Int J Clin Exp Pathol 2017;10(2):1263-1272
www.ijcep.com /ISSN:1936-2625/IJCEP0046126

Original Article

MiR-320a suppresses cell migration and invasion by targeting MRP2 in oral tongue squamous cell carcinoma

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Received December 8, 2016; Accepted December 25, 2016; Epub February 1, 2017; Published February 15, 2017

Abstract: Background: MiR-320a has been found playing important role in tumor progression. However, the molecular mechanisms of miR-320a in oral tongue squamous cell carcinoma (OTSCC) remain unknown. Methods: MicroRNA expression was detected by miRNA microarray in healthy people and OTSCC patients. RT-PCR was further used to verify microarray data. The function of miR-320a was measured by wound healing assay, transwell assay and Western blot in OTSCC cells. The miR-320a target gene was tested by using a luciferase reporter assay, RT-PCR and Western blot. Results: A total of 18 miRNAs showed altered expression in OTSCC patients, and miR-320a was markedly decreased ($P<0.05$). In addition, migration and invasion abilities of OTSCC cells were decreased in the miR-320a group ($P<0.05$); the luciferase activity of the MRP2-3'-UTR plasmid was inhibited following miR-320a binding ($P<0.05$). Conclusion: MiR-320a expression was significantly decreased in patients with OTSCC, indicating that miR-320a would be a potential antioncogene partly through targeting MRP2, it would become a therapeutic target for OTSCC.

Keywords: miR-320a, MRP2, migration, invasion, oral tongue squamous cell carcinoma

Introduction

Head and neck/oral cancer (HNOC) is a common malignant tumor. Head and neck squamous cell carcinoma (HNSCC), which originates from the epithelium lining of this region, accounted for majority (over 90%) of HNOC. Oral tongue squamous cell carcinoma (OTSCC) is one of the most invasive form of HNSCCs, which displays a tendency for rapid local invasion and spread [1], has a typical lymph node metastasis pattern [2, 3]. OTSCC patients also had higher recurrence rate, and the five year survival rate of patients are about 50% [4, 5]. Therefore, it is very urgent to investigate the mechanism of metastasis and find a more effective strategy to therapy OTSCC.

Multidrug resistance-associated protein 2 (MRP2) which is a member of the ABC transporter superfamily, has been observed in many kinds of tumors and play a critical role in drug resistance [6, 7]. The expression level of MRP2 change or function alter can cause altered exposure to drugs which has been shown in pravastatin and methotrexate [8-10]. Therefore we suggested that MRP2 may play an important role in OTSCC development.

MicroRNAs (miRNAs) are a new family of endogenous, conserved and small RNAs. They regulate gene expression through binding to the 3'-untranslated region (3'-UTR) of their target messenger RNAs (mRNAs), causing degradation or translational suppression [11-13]. Increasing research has showed that miRNAs play a critical role in many biological processes such as cell development, invasion, proliferation, differentiation, metabolism, apoptosis as well as migration [14-19]. A growing body of evidence has showed that dysregulated the expression level of miRNA is associated with tumor initia-

tion, development and tumor death through regulating tumor inhibitor gene or oncogene [20-23]. Early research has shown that miR-320a played an important role in the tumor progress [24-27]. For example, Qi X et al found that miR-320a inhibits cell proliferation, migration and invasion by targeting BMI-1 in nasopharyngeal carcinoma [28]. Zhao H et al illustrated that miR-320a suppresses colorectal cancer progression by targeting Rac1 [29]. However, the mechanism of miR-320a suppressing OTSCC development was still unknown. In this study, miRNA microarray analysis was performed on serum samples from healthy people and patients with OTSCC. It is interesting to note that a total of 21 miRNAs showed significant differences expression (all $P<0.05$ and fold change ≥ 2). The expression levels of 4 miRNAs were corroborated by qRT-PCR, and miR-320a showed a significantly decreased in OTSCC. Besides, miR-320a inhibited OTSCC cell migration and invasion in vitro. More importantly, MRP2 was validated as a direct target gene for miR-320a in OTSCC.

Materials and methods

Patients and tissue samples

A total of 48 patients with OTSCC alongside 50 healthy volunteers were enrolled from 2013 to 2014 at our hospital. All the tumor patients were diagnosed and obtained appropriate treatment according to ESMO Clinical Practice Guidelines. Serum samples were gathered from all the patients before any treatment. Tumor tissues and the related non-tumor tissues were collected from 48 patients after surgery. A total of 5 ml venous blood was assembled into a serum-separator tube, and centrifuged for serum extraction within 2 hours; all tissue samples were diagnosed and confirmed by pathological examination, immediately temporary frozen in liquid nitrogen, and stored at -80°C. Each patient agreed to participate in this study and was given written informed consent and the research was approved with Declaration of Helsinki and the ethics committee of our hospital.

MicroRNA microarray

Serum samples were randomly selected from different groups (the healthy and OTSCC groups on one hand, survival and death groups on the other hand). Total RNA was isolated with mirVana Kit (Applied Biosystems, CA) following the handling instructions. Reverse transcription was carried out for miRNA detection. The RNA quality and amounts were detected by standard electrophoresis and spectrophotometric methods, total RNA was labeled and hybridized to the miRNA microarray, according to operating instruction provided by CapitalBio Corporation. Scanned images were then imported into a confocal LuxScan scanner (CapitalBio Corp). The SpotData Pro software (CapitalBio Corp) was used for data analysis.

RNA extraction and quantitative real-time PCR

Total RNA was isolated from clinical tissue samples or cell lines by using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) according to the operation instructions. RNA was measured by using UV absorbancies at 260 and 280nm (A260/280). Then the RNA was reverse-transcribed into cDNA using reverse transcription system (Thermo Scientific, CA, USA). The specificity of the primer sequences was determined by dissociation curve, and the $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression levels of miRNA and mRNA. All the PCR reactions were measured in triplicate for both the U6 control and each miRNA.

Cell lines cultured

The human OTSCC cell lines SCC4, SCC1, Cal-27 and the normal oral keratinocyte cell lines (NHOK) were purchased from the American Type Culture Collection (ATCC). All the cells were cultured in the DMEM/F12 medium supplemented with heat-inactivated 10% FBS (GIBCO, Grand Island, NY, USA) and penicillin/streptomycin (100 U/ml and 100 mg/ml, respectively) at 37°C in a humidified atmosphere of 5% CO_2.

Plasmid constructionand infection

The eukaryotic expression vector pcDNA3.1(+) was subcloned with full-length MRP2 cDNA which lacking the 3'-UTR (Invitrogen, Carlsbad, California, USA). The MRP2 3'UTR target site for miR-320a was amplified by PCR and cloned into the XbaI site of pGL3 control (Promega, Madison, USA). This vector was called WT MRP2 3'UTR. The Quick-change mutagenesis kit (Strata-gene, Heidelberg, Germany) was used to carry out the site-directed mutagenesis of the miR-320a target-site in the MRP2 3'UTR and known as Mut MRP2 3'UTR. All the con-

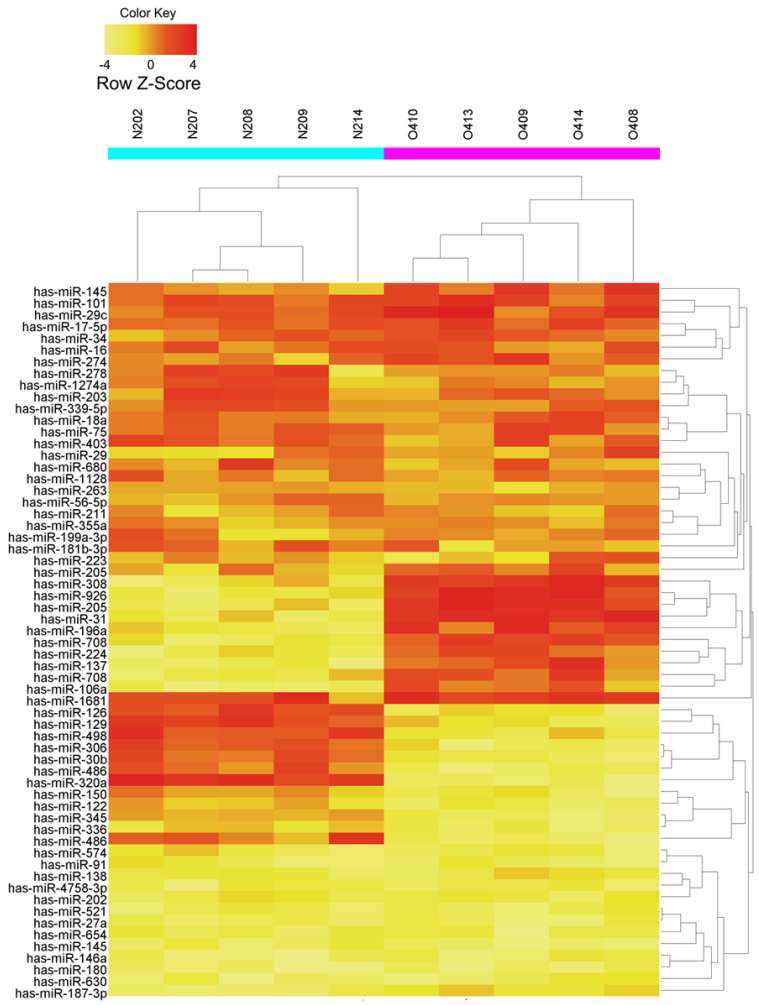

Figure 1. MiRNA expression spectrum clustering map between two groups. Each 5 serum samples were randomly chosen from healthy people (indicates N202, N207, N208, N209, N214) and patients with OTSCC (indicates O410, O413, O409, O414, O408). The yellow bar means downregulated, red part means upregulated.

structs were examined by sequencing. OTSCC cells were planted into 24 well-plates and at a density of 2×10^5 per well. After cultured 24 h, they were transiently transfected with miR-320a mimics or negative controls (Gene-Chem, Shanghai, China) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California, USA) according to the operating instructions.

Dual-luciferase reporter assay

Cells were incubated into 96-well plates and transfected with miR-320a mimics. Wild-type (WT) 3'UTR of MRP2 or the Mut 3'UTR were inserted into the pGL3 control vector (Promega, Madison, USA), respectively. Cells were co-transfected with miR-320a mimics or mimic control and pGL3-MRP2-3'UTR or MUT 3'UTR for 48 h and detected by the Dual-Luciferase Reporter Assay System (Promega, Madison, USA) according to the manufacturer's instruction.

Western blot

Total protein was isolated by using 1% RIPA Lysis Buffer (Beyotime, China) and quantified with the BCA protein assay kit. The total protein samples were separated by SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF, Millipore, Boston, MA, USA), which were blocked with Tris buffer containing 0.1% Tween-20 and 5% nonfat milk at 4°C. Then, the membranes were probed with anti-MRP2 (1:1000, Cell Signaling Technology, CA, USA) and anti-β-actin (1:1000, Cell Signaling Technology, CA, USA) antibodies overnight at 4°C, and followed by incubation with secondary antibodies (1:2000, Cell Signaling Technology, CA, USA) for 1 h at room temperature. The protein signal was detected by an ECL kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions.

Wound healing analysis

The cells were seeded into 6-well plates and cultured with miR-320a or miR-320a NC for 24 h. Then, a wound was generated by a sterile pipette tip and plates were washed using pre-warmed PBS to remove cellular debris. Wound images were acquired at 0 h and 48 h after wounding. The wound width was measured by using microscope (Nikon, Tokyo, Japan).

Cell invasion assay

For invasion assay, cells were cultivated in the upper chamber with a membrane that was pre-treated with matrigel (100 μg per well, BD Biosciences, San Jose, CA, USA). In the upper

Table 1. Differentially expression miRNAs with >2-fold change

MiRNA expression profiles			
Up-regulated miRNAs		Down-regulated miRNAs	
MiRNA name	Fold change	MiRNA name	Fold change
has-miR-205#	10.5	has-miR-126#	20.8
has-miR-308#	20.6	has-miR-129#	9.5
has-miR-926#	25.4	has-miR-498#	10.4
has-miR-31#	32.9	has-miR-306#	12.3
has-miR-196a#	18.7	has-miR-30b#	15.9
has-miR-708#	15.4	has-miR-486#	20.3
has-miR-224#	23.2	has-miR-320a#	34.9
has-miR-137#	9.8	has-miR-150*	17.3
has-miR-106a*	4.9	has-miR-122#	20.4
		has-miR-345#	19.6
		has-miR-336*	15.7
		has-miR-486#	30.5

The expression of miRNAs in serum samples from patients with OTSCC and healthy people. Serum samples from OTSCC patients were collected before surgery. *P<0.05, #P<0.01.

chamber, medium without FBS was added while in the lower portion of the chamber, 10% FBS was added. After the cells were incubated for 24 h at 37°C, we carefully removed the non-invading cells in the upper chamber. Invaded cells were fixed with 4% formaldehyde, stained with 0.5% crystal violet, and counted under a microscope (Nikon, Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± SD (standard deviation) from three independent experiments. Statistical analyses were performed with the SPSS20.0 software. Group differences were assessed by Student's t-test (two groups) or one-way analysis of variance (ANOVA) (>2 groups). P<0.05 was considered to be statistically significant differences.

Results

MicroRNA expression profile is altered in OTSCC, miR-320a is downregulated in OTSCC

In order to find the expression characteristic of miRNAs in OTSCC, miRNA microarray analysis was performed on tissues from patients with OTSCC and healthy volunteers. A total of 5 subjects were randomly chose to form the above two groups, respectively. Then, the expression levels of miRNA were measured in the two groups, using the μParafloTM microRNA microarray assay (covering Sanger miRBase release 18.0). It is interesting to note that, 21 miRNAs displayed obviously different expression levels (P<0.05 and fold change ≥2), including 9 overexpressed miRNAs and 12 low expressed ones (**Figure 1**; **Table 1**). It is interesting that miR-320a showed the highest downregulation. In order to verified the microarray findings, four miRNAs, including miR-320a, miR-129, miR-106a and miR-31 in OTSCC samples and the health people were detected by quantitative real-time reverse transcription-PCR (qRT-PCR) (**Figure 2**). The expression trends were in line with microarray findings (P<0.05); U6 snRNA was used as the normalized control.

MRP2 is upregulated in OTSCC

In order to further illustrate the molecular mechanisms of miR-320a in oral tongue squamous cell carcinoma, we used TargetScan to found that MRP2 was a potential target gene of miR-320a, and has been found to be involved in tumor progression. Therefore, the levels of miR-320a and MRP2 mRNA were measured in OTSCC tissues by RT-PCR. Results showed that the level of miR-320a in OTSCC samples was markedly decreased than in the corresponding normal tissues (**Figure 3A**), which was consistent with the above serum findings. Furthermore, MRP2 gene expression was increased in OTSCC samples (**Figure 3B**).

MiR-320a inhibits OTSCC cell migration and invasion in vitro

Based on the above experimental results, *migration and invasion* assays were performed to research the biological function of miR-320a. The expression level of miR-320a was measured by RT-PCR in the normal oral keratinocyte cell lines as well as oral tongue squamous cell carcinoma cell lines. As shown in **Figure 4A**, the levels ofmiR-320a were markedly reduced in the oral tongue squamous cell carcinoma cell lines (P<0.05). Then, miR-320a mimics and the mimic control were successfully transfected into OTSCC cells for the subsequent studies. As shown in **Figure 4B**, miR-320a expression levels in SCC4 cells were dramatically increased after transfection with

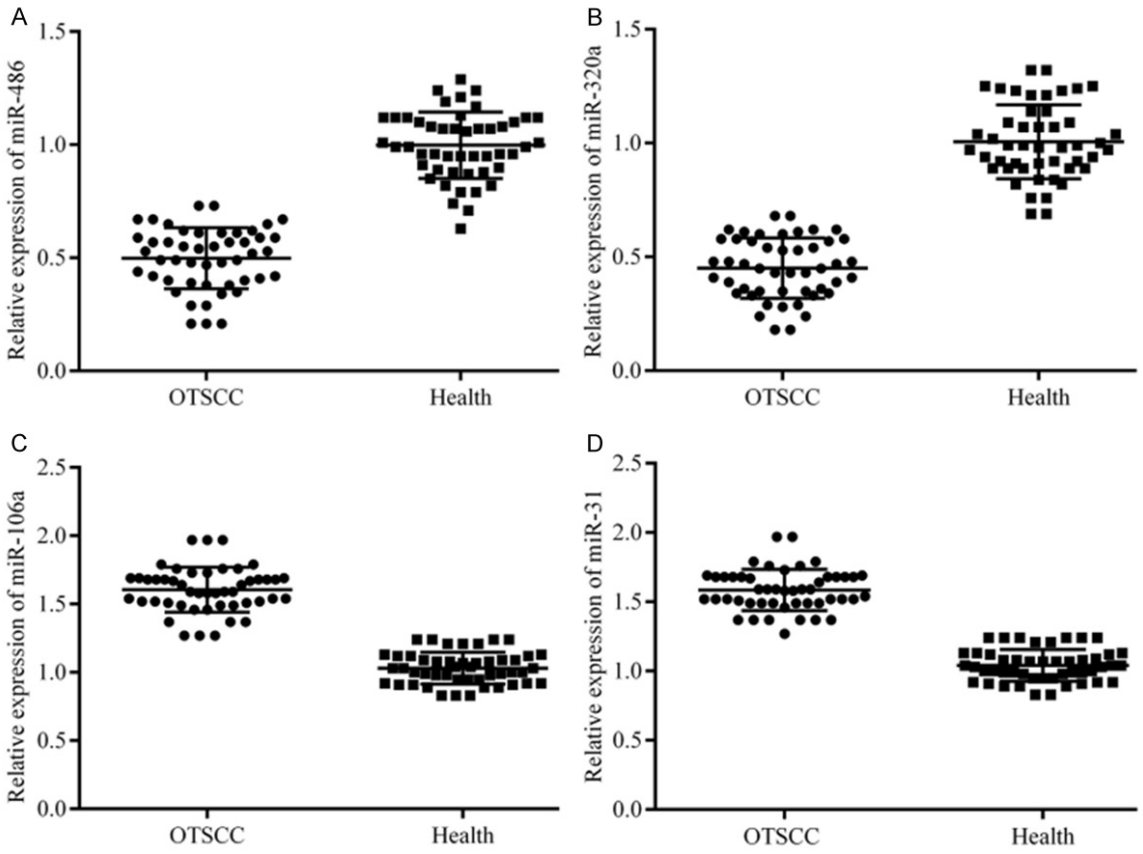

Figure 2. Expression of four miRNAs in OTSCC. The expression level of four miRNAs was tested by qRT-PCR, showing that miR-106a and -31 were upregulated, while miR-496 and -320a were downregulated in serum samples from OTSCC patients compared with healthy people.

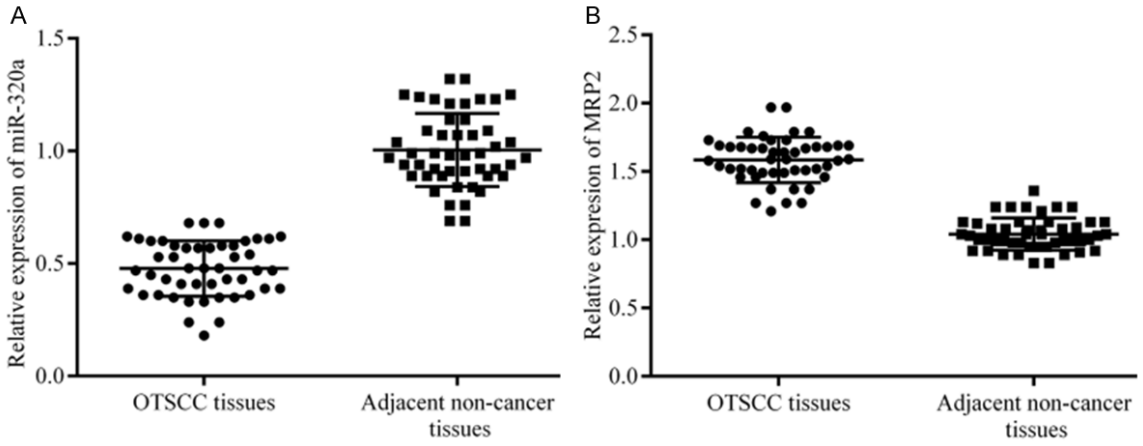

Figure 3. The mRNA expression level of MiR-320a and MRP2 in OTSCC tissues. A: The expression of miR-320a was markedly decreased in OTSCC tissues when compared with the normal tissue. B: The expression level of MRP2 mRNA in OTSCC tissues was significantly increased than in normal tissues.

miR-320a mimics ($P<0.05$) when compared with the miR-negative control group. Then, the effects of miR-320a on SCC4 cell migration and invasion were measured. Wound healing assay illustrated that the migration ability of OTSCC cell was significantly suppressed with

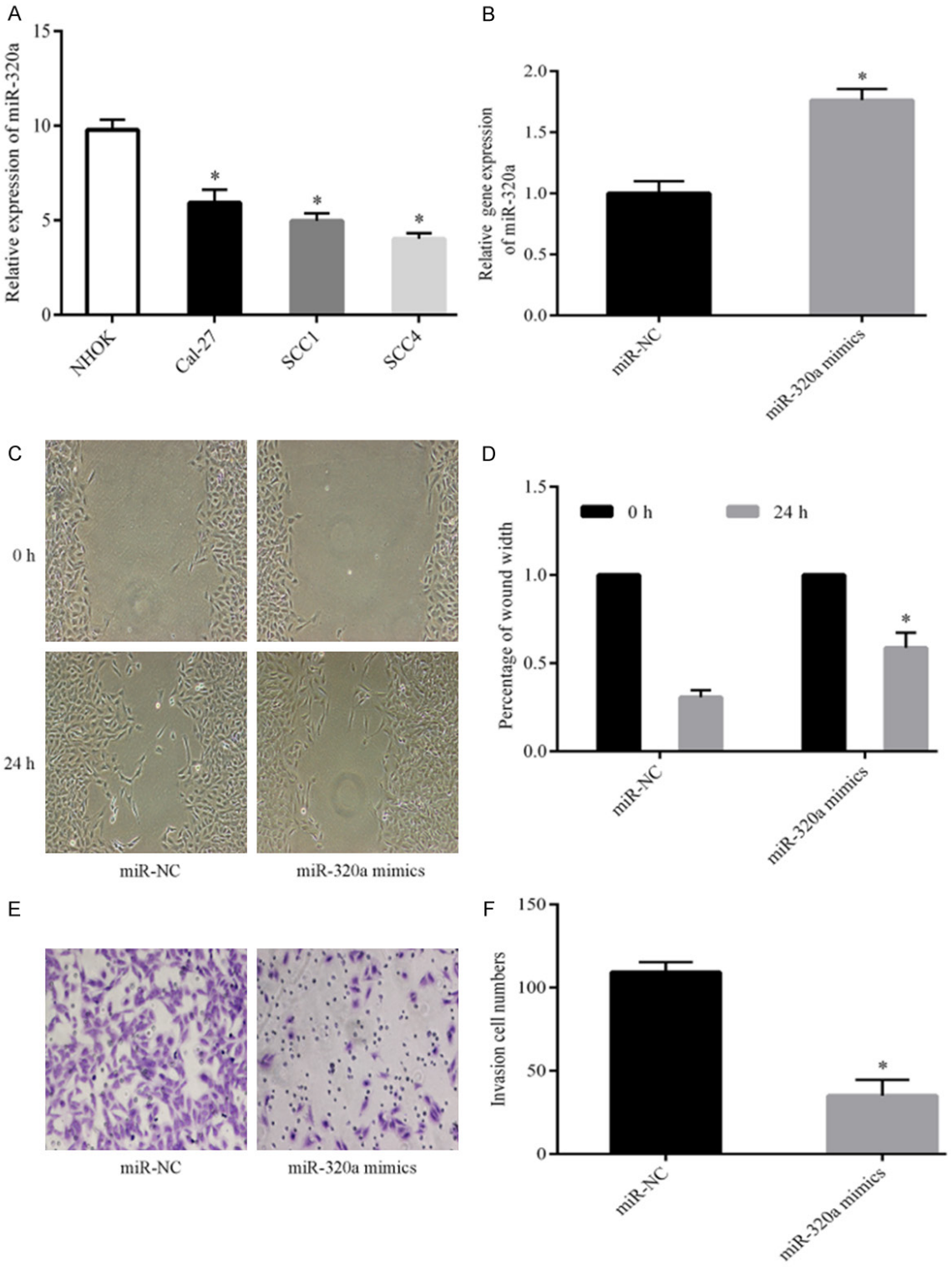

Figure 4. MiR-320a suppressed SCC4 cell migration and invasion in vitro. A: miR-320a was significantly downregulated in different OTSCC cell lines when compared with normal oral keratinocyte cell lines (*P<0.05). B: miR-320a was overexpression in SCC4 cells when transfected with miR-320a mimics, compared with miR-NC. C, D: Wound healing assay showed that when transfected with miR-320a mimics the migration ability inhibited than the cells transfected with miR-NC (*P<0.05). E, F: Transwell migration assay demonstrated that miR-320a remarkably inhibited cell invasion (*P<0.05).

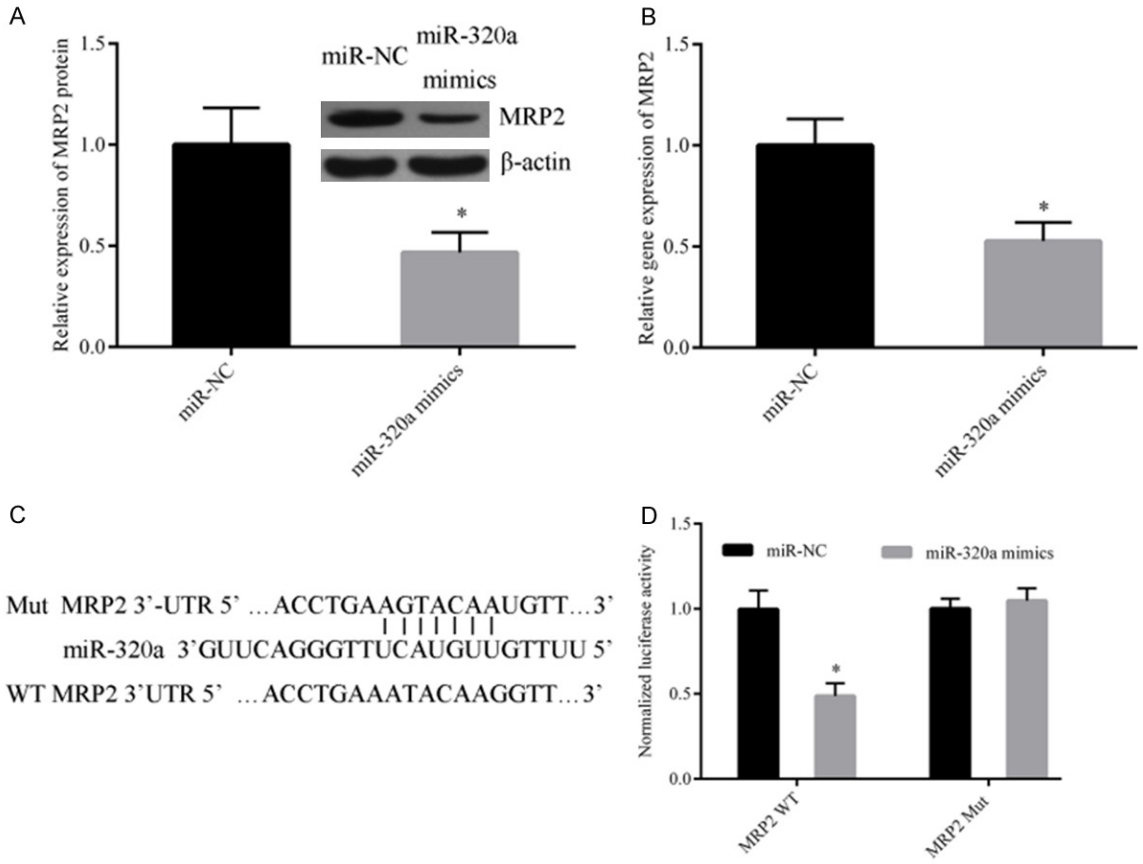

Figure 5. MiR-320a directly targeted WRP2 in SCC4 cells. A, B: Western blot and RT-PCR showed that the expression of MRP2 was significantly suppressed in miR-320a mimics group (*P<0.05). C: Sequence of miR-320a and the MRP2 3'-UTR, which contains a predicted miR-320a biding site. D: Luciferase assay in SCC4 cells co-transfected with miR-320a mimics and mimic control which containing the MRP2 3'-UTR (WT) or a mutant (Mut) (*P<0.05).

miR-320a upregulated (P<0.05, **Figure 4C** and **4D**). Transwell invasion assay demonstrated that increased the level of miR-320a dramatically reduced the cells invaded when compared with the cells transfected with mimics controls (**Figure 4E** and **4F**, P<0.05). To sum up, these data suggested that miR-320a suppressed migration and invasion in SCC4 cells in vitro.

MRP2 is a direct target of miR-320a in OTSCC cell

In order to verify the hypothesis that miR-320a directly reduces WRP2 expression through its mRNA's 3'UTR, the expression level of MRP2 was assessed in miR-320a mimics and miR-NC by Western blot. Results showed that the level of MRP2 was significantly reduced in the miR-320a mimic group than the mimic controls (**Figure 5A**, P<0.05). The gene expression of MRP2 was consistent with the protein level (**Figure 5B**). The luciferase reporter assay was then performed for further validation. MRP2 wild-type (WT) or mutant 3'-UTR was subcloned into a luciferase reporter vector and co-transfected with miR-320a mimics or mimic controls into SCC4 cells, results demonstrated that in SCC4 cell lines the luciferase activity of MRP2 WT 3'-UTR was markedly suppressed by miR-320a but had no influence on the mutant (**Figure 5C** and **5D**).

Discussion

Over the past decade, studies have shown that miRNAs can be functional as a tumor regulator, either as cancer suppressor or oncogene [30, 31]. Even more interesting is that some studies had showed that miR-320 family members could function as tumor inhibitors in several cancers, such as prostate cancer, colorectal cancer, colon cancer and cervical cancer

[32-35]. These discoveries suggested that miR-320a may have an important role in tumor migration and invasion.

In this study, we found that the expression level of miR-320a was downregulated in OTSCC tissues when compared with the corresponding adjacent normal tissues. In addition, we also demonstrated that miR-320a expression was also decreased in OTSCC cell lines than the normal oral keratinocyte cell (NHOK). Furthermore, in order to confirm the effect of miR-320a on migration and invasion, we used miR-320a mimics and mimic control transfected into OTSCC cells, results showed that the expression level of miR-320a can be regulated in OTSCC cells and upregulation of miR-320a dramatically suppressed OTSCC cell migration and invasion than the cells transfected with miR-NC. What is more, in order to investigate the molecular mechanism of the cancer inhibitor role of miR-320a in OTSCC we made use of luciferase reporter assay and western blot verified that MRP2 as a potential target gene of miR-320a in OSCC cell. Moreover, both the qRT-PCR and western blot showed that the level of MRP2 can be negatively regulated by miR-320a, which was play the role by binding with a site in the MRP2 3'-UTR.

In conclusion, our results verified that the expression level of miR-320a was decreased in oral tongue squamous cell carcinoma tissues and cell lines. Overexpression of miR-320a suppressed the oral tongue squamous cell carcinoma cells migration and invasion. We also demonstrated that MRP2 was a potential target gene of miR-320a. This study revealed that miR-320a play as a tumor suppressor in the development and progression of OTSCC by targeting MRP2, it may serve as a predictor for prognosis and a therapeutic target for OTSCC patients.

Acknowledgements

This study was founded by the National Natural Science Foundation of China (No.79632421).

Disclosure of conflict of interest

None.

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