 growth arrest in vitro and in vivo by targeting Rac1 [17]. More recently, Shang and colleagues reported that miR-320a is downregulated in human bladder transitional cell carcinoma specimens and that overexpression of miR-320a inhibits invasion of T24 cells [20]. On the other hand, Yao et al. found that the tumor suppressive role of GNAI1 on hepatocellular carcinoma cell migration and invasion is post-transcriptionally regulated by miR-320a/c/d [21], suggesting that miR-320a exhibits oncogenic properties in this cancer. These contradictory results suggest that miR-320a might be expressed in a tissue-specific pattern and have cell content-dependent functions. Liu et al. recently performed miRNA microarray analysis and the

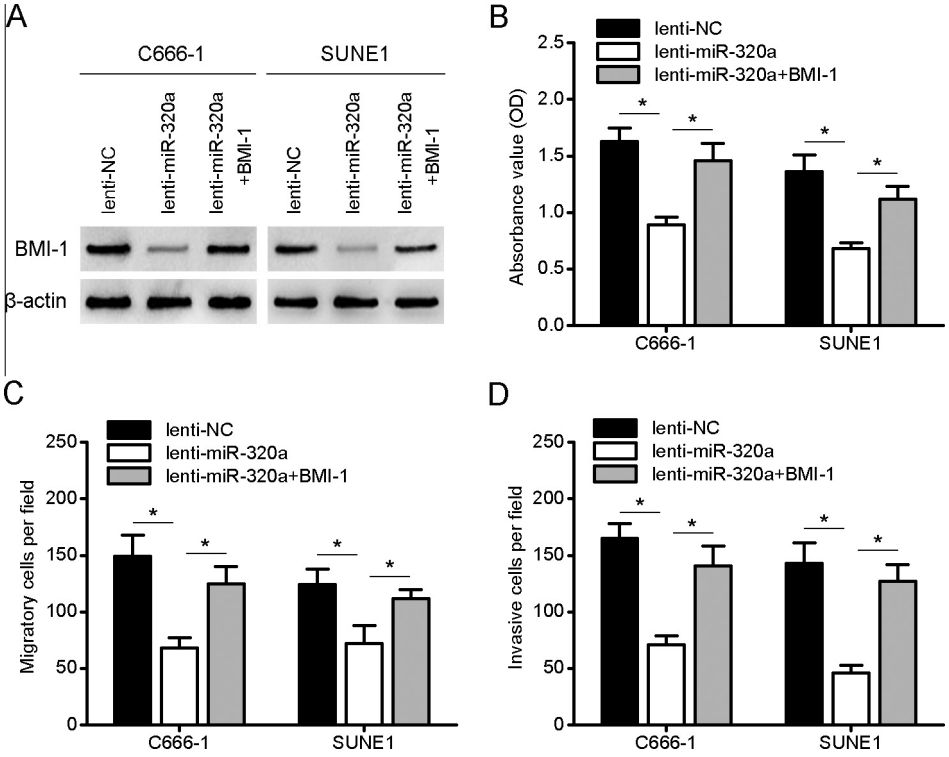


**Fig. 5.** BMI-1 can reverse the suppressive effect of miR-320a. C666-1- and SUNE1-miR-320a cells were transfected with or without pcDNA3.1-BMI-1. (A) BMI-1 protein level was determined using Western blotting. Overexpression of BMI-1 rescued the effects of miR-320a on NPC cell proliferation (B), migration (C) and invasion (D). Data are expressed as mean ± S.D. of three independent experiments (*$P < 0.05$).

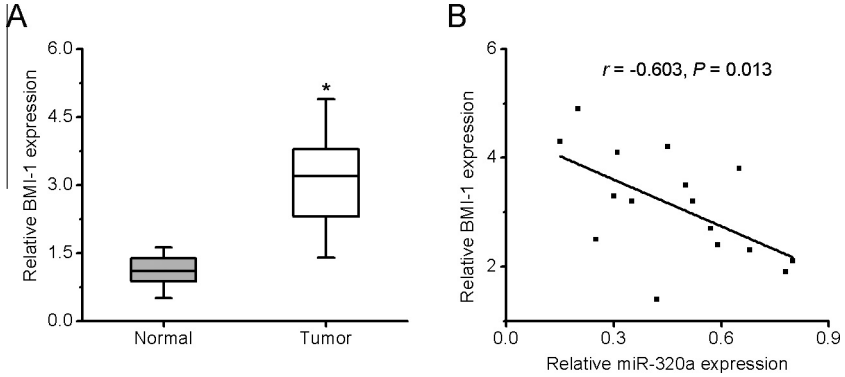


**Fig. 6.** BMI-1 expression is up-regulated in NPC tissues and negatively correlated with miR-320a expression. (A) The miR-320a expression levels in NPC specimens and normal nasopharyngeal tissues were analyzed by qPCR. (B) Spearman's correlation analysis of miR-320a and BMI-1 mRNA levels in NPC tissues ($r = -0.603$; $P = 0.013$). Data are expressed as mean ± S.D. of three independent experiments (*$P < 0.05$).

result showed that miR-320b was significantly downregulated in NPC relative to non-cancer nasopharyngitis tissues, suggesting that miR-320 may play a role in NPC progression [22]. However, to date, no functional evidence of miR-320a in NPC has been documented.

In this study, we found that the expression level of miR-320a was significantly reduced in NPC tissues and cell lines. Overexpression of miR-320a significantly inhibited cell proliferation in both C666-1 and SUNE1 cell lines. Furthermore, Transwell migration and Matrigel invasion assay showed that miR-320a greatly suppresses cell migration and invasive capability. We also explored the role of miR-320a in vivo using a xenograft mouse model and found that tumorigenesis and tumor growth were strongly suppressed when miR-320a was overexpressed. These data suggest a tumor-inhibiting role of miR-320a in NPC cells both in vitro and in vivo.

The mechanism by which miR-320a exerts its influence on the development of NPC was also investigated in this study. Public bioinformatic algorithms predicted BMI-1 to be a theoretical target gene of miR-320a. We found that miR-320a directly targeted the 3′-UTR of BMI-1 mRNA and repressed its expression. Restoring the expression of BMI-1 can reverse the suppressive effects of miR-320a. In addition, we found a negative correlation between the expression of miR-320a and BMI-1 clinically. These data suggest that BMI-1 is a direct and functional target of miR-320a in NPC cells.

BMI-1 is a member of the *Polycomb Group* (*PcG*) family, which is overexpressed in various types of human cancer [23,24]. It also plays an oncogenic role in the development and progression of cancers, including pancreatic cancer, breast cancer, lung cancer, and gliomas [25–29]. Song et al. showed that BMI-1 expression

was markedly up-regulated in pancreatic cancer cell lines and tissues and that stable down-regulation of BMI-1 suppressed cell growth, delayed the G1/S transition, and induced cell apoptosis [26]. Guo et al. showed that BMI-1 promoted invasion and metastasis, and its elevated expression was correlated with an advanced stage of breast cancer [27]. It was previously reported that BMI-1 was overexpressed in NPC cell lines and that high BMI-1 expression positively correlated with poor prognosis of NPC patients [30]. This result is consistent with our findings that BMI-1 is a functional target of miR-320a.

Taken together, our findings suggest that miR-320a is down-regulated in NPC, and functions as a novel tumor suppressor to regulate the proliferation, migration, invasion and tumorigenicity of NPC cells by targeting BMI-1. Therefore, miR-320a may be a novel molecular therapeutic target for the treatment of NPC.

## Conflict of interest

We declare that we have no conflict of interest.

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