

miR-10a-5p inhibits the migration and invasion of human oral carcinoma cells by targeting PIK3CA through PI3K/AKT/mTOR pathway

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Received 26 Oct 2021, Accepted 20 Feb 2022
Available online 25 May 2022

ABSTRACT: Oral carcinoma (OC) is one of the most common malignant tumors in the Chinese population, and cancer metastasis is one of the leading causes of death in OC patients. This study is designed to investigate the correlation between miR-10a-5p and OC cell metastasis. MiR-10a-5p expression was detected by real-time fluorescence quantitative polymerase chain reaction in 93 pairs of OC and adjacent normal tissues, while protein expression was detected using western blot. Transwell assay was performed to assess the metastatic ability of OC cells. In this study, we found that miR-10a-5p expression was low in OC tissues, and the expression level decreased with the increase of histological grade and tumor node metastasis (TNM) stage. Compared with OC tissues without lymph node metastasis, miR-10a-5p was expressed less in OC tissues with lymph node metastasis. OC patients with low miR-10a-5p expression have a shorter 5-year overall survival after surgery. The luciferase gene reporter system confirmed that miR-10a-5p targeted inhibition of PIK3CA expression in OC cells. In addition, miR-10a-5p negatively regulated PI3K, P-AKT/AKT and P-mTOR/mTOR expression and inhibited the invasion and migration of SAS cells *in vitro*. All in all, the present study indicated that miR-10a-5p was lowly expressed as a tumor suppressor gene in OC tissues and suppressed the metastasis of OC cells *in vitro* by targeting PIK3CA via the PI3K/AKT/mTOR pathway.

KEYWORDS: miR-10a-5p, PIK3CA, oral carcinoma, cell migration, cell invasion

INTRODUCTION

Oral carcinoma (OC) is a malignant tumor with a high incidence, and its clinical treatment is mainly surgical resection, adjuvant radiotherapy and chemotherapy [1, 2]. In recent years, although the continuous in-depth research on oral cancer has reduced the 5-year mortality rate of patients with oral cancer, its mortality rate is still at the forefront of malignant tumors. It is one of the primary diseases causing human death. Previous studies have shown that cancer cells that metastasize to lymph nodes or distal tissues and organs are one of the leading causes of death in oral carcinoma patients. The survival rate of oral carcinoma patients with cancer cell metastasis is also significantly low [3]. Therefore, it is of great significance to study the specific molecular mechanism of oral carcinoma cell metastasis to improve the clinical therapeutic effects and prolong the survival time of oral carcinoma patients.

MicroRNAs (miRNAs) are non-coding, single-stranded RNAs encoded by 20–25 ribonucleotides that can regulate cell proliferation, differentiation, apoptosis, metabolism, and invasion/migration through targeted regulation of specific gene translation and transcription [4, 5]. It is not only a biomarker for diagnosing multiple diseases but also plays an essential

role in disease development [6]. In recent years, several studies have shown [7, 8] that the development of OC cancer is related to the abnormal expression of miRNA. MiR-10a-5p is abnormally expressed in many malignant tumor tissues and can be exerted as a tumor suppressor gene or as a proto-oncogene. For example, miR-10a-5p plays a role as a tumor suppressor gene in advanced renal cell carcinoma [9], gastric cancer [10], prostate cancer [11], and cervical cancer [12]. In contrast, it takes on the part of a proto-oncogene in pancreatic ductal carcinoma [13] and acute leukemia [14]. Thus, miR-10a-5p plays different roles in different tumors. However, miR-10a-5p expression levels in OC tissues and their function in OC cell metastasis remain unknown.

This study aims to investigate the expression of miR-10a-5p in OC tissues and cells and explore the effect of miR-10a-5p on the OC cell metastasis *in vitro* and its relationship with the PIK3CA/PI3K/AKT/mTOR pathway.

MATERIALS AND METHODS

Tissue and ethics statement

This study collected 93 pairs of OC tissue and adjacent cancer normal tissues after surgery in the Nanjing Stomatological Hospital. The 93 OC patient cases

Table 1 Relationship between miR-10a-5p and clinical features of OC patients.

Clinical feature	n	miR-10a-5p expression		χ^2	p-value
		High (n=42)	Low (n=51)		
Age (years)					
≤50	30	13	17	0.060	0.807
>50	63	29	34		
Gender					
Male	60	25	35	0.834	0.361
Female	33	17	16		
Smoking					
No	26	12	14	0.029	0.864
Yes	67	30	37		
TNM stage					
0-II	57	35	22	15.685	<0.001
III-IV	36	7	29		
Histological grade					
G1	31	20	11	9.696	0.008
G2	37	15	12		
G3	25	6	19		
Lymph node metastasis					
No	61	35	26	10.682	0.001
Yes	32	7	25		

were aged from 32 to 71 years with a median age of 55 years. Other clinical data are listed in Table 1. In addition, all subjects (or their guardians) included in this study consented to the research protocol and signed an informed consent form. The Ethics Committee of Nanjing Stomatological Hospital approved this research protocol.

Real-time quantitative PCR

The levels of miR-10a-5p were detected by RT-qPCR as previously described with the GoTaq® qPCR Master Mix (A6002, Promega, Beijing, China) and amplified using ABI 7500 fluorescence quantitative PCR instrument. Reverse transcription was performed using a PrimeScript RT reagent Kit with gDNA Eraser (RR047B, Takara, Da Lian, China) with the program: 37°C-15 min, 85°C-5 s, 4°C-2 min. RT-qPCR parameter settings: 95°C for 30 s, 40 cycles of 90°C for 5 s and 65°C for 30 s. PCR primers: miR-10a-5p-F: 5'-ACACTCCAGCTGGGTACCCTGTAGATCCGAA-3', miR-10a-5p-R: 5'-TGGTGTCGTGGAGTCCG-3'. U6 acted as a loading control for miR-10a-5p. The $2^{-\Delta\Delta t}$ method was used to calculate the relative expression levels of genes, GAPDH was used as a reference gene for mRNA and U6 as a reference gene for miRNA.

Cell culture, treatment and transfection

Both SAS and FaDu OC cell lines were all purchased from the American type culture collection (Virginia, USA) and cultured with DEME medium (61870044, ThermoFisher Scientific, CA, USA) containing 10% Fetal bovine serum (10437028, Invitrogen, CA, USA) at 37°C and 5% CO₂. MiR-10a-5p-NC (5'-CUGACCGGUCAACAAAGAGGCGUC-3'), miR-10a-5p-mimic (5'-UUGUAUAUAUACAUAACAGGGUA-3') and miR-10a-5p-inhibitor (5'-AACAUUAUAUGUAUUGUC

CCAU-3') were directly transferred into cells by the Lipofectamine™ 2000 transfection reagent (1668019, ThermoFisher Scientific), and WT-PIK3CA or MUT-PIK3CA was first integrated into the pmirGLO plasmid (49380, Addgene, MA, USA) and then transferred into cells. The experiments were done 48 h after transfection.

Western blot analysis

The levels of PI3K, p-AKT, AKT, p-mTOR and mTOR proteins were detected by western blotting as previously described [13]. All antibodies were purchased from ABCAM (Cambridge, UK) and diluted at a given ratio according to the instructions. The blocking solution was 5% TBST diluted skim milk powder, which was used for blocking at room temperature for 1 h. The primary antibody was diluted with the blocking solution and incubated for 2 h at room temperature. Then the secondary antibody was diluted with blocking solution and incubated for 1 h at room temperature. Imag J (NIH, USA) was used to analyze the gray values of the protein bands, and β -actin was used as the reference protein for normalization. The information of ABCAM antibodies were shown as follows: Anti-PIK3CA antibody (1:1000, ab183957), Anti-PI3K antibody (1:1000, ab32089), Anti-AKT antibody (1:2000, ab8805), Anti-AKT (phospho T308) antibody (1:500, ab38449), Anti-mTOR antibody (1:1000, ab32028) and Anti-mTOR (phospho S2448) antibody (1:500, ab109268).

Cell invasion and migration assay

A 24-well Transwell chamber was used to evaluate cell motility. First, 100 μ l of Matrigel (BD354248, Becton Dickinson, MD, USA) was pipetted into an ice-cold 300 μ l serum-free medium with an ice-cold pipette

and mixed well. The above diluted Matrigel (25 μ l) was added to the upper chamber of the Transwell plate (140644, Costar, CA, USA). The entire polycarbonate film was coated for 30 min at 37 °C to allow the polymerization of Matrigel into the gel. For cell migration assay, the above diluted Matrigel was not added to the Transwell plate upper chamber. In the following tests, the methods of cell migration and invasion tests was the same. Then, 0.5×10^5 cells/100 μ l of medium were seeded in the upper chamber of the Transwell plate. 600 μ l of DMEM medium containing 2.5% (v/v) fetal bovine serum was added to the lower chamber. After incubating for 24 h at 37 °C in a 5% (v/v) CO₂ atmosphere, we removed the cell culture medium in the Transwell lower chamber, washed it twice with sterile PBS, and added formaldehyde for 30 min at room temperature to fix the cells. Then, 0.1% (v/v) crystal violet was added for 20 min at room temperature to stain the cells and washed three times with PBS. Lastly, the cells were quantified under a bright field microscope.

Dual-luciferase reporter assay

The Dual-Lucy Assay kit (D00100; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was used to detect luciferase activity following the manufacturer's protocol. Briefly, cells were collected at 48 h after transfection and lysed for 5 min on ice before centrifugation (12 000 \times g for 1 min at room temperature) to collect the cell supernatant. Five volumes of firefly luciferase reaction solution or Renilla luciferase reaction solution were added to the cell lysate to detect enzyme activity. Protein concentration of cell lysates was also assayed to normalize fluorescence. The protein concentration of the cell lysates was detected to normalize fluorescence.

Statistical analysis

Data were statistically analyzed by SPSS 20.0. A paired *t*-test was used to compare the differences between miR-10a-5p expression in the normal and OC tissues. Differences between the other two groups were compared using the Student's *t*-test or chi-square test. The comparison between multiple groups was performed using the single factor ANOVA method. The Cox regression model was used for univariate and multivariate analysis of OC patient survival time testing. Survival curves of OC patients based on miR-10a-5p expression were plotted by the Kaplan-Meier method. The log-rank test was used to compare differences in survival curves. $p < 0.05$ indicated a significant difference.

RESULTS

MiR-10a-5p was lowly expressed in OC tissues

Ninety-three pairs of OC and adjacent normal tissues were collected after surgery, and the miR-10a-5p ex-

pression was measured. As shown in Fig. 1A, miR-10a-5p expression in OC tissues was significantly lower than in normal tissues. According to the histological grade, 92 OC patients were divided into 31 cases of G1 stage, 37 cases of G2 stage and 25 cases of G3 stage. The highest expression of miR-10a-5p in OC tissues was the G1 stage while the lowest expression was the G3 stage (Fig. 1B). Similarly, there were 57 cases of stage 0–II and 36 cases of stage III–IV according to the TNM grade, and miR-10a-5p had the highest expression in OC tissues with stage 0–II and the lowest expression in OC tissues with stage III–IV (Fig. 1C). Moreover, miR-10a-5p was expressed less with lymph node metastasis compared with OC tissues without lymph node metastasis (Fig. 1D). We also analyzed the correlation between miR-10a-5p expression and clinical pathology of OC patients (Table 1), it was found that miR-10a-5p expression in OC tissues was related to histological grade ($p = 0.008$), lymph node metastasis ($p = 0.001$) and TNM stage ($p < 0.001$).

MiR-10a-5p was associated with the overall survival of OC patients

All OC patients who measured miR-10a-5p were visited after surgery for 5 years or until death. According to miR-10a-5p expression level, 93 OC patients were divided into two groups, the high miR-10a-5p group (miR-10a-5p expression \geq median value) and the low miR-10a-5p group (miR-10a-5p expression $<$ median value). As shown in Fig. 2, compared with OC patients in the high miR-10a-5p group, OC patients in the low miR-10a-5p group had a significantly longer overall survival time. In addition, the results of univariate and multivariate cox regression analysis showed that the level of miR-10a-5p expression levels in OC tissues affected OC patient prognosis (Table 2).

MiR-10a-5p targeted inhibition of PIK3CA expression in OC cells *in vitro*

We predicted the target genes of miR-10a-5p on www.targets.org and found that PIK3CA and miR-10a-5p have a sequence that binds to each other (Fig. 3A). We used the luciferase gene reporter system to confirm that miR-10a-5p could regulate PIK3CA expression by binding to the PIK3CA 3'-UTR end. The results showed that the transfection of miR-10a-5p-mimic significantly decreased the WT 3'-UTR luciferase activity ($p < 0.001$), and the miR-10a-5p-inhibitor significantly increased in SAS and FaDu cells (Fig. 3B-C). However, this did not work in MUT (the mutant for PIK3CA). Furthermore, as shown in Fig. 3D-E, miR-10a-5p targeted inhibition of PIK3CA expression in OC cells *in vitro*.

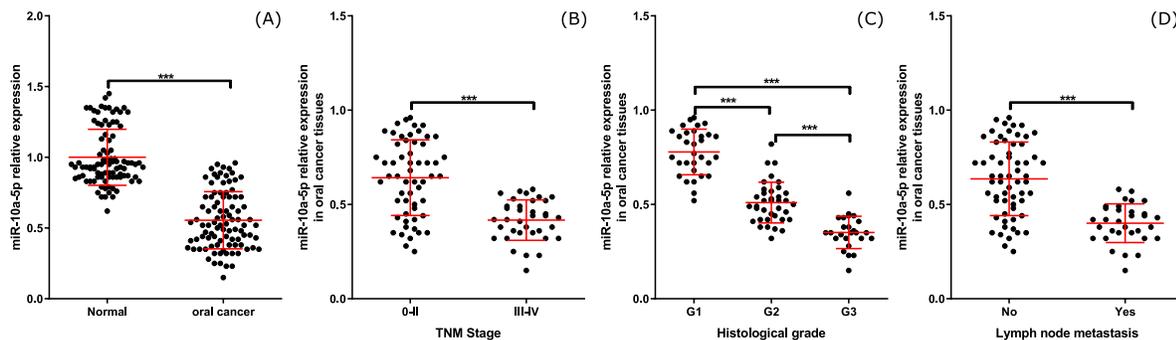


Fig. 1 miR-10a-5p expression in normal tissues and OC tissues measured by RT-qPCR. A, miR-10a-5p expression in normal tissue and OC tissue; B–D, Analysis of miR-10a-5p expression in different OC patients. *** was $p < 0.001$.

Table 2 Univariate and multivariate Cox regression analysis for the prognosis of OC patients.

Clinical parameter	Univariate analysis			Multivariate analysis		
	OR	95%CI	p-value	OR	95%CI	p-value
Age	1.025	0.524–3.228	0.638		0.624	
Gender	1.325	0.894–5.328	0.128		0.635	
smoking	0.976	1.164–6.248	0.084		0.257	
Histological grade	4.025	0.563–8.237	0.018	2.764	1.167–9.435	0.025
Lymph node metastasis	4.425	1.297–6.357	<0.001	2.695	1.432–9.867	0.018
TNM stage	4.024	2.321–7.628	<0.001	4.201	1.045–9.721	0.041
MiR-10a-5p level	6.927	1.032–13.521	<0.001	6.317	3.201–9.328	<0.001

OR = odds ratio, 95%CI = 95% confidence interval; SPSS statistical analysis software was used for multivariate Cox regression analysis. When p was less than 0.05, the results did not show the OR and 95%CI.

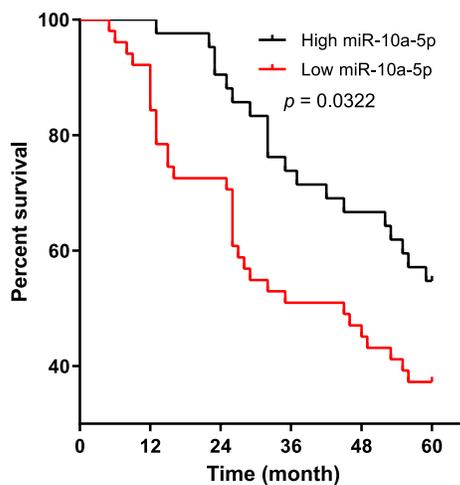


Fig. 2 Effect of miR-10a-5p expression on the 5-year overall survival in OC patients.

MiR-10a-5p inhibited metastasis of OC cells *in vitro*

The expression of miR-10a-5p was regulated by transfecting miR-10a-5p-inhibitor or miR-10a-5p-mimic into SAS cells and measured the related protein expression. We found that miR-10a-5p-inhibitor could significantly increase PI3K, p-AKT(phospho T308)/AKT

and p-mTOR (phospho S2448)/mTOR protein expression in SAS cells, while miR-10a-5p-mimic decreased them (Fig. 4). In addition, miR-10a-5p-inhibitor could significantly increase, while the mimic significantly decreased the number of SAS cells entering the lower chamber of the Transwell in the migration and invasion assay (Fig. 5).

DISCUSSION

Although miRNA is a small non-coding RNA, it plays a vital role in regulating the transcription of genes. It can regulate cell proliferation, differentiation, apoptosis, metabolism, and invasion/migration activities by targeting their target genes [15]. Previous studies have shown that miRNAs play an important regulatory role in the progression of many malignancies [16], such as prostate cancer [17, 18], gastric carcinoma [19], colorectal cancer [20], breast cancer [21], etc. More importantly, dysregulated miRNAs in tumor tissues have been used as targets for developing cancer therapeutic drugs [22].

In the present study, we found that miR-10a-5p was lowly expressed in OC tissues and the expression level decreased with increasing histological grade and TNM stage. Moreover, miR-10a-5p expression was less in OC tissues with lymph node metastasis compared with those tissues without. And OC patients with low miR-10a-5p expression had a shorter 5-year overall survival after surgery. These results suggest that miR-

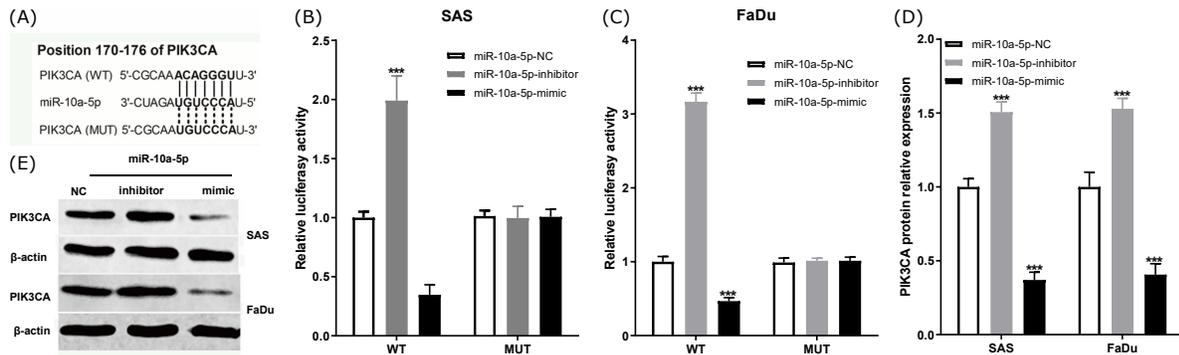


Fig. 3 Effect of miR-10a-5p expression on PIK3CA protein expressions *in vitro*. A, Predicted consequential pairing of PIK3CA and miR-10a-5p; B-C, Luciferase activity was detected in SAS cells and FaDu cells with different treatment; D-E, PIK3CA protein expression in SAS cells and FaDu cells with different treatment. Data were from 3 independent experiments. *** $p < 0.001$ vs. miR-10a-5p-NC group.

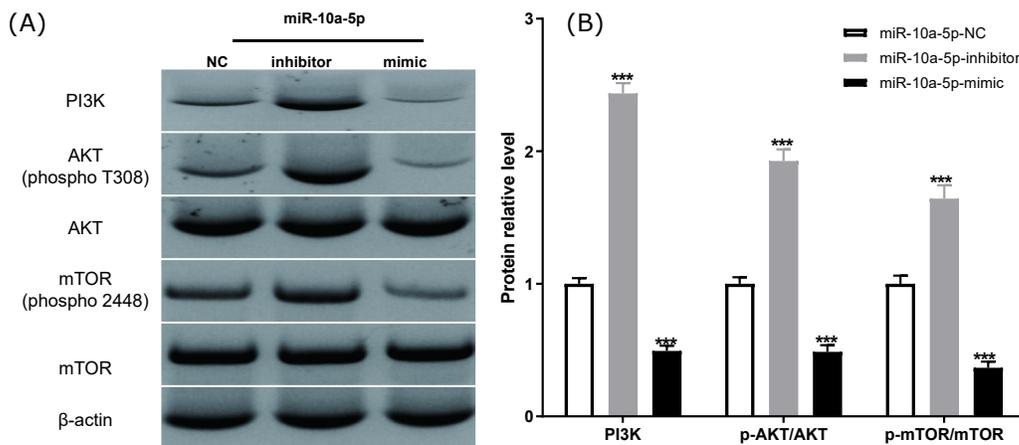


Fig. 4 Effect of miR-10a-5p expression on the PI3K/AKT/mTOR pathway *in vitro*. A, Representative protein band displayed in SAS cells with different treatment; B, Effect of miR-10a-5p expression on PI3K, p-AKT(phospho T308)/AKT and p-mTOR (phospho S2448)/mTOR expression in SAS cells with different treatment. Data were from 3 independent experiments. *** $p < 0.001$ vs. miR-10a-5p-NC group.

10a-5p might act as a tumor suppressor gene in OC. Similarly, Arai et al [9] found that miR-10a-5p was under-expressed in advanced renal cell carcinoma and that low expression of miR-10a-5p inhibited renal cancer cell metastasis by targeted inhibition of SKA1 protein expression. Lu et al [10] reported that miR-10a-5p was essential for lymph node metastasis of gastric cancer cells, and miR-10a-5p inhibited the metastasis of such cancer cells by targeted inhibition of MAPK8IP1 protein expression *in vitro*.

To further investigate the role of miR-10a-5p in the development of OC, we predicted the target genes of miR-10a-5p on www.targetscan.org and verified them by luciferase gene reporter system. We found that PIK3CA is a target gene of miR-10a-5p and miR-10a-5p targets inhibition of PIK3CA protein expression in OC cells. Although the relationship between miR-10a-5p and PIK3CA in OC is unknown, PIK3CA has

been well studied in this type of cancer. The PIK3CA gene was detected by *in situ* hybridization, it is located on chromosome 3q26.3 and is 34 kb in length, contains 20 exons encoding 1068 amino acids and produces a set of 124 kD proteins [23]. PIK3CA is an oncogene expressed in the normal brain, lung, breast, gastrointestinal, cervix, ovary and other tissues. It has a variety of critical physiological functions such as regulating somatic cell proliferation, differentiation and survival [24]. However, PIK3CA is mainly in an inactive form and is usually not easily detected, but its mutated gene and its protein could be overexpressed and detected [24].

Previous studies have shown that mutations in the PIK3CA gene cause an increase in the catalytic activity of P13Ks and promote cell carcinogenesis [25, 26]. Research data showed [27, 28] that about 30% of solid human tumors have mutations in the

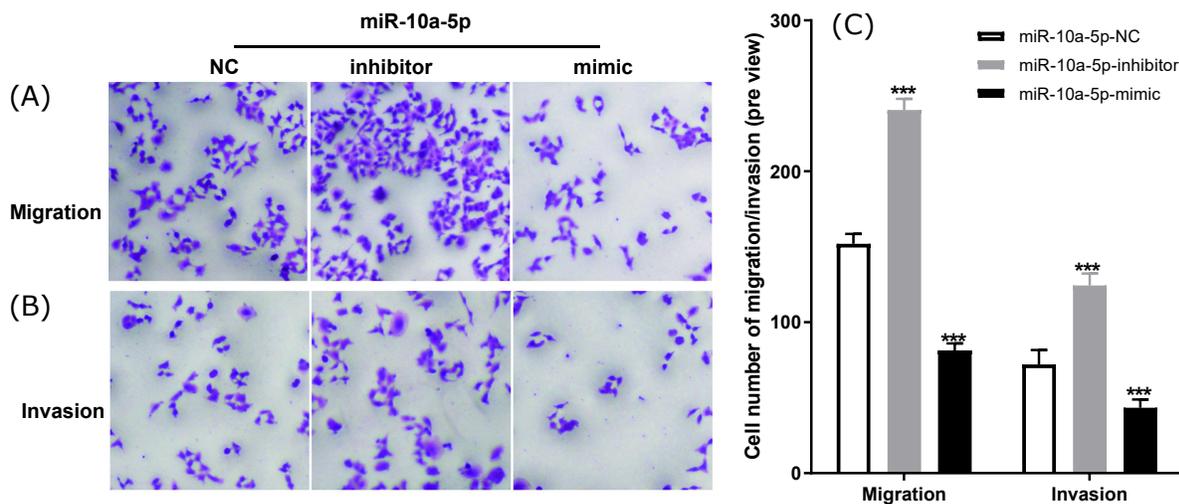


Fig. 5 Effect of miR-10a-5p expression on metastasis of OC cell *in vitro*. Transwell was used to evaluate the invasion and migration of SAS. Scale bar = 50 μ m. 3 independent experiments were performed for each treatment, and *** was $p < 0.001$ vs miR-10a-5p-NC group.

oncogene PIK3CA, and the proportion of mutations in colon cancer, glioblastoma, gastric cancer, breast cancer and lung cancer was about 32%, 27%, 25%, 8% and 4%, respectively. In oral carcinoma, only 16% of patients were found to have mutations in the PIK3CA gene [29]. It has also been identified as a potential target for a new treatment strategy for cetuximab-resistant oral squamous cell carcinoma [30]. Our study showed that miR-10a-5p negatively regulated PI3K, p-AKT(phospho T308)/AKT and p-mTOR (phospho S2448)/mTOR expression in SAS cells. PIK3CA encodes the p110 catalytic subunit of class I phosphatidylinosine-3-kinases (PI3Ks), PI3Kp110 α . Elevated PIK3CA protein (PI3Kp110 α) expression enhances PI3K protein activity, whereas activated PI3K could produce a second messenger PIP3 on the plasma membrane that binds to AKT, translocating AKT from the cytoplasm to the cell membrane and simultaneously undergoing a conformational change (phosphorylation modification) [31, 32]. When the modified phosphorylation site of AKT protein is T308, it is considered to be related to the invasion and migration of tumor cells [31, 32]. When the AKT protein enters the nucleus, it could regulate the expression and modification of the downstream gene mTOR, thereby regulating cell proliferation, differentiation, apoptosis, invasion and migration [31, 32]. Therefore, these results indicate that miR-10a-5p negatively regulates the PI3K/AKT/mTOR pathway by targeting the inhibition of PIK3CA in OC cells.

It is well known that PI3K/AKT/mTOR pathway plays a vital role in the metastasis of cancer cells. Previous studies showed that miRNA-375 inhibited colorectal cancer growth by targeting PIK3CA [33]. MiR-19a inhibited renal cancer cell transfer by targeting

PIK3CA [34]. Fortunately, in the current study, we also found that miR-10a-5p could reduce the number of SAS cells that invaded or migrated into the lower Transwell chamber. It suggests that miR-10a-5p suppressed the metastasis of OC cells *in vitro*.

In conclusion, miR-10a-5p, down-expressed in OC tissues, is a tumor suppressor gene that suppresses the metastasis of OC cells by targeted inhibition of PIK3CA through the PI3K/AKT/mTOR pathway.

Acknowledgements: This study was supported by the Natural Science Foundation of Jiangsu Province (No. BK20201119).

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