RESEARCH ARTICLE

ATAD2 is Highly Expressed in Ovarian Carcinomas and Indicates Poor Prognosis

Wei-Na Wan, Yi-Xia Zhang, Xue-Mei Wang*, Yan-Jun Liu, Yu-Qin Zhang, Yan-Hong Que, Wen-Jing Zhao

Abstract

The purpose of this study was to explore the expression of ATAD2 in ovarian tumor tissue as well as its relationship with degree of malignancy. Tumor tissue from 110 cases of ovarian cancer was collected in accordance with the Declaration of Helsinki for evaluation of ATAD2 expression immunohistochemistry, quantitative PCR (qPCR) and Western blotting. The correlation between the ATAD2 expression and and the prognosis of ovarian cancer was evaluated by Cox regression model. In addition, HO-8910 and OVCAR-3 cells were transfected with two siRNAs targeting ATAD2. Cell viability was evaluated with MTT assay, and cell migration by transwell migration assay. ATAD2 was shown to be highly expressed in 65.5% (72/110) of ovarian cancer cases, both at transcriptional and protein levels. Moreover, highly expression was positively correlated with degree of malignancy. Knock-down of ATAD2 in HO-8910 and OVCAR-3 cells was found to reduce cell migration. In addition, follow-up visits of the patients demonstrated that the 5-year survival rate was lower in patients with high expression of ATAD2. Our study suggested that ovarian tumor tissue may have highly expressed ATAD2, which is associated with tumor stage, omentum-metastasis, ascites and CA-125. Increased ATAD2 may play important roles in tumor proliferation and migration. ATAD2 could serve in particular as a prognostic marker and a therapeutic target for ovarian cancer.

Keywords: ATAD2 - ovarian cancer - 5-year survival rate - CA-125 - prognosis

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INTRODUCTION

Ovarian cancer, as one of the most common gynecologic malignancies, is the deadliest cancer for the female. In 2005, over 16,000 women were diagnosed with this disease in the United States in 2005 (Jemal et al., 2005). According to a fast stats conducted by World Health Organization (WHO) updated in 2013, the five-year survival rate of the 224,747 cases which were reported worldwide in 2008 is no more than 40% (Kobayashi et al., 2012).

Most patients with ovarian cancer will not realize the disease until it progresses to an advanced stage. When the diseases spread beyond ovaries, accompanying with a grossly enlarging tumor and extensive ascites fluid (Kobayashi et al., 2012). On the other hand, early- and middle-stage ovarian cancer is rarely detected due to the lack of reliable tools and biomarkers (Lutz et al., 2011). Even for the advanced-stage disease, being short of sensitive biomarkers has become a big obstacle for the estimate of intervention therapies and the monitoring of metastatic tumor tissue. Therefore, the identification of reliable biomarkers is of great importance for not only the screening and diagnosis but also for the therapy and prognosis of ovarian cancer. ATAD2 (ATPase family, AAA domain containing 2), also known as ANCCA (AAA+ nuclear coregulator cancer associated), is a recently identified member of the AAA+ ATPase family (Zou et al., 2007). ATAD2 contained two AAA (ATPases Associated with diverse cellular Activities) domains, and was identified as an AR (androgen receptor) coactivator (Zou et al., 2007). ATAD2 contained two AAA (ATPases Associated with diverse cellular Activities) domains, and was identified as an AR (androgen receptor) coactivator (Zou et al., 2007). ATAD2 contained two AAA (ATPases Associated with diverse cellular Activities) domains, and was identified as an AR (androgen receptor) coactivator (Zou et al., 2007). ATAD2 contains two AAA (ATPases Associated with diverse cellular Activities) domains, and was identified as an AR (androgen receptor) coactivator (Zou et al., 2007). ATAD2 is directly regulated by oncoproteins like ACTR, AIB1 and SRC-3, and is highly expressed in prostate cancer cells as a critical factor for prostate cancer cell proliferation and survival (Zou et al., 2007; Zou et al., 2009). Moreover, ATAD2 can regulate downstream genes such as cyclin D1, c-Myc, and E2F1, which contributed directly or indirectly to cell proliferation (Revenko et al., 2010). High levels of ATAD2 correlate with poor survival and disease recurrence in patients with prostate and breast cancers, while RNAi-mediated knockdown of ATAD2 strongly inhibited hormone-dependent cancer cell proliferation (Hsia et al., 2009; Kalashnikova et al., 2010; Revenko et al., 2010). ATAD2 has been reported to be overexpressed, as an important risk factor, in several cancers such as...
prostate, breast and lung cancer (Ciró et al., 2009; Zou et al., 2009; Caron et al., 2010; Hsia et al., 2010). Recently, a few of studies have linked ATAD2 gene to ovarian cancer (Wrzeszczynski et al., 2011; Raeder et al., 2013).

In this research, we aimed to study the diversity of ATAD2 level between ovarian tumor tissue and normal tissue, assess the impact of ATAD2 presence on cell proliferation and migration, and evaluate the relationship between ATAD2 level and the grade of malignance including the survival rate of patients with ovarian cancer.

Materials and Methods

**Splices and tissue specimens**

This study was approved by the ethics committee of the University of China Medical University. A total of 110 cases of ovarian tumor tissues were obtained from the First Affiliated Hospital, China Medical University, 60 of which were obtained from the Department of Gynecology along with corresponding normal ovarian tissues/tumor-adjacent tissues, while the rest were obtained from the Department of Pathology. Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki. All patients were given surgery without preoperative radiotherapy or chemotherapy. Tissue slices were stained by hematoxylin-eosin staining after fixing in formalin and embedding in paraffin. Clinical stage and grade were determined by experienced pathologist, according to the TNM/FIGO classification of malignant tumors which revised by American Joint Committee on Cancer (AJCC) in 2010 (Horn et al., 2010). Omentum metastasis was diagnosed by pathological examination. Clinical characteristics of all patients, including tumor size, histological differentiation grade, stage, cancer antigen 125 (CA-125), and omental metastasis were described in Table 1.

**Antibodies**

Anti-ATAD2 antibodies were purchased from Sigma-Aldrich co. ltd. for immunohistochemical analysis (polyclonal; #H0A029424) and western blot (monoclonal; #AMAB90541). Anti-actin antibody was purchased from Santa Cruz, Inc. (monoclonal; #sc-47778).

**Immunohistochemical**

Immunohistochemical (IHC) analysis was performed on unstained sections of formalin-fixed paraffin-embedded tissue. All sections were deparaffinized in xylene and graded alcohols followed by incubation in 1X sodium citrate buffer (diluted from 10X heat-induced epitope retrieval buffer, Ventana-Bio Tek Solutions, Tucson, AZ) before steaming for 20 minutes at 80°C. For immunohistochemical staining, the sections were blocked by 5% goat serum and then incubated with 1X ATAD2 polyclonal antibody and appropriate secondary antibody. Normal tissues were stained as control. Immunolabeling was detected using a Diaminobenzidine (DAB) Detection Kit (Maixin biotechnology, inc., Fuzhou, China). The percentage of positively stained nuclei was graded as follows: 0%, score 0; 1% to 50%, score 1; 51% to 75%, score 2; ≥76%, score 3, and the immunoreactivity was graded as score 1 (weak) or score 2 (strong) in least five different high-power fields (4×100). The total score of each section was determined as the product of the two indicators. Representative images were shown in figure 1, in which total IHC score was determined as negative (score=0), low expression (scores<3) and high expression: (score> 3).

**Cell Culture and RNA Interference**

Ovarian cancer cell lines OVCAR-3 and HO-8910 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in DMEM/RPMI1640 (Life technology, USA) containing 10% fetal bovine serum (Life technology, USA) and incubated at 37°C in a humidified atmosphere of 5% CO₂.

All siRNAs were synthesized by Dharmacon, Inc. (Lafayette, CO, USA). Two target sequences were used for knockdown of ATAD2: ATAD2siRNA-1: 5'-GGACAUCUCUUCAAUAAUTTAUAUAUGAGAGAUCCTT-3'; ATAD2siRNA-2: 5'-GGUCGGUGGAAGUGUUGUAGGATTUUCUAACAUUCGAGCACCCTT-3'.

The double-stranded siRNA duplex was dissolved in diethyl pyrocarbonate-treated water. For RNA interference, cells were incubated with siRNA in appropriate volum of medium containing DharmaFECT1 (Thermo-fisher, USA). Forty-eight hours after interference, mRNA and protein were harvested.

**Quantitative Real-time Reverse Transcription-PCR**

Total RNA was extracted from 30 randomly selected specimens respectively using TRizol reagent (Takara, Dalian, China). First-strand cDNAs were synthesized using the PrimeScriptTM RT reagent Kit (#RR047Q, Takara, Dalian, China) according to the user’s manual. Quantitative real-time PCR was conducted for ATAD2 using the SYBR® Premix Ex TaqTM II (#RR820A, Takara, Dalian, China) according to the user’s manual. GAPDH was amplified at the same time as a reference gene. The primer sequences for human ATAD2 were 5'-GGAATCCCAAAACCCTGGACA-3' (forward) and 5'-GGTACGCGTCGTCGTAACGACA-3' (reverse). The primer sequences for reference gene GAPDH were 5'-ATAGCACAGCCTGGATAGCAACGTAC-3' (forward) and 5'-CACCTTCTCAAATGAGCTGCGT-3' (reverse). Relative quantification with the comparative threshold cycle (Ct) was done using the Ct method. The amount of ATAD2 expression was normalized to the endogenous reference gene GAPDH.

**Western blot**

Cells or tissues were harvested, washed in PBS and placed in appropriate volume of lysis buffer (Beyotime, Beijing, China) with 1% Ipegal/Protease Inhibitor cocktail (Roche, Basel, Switzerland). The protein lysates (50 µg) were boiled in SDS-PAGE sample buffer for 10 min, then loaded into 12% SDS-PAGE gels and transferred to PVDF membranes (Millipore Corp., Billerica, MA, USA) after electrophoresis. Membranes were incubated with primary antibodies and corresponding HRP-secondary antibodies for appropriate time. Detection was performed
ATAD2 is Highly Expressed in Ovarian Carcinoma and Indicates Poor Prognosis

Figure 1. ATAD2 was Highly Expressed in Ovarian Tumor Tissue (A; 10×), and was rarely detected in tumor-adjacent tissues (pic:C, 10×). B and D (40×) were the magnification of A and C, respectively

with chemiluminescent agents. Signals were visualized and analyzed in the Image Studio software program (UVP, LLC., CA, USA). The protein levels were analyzed by densitometric analysis. GAPDH was detected as a reference.

Colony-forming assay
Cells were seeded in 6-well plates at 1000 cells/well after transfection, 48h later. After 12 days of culture, the media was removed, and washed 3 times with PBS. The plates were stained by Giemsa staining. The stained colonies were examined under a microscope. Colonies that contained no less than 50 cells were included to be counted and analyzed.

MTT assay
Cells were seeded in 96-well plates at 3000 cells/well after transfection. At 24, 48, 72 and 96 hours after seeding, MTT was added in corresponding wells to detect cell growth. Cell viability was evaluated with 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. MTT was added to each well at a final concentration of 0.5 mg/ml, and the cells were incubated for an additional 1 h under the same conditions. The medium was then removed from the cultures, and MTT was solubilized by adding 100 μl of dimethyl sulfoxide to each well. The data was collected with an automatic microplate reader (MD Co., USA) at a test wavelength of 450 nm.

Transwell migration assay
Transwell units with 8.0-μm pore-size polycarbonate filters (Corning Costar, USA) were used to investigate cell migration. Forty-eight hours after transfection with or without siRNA, cells were harvested and diluted. 100μL serum-removed media containing 300, 000 cells were added into the upper compartment of the transwell unit. The units were transferred to wells containing 600 µL RPMI 1640 medium with 10% FBS as a chemoattractant.

Figure 2. 6 Pairs of Specimens were Randomly Selected to Evaluate the ATAD2 Expression. T and N represent tumor tissue and normal/tumor-adjacent tissue of one patient, respectively. GAPDH was evaluated as reference. ATAD2 was highly expressed in ovarian tumor tissue, though it was also detected in normal tissue

and further incubation was conducted for 16 hours. After removing the cells which remained on the upper surface of the membrane with a cotton bud, cells migrated were fixed in 4% paraformaldehyde and stained with hematoxylin. Cells on the underside were counted under a light microscopy in ten randomly selected fields per insert. Three replicates were tested in each group.

Statistical Analysis
Significant difference post hoc test (SPSS 17.0 for Windows) was used in Statistical Analysis to test for the expression of ATAD2 in carcinoma. T-test analysis was used to test for Optical density digital analysis and difference in cell growth. Kaplan-Meier was used to test for survival probability. Log-rank test was used in analysis. Cox regression model was used to study the effects of different variables on survival. All statistical tests were two-tailed, and difference was considered statistically significant when \( p < 0.05 \).

Results
ATAD2 is highly expressed in ovarian tumor tissue
Immunoreactivity for ATAD2 was readily detected in the nuclei and plasma of ovarian tumor tissues, whereas little or no staining was observed in the normal ovarian tissues or tumor-adjacent stroma. Representative photomicrographs of ATAD2 in ovarian carcinoma are shown in Figure 1. All specimen were evaluated, ATAD2 was shown to be highly expressed in 65.5% (72/110) of ovarian tumor tissues. Same conclusion was demonstrated by western blotting. Proteins were harvested from frozen-fresh tissues and subjected to Western blot analysis of ATAD2 expression. ATAD2 expression in carcinoma tissue is significant higher than normal ovarian tissues (Figure 2).

Quantitative Real-time PCR was conducted to determine ATAD2 expression at transcriptional level. The results suggested ATAD2 mRNA was also highly expressed in 63.3% (19/30) of those ovarian cancer tissues (Figure 3). ATAD2 mRNA in carcinoma tissue (4.67±0.09)
ATAD2 is related to the malignant degree of ovarian and median survival rate of patients

The expression of ATAD2 is associated with tumor stage, Omentum-metastasis, ascites and CA-125 (Table 1, *p* < 0.05). No statistical evidence suggests the relationship among ATAD2 expression and other pathologic characteristics (Table 1, *p* > 0.05).

110 patients (38 in Group A VS 72 in Group B, respectively), ranged from 28 to 83 (50 average) years old, were follow-up visited and included in survival rate analysis. Selected important patients and tumor characteristics are described in Table 1. Patients in Group A (blue line) were all have low level of ATAD2, while Group B (green line) have high expression of ATAD2. All patients were received cytoreductive surgery (CDS) before the follow-up visit, then 6-8 courses of chemical therapy was conducted within the duration of our follow-up visit. The recorded survival time represents the period from the confirmation of diagnosis until death or until the last follow-up visit. The overall median survival period was 13 months, with 38% mortalities. The 5-year survival rate of group A and group B are 28.9%, 8.0%, respectively. Overall survival curve suggested ATAD2 may be a critical influence factor of prognosis (Figure 4). Low expression of ATAD2 may link to a longer survival time.

The results of cox regression model multi-factor analysis showed that ATAD2 expression and CA-125 (Table 1, *p* < 0.05) were the key factors of survival rate. Other histopathologic characteristics listed in Table 2 had no significant relationship with survival rate. The result of hazard ratio (HR) also revealed that ATAD2 expression, Omentum metastasis and CA-125 are risk factors with overall survival rate.

ATAD2 was highly expressed in HO-8910 and OVCAR-3 cells, knocking-down ATAD2 influent cell

Table 2. Univariate and Multivariate Analyses of Individual Parameters for Correlations with Overall Survival Rate: Cox Proportional Hazards Model

<table>
<thead>
<tr>
<th>Variables</th>
<th>HR</th>
<th>CI(95%)</th>
<th>P value</th>
<th>HR</th>
<th>CI(95%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATAD2</td>
<td>2.238</td>
<td>1.413-3.545</td>
<td>0.001*</td>
<td>1.838</td>
<td>1.152-2.934</td>
<td>0.011*</td>
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<tr>
<td>Age</td>
<td>1.066</td>
<td>0.621-1.830</td>
<td>0.815</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual tumor size</td>
<td>1.188</td>
<td>0.790-1.787</td>
<td>0.408</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ascite</td>
<td>1.359</td>
<td>0.902-2.047</td>
<td>0.143</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td>0.736</td>
<td>0.504-1.074</td>
<td>0.112</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Omentum metastasis</td>
<td>1.666</td>
<td>1.094-2.538</td>
<td>0.017*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathology</td>
<td>0.817</td>
<td>0.462-1.445</td>
<td>0.487</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA125</td>
<td>3.483</td>
<td>1.670-7.263</td>
<td>0.001*</td>
<td>2.819</td>
<td>1.331-5.972</td>
<td>0.007*</td>
</tr>
<tr>
<td>Tumor stage</td>
<td>1.432</td>
<td>0.915-2.240</td>
<td>0.116</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HR, Hazard Ratio; CI, Confidence Interval; *Statistically significant (*p* < 0.05)
proliferation and migration.

After 96 hours after RNA interference, the OD 450 of ATAD2-knockdown HO-8910 and OVCAR-3 cells was 1.236±0.026, 1.005±0.037, respectively, while the average OD450 of control HO-8910 and OVCAR-3 cells was 1.528±0.022, 1.537±0.029, respectively. The results revealed that in HO-8910 and OVCAR-3 cells, knocking-down of ATAD2 can significantly inhibit cell growth (Figure 5).

Transwell assay was used to study the association of ATAD2 with cell migration. In ATAD2-knockdown HO-8910 and OVCAR-3 cells, the average counted cells per field were 37±2.2, 55±3.1 respectively, while in HO-8910 and OVCAR-3 cells transfected with negative siRNA (NC), the average counted cells per field were 87±3.3, 109±4.6, respectively. The results suggested that block of ATAD2 expression can significantly inhibit HO-8910 and OVCAR-3 cells migration (Figure 6 and 7).

Discussion

In this study, we found ATAD2 was overexpressed in ovarian cancer, which may even correlate with the survival time. In ovarian tumor HO-8910 and OVCAR-3 cells, ATAD2-knockdown suppresses ATAD2 expression, which arrest cells in G1, and inhibit cell proliferation and migration. The overexpression of ATAD2 was associated with tumor stage, omentum-metastasis, ascites and CA-125. These results were similar with the previous studies on prostate and breast cancers (Zou et al., 2007; Zou et al., 2009).

We have known from previous studies that E2-stimulated breast cancer cell proliferation involves ERα-mediated regulation of several key cell cycle regulatory genes (Coser et al., 2003; Ngwenya et al., 2003; O’Lone et al., 2004; Eeckhoute et al., 2006). Aberrant DNA methylation of tumor suppressor genes has been reported in many major types of cancer with potential involvement in the inactivation of regulatory cell cycle and apoptosis genes. Study by Bodoor et al. (2014) highlighted an essential role of DAPK methylation in chronic leukemia, suggesting possible synergistic epigenetic disruption of different phases of the cell cycle or between the cell cycle and apoptosis. Nuclear Factor Kappa B (NF-κB) inhibits apoptosis through induction of antiapoptotic proteins and suppression of proapoptotic genes. Various chemotherapy agents induce NF-κB translocation and target gene activation (Prajoko & Aryandono, 2014). E2 acts by preventing NF-κB nuclear translocation induced by LPS or tumor necrosis factor alpha (TNF-α), without altering IKK activity. And this mechanism is specifically mediated by ERα through a nongenomic mechanism, thus indicating that the E2-ERα signaling pathway plays a central role in the immediate-early inflammatory response (Ghisletti et al., 2005). Zou et al. found ATAD2 directly interacts with ERα and ACTR, and plays an important role in the recruitment or assembly of ERα-CBP complex at the
chromatin and enhance the histone modifications mediated by the complex. Furthermore, ATAD2 plays roles for cancer cell proliferation through ERα target genes such as cyclin D1, c-Myc, and E2F1 (Hsia et al., 2009; Revenko et al., 2010). The ATPase domain of ATAD2 also enhances the E2 induction of cyclin D1 and E2F1 expression. ATAD2 act as a coactivator of ERα, thus knocking-down ATAD2 inhibits hormone-dependent cancer cell proliferation. In addition, ATAD2 was also directly associated with AR, coactivated AR-mediated transcription and was required for the expression of androgen-induced genes that control cancer cell proliferation and survival.

As hormone-sensitive cancer, a large subset of ovarian cancers is associated with the deregulation of ERα or AR (Rao et al., 1991; Chan et al., 2008). Our study demonstrated that ATAD2 is overexpressed in ovarian cancers. This result in accord with the recent studies which find ATAD2 overexpressed in ovarian cancer recently (Wrzeszczynski et al., 2011; Raeder et al., 2013). Moreover, our study indicated that high level of ATAD2 directly correlate with poor survival and disease recurrence in the patients with ovarian cancer, which is in line with the previous study of breast cancer (van ’t Veer et al., 2002; Kalashnikova et al., 2010).

On the other hand, Kalashnikova found ATAD2 overexpression correlated strongly with the triple-negative subtype of breast cancer. High levels of ATAD2, which directly regulated B-Myb and EZH2, were required to maintain proliferation and survival of TNBC cells. EZH2 was known to promote cell proliferation. High level of EZH2 stimulated tumor metastasis (Kalashnikova et al., 2010). Silencing of ATAD2 markedly inhibited EZH2 and B-Myb expression at both RNA and protein levels in TNBC cells. ATAD2 deregulated may contribute to EZH2 overexpression in the tumors. In addition, the expression of survival pathway proteins such as IRS2, SGK1, vascular endothelial growth factor α (VEGFα), Akt, and phosphorylated Akt was also significantly affected by ATAD2 knockdown (Kalashnikova et al., 2010). These results suggest that ATAD2 is also involved in cell migration and proliferation in ERα-negative cells, which means ATAD2 may be involved in tumorigenesis, cell growth and migration dependently or independently of ERα. Thus, ATAD2 overexpression may correlate with ovarian cancer whether it is hormone-related or not.

Meanwhile, ATAD2 has the potential to be a sensitive and common marker for both ER-positive and ER-negative ovarian cancer. Currently, cancer antigen 125 (CA-125) is the most common biomarker for ovarian screening. However as an individual marker on a single occasion, CA-125 is not sufficiently sensitive and specify to detect most cases of ovarian cancer (Gadducci et al., 2004; Nossov et al., 2008). With appropriate statistical models, combinations of several markers such as leptin, prolactin, osteopon-tin, insulin-like growth factor II (IGF-II), macrophage inhibitory factor (MIF) and CA-125 have become more effective and accurate means for the detection of ovarian tumorigenesis (Visinint et al., 2008; Li et al., 2009; Sorensen et al., 2011), while no reliable method can be used to distinguish tumor stages. ATAD2 is one of the 76 genes identified for prediction of distant metastasis of ER-positive, lymph node-negative, primary breast cancer, which was later validated in a multicenter study (Wang et al., 2005; Foekens et al., 2006; Desmedt et al., 2007; Haibe-Kains et al., 2008). In addition, ATAD2 protein levels alone was also reported to predict tumor grades, a high level of ATAD2 transcription is a constituent of the genetic grading signature that can reclassify histological grades of breast cancer (Ma et al., 2003; Ivshina et al., 2006; Teschendorf et al., 2006). These reports indicated the potential of ATAD2 to be a biomarker in screening, monitoring and reclassifying histological grades of ovarian cancer. In this study, we found ATAD2 is related to the malignant degree of ovarian and median survival rate of patients. The level of ATAD2-CA-125 would be a potential indicator to classify the tumor stage in ovarian cancer.

In summary, ATAD2 was overexpressed in not only prostatic, breast and lung cancer but also in ovarian cancer. The level of ATAD2 was associated with the malignant degree of cancer. Knocking-down of ATAD2 inhibited cell proliferation and migration in vitro. Our results suggested the potential of ATAD2 to be a sensitive therapeutic target as well as a reliable biomarker for the therapy and detection of ovarian cancer.

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References


