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ORIGINAL ARTICLE

Influence of hsa-miR-6727-5p on the proliferation, apoptosis, invasion and migration of Caski, Hela and SiHa cervical cancer cells

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Summary

Purpose: *To study the influence of hsa-miR-6727-5p on the proliferation, apoptosis, invasion and migration of Caski, Hela and SiHa cervical cancer cells.*

Methods: *The CCK8 test, flow cytometry, Transwell and scratch healing experiments were used to measure the influence of hsa-miR-6727-5p on the proliferation, apoptosis, invasion and migration of Caski, Hela and SiHa cells.*

Results: *Compared with the negative control (empty vector) group, the hsa-miR-6727-5p expression in the cells transfected with hsa-miR-6727-5p mimics showed a significant increase ($p<0.01$). CCK8 results showed that the hsa-miR-6727-5p overexpression enhanced significantly the proliferation of cervical cancer cells. Annexin V FITC/PI staining cell results showed that hsa-miR-6727-5p overexpression strongly inhibited the cervical cancer cells apoptosis. Transwell invasion in vitro test showed that hsamiR-6727-5p overexpression significantly enhanced the invasion ability of cervical cancer cells. The scratch healing test suggested that, compared with the control group, the migration ability of cells transfected with hsa-miR-6727-5p mimics in the experimental group was stronger.*

Conclusion: *We confirmed at the cellular level that hsa-miR-6727-5p promoted the proliferation, invasion and migration of cervical cancer cells and inhibited the apoptosis through the overexpression or inhibition of hsa-miR-6727-5p. These results indicated that hsamiR-6727-5p played a role in promoting the cervical cancer.*

Key words: *Caski, cervical cancer, Hela, hsa-miR-6727-5p, SiHa*

Introduction

Studies have shown that the incidence of cervical cancer is about 0.1 % [1], and it is rising rapidly by a rate of 0.6 % each year. Statistics have suggested [2] that, in China, the incidence of cervical cancer is generally higher in remote mountain areas and other underdeveloped areas where the cure rate is extremely low due to poor medical conditions [3]. The current treatment methods for cervical cancer are surgery and radiation therapy [4]; however, invasion and metastasis may lead to a high relapse rate. Recent studies have shown that microRNAs (miRs) can regulate gene expression in humans [5]. A miR is typically a nucleotide fragment with 18-25 nucleotides, with relatively conserved sequence. Previous studies showed that miRs in cells can effectively regulate gene silencing by blocking the expression of mRNAs or degrading the target mRNAs [6]. Using gene chip technology, we discovered that certain differences existed between the expression of miR-6727-5p in

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normal cervical cells and that in cervical cancer cells, but there was no clear reports about specific effects of miR-6727-5p in different cervical cancer cells or the relationship between miR-6727-5p and cervical cancer. In this study, the miR-6727-5p expression in different cervical cancer cells was evaluated and its effects on cervical cancer cells including Caski, Hela and SiHa were studied.

Methods

Materials

Cell lines: the cervical cancer cell lines studied in this research included Caski, Hela and SiHa cell lines that are stored in our laboratory.

Main reagents and materials: mirVana mirRNA Isolation (Ambion,USA); RNA extraction kit (TAKARA, China); TaqMan MicroRNA Reverse Transcription Kit (ABI, USA); hsa-miR-6727-5p primers synthesized by Shanghai Biological Engineering Co., Ltd. (Shanghai, China); transfection kit (Invitrogen, USA); fetal calf serum (HYCLONE, USA); antibiotics (including penicillin, streptomycin, etc.) by Sinopharm Group (Shanghai, China).

Main equipment: low-temperature high-speed centrifuge (Thermo, Germany); cell incubator (Thermo, Germany); Super Clean Bench (Suzhou Purification Equipment Co. Ltd., Suzhou); IX70 inverted microscope (Olympus, Japan); PCR (Thermo, Germany); t-race nucleic acids analyzer (Thermo, Germany); microplate reader (Thermo, Germany).

Cell transfection

Caski, Hela and SiHa cervical cancer cell lines (Shaghai Cell Model Bank, Shanghai, China) were cultured at 37°C and 5% CO_2 till cell lines were mixed up by 72-85%. Each cell line type was divided into three groups and each group was transfected with hsa-miR-6727-5p mimics, hsa-miR-6727-5p inhibitor and empty plasmids, respectively. The transfection efficiency was measured by closely following the instructions provided by the kit's manufacturer (Invitrogen, Carlsbad, CA, USA).

Cell culture and collection

Cell lines were cultivated at 37°C and 5% CO_2. Cells were collected when they reached 90% confluence, were centrifuged at 2000 rpm for 10 min at 4°C, and were then stored at -80°C.

Fluorescent quantitative PCR

Extraction [7]

A cell sample (0.2 g) was placed on ice. Subsequently, 0.5 ml RNAPlus was added and the sample was crushed quickly in the pre-cooled mortar. Sample was then transferred into an RNA-clean EP tube (Corning, NY, USA). Mortar was cleaned with 0.25 ml RNAPlus. The solution with 0.25 ml RNAPlus was transferred into a tube and 200 µl chloroform were added, the sample was shaken for 15, and placed on ice for 15 min. It was then was centrifuged at 12000 rpm at 4°C, and the supernatant was transferred into the RNase-free EP tube, and an equal volume of isopropanol was added and mixed well. The sample was placed on ice for 15 min followed by centrifugation at 12000 rpm for 10 min at 4°C. The supernatant was removed and 750 µl of 75 % ethanol were added to the sample and mixed gently followed by centrifugation at 12000 rpm for 10 min at 4°C. The supernatant was removed and ethanol was cleaned. A proper amount of RNase-free water was added and the weight of the extracted RNA was measured [8].

Fluorescent quantitative PCR

We followed the instructions provided by TAKARA fluorescent quantitative PCR. Fluorescent quantitative PCR primers were synthesized by Shanghai Biological Engineering Technology Co., Ltd. and the primer sequences are shown in Table 1.

Table 1. Fluorescent quantitative PCR primers

Primer ID	*Sequence*
hsa-miR-6727-5p-F	CTCGGGGCAGGCGGCTGGGAGCG
hsa-miR-6727-5p-R	CTCGGGGCAGGCGGCTGGGAGCG
GAPDHF	GTCGATGGCTAGTCGTAGCATCGAT
GAPDHR	TGCTAGCTGGCATGCCCGATCGATC

CCK8 cell proliferation experiment [8]

The CCK8 test was used to measure the proliferation of the cells that underwent different types of processing. Cells were inoculated into 48-well plates (10^3/well), and each step was set with 3 replicates. Ten µl CCK8 reagent were added and cells were cultured at 37°C in the presence of 5% CO_2 for 2 hrs. Finally, absorbance was measured at 450 nm (OD450).

Transwell experiment [9]

Cervical cancer cells that were subjected to different types of processing were cultured in serum-free medium at 37°C in presence of 5% CO_2. The BD chamber (BD Biocoat™ Matrigel™ Invasion Chambers) contained polyethylene terephthalate (PET) membrane (diameter=6.5mm, aperture=8µm) coated with Matrigel. Caski, Hela and SiHa cells were inoculated into the upper layer of a small chamber (5×105/ ml). A medium with 1% FBS and a medium (chemotaxin) with 10 % FBS were added respectively to the upper and lower layer of the small chamber. Cells were then cultured in the presence of 5% CO_2 at 37°C for 48 hrs. Methanol 100 % was used to fix the cells for 2 min, hematoxylin was used to stain the cells for 1 min, distilled water was used to remove the redundant dye fluid, and gradient ethanol (80, 95 and 100 %) was used for dehydration. Cells were air-dried.

Photomicrographs of the upper chamber cells were taken. Photomicrographs of the lower chamber cells (which had invaded and migrated to the lower layer cells) were taken after the upper layer cells were removed with a wet swab. Cells were observed under mi-

croscope (400x) and their migration rate within 48 hrs was calculated as follows: cell migration rate = Lower layer cell number / (upper layer cell number + lower layer cell number) × 100.

Scratch test [10]

Caski, Hela and SiHa cervical cancer cells were inoculated into 6-well plates (2×105 cells/well) and cultured at 37°C in presence of 5% CO_2. The incubator working volume was 2 ml/well. After 24 hrs, when cells were mono-layered, the DMEM nutrient solution with 10% FBS was replaced by DMEM with 1 % FBS for 24-hr cell synchronization. Ten μl sterile pipette tip was laid in a vertical position to the plate to gently cut through the plate to form a 300 to 500 μm non-cell wound patch. PBS was used to wash the cells twice and DMEM with 10% FBS was then added for subsequent cell culture. Cells were photographed at 0 hr and 24 hrs after the scratch. The scratch healing speed was calculated within 24 hrs as follows: mean healing speed (unit:μm) = 0 hr width of noncellular region on scratch surface – 24 hrs width of noncellular region on scratch surface.

Cell apoptosis detection with flow cytometry

Cells (1-5 x10^6/ml) under different conditions were collected and centrifuged at 4500 rpm. After discarding the supernatant, cells were washed with PBS (pH=7.2) 2-3 times, and 70% cold ethanol was added for fixing. After cells were fixed at 4°C for 2 hrs, the fixing solution was discarded and 2-3 ml PBS were added to suspend the solution for 3-5min. The solution was then filtered twice with a 400 mesh sieve and then centrifuged for 5 min at 3500 rpm. The supernatant was discarded, and cells were stained with propidium iodide (PI) dye (ABM) and annexin (ABM) for 30 min at 4°C. Using flow cytometry (Beckman) apoptosis was measured (the exciting light wave was 488 nm and the emitting light wave was 630 nm).

Statistics

SPSS 20.0 software was used for statistical analysis. Experimental data was displayed as mean ± standard deviation ($\bar{x}$±s). Comparison between groups was done using oneway ANOVA test followed by post hoc test (Least Significant Difference). p<0.05 indicated significant difference.

Results

hsa-miR-6727-5p expression measurement in different cervical cancer samples

hsa-miR-6727-5p expression level in cervical cancer tissues and normal cervical tissue was measured using fluorescent quantitative PCR. Compared with the hsamiR-6727-5p expression level in normal tissue the expression level in cancer tissues was much higher (p<0.05) (Figure 1). The results were consistent with results obtained with gene chip test results.

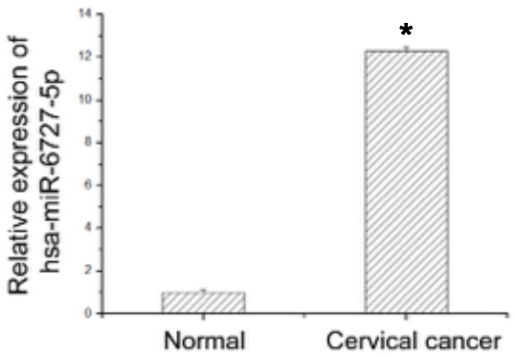

Figure 1. hsa-miR-6727-5p expression measurement of different cervical cancer cell samples. *p<0.05 indicates significant differences between groups.

Results after Caski , Hela and SiHa cell lines went through hsa-miR-6727-5p mimics and hsa-miR-6727-5p inhibitor

The hsa-miR- 6727-5p expression levels in different groups were measured with fluorescent quantitative PCR. hsa-miR-6727-5p expression in the cervical cancer cells processed with hsa-miR-6727-5p mimics was much higher than that of the hsamiR-6727-5p in the cells processed with hsa-miR-6727-5p inhibitor (p<0.01) (Figure 2). This suggested that hsa-miR-6727-5p mimics could promote hsa-miR-6727-5p expression in cervical cancer cell lines while hsa-miR-6727-5p inhibitor could inhibit the hsa-miR-6727-5p expression in cervical cancer cell lines.

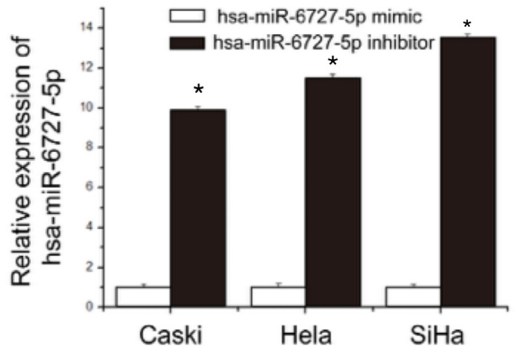

Figure 2. Results after Caski, Hela and SiHa cell lines went through hsa-miR-6727-5p mimics and hsa-miR-6727-5p inhibitor. *p<0.05 indicates significant differences between groups.

CCK8 test results of the proliferation of Caski, Hela and SiHa cells

Caski, Hela and SiHa cervical cancer cells that were subjected to different types of processing were studied. Their proliferation was measured by CCK8 test, as shown in Figure 3. The results showed that the proliferation of cancer cells processed with hsamiR-6727-5p mimics was much stronger than the proliferation of cells in the con-

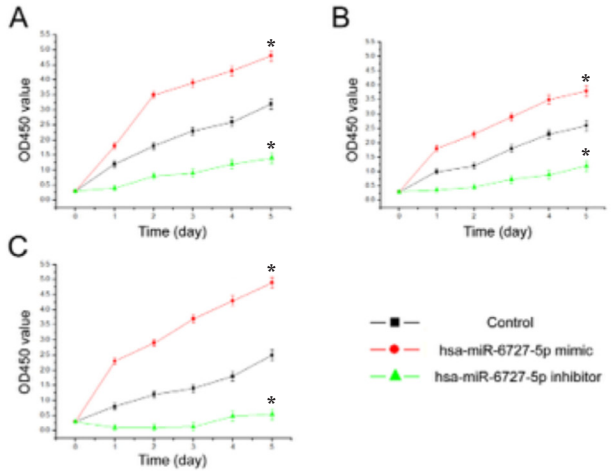

Figure 3. CCK8 test results of the proliferation of Caski, HeLa and SiHa cells. **A:** Caski cervical cancer cell line; **B:** HeLa cervical cancer cell line; **C:** SiHa cervical cancer cell line. *p<0.05 indicates significant differences between groups.

trol group (p<0.01). The proliferation of cancer cells processed with hsa-miR-6727-5p inhibitor was much lower than the proliferation of cells in the control group (p<0.01).

Flow cytometry test results of the apoptosis of Caski, Hela and SiHa cells

In this study Caski, Hela and SiHa cells that were treated with hsa-miR-6727-5p mimics and hsa-miR-6727-5p inhibitor processing methods were tested by flow cytometry. As shown in Figure 4A, hsa-miR-6727-5p mimics significantly inhibited the apoptosis in various cervical cancer cell lines. Figure 4B indicates the same results.

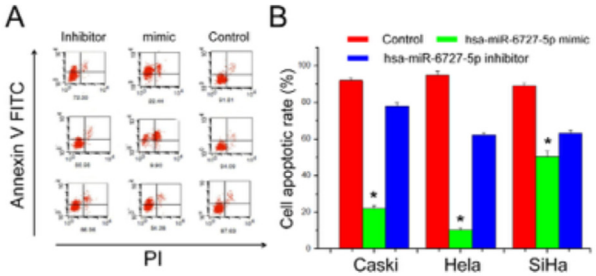

Figure 4. Flow cytometry test results of Caski, Hela and SiHa cells. *p<0.05, compared with control group.

Transwell assay results of Caski, Hela and SiHa cells

Caski, Hela and SiHa cell lines that were were subjected to hsa-miR-6727-5p mimics and hsa-miR-6727-5p inhibitor processing methods were studied. These cell lines were assessed by the Transwell assay to measure the invasion ability under different processing conditions (Table 2). As shown in Table 2, the cancer cell lines (Caski, Hela and SiHa) that underwent the hsa-miR-6727-5p mimics processing had stronger invasion ability than the control group. Cancer cell lines (Caski, Hela and SiHa) treated with hsa-miR-6727-5p inhibitor processing were much less invasive compared with the control group.

Table 2. Transwell test results (invasion of Caski, Hela and SiHa cells)

	Caski	*Hela*	*SiHa*
hsa-miR-6727-5p mimics processing	120.67±4.52	120.67±4.52	120.67±4.52
hsa-miR-6727-5p inhibitor processing	9.53±1.26	11.5±2.18	8.76±1.83
Control group	43.4±3.21	52.1±4.82	38.5±2.63
P	0.004	0.0032	0.0052

Scratch test results of Caski, Hela and SiHa cells

Caski, Hela and SiHa cell lines treated with hsa-miR-6727-5p mimics and inhibitor processing methods were studied. These cell lines were cultured at 37°C with 5% CO_2. When cells reached confluence, the culture condition was changed to 7°C and 5% CO_2 for 24 hrs. Scratch healing ability of Caski, Hela and SiHa cervical cancer cells that received the hsa-miR-6727-5p mimics processing was much stronger than that of cancer cells treated with hsa-miR-6727-5p inhibitor (Figure 5). The measurement of the migration distance of the cervical cancer cells that underwent different processing suggested that hsa-miR-6727-5p mimics could facilitate the migration of different cervical cancer cell lines (Table 3).

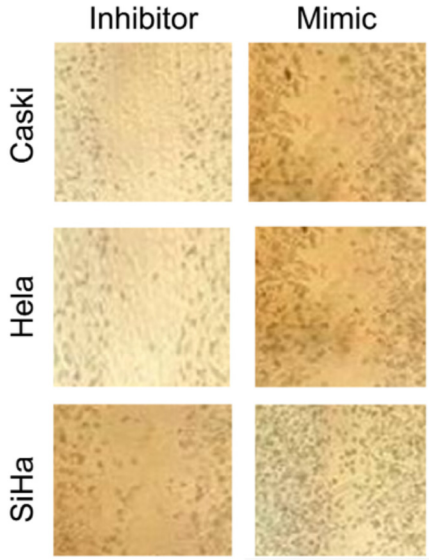

Figure 5. Cell scratch test results of Caski, Hela and SiHa cells. hsa-miR-6727-5P mimic processing was much stronger than that of cancer cells treated with hsamiRs-6727-5P inhibitor.

Table 3. Migration distances of cervical cancer cells in different processing methods

	Caski	*Hela*	*SiHa*
hsa-miR-6727-5p mimics processing (mm)	4.8	4.2	3.94
hsa-miR-6727-5p inhibitor processing (mm)	1.63	1.29	1.23

Discussion

A miR is a noncoding RNA that has 21-23 bp [11]. A previous study [12] has shown that miRs have the ability to inhibit target's gene expression by binding to mRNAs. For example , it has been confirmed that miR-1246s (sequence: AAUGGAUUUUGGAGCAGG) can participate in regulating tumor-related genes in human cells [13]. It was also shown that the miR-1246 could activate Tang syndrome associated proteins by target-regulating p53 gene and ultimately enable the migration of tumor vascular cells [14]. Through analysis made on miR gene chips of cervical lesions and normal cervical tissues [15], we discovered that there were significant differences between the hsa-miR-6727-5p expression levels in cervical lesions and those in normal cervical tissues. Using bioinformatics techniques, we discovered that hsa-miR-6727-5p is a 23 bp small non-coding RNA (http://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MI0022572) [16]. Results obtained from other studies revealed that hsa-miR-6731, hsa-miR-1233-2 and hsamiR-5088 were linked to human cancers such as breast cancer [17], and also in uterine fibroids [18]. However, reports about the correlation between hsamiR-6727-5p and tumors are rare [19].

Fluorescent quantitative PCR results were consistent with the results obtained in gene chip test [20]. We used CCK8 to measure the influence of hsa-miR-6727-5p on the proliferation of Caski, Hela and SiHa cells. The Annexin V FITC/PI double staining cell experiment was performed to measure the influence of hsa-miR-6727-5p on the apoptosis of Caski, Hela and SiHa cells. Transwell test was used to measure the influence of hsa-miR-6727-5p on the invasion ability of Caski, Hela and SiHa cells, and scratch test was applied to measure the influence of hsa-miR-6727-5p on the migration ability of these cells. The results suggested that, compared with the negative control group, the hsa-miR-6727-5p expression in hsa-miR-6727-5p mimics transfected cells had a significant rise ($p<0.01$).

CCK8 results showed that the overexpression of hsa-miR-6727-5p significantly enhanced the proliferation of cervical cancer cells. Annexin V FITC/PI double staining experiments showed that the hsa-miR-6727-5p overexpression significantly inhibited the apoptosis of cancer cells, and the Transwell results showed that the overexpression of hsa-miR-6727-5p significantly enhanced the invasiveness of cancer cells. Scratch healing results showed that, compared to the control group, cells transfected with hsa-miR-21-3p mimics had stronger migration capability. We observed that the expression of hsa-miR-6727-5p was different in the various cervical cancer cell lines.

The hsa-miR-6727-5p expression in Hela cell line was higher than that in Caski and SiHa cell lines, and the influence of the overexpression of hsa-miR-6727-5p on the proliferation and cell migration was different in the various cervical cancer cell lines.

Acknowledgements

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Conflict of interests

The authors declare no confict of interests.

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