EJGO European Journal of Gynaecological Oncology

Upregulation of ATF3 expression inhibit the proliferation of human uterine serous carcinoma in vitro

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Summary

Objects: To investigate the effects of ATF3 upregulation on the proliferation of human uterine serous carcinoma line SPEC-2 and its mechanism. *Materials and Methods:* Ampho-293 and HEK-293 Fast cells were used to prepare the virus. SPEC-2 cells were infected by polybrene. Western blot experiments were used to verify the success of transfection. The proliferation of cells was detected by MTT method. At different time points, the absorbance of the cells was detected at the wavelength of 490 nm at the enzyme linked immunosorbent assay. The expression of MTP53, ATF3, P21, and CyclinD1 in the cells was detected by Western blot. *Results:* In negative control and blank control groups, the expression of ATF3 cells was lower in SPEC-2 cells, while the expression of ATF3 protein in the transfection group was significantly higher ($p < 0.05$).The results of MTT showed that the growth rate of SPEC-2 cells was significantly lower than that in the control group. From the experimental results, it can be seen that the expression level of P21 increased, while the expression of MTP53 and CyclinD1 decreased significantly ($p < 0.05$). *Conclusions:* High expression of ATF3 in SPEC-2 cells could inhibit the proliferation in vitro. The expression of P21 was activated and the expression of mutant type P53, and CyclinD1 was inhibited which might be the molecular mechanism of inhibition of cell proliferation.

Key words: Uterine serous carcinoma; ATF3; SPEC-2; Proliferation.

Introduction

Uterine serous carcinoma is one kind of endometrial cancer with special biological behavior which has a high degree of malignancy, accounting for 50% of all endometrial cancers. There is still no effective treatment method. Based on the results of the authors' previous research, the expression of ATF3 in uterine serous carcinoma tissues was lower and the expression of ATF3 was negatively correlated with the expression of MTP53. In this experiment, the expression level of ATF3 in SPEC-2 cells was upregulated. The effect of ATF3 on the proliferation of SPEC-2 and the mechanism in vitro was studied.

Materials and Methods

A frozen tube was removed from the liquid nitrogen tank and rapidly placed into a 37°C water bath to melt it. One minute later, the tube was removed from the water bath tank, diluted ten times with the culture liquid, centrifuged at low speed for ten minutes, the supernatant was discarded, fresh cell culture medium was added, and transferred to cell culture dish to continue culture. After digestion with 0.25% trypsin for five minutes, the digestion was terminated and centrifuged for ten minutes (1000 r/m). The supernatant was discarded and cells were prepared by adding 10% fetal bovine serum (MEM) into the cell culture medium suspension. Cell count was taken after 1×10^4/ml density passages.

The experiment was divided into negative control, blank control, and transfection groups. PBabe-ATF3 / pBabe-puro and p-SIH-shATF3 / p-SIH-shLuc viral vector were transfected into the transfection group. The cells were cultured with ampho-293 and HEK-293 Fast cells, respectively. The SPEC-2 cells were infected with polybrene as a synergist. Twenty-four hours later, fresh cells were cultured for 24 hours. After the cells were incubated with puromycin for 24 hours, growth conditions were assessed, and the timely replacement of the same concentration of puromycin culture medium to remove dead cells was performed. After one week of culture, the cells were selected for amplification and the expression level of ATF3 was identified by Western blot.

The logarithmic phase SPEC-2 cells were digested by trypsin to prepare cell suspension, and the cell count was adjusted to 5×10^4 / ml. The prepared cell suspension was gently mixed and inoculated into 96-well cell culture board. The amount of cell suspension added per well was 100 µl and the marginal wells were filled with sterile PBS. Then the transfection of ATF3 was placed in 5% CO_2 concentration in a 37°C cell incubator. Ten ul MTT solution was added to each well at 0, 12, 24, 36, and 48 hours after transfection, and the culture was terminated after one hour of incubation. The supernatant was removed from each hole and 150 ul dimethyl sulfoxide was added, in the shaker at low speed oscillation for two minutes, while fully dissolved crystals. The absorbance values of the wells were measured at a wavelength of 490 nm by the enzyme immunoassay. The transfection group and the control group were repeatedly detected three times per hole on average, and then the cell growth curve was drawn.

All proteins were extracted from cells. After centrifuging at 12,000 g for ten minutes at 4°C, the concentration of protein was determined. About 30 µg protein was loaded in each lane and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then the proteins were transferred to polyvinylidene fluoride membranes and blocked with 5% milk for two hours at

Revised manuscript accepted for publication November 13, 2017

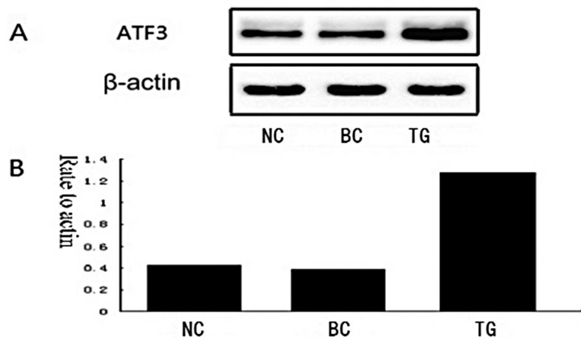

Figure 1. — ATF3 expression in SPEC-2 cells of each group after transfection. A) Experimental results show the expression of ATF3 protein in each group. B) the ratio of the expression of ATF3 and beta-actin protein in each group. Compared with the negative control and the blank control groups, $^{*}p < 0.05$. NC: negative control group, BC: blank control group, TG: transfection group.

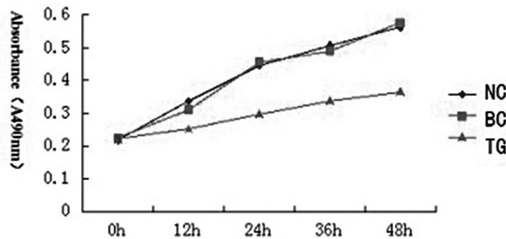

Figure 2. — Cell growth curves in vitro. NC: negative control group, BC: blank control group, TG: transfection group.

room temperature. The expression of ATF3, MTP53, P21, and CyclinD1 in the control, negative control, and transfection groups was detected by the aforementioned experimental procedure. β-actin was used as the loading control.

Statistical analysis was performed using SPSS 17.0 software. Statistical analysis between groups of data using *t*-test. $P < 0.05$ was statistically considered statistically significant.

Results

ATF3 was transfected into SPEC-2 cells by virus vector, and the expression of ATF3 cells was examined by Western blot assay. In the negative control group and blank control group, a certain amount of ATF3 was expressed by SPEC-two cells, but the protein level of transfected cells in ATF3 increased significantly ($p < 0.05$). The authors compared the negative control with the control groups, and no significant difference in ATF3 protein levels was seen ($p > 0.05$) (Figure 1).

After SPEC-2 cells were transfected with ATF3, the cell growth curve was drawn based on the absorbance of the cells in each group at each time point (Figure 2). MTT colorimetric experiment results showed that compared with the negative control and blank control groups, the growth

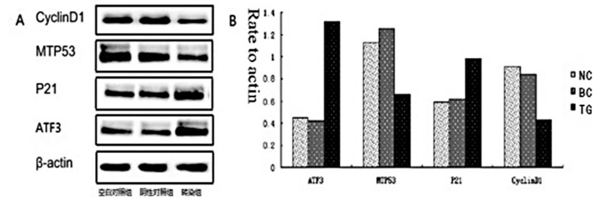

Figure 3. — The expression of ATF3, MTP53, P21, and CyclinD1 in each group. A) Western blot experimental results showed that the expression of ATF3, MTP53, cell P21, and CyclinD1 protein. B) The ratio of corresponding protein compared with beta-actin expression. Compared with the negative control and blank control groups, $^{*}p < 0.05$. NC: negative control group, BC: blank control group, TG: transfection group.

rate of SPEC-2 cells was significantly decreased after the expression of ATF3 was upregulated. With the prolongation of the culture time in vitro, the extent of cell growth in vitro was increased (Table 1). After *t*-test, the transfection group was significantly different from the control and the blank control groups compared with the negative control group in vitro culture at 12, 24, 36, and 48 hours ($p < 0.05$). Compared with the control group, there was no significant difference between the negative control and the blank control groups at each time point ($p > 0.05$).

After the cells were cultured in vitro for 48 hours, the proteins in the cells were extracted, and the expression of ATF3, MTP53, P21, and CyclinD1 were analyzed by Western blot (Figure 3). The experimental results showed that the transfection group compared with the negative control and the blank control groups, ATF3 expression was upregulated, the expression of P21 was increased, and the expression of CyclinD1 and MTP53 were decreased in different degrees. By statistical analysis, the differences were significant ($p < 0.05$). This showed that the expression of P21, MTP53, and CyclinD1 was related to the expression level of ATF3. There was no significant difference between the blank control and the negative control groups ($p > 0.05$).

Discussion

Whether normal or tumor cells, cell proliferation is closely related to cell cycle. Cell cycle is a complex process involved in many regulatory factors. It is divided into four stages: G1 phase (the early stage of DNA synthesis), S phase (the period of DNA synthesis), G2 phase (the late stage of DNA synthesis), and M phase (the mitotic stage). Once the cell is out of the cell cycle, then the cell will enter the G0 period (rest period) and no longer copy. However

Table 1. — *Results of MTT colorimetric experiments* (± *s*).

Group \ Time	0 hours	12 hours	24 hours	36 hours	48 hours
NC	0.218 ± 0.056	0.337 ± 0.042	0.446 ± 0.053	0.508 ± 0.058	0.563 ± 0.054
BC	0.226 ± 0.082	0.311 ± 0.023	0.457 ± 0.029	0.491 ± 0.056	0.576 ± 0.052
TG	0.221 ± 0.074	0.253 ± 0.071*	0.298 ± 0.026*	0.339 ± 0.061*	0.365 ± 0.044*

*NC: negative control group; BC: blank control group; TG: transfection group. Compared with NC and BC, *$p < 0.05$.*

with external stimulation factors, the cell can reenter the G1 phase to participate in cell proliferation [1-3]. CyclinD1 gene is a cancer gene, and it is the promoter of cell cycle progression, which can also promote cell proliferation and transformation with a variety of cancer genes. CyclinD1 protein was cloned and identified successfully in 1991, which is a positive regulatory protein of cell cycle G1 [4]. The cell cycle theory at present suggests that the various signal transduction pathways promote cell proliferation by stimulating factor inside and outside of cells, while enhancing the expression of CyclinD1 protein in early G1 phase function, and the corresponding cell cycle protein kinase (CDK4/6) binding protein complex formation. Activation of retinoblastoma protein (pRb), which is in the high state, and releases factor of E_2F phosphorylation. E_2F, as a transcription factor into the nucleus, with a variety of gene promoter sequence binding, promotes the expression of the corresponding gene. The product of mitosis related protein and DNA synthesis, cells, cells driven by G1/S levels, the G1 phase of the cell cycle in S phase, and play the role of promoting cell proliferation [5-8]. The pRb-E2F pathway is a classic pathway G1/S transition of cell cycle. Once the cells undergo this cycle, the cell will no longer have anti-proliferative effects of exogenous cytokines or drug reaction, and will no longer depend on foreign growth factor stimulation, and will independently complete a cycle [9]. P21 is a tumor suppressor gene and its protein expression product is a negative regulator of cell cycle. P21 could combine protein kinase (CDK4/6) in competition with CyclinD1 and then inhibit protein kinase activity, causing cell cycle arrest at the G1/S phase checkpoint and the cell proliferation process is blocked [10, 11].

P53 gene is located on the short arm of human chromosome 17 and encodes 393 amino acids, which is divided into the wild type and the mutant type according to the amino acid composition. WTP53 is a tumor suppressor protein, which contains a section of amino acid residues with transcriptional activity, which is involved in DNA repair, cell cycle regulation, and induces apoptosis by binding to specific DNA sequences and activation of downstream related genes [12]. MTP53 protein belongs to cancer and has a close relation with nearly half of the malignant tumor, MTP53 protein promotes tumor development in three mechanisms: 1) MTP53 loses the ability of wild-type P53 to combine with the specific DNA sequence, and loses the normal tumor suppressor function. 2) MTP53 and WTP53 are combined to form a heterodimer, which inhibits the activity of the latter as a transcription factor. 3) MTP53 obtains a new carcinogenic function, which promotes the malignant transformation of cells [13-15]. Among them, a new cancer-causing function of MTP53 is to promote the proliferation of tumor cells [16-17]. Zhou *et al.* found that MTP53 can inhibit the proliferation of head and neck cancer cells in vitro by affecting the activation of protein kinase signal transduction pathway [18]. This part of the experiment found a high expression of MTP53 and CyclinD1 in SPEC-2 cells in the blank control and in the negative control groups,while the expression of P21 was weak. This result suggested that MTP53 may be involved in the regulation of cell cycle in SPEC-2 cells, which is related to the ability of proliferation in vitro.

ATF3 belongs to the rapid induction of genes and a series of stress signal can be rapidly induced in response to stress. ATF3 is not only a key regulatory factor; it is also widely involved in homeostasis, cell adhesion, wound healing, cell apoptosis, and signal transduction of physiological and pathological processes [19-21]. The study found that the expression of ATF3 can affect the proliferation of a variety of malignant tumor cells in vitro. SiRNA knockdown of in colorectal cancer cell ATF3 gene, the results in vitro increased tumor metastasis, promoted the growth of the tumor, and the ability to transfer to the liver [22]. Wang *et al.* found that the deletion of ATF3 gene can enhance the proliferation of prostate cancer cells in vitro [23]. The proliferation of human esophageal cancer cell line EC171 with high expression of ATF3 decreased in vitro, and inhibited the growth of transplanted tumor [24]. ATF3 is an important regulatory factor of WTP53. ATF3 could block the degradation pathway of WTP53 by direct binding of WTP53 protein. In HPV positive cervical cancer cells, ATF3 could inhibit the WTP53 degradation pathway mediated by HPV-E6 in HPV, induce the expression of downstream gene p21, and cause cancer cell apoptosis. The possible mechanism is that ATF3 and WTP53 protein binding causes changes in its conformation, making it easier to combine with DNA sequence or other transcription activating factor or ATF3 direct competitive binding P53 gene promoter sequence to enhance the transcriptional activity of P53 [25]. In vitro experiments showed that ATF3 was able to completely inhibit the activity of MTP53 [26]. In the study of non-small cell

lung cancer, it was found that ATF3 can directly bind to MTP53 and inhibit tumor metastasis [27]. In this study, the authors found that the high expression of ATF3 by in vitro viral transfection of SPEC-2 cells, the proliferation was significantly inhibited. At the same time, the results of Western blot showed that the expression of MTP53 and CyclinD1 decreased significantly, while the expression of P21 was significantly increased. The results suggested that ATF3 may be expressed by inhibiting MTP53 expression, then weaken the inhibitory effect of MTP53 on the expression of P21, so that the latter and CyclinD1 competitively bind CDK4/6. The cells arrested at the G1/S phase checkpoint in vitro and the proliferation capacity was inhibited.

Acknowledgements

Project of Natural Science Foundation of Shandong Province (ZR2015HQ013).

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