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ORIGINAL ARTICLE

MiR-218 restrains proliferation of cervical cancer cells *via* targeted regulation of HMGB1/RAGE pathway

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Summary

Purpose: *To explore the role of micro ribonucleic acid (miR)-218 in cervical cancer (CC) and the regulatory mechanism between the high mobility group box 1 (HMGB1)/receptor for advanced glycation end-product (RAGE) pathway and miR-218.*

Methods: *The CC HeLa cells were first transfected with miR-218 mimic (miR-218 mimic group) or miR-218 negative control (NC group) using Lipofectamine 2000 transfection reagent, and those only added with Lipofectamine 2000 transfection reagent were taken as Control group. Then, quantitative real-time polymerase chain reaction (qRT-PCR) was performed to determine the level of miR-218 in CC cell line. Besides, the migration and invasion abilities of the cells were measured via Transwell chamber assay, and the apoptosis was analyzed using a flow cytometer. Finally, the protein levels of HMGB1 and RAGE were determined via Western blotting.*

Results: *The expression of miR-218 declined in the CC HeLa cell line. After overexpression of miR-218, the proliferation ability of the CC cells was weakened, and the migration and invasion of CC cells were repressed. Moreover, miR-218 was observed to directly regulate the HMGB1/RAGE signaling pathway in a targeted manner to affect the proliferation and migration of CC cells.*

Conclusions: *MiR-218 inhibits the HMGB1/RAGE pathway to suppress the proliferation, migration and invasion of CC cells.*

Key words: *miR-218, HMGB1/RAGE signaling pathway, cervical cancer, HeLa cells*

Introduction

As one of the four most common cancers in women, cervical cancer (CC) remains the leading cause of deaths in females [1-5]. Its morbidity rate is gradually increasing with the age, endangering the health of females [6]. Currently, surgery, radiotherapy, hormone therapy, targeted therapy and chemotherapy are the primary strategies to treat cancers. However, chemoresistance and toxic side effects pose grave challenges to the clinical application of chemotherapy [7]. Therefore, there is an urgent need of developing a novel non-toxic method for the prevention and treatment of CC.

Various non-coding ribonucleic acids (RNAs) and micro RNAs (miRNAs), which regulate the expressions of oncogenes and tumor suppressor genes, play a pivotal role in the development and progression of CC [8]. Hence, identifying these oncogenic signaling pathways miRNAs may be a new way for the treatment of CC. For example, the study of Zubillage-Guerrero et al [9] demonstrated that miR-16-1 inhibits cyclin E1 (CCNE1) gene to be closely involved in CC cell cycle, and that the gene expression of CCNE1 modulates the post-transcriptional G1-to-S phase transition of cells. MiR-218,

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a tumor suppressor miRNA, represses the proliferation of glioma cells [10], the osteogenic differentiation of synovial mesenchymal stem cells [11] and the tumor angiogenesis in prostate cancer [12]. Besides, miR-218 is down-regulated in renal cell carcinoma tissues, and it is implicated in the development and progression of many cancers, such as breast cancer [13], osteosarcoma [14] and colorectal cancer [15]. Tu et al [16] reported that miR-218 is dramatically down-regulated in NSCLC, and that the overexpression of miR-218 substantially represses tumor growth and metastasis through regulating IGF1R. It can be in-ferred from these studies that miR-218 may work as a tumor suppressor gene in these cancers. Since miR-218 serves as an important therapeutic target for cancers, the present study corroborated the cor-relation between miR-218 and CC and explored the possible regulatory axis therein.

According to recent studies, the receptor for advanced glycation end-product (RAGE) signals is essential player in the pathogenesis of diverse human diseases, including diabetes, osteoarthritis, atherosclerosis and cancers. RAGE and its ligand have been discovered to be overexpressed in various types of cancers [17,18]. High mobility group box 1 (HMGB1) is a highly conservative nucleoprotein that can function as a chromatin-binding factor to bend deoxyribonucleic acids (DNAs) and promote the access to the transcriptional protein components of specific targets. Not only does HMGB1 work in the nucleus, but it can also act as an extracellular signaling molecule [19]. HMGB1, once released, mediates numerous reactions by binding to several receptors, including RAGE and Toll-like receptor (TLR)-2/4, further triggering pleiotropic responses, such as cell proliferation, differentiation, death, inflammation and immunity [20]. Interestingly, HMGB1 may interact with RAGE mainly in tumor cells, instead of non-tumor cells [21]. However, the exact role of the HMGB1/RAGE pathway has not yet been elucidated. The present study, therefore, aimed to deepen the understanding of the role of the HMGB1/RAGE signaling pathway-associated molecular mechanism in the targeted therapy of CC.

Methods

Cell culture

Human endometrial microvascular endothelial cells (HEMECs) and HeLa cells were purchased from the National Laboratory and cultured using the Roswell Park Memorial Institute (RPMI) 1640 medium (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA) and 1% penicillin-streptomycin in a humid environment with 5% CO_2 at 37°C. Finally, the cells in the logarithmic growth phase were harvested for the subsequent experiments.

Main reagents and instruments

The following reagents and instruments were used in this study: RPMI-1640 medium, trypsin-EDTA and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA), and cell counting kit (CCK)-8 from Dojindo Laboratories, Dojindo, Kumamoto, Japan, Annexin V-FITC cell apoptosis assay kit I was purchased from Roche, Basel, Switzerland, 4% paraformaldehyde and penicillin-streptomycin from Solarbio Science Technology Co., Ltd. (Beijing, China), and ultrapure water system for double distillation of water from Hangzhou Yongjieda Purification Technology Co., Ltd. (Hangzhou, China).

Cell transfection

First, the expression level of miR-218 was determined in the above-mentioned cell lines. MiR-218 mimic (miR-218 mimic group) and negative control (NC) sequences (NC group) were then synthesized and separately transfected into HeLa CC cells using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). Moreover, the HeLa cells only added with Lipofectamine 2000 transfection reagent were set as Control group. Following digestion, the HeLa cells in the logarithmic growth phase were re-suspended and incubated in 6-well plates at a density of 1×10^6 cells/well. After

Table 1. Primer sequences

Gene	*Primer sequence*
MiR-218 mimic	F: 5'-TGGACAACATCGCTCTGTGGA-3'
	R: 5'-TCAAACAGAGGTCGCATGCTG-3'
HMGB1	F: 5'-CATCTCAGGGCCAAACCGAT-3'
	R: 5'-TGACATTTTGCCTCTCGGCT-3'
RAGE	F: 5'-GCTTGGAAGGTCCTGTCTCC-3'
	R: 5'-CCTCTGACACACATGTCCCC-3'
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	F: 5'-ATGAAGATCCTGACCGAGCGTG-3'
	R: 5'-CTTGCTGATCCACATCTGCTGG-3'

18-24 h of incubation, the cell confluence reached 80-90%, and the medium without serum and antibiotics was then added into the plates. Finally, the cells were transfected using Lipofectamine 2000 and incubated for 48 consecutive hours.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were extracted from cells using TRIzol method (Invitrogen, Carlsbad, CA, USA). Then, qRT-PCR was conducted with 2× Power Taq PCR MasterMix and SYBR Green (BioTeke, Beijing, China) using Exicycler™ 96 qRT-PCR system. Besides, total RNAs were isolated from the tissues using TRIzol, and their concentration and purity were determined using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, 1 μg of total RNAs was taken to synthesize complementary DNAs (cDNAs) using reverse transcriptase (Fermentas, Waltham, MA, USA) and Oligo-dT primers. Afterwards, PCR amplification was performed in a PCR system (50 μL) composed of reaction buffer, Taq DNA polymerase, dNTPs, forward and reverse primers (1 μL each) and 3 μL of reverse transcription product cDNAs as the template. Upon completion of loading, the samples were transiently centrifuged and amplified in 7900 qPCR system (Applied Biosystems, Foster City, CA, USA) with the following cycle parameters: 95°C for 5 min ×1 cycle and 95°C for 10 s and 60°C for 20 s ×40 cycles. The PCR conditions were recommended as denaturalization at 94°C for 20 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s for 30 cycles, and extension at 72°C for 30 cycles. The sequences are shown in Table 1.

Cell counting kit-8 (CCK-8) assay

The influence of transfection with miR-218 mimic on the viability of HeLa cells was analyzed using CCK-8 assay (Dojindo, Kumamoto, Japan). The cells in each group were seeded into a 96-well plates at a density of 5×10^3 cells/well, with triplicate wells set in each group. After incubation at 37°C for 24 h, 20 μL of CCK-8 reagent was added into each well, followed by incubation at 37°C for another 1 h. Finally, the optical density (OD) was read by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm.

Annexin-V/propidium iodide (PI) assay

The impact of transfection with miR-218 mimic on the apoptosis of HeLa cells was determined using the Annexin-V/PI assay kit (Roche, Basel, Switzerland). First, each group of cells was inoculated into 6-well plates at 5×10^4 cells/well and reacted with 5 μL of Annexin-V and 5 μL of Propidium Iodide (PI) at 37°C in the dark for 5 min. Finally, the cell apoptosis rate was measured by a flow cytometer.

Western blotting

The cells were lysed using radioimmunoprecipitation assay (RIPA) lysis buffer to isolate proteins, and the sample was then placed on ice for 30 min. The resulting lysate was centrifuged at 12,000 relative centrifugal force (RCF) for 10 min to obtain proteins. Subsequently, the proteins were quantified using bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China)

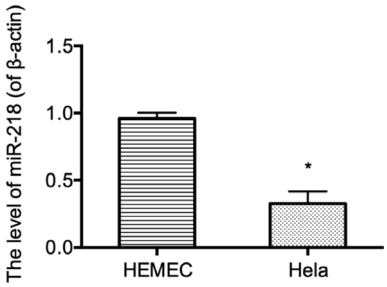

Figure 1. Compared with HEMECs, HeLa cells have an obviously decreased level of miR-218 (*p<0.05 *vs.* HEMECs).

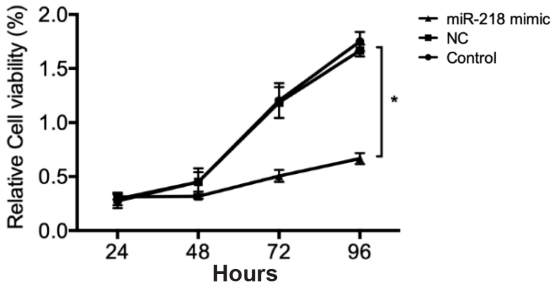

Figure 2. Overexpression of miR-218 can repress the growth of CC cells (*p<0.05: Control group *vs.* NC group).

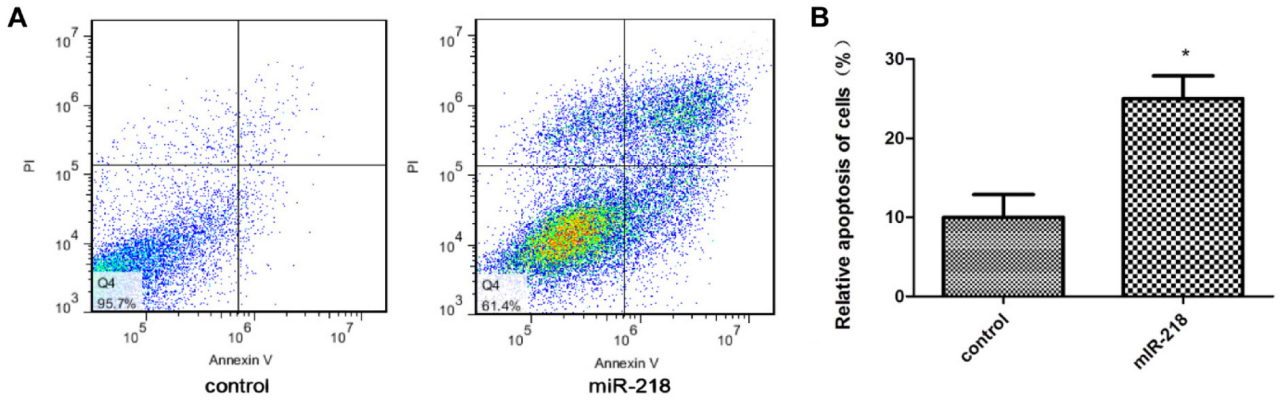

Figure 3. Overexpression of miR-218 can promote the apoptosis of HeLa CC cells. **(A)** Flow cytometry results of cell apoptosis, and **(B)** statistics of cell apoptosis rate (*p<0.05 *vs.* Control group).

based on the standard protein concentration curve. Protein samples were then prepared and isolated *via* sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Subsequently, the separated proteins were transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA), and incubated with the primary antibodies against HMGB1 (1:1000), RAGE (1:1000) and GAPDH (1:25000). The resulting membrane was washed by Tris buffered saline and probed with secondary antibodies at room temperature (20-25°C) for 30 min. Finally, the protein bands were detected using ImageLab software system (Bio-Rad, Hercules, CA, USA).

Detection of cell migration and invasion

The migration and invasion of cells were detected using Matrigel-coated Transwell chambers. First, the cells were seeded into the serum-free 24-well insert in the upper chamber at a density of 1×10^5 cells/well, while the lower chamber was added with 500 μL of DMEM supplemented with 10% fetal bovine serum. After incubation for 24 h, non-invasive cells were removed from the top of the wells using cotton swabs, whereas the cells at the bottom were trypsinized and re-seeded into a 96-well insert, followed by determination of cell viability using MTT assay. Finally, the optical density (OD) of each well was measured using the microplate reader at a wavelength of 490 nm.

Statistics

All the data were presented as mean ± standard deviation, and SPSS 11.0 software (SPSS Inc., Chicago, IL, USA) was used for analyses. Differences between two groups were analyzed by using the Student's t-test. Comparison between multiple groups was done using One-way ANOVA test followed by *post hoc test* (least significant difference). P<0.05 was considered statistically significant.

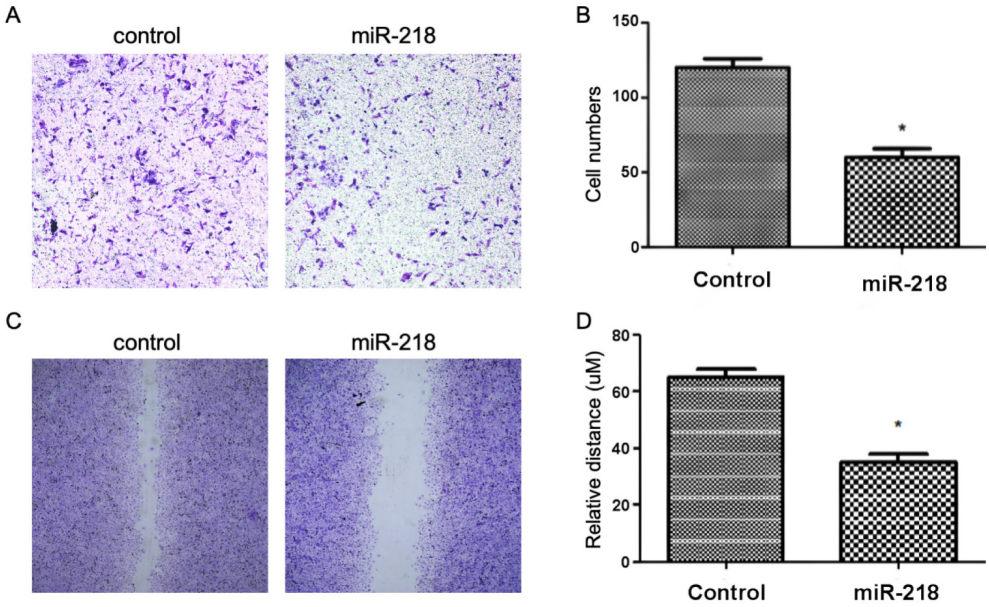

Figure 4. Detection results of HeLa cell migration and invasion: **A:** HeLa cell invasion results (magnification: 200×). **B:** Statistics of invasive cell number. **C:** HeLa cell migration results; and **D:** Statistics of HeLa cell migration (*p<0.05 *vs.* Control group).

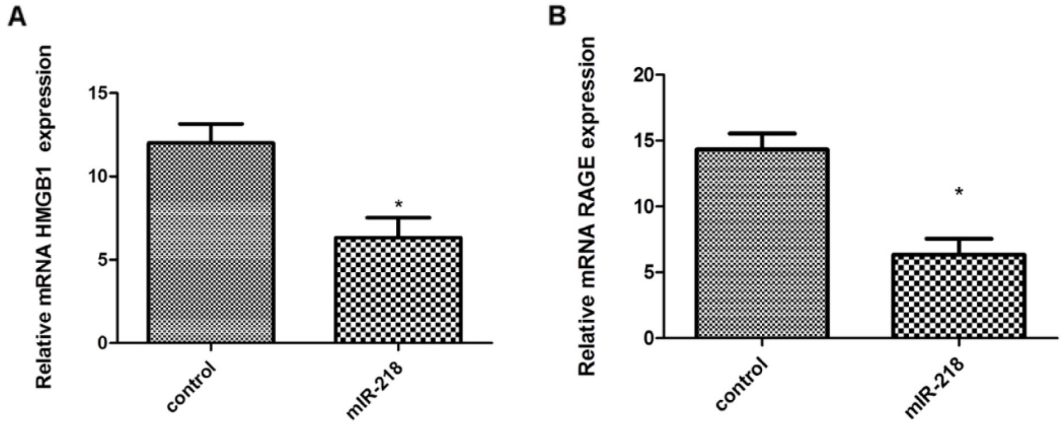

Figure 5. MRNA expressions of HMGB1 and RAGE in HeLa CC cells: **A:** Expression of HMGB1 in each group. **B:** Expression of RAGE in each group (*p<0.05 *vs.* Control group).

Results

MiR-218 expression level in HEMECs and HeLa cells

The expression level of miR-218 in CC cells and HEMECs was determined *via* qRT-PCR, and it was found that the expression level of miR-218 declined substantially in CC cells (Figure 1).

CCK-8 assay results

To elucidate the role of miR-218 in the development of CC, HeLa cells were transfected with miR-218 mimic or NC sequences, and the expression of miR-218 was up-regulated in HeLa cells to observe the inhibitory effect of miR-218 on cell growth. In comparison with that in Control and NC groups, the proliferation of HeLa cells was evidently inhibited after overexpression of miR-218, and showed no notable difference between Control group and NC group (Figure 2). Therefore, miR-218 mimic group and Control group were compared in subsequent experiments.

Cell apoptosis

According to the flow cytometry results, compared with those in Control group, the cell viability was notably repressed, while the cell apoptosis was promoted in miR-218 mimic group ($p<0.05$) (Figure 3).

Detection results of cell migration and invasion

The influences of miR-218 on the invasion and migration of HeLa cells were identified *via* Transwell chamber assay. Based on the results (Figure 4A-4D), compared with those in Control group, the migration and invasion abilities of HeLa cells declined by 55% ($p<0.05$) and 38% ($p<0.05$), respectively.

QRT-PCR results

The messenger RNA (mRNA) expression levels of HMGB1 and RAGE in HeLa cells were determined using qRT-PCR. According to the results (Figure 5A and 5B), the mRNA expression levels of HMGB1 and RAGE in HeLa cells were obviously decreased after overexpression of miR-218 compared with those in Control group.

Western blotting results

The expressions of HMGB1 and RAGE were detected *via* Western blotting. It was found that the mRNA expression levels of HMGB1 and RAGE were decreased in HeLa cells after overexpression of miR-218 compared with those in Control group (Figure 6A and 6B).

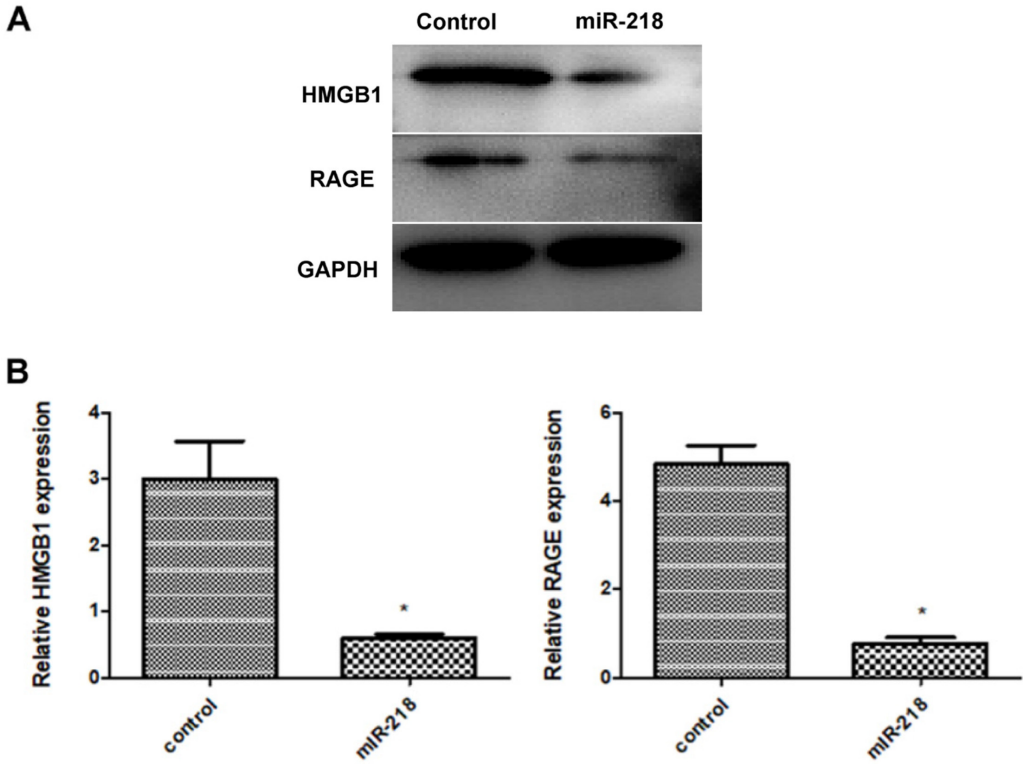

Figure 6. Overexpression of miR-218 weakens the survival ability of CC cells through the HMGB1/RAGE pathway: **A:** Western blotting bands, and **B:** Statistics of HMGB1 and RAGE protein expressions (*$p<0.05$ *vs.* Control group).

Discussion

CC, one of the most common malignancies, is the leading cause of cancer deaths among the malignancies in the female reproductive system [22-26]. As diagnostic techniques and medical technologies develop, the prognosis of CC patients has been obviously improved. However, the prognosis is still very poor in the patients with local and distant metastases. Many tumor suppressor genes and oncogenes are aberrantly expressed in such a complex disease [27,28]. Although the existing genes have provided new insights, more information will be obtained through specific studies on miRNAs.

MiRNAs encode the peptides or proteins involved in a series of biological processes, such as differentiation, metabolism and neuronal signaling, to carry genetic information [29]. They mainly bind to the 3'-untranslated region of the target mRNA through complementary effect. After base pairing, the mature miRNAs degrade the target mRNAs or inhibit protein synthesis, thereby regulating the post-transcriptional gene expression [30,31]. MiRNAs can act as both oncogenes and tumor suppressors to exert vital effects in cancer development and cell processes, including proliferation, apoptosis and migration. Moreover, the aberrant expression of miRNAs has been proven to be associated with the development and progression of cancers [32]. According to a previous study report, miR-218 is considerably down-regulated in esophageal squamous cell carcinoma (ESCC) tissues compared with that in the adjacent non-cancer tissues [33]. In addition, the latest studies have demonstrated that miR-218 restrains the development of cancers and mainly binds to oncogenes to inhibit the proliferation and invasion of cancer cells in different types of cancers.

HMGB1, a chromatin-binding factor targeting DNAs, is able to promote the assembly of transcription proteins. As a damage-associated molecular pattern, highly affinitive HMGB1 binds to various receptors, such as RAGE and TLR-2/4/9, to mediate immune responses to necrosis and the invasion of immune cells in traumas, pathogens and sepsis [34]. Furthermore, the overexpression of HMGB1 is associated with the characters of several types of cancers. After binding to RAGE, HMGB1 enhances cell migration and tumor metastasis, thereby promoting the progression of cancers [35,36].

Conclusions

In this study, it was found that the expression of miR-218 in HeLa CC cells was obviously lower than that in HEMECs. Based on the further study results, after overexpression of miR-218, the proliferation, invasion and migration of HeLa cells were inhibited, and the apoptosis rate rose substantially, suggesting that overexpression of miR-218 can inhibit carcinogenicity. In conclusion, the results of this study provide a novel biological target for the treatment of CC, which may become a molecular target helping develop the treatment methods for CC.

Conflict of interests

The authors declare no conflict of interests.

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