Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Effect of MiR-10b on cervical cancer rats through mTOR/P70S6K signaling pathway

Qiaoling Tong[#], Man Hu[#], Meixian Deng[*]

Department of Gynaecology, Wuhan Fifth Hospital, Wuhan, 430050, Hubei Province, China

[#]These authors contributed equally to this work as co-first author

ARTICLE INFO

Original paper

Article history:
Received: August 12, 2022
Accepted: September 19, 2022
Published: September 30, 2022

Keywords:

miR-10b, mTOR/P70S6K signaling pathway, rats, cervical cancer, immune, oxidative stress, apoptosis

ABSTRACT

The purpose of this experiment was to observe the biological effect and mechanism of miR-10b on cervical cancer (CC) rats. For this purpose, the rat model of CC was established and divided into three groups (Inhibitors/ Mimics/Control). The miR-10b transfection efficiency was analyzed via RT-PCR in cervical tissues in each group. The content of CD3+, CD4+, and CD8+ was detected. The levels of IL-8, TNF-β, IL-6, (CAT, SOD, and MDA were determined via ELISA, and the apoptosis of cervical tissues was detected usingTUNEL assay. The expressions of Caspase-3, Bcl-2, and the mTOR/P70S6K pathway genes and proteins were detected by qRT-PCR and Western blotting. Results showed that miR-10b was significantly increased in the Mimics group and decreased in the Inhibitors group. The content of IL-8, TNF-β, IL-6, CAT and MDA was raised, while that of SOD notably declined in the Inhibitors group. There were remarkably more apoptotic cells in the Mimics group, dominated by gliocytes, and fewer apoptotic cells in the Inhibitors group, with increased content of CD3+, CD4+ and CD8+. The Bcl-2, mTOR, and P70S6K mRNA expressions in the Inhibitors group were up-regulated than those in the other two groups, and the Caspase-3 gene in the Mimics group was increased and close to that in the control group. In the Mimics group, the mTOR and P70S6K protein were remarkably lower than those in the Inhibitors group. In conclusion, miR-10b can inhibit the occurrence and development of CC in rats by suppressing mTOR/P70S6K signaling, reducing the level of inflammation and oxidative stress, and increasing the level of immune factors.

Doi: http://dx.doi.org/10.14715/cmb/2022.68.9.10

Introduction

Cervical cancer (CC) is the most common gynecological tumor, only next to breast cancer. About 500,000 CC cases are diagnosed each year, and 45% of them are fatal (1-2). The morbidity rate of CC gradually increases with increasing age, seriously threatening women's health and life including in China (3). The development of CC is extremely complex and has not yet been fully elucidated (4). Early detection of cancer is crucial for the therapeutic effect. The number of new cases is gradually increasing and the death cases also increase sharply every year, and the operation, radiation therapy, or chemotherapy will eventually fail in approximately 30% of CC patients (5). Therefore, the discovery and identification of new biomarkers for early CC are urgently needed for the early detection and individualized treatment of CC. High-risk human papillomavirus detection will be an important detection means for CC (6). Furthermore, cisplatin-based radiotherapy and chemotherapy regimens can improve the disease condition, which is also considered to improve the tumor-free and progression-free survival of female patients, but there are serious side effects, and the long-term chemotherapy seriously threatens patients' lives and health, at a high cost, increasing the living burden of living for patients and their family (7-8). Therefore, searching for new therapeutic methods for CC has become the focus of scientists.

In recent years, with the progress in gene study, researchers have paid attention to microribonucleic acids (miRNAs) and have begun to study their specific biological roles (9-10). Increasingly more studies have confirmed that 1/3 of genes could be regulated by miRNAs in human (11-12). MiRNAs have become important regulators of gene expression in many diseases including cancer (13). It is reported that miR-10b is down-regulated in advanced small cell carcinoma of the cervix (SCCC) compared with that in patients with early SCCC (14). However, the role of miR-10b in CC remains unclear currently.

The mammalian target of rapamycin (mTOR) belongs to the protein kinase in the phosphokinase-associated kinase family, and it mainly regulates p70S6 kinase (P70S6K) in mammals (15). mTOR -mediated P70S6K phosphorylation is believed to improve the mRNA translation of cell growth-related proteins and the mTOR abnormal activation has been found in a variety of cancers (16-18), but the mTOR signaling pathway function in CC has not been well revealed.

MiR-10b could exert a therapeutic effect on CC in rats through the mTOR/P70S6K signaling pathway. Our results revealed the therapeutic effect of miR-10b on rats CC, and provide an experimental basis for the follow-up research of new drugs.

* Corresponding author. Email: ruluan188054@163.com

Materials and Methods

Commonly used reagents and instruments

SIHA cell lines (ATCC, USA), chloral hydrate (Sigma), tissue homogenizer (FLUKO), microplate reader (Thermo, USA), qPCR instrument (Applied Biosystems), SPT rats (Biological Sciences, Shanghai), SuperScript III RT kit, SYBR qPCR Mix (ABI), IL-1 and IL-6 ELISA kits (Wuhan Hanbio Co., Ltd.), radio RIPA lysis buffer (Beyotime), TUNEL fluorescence staining kit (Roche, Germany), BCA protein concentration assay kit (Biosharp), and β-actin, secondary, and primary antibodies (CST).

Animal grouping and modeling

After the concentration was adjusted, 150 μL of SIHA cell suspension (2.0×106/L) was taken and subcutaneously inoculated at the axilla to establish the CC rat model. MiR-10b was transferred into rats. Rats were divided into the miR-10b inhibitors group (Inhibitors group), miR-10b mimics group (Mimics group) and normal group (Control group), and each group has 15 rats. All operations were performed accordioning to the regulations in the NIH Laboratory Animal Guide. The serum of the rat was collected and stored at -80°C, and the biochemical indexes detached. The rats were anesthetized with pentobarbital sodium. Two samples of cervical tissues were taken, one for TUNEL staining and the other stored at -80°C for detecting the genes and proteins expression levels.

Transfection efficiency of miR-10b

To deeply explore the miR-10b function in CC, our team transfected miR-10b into rats using the adenovirus and detected the miR-10b transfection efficiency in cervical tissues was via RT-PCR. Chloral hydrate was injected into the abdominal cavity of rats for anesthesia, and an appropriate number of cervical tissues was carefully taken and mashed with tissue homogenizer to analyze miR-10b expression, to prepare for the in-depth study of the miR-10b regulatory mechanism in CC.

Inflammatory factors detection

The expression of inflammatory factors in serum was detected by ELISA. According to the actual conditions and instructions, use the kit to detect the changes in each index. Finally, the inflammatory factors absorbance in rats was detected by the microplate reader.

Detection of the differentiation cluster 3 + CD3+, CD4+ and CD8+

The frozen samples were collected in advance, and the supernatant was centrifuged (2000 g, 15 min, room temperature). The serum immune indexes (such as CD3+) were detected by flow cytometer (BD). Record the level of index (CD3+, CD4+ and CD8+) in detail and analyze their changes.

MDA, SO) and CA) detection

The rats were killed under anesthesia and 0.2 g of cervical tissues were taken. Pyrolysis, 1500 g centrifugal 10 min. The supernatant was taken to check SOD, CAT, and MDA. According to the instructions, the change of each index content was measured by microboard analyzer.

TUNEL apoptosis assay

We used an apoptosis assay kit (Roche, Germany) to analyze the paraffin sections apoptosis. The sections were dewaxed, sealed (containing protease K), fixed and rinsed, labeled with a TUNEL detection kit, and counted TUNEL-positive cells under a fluorescence microscope.

RT-PCR

The total RNA was taken using the Trizol reagent. The genes primer sequences were designed according to those of GenBank listed in Table 1. The target genes expression was calculated using the 2-ΔΔCt method.

Western blotting

The cervical tissues were frozen broken on ice and the mitotic protein of modified RIPA buffer (protease inhibitor and) was added. The protein concentration was analyzed by BCA kit. Total protein was separated with 12% gel, transferred to PVDF membrane, sealed with 5% skim milk (1.5 h, room temperature), and incubated with the primary/secondary antibody (1:1000). Gel was imaged with the imaging system. The gray value of the protein band was analyzed, and the experiment was repeated 3 times.

Statistical analysis

SPSS 20.0 software was used for the processing of raw experimental data, and multiple comparisons were performed for the data. The results were expressed as mean ± standard deviation ($\chi \pm SD$). $p<0.05$ suggested the statistically significant difference. Each experiment was performed at least 3 times.

Table 1. Primer sequences of indexes in RT-PCR.

Target gene	Primer sequence (5'-3')
GAPDH	F: 5'-TGACTTCAACAGCGACACCCA-3' R: 5'-CACCCTGTTGCTGTAGCCAAA-3'
Caspase-3	F: 5'-CTACCGCACCCGGTTACTAT-3' R: 5'-TTCCGGTTAACACGAGTGAG-3'
B-cell lymphoma-2 (Bcl-2)	F: 5'-GGTGCTCTTGAGATCTCTGG-3' R: 5'-CCATCGATCTTCAGAAGTCTC-3'
mTOR	F: 5'-CTG GGA CTC AAA TGT GTG CAG TTC-3' R: 5'-GAA CAA TAG GGT GAA TGA TCC GGG-3'
P70S6K	F: 5'-TACTTCGGGTACTTGGTAA-3' R: 5'-GATGAAGGGATGCTTTACT-3'
miR-10b	F: 5'-AGCTGTTCAGTGCACTACAGA-3' R: 5'-GTGCTACCCTGTAGAAC-3'

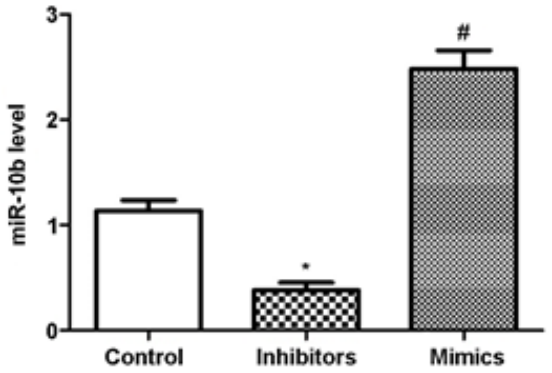

Figure 1. Transfection effect of miR-10b. The expression of miR-10b is increased in the Mimics group (p<0.05) and decreased in the Inhibitors group (p<0.05). $^{*}p$<0.05 vs. Control group, $^{\#}p$<0.05 vs. Inhibitors group.

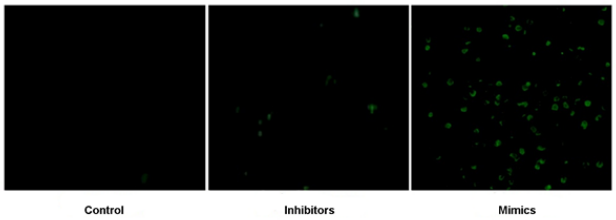

Figure 2. TUNEL staining. No obvious positive cells are observed in the Inhibitors group, and the number of positive cells was increased in the Mimics group (p<0.05).

Results

miR-10b expression in each group

To detach the miR-10b transfection efficiency in each group, its expression level was detected. MiR-10b was up-regulated in the Mimics group (p<0.05) and decreased in the Inhibitors group (p<0.05), indicating that the transfection effect is obvious and subsequent experiments can be performed (Figure 1).

Levels of immune indexes CD3 +, CD4 +, and CD8+

CD3 +, CD4 + and CD8 + levels in the peripheral blood obviously decreased in the Inhibitors group, while they were increased in the mimic group (p<0.05) (Table 2).

Serum TNF-β, IL-8 and IL-6 content

The level of IL-8, IL-6 and TNF-β was increased in the Inhibitors group (p<0.05), while it declined in the Mimics group (p<0.05) (Table 3).

Levels of oxidative-antioxidant indices SOD, CAT, and MDA in cervical tissues determined by ELISA

The content of CAT and MDA in the Inhibitors group increased (p<0.05), while these declined in the Mimics group (p<0.05), while the contents of SOD were on the contrary trends (p<0.05) (Table 4).

TUNEL apoptosis assay

In the Mimics group, the TUNEL-positive cells number was larger than in the Control group (p<0.05), and there were fewer positive cells in the Inhibitors group (Figure 2), suggesting that the abnormal cell proliferation occurs and the apoptosis declines in CC and the miR-10b can promote the apoptosis of CC cells.

Expressions of related genes

The inhibitor group had significantly higher Bcl-2, mTOR and P70S6K (p<0.05), and a remarkably lower expression of Caspase-3 than the control group (p<0.05), while the expressions in the Mimics group displayed the opposite trends (p>0.05) (Figure 3).

Table 2. Levels of CD3+, CD4+ and CD8+ (%).

Group	CD3+	CD8+	CD4+
Control	70.5±2.1	38.4±1.1	45.7±2.1
Inhibitors	31.4±3.4*	15.4±1.9*	14.4±1.8*
Mimics	68.8±2.5#	32.5±1.1#	40.6±2.1#

Note: The levels of CD3+, CD4+ and CD8+ in the peripheral blood decline in the Inhibitors group, while they are increased in the Mimics group (p<0.05). $^{*}p$<0.05 vs. Control group, $^{\#}p$<0.05 vs. Inhibitors group.

Table 3. Content of serum TNF-β, IL-8 and IL-6.

Group	TNF-β (fmol/mL)	IL-6 (mg/L)	IL-8 (mg/L)
Control	15.2±3.0	19.0±1.1	21.0±4.1
Inhibitors	48.1±2.0*	84.4±1.4*	96.8±2.4*
Mimics	19.7±3.5#	26.1±3.0#	29.5±3.1#

Note: The content of IL-8, IL-6 and TNF-β is increased in the Inhibitors group (p<0.05), while it declines in the Mimics group (p<0.05). $^{*}p$<0.05 vs. Control group, $^{\#}p$<0.05 vs. Inhibitors group.

Table 4. Levels of CAT, SOD and MDA in cervical tissues.

Group	CAT (IU/mL)	SOD (μ/mg)	MDA (μ/mg)
Control	5.7±2.3	290.8±1.9	5.0±0.4
Inhibitors	38.9±1.0*	109.7±2.1*	30.4±2.1*
Mimics	8.3±1.2#	268.5±1.1#	9.3±1.1#

Note: The content of CAT and MDA is increased in the Inhibitors group (p<0.05) and declines in the Mimics group (p<0.05), while that of SOD shows the opposite trend (p<0.05). $^{*}p$<0.05 vs. Control group, $^{\#}p$<0.05 vs. Inhibitors group.

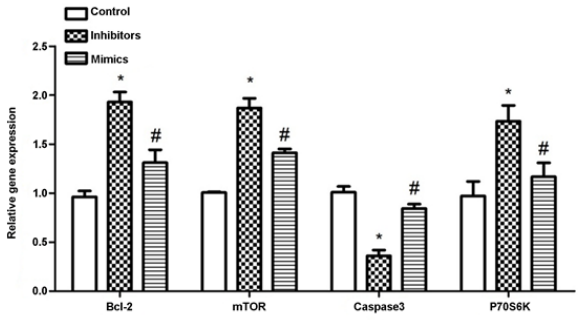

Figure 3. Results of RT-PCR of related genes. The inhibitors group has remarkably higher mRNA expressions of Bcl-2, mTOR and P70S6K (p<0.05) and a remarkably lower expression of Caspase-3 (p<0.05). $^{*}p$<0.05 vs. Control group, $^{\#}p$<0.05 vs. Inhibitors group.

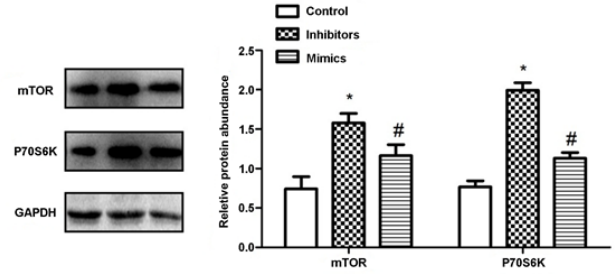

Figure 4. Western blotting results. The protein expressions of mTOR and P70S6K are significantly increased in the Inhibitors group (p<0.05), while they significantly decline in the Mimics group (p<0.05). $^{*}p$<0.05 vs. Control group, $^{\#}p$<0.05 vs. Inhibitors group.

Western blotting results

Compared with the Control group, the mTOR and P70S6K were significantly increased in the Inhibitors group (p<0.05), while they significantly declined in the Mimics group (p<0.05) (Figure 4).

Discussion

CC seriously threatens women's health and lives in developing countries and some developed countries. CC can infiltrate and metastasize into normal tissues, and this process involves the abnormal expression of cancer-related genes. Currently, the therapeutic methods for CC include operation, radiotherapy, and chemotherapy, but these methods will eventually fail in about 30% of CC patients. Moreover, there are great side effects, and long-term chemotherapy seriously threatens patients' lives, with a high cost, increasing the living burden of patients and their families (19-20). Therefore, searching for new therapeutic methods for CC has become the top priority.

In this study, the CC rat model was established. Our results confirmed that miR-10b was up-regulated in rat CC. Its role and function in CC rats needed further research.

CD8+ cells can control tumor growth and are usually as potential targets for cancer immune monitoring. High levels of CD3+, CD4+ and CD8+ with a better efficacy in ovarian patients (21-22). During and after chemotherapy, there is a strong correlation between clinical tumor response and CD8+/CD4+ cell functions. In addition, Monitoring the T-cells general functions during conventional therapy of advanced malignant tumors can provide better outcomes for immunotherapy (23-24). In our study, the CD3+, CD4+ and CD8+ levels in the peripheral blood declined in the Inhibitors group, while they were increased in the Mimics group, consistent with the above studies.

IL-6 can accelerate the other inflammatory mediators' production (25). Excessive responses to inflammatory and oxidative stress are found in our mouse models, including neutrophils, IL-6, and TNF. The content of IL-8, IL-6 and TNF-β was up-regulated in the inhibitors group, while it was reduced in the mimics group. In addition, oxygen-free radicals participate in CC and affect subsequent resuscitation. SOD exists widely, and MDA could resist the function of SOD, with cytotoxicity. Antioxidant therapy can alleviate organ oxidative stress and achieve better results (26-27). It was found that the content of CAT and MDA was raised in the Inhibitors group and decreased in the Mimics group, while that of SOD showed opposite trends, similar to those of the above studies.

MTOR mainly regulates P70S6K in mammals. The mTOR signaling pathway involves many cancers occurrence and development (28), but its role of it in human CC remains unclear. MiRNAs have attracted much attention in recent years. However, the role of miR-10b in CC remains unclear. In this study, no positive cells were observed in the Control group, the TUNEL positive cells number in the Mimics group was larger than in the Control group, and there were fewer positive cells in the Inhibitors group, indicating that abnormal cell proliferation occurs and the apoptosis declines in CC, and miR-10b overexpression of miR-10b can enhance apoptosis of CC cells. Furthermore, at the Inhibitors group, higher mRNA expressions of Bcl-2, mTOR and P70S6K and a significantly lower expression of Caspase-3 than the Control group, while the expressions in the Mimics group showed opposite trends. The mTOR and P70S6K protein expressions were significantly up-regulated in the Inhibitors group, while they significantly declined in the Mimics group. All these results are similar to previous research (29-30). In summary, miR-10b could block CC occurrence and development by suppressing the mTOR / P70S6K signaling pathway activation. Nextly, such an effect will be further verified through cell experiments.

In conclusion, a series of pathological changes will occur in CC, such as cellular oxidative stress, apoptosis, and inflammation, but miR-10b can block the occurrence of disease by restraining the mTOR/P70S6K signaling pathway. Its effect can be further explored using more molecular means in the future. This study not only provides a solid theoretical basis for CC prevention but also provides experimental bases for further research.

Declaration of interest

The authors declare that they have no conflict of interest.

References

1. Ferlay J. Estimates of worldwide burden of cancer in 2008:GLOBOCAN 2008. Int J Cancer2010;127 (12): 2983-2987.
2. Arbyn M, Castellsague X, De Sanjose S.Worldwide burden of cervical cancer in 2008. Ann Oncol 2011;22(12):2675-2686.
3. Bayo S, Bosch FX, de Sanjose S. Risk factors of invasive cervical cancer in Mali. Int J Epidemiol 2002;31:202–209
4. Forouzanfar MH, Foreman KJ, Delossantos AM. Breast and cervical cancer in 187 countries between 1980 and 2010: A systematic analysis. Lancet 2011;378:1461–1484
5. Duenas-Gonzalez, ACetina, L,Mariscal I,de la Garza J. Modern

management of locally advanced cervical carcinoma. Cancer Treat Rev 2003; 29:389–399.
6. Colombo N,Carinelli S,Colombo A,Marini C,Rollo D,Sessa C,ESMO guidelines working group. Cervical cancer: Esmo clinical practice guidelines for diagnosis, treatment and follow-up. Ann. Oncol 2012; 23: 27–32.
7. Pimple S, Mishra G, Shastri S. Global strategies for cervical cancer prevention. CurrOpinObstetGynecol 2016;28(1):4-10.
8. Dueñas-González A, Campbell S. Global strategies for the treatment of early-stage and advanced cervical cancer. Curr Opin Obstet Gynecol 2016;28(1):11-17.
9. Nagao Y, Hisaoka M, Matsuyama A, Kanemitsu S, Hamada T, Fukuyama T, Nakano R, Uchiyama A, Kawamoto M, Yamaguchi K, Hashimoto H.. Association of microRNA-21 expression with its targets, PDCD4 and TIMP3, in pancreatic ductal adenocarcinoma. Mod Pathol 2012; 25: 112–121.
10. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281-297.
11. Carleton M, Cleary MA, Linsley PS. MicroRNAs and cell cycle regulation. Cell Cycle 2007;6:2127-2132.
12. Wang L, Song G, Liu M, Chen B, Chen Y, Shen Y, Zhu J, Zhou X. MicroRNA-375 overexpression influences P19 cell proliferation, apoptosis and differentiation through the Notch signaling pathway, Int. J Mol Med2016; 37:47–55.
13. Wang S, He W, Wang C. MiR-23a regulates the vasculogenesis of Coronary artery disease by targeting epidermal growth factor receptor, Cardiovasc. Ther 2016;34:199–208.
14. Long H, JiaXin L, YanHong Y. Downregulation of Six MicroRNAs Is Associated with Advanced Stage, Lymph Node Metastasis and Poor Prognosis in Small Cell Carcinoma of the Cervix. PLoS ONE 2012; 7(3):e33762.
15. Paavonen J.Human papillomavirus infection and the development of cervical cancer and related genital neoplasias.Int J Infect Dis 2007;11(supp-S2):S3-S9.
16. Dorff T, Mack P C. The role of mTORinhibitors and PI3K pathway blockade in renal cell cancer. Kidney Cancer. 2015.
17. Hou G, Xue L, Lu Z, Fan T, Tian F, Xue Y.An activated mTOR/p70S6K signaling pathway in esophageal squamous cell carcinoma cell lines and inhibition of the pathway by rapamycin and siRNA against mTOR. Cancer Lett 2007;253:236–248.
18. Zhang YJ, Dai Q, Sun DF. MTOR signaling pathway is a target for the treatment of colorectal cancer. Ann SurgOncol 2009;16:2617–2628.
19. GarcíaMaceira P, Mateo J. Silibinin inhibits hypoxia-inducible factor-1alpha and mTOR/p70S6K/4E-BP1 signalling pathway in human cervical and hepatoma cancer cells: implications for anticancer therapy. Oncogene 2009; 28(3):313.
20. Pang X, Yi Z, Zhang X. Acetyl-11-keto-beta-boswellic acid inhibits prostate tumor growth by suppressing vascular endothelial growth factor receptor 2-mediated angiogenesis. Cancer Res 2009; 69(14):5893-5900.
21. Maecker B, Anderson K, von Bergwelt-Baildon M.Viral antigen-specific CD8+ T-cell responses are impaired in multiple myeloma. Br J Haematol 2003;121:842–848
22. Batrla R, Linnebacher M, Rudy W. CD40-expressing carcinoma cells induce down-regulation of CD40 ligand (CD154) and impair T-cell functions. Cancer Res 2002;62:2052–2057
23. Conejo-Garcia J, Benencia F, Courreges M. Ovarian carcinoma expresses the NKG2D ligand Letal and promotes the survival and expansion of CD28 antitumor T cells. Cancer Res 2004;64:2175–2182.
24. Coleman S, Clayton A, Mason M D. Recovery of CD8+ T-cell function during systemic chemotherapy in advanced ovarian cancer. Cancer Res2005; 65(15):7000-7006.
25. Xu DQ, Gao C, Niu W. Sodium hydrosulfde alleviates lung inflammation and cell apoptosis following resuscitated hemorrhagic shock in rats. ActaPharmacol Sin 2013;34:1515–1525
26. Broechner AC, Toft P. Pathophysiology of the systemic inflammatory response after major accidental trauma. Scand J Trauma ResuscEmerg Med 2009;17:43
27. Panteli ES, Fligou F, Papamichail C, Papapostolou I, Zervoudakis G, Georgiou CD. Quantification of superoxide radical production in 4 vital organs of rats subjected to hemorrhagic shock. Am J Emerg Med 2012;30:476–480.
28. Shin J M, Jeong Y J, Cho H J.Melittin Suppresses HIF-1α/VEGF Expression through Inhibition of ERK and mTOR/p70S6K Pathway in Human Cervical Carcinoma Cells. PLOS ONE2013; 8.e69380.
29. Zou D, Zhou Q, Wang D.The downregulationof microRNA-10b and its role in cervical cancer.Oncol Res 2016; 24(2):99-108.
30. Wang F, Tan W H, Liu W. Effects of miR-214 on cervical cancer cell proliferation, apoptosis and invasion via modulating PI3K/AKT/mTOR signal pathway. Eur Rev Med PharmacolSci 2018; 22(7):1891-1898.