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Down-regulating ATDC inhibits the proliferation of esophageal carcinoma cells

W. LAI[1], X. ZHENG[2], Q. HUANG[1], X. WU[3], M. YANG[1]

[1]Department of Rheumatology; [3]Department of Thoracic and Cardiovascular Surgery, Nanfang Hospital, The Southern Medical University, Guangzhou, China.
[2]Department of Thoracic Surgery, General Hospital of Chengdu Military Command, Chengdu, Sichuan Province, China

Weinan Lai, *Xiushan Zheng* and *QinHuang* contributed equally to this work

Abstract. – OBJECTIVE: Our previous study showed that Ataxia-Telangiectasia Group D Complementing gene (ATDC) expression was correlated with tumor aggressiveness and poor prognosis in patients with esophageal squamous cell carcinoma (ESCC). However, the roles of ATDC on the esophageal carcinoma cells remain unclear. The present study aimed to explore the effects of ATDC gene silencing on the growth and proliferation of esophageal carcinoma cell lines EC109 and EC9706 in vivo and *in vitro*.

MATERIALS AND METHODS: The lentivirus-mediated siRNA targeting ATDC was constructed and transfected into EC109 and EC9706 cells. After the gene silencing effects were confirmed by Western blot analyses, the growth and proliferation were determined by MTT, palte clone and flow cytometry analyses.

RESULTS: The lentivirus-mediated siRNA markedly inhibited the expression of ATDC in both EC109 and EC9706 cells. MTT, plate colony formation and Xenograft assays illustrated that down-regulation of ATDC significantly repressed the growth of cells *in vitro* and *in vivo*. Furthermore, western blot assays revealed that ATDC down-regulation could decrease the expression of cyclinE in ESCC cell lines.

CONCLUSIONS: We demonstrated that down-regulation of ATDC could inhibit the growth and proliferation partly through regulation of cyclin E.

Key Words:
ATDC gene, Esophageal squamous cell carcinoma, siRNA, Growth.

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignant digestive cancers in China and most frequent causes of cancer-related death worldwide[1,2]. Although the progress includes surgical techniques and perioperative management for patients, the prognosis of ESCC patient is still poor. To improve the prognosis of ESCC, it is pivotal to find potential biomarkers and molecular therapeutic targets.

The Ataxia-Telangiectasia Group D Complementing gene (ATDC), also named TRIM29, is located on chromosome 11q23[3], which belongs to the TRIM family that include multiple zinc finger motifs and an adjacent leucine zipper motif with various functions such as transcriptional regulatory factors for the malignant progression of tumors[4]. ATDC has various functions such as transcriptional regulation, cell growth, apoptosis and carcinogenesis[5]. ATDC was found to play oncogenic function in pancreatic cancer through activatiing the Wnt and β-catenin pathway[6], which is also a marker for predicting the lymph node metastasis of gastric cancer[7]. Our previous study showed that increased ATDC expression correlated with advanced stage, poor differentiation and lymph node metastasis in ESCC[8], indicating that ATDC might play a critical role in the malignant progression of ESCC. However, the roles of ATDC in the tumorigenesis of ESCC are still unclear.

In this study, the lentivirus-mediated siRNA targeting ATDC was transfected into ESCC cell lines EC109 and EC9706. After the gene silencing effects were confirmed by Western blot analyses, the growth and proliferation were determined by MTT, palte clone and flow cytometry analyses.

Materials and Methods

Cell Lines

The human ESCC cell lines EC109 and EC9706 were obtained from the Chinese Academy of Medical Science (Beijing, China)[9], which

Corresponding Author: Dr Xu Wu, PhD; e-mail: yangmin@fimmu.com

were maintained in our Laboratory. Both the two cell lines were cultured in DMEM (Dulbecco's Modified Eagle's Medium) 1640 with 10% fetal bovine serum (FBS) at 37°C in an incubator with 5% CO_2. The culture solution also contains 10 units/ml penicillin-G, and 10 mg/ml streptomycin.

siRNA Construction and Transfection

The siRNA targeting ATDC (M-012409-01), and control siRNA (D-001810-01) targeting none of the known genes were purchased from Dharmacon (Lafayette, CO, USA), which were transfected into EC109 and EC9706 cells using Attractene Transfection reagent (Qiagen, Hilden, Germany) following the introduction of the company. The transfected cell lines were named si-EC109, Con-EC109, Si-EC9706 and Con-EC9706 cells respectively (Si-cells were transfected with ATDC siRNA, and Con- cells were transfected with control siRNA).

Western Blot Analysis

The western blot analyses were carried out as previously described[8]. Briefly, the total protein of each cell lines was extracted using lysis buffer (Thermo Fisher Scientific, Rockford, IL, USA), and quantified using the Bradford method, which was separated using SDS-PAGE and transferred onto an NC membrane (0.45 mm, Millipore, Billerica, MA, USA). Then the membranes were incubated overnight at 4°C with the following antibodies: ATDC (1:500, Abcam: Cambridge, MA, USA), cyclinE (1:50, Santa Cruz, CA, USA), and-actin (1:3000, Sigma, St Louis, MO, USA).

MTT Assays

MTT assay was performed as described previously[10]. The cells in log phase were made into single cell suspension, and palted into 96-well plates at a density of 1×10^4/well in 200 μl culture medium. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution was added into each well at each time point including 1, 2, 3, 4, 5, 6 and 7 days. Then 150 μl DMSO (dimethyl sulfoxide) was added into each well to dissolve the crystals by agitation, and the absorbance value was evaluated using an ELISA reader (wave length: 490 nm). Each time point was repeated three times.

Plate Colony Formation Assay

The plate colony formation assay was performed to detect the growth of cells. Each cell line was planted into 6-well plates at a density of 1×10^3/well, and incubated for 14 days. Then each plate was washed with phosphate buffered saline (PBS) for three times, and stained with Giemsa for 10 min. After 5-washes by PBS, the number of colonies with more than 50 cells was counted using optical microscope. Each time point was repeated three times.

Flow Cytometry

The cell cycles were measured by flow cytometry following previous study [10]. Briefly, each cell line in log stage was harvested, and fixed in 4% paraformaldehyde for 10 min. Then cells were washed with PBS for three times and stained with 5 mg/ml propidium iodide (PI) in PBS supplemented with RNase A for 30 minutes. Then the cell cycles were analyzed by a flow cytometry system. Each was repeated for time three times.

Animal Experiments

To study the effects of ATDC gene silencing on the growth *in vivo*, BALB/c nu/nu mice were used to subcutaneous tumorigenesis experiments according to the NIH Animal Care and Use Committee guidelines. Each cell lines were prepared, and injected subcutaneously with 2×10^6 cells in 200 μl medium without fetal calf serum (FCS) into both upper backs (right: siRNA transfected cells; left: controls). Both the EC109 and EC9706 groups were contained 3 mice. The mice were cultivated for 30 days. The tumor volume was determined as:$0.5\times\text{length}\times\text{width}^2$.

Statistical Analysis

Each p-value was calculated using SPSS 17.0 software package (SPSS, Chicago, IL, USA), with a $p < 0.05$ designating statistical significance. The one-way ANOVA method was used for comparisons between three groups, and Student's *t*-test was used for two groups.

Results

The Effects of siRNA on the ATDC Expression

The lentivirus-mediated siRNA targeting ATDC was transfected into ESCC cell lines EC109 and EC9706. Compared with con-EC109 and EC109 cells, the expression of ATDC in Si-EC109 cells was markedly down-regulated. Similarly, the expression of ATDC in Si-EC9706 cells was obviously inhibited compared with Con-EC9706 and EC9706 cells (Figure 1).

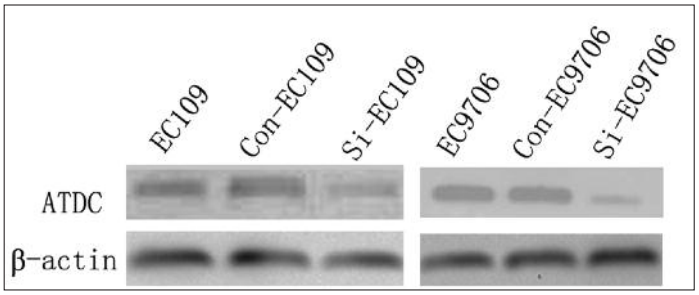

Figure 1. The siRNA targeting ATDC was transfected into ESCC cell lines EC109 and EC9706. Western blot analyses showed that the expressions of ATDC in both EC109 and EC9706 cells were markedly decreased compared with matched controls (Si-EC109 versa EC109 or Con-EC109; Si-EC9706 versa EC9706 or Con-EC9706. β-actin was used as the loading controls.

Inhibiting ATDC Repressed the Growth of Cells

The expression of ATDC was markedly down-regulated in both EC9706 and EC109 cells. MTT assays revealed that the growth of Si-EC9706 was significantly inhibited than that of EC9706 or Con-EC9706 cells (Figure 2A); Similarly, the growth of Si-EC109 was significantly inhibited than that of EC109 or Con-EC109 cells (Figure 2B). The significant inhibitive effects were observed from the third day.

The plate colony experiment showed that the colony number of Si-EC109 cells was significantly decreased than that in EC109 and Con-EC109 cells ($p < 0.05$), and the colony number of Si-EC9706 cells was significantly decreased than that in EC9706 and Con-EC9706 cells ($p < 0.05$; Figure 3).

Inhibiting ATDC Repressed the Cell Cycle Progression Through Regulation Cyclin E

Flow cytometry assays were used to evaluate the effects of ATDC down-regulation on the cell cycle of ESCC cells. As shown in Figure 4a, the mean percentage of Si-EC109 cells in the G1 stage was significantly increased, but significantly decreased in the S stage compared with EC109 and Con-EC109 cells ($p < 0.05$). This finding was further strengthened in EC9706 cells, indicating that repressing ATDC inhibited the cell cycle progression in ESCC cells.

We further asked the possible molecular mechanism that ATDC involved in the cell cycle regulation. Western blot analyses were performed to detect the alteration of cell cycle related proteins cyclin E in ESCC cells transfected with lentivirus-mediated siRNA targeting ATDC. We found that the cyclin E expression were markedly decreased in siRNA transfected cells compared with matched controls, revealing that ATDC influenced ESCC cell cycle partly through regulating cyclin E expression.

Knockdown ATDC Inhibited the Growth of Cells in vivo

The subcutaneous tumor formation assay was performed to study the effects of ATDC gene silencing on tumor formation in BALB/c nu/nu

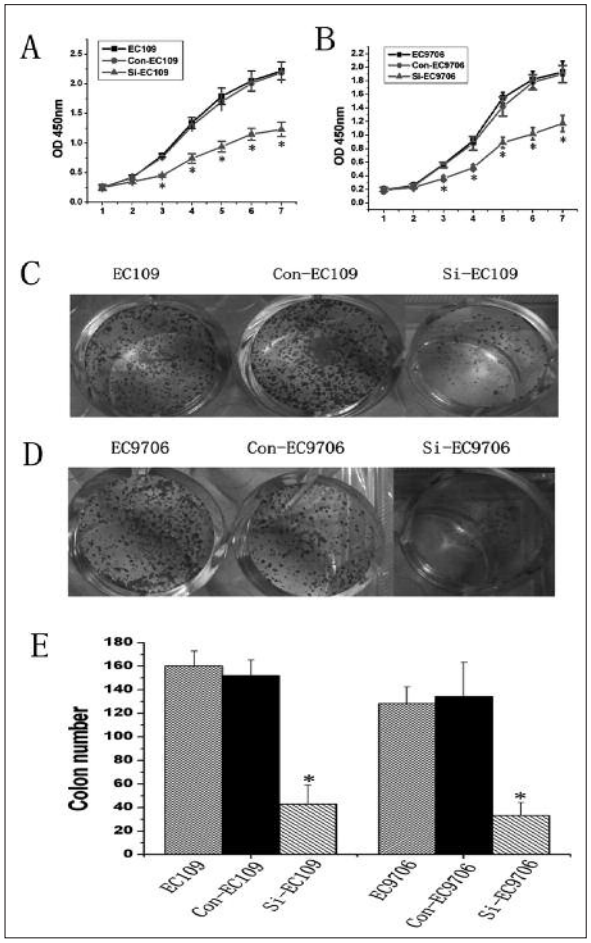

Figure 2. Knockdown ATDC significantly inhibited the growth of ESCC cells ***(A*** and ***B)***, The growth curves of ATDC siRNA transfected cells and controls were detected by MTT. The OD values shown is the mean of three times. ***C*** and ***D,*** The representatives of colony formation for ATDC siRNA transfected cells and controls were shown. ***E,*** The mean colony numbers were presented. *Statistical significance ($p < 0.05$, Si-EC109 or Si-EC9706 versa matched controls).

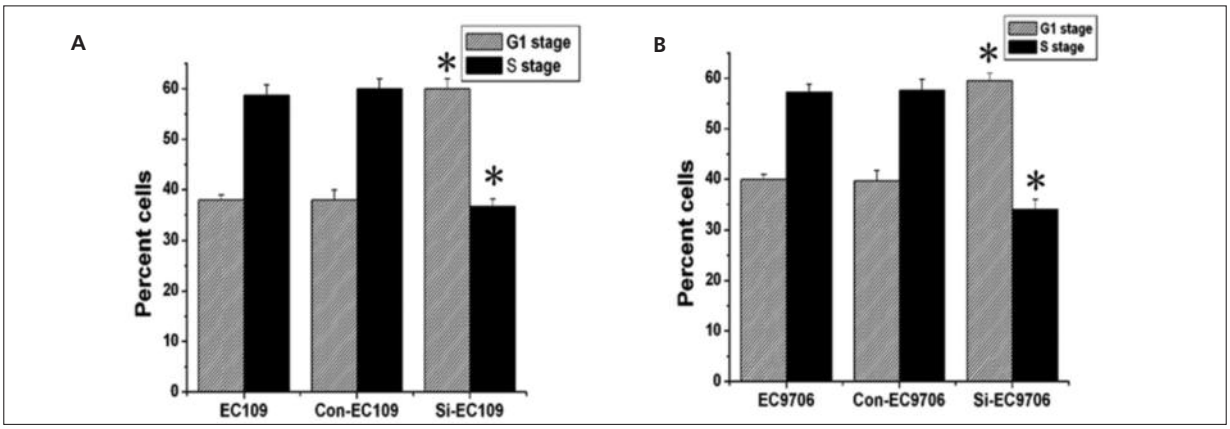

Figure 3. Knockdown ATDC induced of cell cycle arrest and inhibited the tumor growth *in vivo*. The flow cytometry analyses showed that both the EC109 and EC9706 cells transfected with ATDC siRNA have significantly higher percent cells in G1 stage and decreased S stage, compared with matched controls.

mice. The mean tumor volumes of Con-EC109 and Si-EC109 were 1.87±0.20 and 0.37±0.21 cm^3 (Figure 5A), and the mean tumor volumes of Con-EC9706 and Si-EC9706 were 1.52±0.14 and 0.33±0.15 cm^3 (Figure 5B), respectively. Statistical analyses showed that the mean tumor volumes of Si-EC109 and Si-EC9706 were significantly decreased compared with matched controls Con-EC109 and Con-EC9706 ($p < 0.05$; Figure 5C), indicating that down-regulation of ATDC can induce the inhibitive effects of tumor growth *in vivo*.

Discussion

ESCC is one of the common malignant cancers, also one of the leading causes of cancer death, due to its aggressive and malignant phenotypes and resulting in poor clinical outcomes. Recently years, the pathogenesis of ESCC has interested scientists and clinicians; however, the molecular mechanism involved in the development of ESCC is still far from being cleared. Previous studies showed that many molecules such as gankyrin and fascin were involved in the ma-

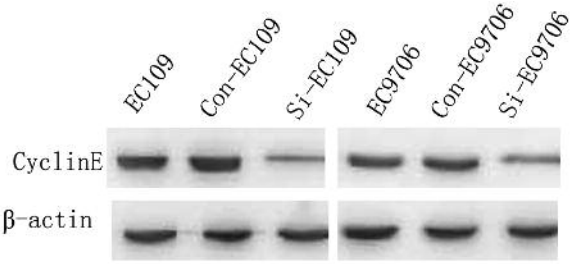

Figure 4. Western blot analysis of cell cycle-related protein cyclinE in siRNA transfented cells and controls. β-actin was used as the loading controls. The expression of cyclinE in siRNA transfected cells was markedly decreased compared with matched controls.

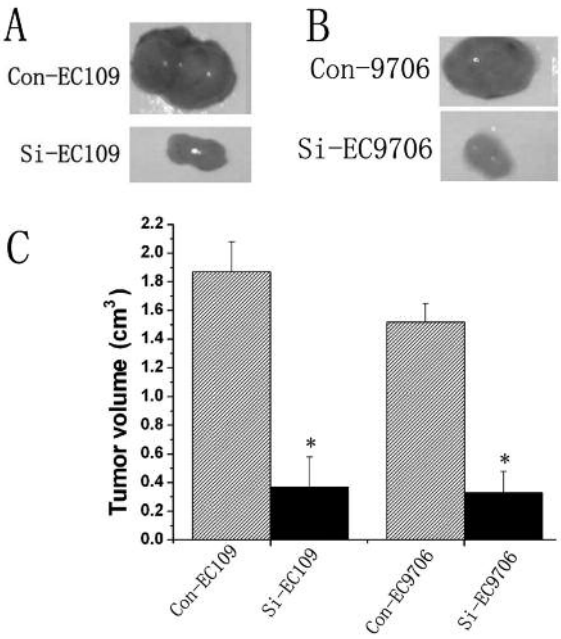

Figure 5. Knockdown ATDC induced inhibited tumor growth *in vitro*. ***A,*** and ***B,*** After cultured for 30 days, the tumors were resected from the mice. The tumors formed by ATDC siRNA transfected cells were obviously smaller compared with matched controls. ***C,*** The tumor volumes were calculated using the formula: 0.5 × length ×width 2, and the tumors formed by ATDC siRNA transfected cells were significantly smaller than controls. *Statistical significance ($p < 0.05$, Si-EC109 or Si-EC9706 versa matched controls).

lignant progression of human ESCC[11,12], which might be a therapeutic strategy and the individualization of intervention.

The genome-wide microarrays showed that ATDC expression was significantly increased in bladder cancer, endometrial cancers and multiple myeloma[13-15]. Our previous study showed that ATDC expression was correlated with tumor aggressiveness and poor prognosis in patients with ESCC, indicating that ATDC might be a target for ESCC therapy. We used the siRNA technology, which presented highly perturb targeted gene expression with high specificity and low toxicity[16], to down-regulate the ATDC expression in ESCC cells. After siRNA transfection, western blot analyses showed that ATDC expression was markedly inhibited in both EC109 and EC9706 cell lines. *In vitro* assays including MTT, plate colony formation and Xenograft study in nude mice illustrated that the growth and proliferation of EC109 and EC9706 cells *in vitro* and *in vivo*, which is consistent with our previous study that ATDC expression was correlated with tumor aggressiveness and poor prognosis in patients with ESCC, and also consistent with the result that ATDC promotes lung cancer cell proliferation by activating NF-kB pathway[17]. In addition, ATDC was found to be as a protein highly expressed in the majority of human pancreatic adenocarcinomas and pancreatic cancer precursor lesions, and expression of ATDC in pancreatic cancer cells promoted cellular proliferation and enhanced tumor growth and metastasis in pancreatic cancer cells[6].

Knockdown ATDC induced more percent of cells in the G1 stage, and lesser in the S stage, leading to the cell cycle G1-S progression, indicating that ATDC might be involved in the regulation of cell cycle-related proteins. Therefore, we detected the effects of ADTC siRNA on the expression of cyclin E. The result showed that knockdown ATDC markedly decreased the expression of cyclin E, suggesting that ATDC promotes the malignant progression of ESCC partly through regulation of cyclin E. ATDC has oncogenic activities through chromosomal translocations, which might be involved in the regulation of cyclin E and phosphorylated Rb[18,19]. This finding is also consistent with that ATDC can increase in cell proliferation through inhibiting p53 nuclear activities, and decreasing the expression of p53-regulated genes including p21 and NOXA[20]. Cyclin E belongs to the cyclin-dependent kinases (CDKs) family that plays critical roles in controlling the cell cycle progression for G1 to S phase transition, and regulating cell proliferation[21]. Previous studies showed that cyclinE are associated with various malignant cancers[22].

Conclusions

Knockdown ATDC expression significantly inhibits the growth and proliferation of ESCC cells partly through regulation of cyclin E. These data suggest ATDC is a potential therapeutic target for ESCC treatment, which needs to be further evaluated by clinical and experimental studies.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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