MIR-184 INHIBITS PROLIFERATION AND INVASION OF GASTRIC CANCER CELLS BY TARGETING STC2

Qing Qiao[#], Jia-Xing He[#], Ke Wang, Shuai Zhou, Ling Dang, Bo Zhang[*], Nan Wang[*]
Department of General Surgery, Tangdu Hospital, the Air Force Medical University, Xi'an 710038, China
[#]*These authors contributed equally to this work*

ABSTRACT

Objective*: To investigate the regulatory mechanism of miR-184 in blocking gastric cancer (GC) progression through targeting stanniocalcin 2 (STC2).*

Methods: *Data analysis was performed on clinical samples in the dataset GSE63121 and the UALCAN database, and the expression levels of miR-184 and STC2 in GC patients were calculated. Over- or low-expression cell strains of miR-184 and STC2 were established by cell transfection of miR-184 mimics, miR-184 inhibitors, STC2 plasmids, and STC2 siRNA, respectively. Real-time quantitative polymerase chain reaction (RT-qPCR) was used to detect the expression levels of miR-184 and STC2 in GC cell lines. The expression of STC2 was detected by Western blot. The MTT and transwell methods were used to detect cell proliferation and invasion. In addition, the target binding sequence for binding miR-184 to STC2 was predicted from the Targetscan database, and a double luciferase reporter experiment was used to confirm the binding site on STC2.*

Results: *The expression of miR-184 was significantly down-regulated in gastric cancer patients and the expression of STC2 was significantly up-regulated in both GC cell lines. MiR-184 overexpression significantly inhibited the expression of STC2 in MGC803 GC cells and functionally inhibited the proliferation and invasion of MGC803 cells. Whereas, miR-184 low-expressing MGC803 GC cells showed the opposite results. The proliferation and invasion ability of low-expressing MGC803 cells was significantly reduced. The double luciferase reporter gene experiment and Western blotting results showed that miR-184 could directly and negatively regulate the expression of STC2. In addition, overexpression of STC2 completely reversed the inhibitory effect of miR-184 on GC cells.*

Conclusions: *Th results indicate that miR-184 can inhibit the proliferation and invasion of GC cells by targeting STC2 in GC, thus providing a new therapeutic target for GC.*

Keywords: *Gastric cancer, STC2, miR-184, proliferation, invasion.*

DOI: 10.19193/0393-6384_2021_2_196

Received *March 15, 2020;* ***Accepted*** *October 20, 2020*

Introduction

Gastric cancer (GC) is one of the most common malignancies in humans. Although its morbidity and mortality have been steadily decreasing globally, gastric cancer is still a major burden on human health and the third leading cause of cancer-related deaths[1, 2]. Research reports show that the region with the highest incidence of GC is East Asia, especially China, with an annual incidence of about 4-6 cases per 10,000 people[3, 4]. In addition, data from the National Cancer Institute indicate that the incidence and diagnosis of the disease occur mainly between 60 and 70 years of age and that most patients have a poor prognosis due to diagnosis at an advanced stage of the disease[5, 6]. Although a large number of patients benefit from surgery and adjuvant treatment strategies, their 5-year survival rate is still low and the recurrence rate is high. Therefore, understanding the pathological and molecular events involved in GC development is essential on order to cure this malignancy[7].

MicroRNA (miRNA) is a non-coding RNA with a length of about 22 bp which regulates gene

expression at the post-transcriptional level[8]. Known miRNAs regulate about 30% of the genes in the human genome, and these genes are involved in human development, cell proliferation and differentiation, hematopoietic action, and apoptosis[9, 10]. Nearly 50% of miRNAs are located in tumour-associated genomic regions, including fragile sites, chromosome amplification, and loss of heterozygous chromosomal regions. Therefore, miRNA could significantly affect the entire progression from tumorigenesis to tumour metastasis[11, 12]. The abnormal expression of miRNA is also related to the occurrence and development of GC and could regulate the expression of various oncogenes or anti-oncogenes, thereby affecting tumour cell proliferation, migration, invasion, apoptosis, and other biological characteristics[13, 14]. As one of the newly discovered miRNAs, miR-184 has been shown to have pluripotent pathophysiological activity and can participate in metabolic disorders outside of tumorigenesis mechanisms[15, 16].

In this paper, we found that miR-184 was negatively correlated with STC2 through clinical sample data analysis and further verified this finding in GC cell lines. The luciferase reporter gene experiment proved that miR-184 can negatively regulate its expression by binding to 3'-UTR on STC2. MiR-184 overexpression and miR-184 inhibitors proved that miR-184 inhibits the proliferation and invasion of GC cells by down-regulating the expression of STC2. GC cells with low STC2 expression showed the same results, and forced overexpression of STC2 can reverse the inhibitory effect of miR-184 on GC cells. In summary, the results showed that miR-184 inhibits the proliferation and invasion of GC cancer cells by negatively regulating STC2.

Methods

Cell culture and cell transfection

The human normal gastric epithelial cell lines GES1 and AGS and the MGC803 gastric cancer cell lines used in this experiment were purchased from the Shanghai Institute of Cells, Chinese Academy of Sciences. All cells were seeded in RPMI-1640 medium (Gibco, Rockville, MD, USA) containing 10% foetal bovine serum (FBS) and cultured in an incubator at 37 °C and 5% CO_2. The MiR-184 mimics and inhibitors, STC2 siRNA (si-STC2), were purchased from GenePharma (Shanghai, China). They were transfected into MGC803 cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

RT-qPCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA), and RT-qPCR was performed on the ABI StepOne Plus system using SYBR Green Master Mix (Roche, Basel, Switzerland). GAPDH was used as a control for miR-184 and STC2, and its contents were calculated via the $2^{-\Delta\Delta ct}$ method.

The primers used for miR-184 were the forward primer 5'- TCGACCCCTACCTGCTTAGT-3 ', and the reverse primer 5'- TGGGATACCAGTGTGTCATA-3'. For STC2, the forward primer 5'-ATGCTACCTCAAGCACGACC-3 ' and reverse primer 5'-TCTGCTCACACTGAACCTGC-3' were used. GAPDH had the forward primer,5'-ACATCGCTCAGACACCATG-3 ', and reverse primer 5'-TGTAGTTGAGGTCAATGAAG-3'.

Determination of luciferase activity

The 3'-UTR for the control and mutant STC2s was inserted into a pGL3 promoter vector (Invitrogen, USA) for a luciferase reporter gene experiment. The vector and miR-184 mimic were then transfected into MGC803 cells. Finally, a luciferase assay was performed using the Double Luciferase Reporting Test (Promega, USA).

MTT experiment

MTT experiment

Transfected cells (1×10^3/well) were seeded in 96-well plates. MGC803 cells containing miR-184 mimics or inhibitors were incubated for 24-96 hours. After incubation, they were incubated with MTT (Sigma-Aldrich, USA) for 4 hours at 37 °C. The absorbance at 490 nm was measured with a spectrophotometer (OD=490 nm).

Transwell experiment

The upper chamber of the cell culture dish was coated with matrix gel to detect cell invasion, 5×10^5 cells were seeded into the upper chamber (8 μm pore size), and a 20% foetal bovine serum medium was placed in the lower chamber. The cells were incubated for 18 hours at 37 °C and 5% CO_2. The lower surfaces of the infiltrated cells were then fixed with 4% paraformaldehyde (PFA), stained with 0.1% crystal violet, and the cells were counted using a light microscope.

Western blotting

Protein samples were obtained by lysing cells using radioimmunoprecipitation analysis (RIPA) lysis buffer (Beyotime, Shanghai). Proteins were

separated using 10% sodium lauryl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After blocking with 5% skim milk, they were transferred to a polyvinylidene fluoride (PVDF) membrane at room temperature (Millipore, USA). They were incubated with primary antibodies at 4 °C overnight and then with HPC-labelled secondary antibodies. Finally, the protein expression level was measured via the enhanced chemiluminescence (ECL) method.

Statistical analysis

All data are expressed as mean ± standard deviation. Statistical analyses were performed using GraphPad Prism 8.0 for analysis and mapping. Differences between groups were determined via the Student's t test and One-Way ANOVA test, and post hoc tests were performed. Overall, $p<0.05$ was considered statistically significant.

Results

MiR-184 and STC2 are negatively correlated in GC patients

First, we analysed clinical samples of GC patients and healthy people in the dataset GSE63121, and the results showed that miR-184 expression in GC patients was significantly lower than that in normal people (Figure 1A). Clinical samples were in the UALCAN database were used to analyse the expression level of STC2 in clinical GC patients, and the results showed that the expression of STC2 in these patients was significantly increased (Figure 1B). RT-qPCR detection of miR-184 and STC2 transcription levels in the GC cell lines MGC803 and AGC showed that their transcription levels were significantly reduced in these cells, while STC2 transcription levels were significantly increased (Figure 1C and Figure 1D). It can be seen from the above results that miR-184 and STC2 have a significant negative correlation in GC patients.

MiR-184 inhibits the proliferation and invasion of gastric cancer cells

We transfected MiR-184 mimics or inhibitors into MGC803 cells and explored their role in GC. RT-qPCR experiments were first used to test the transfection efficiency of MiR-184 mimics and inhibitors (Figure 2A). The MTT and transwell methods were used to detect the proliferation and invasion ability of transfected MGC803 cells.

The results showed that compared with the control group, the proliferation ability of miR-184 over-expressing MGC803 cells was significantly reduced, while that of miR-184 underexpressing MGC803 cells was significantly enhanced (Figure 2B). Similarly, overexpression of miR-184 inhibited the invasion ability of MGC803 cells, while down-regulation of miR-184 can significantly promote the invasion of MGC803 cells (Figure 2C). The above results showed that miR-184 can inhibit the proliferation and invasion of GC cells.

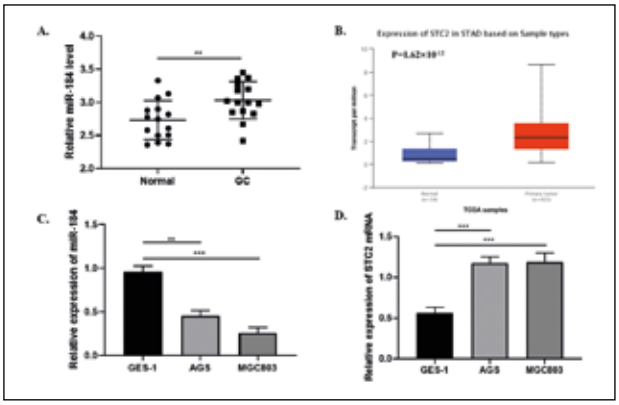

Figure 1: MiR-184 and STC2 are negatively correlated in GC patients.
A. Data analysis of clinical samples in the dataset GSE63121 was used to detect the expression of miR-184 in GC patients and normal people. B. Analysis of STC2 expression in GC patients and normal people utilizing the UALCAN database. C. Detection of MiR-184 expression in MGC803, AGS, and GES-1 (control) cells via qRT-PCR. D. Detection of STC2 expression in MGC803, AGS, and GES-1 (control) cells via qRT-PCR. **, $p<0.01$; ***, $p<0.001$.

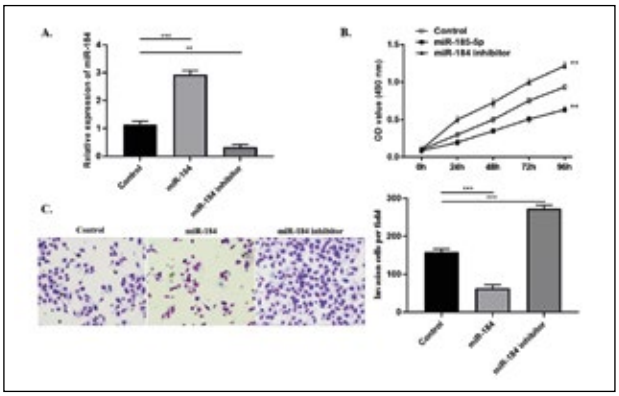

Figure 2: MiR-184 inhibits the proliferation and invasion of gastric cancer cells.
A. MiR-184 expression in MGC803 cells containing MiR-184 mimics or inhibitors was detected via RT-qPCR. B. Proliferation of MGC803 cells over- or under-expressed using miR-184 was detected via MTT. C. The transwell test was used to detect the invasion ability of each group of transfected cells and in the statistical analysis. ***, $p<0.001$.

MiR-184's direct target STC2

In order to explore the relationship between STC2 and MiR-184, we performed Targetscan prediction (http://www.targetscan.org/), and the re-

sults showed that STC2 might be a potential target for miR-184 (Figure 3A). This prediction was further verified by the double luciferase reporter gene experiment. As expected, the luciferase activity of MGC803 cells was significantly reduced after using a miR-184 mimic and wt-STC2 vector.

However, there was little change in MGC803 cells when using a miR-184 mimic and mut-STC2 vector (Figure 3B). Through Western blotting experiments, we also found that the protein expression of STC2 was reduced by miR-184 mimics and enhanced by miR-184 inhibitors (Figure 3C). Therefore, we believe that miR-184 can directly target STC2 and negatively regulate its expression in GC.

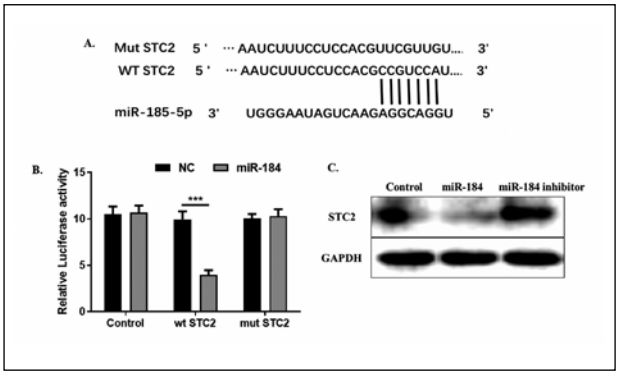

Figure 3: MiR-184 directly targets STC2 in GC.
*A. Targetscan predicts the binding sequence of MiR-184 to 3'-UTR on STC2. B. Dual luciferase reporter assay. C. Western blotting was used to detect the protein expression level of STC2 in each group of transfected cells. ***, p<0.001.*

STC2 promotes the proliferation and invasion of GC cells

In order to further study the biological function of STC2 in GC, we constructed MGC803 cells with low STC2 expression via si-STC2 transfection and verified the transfection efficiency in RT-qPCR experiments (Figure 4A). The proliferation ability of each group of cells was tested via MTT experiments. The results showed that the cell proliferation ability of the si-STC2 group was significantly lower than that of the MGC803 control group (Figure 4B).

Transwell results showed that si-STC2 also inhibited the invasion ability of MGC803 cells (Figure 4C). These results indicate that STC2 acts as an oncogene in GC and promotes the value-added and invasive capacity of GC cells.

MiR-184 regulates the proliferation and invasion of GC cells by inhibiting STC2

STC2 was overexpressed in miR-184 overexpressing MGC803 cells to verify their interaction in GC. RT-qPCR results showed that forced overexpression of STC2 can restore the miR-184 mimetic-induced decrease in STC2 mRNA and protein expression in MGC803 cells (Figures 5A and 5B). MTT results showed that forced overexpression of STC2 disrupted the inhibitory effect of miR-184 mimics on GC cell proliferation (Figure 5C).

Transwell results showed that miR-184 completely inhibited cell invasion in the STC2 forced overexpression group (Figure 5D).

All these results indicate that miR-184 inhibits cell proliferation and invasion by regulating the expression of STC2 in GC.

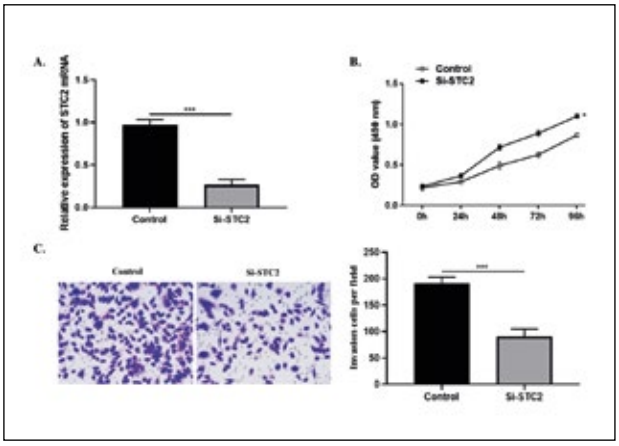

Figure 4: STC2 promotes the proliferation and invasion of GC cells.
*A. The mRNA transcription levels of STC2 in each group of transfected cells were detected by RT-qPCR. B. The MTT method was used to detect the proliferation level of transfected cells in each group at different time points. C. The transwell test was used to detect the invasion ability of each group of transfected cells and in the statistical analysis. *, p<0.05; ***, p<0.001.*

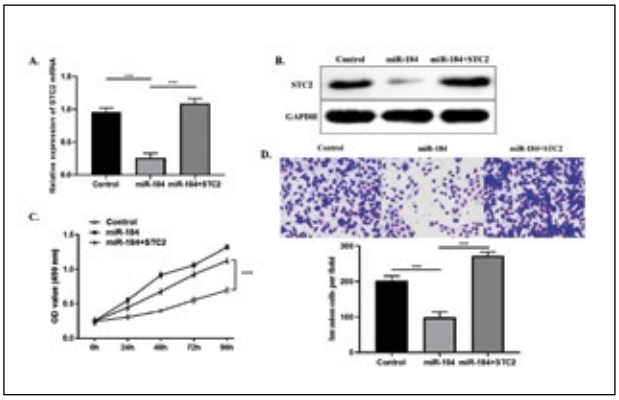

Figure 5: MiR-184 regulates the proliferation and invasion of GC cells by inhibiting STC2.
*A. The mRNA transcription levels of STC2 in each group of transfected cells were detected by RT-qPCR. B. Western blotting was used to detect the protein expression level of STC2 in each group of transfected cells. C. The MTT method was used to detect the proliferation level of transfected cells in each group at different time points. D. The transwell test was used to detect the invasion ability of each group of transfected cells and in the statistical analyses. ***, p<0.001.*

Discussion

GC is a heterogeneous disease, and its occurrence and development are related to a variety of environmental factors and cancer pathways. External factors include H. pylori and Epstein-Barr virus infections, alcoholism, body mass index, and physical activity[17], while internal factors are manifested genetically[18]. In the treatment of early GC, surgical resection is still the main treatment strategy[19]. During the postoperative period, other comprehensive treatments can be used, such as adjuvant chemotherapy; however, the overall outcome of GC is still unfavourable[20]. In recent years, molecular targeted therapy has gradually become a research focus, and more and more molecular targeted drugs have been used in clinical trials. Therefore, exploring new molecular markers or targets will help in the diagnosis and treatment of GC[21, 22].

The STC2 gene is usually found in the human heart, spleen, kidneys, and pancreas, and its encoded protein contains 302 amino acids[23]. It has also been found that changes in STC2 expression affect the biological activities of several cancer cells, including its upregulation in hepatocellular carcinoma and promotion of cell proliferation and migration in GC[24]. In contrast, STC2 has been reported to inhibit breast cancer cell proliferation[25]. Studies have found that STC2 was significantly up-regulated in GC and inhibits the proliferation of GC cells, thus proving that STC2 could be used as a prognostic indicator of GC[26]. In addition, research has also found that downregulation of STC2 can inhibit the proliferation and invasion of GC cells[27], and Shen et al. discovered that STC2 can inhibit the invasion of cervical cancer cells[28]. These findings suggest that STC2 can be used as an oncogene for GC.

In this paper, we analysed the clinical data in the GSE63121 dataset and the UALCAN database. The results showed that the expression of MiR-184 was significantly down-regulated in GC patients, while the expression of STC2 was significantly up-regulated in these patients. At the same time, the RT-qPCR results regarding expression of MiR-184 produced the same results in GC cell lines, indicating a significant negative correlation. The overexpression of miR-184 significantly inhibited the expression of STC2 in MGC803 cells and functionally inhibited the proliferation and invasion of MGC803 cells, while the low expression of miR-184 significantly promoted the proliferation and invasion of MGC803 cells. STC2 low-expressing MGC803 cells constructed using siRNA showed a significant reduction in proliferation and invasion capacity. According to the Targerscan prediction, STC2 may be used as a direct target for miR-184. At the same time, double luciferase reporter gene experiments and Western blotting results showed that miR-184 can directly and negatively regulate STC2 expression. In order to further confirm our conjecture, by forcibly overexpressing STC2, the value-added and invasion ability of each group of transfected cells was tested, and the results showed that forced overexpression of STC2 completely reversed the inhibitory effect of miR-184 on GC cells. In summary, the results showed that miR-184 can inhibit the proliferation and invasion of GC cells by targeting STC2, thereby providing a new therapeutic target for GC.

References

1) Parkin DM, Hämmerl L, Ferlay J, Kantelhardt EJ. Cancer in Africa 2018: The role of infections. Int J Cancer 2020; 146: 2089-2103.
2) Jee H, Kim JH. Gender difference in colorectal cancer indicators for exercise interventions: the National Health Insurance Sharing Service-Derived Big Data Analysis. J Exerc Rehabil 2019; 15: 811-818.
3) Kolacinska A. How Can We Improve Education of Breast Surgeons Across Europe? Chirurgia (Bucur) 2017; 112: 365-366.
4) Soares FA, Coimbra FJ, Pelosof AG, Freitas HC, Begnami MD, et al. Genomics and epidemiology for gastric adenocarcinomas. Appl Cancer Res 2017; 37: 7.
5) Li B, Zhou SL. Dr. Samuel J. Klempner: collaborations with Asian countries speed up gastric cancer research. Hepatobiliary Surg Nutr 2019; 8: 82-83.
6) Vivacqua A, De Marco P, Belfiore A, Maggiolini M. Recent Advances on the Role of microRNAs in both Insulin Resistance and Cancer. Curr Pharm Des 2017; 23: 3658-3666.
7) Wu W. MicroRNA, Noise, and Gene Expression Regulation. Methods Mol Biol 2018; 1699: 91-96.
8) Liu B, Sun G. Transcriptome and miRNAs analyses enhance our understanding of the evolutionary advantages of polyploidy. Crit Rev Biotechnol 2019; 39: 173-180.
9) Li Q, Li J, Dai W, Li YX, Li YY. Differential regulation analysis reveals dysfunctional regulatory mechanism involving transcription factors and microRNAs in gastric carcinogenesis. Artif Intell Med 2017; 77: 12-22.
10) Mirghasemi A, Taheriazam A, Karbasy SH, Torkaman A, Shakeri M, et al. Retraction Note: Down-regulation of miR-133a and miR-539 are associated with unfavorable prognosis in patients suffering from osteosarcoma. Cancer Cell Int 2016; 16: 84.

11) Yang PW, Lu ZY, Pan Q, Chen TT, Feng XJ, et al. MicroRNA-6809-5p mediates luteolin-induced anticancer effects against hepatoma by targeting flotillin 1. Phytomedicine 2019; 57: 18-29.
12) Lv LY, Wang YZ, Zhang Q, Zang HR, Wang XJ. miR-539 induces cell cycle arrest in nasopharyngeal carcinoma by targeting cyclin-dependent kinase 4. Cell Biochem Funct 2015; 33: 534-540.
13) Zhao H, Dinh-Sac P, Song Y, Do TD, Li S. Seven new species of Pinelema from Vietnam (Araneae, Telemidae). Zookeys 2018; 734: 13-42.
14) Gu L, Sun W. MiR-539 inhibits thyroid cancer cell migration and invasion by directly targeting CARMA1. Biochem Biophys Res Commun 2015; 464: 1128-1133.
15) Gaudreault C, Salvas J, Sirois J. Savitzky-Golay smoothing and differentiation for polymerase chain reaction quantification. Biochem Cell Biol 2018; 96: 380-389.
16) Singh S, Jha HC. Status of epstein-barr virus coinfection with Helicobacter pylori in gastric cancer. J Oncol 2017; 2017: 3456264.
17) Jin H, Wang W. MicroRNA-539 suppresses osteosarcoma cell invasion and migration in vitro and targeting Matrix metallopeptidase-8. Int J Clin Exp Pathol 2015; 8: 8075-8082.
18) Lu W, Kang Y. Epithelial-Mesenchymal Plasticity in Cancer Progression and Metastasis. Dev Cell 2019; 49: 361-374.
19) Oh E, Kim JY, Sung D, Cho Y, Lee N, et al. Inhibition of ubiquitin-specific protease 34 (USP34) induces epithelial-mesenchymal transition and promotes stemness in mammary epithelial cells. Cell Signal 2017; 36: 230-239.
20) Pei XH, Lv XQ, Li HX. Sox5 induces epithelial to mesenchymal transition by transactivation of Twist1. Biochem Biophys Res Commun 2014; 446: 322-327.
21) Zhang D, Liu S. SOX5 promotes epithelial-mesenchymal transition in osteosarcoma via regulation of Snail. J BUON 2017; 22: 258-264.
22) Han C, Zhou Y, An Q, Li F, Li D, et al. MicroRNA-1 (miR-1) inhibits gastric cancer cell proliferation and migration by targeting MET. Tumour Biol 2015; 36: 6715-6723.
23) Hasan MM, Ushio H, Ochiai Y. Expression levels of myoglobin in muscle and non-muscle tissues of rainbow trout Oncorhynchus mykiss, a hypoxia intolerant species. Comp Biochem Physiol B Biochem Mol Biol 2018; 225: 48-57.
24) Zhang M, Li Q, Zhang L, Wang Y, Wang L, et al. RASSF7 promotes cell proliferation through activating MEK1/2-ERK1/2 signaling pathway in hepatocellular carcinoma. Cell Mol Biol (Noisy-le-grand) 2018; 64: 73-79.
25) Hernandez Cordero AI, Gonzales NM, Parker CC, Sokoloff G, Vandenbergh DJ, et al. Genome-wide Associations Reveal Human-Mouse Genetic Convergence and Modifiers of Myogenesis, CPNE1 and STC2. Am J Hum Genet 2020; 106: 138.
26) Hu Q, Masuda T, Sato K, Tobo T, Nambara S, et al. Identification of ARL4C as a Peritoneal Dissemination-Associated Gene and Its Clinical Significance in Gastric Cancer. Ann Surg Oncol 2018; 25: 745-753.
27) Haehl E, Nicolay NH. Omission of postoperative radiation to pathologically tumor-free cervical lymphatic pathway: long-term results of a prospective phase II study. Strahlenther Onkol 2020; 196: 101-103.
28) Nilsen A, Jonsson M, Aarnes EK, Kristensen GB, Lyng H. Reference MicroRNAs for RT-qPCR Assays in Cervical Cancer Patients and Their Application to Studies of HPV16 and Hypoxia Biomarkers. Transl Oncol 2019; 12: 576-584.

Acknowledgements:
This paper was supported by Social Development Projects of Shaanxi Province (2019SF-004).

Corresponding Authors:
Bo Zhang, Nan Wang
Email: wangnandoc@163.com
(China)