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MicroRNA-409 inhibits the proliferative ability of cervical carcinoma cells by regulating AKT

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Abstract. – OBJECTIVE: To observe the microRNA-409-3p expression in cervical carcinoma and its effect on the growth and proliferative ability of cervical carcinoma cells.

PATIENTS AND METHODS: The microRNA-409-3p level in 62 cases of cervical carcinoma and 38 cases of normal cervical tissue was detected by qRT-PCR. The association between the microRNA-409-3p level and clinicopathological features of cervical carcinoma was investigated. Knockdown and overexpressed microRNA-409-3p in cervical carcinoma cells, HeLa and SiHa, were used to detect the cell cycle and the activity of cervical carcinoma cells. Subsequently, potentially target genes of microRNA-409-3p were predicted by bioinformatics website. Western Blot and luciferase assay were used to confirm their correlation.

RESULTS: We observed a significant lower microRNA-409-3p level in cervical carcinoma tissues than that in normal cervical tissues. Significant correlation was found between the microRNA-409-3p level in patients with cervical carcinoma and the overall survival rate, tumor size and TNM stage ($p<0.05$), but correlations with age, pathological type and lymph node metastasis were not found ($p>0.05$). After silencing the expression of microRNA-409-3p in cervical carcinoma cells, the proliferative ability of cervical carcinoma cells was greatly promoted. In addition, microRNA-409-3p was targeting on protein kinase B (AKT) and had a negative correlation with AKT expression.

CONCLUSIONS: The microRNA-409-3p level was lower in cervical carcinoma tissues, and was significantly affected the overall survival, tumor size and TNM staging in clinical patients. Low expression of microRNA-409-3p could promote the proliferative ability of cervical carcinoma cells through the target gene AKT.

Key Words
Cervical carcinoma, MicroRNA-409-3p, AKT, Proliferative ability.

Introduction

Cervical carcinoma is one of the most common gynecologic malignancies. There are about 500,000 newly diagnosed cases and about 300,000 deaths each year in the world. In recent years, reports of cervical carcinoma showed a younger trend. The incidence of cervical carcinoma is known to be associated with high-risk HPV infection[1]. However, it is undeniable that the abnormal expressions of oncogenes and tumor suppressor genes are crucial for the development of cervical carcinoma[2]. The mechanism of development and progression of cervical carcinoma has been the hot topic and could provide more effective approach in diagnosing and treating cervical carcinoma. MicroRNA (MiRNA) is a non-coding single-stranded RNA molecule encoding approximately 22 nucleotides in length and widely distributed in plants and animals. It mainly functions in the regulation of gene expression *in vivo* and presents an extremely important function in regulation and control of tissues and cells[3]. The miRNAs can be involved in the regulation of various life activities, such as cell morphology, proliferative ability, cell cycle, angiogenesis, metabolism, etc. The abnormal expression of miRNA functions a lot in the process of tumor growth, invasion and apoptosis[4]. microRNA-409-3p is a member of miRNA; recent investigations illustrated that it was involved in many biological processes and closely related to the occurrence of multiple tumors. Researches have also indicated that microRNA-409-3p functioned in cell proliferative ability, apoptosis and invasion, abnormal metastasis and signal transduction[5-7], but studies on microRNA-409-3p and pathogenesis of cervical carcinoma were not clearly explored. In this study, cervical carcinoma cell lines, SiHa and HeLa, were used as cell models

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to explore the microRNA-409-3p on proliferative ability and apoptosis of cervical carcinoma cells, as well as the possible molecular mechanisms for preventing and treating cervical carcinoma, which provided a new treatment strategy.

Patients and Methods

Patients Information

A total of 62 cases of cervical carcinoma and 38 normal cervical tissue samples were used from patients admitted in Anhui Medical University Affiliated First Hospital. Both patients' consent for sample collection and informed consent form were obtained from all participants before the study. After the sample was collected, it was stored in liquid nitrogen tank. The general information of patients was listed in Table I. microRNA-409-3p expression characteristics were based on the overall survival rate, staging and tumor size of cervical carcinoma patients. This investigation was approved by the Ethics Committee of Anhui Medical University Affiliated First Hospital.

Cell Culture

Human normal cervix epithelial cells (HcerEpic), and cervical carcinoma cells HeLa and SiHa, were incubated in the 1640 culture medium supplemented with 100 μL/mL fetal bovine serum (FBS) in a cell incubator at 37°C and 50 mL/L CO_2 atmosphere.

Cell Transfection

Cells in the logarithmic growth phase were seeded in 6-well plates with 3.5*10^5 per well. When the adherent growth of cells was confluent to 50%, 100 pmol of mimic of mature microRNA-409-3p, inhibitory of mature microRNA-409-3p and si-AKT, respectively, and 5 μL of Lipofectamine 2000, were mixed and transfected into Hcer Epic, HeLa and SiHa cells. After the transfection of NC as a negative control, the specific operation was according to instructions of Lipofectamine 2000.

RNA Extraction and qRT-PCR

Cells were lysed with TRIzol and total RNA was extracted from each group; reverse transcription was performed according to the reagent instructions. qRT-PCR was used to detect the expressions of microRNA-409-3p and protein kinase B (AKT). Each experiment was repeated for three times.

Cell Viability Assay

Transfected cells for 24 h were digested and counted, and cultured in 96-well plates with 3*10^3 cells/per well. 5 repeated wells in each group were set, 10 μl of cell counting kit-8 (CCK8) reagent were added before the detection; the incubation was for 1 h at 37°C without light, gently shake. OD values were detected in the microplate reader at 450 nm wavelengths. Vitality of cervical carcinoma cells was observed.

Table I. Correlation between microRNA-409-3p expression and clinicopathological information in patients with cervical carcinoma (n = 62).

Clinicopathologic features	Number of cases	miR-409-3p expression		*p*-value
		Low (n=31)	High (n=31)	
Age (years)				
< 50	36	15	21	0.1225
≥ 50	26	16	10	
Histology				
Squamous	25	12	13	0.7957
Adenocarcinoma	37	19	18	
Tumor size				
< 4 cm	33	11	22	0.0051*
≥ 4 cm	29	20	9	
FIGO stage				
I-II	38	14	24	0.0155*
III-IV	25	17	8	
Lymph node metastasis				
No	34	16	18	0.6098
Yes	28	15	13	

*$p<0.05$.

Cell Cycle Assay

Cells were harvested by trypsin digestion, fixed with 70% ethanol overnight at 4°C; the supernatant was removed, resuspended in 50 μg/mL RNAse A solution and incubated at room temperature for 30 min. The propidium bromide was used for staining, and then incubated in the dark at 40°C for 30 min. Flow cytometry was utilized to detect the cell cycle, and the ratio of cells in G1, S and G2 phases was detected. The experiment was performed in triplicate.

Prediction and Validation of Downstream Target Genes

Three common bioinformatics tools, TargetScan, miRanda and PicTar3, were utilized to predict target genes of microRNA-409-3p. To further confirm their relationship, we constructed a dual luciferase reporter vector to verify the binding relationship, and then used qRT-PCR and Western blot to verify the target gene expressions.

Western blot

Total protein was extracted after cells transfection. Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to membranes in ice bath for 60 min at 80 V. After blocking for 2 h at room temperature, primary antibodies (1:1000 dilution of both AKT and GAPDH) and secondary antibody (1:10000 of horseradish peroxidase (HRP)-labeled IgG) were used for incubation for 1-2 h at room temperature. Chemiluminescence detection and results were analyzed.

Statistical Analysis

We used statistical product and service solutions (SPSS) 17.0 software (SPSSS Inc., Chicago, IL, USA) for statistical analysis. Data were expressed as mean ± SD, classification data were analyzed by the x^2-test, comparison data between groups were analyzed by *t*-test, survival analysis was analyzed by Kaplan-Meier survival curve, and measurement data comparison was analyzed using *t*-test. $p<0.05$ indicated significant difference. *$p<0.05$, **$p<0.01$, *** $p<0.001$.

Results

Relationship Between microRNA-409-3p Expression and Clinical Data in Patients with Cervical Carcinoma

In comparison with normal cervical tissue, microRNA-409-3p expression was significantly reduced in cervical carcinoma tissues (Figure 1A, $p<0.001$). Next, we analyzed the clinical data of patients; there was a higher overall survival rate in microRNA-409-3p high expression group in comparison with that of low expression group (Figure 1B, $p=0.0206$, HR=0.4439). Based on the tumor stage, microRNA-409-3p level in FIGO III+IV was lower than that in I+II (Figure 1C, $p<0.001$). In addition, microRNA-409-3p level in cervical carcinoma, which was greater than or equal to 4 cm, was lower than that in tumors less than 4 cm (Figure 1D, $p<0.001$). The above presented that microRNA-409-3p was downregulated in cervical carcinoma tissue. It provided a foundation for further research of its biological function.

Cell Line Expression and Screening of Transfection Sequences

The normal human cervical epithelial cells were used as a control, total RNA was extracted from cervical carcinoma cells, HeLa and SiHa; then, the relative expression of microRNA-409-3p was detected by qRT-PCR. The microRNA-409-3p level in the cervical carcinoma cell lines was significantly reduced (Figure 2A). Next, we constructed the transfection sequences (mimic, inhibitor) and selected the most suitable sequence based on the transfection results (Figure 2B, 2C).

Knockdown of microRNA-409-3p Promotes Proliferative Ability of Cervical Carcinoma Cells

CCK8 results showed that, after culturing HeLa and SiHa cells for 24 h, the OD450 value of microRNA-409-3p was significantly decreased compared with NC group (Figure 2D). In addition, flow cytometry results showed that, compared with the negative control group, after transfecting with microRNA-409-3p mimics into cells, the ratio of cells in the G1 phase decreased, but the ratio of cells in S-phase increased. It illustrated that overexpression of microRNA-409-3p blocked the cell cycle of cervical carcinoma cells. Opposite results were obtained after transfecting the microRNA-409-3p inhibitor (Figure 2E). The above results revealed that low expression of microRNA-409-3p could promote proliferative ability of cervical carcinoma cells.

microRNA-409-3p Directly Regulates AKT

Based on bioinformatics prediction analysis of TargetScan, miRanda and PicTar3, AKT was a potential target gene of microRNA-409-3p. Correlation curves showed that microRNA-409-3p

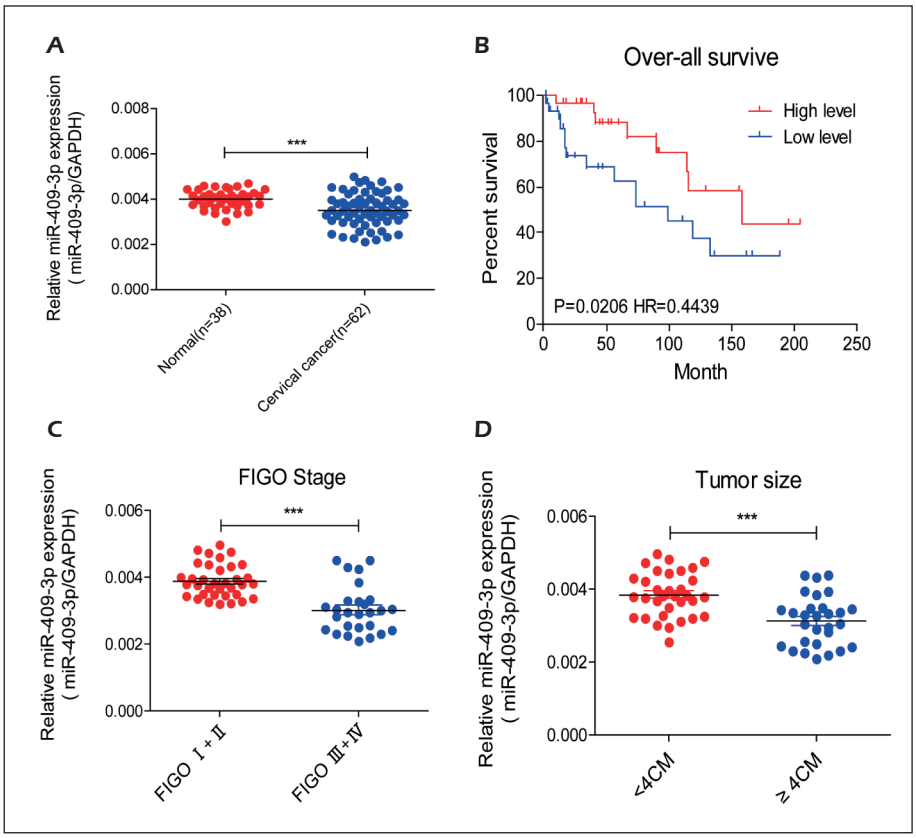

Figure 1. Low-expression of microRNA-409-3p in cervical carcinoma samples. **A**, The expression of microRNA-409-3p in cervical carcinoma tissues was lower than that in normal cervical tissues. **B**, The overall survival rate in microRNA-409-3p high expression group was higher than that of low expression group. **C**, The expression of microRNA-409-3p in FIGO III+IV was lower than that in I+II. **D**, The expression of microRNA-409-3p in large cervical carcinoma tissues (with a diameter > 4 cm) was lower than that in small tumors (with a diameter < 4 cm).

level was negatively related to AKT expression in tissue samples (Figure 3, $p<0.0001$, $R^2=0.5031$). Subsequently, we sequenced the WT 3'UTR sequence of AKT gene (including the entire predicted microRNA-409-3p binding site) and the mutant 3'UTR sequence of the AKT gene (deleted the entire predicted microRNA-409-3p binding site), which were cloned into the dual luciferase reporter plasmid (Figure 3A). Dual luciferase reporter assay pointed out that microRNA-409-3p inhibited luciferase activity through AKT-WT 3'UTR (Figure 3B). When co-transfected with microRNA-409-3p and AKT-mutant 3'UTR, there was no inhibitory effect on the activity (Figure 3C). Western blot revealed that protein expression of microRNA-409-3p was negatively related to AKT protein expression in cervical carcinoma cells (Figure 3D). Taken together, it revealed that microRNA-409-3p had a direct regulatory effect on AKT expression.

Knockdown of AKT Inhibits Proliferative Ability of Cervical Carcinoma Cells

We further examined the effect of AKT, the target gene of microRNA-409-3p, on the metastasis of cervical carcinoma cells. Results suggested that the protein expression of AKT in HeLa and SiHa cells transfected with si-AKT decreased significantly, and the proliferative ability of cervical carcinoma cells was significantly inhibited (Figure 3E, F).

Discussion

MicroRNAs are endogenous, non-coding small RNAs that target the 3'-untranslated region of a messenger RNA (mRNAs), and negatively regulate gene expression, including mRNA degradation and translational inhibition[8]. Targeting of miRNAs affects approximately 30% of human genes[9]. More evidence showed the importance of miRNA in gene expression, including tumor growth, invasion and apoptosis[10,11]. Dysregulated miRNAs play an important role in tumor cell growth, invasion and apoptosis[12-14]. It has been reported[15-17] that multiple miRNAs were expressed in cervical carcinoma tissues and played a regulatory role. microRNA-409-3p is a member of the miRNA family. Current investigations[5,18,19] have shown that microRNA-409-3p was involved in different biological processes and was closely related to the occurrence of multiple tumors.

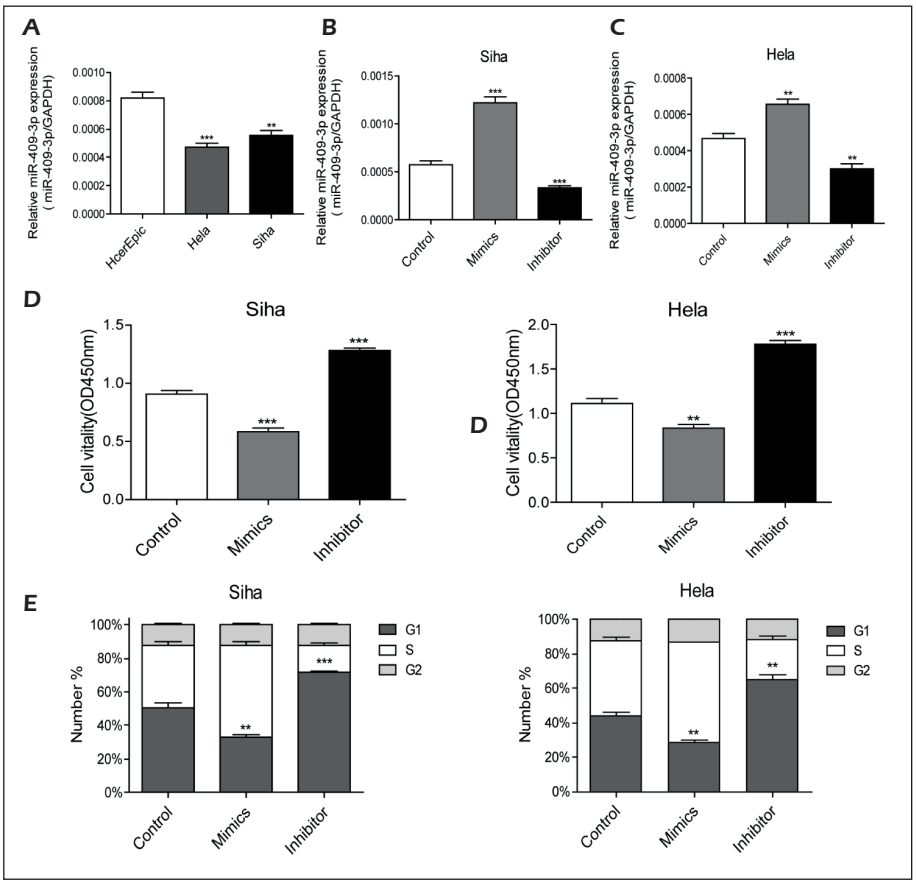

Figure 2. microRNA-409-3p inhibits the growth of cervical carcinoma cells. **A**, Expression of microRNA-409-3p in HcerEpic, HeLa and Si-Ha cells. **B**, Expression of microRNA-409-3p in SiHa cells after transfecting mimic and inhibitory of microRNA-409-3p. **C**, Expression of microRNA-409-3p in HeLa cells after transfecting mimic and inhibitory of microRNA-409-3p. **D**, Cell viability of SiHa and HeLa after transfecting mimic and inhibitory of microRNA-409-3p was detected by CCK8. E. Cell cycle change of SiHa and HeLa after transfecting mimic and inhibitory of microRNA-409-3p was detected by flow cytometry.

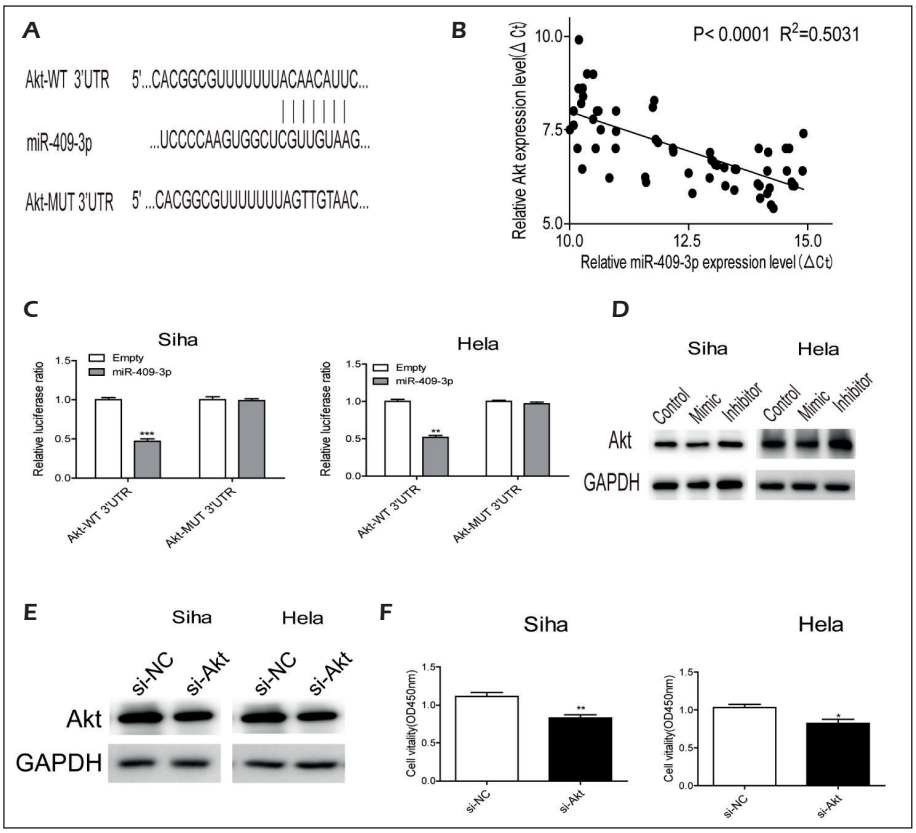

Figure 3. AKT is predicted to be the target gene for microRNA-409-3p. **A**, Bioinformatics predicted that AKT can serve as a potential target gene for microRNA-409-3p. The sequence of its binding site was shown in the figure. **B**, Pearson correlation analysis showed that microRNA-409-3p and AKT were negatively correlated in clinical samples. **C**, In SiHa and HeLa cells, luciferase reporter assays tested the activity of WT AKT 3'UTR and MUT AKT 3'UTR. **D**, Protein expression changes of AKT after transfection of mimics and inhibitors in SiHa and HeLa cells. **E**, Protein expression of AKT after knockdown of AKT in SiHa and HeLa cells. **F**, Viability of both cell lines after knockdown of AKT in SiHa and HeLa cells were detected by CCK8.

Previous works indicated that microRNA-409-3p expression in gastric carcinoma cell SGC-7901 significantly inhibited cell proliferative ability and induced apoptosis[20]. Ma et al[21] found that the microRNA-409-3p level in breast carcinoma tissues was significantly down-regulated compared with that in paracarcinomaous tissues. It was proved to be associated with poor prognosis in breast carcinoma. In addition, another research[22] found that, compared with the adjacent non-tumor group, microRNA-409-3p expression in osteosarcoma cells decreased, and the expression was related to the migration of osteosarcoma. In this study, the microRNA-409-3p level was significantly decreased in cervical carcinoma tissues, and significantly affected the overall survival, tumor size and TNM stage in clinical patients. It indicated that microRNA-409-3p may play an important role in the occurrence of cervical carcinoma. To further investigate the impact of microRNA-409-3p on the biological function of cervical carcinoma cell lines *in vitro*, miRNA-300 levels in cervical carcinoma cell lines were altered. Here, CCK8 assay pointed out that microRNA-409-3p was downregulated, which improved the proliferative ability of cervical carcinoma cells. Flow cytometry showed that knockdown of microRNA-409-3p can decrease the proportion of cells in G1 phase. Therefore, low expression of microRNA-409-3p in cervical carcinoma can significantly promote the proliferative ability of tumor cells. Subsequently, we predicted by bioinformatics that AKT was a potential target gene for microRNA-409-3p. Dual luciferase reporter assay initially demonstrated the functional role of microRNA-409-3p in AKT. Finally, qPCR and Western blot suggested that microRNA-409-3p had the ability to inhibit AKT expression. AKT presents serine threonine kinase activity, which is an essential downstream target kinase in the PI3K signal transduction pathway. It promotes the abnormal differentiation and proliferative ability of cells. Related studies have confirmed that high protein expressions of PI3K and AKT were related to proliferative ability, invasion and metastasis of cervical carcinoma cells[23]. Yih et al[24] observed that inhibition of AKT can promote apoptosis of cervical carcinoma cells induced by arsenic trioxide. Thus, AKT may have importance for the formation, diagnosis and treatment of cervical carcinoma. This study showed that protein expression of AKT increased after transfected with microRNA-409-3p inhibitor. By inhibiting the expression of AKT protein, the proliferative ability of cervical carcinoma cells weakened. Thus, microRNA-409-3p may inhibit proliferative ability of cervical carcinoma cells through AKT. Taken together, our study proved that microRNA-409-3p was downregulated in cervical carcinoma tissues and targeted to regulate AKT. Low expression of microRNA-409-3p can up-regulate the expression of AKT and inhibit the proliferative ability of cervical carcinoma cells. Therefore, we provide new reference to the diagnosis and treatment of cervical carcinoma and enhance our knowledge of the post-transcriptional regulation of AKT.

Conclusions

We showed that the microRNA-409-3p level was low in cervical carcinoma tissues and closely related to the total survival, tumor size and TNM stage in clinical patients. Down-regulation of microRNA-409-3p resulted in the increased AKT, early diagnosis, and targeted therapy, may provide potential molecular targets.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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