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Original Article

ATR inhibition sensitizes liposarcoma to doxorubicin by increasing DNA damage

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Abstract: Liposarcomas account for approximately 20% of all adult sarcomas and have limited therapeutic options outside of surgery. Inhibition of ataxia-telangiectasia and Rad3 related protein kinase (ATR) has emerged as a promising chemotherapeutic strategy in various cancers. However, its activation, expression, and function in liposarcoma remain unkown. In this study, we investigated the expression, function, and potential of ATR as a therapeutic target in liposarcoma. Activation and expression of ATR in liposarcoma was analyzed by immunohistochemistry, which was further explored for correlation with patient clinical characteristics. ATR-specific siRNA and the ATR inhibitor VE-822 were applied to determine the effect of ATR inhibition on liposarcoma cell proliferation and anti-apoptotic activity. Migration activity and clonogenicity were examined using wound healing and clonogenic assays. ATR (p-ATR) was overexpressed in 88.1% of the liposarcoma specimens and correlated with shorter overall survival in patients. Knockdown of ATR via specific siRNA or inhibition with VE-822 suppressed liposarcoma cell growth, proliferation, migration, colony-forming ability, and spheroid growth. Importantly, ATR inhibition significantly and synergistically enhanced liposarcoma cell line chemosensitivity to doxorubicin. Our findings support ATR as critical to liposarcoma proliferation and doxorubicin resistance. Therefore, the addition of ATR inhibition to a standard doxorubicin regimen is a potential treatment strategy for liposarcoma.

Keywords: ATR, liposarcoma, prognostic marker, therapeutic target, VE-822

Introduction

Liposarcomas are the second most common soft tissue sarcoma in adults and are classified into four distinct histological subtypes: well differentiated, dedifferentiated, myxoid, and pleomorphic [1, 2]. Although extensive resection can be achieved, retroperitoneal liposarcomas or those adjacent to vital neurovascular structures are commonly unresectable, recurrent, and deadly [3, 4]. To address this, neo-adjuvant chemotherapeutics such as doxorubicin are often used alongside surgery for recurrent or metastatic liposarcomas, but results are limited [5-7]. For those with recurrent or metastatic liposarcoma, survival rates have plateaud for almost four decades [6, 8]. Currently, the median overall length of survival in metastatic liposarcoma is approximately 12 months [9, 10]. Therefore, there is a clear need for improved therapies for liposarcoma.

Ataxia-telangiectasia and Rad3 related (ATR) is a serine/threonine kinase and belongs to the phosphoinositide 3-kinase related protein kinases (PIKKs), particularly to the ataxia telangiectasia mutated (ATM) subfamily [11]. ATR activity is required to ensure proper DNA repli-

cation and genomic stability of proliferating cells in response to replication stress and DNA damage. Specifically, activated ATR (phosphorylated, p-ATR) phosphorylates downstream targets including checkpoint kinase 1 (CHK1) to promote DNA damage repair and stabilization. Previous studies have shown homozygous knockout of ATR or CHK1 is lethal in early embryonic life, and highlights the vital role of these protein kinases during cellular proliferation [11, 12]. Conversely, overexpression of activated p-ATR has been reported in various malignancies, perhaps due to their hyperproliferative state [11, 13]. Aberrant expression and activation of ATR have been associated with higher tumor stage, progression, mitotic index, pleomorphism, invasion, and shorter survival in cancer patients [14, 15]. Knockdown of aberrant ATR expression or activity leads to the lethal accumulation of DNA damage and therefore has chemotherapeutic effects [11, 12]. Inhibition of ATR has been shown to decrease metastasis *in vitro* and *in vivo* and enhance sensitivity to immune checkpoint inhibitors or radiation therapy [16-19]. Several ongoing phase I and II clinical trials are utilizing selective ATR inhibitors such as VE-822 (also known as Berzosertib, VX-970 or M6620), AZD6738, or BAY1895344 in solid tumors and leukemia, with some showing positive results [11, 20-22]. Despite the growing body of evidence in several cancers, the importance of ATR in recalcitrant liposarcoma remains unknown. We, therefore, investigated the expression, function, and potential of ATR as a therapeutic target in liposarcoma. Specifically, we analyzed the ATR expression in liposarcoma patient specimens, correlated it with clinical outcomes, and followed up with targeted liposarcoma cell line studies to discern the roles of ATR in liposarcoma proliferation, spheroid growth, colonization, and doxorubicin sensitivity.

Materials and methods

Liposarcoma tissue microarray (TMA) and immunohistochemistry (IHC) assay

The human liposarcoma TMA was purchased from Novus Biologicals, LLC (Littleton, CO, USA), and used in accordance with the policies of the institutional review board of the Partners Human Research Committee (IRB protocol #2007P-002464) as previously described [23, 24]. All methods were carried out in accordance with relevant guidelines and regulations. The TMA contained 53 samples from 42 liposarcoma and 11 lipoma patients, and included clinicopathological data on age, sex, tumor location, diagnosis, tumor tissue pathological subtypes, follow-up time and results, and cause of death. The expression of activated p-ATR was accessed by IHC according to the instructions of manufacturer (Cell Signaling Technology, MA, USA) as previously described [25]. First, the paraffin-embedded slide was baked at 60°C for 1 hour before xylene deparaffinization. The slide was subsequent rehydration through the graded ethanol (100% and 95%). Three percent of hydrogen peroxide (H_2O_2) solution was used to quench endogenous peroxidase activity after antigen retrieval. After that, the slide was blocked for 1 hour with goat serum, and incubated with rabbit antibody to human p-ATR (Cell Signaling Technology, 1:100 dilution), in 1% bovine serum albumin (BSA) of PBS overnight at 4°C. The next day, SignalStain® Boost Detection Reagent (Cell Signaling Technology) and SignalStain® DAB (Cell Signaling Technology) were then applied to determin the HRP-bound antibody. Hematoxylin QS (Vector Laboratories, CA, USA) was utilized counterstain of the liposarcoma cell nuclei before preservation by using VectaMount AQ (Vector Laboratories) mounting.

Evaluation of IHC staining in the liposarcoma TMA

The degree of immunostaining on TMA slide was scored based on the percentage of nuclear p-ATR immunostaining, as reviewed and determined by two investigators who had no knowledge of the histopathological features or patient information of the tissues. Levels of p-ATR expression were then divided into six groups based on the percentage of cells showing positive nuclear staining as follows: 0, negative, no nuclear staining; 1+, <10% of positive cells; 2+, 10-25% of positive cells; 3+, 26-50% of positive cells; 4+, 51-75% of positive cells; 5+, >75% of positive cells. The low-p-ATR expression included groups 0, 1+, and 2+, while the high p-ATR expression included groups 3+, 4+, and 5+. Staining images were captured using a Nikon microscope (Nikon Instruments Inc., Melville, NY, USA).

Cell lines and cell culture

The human liposarcoma cell lines 94T778 and SW872 were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The 94T778 cell line is a well-differentiated liposarcoma derived from the retroperitoneum of a 69 year old female [26]. The SW872 cell line is a dedifferentiated liposarcoma cell line derived from a 36 year old male [27]. Both the liposarcoma cell lines were cultured in a humidified 5% CO_2 atmosphere at 37°C in RPMI 1640 medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, MO, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, MA, USA).

ATR siRNA knockdown and cell proliferation assay

ATR knockdown in liposarcoma cells was performed by transfection of synthetic human ATR siRNA (Sigma-Aldrich). The siRNA sequence targeting ATR corresponded to coding regions (5'-GAUCCUACAUCAUGGUACA-3', catalog # (#SASI_Hs01_00176271) of the ATR gene. The human nonspecific siRNA oligonucleotides (MISSION siRNA Universal Negative Control, catalog #SIC001, Sigma-Aldrich) were used as a negative control. Liposarcoma cells 94T778 and SW872 were grown at a concentration of 2×10^3 cells/well in 96-well plates or at 4×10^4 cells/well in 12-well plates and transfected with increasing concentrations (0, 10, 30, 60 nM) of ATR siRNA by using Lipofectamine RNAiMax transfection reagent (Invitrogen, CA, USA). Nonspecific control siRNA was utilized as a negative control. Four days after transfection with the ATR siRNA, the total proteins of 94T778 and SW872 were extracted to Western blotting, or assessment of cell growth and proliferation by MTT assay. In brief, 20 μL of MTT (5 mg/mL) was added to each well of the 96-well plates and incubated at 37°C in 5% CO_2 atmosphere for 4 h. The formazan products were solubilized by adding 100 μL of acid isopropanol and the absorbance was measured at a wavelength of 490 nm on the SpectraMax Microplate® Spectrophotometer (Molecular Devices LLC, CA, USA). All MTT assays were performed in triplicate.

ATR inhibitor and cell proliferation assay

The function role of activated ATR expression in liposarcoma cell growth and proliferation was analyzed by a potent and selective ATR inhibitor VE-822 (Selleckchem Inc. TX, USA). VE-822 decreases cell-cycle checkpoints, increases DNA damage, and decreases homologous recombination in cancer cells. VE-822 has been verified to inhibit ATR activation in several cancer cells *in vitro,* and demonstrates antitumor activity in xenograft tumor models *in vivo* [19, 20, 28]. Liposarcoma cells 94T778 and SW872 were seeded separately into 96-well plates at a density of 4×10^3 cells/well or 6-well plates at a density of 6×10^5 cells/well and incubated with increasing concentrations (0, 0.10, 0.25, 0.50, 1.00 μM) of VE-822 for 5 days prior to quantification. After VE-822 treatment for 5 days, the growth and proliferation of 94T778 and SW872 cells was assessed via MTT assay. In addition, a Nikon microscope (Diagnostic Instruments Inc., NY, USA) was used to check the morphological changes of the 94T778 and SW872 cells after VE-822 treatment.

Protein preparation and Western blot

Protein lysates of the liposarcoma cell lines 94T778 and SW872 were extracted with 1×RIPA lysis buffer (Upstate Biotechnology, VA, USA) supplemented with protease inhibitor cocktail tablets (Roche Applied Science, IN, USA) after incubation with ATR siRNA or ATR inhibitor VE-822. The concentration of the protein were determined by the DC Protein Assay reagents (Bio-Rad, Hercules, CA, USA) with a spectrophotometer (Molecular Devices, Inc., CA, USA). Western blotting were performed as follows: equal amounts of denatured protein were run and separated in NuPAGE 4-12% Bis-Tris Gel (Thermo Fisher Scientific) and then transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked in 5% non-fat milk for 1 hour, and then incubated with specific primary antibodies overnigh with specific primary antibodies at 4°. The antibodies used in this study as follow: rabbit monoclonal antibodies to human ATR, p-ATR (Ser 428, 1:1000 dilution, #2853, Cell Signaling Technology), p-CHK1 (pCHK1, Ser 345, 1:1000 dilution, #2348, Cell Signaling Technology), phosphohistone H2AX (γ-H2AX, Ser 139, 1:1000 dilution, #9718, Cell Signaling Technology) and tubulin (1:2000 dilution, Sigma-Aldrich). Following overnight incubation, membranes were washed with Tris-buffer saline tween 20 (TBST) three times (5 min each, room temperature). After that, goat anti-rabbit IRDye 800CW (1:5000 dilution) or goat anti-mouse IRDye 680LT sec-

ondary antibody (1:15000 dilution) (Li-COR Biosciences, Lincoln, NE) was incubated for 2 hour at room temperature followed by another TBST wash (again 5 min each, room temperature). Protein bands were detected by Odyssey CLx Near-Infrared Fluorescence Imaging System (Li-COR Bioscience).

Immunofluorescence detection of p-ATR expression

To visualize immunofluorescence of p-ATR expression, 94T778 and SW872 cells were transfected with nonspecific siRNA or ATR siRNA in 12-well chambers for 60-65 hours. The cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 15 min at room temperature. The cells were then permeabilized with ice-cold methanol (Sigma-Aldrich) for 10 min and blocked in 1% BSA (Sigma-Aldrich) in PBST for 30 min to block unspecific binding of the antibodies. Following incubation with p-ATR primary antibody (1:200 dilution, Cell Signaling Technology) or β-actin (1:200 dilution, Sigma-Aldrich) at 4°C overnight, the cells were incubated with Alexa Fluor 488 (Green) conjugated goat anti-rabbit antibody (A-11034; Invitrogen) and Alexa Fluor 594 (red) goat anti-mouse antibody (A-11032; Invitrogen) for one hour. Finally, Hoechst 33342 (1 μg/ml, Invitrogen) was added to counterstain the cell nucleus. Cells were then imaged by a Nikon fluorescence microscope.

Cell migration activity and clonogenic assay

Effect of VE-822 on the cell migration was evaluated by a wound healing assay. In brief, 2×10^5 cells of 94T778 and SW872 were seeded onto 12-well plates and treated with VE-822 at the concentration of 0.10 μM. After the cells reached 100% confluence, they were wounded by scraping three parallel lines with a 200 μl tip, and then washed three times in serum-free medium and incubated in regular medium. Wounds were observed at 0, 24, 48 and 72 hours, respectively. Three images were recorded per well at each time point using a Nikon microscope (10× objective) to monitor the cell repair process, and the distance between the two edges of the scratch (wound width) which was measured at three random sites per microscopic image. The cell migration distance was calculated by subtracting the wound width at each time point from the wound width at the 0 hour time point.

Clonogenic assays were used to access the effect of ATR inhibition on liposarcoma cell growth and proliferation by VE-822. Liposarcoma cells 94T778 and SW872 were prepared in 12-well plates at 100 cells/well, treated with VE-822 at increasing concentrations (0, 0.25, and 0.50 μM). After a 6-day incubation period at 37°C, the colonies were fixed with methanol for 10 minutes, washed three times with PBS, then subsequently stained for 20 minutes with a 10% Giemsa stain (MilliporeSigma). The stained colonies of treated cells were then washed with water and dried, and a digital camera (Olympus, Tokyo, Japan) was used to photograph the stained colonies.

Three-dimensional (3D) cell culture

Three-dimensional (3D) cell culture can mimic cell growth *in vivo* environment to better access growth behavior. A hydrogel 3D cell culture assay was used to evaluate the effect of ATR inhibition on liposarcoma cell growth and proliferation. Spheroids formed from the liposarcoma cell lines 94T778 and SW872 in 24-well VitroGel™ 3D cell culture plates at a density of 2×10^5 cells/well, and were set up according to manufacturer protocol (The Well Bioscience Inc., NJ, USA). Next, 0.10 μM of VE-822 was added into the cell medium, with the untreated 94T778 and SW872 cells serving as control. The liposarcoma cell plates were incubated in a humidified 5% CO_2 atmosphere at 37°C, and the medium was changed every 24-48 h to provide enough nutrients for cell growth. The spheroids were photographed every 2 days with a Nikon microscope. At the 12 day point, the spheroids were harvested from the bottom of the plate by gentle pipetting of 100 μL PBS into each well. The spheroids were imaged on a Nikon fluorescence microscope after 15 minutes of incubation with 0.25 μM Calcein AM (Life Technologies).

Effect of VE-822 in combination with doxorubicin in liposarcoma cell lines

Doxorubicin is the most commonly used chemotherapeutic in liposarcoma [6, 8]. We therefore investigated the effect of VE-822 on doxorubicin response in liposarcoma cell lines.

94T778 and SW872 cells were cultured in 96-well plates as previously described and incubated with different concentrations of doxorubicin and VE-822. At the end of the 5-day treatment, MTT assays were used to determine cytotoxic effects in both cell lines as previously described [25]. The synergistic effect of VE-822 was further evaluated by SynergyFinder 2.0, a well-established web-application for multi-drug combination synergy analysis 29 (https://synergyfinder.fimm.fi). The degree of combination synergy was evaluated using a zero interaction potency (ZIP) model 30, which defines drugs interactions as either synergistic (synergy score >10), additive (synergy score -10-10), or antagonistic (synergy score <-10).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.0 software (GraphPad Software, San Diego, CA, USA). Data were expressed as mean ± SD. Student's t-tests were performed to analyze the differences between groups. Differences in survival were analyzed by Kaplan-Meier plots and log-rank tests. The relationship between p-ATR expression and liposarcoma patient clinicopathological features was evaluated by the χ^2 test. All results are presented as mean ± SD, and p values <0.05 are considered statistically significant.

Results

ATR activation and expression correlate with liposarcoma patient outcome

ATR exists in a constitutively active state (p-ATR) in cancer cells. Phosphorylation of ATR at serine 428 has been confirmed as a major form of p-ATR [11, 29, 30]. First, we evaluated the expression of p-ATR in a liposarcoma TMA. Of these 42 liposarcoma patient tissues, 37 of 42 (88.1%) showed positive p-ATR expression, ranging from staining group 0 (5 of 42, 11.9%); 1+ staining (3 of 42, 7.1%); 2+ staining (5 of 42, 11.9%), 3+ staining (10 of 42, 23.8%) 4+ staining (12 of 42, 28.6%), 5+ staining (7 of 42, 16.7%), (**Figure 1A**, **1B**). These stained specimens were then subdivided into two categories: 0, 1+, and 2+ were defined as the low p-ATR expression group (31.0%), whereas the 3+, 4+, and 5+ staining groups were defined as the high p-ATR expression group (69%) (**Figure 1A**, **1C**).

To evaluate the clinical significance of p-ATR, we compared p-ATR levels in our liposarcoma TMA to patient clinical characteristics and outcome. We found expression of p-ATR in patients who died (non-survival) from liposarcooma was significantly higher than in those who survived (survival) (P<0.0467, independent two-tailed Student's t-test) (**Figure 1D**). Next, we further evaluated the association between p-ATR expression and patient overall survival using Kaplan-Meier analysis. Those patients with high p-ATR expressing tumors had significantly worse overall survival rates compared to patients with low p-ATR expressing tumors (P<0.0066, log-rank test) (**Figure 1E**). Further analysis showed p-ATR expression was unrelated to other patient clinicopathological features, including age, gender, and tumor site (**Table 1**).

ATR knockdown by siRNA decreases liposarcoma cell growth and proliferation

To evaluate the role of ATR expression and activation in liposarcoma cell growh and proliferation, we used ATR siRNA to knockdown ATR expression in 94T778 and SW872 cell lines. Four days post ATR siRNA transfection, the MTT assay showed a significant 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 (**Figure 2A**, **2B**). We also observed morphologic changes and diminished cell proliferation after siRNA transfection during this period (**Figure 2C**).

Effect of ATR knockdown on ATR singling and DNA damage in liposarcoma cell

The activation of ATR associated DNA damage response is a multi-component network of signaling pathways regulating DNA damage repair, cell cycle checkpoints, and apoptosis [11, 12]. To investigate the ATR signaling pathway, we measured the expression of several ATR downstream proteins via Western blot following ATR siRNA transfection. CHK1 phosphorylation (p-CHK1) was used to indicate ATR activity, with γ-H2AX foci expression as evidence of DNA damage. Knockdown of ATR decreased levels of p-ATR and p-CHK1 in both in 94T778 and SW872 cell lines, strongly indicative of a G2-M cell cycle arrest (**Figure 2D**, **2E**

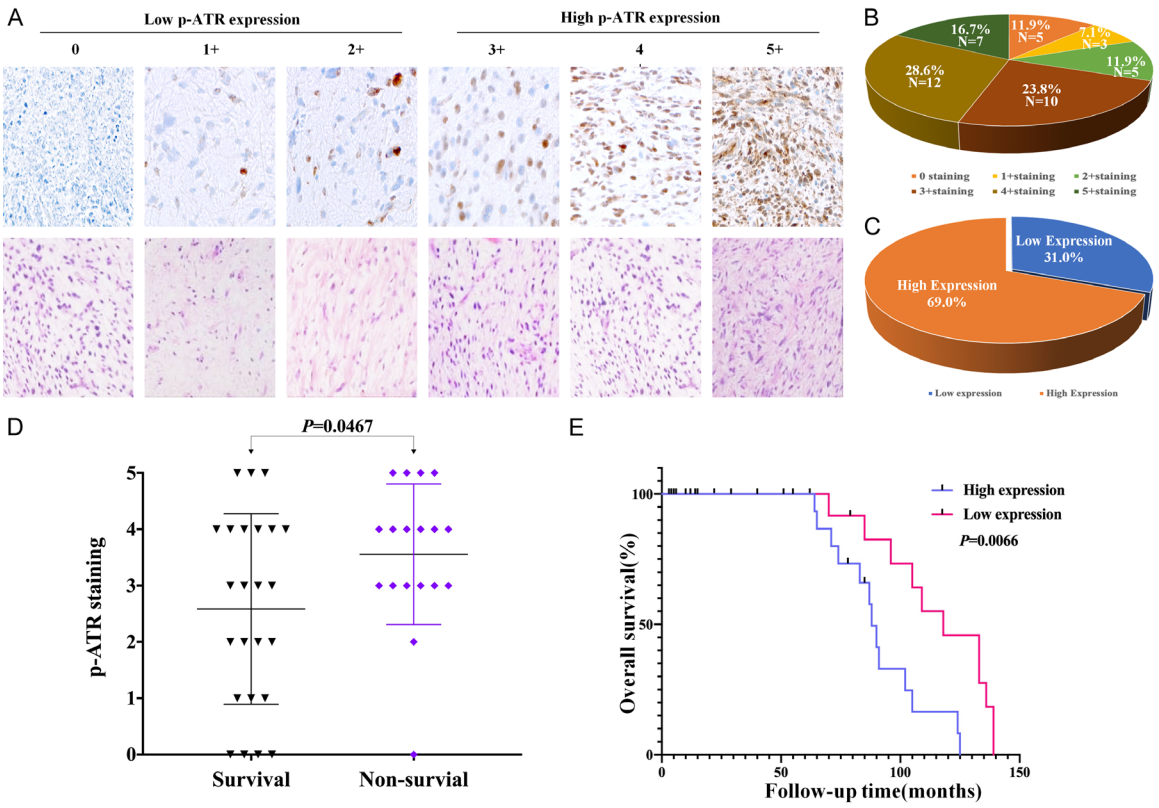

Figure 1. P-ATR is overexpressed in liposarcoma and correlates with poor patient prognosis. A. Representative images of p-ATR staining along with hematoxylin and eosin (HE) staining in liposarcoma tissues. P-ATR staining intensity patterns were divided into 6 groups: no staining (0); <10% positive cells (1+); 10-25% positive cells (2+); 26-50% positive cells (3+); 51-75% positive cells (4+); >75% positive cells (5+). B. Pie chart representing relative frequency of different p-ATR expression levels in liposarcoma TMA. C. Tumor with the staining score of ≤2+ were defined as the low p-ATR expression group (blue), ≥3+ were defined as the high p-ATR expression group (orange). Pie chart representing relative frequency of the two groups in the liposarcoma TMA. D. Comparison of p-ATR IHC staining scores between surviving and non-surviving patient groups. E. Kaplan-Meier overall-survival curve of patients with liposarcoma were sub-grouped as either p-ATR low-expression group (staining score ≤2+) or high-expression group (staining score ≥3+). Compared with the low-expression group, the patients with high p-ATR staining had a shorter overall survival.

and **2G**). The apoptotic signifier γ-H2AX, which indicates DNA damage and replication fork stress, was elevated with increasing concentrations of ATR siRNA (**Figure 2D**, **2E** and **2G**). Taken together, these results show knockdown of ATR causes an accumulation of liposarcoma DNA damage, reduces cell viability and proliferation, and induces apoptosis and cell death.

ATR inhibitor suppresses liposarcoma cell viability and proliferation

VE-822 is an ATR-selective inhibitor that attenuates the ATR signaling pathway and reduces survival in cancer cells. Importantly, it is well tolerated in mice and does not enhance toxicity in normal cells and tissues. 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 liposarcoma cells, we treated the liposarcoma cell lines 94T778 and SW872 with VE-822 over 5 days and subsequently observed a dose-dependent reduction in cell viability (**Figure 3A**, **3B**). Over a 3 days culture period with increasing VE-822 doses, we observed morphological changes and decreased cell proliferation in both cell lines (**Figure 3C**). Assessment of ATR signaling proteins by Western blot after VE-822 treatment showed p-ATR/p-CHK1 were concomitantly decreased (**Figure 3D-G**). Similar to our findings with ATR-siRNA treatment, increased levels of γ-H2AX was also observed (**Figure 3D-G**). These results indicate VE-822 suppresses ATR signaling via a blockade of ATR protein activation, thus induc-

Table 1. Summary of the clinicopathological characteristics of lipomatous tumors patients

Clinicopathological features		Number (n, %)	p-ATR Expression		*P* value
			Low (n, %)	High (n, %)	
All		53 (100.0)	22 (41.5)	31 (57.4)	
Age	Mean	52.1 (28-88) y			
	≤40 y	11 (20.8)	5 (45.5)	6 (54.5)	0.765
	>40 y	42 (79.2)	17 (40.4)	25 (59.5)	
Gender					
Male		24 (45.3)	8 (33.3)	16 (66.7)	0.097
Female		29 (54.7)	14 (48.3)	15 (51.7)	
Tumor site (Liposarcoma n=42)					
Neck/axilla/chest/abdomen/buttock		10 (21.4)	4 (40.0)	6 (60.0)	0.410
Mediastinum		1 (2.4)	0	1 (100.0)	
Abdominal cavity		10 (26.2)	1 (10.0)	9 (90.0)	
Retroperitoneal		10 (23.8)	3 (30.0)	7 (70.0)	
Extremities		11 (26.2)	5 (45.5)	6 (54.5)	
Pathology (n=53)					
Lipoma		11 (20.8)	9 (81.8)	2 (18.2)	
Liposarcoma, well differentiated		19 (35.8)	13 (68.4)	6 (31.6)	<0.001*
Liposarcoma, dedifferentiated		5 (9.4)	0	5 (100.0)	
Liposarcoma, myxoid		15 (28.3)	0	15 (100.0)	
Liposarcoma, pleomorphic		3 (5.7)	0	3 (100.0)	
Survival (Liposarcoma n=42)					
Alive		24 (57.1)	11 (45.8)	13 (54.2)	0.016*
Dead		18 (42.9)	2 (11.1)	16 (88.9)	

*Stand for *P* value less than 0.05 and is statistically significant.

ing liposarcoma cell apoptosis and an accumulation of toxic DNA damage.

ATR knockdown induces cell death and decreases p-ATR expression as confirmed by immunofluorescence

The effect of ATR activation and expression on liposarcoma cell growth and the subcellular localization of p-ATR were further evaluated by immunofluorescence assay. Consistent with the results of our liposarcoma TMA, the immunofluorescence assay also showed that the p-ATR protein was mainly localized in the nucleus of liposarcoma cells, and supports p-ATR as an activated form of ATR involved in DNA damage repair within the nucleus (**Figure 4**). The p-ATR immunofluorescence analysis further confirmed a significant reduction of cell viability in both 94T778 and SW872 cell lines with ATR siRNA transfection relative to cells transfected with nonspecific siRNA. Liposarcoma cells also expressed much lower levels of p-ATR after being transfected with ATR siRNA. These data are comparable with p-ATR expression in other human tumors which have also shown nuclear localization [11, 15, 31].

Inhibition of ATR reduces liposarcoma cell motility, clonogenicity, and spheroid growth

Cancer cell motility is essential for invasion and metastasis. We, therefore, investigated the effect of ATR inhibition on liposarcoma cell migration. Wound healing assays were performed after treatment with VE-822. Relative cell migration distance was evaluated at 0, 24, 48 and 72 hours. After treatment by the scratch assay as described, we observed a marked inhibition of migratory potential in both 94T778 and SW872 cell lines compared with control groups (**Figure 5A**). In contrast, wounds were almost fully recovered after the 72-hour migration in control group cells. These data demonstrate that inhibition of ATR impairs liposarcoma cell motility.

We next assessed the effects of VE-822 on the colony-forming ability of liposarcoma cells with a clonogenic assay. After six days of VE-822

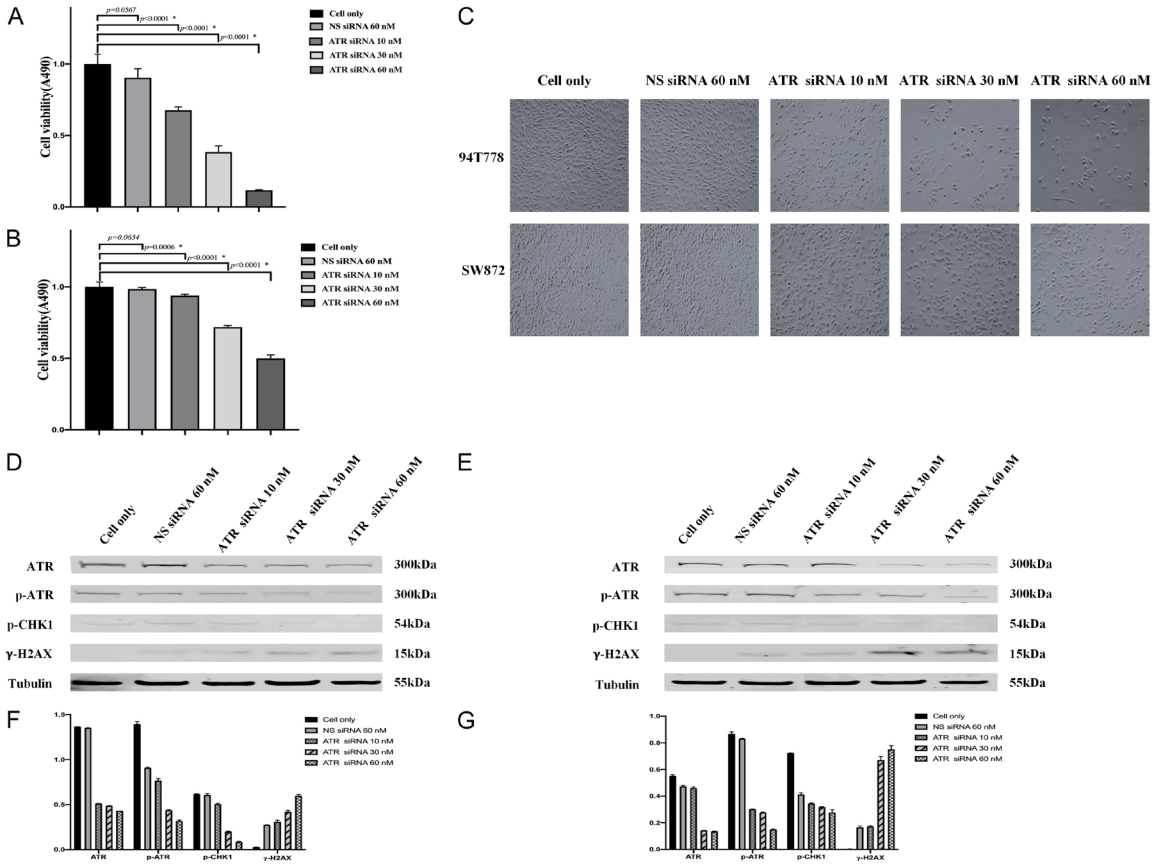

Figure 2. ATR inhibition by siRNA decreased liposarcoma cell proliferation. (A and B) Cell viability of 94T778 (A) and SW872 (B) determined by MTT assays after four days of ATR siRNA and negative control (NS) siRNA transfection. The data are presented as mean ± SE of the 2 experiments carried out in triplicate. (C) Microscopy images of morphologic changes and a reduction in cell number after 72 h of ATR siRNA transfection. (D and E) The expression of proteins ATR, p-ATR, p-CHK1, and γ-H2AX in the ATR-associated signaling pathway as measured by Western blotting in the liposarcoma cell lines 94T778 (D), and SW872 (E) after 72 h of siRNA transfection. (F and G) Semiquantitative analysis of (D) and (E) by densitometry relative to tubulin. The data are mean ± SE of the experiment carried out in triplicate.

treatment, the clonogenicity of 94T778 and SW872 decreased in a dose-dependent manner with no changes seen in the untreated group (**Figure 5B**). Additionally, because flat 2D culture systems may not adequately mimic the *in vivo* conditions by which liposarcoma cells attach, spread, and grow three dimensionally, we evaluated how ATR alters liposarcoma tumorigenicity within a simulated *in vivo* 3D culture environment. Observations of spheroid size were recorded at several time points, and although the spheroids continuously grew, the diameters in ATR inhibitor-treated 94T778 and SW872 cell spheroids were significantly smaller than the untreated cell spheroids (**Figure 5C**). Collectively, our results further support ATR to have a crucial role in liposarcoma growth and progression.

Inhibition of ATR enhances doxorubicin efficacy in liposarcoma cell lines

High level activation and expression of ATR may contribute to the survival advantage of liposarcoma cells, in part through anti-apoptotic mechanisms in response to DNA damage. ATR inhibitors have known anti-cancer effects when combined with additional chemotherapy or radiation therapy. We therefore hypothesized that inhibition of the ATR pathway in liposarcoma may lower the apoptotic threshold and increase sensitivity to doxorubicin, as compared to cells treated with either doxorubicin or VE-822 alone. MTT assays were used to compare viabilities of 94T778 and SW872 treated with increasing concentrations of doxorubicin in combination with ATR inhibitor VE-822. We

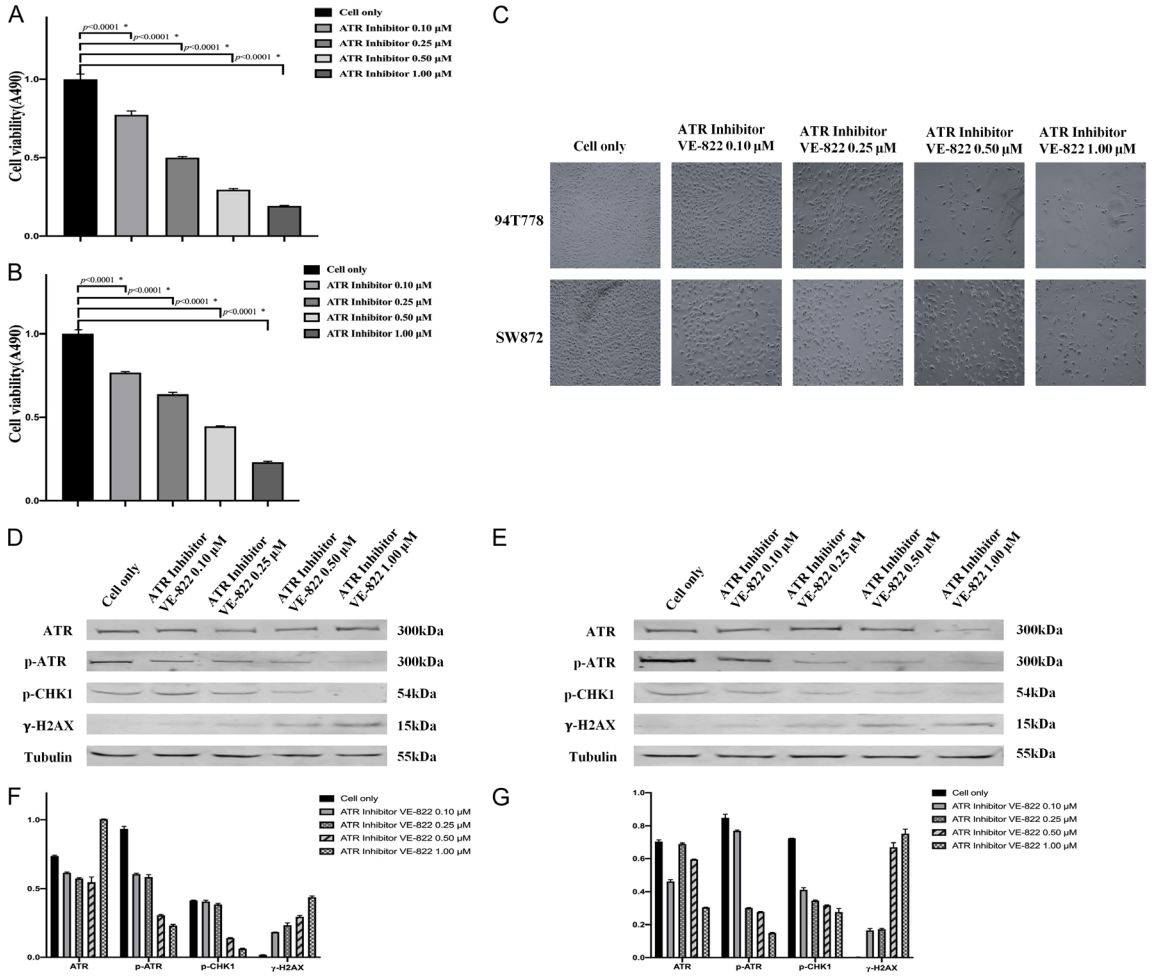

Figure 3. ATR inhibition by VE-822 decreased liposarcoma cell proliferation. (A, B) VE-822, at the indicated concentrations, inhibited liposarcoma cell lines 94T778 (A) and SW872 (B) cell proliferation, determined by MTT assay. The data represent the mean ± SE of 2 experiments carried out in triplicate. (C) Microscopy images of morphologic changes and a reduction in cell number after 72 h of VE-822 treatment. (D and E) The expression of respective proteins ATR, p-ATR, p-CHK1, and γ-H2AX in the ATR-associated signaling pathway was measured by Western blotting in the liposarcoma cell lines 94T778 (D), and SW872 (E) after 72 h of VE-822 treatment. (F and G) Semiquantitative analysis of (D) and (E) by densitometry relative to tubulin. The data are mean ± SE of the experiment carried out in triplicate.

found increasing concentrations of VE-822 decreased the IC_{50} of doxorubicin in both 94T778 and SW872 in a dose-dependent manner (**Figure 6A** and **6E**). The combination of VE-822 and doxorubicin significantly inhibited liposarcoma cell growth and survival compared to those from each treatment alone in SW872 and 94T778 cell lines (**Figure 6B** and **6F**). A synergistic analysis revealed VE-822 had a synergistic anticancer effect alongside doxorubicin in both 94T778 (ZIP synergy score =13.915; **Figure 6C** and **6D**) and SW872 (ZIP synergy score =19.789; **Figure 6G** and **6H**). Thus, our results show that ATR inhibition enhances the efficacy of doxorubicin in liposarcoma cell lines.

Disussion

We found that activated p-ATR is expressed in the majority of liposarcoma patient tissues, and higher expression correlates with a worse prognosis. These findings are consistent with previous reports in other cancer types showing the role of p-ATR in tumor progression [11, 12]. We then investigated the roles of ATR in liposarcoma cell lines with *in vitro* loss-of-function studies using ATR siRNA or the small molecule inhibitor VE-822. A pronounced reduction of cell proliferation, motility, increased apoptosis, and doxorubicin sensitivity occurred in a dose-dependent manner was found with ATR siRNA or VE-822.

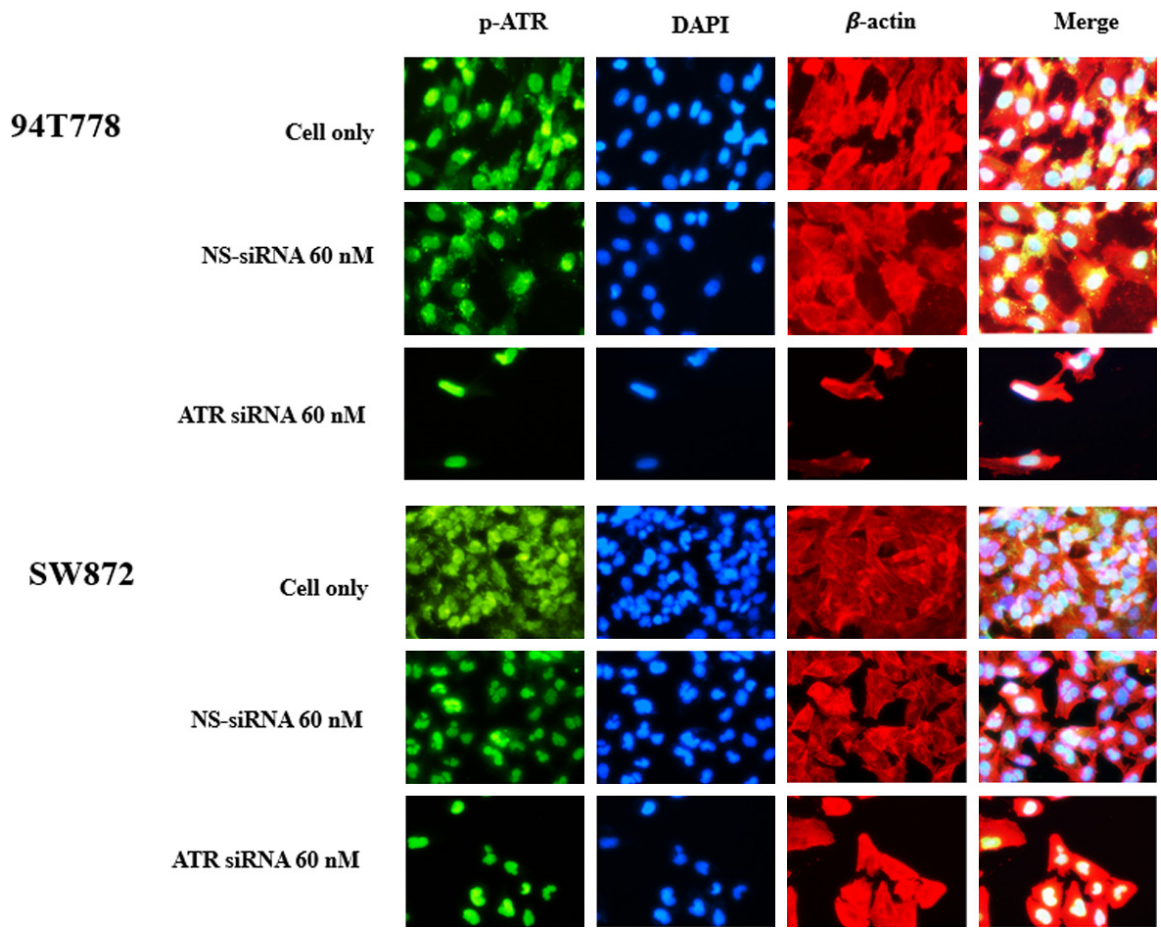

Figure 4. ATR knockdown induces cell death and decreases p-ATR expression as shown by immunofluorescence. p-ATR expression in liposarcoma cell lines, with groups including cells only and transfection with non-specific siRNA (60 nM) or ATR siRNA (60 nM). Immunofluorescence signals include p-ATR (green), β-actin (red in cytoplasm) and Hoechst 33342 (blue in nuclei). The green fluorescence signal indicats p-ATR protein is localized in the nucleus of liposarcoma cells and was clearly inhibited by ATR siRNA.

The antitumor activity we observed occurred in the setting of reduced ATR and pCHK1 expression with a concomitant increase in γ-H2AX. These findings are expected in ATR inhibition, as ATR phosphorylates CHK1 hence stabilizing the genome and allowing for cancer survival [32]. The γ-H2AX protein is a sensitive indicator of DNA damage and replication stress, and detectable in the later stages of apoptosis during DNA fragmentation [22, 33]. As the transcriptional roles of ATR require phosphorylation of CHK1 and γ-H2AX, these proteins are markers of ATR associated DNA damage in experimental settings [12, 18]. Our liposarcoma findings are consistent with previous reports showing similar trends in pCHK1 and γ-H2AX in ATR cancer signaling [12, 15, 16, 22, 32].

ATR is a verified and suppressible target capable of reducing tumor cell proliferation, migration, and invasion [11, 34]. As clonogenic cell survival assays can accurately measure the ability of cancer to rapidly colonize from a single cell [35, 36], we used this method to assess liposarcoma cells treated with VE-822. VE-822 treated 94T778 and SW872 liposarcoma cell lines showed a significantly decreased colony count. We next implemented 3D cell culture to assess the effects of ATR inhibition, as it has been shown to more accurately model tissue and *in vivo* [37, 38]. We found the spheroid diameter of cells treated with VE-822 was significantly smaller compared to the untreated cells. Collectively, our results indicate ATR has roles in the growth and proliferation of liposarcoma cells through attenuating DNA damage.

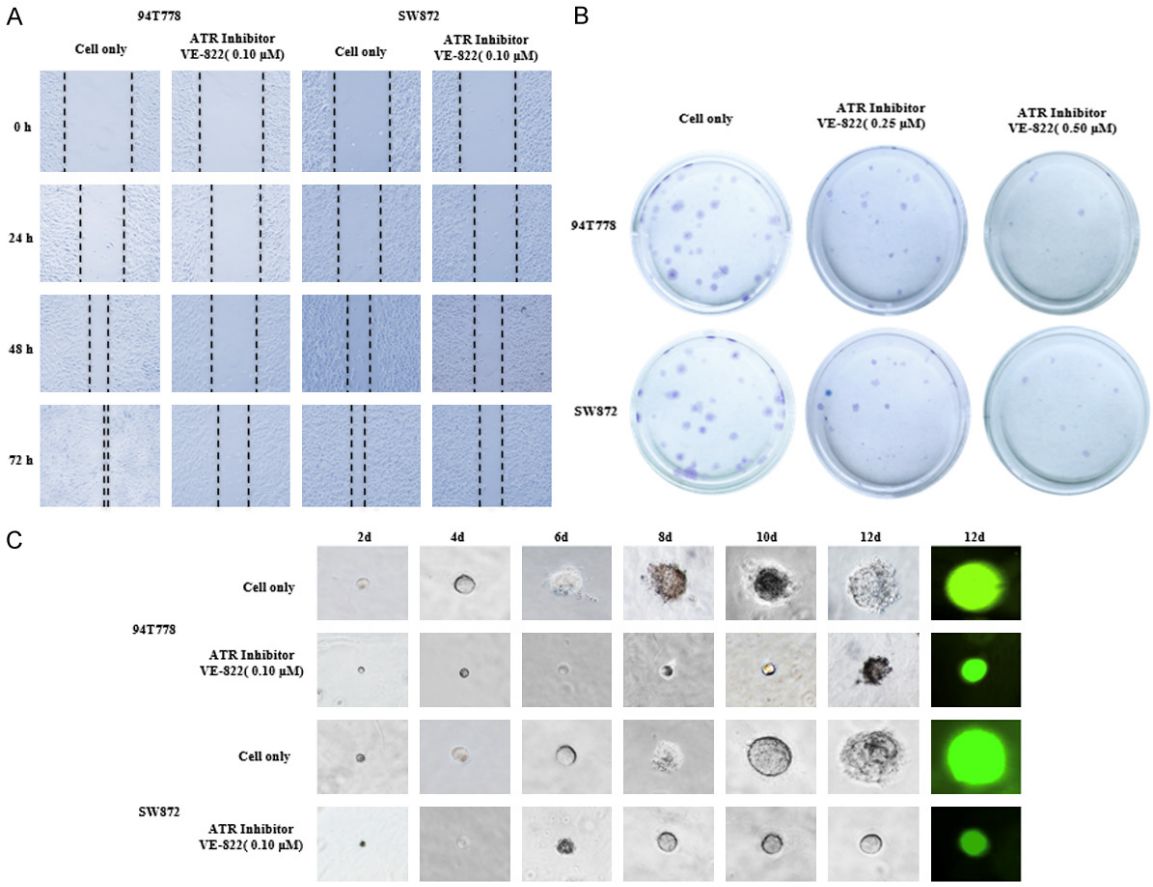

Figure 5. ATR inhibition reduced liposarcoma cell migration and clonogenicity *in vitro* and decreased the spheroid diameter in a 3D cell culture. A. 94T778 and SW872 cell line migration distances after VE-822 treatment for 24 h, 48 h, and 72 h. B. Representative results of colony formation in 94T778 and SW872. The numbers of colonies and their sizes were markedly decreased in cells treated with VE-822. C. Spheroid formation of 94T778 and SW872 were significantly smaller than untreated cells at all observation points. Cell fluorescence images of spheroid formation were recorded after 12 days of cultivation.

Finally, we found that inhibition of ATR with VE-822 sensitized liposarcoma cells to doxorubicin, which is consistent with previous reports on chemotherapeutics such as doxorubicin, 5-FU, gemcitabine, taxanes, cisplatin, topotecan, and PARP inhibitors in other cancers [11, 16, 20, 31, 39-42]. This finding is expected given cancer cells can resist chemotherapeutics by activating ATR associated DNA damage response pathways [43-45]. While ATR inhibitors have shown efficacy as monotherapy in preclinical models, their full clinical value will likely result when they are integrated with established chemotherapeutic regimens or radiotherapy. Recently, a combination of VE-822 with gemcitabine proved to be synergistic in inducing apoptosis in soft tissue sarcomas [28]. This combination therapy increased γ-H2AX expression as a result of DNA damage. *In vivo*, this combination inhibited tumor growth and progression-free survival in a model of undifferentiated pleomorphic sarcoma [28]. Targeting of ATR is also a promising strategy for overcoming resistance to chemotherapeutics such as PARP inhibitors [46-48]. A combination regimen of chemotherapeutics such as doxorubicin with ATR should be considered for clinical trials in liposarcoma.

Doxorubicin is one of the commonly used chemotherapy for the treatment of wide range of malignancies include breast cancer, hematological cancers and sarcomas [49]. Doxorubicin inhibits topoisomerase II enzyme functionality, thus inducing structural damages of DNA. Cancer cells respond to DNA damages by activating the ATR-CHK1 and/or the ATM-HK2 pathway, whose function is to promote damage

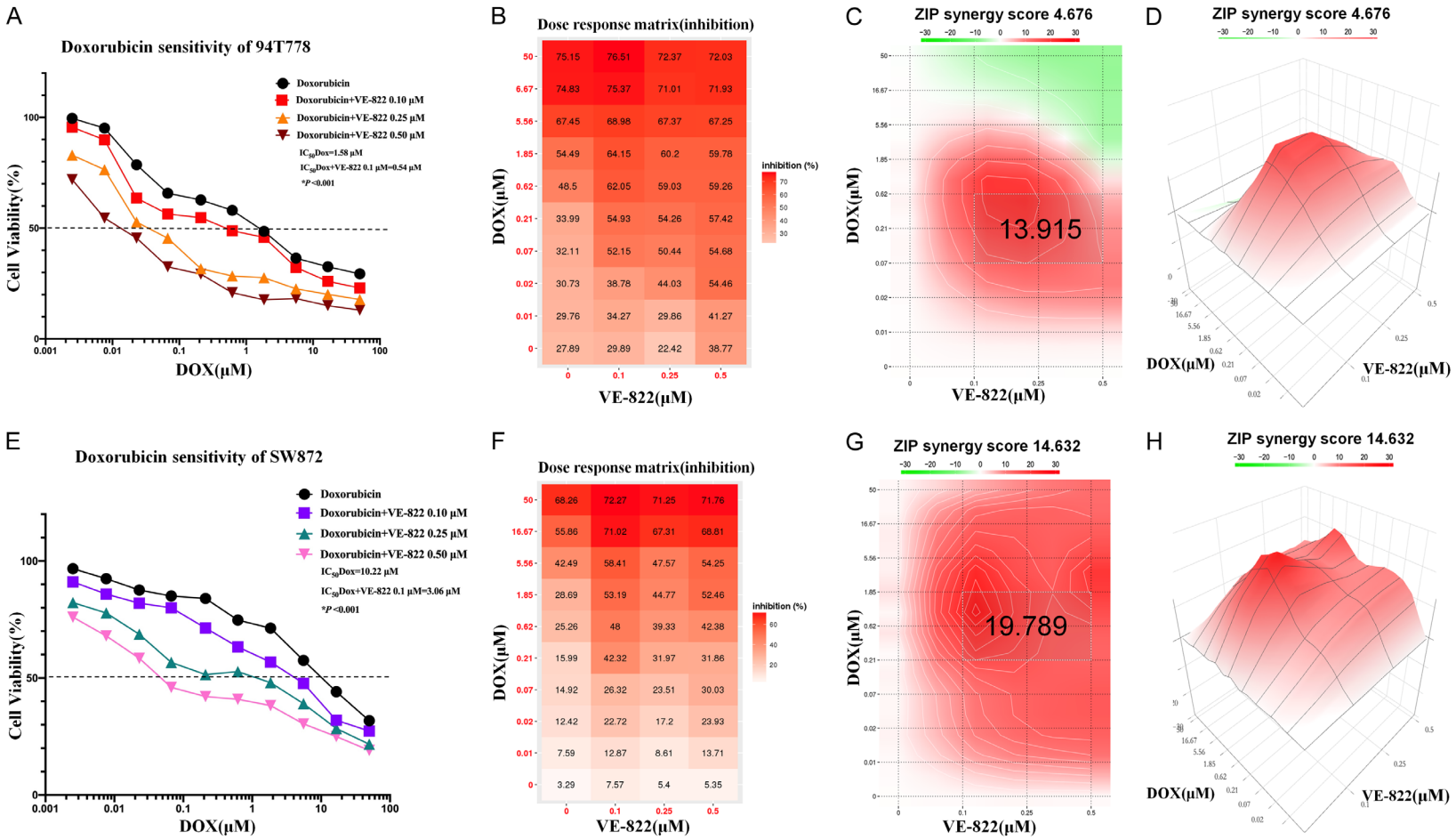

Figure 6. VE-822 is synergistic with doxorubicin in liposarcoma cells. (A and E) Dose-response curve of doxorubicin sensitivity in 94T778 (A) and SW872 (E) treated with different concentrations of VE-822. Cell viability significantly decreased with increasing concentrations of VE-822. The data represent mean ± SD of the independent triple experiment. (B and F) Dose-response matrix of doxorubicin combined with VE-822 in 94T778 (B) and SW872 (F), as analyzed by SynergyFinder 2.0. (C and G) Two-dimensional synergy map showing synergistic effects of VE-822 combined with doxorubicin in 94T778 (C) and SW872 (G) with Zero Interaction potency (ZIP) scores. The most synergistic area in the interaction map was 0.1-0.5 μM of VE-822 and 0.07-0.62 μM of doxorubicin in 94T778 (C), with ZIP score 13.915; and 0.1-0.5 μM of VE-822 and 0.21-1.85 μM of doxorubicin in SW872 (G) with ZIP score 19.789. (D and H) Three-dimensional synergy illustration depicting the results from (C and G).

repair, and to control apoptosis [50, 51]. However, a major side-effect of doxorubicin treatment is a dose-dependent cardiotoxicity. New formulations and novel combination strategies have been developed to enhance the clinical efficacy doxorubicin and decrease the side-effect of toxicity. Improved formulations, such as the doxorubicin HCl liposome (Doxil), increase doxorubicin internalization in cancer cells while reducing the amount of drug needed to obtain therapeutic efficacy. Doxil demonstrated favorable toxicity profiles with better cardiac safety and less myelosuppression compared with the conventional doxorubicin [49]. A recent study demonstrated that doxorubicin followed by ATR-CHK1 inhibitors can increase doxorubicin cytotoxicty against acute lymphoblastic leukemia (ALL) cells, while using lower drug doses. This phenomenon was associated with the abrogation of the G2/M cell cycle checkpoint with changes in the expression of pCDK1 and cyclin B1, and cell entry in mitosis, followed by the induction of apoptosis [50]. Several phase I/II trials of ATR inhibitor VE-822 in combination with radiotherapy or chemotherapy are currently ongoing [20, 21, 52-55]. The results from these ongoing and future clinical trials will help to elucidate the role of ATR inhibitors in alleviate potential doxorubicin side effects.

In summary, this is the first study to demonstrate the overexpression and function of ATR in liposarcoma. Elevated p-ATR expression was associated with worse outcome, and is therefore a promising prognostic and predictive biomarker for liposarcoma. Inhibition of ATR significantly decreased cell growth and motility in liposarcoma cell lines, and had a synergistic effect in combination with doxorubicin. Our findings suggest that ATR is a potential prognostic biomarker and therapeutic target for liposarcoma and warrants future investigation.

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Disclosure of conflict of interest

None.

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