Original Research Article

Suppressive effect of Hsa-miR-125a-5p on cervical cancer proliferation and invasion via regulation of Rab25-PI3K/AKT pathway

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Abstract

Purpose: To study the suppressive effect of Hsa-miR-125a-5p on the proliferation and invasion of cervical cancer (CC) cells through the modulation of the Rab25-PI3K/AKT pathway.

Methods: 66 pairs of CC and neighboring cell samples were harvested from patients who underwent surgical resection in the Oncology Department from August 2017 to December 2018. The expression levels of Hsa-miR-125a-5p in CC and neighboring cell samples were assessed by quantitative polymerase chain reaction (qPCR). The overexpression vector was transfected into CC cells (siHa cell line), and the proliferation, invasion, migration, and apoptosis of siHa cells were determined. The regulatory role of Hsa-miR-125a-5p in PI3K/AKT pathway was determined using Western blotting.

Results: CC tissues, relative to the neighboring tissues, exhibited remarkably lower Hsa-miR-125a-5p levels (p < 0.05), and their upregulation significantly inhibited the growth, migration, and intrusion of siHa cells while repressing apoptosis (p < 0.05). Dual-luciferase reporter assay (DLRA) data indicate that luciferase activity decreased when Hsa-miR-125a-5p was bound to Rab25 (p < 0.05). The Hsa-miR-125a-5p mimics group had significantly lower PI3K/AKT levels, relative to the negative control mimics group (p < 0.05).

Conclusion: Hsa-miR-125a-5p represses CC cell proliferation and invasion through the modulation of Rab25-PI3K/AKT pathway, thus suggesting that this pathway is a target for drug development.

Keywords: Hsa-miR-125a-5p, Rab25, PI3K/AKT pathway, Cervical cancer, Cell proliferation

INTRODUCTION

Cervical cancer (CC) is a primary gynecological cancer characterized by abnormal cell division and female cervical tissue infiltration [1]. In addition, it remains the fourth most frequently occurring gynecological cancer worldwide [2], with high morbidity and mortality rates [3]. There are 5.28 × 10^5 new cases of CC and 2.66 × 10^5 associated deaths worldwide each year, and these numbers are higher than those of other gynecologic cancers [4]. Although early CC can be cured through radical and fertility-preserving surgeries, several patients still face unsatisfactory prognosis, limited treatments, and incurable diseases [5]. The present standards for
treatment of CC include surgery, radiation, and chemotherapy [6]. However, conventional chemotherapy is incapable of eliciting therapeutic responses, and triggers serious systemic toxicity. In addition, drug resistance to chemotherapy/radiotherapy induced by long-term treatment, leads to cancer invasiveness. Therefore, the development of novel treatment methods for CC is required [7].

MicroRNAs (miRNAs) are a class of 22-nucleotide-long, endogenous, noncoding RNAs [8] that are present in nearly all eukaryotes, which is essential in every cell process, especially in determining cell fate during the development and regulation of the cell cycle [9]. Under normal physiological conditions, miRNAs maintain cell proliferation, differentiation, and apoptosis [10].

The motility and invasion of cancer cells play an essential role in the mortality of patients with CC [11]. Previous evidence showed that hsa-miR-125a-5p has been found to be abnormally expressed in various cancers. For example, miR-125a-5p overexpression has been observed in non-small-cell lung cancer (NSCLC), and it is a noninvasive biomarker for the diagnosis of NSCLC [12]. In addition, miR-125a-5p is a critical regulator of the proliferation, migration, and differentiation of various cells, and it exhibits a high expression level in vascular smooth muscle cells, and decreased levels after vascular injury [13]. This study aims to examine the suppressive role played by Hsa-miR-125a-5p in the proliferation and invasion of cervical cancer (CC) cells through the modulation of the Rab25-PI3K/AKT pathway.

EXPERIMENTAL

Sample collection

Sixty-six pairs of CC and neighboring samples were harvested from patients who received surgical resection in the Oncology Department of Sanming First Hospital, Affiliated Hospital of Fujian Medical University from August 2017 to December 2018.

Exclusion and inclusion criteria

Inclusion criteria were patients diagnosed with CC who had complete clinical data and normal mentality. This study was approved by the ethics committee of Sanming First Hospital, Affiliated Hospital of Fujian Medical University, and followed International Human Research and Ethics Standards [14]. Patients and their family members signed an informed consent form. Exclusion criteria included patients who previously received CC treatment or who had other tumors, severe hematologic diseases, or severe hepatic and renal insufficiencies.

Main materials and equipment used

The CC cell line, siHa (Shanghai Qiming Biotechnology Co., Ltd., Shanghai, China); a real-time fluorescence quantitative polymerase chain reaction (qRT-PCR) instrument (Guangzhou Huafeng Biotechnology Co. Ltd, Guangzhou, China); an apoptosis kit (Beijing Bionega Technology Co. Ltd, Beijing, China); TRizol reagent (Beijing Baolebo Technology Co. Ltd, Beijing, China); 10 % fetal bovine serum (FBS; Shanghai Lianshuo Biotechnology Co. Ltd, Beijing, China); Dulbecco’s modified eagle medium (DMEM; Qingdao Jisskang Biotechnology Co. Ltd, Qingdao, China); lipofectamine TM2000 transfection reagent (Suzhou Yuheng Biotechnology Co., Ltd., Suzhou, China); Cell-Counting Kit 8 (CCK-8; Beijing G-Clone Biotechnology Co., Ltd., Beijing, China); an ultraviolet spectrophotometer (Shanghai Clinx Science Instrument Co., Ltd, Shanghai, China); BD flow cytometer (Beijing DERICA Biotechnology Co. Ltd, Beijing, China); Transwell (Shanghai Shengbo Biotechnology Co. Ltd, Shanghai, China); reverse transcriptase (Shanghai Kang Lang Biotechnology Co. Ltd., Shanghai, China); a microplate reader (Beijing Image Trading Co. Ltd, Beijing, China); paraformaldehyde (Zibo Qixing Chemical Technology Co. Ltd, Zibo, China); and crystal violet (Shenzhen XYS Industry Co. Ltd, Shenzhen, China). Hsa-miR-125a-5p and internal reference were synthesized by Suzhou Syn Biotechnology Co. Ltd. (Suzhou, China).

Determination of Hsa-miR-125a-5p levels

The Hsa-miR-125a-5p levels in CC and neighboring cell samples were independently measured using qRT-PCR. Total RNA was extracted using TRizol reagent, and its concentration and purity were detected using an ultraviolet spectrophotometer. RNA with an OD260/OD280 ratio of 1.8:2.0 was obtained and synthesized to cRNAs using reverse transcriptase and oligonucleotide. The transcription system (20 μL) consisted of the following: 4 μL ProtoScript® II Reverse Transcriptase Reaction buffer, 2-μL reverse transcriptase, 2 μL total RNA, and 12 μL RNase-free water. Reaction conditions included a 42°C water bath for 1 h and a 95 °C water bath for 5 min. Amplification was performed using PCR and glyceraldehyde phosphate dehydrogenase as an internal control, and the Hsa-miR-125a-5p level
was measured through its specific primers on a quantitative fluorescence RT-PCR instrument. The PCR reaction system comprised of upstream and downstream primer (0.4 μL each), Taq DNA polymerase (0.5 μL), and double-distilled H2O in a final volume of 20 μL. PCR conditions were 94 °C for 10 s, followed by 40 cycles of 94 °C for 5 s, and annealing at 52 °C for 0.5 min and 72 °C for 15 s. Three replicates were set up for each sample, and each test was repeated three times. The test outcomes were quantitatively analyzed, and the 2−△△ct method was applied for calculating the relative expressions of Hsa-miR-125a-5p. The primer sequences used are shown in Table 1.

**Cell culture and transfection**

Conventional subculturing was performed in DMEM (high glucose) containing 10 % FBS, but without double antibody at 37 °C and 5 % CO2. Cells at a density of 60 – 70 % were seeded in six-well plates. Hsa-miR-125a-5p mimic and negative control mimic vectors were transfected in cells with the lipofectamine TM2000 kit.

**Cell growth studies**

Transfected siHa cells were prepared in a suspension and inoculated into 96-well plates (100 μL/well). Each sample was tested in three parallel wells and cultivated for 1, 2, 3, and 4 days, separately. Afterwards, the CCK-8 solution (0.02 mL) was transferred into each well 2 h before the completion of the culture, and placed into an incubator at 37 °C and 5 % CO2. Two hours later, A490 was determined in a full-automatic microplate reader for evaluating cell proliferation. The test was repeated three times.

**Evaluation of cell migration and invasion**

The cells were trypsinized and then resuspended in a serum-free culture medium. Resuspension (200 μL) was performed for the determination of migration. Cells (5 × 10⁶) were placed in the apical chamber of the Transwell set-up, and 1 mL of the medium containing FBS was transferred into the basolateral chamber. Following conventional culturing for 1 day, the cells in the upper chamber were wiped off with cotton buds, and those moved to the basolateral chamber were stained with 4 % POM and 0.1 % crystal violet. After the Transwell insert was dried, the membrane was blocked, and migrated/invaded cells were counted under an optical microscope. The test was repeated in triplicate.

**Determination of cell apoptosis**

Cell apoptosis was determined using an apoptosis detection kit. A BD flow cytometer was employed for detecting the transfected cells for 48 h in the six-well plate and stained with Annexin V/PI, and the test was repeated three times.

**Dual-luciferase reporter assay (DLRA)**

The DNA fragment of Rab25 mRNA 3′-UTR containing the Hsa-miR-125a-5p putative binding site was subcloned to Xho I and Not I restriction sites downstream of the Renilla luciferase coding site of the psiCHECK-2 vector. Sequencing was performed by Sangon Biotech. PsiCHECK2-miR-125a-5p wild type (wt) and PsiCHECK2-miR-125a-5p-mutant (mut) were constructed and transfected into siHa cells separately using lipofectamine TM2000. Cells were transfected for 2 days and harvested, and the Renilla and firefly luciferases activities were determined through DLRA (Promega, Madison, WI, USA).

**Western blotting**

The transfected cells were lysed using cell lysis buffer (Qincheng Biotech, Shanghai, China; Cat no: QC25-05099). The same amount of protein was isolated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10%) and then added into a polyvinylidene fluoride membrane (BSP0161; Zeping Bioscience & Technologies Co., Ltd., Beijing, China) and sealed for 1 h. The membrane was sealed with 5 % bovine serum albumin sealing solution (QN1119-ANF; Biolab Technology Co., Ltd., Beijing, China) at ambient temperature for 1 h and then incubated overnight with PI3K/AKT signalling pathway combination kit (Abcam, ab283852, 1:1000) at 4 °C. The washed membrane was incubated with Universal secondary antibody (goat anti-rabbit, Shanghai Yuanmu Biotechnology Co., Ltd., Shanghai, China) at room temperature for a 120-min reaction.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Upstream primer (5′-3′)</th>
<th>Downstream primer (5′-3′)</th>
</tr>
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<tbody>
<tr>
<td>miR-125a-5p</td>
<td>GGCGGTCCCTGAGCCCTTTAC</td>
<td>ATCCAGTGACGGGTCGCCAGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AGGTTCGTCATGGGTTGAA</td>
<td>GTGTCAAGAGCATGGGGT</td>
</tr>
</tbody>
</table>
After rinsing three times, the member was fixed and developed with enhanced chemiluminescence.

Statistical analysis

Differences were verified using SPSS statistical analysis software (version 26.0). The results were analyzed using t-test and are displayed as mean ± standard deviation (SD). Comparison among multiple time points was performed using repeated-measures analysis of variance (denoted by F). \( P < 0.05 \) was considered statistically significant.

RESULTS

HSA-miR-125a-5p level in CC and neighboring samples

Results showed that CC cell samples exhibited significantly lower Hsa-miR-125a-5p levels compared with the neighboring cell samples (0.51 ± 0.13 vs. 1.13 ± 0.32; \( P < 0.05 \)). Details are shown in Figure 1.

Effect of transfection on Hsa-miR-125a-5p levels

The experiment group, relative to the negative control mimics group (NC group), exhibited a higher relative Hsa-miR-125a-5p level (1.25 ± 0.23 vs. 0.54 ± 0.14; \( P < 0.05 \)), indicating that Hsa-miR-125a-5p was significantly upregulated after transfection of the mimics (Figure 2).

Growth of transfected siHa cells

No significant difference was noted in siHa cell growth at 24 h between the Hsa-miR-125a-5p mimics (experimental group) and NC group (\( P > 0.05 \)), whereas the experimental group had significantly lower growth from 48 h to 96 h (\( P < 0.05 \)). A comparison among the different time points revealed that cell growth in both groups had a significant upward trend (\( P < 0.05 \)) (Figure 3).

Migration and intrusion of transfected siHa cells

After transfection, the number of migrated cells in the experimental group was significantly lesser than in the NC group (68.24 ± 12.56 vs. 135.68 ± 14.67; \( P < 0.05 \)). Similarly, after transfection, the number of intrusive cells in the experiment group was significantly lesser (56.58 ± 7.29 vs. 124.13 ± 11.43; \( P < 0.05 \)). Therefore, the Hsa-miR-125a-5p upregulation inhibited the migration and intrusion of siHa cells (Figure 4 and Figure 5).

Apoptosis of transfected siHa cells

Following transfection, the experimental group had a significantly higher apoptotic rate compared to the NC group (28.45 ± 0.464 % vs. 5.81 ± 1.43 %; \( P < 0.05 \)), suggesting that Hsa-miR-125a-5p upregulation accelerated the apoptosis of siHa cells. See Figure 6 for details.
Figure 4: Migration of siHa cells after transfection. After transfection, the number of migrated cells in the Hsa-miR-125a-5p mimics group was significantly lower than that in the NC mimics group. *P < 0.05 vs. the NC mimics group.

Figure 5: Invasion of siHa cells after transfection. After transfection, the number of invaded cells in the Hsa-miR-125a-5p mimics group was significantly lower than those in the NC mimics group. *P < 0.05 vs. the NC mimics group.

Figure 6: Apoptosis of siHa cells after transfection. After transfection, the apoptotic ratio in Hsa-miR-125a-5p mimics group was significantly higher than that of the NC mimics group. *P < 0.05 vs. the NC mimics group.

Hsa-miR-125a-5p targets Rab25

Binding sites of Rab25 and miR-125a-5p were identified using starBase prediction. Moreover, DLRA demonstrated that Hsa-miR-125a-5p mimics reduced the luciferase activity of Rab25-wt, but that of Rab25-mut revealed no changes, indicating that the luciferase activity was significantly reduced when miR-125a-5p was bound to Rab25 (p < 0.05, Figure 7).

Figure 7: Dual-luciferase reporter gene assay. The fluorescence intensity of miR-125a-5p was reduced when it was binding to Rab25. *P < 0.05 vs. the NC mimics group.

Regulatory role of Hsa-miR-125a-5p in PI3K/AKT

The PI3K level was 0.24 ± 0.03 in the experimental group and 1.13 ± 0.12 in the NC group; the corresponding AKT levels were 0.26 ± 0.04 and 1.10 ± 0.11, respectively. Therefore, the experiments group exhibited lower PI3K and AKT levels relative to the NC group (p < 0.05, Figure 8).

Figure 8: Regulatory effect of HSA-mir-125a-5p on PI3K/AKT. Levels of PI3K and AKT in the Hsa-miR-125a-5p mimics group were significantly lower than those in the NC mimics group; *p < 0.05 vs. the NC mimics group.

DISCUSSION

The qPCR results showed that CC tissues exhibited lower levels of Hsa-miR-125a-5p relative to the neighboring tissues, indicating the downregulation of Hsa-miR-125a-5p in CC [15]. Another study found that the miR-125a level was decreased in CC, and was lower relative to the...
tumor size [16]. The miR-125a-5p level in patients with breast cancer (BC) with short-term survival are relatively lower than those with long-term survival, which reveals that serum miR-125a-5p may be used as a prognosis biomarker, with a novel treatment approach targeting HDAC4[17].

When the miR-125a-5p levels in BC samples is lower than that of the reference patients, the survival rate of patients with BC are lower than that of reference patients [18]. These observations demonstrated that miR-125a-5p is a carcinogen, and it may be downregulated in CC. A previous study showed that miR-125a-5p is downregulated in CC cells and human cervical cancer, and miR-125a-5p upregulation inhibits cancer proliferation and migration in vitro and CC transplantation in vivo [18]. In addition, the Hsa-miR-125a-3p level decreased in both chemically-resistant BC samples and experimentally established chemically-resistant BC cells, and its knockdown promotes cell proliferation and damages docetaxel-induced cell death; whereas, its overexpression reduces docetaxel tolerance in BC cells [20]. The miR-125a-5p level in pulmonary adenocarcinoma is reportedly lower than that of the neighboring healthy tissues, and the relative level is significantly associated with cell growth, apoptosis, and lymph node metastasis [21].

Moreover, the proliferation and intrusion into two LA cell lines subjected to miR-125a-5p mimics were significantly decreased, and apoptosis was enhanced [22]. MiR-125a-5p is specifically decreased in colon cancer tissues and cells, and its overexpression represses cell proliferation and elicits apoptosis, which confirms the suppressive role played by miR-125a-5p [23]. Therefore, it can be concluded that miR-125a-5p upregulation inhibits siHa cell growth and migration and promotes apoptosis.

Rab25 is a small GTPase of the Rab protein family that plays a pivotal part in cancer pathophysiology [24]. It can restore cell surface receptors and activate cell-signaling pathways, thereby regulating various cell functions such as proliferation, motility, and death [25]. MiR-125a-5p was found to share binding sites with Rab25 through starBase prediction. Moreover, DLRA demonstrated that the fluorescence activity of miR-125a-5p was inhibited when attempting to bind to Rab25. The upregulated miR-125a-5p also markedly decreased PI3K and AKT levels. There was therefore the suspicion that Hsa-miR-125a-5p inhibited siHa cell proliferation and intrusion through the modulation of the Rab25-PI3K/AKT pathway.

Previous evidence has shown that inhibition of the PI3K/AKT pathway reduces the expression of the Rab25 gene, and mediates the sensitivity of ovarian cancer to cisplatin. In addition, Rab25 knockdown shows an effect equivalent to the blocking of the PI3K/AKT pathway [26]. According to Ding et al, Rab25 knockdown significantly inhibited the phosphorylation of PI3K and AKT in glioblastoma multiforme cells [27]. Therefore, Hsa-miR-125a-5p repressed siHa cell proliferation and invasion by regulating PI3K/AKT pathway and targeting Rab25.

**CONCLUSION**

Hsa-miR-125a-5p in CC is suppressed, and its upregulation inhibits siHa cell growth and invasion, as well as induces apoptosis. The mechanism of action is via modulation of PI3K/AKT pathway and targeting of Rab25, thereby indicating that they are potential targets for the development of drugs for the treatment of cervical cancer.

**DECLARATIONS**

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**Conflict of Interest**

No conflict of interest associated with this work.

**Contribution of Authors**

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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