

Original Research Article

Adiponectin exhibits proliferative and anti-apoptotic effects on ovarian cancer cells via PI3K/Akt and Raf/MEK/ERK pathways

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Sent for review: 15 August 2018

Revised accepted: 26 October 2018

Abstract

Purpose: To elucidate the effects and the underlying mechanism of adiponectin on human ovarian cancer cells.

Methods: The level of adiponectin, adiponectin receptor-1, caspase-3 and bcl-2 in the serum and ascites of the patients were measured with enzyme-linked immunosorbent assay (ELISA), qPCR and Western blotting. The human ovarian cancer cell lines (Caov3 and SKOV3) were enumerated using 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide (MTT) assay. Western blotting was also used to determine the levels of p-Akt, p-ERK and cyclin B.

Results: Serum and ascite levels of adiponectin were significantly higher in ovarian cancer patients than in healthy patients ($p < 0.05$). Expression of adiponectin in the serum and ascites of patients in FIGO stage IV was remarkably higher than in earlier stages ($p < 0.05$). The proliferative effect of adiponectin on ovarian cells was dose-dependent. Adiponectin treatment significantly increased the expression of cyclin B in Caov3 and SKOV3, and reduced the levels of caspase-3 and bcl-2. Inhibitors of PI3K and MEK pathways significantly reduced the proliferation of attenuated Caov3 and SKOV3 by up-regulating cyclin B upon adiponectin treatment ($p < 0.05$), and thus alleviated the inhibitory effect of adiponectin on the expressions of caspase-3 and bcl-2.

Conclusion: The findings demonstrate that adiponectin promotes proliferation of the cells via the PI3K/Akt and Raf/MEK/ERK pathways, and also provide new insights into ovarian cancer treatment.

Keywords: Adiponectin, Ovarian cancer, Proliferation, Apoptosis, PI3K/Akt pathway

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INTRODUCTION

Adiponectin, one of the cytokines secreted by adipose tissue[1,2], is a lipid-like and collagen-like protein encoded by apM1 gene [3]. The role of adiponectin in the occurrence and development of tumors is multiple and complex

[4,5]. For example, it inhibits the proliferation and migration of tumor cells while promoting their apoptosis via activation of the cAMP/PKA and AMPK pathways [6,7]. It also down-regulates the expression of vascular endothelial growth factor while suppressing the blood vessel formation [8,9].

Recently, increasing literatures have indicated that adiponectin may act as a tumor suppressor gene. Adiponectin suppresses the proliferation and migration of hepatocellular carcinoma cells [10], gastric cancer cells [11], breast cancer cells [13], prostate cancer cells [14] and endometrial cancer cells [15]. Adiponectin can activate different pathways by binding to its receptors; it regulates the activity of some growth factors and regulates the expression of oncogenes or tumor suppressor genes. Adiponectin stimulates the expression of nicotinamide adenine dinucleotide-dependent histone deacetylase (Sirt1) via the promotion of the phosphorylation of AMP-activated protein kinase (AMPK), and inhibition of apoptosis in pancreatic cancer cells [17]. Adiponectin also promotes the migration and invasion of breast cancer cells via activating MAPK and PI3K pathways [7,18], and promote the phosphorylation of signaling molecules such as STAT3, ERK1/2 and Akt in 786-O renal carcinoma cells.

Ovarian cancer is one of the most common gynecological malignancies since patients do not present obvious early symptoms and its diagnosis is usually poor. More than 70 % of ovarian cancer patients are identified and confirmed at the terminal stage of the disease [19]. In a previous study, the expression ratio of adiponectin receptor 1/2 in follicular cells of polycystic ovary syndrome patients was significantly higher compared with that in normal ovaries [20], but there are few studies on the role of adiponectin in ovarian cancer's development and progression. This study aims to investigate the effects and underlying mechanism of adiponectin on ovarian cancer cells.

EXPERIMENTAL

Materials and reagents

Adiponectin ELISA kit and recombinant protein were produced by R&D, USA; rabbit anti-human adiponectin receptor 1 and bcl-2 antibodies were purchased from Abcam, USA; HRP labeled goat anti-mouse IgG and goat anti-rabbit IgG antibodies were obtained from ZSGB-BIO, China. Polymerase chain reaction (PCR) primers and MTT assay kit were purchased from Sangon Biotech, China. Rabbit anti-human Akt, p-Akt, ERK, p-ERK primary antibodies, and rabbit anti-human caspase-3 were products of Cell Signaling Technology, USA. Mouse anti-human GAPDH antibody was obtained from Biotime, China, while rabbit anti-human cyclin antibody was produced by Santa Cruze, USA. Trizol reagent was obtained from Sigma Aldrich Co., Germany.

Patients and clinical data

Sixty three patients in total were included for this one year study. All the patients were divided into two groups: an observation group consists of 46 patients with ovarian cancer, and a control consists of 17 healthy patients. Patients in the observation group were aged between 40 and 59 years (mean age = 53.00 ± 9.50 years), while those in control were aged between 30 and 55 years (mean age = 43 ± 12.50 years). Patients in the observation group were diagnosed pathologically and met the following conditions: (1) confirmed FIGO stage; (2) absence of hypertension; and (3) absence of diabetes or other chronic diseases. The FIGO stages were I, II, III, and IV. Patients in the control group were subjected to physical examinations. Inclusion criteria were: (1) healthy women with no cancer; and (2) absence of gynecological diseases, hypertension, diabetes or other chronic diseases. This research was approved by Ethical Committee of Department of Biochemistry and Molecular Biology, China Medical University, Shenyang, Liaoning, China (approval no. 20150457), and followed the guidelines of Declaration of Helsinki promulgated in 1964 as amended in 1996 [21]. The patients and their family members signed written informed consent.

Enzyme-linked immunosorbent assay (ELISA)

Serum and ascites samples obtained from the patients were incubated overnight at 4°C after the addition of enzyme-labeled primary antibody. The blocking condition was placing in room temperature for one hour. The mixture was further added with enzyme-labeled secondary antibody and kept at room temperature for one hour. Positive and negative controls were routinely employed.

Cell culture

Human ovarian cancer cell lines (Caov3 and SKOV3) were cultured with complete culture medium (FCS-RPMI 1640 medium), spiked with 10 % fetal bovine serum and kept at 37°C in a humidified incubator in an atmosphere of 5 % CO_2 . The entire operation was carried out in 10,000-grade purification room in sterile conditions.

MTT assay

The cells at the concentration of 1×10^4 cells/well were inoculated into a 96-well plate in DMEM, and were incubated for 24 h. Different concentrations of adiponectin (0 – 200 ng/mL) was spiked to the cells and the mixtures were

further incubated for 24, 48, and 72 h, respectively. After 72 h, 20 μ L of MTT solution (5 mg/mL) was supplemented to each well, and then incubated for another 4 h. The medium was finally replaced with dimethyl sulfoxide (DMSO) solution and agitated (50 oscillations/min, 10 min). A microplate reader were used to determine the absorbances of the mixtures at 570 nm. MTT assays of cells in each group were conducted in quintuplicate.

Real-time quantitative PCR

Trizol reagent was used to extract the total RNA from the cells. The RNA was then reverse-transcribed to cDNA and subjected to real-time quantitative PCR (qPCR). The reactions were performed in a final 25- μ L reaction mixture volume. Reaction conditions were listed as follow: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 94 °C for 20 sec, 61 °C for 20 sec, 72 °C for 20 sec, and a final elongation at 72 °C for 5 min. The following protocol of melting curve was used: 10 seconds at 56 °C, and then 10 seconds at a 0.5 °C increment to a final temperature of 95°C. The resultant PCR products were confirmed by subjecting them to gene sequencing. The examination of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The primer sequences were listed in Table 1.

Table 1: Primer sequences used

Primer	Sequence
GAPDH	5'-AAGGTGAAGGTCGGAGTCAAC-3' 5'-GGGGTCATTGATGGCAACAATA-3'
AdipoR1	5'-TGTTCTCTTAATCCTGCCCA-3' 5'-CCAACCTGCACAAGTTCCTT-3'
Cyclin B	5'-GTTAGGGTGTCTTCTCGAATCGG-3', 5'-TTTCTGCGTTAATTTTCGTGTTCT-3'

Western blotting

We used the phosphate-buffered saline (PBS) to wash the cells treated with varied concentrations of adiponectin. After washing, the clean cells were lysed with ice-cold radio-immunoprecipitation assay buffer (RIPA). The BCA protein assay kit was used to determine the protein content. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to isolate the protein. The protein bands were transferred to PVDF membranes and incubated with primary antibodies (Akt, p-Akt, ERK, p-ERK, cyclin B and caspase-3). The

membranes were then washed and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies. We attenuated the primary and secondary antibodies w at a ratio of 1 to 500 overnight at 4 °C. The densities of the bands were estimated using Chemi-doc XRS imaging system.

Statistical analysis

All statistical analyses were performed with SPSS (13.0). Significant differences between the observation group and the control group were estimated using *t*-test. The data are presented as mean \pm standard deviation (SD). Statistically significant were accepted when $p < 0.05$.

RESULTS

Expression of adiponectin

The levels of serum and ascites adiponectin expression were significantly higher in the patients belonging to the observation group than the control group ($p < 0.05$). The expression of adiponectin in the serum of patients in FIGO stage IV was significantly higher than that in stage I ($p < 0.05$), but no significant difference was observed in the corresponding expressions between the other stages ($p > 0.05$; Figure 1 A). The expressions of adiponectin in ascites of patients in stages III and IV were significantly higher than the corresponding expressions in the other stages ($p < 0.05$; Figure 1 B).

Expressions of adiponectin receptor-1 in human ovarian cancer cell lines

The expression of adiponectin receptor-1 was significantly higher in SKOV3 than in Caov3 ($p < 0.05$; Figure 2 A and B).

Effect of exogenous adiponectin on proliferation of Caov3 and SKOV3

The proliferation of Caov3 and SKOV3 cells was dose-dependent and significantly higher than the corresponding proliferation in the control group ($p < 0.05$ Figures 3 A and B).

Expression of cyclin B in Caov3 and SKOV3 48 h after treatment with adiponectin

After 48 h adiponectin treatment, the expressions of cyclin B in Caov3 and SKOV3 were significantly increased ($p < 0.01$; Figure 4 A and B).

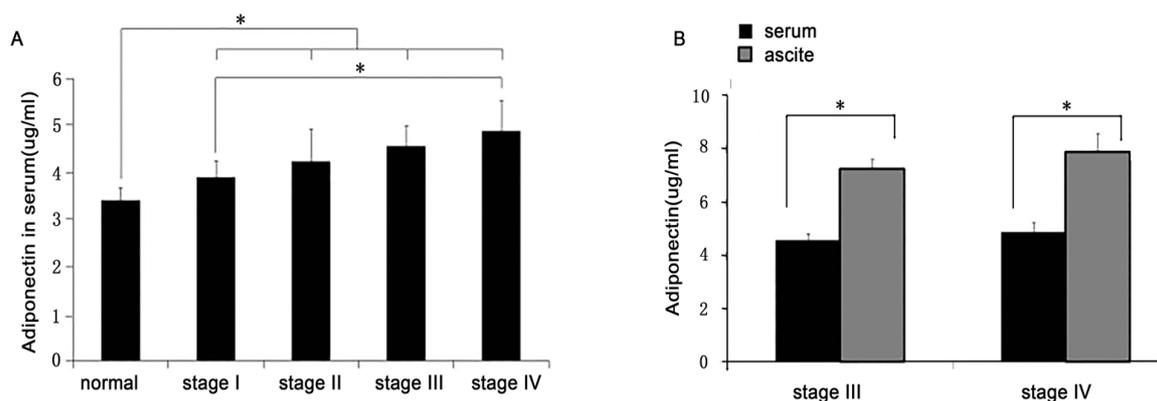


Figure 1: Expression of adiponectin in the two groups and FIGO stages. A: Expression of adiponectin in serum of patients in the observation and control groups; B: Expressions of adiponectin in ascites of patients in FIGO stages III and IV

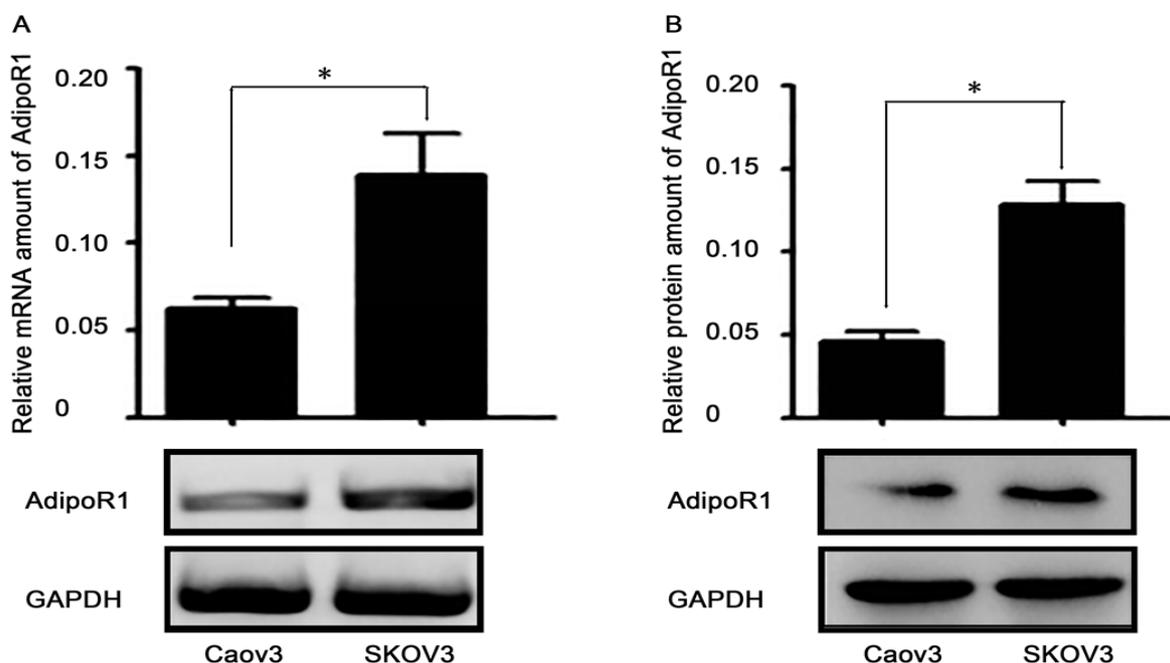


Figure 2: Expression of adiponectin receptor-1 in ovarian cancer cells. A: Expression of adiponectin receptors-1 in Caov3 and SKOV3, as measured using real-time qPCR; B: Expression of adiponectin receptor-1 in Caov3 and SKOV3, as measured using Western blotting

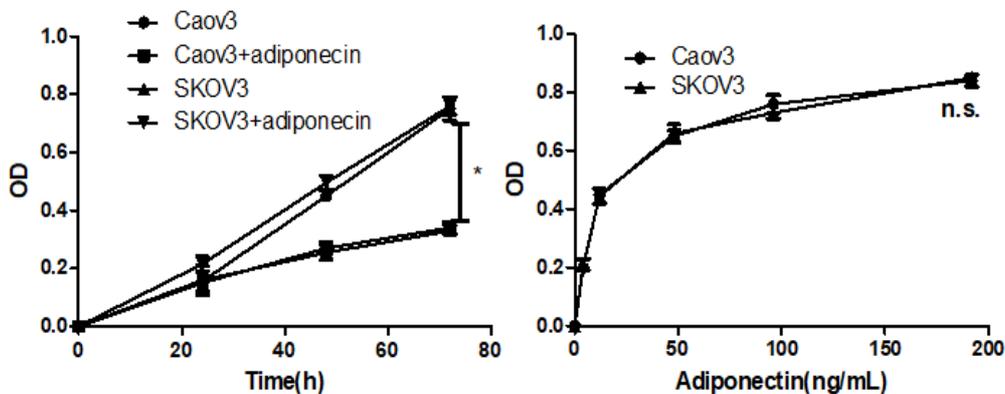


Figure 3: Effect of exogenous adiponectin on proliferation of ovarian cancer cells. A: After stimulation of adiponectin for different periods; B: 48 h after stimulation with varied concentrations of adiponectin

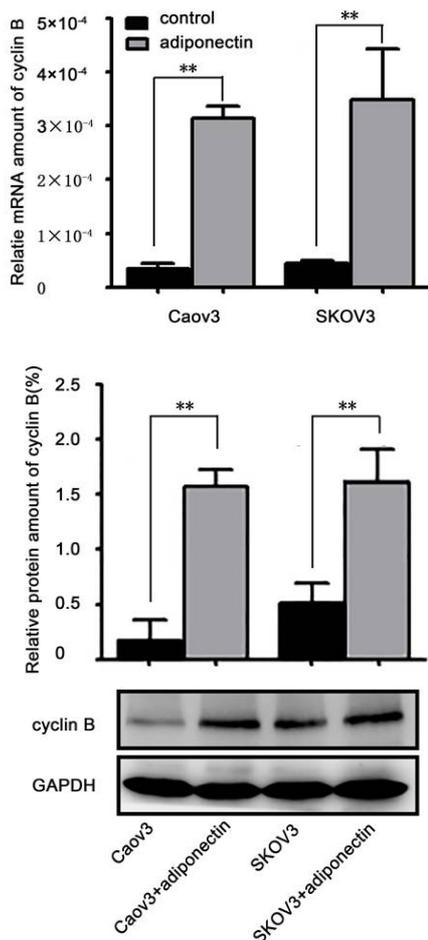


Figure 4: Expression of cyclin B in Caov3 and SKOV3 48 h after treatment with adiponectin. *Top:* Relative mRNA expression of cyclin B before and after treatment with adiponectin; *bottom:* Relative protein expression of cyclin B before and after adiponectin treatment

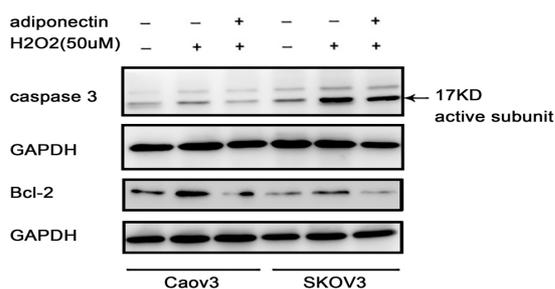


Figure 5: Expressions of apoptosis-related proteins after treatment with adiponectin

Expression of apoptosis-related proteins after treatment with adiponectin

The expressions of caspase-3 and bcl-2 in Caov3 and SKOV3 were determined before and after treatment with adiponectin. The expressions of caspase-3 and bcl-2 were up-regulated in Caov3 and SKOV3 treated with H₂O₂ (50 μM). However, after treatment with both H₂O₂ (50 μM)

and adiponectin, there were significant reductions in the expressions of caspase-3 and bcl-2 (Figure 5).

Effect of adiponectin on phosphorylation of Akt and ERK in ovarian cancer cells

A significant increase was observed in the expression of p-Akt after the adiponectin treatment. The expression peaked 10 and 30 min after treatments of Caov3 and SKOV3, respectively, with adiponectin. The expression of p-ERK also increased after adiponectin treatment and peaked 60 min and 30 min after the treatment of Caov3 and SKOV3, respectively (Figure 6).

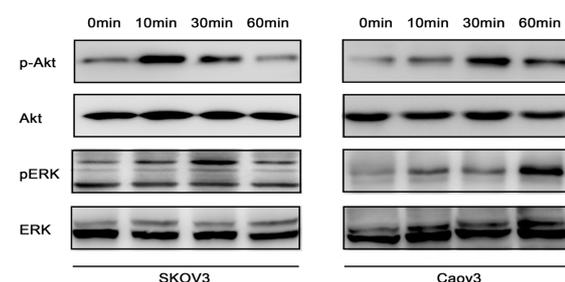


Figure 6: Effects of adiponectin on phosphorylation of Akt and ERK in SKOV3 and Caov3 treated with adiponectin

Effect of inhibitors of PI3K and MEK pathways on adiponectin-induced proliferation of human ovarian cancer cell lines

Inhibitors of PI3K (2-morpholin-4-yl-8-phenylchromen-4-one (LY294002)) and MEK (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059)) pathways were used to determine the effect of adiponectin on the proliferation of Caov3 and SKOV3. inhibitors of PI3K and MEK pathways significantly reduced the proliferation of Caov3 and SKOV3 after 48 or 72 h of treatments ($p < 0.05$, Figure 7).

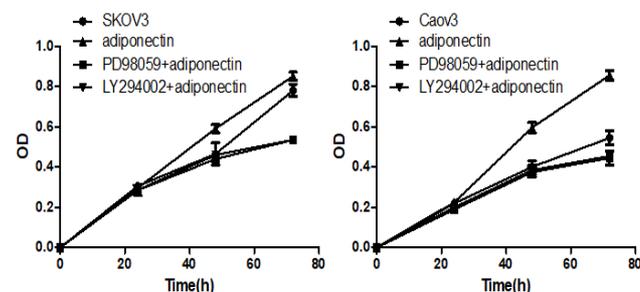


Figure 7: Effects of inhibitors of PI3K and MEK pathways on adiponectin-induced proliferation of human ovarian cancer cell lines. A: SKOV3; and B: Caov3

Effect of inhibitors of PI3K and MEK pathways on adiponectin-induced inhibition of apoptosis

The inhibitors of PI3K and MEK pathways significantly attenuated the up-regulation of cyclin B upon adiponectin treatment ($p < 0.05$). After treatment with inhibitors of PI3K and MEK pathways, the inhibitory effect of adiponectin on the expressions of caspase-3 and bcl-2 was also attenuated (Figure 8).

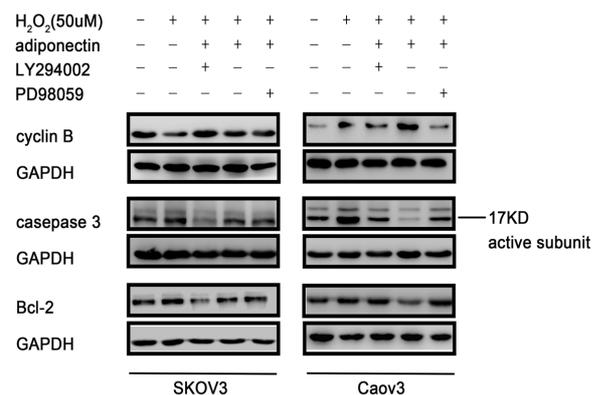


Figure 8: Expressions of cyclin B, caspase-3 and bcl-2 after treatment with H₂O₂, adiponectin, and inhibitors of PI3K and MEK pathways

DISCUSSION

Adiponectin is a fat factor that is relatively abundant in plasma [22] and mediates a variety of biological effects through its receptors expressed in cells of different tissues. Several studies have shown that there is a degree of correlation between its expression in the systemic circulation and the incidence of malignant tumors [23, 24].

In the present study, the expression of serum and ascites adiponectin was significantly higher in the observation group patients than the control group patients. The expression of adiponectin in the serum of patients in FIGO stage IV was significantly higher than that of stage I, but there were no significant differences of the corresponding expressions among other stages. The expressions of adiponectin in ascites of patients in stages III and IV were significantly higher than the corresponding expressions in the other stages. These results suggest that adiponectin may act as an oncogene in ovarian cancer. Our result is inconsistent with findings of Chen *et al* [25] and Arano *et al* [26], indicating that adiponectin may increase the risk of hepatocellular carcinoma.

It has also been reported that an increase in the serum level of adiponectin is associated with the

late stage and poor differentiation of GC. Some authors have reported a negative correlation between the expression of adiponectin in the serum and the incidence of malignant tumors such as GC, breast cancer, endometrial cancer and prostate cancer [27,28]. High level of expression of adiponectin is an indication of better outcomes for patients with tumor metastasis [29]. Studies have shown that adiponectin can act as an oncogene or tumor suppressor gene in different tumors. However, some researchers have speculated that it plays contradictory roles in the development of tumors, regulation of metabolism, changes in cell microenvironment and direct influence on tumor cells [4]. A meta-analysis of patients with breast cancer revealed inconsistent results. So far, it is difficult to determine whether adiponectin acts as an oncogene or not [30].

In this study, the expression of adiponectin receptor-1 was significantly higher in SKOV3 than in Caov3. These results suggest that adiponectin may exert its biological effects by interacting with adipoR1 on the surface of ovarian cancer cells. In a previous study it was reported that 87.30 % of patients with epithelial ovarian cancer expressed adipoR1, an indication that adipoR1 is highly expressed in obesity-related cancers [31]. Some authors have reported that the ratio of adipoR1 to R2 in follicular cells of patients with polycystic ovary was significantly higher than that in normal tissues [32]. In their studies, adiponectin inhibited the synthesis of androstenedione in follicular cells, luteinizing hormone (LH) receptors and the key enzymes of androgen synthesis. In this study, the effect of adiponectin on proliferations of Caov3 and SKOV3 cells were in a dose-dependent manner and the level of proliferations in the observation group was significantly higher than that of control.

The expressions of cyclin B in Caov3 and SKOV3 were significantly increased after treatment with adiponectin. These results suggest that adiponectin up-regulated the expression of cyclin B. Apoptosis is the orderly programmed death of cells regulated by genes and enzymes so as to maintain the stability of the cell inner microenvironment. Cytochrome C released into the cytosol binds to apoptosis-related factor 1 (Apaf-1) and promotes the formation of apoptotic bodies by recruiting caspase-9, which is followed by the activation of other caspases such as caspase-3, resulting in initiation of apoptosis.

It is believed that activated caspase-3 initiates caspase-chain reaction in the apoptotic pathway.

In the present study, the expressions of caspase-3 and bcl-2 were up-regulated in Caov3 and SKOV3 treated with H₂O₂. However, after treatment with H₂O₂ and adiponectin, significant reductions in their expressions were detected. These results are consistent with a previous study which showed that increased serum level of adiponectin significantly inhibited apoptosis in pancreatic cancer cells [33]. Recent studies have also shown that AMPK may play an important role in the suppression of adiponectin-induced proliferation of tumor cells [34].

Some authors have suggested that adiponectin inhibits leptin-induced proliferation of hepatocellular carcinoma by blocking STAT-3, AKT, and m-TOR pathways [35, 36]. Proteins such as AdipoRs, APPL1, AMPK, JNK, AKT, MAPK-glucose transporter 4 (MAPK-GLUT4), GSK-3 β , I κ B kinase, NF- κ B and caspases are involved in tumorigenesis induced by adiponectin [37]. Adiponectin may promote the metastasis and invasion of breast cancer cells through MAPK and PI3K pathways, and induce the expression of VEGF in macrophages via the ERK pathway [7,38].

The results of this study showed that adiponectin promote the phosphorylation of Akt and ERK. After treatment with inhibitors of PI3K and MEK pathways, the proliferations of Caov3 and SKOV3 at 48 or 72 h after the treatment were significantly reduced, an indication that adiponectin inhibit apoptosis in ovarian cancer cells and attenuate the down-regulation of casepase-3 and bcl-2. These results suggest that adiponectin may promote the proliferation of ovarian cancer cells by inducing apoptosis via the PI3K/Akt and the Raf/MEK/ERK pathways. In recent years, the role of PI3K/Akt pathway in tumorigenesis has attracted huge attention, probably because it plays an important role in cell proliferation, apoptosis, migration, adhesion and degradation of extracellular matrix.

The molecule PI3K is a key in many physiological processes, having both serine/threonine protein kinase and phosphatidylinositol kinase activities. Activation of PI3K by cytokines promotes the binding of Akt to phosphatidylinositol to the serosal membrane through its PH domain, thereby activating it. The activated Akt is released into the cytosol where it activates downstream factors causing them to regulate cell proliferation, differentiation, growth and migration. The Ras/Raf/MEK/ERK pathway is considered to be a classical MAPK pathway. The MAPK pathway is at the center of several signaling pathways, and plays an important role in receiving signals from the membrane and

transferring them to the cell nucleus. It also plays the leading role in many pathways related to cell proliferation.

MAPK family includes three major members of ERK, C-JUN amino-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38MAPK). ERK pathway can be activated by receptor tyrosine kinase, G protein-coupled receptors and partial cytokine receptors. Studies have shown that MAPK pathway mediates signal transduction of several cytokines, thus acting on transcription factors to regulate cell proliferation, differentiation and apoptosis [39]. This is important in malignant transformation of cells.

CONCLUSION

The findings of this study demonstrate that adiponectin promotes proliferation of the cells via PI3K/Akt and Raf/MEK/ERK pathways, and thus provide new insights into ovarian cancer treatment.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

This work was done by the authors named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Xuemei Wang; Fengjin Hao and Weina Wan collected and analysed the data. Yueqin Feng wrote the text. All authors have read and approved the text prior to publication.

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