ATR and p-ATR are Emerging Prognostic Biomarkers and DNA Damage Response Targets in Ovarian Cancer

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Abstract
Background: Although ATR has an established role in DNA damage response in various cancers, its clinical and prognostic significance in ovarian cancer remains largely unknown. The aims of this study are to assess the expression, function and clinical prognostic relationship of ATR, p-ATR in ovarian cancer.

Methods: We confirmed ATR and p-ATR expressions by immunohistochemistry in a unique ovarian cancer tissue microarray constructed of paired primary, recurrent and metastatic tumor tissues from 26 individual patients. ATR specific siRNA and ATR inhibitor VE-822 were applied to determine the effect of ATR inhibition on ovarian cancer cell proliferation, apoptosis, and DNA damage. ATR expression and the associated proteins of the ATR/Chk1 pathway in ovarian cancer cell lines were evaluated by Western blotting. The clonogenicity was also examined using clonogenic assays. A 3D cell culture model was performed to mimic the in vivo ovarian cancer environment to further validate the effect of ATR inhibition on ovarian cancer cells.

Results: We show recurrent ovarian cancer tissues express higher levels of ATR and p-ATR than their patient-matched primary tumor counterparts. Additionally, higher expression of p-ATR correlates with decreased survival in ovarian cancer patients. Treatment of ovarian cancer cells with ATR specific siRNA or ATR inhibitor VE-822 led to significant apoptosis and inhibition of cellular proliferation, with reduced phosphorylation of Chk1 (p-Chk1), Cdc25c (p-Cdc25c), Cdc2 (p-Cdc2), and increased expression of cleaved PARP and γH2AX. Inhibition of ATR also suppressed clonogenicity and spheroid growth of ovarian cancer cells.

Conclusion: Our results support the ATR and p-ATR pathway as a prognostic biomarker, and targeting the ATR machinery is an emerging therapeutic approach in the treatment of ovarian cancer.

Introduction
Ovarian cancer accounts for 2.5% of all malignancies in females and is the leading cause of gynecologic cancer related death[1, 2]. The 5-year survival rate for ovarian cancer patients is grim, especially given the majority of patients present to clinic with advanced stage disease. Late-stage III or IV patients have a 5-year relative survival rate of 29% whereas patients presenting with early-stage
disease have a 70% survival rate[2]. Currently, the standard treatment protocol for ovarian cancer consists of tumor debulking surgery followed by platinum–taxane chemotherapy, and (rarely) radiotherapy[3]. Although a small proportion of patients may attain complete response, approximately 25% of these patients will develop platinum-resistant cancer recurrence within six months[4]. With respect to tumor biomarkers, several have been reported in ovarian cancer, including the famous carbohydrate antigen 125 (CA125)[3]. However, CA125 has low sensitivity in the early stages of ovarian cancer and is therefore not a useful screening tool[5] and increased CA125 levels are found in a wide range of other conditions such as menstruation, pregnancy, and endometriosis[6]. At present, there are no reliable prognostic biomarkers in ovarian cancer and current therapeutic options are quite limited, especially after tumor recurrence. There is, therefore, an urgent need for biomarkers and potent and novel therapeutic targets to advance ovarian cancer treatment.

Genomic instability is a hallmark of cancer[7]. In principle, oncogene activation promotes replication stress and abundant DNA damage, overcoming physiologic anti-cancer defenses[8]. Interestingly, cancer treatments such as radio- and chemotherapies rely on a similar mechanism of DNA damage, whereby highly proliferative cancer cells undergo an excessive amount of DNA damage causing toxicity to cancer cells. These cells can, however, resist lethal effects by activating DNA damage response pathways[9-11] which repair and transiently arrest the cell cycle to ensure genomic stability and survival[12, 13]. Regulators of DNA damage response have therefore emerged as attractive targets in cancer therapy.

Ataxia-telangiectasia and Rad3 related (ATR) is a serine/threonine kinase and a member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family, particularly the ataxia telangiectasia mutated (ATM) subfamily. In response to replication stress and DNA damage, phosphorylated ATR acts via its downstream targets including the checkpoint kinase 1 (Chk1) to promote DNA damage repair and stabilization, as well as to restart stalled replication forks and transient cell cycle arrest[14]. Mechanistically, post-translational modifications of ATR contribute to ATR regulation and autophosphorylation and potentiate its action[15]. The phosphorylation site of ATR is located at Ser428 and is crucial for proper ATR function[15]. In a series of breast cancer studies, high ATR
expression and activation were significantly associated with higher tumor stage, mitotic index, pleomorphism, lymphovascular invasion and poor survivorship[16-18]. In turn, additional works have demonstrated loss of ATR function increases cancer cell sensitivity to oncogene-induced replication stress while decreasing tumor growth and inducing apoptosis and overall cell death[19-21]. A review of the literature shows inhibition of ATR significantly enhances platinum drug response in endometrial, cervical and ovarian cancer cell lines, whereas inhibition of ATM does not enhance the response to platinum drugs[22]. Of note, ATR inhibition sensitizes ovarian cancer cells to chemotherapy irrespective of BRCA status[23]. These promising preclinical results and others have led to a number of clinical trials utilizing ATR-selective small-molecule inhibitors such as AZD6738, BAY1895344, and VE-822 (VX-970, M6620), which are currently within phase I/II clinical trial stages in solid tumors and leukemia[24]. Surprisingly, however, the expression of ATR, clinical and prognostic significance, biological functions, and the efficacy of its therapeutic targeting in ovarian cancer is unclear. Few studies have investigated ATR and p-ATR expression in ovarian cancer patients with long-term follow up and no ATR studies have used paired primary, recurrent and metastatic tumor tissues from each individual ovarian cancer patient. We therefore examined ATR and phospho-ATR ser428(p-ATR) expression in ovarian cancer patient specimens and correlated their expression to clinical prognosis. We also expand upon the function of ATR in ovarian cancer cell proliferation, colonization, tumor spheroid growth, as well as the stepwise ATR signaling pathways.

Materials And Methods

Ovarian cancer TMA construction and Immunohistochemistry (IHC)

Seventy-eight formalin-fixed paraffin-embedded tumor specimens were obtained from 26 ovarian cancer patients, comprising primary, synchronous metastasis, and metachronous recurrence from the same patient collected at the time of tumor recurrence after combination paclitaxel and platinum therapy. Tissue microarray (TMA) construction and IHC staining were conducted as previously described[25-27]. Twenty-one patients were grade 3, four patients were grade 2 and one patient was grade 1 at time of diagnosis. All the patients were disease stage III to IV with various pathological types, including serous, clear cell, transitional cell, endometroid, and undifferentiated cell. The time
range of disease-free survival (DFS) was between 5.3 months and 53.3 months; the shortest overall survival (OS) of a patient was 12 months, and the longest follow-up of a living patient was 162.3 months (Supplementary Table 1).

**Evaluation of immunohistochemistry staining in TMA**

Assessment of immunohistochemical staining was performed separately by two independent investigators blinded to clinical information. For total ATR, the staining intensity pattern was scored as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; and 3+, intense staining. p-ATR mainly resided in the nucleus and was scored according to the percentage of cancer cells with positive nuclear staining, the staining patterns were categorized into six groups: 0, no nuclear staining; 1+, <10% of cells stained positive; 2+, 10% to 25% positive cells; 3+, 26% to 50% positive cells; 4+, 51% to 75% positive cells; 5+, >75% positive cells. ATR and p-ATR staining images were obtained using a Nikon Eclipse Ti-U fluorescence microscope (Diagnostic Instruments Inc., NY, USA) with a SPOT RTTM digital camera (Diagnostic Instruments Inc.).

**Cell lines and cell culture**

Human ovarian cancer cell lines Skov3 (ATCC® HTB-77™), and Caov-3 (ATCC® HTB-75™) were purchased from the American Type Culture Collection (Rockville, MD). A2780 (ECACC 93112519) was obtained from the European Collection of Authenticated Cell Cultures. Dr. Patricia Donahoe (Massachusetts General Hospital, Boston, MA) provided the human IGROV-1, OVCAR5, and OVCAR8 ovarian cancer cell lines. These cell lines were maintained in RPMI 1640 (GE Healthcare Life Sciences, Logan, Utah, USA) medium supplemented with 10% FBS (MilliporeSigma, Burlington, MA, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) in a humidified incubator containing 5% CO₂ at 37°C. Cells were resuspended with 0.05% trypsin-EDTA (Life Technologies Corporation, NY, USA) before subculture.

**Protein extraction and Western blotting**

Cell lysates were prepared with 1× RIPA lysis buffer (EMD Millipore Corporation, CA, USA) and protease inhibitor cocktail tablets (Roche Applied Science, IN, USA). Lysates were centrifuged and collected as supernatants, then total protein concentration was determined by Bio-Rad DC Protein
Assay reagents (Bio-Rad, Hercules, CA, USA) following the manufacturer’s instructions. Antibodies directed against ATR, Chk1, p-Chk1(Ser345), p-Cdc25c (Ser216), p-Cdc2(Tyr15), PARP, γH2AX were purchased from Cell Signaling Technologies (Sampler Kit #9947, Cambridge, MA). Other antibodies included p-ATR (Ser428) (Catalog #ab178407, Abcam, San Francisco, CA) and a monoclonal antibody to human actin from Sigma-Aldrich (Catalog #A2228, St. Louis, MO). Equal amounts of each protein sample were separated in NuPAGE 4–12% Bis-Tris Gel (Thermo Fisher Scientific), blotted onto nitrocellulose membranes (Bio-Rad), blocked with 5% non-fat dry milk, rinsed, and incubated overnight with the corresponding specific primary antibodies at 4°C. The next day, the membranes were rinsed and incubated with the secondary antibodies: goat anti-rabbit IRDye 800CW and goat anti-mouse IRDye 680LT (1: 10,000 dilution Li-Cor Biosciences, Lincoln, NE, USA) for 1 hour at room temperature with gentle agitation. After washed with 1×PBS, the protein band was detected by Odyssey CLx equipment. Odyssey v.3.0 software (Li-Cor Biosciences) was used to quantify protein bands by optical density measurement.

**Immunofluorescence**

The ovarian cancer cell lines were seeded into 24-well plates at a concentration of 2 × 10⁴ cells/ml for 72 hours and fixed in 4% paraformaldehyde for 15 minutes at room temperature. Following fixation, the cells were washed in 1× PBS (3 times, 5 minutes each), prior to permeabilization with 100% ice-cold methanol in a -20°C refrigerator for 10 minutes. After blocking with 5% goat serum for 1 hour, the cells were then incubated with the primary antibody ATR (1:200, Cell Signaling Technology), p-ATR (1:200, Abcam) and β-Actin (1:1000, Sigma-Aldrich) overnight at 4°C in a humidified chamber. The next day, we removed the primary antibody solution and rinsed before incubation with fluorochrome-conjugated secondary antibody for 1 hour at room temperature in the dark. The secondary antibodies Alexa Fluor 488 (Green) conjugated goat anti-rabbit antibody and Alexa Fluor 594 (Red) conjugated goat anti-mouse antibody were purchased from Invitrogen (NY, USA) and diluted in 5% goat serum at 1:1000. Finally, they were washed and incubated with a DAPI solution (1:10 000) for 5 minutes. Pictures were obtained with a Nikon Eclipse Ti-U fluorescence microscope (Diagnostic Instruments Inc., NY, USA) equipped with a SPOT RTTM digital camera.
siRNAs and in vitro siRNA transfection

We used synthetic ATR siRNA to silence ATR expression in ovarian cancer cells. The ATR siRNA (target sequence: 5'-GAUCCUACAUCAUGGUACA-3'; antisense: 5'-UGUACCAUGUGUAGGAUC-3') was purchased from MilliporeSigma and the non-specific negative control siRNA (Catalog #: AM4637) was purchased from Applied Biosystems. The siRNAs were mixed with antibiotic-free Opti-MEM medium (Life Technologies) and Lipofectamine RNAiMax (Thermo Fisher Scientific). The transfection mix was incubated for 30 minutes at room temperature and then added to the cells at a concentration of 10, 30, and 80 nM. The ovarian cancer cell lines Skov3 and OVCAR8 were prepared at a concentration of 2×10^4 cells/ml for siRNA and methyl thiazolyl tetrazolium (MTT) assay in 96-well plates and 5×10^4 cells/ml for protein extraction in 12-well plates. Non-specific siRNA (80 nM) was used as a negative control. Transfection of siRNA and the MTT assay were performed as previously described[25].

Inhibition of ATR by inhibitor VE-822

The role of ATR in ovarian cancer cell growth and proliferation was further accessed by ATR inhibitor VE-822 (Selleck Chemicals, Houston, TX, USA). The development of specific and potent ATR inhibitors has been historically challenging due to the large size of the ATR protein (310 Kda). The application of a recombinant ATR protein for in vitro kinase assay has revealed several compounds which target ATR without affecting the ATM or DNA dependent protein kinase catalytic subunit (DNA-PKcs). One of the most significant compounds discovered was VE-821, which has since been pharmacologically modified and enhanced to VE-822 and featured in clinical trials as VX-970 (also as known as M6620). VE-822 attenuates the ATR signaling pathway and reduces tumor cell survival via blockade of pChk1 Ser345[28]. In our work, we cultured the ovarian cancer cell lines Skov3 and OVCAR8 (2×10^4 cells/ml) in 96-well plates with VE-822 at increasing concentrations over 5 days in MTT cell proliferation assays. 5×10^4 cells/ml were also grown in 12-well plates with VE-822 at concentrations of 0.05, 0.1, 0.5, 1.0 μM/ml and their protein content was subsequently extracted for Western blot analysis as previously described[25].

Clonogenic assay
The clonogenic assay is a well-established in vitro method for evaluating cell viability and proliferation. The ovarian cancer cell lines Skov3 and OVACAR8 were seeded into 12-well plates at 100 cells per well and treated with increasing VE-822 concentrations (0, 0.1, 0.5 μM) then incubated at 37 °C for 15 days. The suspension was aspirated and the colonies were fixed with methanol for 10 minutes then washed three times with 1×PBS before being stained with 10% Giemsa stain (Sigma-Aldrich) for 20 minutes. Finally, the cell colonies were gently washed with flowing water and dried. Pictures were obtained using a digital camera (Olympus, Tokyo, Japan).

Three-dimensional (3D) cell culture

The 3D cell culture system mimics the in vivo environment and serves as a unique platform to evaluate how ATR is related to in vivo ovarian cancer cell growth. Consistent with the manufacturer’s protocol, the ovarian cancer cell lines Skov3 and OVCAR8 were mixed with 3D VitroGelTM (TheWell Bioscience Inc., NJ, USA) then established in 24-well plates at a density of 1×10⁴ cells/ml. Each well was covered with the same volume of cell culture medium. The experimental group received an additional treatment of VE-822 0.1 μM/ml. The plates were then placed in a 37 °C incubator with a humidified 5% CO₂ atmosphere; the covering medium was changed every 48 hours. Images of the cell spheroids were obtained with a Nikon microscope every three days. After 15 days, calcein-AM (Thermo Fisher Science) was applied to stain the tumor spheroids, and images were obtained with an Eclipse Ti-U fluorescence microscope (Nikon) equipped with a Spot RT digital camera.

Statistical analysis

GraphPad Prism v. 8.0 software and SPSS 24.0 software were used for statistical analysis. Multiple comparisons were performed with one-way ANOVA tests. Analysis of the difference in survival was analyzed with Kaplan-Meier plots and log-rank tests. The relationship between p-ATR expression and clinicopathological parameters in ovarian cancer patients was evaluated by the x² test. The prognostic factors related to overall survival were analyzed with a Cox proportional hazard regression model. Only those factors that had statistical significance with univariate survival analysis (p < 0.05) were employed in Multivariate analysis. The effects of ATR siRNA and inhibitor were evaluated by one-
way ANOVA. In all cases, the results are presented as mean ± SD, and p <0.05 was considered statistically significant.

Results

Analysis of ATR and p-ATR expression in ovarian cancer patient specimens by TMA

We first performed immunohistochemistry on an ovarian cancer TMA to determine ATR and p-ATR expression. Our TMA included primary tumors, synchronous metastasis, and tumors collected at the time of recurrence following a platinum- and taxane-based regimen as previously described[25-27]. The expression pattern varied for ATR and p-ATR, as ATR was mainly located within the cytoplasm and p-ATR resided within cell nuclei (Fig.1). We scored all tumors in the TMA from 0~3+ for total ATR and 0~5+ for p-ATR staining in the nucleus (Fig.1, Supplementary Table S1). There were clear trends towards higher ATR (p=0.007) and p-ATR (p=0.01) expression in the recurrent tumors compared to the matched primary tumors (Fig.2 A&D). In contrast, there was no significant difference between metastatic tumors and their matched primary tumors, with p values of 0.326 for ATR and 0.972 for p-ATR (Fig.2 A&D). These results indicate ATR and p-ATR have roles in ovarian cancer cell survival after first-period treatment and thus likely promote recurrent phenotypes.

To evaluate the association between ATR and p-ATR expression levels with ovarian cancer patient prognosis and clinical characteristics, we defined a staining score of ≤ 2+ as low ATR expression and 3+ as high expression; however, Kaplan-Meier analysis showed no significant difference between low and high expression groups in OS or PFS (Fig.2 B&C). p-ATR is the active form of ATR protein, and its expression in the 26 patient primary tissues were as follows: non-staining 0 (1 of 26, 3.8%); 1+ staining (0); 2+ staining (6 of 26, 23.1%); 3+ staining (5 of 26, 19.2%); 4+ staining (10 of 26, 38.4%); and 5+ staining (4 of 26, 15.4%). The median survival times for patients with scores of 0,2,3,4,5 was 100.7, 63.5, 56.7, 20.7, and 14.2 months, respectively (p=0.005, based on log-rank test) (Table 1). We further defined a staining score of ≤3+ as low p-ATR expression and ≥4+ as high expression. Accordingly, 46.2% (12/ 26) of patients had low p-ATR expression and 53.8% (14/26) of patients had high expression. While the 5-year survival rate for patients with low p-ATR expression was 41.7%, zero patients with high p-ATR expression survived at the 5-year mark. The median survival time for
patients with low p-ATR expression was 63.5 months, whereas those with high p-ATR expression had a median of 18.8 months (Table 1). Kaplan-Meier analysis revealed patients with high p-ATR expression have significantly worse overall survival (OS) (p=0.0002) and progression-free survival (PFS) (p=0.008) by log-rank test (Fig.2 E&F). Taken together, our results show high expression of p-ATR is associated with grim outcomes for ovarian cancer patients, and is consistent with other works in cancer such as esophageal malignancy[29].

We next analyzed the possible correlation between p-ATR levels to ovarian cancer patient clinical characteristics and prognosis. There was no significant difference between p-ATR expression and tumor stage, grade, histologic subtype, or ascitic fluid at surgery (Table 2). In a univariate Cox regression analysis, we found advanced cancer stage, presence of ascites at surgery and high p-ATR expression were associated with decreased ovarian cancer patient survival (Table 3). Notably, the multivariate Cox regression analysis showed p-ATR expression, like stage and ascites, is an independent predictor of survival in ovarian cancer patients (p = 0.001, Cox proportional risk regression model) (Table 3). Collectively, these results support p-ATR expression as an independent predictor of ovarian cancer patient outcomes.

**ATR/Chk1 pathway associated protein expression in ovarian cancer cell lines**

To determine the role of the ATR signaling pathway in human ovarian cancer cells, we performed Western blot to quantify the expression of ATR, p-ATR, Chk1, and p-Chk1 as these proteins are accepted surrogate markers for ATR pathway activation[24]. Our results confirmed that ATR, p-ATR, Chk1, and p-Chk1 are expressed in all tested ovarian cancer cell lines including A2780, OVCAR5, IGROV-1, Skov3, OVCAR8, and Caov-3(Fig.3). p-ATR and p-Chk1 were endogenously activated in the ovarian cancer cell lines. Our results show the ATR signaling pathway activation is responsive to replication stress and elicits sustained genomic stability in ovarian cancer.

**ATR knockdown by siRNA decreases ovarian cancer cell proliferation**

To further evaluate the role of ATR in ovarian cancer cell proliferation, we used ATR siRNA to knockdown ATR expression in Skov3 and OVCAR8 cell lines. As shown in Fig.S1 by immunofluorescence, ATR was located in both the cytoplasm and nucleus in Skov3 and OVCAR8,
whereas p-ATR was mainly located in the nucleus. These results were consistent with the TMA findings (Fig.1), and support p-ATR as an activated form of ATR involved in DNA damage repair within the nucleus. The downregulation of ATR and p-ATR, as well as the decrease of cell proliferation, were observed after ATR-siRNA transfection compared to the untreated control and non-specific siRNA groups (Fig.S1). Similarly, five days post ATR siRNA transfection, the MTT assay showed a sharp reduction of cell viability in both cell lines with increasing ATR siRNA concentrations. No significant changes were observed in the untreated control group or in those cells transfected with nonspecific siRNA (Fig.4A). We also observed morphologic changes and diminished cell proliferation after siRNA transfection during this period (Fig.4B).

The DNA damage response is a multi-component network of signaling pathways regulating DNA damage repair, cell cycle checkpoints, and apoptosis. To further investigate these signaling pathways after ATR knockdown in ovarian cancer, we measured downstream ATR/Chk1 pathway proteins via Western blot (Fig.4C). Knockdown of ATR decreased levels of p-ATR, p-Chk1, p-Cdc25c, and p-Cdc2, strongly indicative of a G2-M cell cycle arrest. The apoptotic signifier cleaved PARP as well as γH2AX, which indicate DNA damage and replication fork stress, were both elevated with increasing concentrations of ATR siRNA. Taken together, these results show knockdown of ATR causes an accumulation of ovarian cancer DNA damage, reduces cell viability and proliferation, and induces apoptosis and cell death.

**ATR inhibitor suppresses ovarian cancer cell viability and proliferation**

VE-822 is an ATR-selective inhibitor that attenuates the ATR signaling pathway and reduces survival in cancer cells[24]. Importantly, it is well tolerated in mice and does not enhance toxicity in normal cells and tissues[30]. Owing to its excellent solubility and pharmacokinetic profile, VE-822 became the first selective ATR inhibitor to enter clinical development. To evaluate its effect in ovarian cancer cells, we treated the ovarian cancer cell lines Skov3 and OVCAR8 with VE-822 over five days and subsequently observed a dose-dependent reduction in cell viability, with IC50 values of VE-822 at 0.077 μM in Skov3 and 0.056 μM in OVCAR8 (Fig.5A). Over a 72-hour culture period with increasing VE-822 doses, we observed morphological changes and decreased cell proliferation in both cell lines.
Assessment of the ATR signaling proteins by Western blot after VE-822 treatment showed p-ATR/p-Chk1/p-Cdc25c/p-Cdc2 were concomitantly decreased (Fig. 5C). Similar to our findings with ATR-siRNA treatment, increased levels of cleaved PARP and γH2AX were also observed. These results indicate VE-822 suppresses ATR signaling via a blockade of protein phosphorylation, thus inducing ovarian cancer cell apoptosis and an accumulation of toxic DNA damage.

Inhibition of ATR reduces ovarian cancer clonogenicity and spheroid growth

The clonogenic assay is an in vitro cell survival assay which measures a single cell’s ability to rapidly grow into a colony of progeny, or "infinite" division. Clinically, this test is often used to determine the efficacy of cytotoxic agents[31]. We performed clonogenic survival assays to determine the effect of VE-822 on the colony-forming ability of ovarian cancer cells. After a 15-day treatment period, Skov3 and OVCAR8 clonogenicity was reduced in a dose-dependent manner, whereas the untreated control cells did not experience this significant change (Fig. 6A).

In two-dimensional (2D) culture systems, flat surfaces cannot adequately mimic the in vivo conditions by which cancer cells attach, spread, and grow[32]. Given this limitation, we applied the 3D culture system, in which cancer cells can naturally form 3D spheroids with the customizability of in vitro experimentation. As shown in Fig. 6B&C, during a 15-day observation period, although the spheroids of Skov3 and OVCAR8 continuously grew, the ATR inhibitor-treated spheroids were significantly smaller than the untreated control group. Collectively, our results further support ATR to have a crucial role in ovarian cancer growth and progression.

Discussion

The expression of ATR in matched ovarian cancer tissues has not been previously reported, and moreover, the clinical significance of ATR expression in ovarian cancer remains largely unknown. In our study, we show that ATR and p-ATR have higher immunohistochemistry TMA staining intensity in recurrent ovarian cancer tumors compared to matched primary tumors. Consistent with its role in the DNA damage response, we found p-ATR to overwhelmingly reside within the nucleus. As predicted, patients with higher p-ATR levels had significantly shorter median survival times and 5-year survival rates. When we conducted additional analysis, p-ATR was an independent predictive biomarker of
poor prognosis in ovarian cancer patients. These results are in line with previous works in breast cancer, endometrial cancer, and esophageal carcinoma [17, 29, 33].

Previous studies have shown ATR activity is required to ensure proper DNA replication and genomic stability in proliferating cells[14]. This response, when unregulated, is instrumental in cancer cell survival and progression. A downstream protein essential in this process is Chk1, a kinase which is activated via phosphorylation by upstream ATR[34, 35]. Homozygous knockout of ATR or Chk1 is lethal in early embryonic life, highlighting the crucial role of these protein kinases[36, 37]. ATR kinase dead cells, characterized by an inactive form of ATR which functions as a dominant negative inhibitor of native ATR function, promotes DNA hypersensitivity without G2–M cell cycle arrest[38]. When combined, ATR and Chk1 inhibit origin firing, stabilize replication forks, facilitate fork repair, and allow for fork restart in cellular DNA. The G2–M checkpoint response of DNA damage is the primary location of ATR and Chk1 regulation[15]. Entry into mitosis requires additional activation of another protein kinase, Cdc2, which is activated by the Cdc25c protein phosphatase[39]. Conversely, activated Chk1 phosphorylates and inactivates Cdc25c phosphatase, thereby inhibiting its ability to activate the Cdc2 Tyr15 residue and ultimately preventing mitosis[39, 40] (Fig.S2). Previous reports show knockdown or inhibition of ATR leads to a general loss of DNA damage checkpoints like this, causing an accumulation of DNA damage and premature entry into mitosis, resulting in mitotic catastrophe and cancer cell death. As predicted, ATR inhibitors have therefore shown effective antitumoral efficacy in several preclinical cancer models[24].

In the present study, we found ATR, p-ATR, Chk1, and p-Chk1 are endogenously expressed in ovarian cancer cells. The proliferation and viability of ovarian cancer cell lines Skov3 and OVCAR8 were significantly suppressed with ATR-siRNA or VE-822 treatment in a dose-dependent manner. Downregulation of ATR and p-ATR was observed after ATR siRNA transfection. Consistent with antitumor activity, this treatment produced a concomitant decrease in expression of pChk1, p-Cdc25c, and p-Cdc2, as well as a stepwise increase in cleaved PARP and γH2AX. Cleaved PARP is a marker of cell death and γH2AX is a well-supported approach used to quantify levels of DNA damage in both experimental and clinical settings[24, 41]. We additionally verified the effect of VE-822 on
clonogenicity and tumor spheroid growth. The Skov3 and OVCAR8 cell lines had significantly reduced colony counts and size following VE-822 treatment. When these cell lines were cultured in 3-D environment, which mimics in vivo growth conditions, there was significantly reduced spheroid formation and growth.

Our study shows ATR and p-ATR are significantly upregulated during the progression of human ovarian cancer, and that high levels of ATR and p-ATR correlate with tumor recurrence. Elevated p-ATR is a prognostic biomarker for grim survival in ovarian cancer. Likewise, knockdown and inhibition of ATR significantly reduces ovarian cancer cell proliferation and induces apoptosis. As is expected, the components of the ATR pathway including Chk1, Cdc25c and Cdc2, are also promising synergistic therapeutic targets alongside ATR knockout. Taken together, our work shows targeting ATR is a potential therapeutic strategy and therefore warrants future clinical trials for patients with ovarian cancer.

Abbreviations
3D, 3-dimensional; ATM, ataxia telangiectasia mutated; ATR, Ataxia-telangiectasia and Rad3 related; CA125, carbohydrate antigen 125; Chk1, checkpoint kinase 1; DNA-PKcs, DNA dependent protein kinase catalytic subunit; IHC, Immunohistochemistry; MTT, methyl thiazolyl tetrazolium; OS, overall survival; PFS, progression-free survival; PIKK, phosphatidylinositol 3-kinase-related kinase; siRNA, small interfering RNA; TMA, tissue microarray;

Declarations
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Author Contributions
Formal analysis, WF; Funding acquisition, ZD; Methodology, WF, JW, YJ; Project administration, HS and ZD; Resources, FH; Software, WF; Supervision, HS and ZD; Writing – original draft, WF, JW, YJ; Writing – review & editing, DD, HS and ZD. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

This study was reviewed and approved by the Ethical Board at the David Geffen School of Medicine at UCLA.

Competing interests

The authors declare that they have no competing interests.

References

1. Howlader N NA, Krapcho M, Miller D, Bishop K, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds). SEER Cancer Statistics Review, 1975-2014, National Cancer Institute. Bethesda, MD, https://seer.cancer.gov/csr/1975_2014/, based on November 2016 SEER data submission, posted to the SEER web site. April 2017.

2. Torre LA, Trabert B, DeSantis CE, Miller KD, Samimi G, Runowicz CD, Gaudet MM, Jemal A, Siegel RL: Ovarian cancer statistics, 2018. CA Cancer J Clin 2018, 68:284-296.

3. Lheureux S, Gourley C, Vergote I, Oza AM: Epithelial ovarian cancer. Lancet 2019, 393:1240-1253.

4. Miller DS, Blessing JA, Krasner CN, Mannel RS, Hanjani P, Pearl ML, Waggoner SE, Boardman CH: Phase II evaluation of pemetrexed in the treatment of recurrent or persistent platinum-resistant ovarian or primary peritoneal carcinoma: a study of the Gynecologic Oncology Group. J Clin Oncol 2009, 27:2686-2691.
5. Jacobs I, Bast RC, Jr.: The CA 125 tumour-associated antigen: a review of the literature. *Hum Reprod* 1989, 4:1-12.

6. Montagnana M, Lippi G, Danese E, Franchi M, Guidi GC: Usefulness of serum HE4 in endometriotic cysts. *Br J Cancer* 2009, 101:548.

7. Hanahan D, Weinberg RA: Hallmarks of cancer: the next generation. *Cell* 2011, 144:646-674.

8. Halazonetis TD, Gorgoulis VG, Bartek J: An oncogene-induced DNA damage model for cancer development. *Science* 2008, 319:1352-1355.

9. Kaelin WG, Jr.: The concept of synthetic lethality in the context of anticancer therapy. *Nat Rev Cancer* 2005, 5:689-698.

10. Myers K, Gagou ME, Zuazua-Villar P, Rodriguez R, Meuth M: ATR and Chk1 suppress a caspase-3-dependent apoptotic response following DNA replication stress. *PLoS Genet* 2009, 5:e1000324.

11. Woods D, Turchi JJ: Chemotherapy induced DNA damage response: convergence of drugs and pathways. *Cancer Biol Ther* 2013, 14:379-389.

12. Jackson SP, Bartek J: The DNA-damage response in human biology and disease. *Nature* 2009, 461:1071-1078.

13. Ciccia A, Elledge SJ: The DNA damage response: making it safe to play with knives. *Mol Cell* 2010, 40:179-204.

14. Weber AM, Ryan AJ: ATM and ATR as therapeutic targets in cancer. *Pharmacol Ther* 2015, 149:124-138.

15. Saldivar JC, Cortez D, Cimprich KA: The essential kinase ATR: ensuring faithful duplication of a challenging genome. *Nat Rev Mol Cell Biol* 2017, 18:622-636.

16. Abdel-Fatah TM, Middleton FK, Arora A, Agarwal D, Chen T, Moseley PM, Perry C, Doherty R, Chan S, Green AR, et al: Untangling the ATR-CHEK1 network for
prognostication, prediction and therapeutic target validation in breast cancer. *Mol Oncol* 2015, **9**:569-585.

17. Di Benedetto A, Ercolani C, Mottolese M, Sperati F, Pizzuti L, Vici P, Terrenato I, Shaaban AM, Humphries MP, Di Lauro L, et al: *Analysis of the ATR-Chk1 and ATM-Chk2 pathways in male breast cancer revealed the prognostic significance of ATR expression*. *Sci Rep* 2017, **7**:8078.

18. Savva C, De Souza K, Ali R, Rakha EA, Green AR, Madhusudan S: *Clinicopathological significance of ataxia telangiectasia-mutated (ATM) kinase and ataxia telangiectasia-mutated and Rad3-related (ATR) kinase in MYC overexpressed breast cancers*. *Breast Cancer Res Treat* 2019, **175**:105-115.

19. Gaillard H, Garcia-Muse T, Aguilera A: *Replication stress and cancer*. *Nat Rev Cancer* 2015, **15**:276-289.

20. Murga M, Campaner S, Lopez-Contreras AJ, Toledo LI, Soria R, Montana MF, Artista L, Schleker T, Guerra C, Garcia E, et al: *Exploiting oncogene-induced replicative stress for the selective killing of Myc-driven tumors*. *Nat Struct Mol Biol* 2011, **18**:1331-1335.

21. Gilad O, Nabet BY, Ragland RL, Schoppy DW, Smith KD, Durham AC, Brown EJ: *Combining ATR suppression with oncogenic Ras synergistically increases genomic instability, causing synthetic lethality or tumorigenesis in a dosage-dependent manner*. *Cancer Res* 2010, **70**:9693-9702.

22. Teng PN, Bateman NW, Darcy KM, Hamilton CA, Maxwell GL, Bakkenist CJ, Conrads TP: *Pharmacologic inhibition of ATR and ATM offers clinically important distinctions to enhancing platinum or radiation response in ovarian, endometrial, and cervical cancer cells*. *Gynecol Oncol* 2015, **136**:554-561.

23. Huntoon CJ, Flatten KS, Wahner Hendrickson AE, Huehls AM, Sutor SL, Kaufmann SH,
Karnitz LM: ATR inhibition broadly sensitizes ovarian cancer cells to chemotherapy independent of BRCA status. Cancer Res 2013, 73:3683-3691.

24. Lecona E, Fernandez-Capetillo O: Targeting ATR in cancer. Nat Rev Cancer 2018, 18:586-595.

25. Wang J, Dean DC, Hornicek FJ, Shi H, Duan Z: Cyclin-dependent kinase 9 (CDK9) is a novel prognostic marker and therapeutic target in ovarian cancer. FASEB J 2019, 33:5990-6000.

26. Guo Y, Nemeth J, O’Brien C, Susa M, Liu X, Zhang Z, Choy E, Mankin H, Hornicek F, Duan Z: Effects of siltuximab on the IL-6-induced signaling pathway in ovarian cancer. Clin Cancer Res 2010, 16:5759-5769.

27. Duan Z, Foster R, Bell DA, Mahoney J, Wolak K, Vaidya A, Hampel C, Lee H, Seiden MV: Signal transducers and activators of transcription 3 pathway activation in drug-resistant ovarian cancer. Clin Cancer Res 2006, 12:5055-5063.

28. Fokas E, Prevo R, Hammond EM, Brunner TB, McKenna WG, Muschel RJ: Targeting ATR in DNA damage response and cancer therapeutics. Cancer Treat Rev 2014, 40:109-117.

29. Shi Q, Shen LY, Dong B, Fu H, Kang XZ, Yang YB, Dai L, Yan WP, Xiong HC, Liang Z, Chen KN: The identification of the ATR inhibitor VE-822 as a therapeutic strategy for enhancing cisplatin chemosensitivity in esophageal squamous cell carcinoma. Cancer Lett 2018, 432:56-68.

30. Fokas E, Prevo R, Pollard JR, Reaper PM, Charlton PA, Cornelissen B, Vallis KA, Hammond EM, Olcina MM, Gillies McKenna W, et al: Targeting ATR in vivo using the novel inhibitor VE-822 results in selective sensitization of pancreatic tumors to radiation. Cell Death Dis 2012, 3:e441.

31. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C: Clonogenic assay of
cells in vitro. Nat Protoc 2006, 1:2315-2319.

32. Gao S, Shen J, Hornicek F, Duan Z: Three-dimensional (3D) culture in sarcoma research and the clinical significance. Biofabrication 2017, 9:032003.

33. Zighelboim I, Ali S, Lankes HA, Backes F, Moore K, Mutch D, Robison K, Behbakht K, Waggoner S, Ghebre RG, et al: Assessing the prognostic role of ATR mutation in endometrioid endometrial cancer: An NRG Oncology/Gynecologic Oncology Group study. Gynecol Oncol 2015, 138:614-619.

34. Lopez-Girona A, Tanaka K, Chen XB, Baber BA, McGowan CH, Russell P: Serine-345 is required for Rad3-dependent phosphorylation and function of checkpoint kinase Chk1 in fission yeast. Proc Natl Acad Sci U S A 2001, 98:11289-11294.

35. Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, et al: Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev 2000, 14:1448-1459.

36. Takai H, Tominaga K, Motoyama N, Minamishima YA, Nagahama H, Tsukiyama T, Ikeda K, Nakayama K, Nakanishi M, Nakayama K: Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice. Genes Dev 2000, 14:1439-1447.

37. Brown EJ, Baltimore D: ATR disruption leads to chromosomal fragmentation and early embryonic lethality. Genes Dev 2000, 14:397-402.

38. Cliby WA, Roberts CJ, Cimprich KA, Stringer CM, Lamb JR, Schreiber SL, Friend SH: Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. Embo j 1998, 17:159-169.

39. Graves PR, Lovly CM, Uy GL, Piwnica-Worms H: Localization of human Cdc25C is
regulated both by nuclear export and 14-3-3 protein binding. Oncogene 2001, 20:1839-1851.

40. Kumagai A, Dunphy WG: Binding of 14-3-3 proteins and nuclear export control the intracellular localization of the mitotic inducer Cdc25. Genes Dev 1999, 13:1067-1072.

41. Chaitanya GV, Steven AJ, Babu PP: PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration. Cell Commun Signal 2010, 8:31.

Tables
Due to technical limitations, Tables 1-3 are provided in the Supplementary Files section.

Figures
Figure 1

The expression pattern varied for ATR and p-ATR, as ATR was mainly located within the cytoplasm and p-ATR resided within cell nuclei.
There were clear trends towards higher ATR (p=0.007) and p-ATR (p=0.01) expression in the recurrent tumors compared to the matched primary tumors. In contrast, there was no significant difference between metastatic tumors and their matched primary tumors, with p values of 0.326 for ATR and 0.972 for p-ATR. (B&C) Kaplan-Meier analysis showed no significant difference between low and high expression groups in OS or PFS. (E&F) Kaplan-Meier analysis revealed patients with high p-ATR expression have significantly worse overall survival (OS) (p=0.0002) and progression-free survival (PFS) (p=0.008) by log-rank test.
Our results confirmed that ATR, p-ATR, Chk1, and p-Chk1 are expressed in all tested ovarian cancer cell lines including A2780, OVCAR5, IGROV-1, Skov3, OVCAR8, and Caov-3.

Figure 4

(A) SKOV3
- Untreated control
- Non-specific siRNA 80 nm
- ATR-specific siRNA 10 nm
- ATR-specific siRNA 30 nm
- ATR-specific siRNA 80 nm

(B) OVCAR8
- Untreated control
- Non-specific siRNA 80 nm
- ATR-specific siRNA 10 nm
- ATR-specific siRNA 30 nm
- ATR-specific siRNA 80 nm

(C) Western blot analysis of SKOV3 and OVCAR8 cells treated with ATR-siRNA and non-specific siRNA.
Figure 4

A. No significant changes were observed in the untreated control group or in those cells transfected with nonspecific siRNA. B. We also observed morphologic changes and diminished cell proliferation after siRNA transfection during this period. C. To further investigate these signaling pathways after ATR knockdown in ovarian cancer, we measured downstream ATR/Chk1 pathway proteins via Western blot.

**Fig. 5**

A. [Graph showing cell viability for SKOV3 and OVCAR8 cell lines treated with different concentrations of ATR inhibitor VE-822.]  
- **SKOV3**: IC50: 0.077, 95% CI: 0.062-0.096  
  - Untreated control  
  - ATR inhibitor VE-822 0.05 µM  
  - ATR inhibitor VE-822 0.1 µM  
  - ATR inhibitor VE-822 0.5 µM  
  - ATR inhibitor VE-822 1.0 µM  

B. [Images showing morphologic changes in SKOV3 and OVCAR8 cells treated with different concentrations of ATR inhibitor VE-822.]  

C. [Additional images not described in the text.]
A. To evaluate its effect in ovarian cancer cells, we treated the ovarian cancer cell lines Skov3 and OVCAR8 with VE-822 over five days and subsequently observed a dose-dependent reduction in cell viability, with IC50 values of VE-822 at 0.077 μM in Skov3 and 0.056 μM in OVCAR8. B. Over a 72-hour culture period with increasing VE-822 doses, we observed morphological changes and decreased cell proliferation in both cell lines. C. Assessment of the ATR signaling proteins by Western blot after VE-822 treatment showed p-ATR/p-Chk1/p-Cdc25c/p-Cdc2 were concomitantly decreased.

**Figure 5**

**Figure 6**
A. After a 15-day treatment period, Skov3 and OVCAR8 clonogenicity was reduced in a dose-dependent manner, whereas the untreated control cells did not experience this significant change. B & C. During a 15-day observation period, although the spheroids of Skov3 and OVCAR8 continuously grew, the ATR inhibitor-treated spheroids were significantly smaller than the untreated control group.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.
Tables of ATR.docx
Supplementary Table S1 Clinical data for ovarian cancer TMA.xlsx
Figure S2.png
Figure S1.png
