

RESEARCH ARTICLE



# MicroRNA-34a inhibits cell invasion and epithelial-mesenchymal transition via targeting AXL/PI3K/AKT/Snail signaling in nasopharyngeal carcinoma

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## Abstract

**Background** MicroRNA-34a (miR-34a) has been reported to inhibit TGF- $\beta$  (transforming growth factor- $\beta$ )-induced epithelial-mesenchymal transition (EMT) in nasopharyngeal carcinoma (NPC). However, the underlying mechanism remain unclear. Using the bioinformatics, we found that the AXL receptor tyrosine kinase (AXL) is a predicted target of miR-34a.

**Objective** we aimed to reveal the relationship between miR-34a and AXL, and investigate the effect and mechanism of miR-34a in NPC progression.

**Methods** The expression patterns of miR-34a and AXL in 30 paired NPC tissues and the adjacent tissues were examined by quantitative real time PCR (qRT-PCR). The target relationship between miR-34a and AXL was evaluated by the luciferase gene reporter assay. Cell migration and invasion were assessed by wound healing and transwell chamber assays, respectively.

**Results** miR-34a level was dramatically decreased in the NPC tissues compared to the adjacent tissues, while AXL expression was increased. Overexpression of miR-34a significantly reduced the luciferase activity of the luciferase vector of AXL (pGL3-AXL-WT), whereas this effect was abrogated when binding sites between miR-34a and AXL were mutated. In addition, ectopic expression of miR-34a dramatically inhibited Sune-1 cell migration and invasion abilities, decreased the levels of N-cadherin and Vimentin and increased E-cadherin and  $\gamma$ -catenin expressions, as well as induced significant reductions in the expressions of p-AKT and Snail. However, these effects were attenuated when the cells were treated with recombinant human AXL protein.

**Conclusions** Our results demonstrate that miR-34a/AXL can inhibit NPC cell migration, invasion and EMT through inhibition of AKT/Snail signaling.

**Keywords** miR-34a · AXL · AKT/Snail signaling · Migration · Invasion

## Introduction

Nasopharyngeal carcinoma (NPC) is a type of tumor derived from the epithelial cells of nasopharynx, with a high morbidity in southern China (Xu et al. 2016). Evidence shows that

the pathogenesis of NPC is complex, which includes dietary, virus infection, genetic susceptibility, and carcinogen hazards (He et al. 2018). At present, radiotherapy combined with chemoradiotherapy remains the primary treatment option for NPC, and has been proved to increase patients' survival (Chen et al. 2010; Cosmopoulos et al. 2009). However, 15 to 30% of patients develop distant metastasis, resulting in low treatment efficiency and poor prognosis (Pratt et al. 2009). Therefore, it is important to further reveal the molecular mechanisms of cancer cell migration and invasion in NPC.

MicroRNAs (miRNAs) are a class of non-coding RNA molecules with ~22 nucleotides. MiRNAs can post-transcriptionally modulate gene expression by directly targeting the 3'-untranslated region (3'-UTR) of target mRNAs (He and Hannon 2004). Many miRNAs are reported to present

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with a dysregulated expression pattern in cancers, whereas they function as suppressor or an oncogene (Pratap et al. 2018; Wang et al. 2017). MiR-34a as one of the earliest known members of the miR-34 family, has been identified to be downregulated and exert a suppressive role in several categories of cancers, including intestinal tumor (Jiang and Hermeking 2017), lung adenocarcinoma (Kasinski and Slack 2012), osteosarcoma (Yan et al. 2012), and colorectal cancer (Pang et al. 2013). In NPC, Huang et al. (2018) reported that miR-34a could efficiently inhibited TGF- $\beta$  (transforming growth factor- $\beta$ )-induced epithelial-mesenchymal transition (EMT), which is a requirement for cancer cell invasion and metastasis via targeting SMAD4 (Kang and Massague 2004). However, the underlying mechanisms by which miR-34a inhibits the EMT and invasion of NPC cells still need to be further elucidated.

The AXL receptor tyrosine kinase (AXL), also known as UFO and ARK, was first found in chronic myeloid leukemia as a transforming gene and is the founding member of the TAM family of receptor tyrosine kinases which contains Tyro3, AXL, and MER (Hafizi et al. 2005; Paccetz et al. 2014). Jiang et al. (2016) showed that AXL expression level was markedly elevated in NPC tissues compared to those in normal nasopharyngeal epithelial tissues, and high level of AXL was correlated with the distant metastasis and advanced TNM stage in NPC, as well as the shorter overall survival. Using the TargetScan software ([http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/)), we predicted that AXL was a potential target for miR-34a-5p (miR-34a) in human. Therefore, we hypothesized that AXL might be a molecular which was regulated by miR-34a and subsequently involved in NPC migration and invasion.

Consistently with hypothesis, we demonstrated that miR-34a significantly repressed NPC cell migration, invasion and EMT via targeting AXL. Our data elucidated a new mechanism by which miR-34a repressed the progression of NPC, which might be explored as a therapeutic target to inhibit the metastasis of NPC.

## Materials and methods

### Ethic statement

All procedures in the current study involving human samples were performed in accordance with the Helsinki declaration and approved by the Ethic Committee of The First Affiliated Hospital of Bengbu Medical College. The informed consent has been obtained from each participant.

### Tissue samples

Thirty freshly frozen NPC tissues and 30 normal nasopharyngeal epithelium samples were obtained from NPC patients who accepted surgery in The First Affiliated Hospital of Bengbu Medical College prior to preoperative therapy.

### Cell culture

Human NPC cell line Sune-1 was purchased from the Shanghai huiying biological technology co., LTD (Shanghai, China) and grown in RPMI-1640 medium (Thermo Fisher Scientific, MA, USA) with 10% FBS (fetal serum bovine; Thermo Fisher Scientific). Sune-1 cells were cultured in a humidified incubator at a stationary temperature of 37 °C, supplemented with 5% CO<sub>2</sub>. To increase AXL expression, Sune-1 cells were incubated with recombinant human AXL protein (100 ng/ml; No. PV6253, Thermo Fisher Scientific) for 24 h.

### Cell transfection

The mimic used to upregulate miR-34a in NPC cells and the negative control mimic (NC mimic) were gained from the Shanghai GenePharma, LTD. (Shanghai, China). They were transfected into cells by using lipofectamine 2000 transfected reagent (Thermo Fisher Scientific). The sequences of the mimic and mimic control was listed as following:

miR-34a mimic: 5'-UGGCAGUGUCUUAGCUGG UUGU-3',

NC mimic: 5'-GUA AUGUUUGGCUCGUGUGCUG-3'.

### Quantitative real time PCR (qRT-PCR)

Total RNAs were extracted from cells or tissues using Trizol Reagent (Thermo Fisher Scientific) and then cDNA was abstained by reverse transcription (RT). Then, the cDNAs were submitted to the qRT-PCR using the SYBR Green Supermix (Takara, Dalian, China) to measure the expression of miR-34a and AXL. The expression level of GAPDH or RNU6B (U6) was used as the internal control for normalization of AXL or miR-34a, respectively. The 2<sup>- $\Delta\Delta$ CT</sup> method was applied to calculate the relative expression levels of miR-34a and AXL mRNA. The sequences of the primers used for q-PCR were listed as followings:

miR-34a-RT sequence: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAA CC-3';

miR-34a-sense: 5'-GCTGTTGGCAGTATCTTAGC-3', antisense: 5'-GTGCAGGGTCCGAGGT-3';

AXL—sense: 5′-CACCCCAGAGGTGCTAATGG-3′, antisense: 5′-GAAGGTTTCCTTCACTGGGCG-3′.

SIRT1 (sirtuin 1)—sense: 5′-TCCTACTGGCCTGAGGTTGAG-3′, antisense: 5′-AAGTCTACAGCAAGGCCGAGC-3′;

CD44—sense: 5′-CACACCCTCCCCTCATTCAC-3′, antisense: 5′-TGGATGGCTGGTATGAGCTG-3′;

CDK6 (cyclin dependent kinase 6)—sense: 5′-CGGAGAGCCGACTGACACT-3′, antisense: 5′-CCTCGAAGCGAAGTCTCAA-3′;

c-MET—sense: 5′-TGGGCACCGAAAGATAAACT-3′, antisense: 5′-ATCTGGGTGTTCCAGCACAG-3′;

Cyclin D1—sense: 5′-CTGATTGGACAGGCATGGGT-3′, antisense: 5′-GTGCCTGGAAGTCAACGGTA-3′;

GAPDH—sense: 5′-CATGGTTCACACCCATGACG-3′, antisense: 5′-CCACTAGGCGCTCACTGTTCTC-3′.

### Western blotting assay

Total protein extraction was carried out using the RIPA lysis (Beyotime) with proteinase inhibitors (Roche, Switzerland) and the protein concentration was measured with the BCA kit (Thermo Fisher Scientific, MA, USA). Then, same amount of total proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred onto the polyvinylidene fluoride membranes (Sigma, MA, USA). Later, the membranes were blocked with 5% BSA for 1 h at room temperature and were probed with the following primary antibodies (4 °C, 10–12 h), including E-cadherin, N-cadherin, Vimentin,  $\beta$ -catenin AXL, p-AKT, AKT and Snail, all purchased from Cell Signaling Technology (MA, USA) with a working concentration of 1:1000 dilution. After that, the membranes were washed with TBST for 3 times and incubated with the horse radish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology) for 1 h (room temperature). The protein expression levels were measured by using the densitometric analysis.

### Luciferase gene reporter assay

3′-UTR of AXL (> hg38\_ncbiRefSeq\_NM\_001699.6; range = chr19:41259905–41261766) including the predicted miR-34a binding sites (pGL3-AXL-WT) or the mutated binding sites (pGL3-AXL-MUT) was inserted into a pGL3-REPORT vector (Promega, Madison, USA). Then, the NPC cells were co-transfected with miR-34a mimic or NC mimic, and pGL3-AXL-WT or pGL3-AXL-MUT, together with the pGL3-REPORT  $\beta$ -gal control plasmid with lipofectamine 2000. After 48 h of the above transfections, the luciferase activities were detected by using the dual-Luciferase Reporter Assay System (Promega).

### Wound healing assay

The NPC cells were plated in 6-well plates (Corning, USA) with adequate numbers to reach 100% confluence in the next day. Then, the wounds were scratched in each well by using a 10  $\mu$ l pipette tip and the non-attached cells were removed through washing with PBS. Next, the cells were grown in FBS-free medium and incubated at 37 °C. Gap size was measured and photographed at 0, 24 and 48 h following the scratch.

### Transwell chamber assay

A transwell chamber (8  $\mu$ m, 24-well format; Corning, USA) precoated with a diluted Matrigel (BD Biosciences, New Jersey, USA) (50% concentration) was used to assess cell invasion. Briefly, the transfected cells ( $5 \times 10^4$ /well) resuspended with FBS-free medium were seeded in the upper chamber, while 600  $\mu$ L cell culture medium with 20% FBS were added into the lower chambers. After the cells were incubated at 37 °C for 24 h, cells in the lower of the upper chambers were stained with 1% crystal violet at room temperature for 10 min. Cell invasion and migration abilities were assessed by counting cell numbers with positive staining in six randomly selected regions.

### Statistical analysis

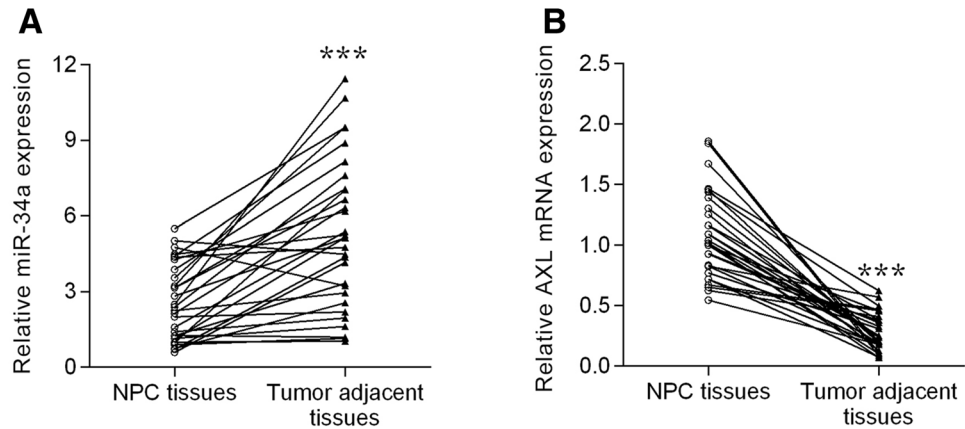
Three independent experiments were carried out for each assay in this study. The data were expressed as the means  $\pm$  standard deviation (SD). Statistical analysis between 2 groups or multiple groups was performed with Student's t-test or One-Way Analysis of Variance (ANOVA) followed by Tukey's test. SPSS 20.0 software was recruited for the statistical analysis. *P* value less than 0.05 was identified as statistically significant.

## Results

### miR-34a is lowly expressed while AXL is highly expressed in NPC tissues

To investigate whether AXL was involved in miR-34a-mediated NPC repression, we first explored the expression levels of miR-34a and AXL in NPC tissues and the adjacent non-tumor tissues. Compared with the normal tissues, miR-34a expression was markedly decreased in the NPC tissues (Fig. 1a), while the expression of AXL was obviously increased (Fig. 1b). These results illustrated that the expression patterns of miR-34a and AXL showed inverse

**Fig. 1** miR-34a expression was decreased and AXL expression was increased in NPC tissues. **a** QRT-PCR analysis of the expression of AXL in 30 paired NPC tissues and the tumor adjacent tissues. **b** QRT-PCR analysis of the mRNA levels of AXL in 30 paired NPC tissues and the tumor adjacent tissues. Three independent experiments were carried out for each assay. (n=30, \*\*\**P* < 0.001)



correlations between NPC tissues and adjacent non-tumor tissues.

### AXL is a target of miR-34a in NPC cells

Next, we explored the relationship between miR-34a and AXL in NPC Sune-1 cells. The putative binding sites between them were shown in Fig. 2a. Transfection with miR-34a mimic dramatically increased the level of miR-34a in Sune-1 cells (Fig. 2b). And, miR-34a overexpression reduced the expression of pGL3-AXL-WT reporter, while this effect was abolished when the putative miR-34a binding site of pGL3-AXL-WT was mutated (Fig. 2c). In addition, overexpression of miR-34a significantly reduced the expression of AXL at both mRNA and protein levels (Fig. 2d, e). Moreover, we detected the mRNA levels of the known targets of miR-34a after Sune-1 cells were transfected with miR-34a mimic (Badi et al. 2018; Hong et al. 2015; Ye et al. 2016; Zuo et al. 2018). The results showed that miR-34a overexpression obviously decreased the mRNA levels of SIRT1, CD44, CDK6 and Cyclin D1, with no significant change in c-MET mRNA level (Supplementary Fig. 1). These results confirm that AXL serves as a target of miR-34a.

### miR-34a overexpression inhibits cell migration, invasion and EMT in NPC via targeting AXL

Then, we explored the effects of miR-34a/AXL axis on cell migration, invasion and EMT via carrying out the loss-of-function assay in Sune-1 cell line. Compared with the control group, cell migration and invasion abilities were all significantly reduced following the transfection with miR-34a mimic, whereas AXL protein treatment rescued these tendencies (Fig. 3a, b). In addition, miR-34a overexpression induced obvious increases in the expressions of E-cadherin and  $\gamma$ -catenin and decreases in the expressions of N-cadherin and Vimentin, whereas these effect induced

by overexpression of miR-34a were obviously impaired following AXL treatment (Fig. 3c). Overall, these results reveal that miR-34a exerts a repressive role in regulating cell invasion, migration and EMT in NPC via targeting AXL.

### miR-34a overexpression inhibits the activation of AKT/Snail signaling via targeting AXL

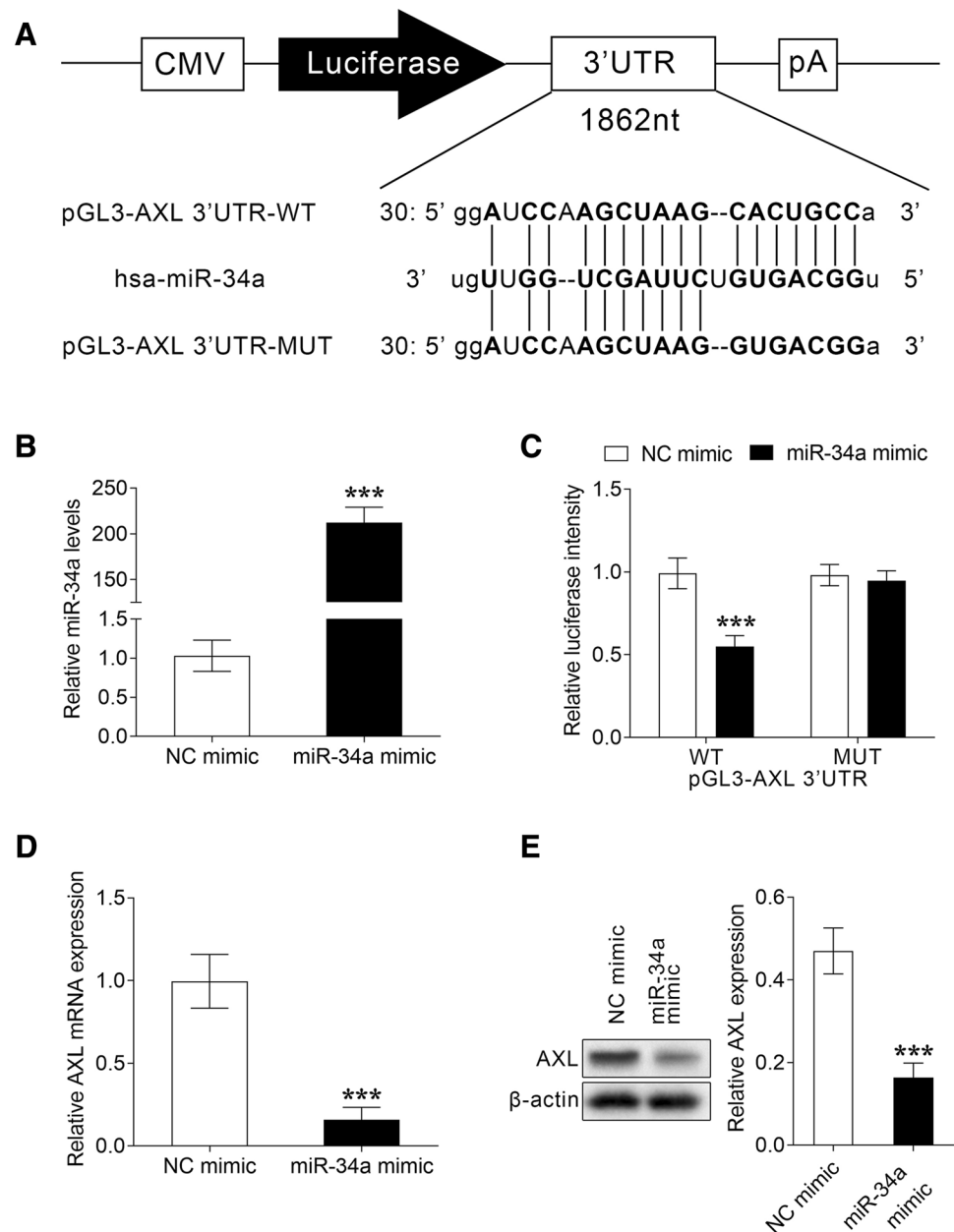
To reveal the underlying mechanism of miR-34a/AXL-induced repressions of cell migration, invasion and EMT in NPC, we explored the effect of miR-34a/AXL on the activation of AKT/Snail signaling. The results showed that the ratio of p-AKT/AKT and the expression level of Snail were obviously decreased when miR-34a was overexpressed in Sune-1 cells, whereas effects were obviously impaired following AXL treatment (Fig. 4), which indicated that miR-34a could induce a repression in the activation of the AKT/Snail signaling.

## Discussion

It has been reported that about 15–30% of patients with NPC would develop distant metastasis despite of the 80–90% 5-year local control rates (Pratt et al. 2009). With the purpose of development of potent methods to avoid or treat the metastasis and invasion in NPC, we further investigate the role of miR-34a. Our results showed that miR-34a was downregulated in NPC tissues, while its target gene AXL expression was upregulated. And, overexpression of miR-34a significantly inhibited cell migration, invasion and EMT in NPC via targeting AXL.

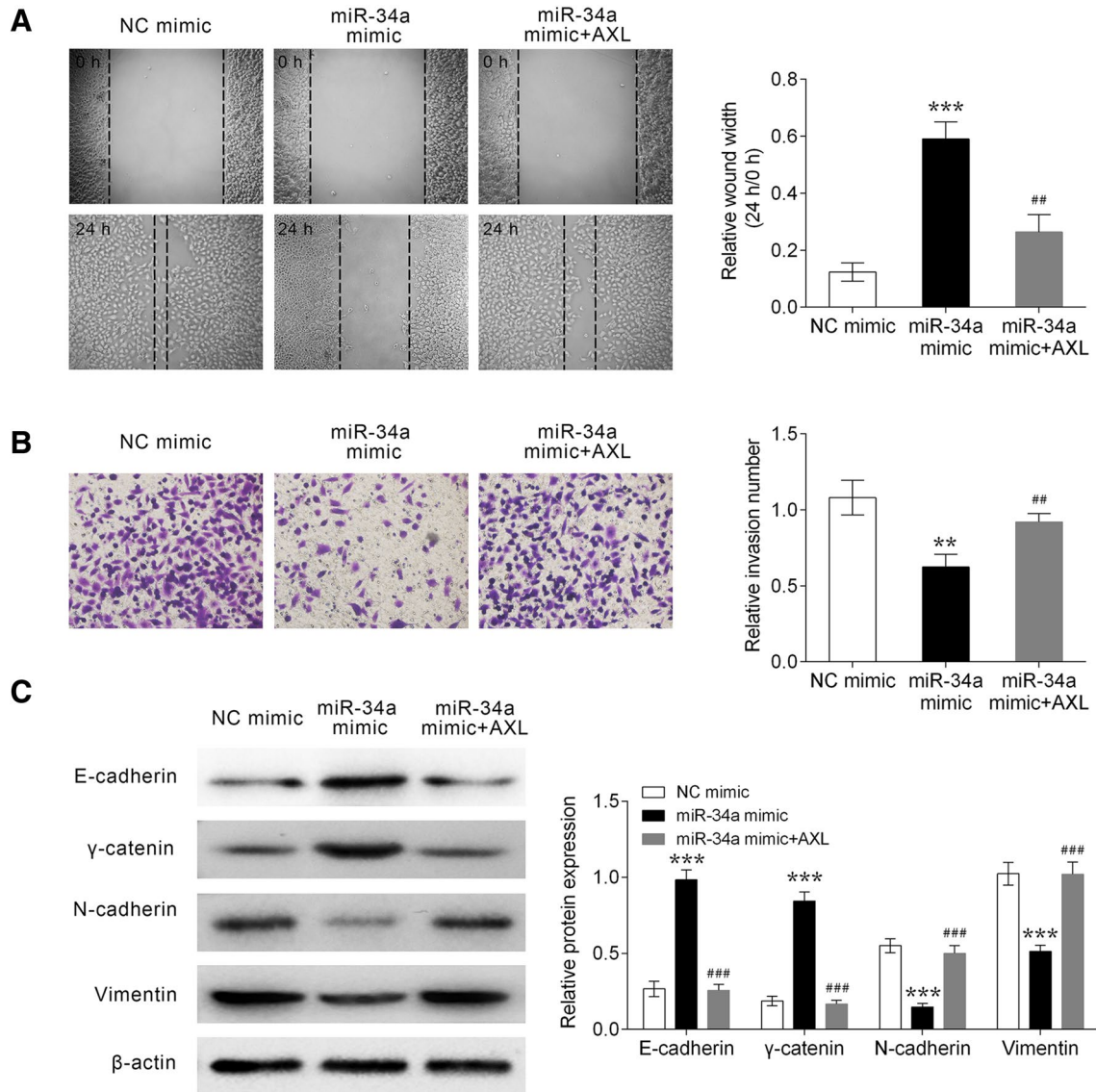
EMT is of great importance for tumor invasion and metastasis in many cancers, which was characterized by the decreased expressions of E-cadherin and  $\gamma$ -catenin and the increased expressions of N-cadherin and Vimentin (Kang and Massague 2004; Sui et al. 2014). miR-34a has been reported to play a role in EMT in variety of cancers

**Fig. 2** AXL was a target of miR-34a in NPC cells. **a** The putative binding sites between miR-34a and AXL was displayed. **b** The expression of miR-34a was detected by using the qRT-PCR assay after Sune-1 cells were transfected with NC mimic or miR-34a mimic. **c** The luciferase intensity of the pGL3-AXL-WT/MUT was determined by the luciferase gene reporter assay after Sune-1 cells were transfected with NC mimic or miR-34a mimic. **d, e** The expression of AXL at both mRNA and protein levels were measured by using the RT-PCR and western blotting assay after Sune-1 cells were transfected with NC mimic or miR-34a mimic, respectively. All the experiments were repeated for at least three times. (n=03, \*\*\**P* < 0.001)



and other diseases. For example, Yamamoto et al. (2019) demonstrated that the introduction of miR-34a mimic into lung cancer A549/ABCA3 cells significantly increased the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), another EMT marker. Repression of miR-34a by the long non-coding RNA (lncRNA) small nucleolar RNA host gene 7 (SNHG7) apparently accelerated EMT in breast cancer cells. Han et al. (2019) reported that miR-34a significantly repressed the EMT in lens epithelial cells, a crucial process in the pathogenesis of posterior capsule opacification by targeting Notch1. In NPC, Long et al. (2014) illustrated that increased expression of miR-34a induced by hypofractionated radiotherapy obviously increased NPC CNE1 and

CNE2 cell apoptosis. Suppression of lncRNA NEAT1 was identified to inhibit several cell functions, such as proliferation, invasion, migration, and EMT via downregulating miR-34a-5p expression in 5-8F NPC cells (Ji et al. 2019). In addition, miR-34a was expressed at a low level in the cancer tissues and suppressed NPC cell metastasis in vivo, as well as inhibit TGF- $\beta$ -induced migration, invasion and EMT via targeting SMAD4 in NPC CNE1 cells (Huang et al. 2018). All of the above findings demonstrated that miR-34a could repress the migration, invasion and EMT in NPC cells. Consistently, this study also showed that upregulation of miR-34a in NPC Sune-1 cells with mimic transfection led to apparent repressions in cell migration, invasion and EMT,



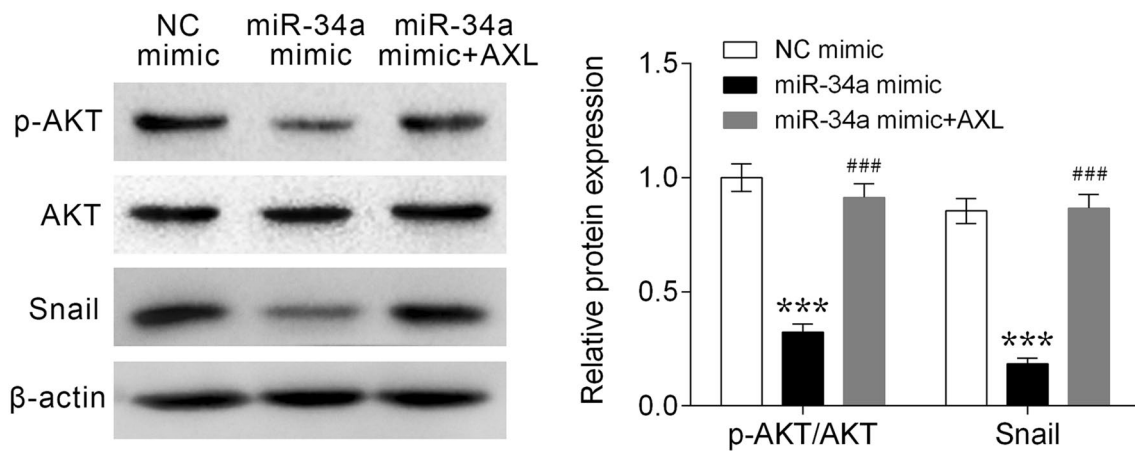
**Fig. 3** miR-34a overexpression repressed NPC cell migration, invasion and EMT via targeting AXL. Sune-1 cells were treated with NC mimic, miR-34a mimic or miR-34a mimic+AXL (100 ng/ml), then the following assays were carried out. **a** Cell migration ability was determined by using the wound healing assay. **b** Cell invasion capacity

was tested by using the transwell chambers. **c** The protein levels of E-cadherin,  $\gamma$ -catenin, N-cadherin and Vimentin were examined by the western blotting assay. Three independent experiments were carried out for each assay. (n=03, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001)

as detected by the wound healing, transwell chambers and western blotting assays.

Mechanistically, the results from the current study confirmed that miR-34a could target AXL and repress AXL expression at both mRNA and protein levels. AXL has been confirmed to play an essential role in modulating cancer cell growth, migration, invasion and chemosensitivity, and was identified to be a promising anti-cancer target in several types of human cancers (Zhu et al. 2019), including non-small cancer (Linger et al. 2013), gastric cancer (Wu et al. 2002), ovarian cancer (Sun et al. 2004) and multiple myeloma (Yan et al. 2019). Jiang et al. (2016) reported that

the AXL expression level in NPC tissues was obviously elevated compared with those in normal nasopharyngeal epithelial tissues, which was closely correlated with the distant metastasis, advanced TNM stage and poor prognosis in NPC patients, revealing that AXL served as a potential marker for the migration and invasion of NPC. In the current study, we found that AXL was overexpressed in NPC tissues and was a target of miR-34a in NPC Sune-1 cells, and overexpression of AXL dramatically impaired the role of miR-34a in the repressions of cell migration, invasion and EMT in NPC, suggesting that miR-34a inhibited NPC migration and invasion via targeting AXL.



**Fig. 4** miR-34a overexpression inhibited the activation of AKT/Snail signal by targeting AXL. After cell treatment with NC mimic, miR-34a mimic or miR-34a mimic + AXL, Sune-1 cells were collected for

the western blotting assay to detect the protein expression levels of p-AKT, AKT and Snail. Three independent experiments were carried out for each assay. (n=03, \*\*\* $P < 0.001$ )

Moreover, we observed that the expressions of Snail and the phosphorylation level of AKT were significantly reduced when Sune-1 cells were transfected with the miR-34a mimic to upregulate miR-34a expression, suggesting that the inhibition of AKT/Snail signaling might play a role in miR-34a-mediated repressions in NPC cell migration, invasion and EMT. The activation of PI3K/AKT/Snail signaling pathway has been verified to exert a positive role in EMT (Zhou et al. 2019). Moreover, AXL knockdown was demonstrated to significantly repress the expressions of PI3K, p-AKT and Snail, resulting in the repressions of EMT and invasion of breast cancer cells (Li et al. 2014). Furthermore, AXL has been identified to be an activator of the PI3K/AKT signaling in cancers (Li et al. 2015; Ma et al. 2018). Therefore, we conjecture that miR-34a represses NPC cell migration, invasion and EMT through repressing the PI3K/AKT signaling via targeting AXL, which will be verified in our next study.

In conclusion, the findings in this study indicated that miR-34a is downregulated in NPC tissues, and can suppress NPC cell migration, invasion and EMT via targeting AXL. This study further points out the important value of miR-34a in the diagnosis and treatment of NPC.

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**Authors' contributions** CYJ and ZQC conceived and designed the experiments, TJ and YJX analyzed and interpreted the results of the experiments, BW performed the experiments.

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## Compliance with ethical standards

**Conflict of Interest** Chengyi Jiang, Zhongqiang Cheng, Tao Jiang, Yajia Xu and Bin Wang state that there are no conflicts of interest to disclose.

**Ethics approval and consent to participate** All procedures in the current study involving human samples were performed in accordance with the Helsinki declaration and approved by the Ethic Committee of The First Affiliated Hospital of Bengbu Medical College. The informed consent has been obtained from each participant.

**Availability of data and materials** All data generated or analyzed during this study are included in this published article.

**Patient consent for publication** Not applicable.

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