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Qi Shi, Luyan Shen, Bin Dong, Hao Fu, Xiaozheng Kang, Yongbo Yang, Liang Dai, Wanpu Yan, Hongchao Xiong, Zhen Liang, Keneng Chen

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Abstract

The activation of ATM (ataxia-telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related), is essential for DNA damage repair and the maintenance of genomic stability. Therefore, ATM or ATR inhibition is considered as a promising strategy for sensitizing cancer cells to chemotherapy. This study is aimed to explore the effect of ATR inhibitor on sensitizing ESCC (esophageal squamous cell carcinoma) cells to cisplatin, and whether ATM deficiency could impact the sensitization. We found that 21.5% of ESCC cases had ATM deficiency and that patients with ATR activation after neoadjuvant chemotherapy had worse chemotherapy response and poorer overall survival than that without ATR activation (32 mons vs. >140mons). Then, it was shown that VE-822 inhibited CHK1 activation, leading to the accumulation of cisplatin-modified DNA. And it inhibited cell proliferation, induced cell cycle arrest in G1 phase and enhanced cell apoptosis. Moreover, VE-822 significantly sensitized ESCC cells to cisplatin, and these two drugs had synergistic effects, especially in ATM-deficient cells, in vitro and in vivo. Our results suggest that ATR inhibition combining with cisplatin is a new strategy for managing patients with ESCC, especially patients with ATM-deficiency.

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The identification of the ATR inhibitor VE-822 as a therapeutic strategy for enhancing cisplatin chemosensitivity in esophageal squamous cell carcinoma

Running title: ATR inhibitor sensitized ESCC cells to chemotherapy

Qi Shi[1*], Luyan Shen[1*], Bin Dong[2], Hao Fu[1], Xiaozheng Kang[1], Yongbo Yang[1], Liang Dai[1], Wanpu Yan[1], Hongchao Xiong[1], Zhen Liang[1], Keneng Chen[1].

Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), 1Department of Thoracic Surgery I, 2Department of Pathology, Peking University Cancer Hospital & Institute, Beijing, People's Republic of China

*Qi Shi and Luyan Shen contributed equally to this work

Corresponding author: Keneng Chen, Department of Thoracic Surgery I, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Peking University Cancer Hospital & Institute, No. 52, Fucheng Rd, Haidian Dist, Beijing 100142, P.R. China. Phone: 86-10-88196536; Fax: 86-10-88196526; E-mail: chenkeneng@bjmu.edu.cn.

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Abstract

Inducing DNA damage is known to be one of the mechanisms of cytotoxic chemotherapy agents for cancer such as cisplatin. The endogenous DNA damage response confers chemoresistance to these agents by repairing DNA damage. The initiation and transduction of the DNA damage response (DDR) signaling pathway, which is dependent on the activation of ATM (ataxia-telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related), is essential for DNA damage repair, the maintenance of genomic stability and cell survival. Therefore, ATM or ATR inhibition is considered as a promising strategy for sensitizing cancer cells to chemotherapy. This study is aimed to explore the effect of ATR inhibitor on sensitizing ESCC (esophageal squamous cell carcinoma) cells to cisplatin, and whether ATM deficiency could impact the sensitization. We found that 21.5% of ESCC cases had ATM deficiency and that patients with ATR activation after neoadjuvant chemotherapy had worse chemotherapy response and poorer overall survival than that without ATR activation (32 mons vs. >140mons). Then, it was shown that VE-822 inhibited ATR-CHK1 pathway activation, leading to the accumulation of cisplatin-modified DNA. And it inhibited cell proliferation, induced cell cycle arrest in G1 phase and enhanced cell apoptosis. Moreover, VE-822 significantly sensitized ESCC cells to cisplatin, and these two drugs had synergistic effects, especially in ATM-deficient cells, in vitro and in vivo. Our results suggest that ATR inhibition combining with cisplatin is a new strategy for managing patients with ESCC, especially those with ATM-deficiency. However, this is an idea that requires further validation.

Highlights:

- ATR activation was associated with efficacy of neoadjuvant

chemotherapy.

- VE-822 promoted cell apoptosis and induced cell cycle arrest in ESCC cells.
- VE-822 sensitized ESCC cells to cisplatin, in vitro and in vivo.
- VE-822 along with cisplatin induces DNA-cisplatin adduct accumulation.

Key words: DNA damage response; chemoresistance; ATM deficiency; ATR inhibitor

1. Introduction

Esophageal carcinoma (EC) is the 8th most common cancer worldwide[1]. EC ranks as the 6th most common cause of cancer-related morbidity and the 4th most common cause of cancer-related mortality in China[2]. EC comprises the following two major pathological types: adenocarcinoma and squamous cell carcinoma. The majority of ESCC cases occur in Asia, particularly in north China. Surgery is known as the main treatment for ESCC, but long-term survival of patients with advanced disease remains poor and unsatisfactory. Currently, the development of comprehensive perioperative therapies has greatly improved the efficacy of ESCC treatment, especially for long-term survival. Platinum-based combination regimen is used most frequently in the clinical practice; however, previous studies have demonstrated that the improved efficacy associated with neoadjuvant therapy is limited to patients who respond to chemotherapy, and the prognosis of non-responders is worse compared with that of patients who received surgery alone[3, 4], probably because of chemotherapy resistance, which is inevitable. Thus, new approaches to conquering chemoresistance to improve chemotherapy effectiveness are urgently needed.

Platinum-based drugs cross-link with double-stranded DNA and form DNA-platinum abduct to induce DNA damage, leading to cell apoptosis[5]. Due to the intrinsic DDR mechanism, the DNA damage could be repaired. DDR initiation relies on the activation of two major kinase systems, namely, ATR/CHK1 and ATM/CHK2 pathways. Sequentially activated ATR and ATM directly phosphorylate the kinases CHK1 and CHK2, respectively, to activate the downstream effectors such as p53 to upregulate cell cycle checkpoint pathways and then repair the DNA damage[6].Therefore, DDR is an important chemoresistance mechanism through which tumor cells escape from DNA damage induced by genotoxic agents and thus avoid cell death[7-10]. It has been reported that many malignant cancers are characterized by the functional loss or deficiencies in key proteins involved in the DDR, most notably ATM and p53[5, 6, 11-14]. ATM or p53 deficiency in cells leads to synthetic lethality in the presence of ATR depletion[15-19]. Therefore, ATR blockades are considered as promising therapeutic targets, as ATR inhibition may have deleterious effects on cancer cells.

Previous studies have demonstrated ATR inhibition is effective for treating cancers combining with chemotherapies in lung adenocarcinoma, gastric cancer, HER2 positive breast cancer and chronic lymphocytic leukemia cells to enhance chemotherapy sensitivity[16-18, 20-22]. VE-822 is an orally, highly specific ATR inhibitor, which has been entered clinical trials. However, we have less knowledge about not only the effect of ATR inhibition in ESCC, but also whether it enhance the chemotherapy sensitivity.

In this study, we firstly examined ATM expression status in ESCC and analyzed the association between ATR activation with chemotherapy response evaluated with TRG and overall survival of patients who underwent neoadjuvant chemotherapy. Then, we investigated the effect of VE-822 or combination with cisplatin in ESCC in vitro and in vivo with the context of

103 endogenous ATM activation or ATM deficiency by CRISPR. It was
104 demonstrated that VE-822 could block the activation of ATR, which
105 consequently increase DNA damage and sensitize tumor cells to
106 chemotherapy, with presenting the synthetic lethality effect, especially when
107 ATM was deficient.
108
109 **2. Materials and methods**
110 *2.1 Patients*
111 All data of the patients included in this study were retrieved form our
112 prospective database for esophageal cancer, which is established beginning in
113 January 2000 at the Department of Thoracic Surgery, Peking University
114 Cancer Hospital (Beijing, China). From January 2000 to December 2012, 954
115 cases of esophageal cancer underwent surgery, of which, 651 cases were
116 diagnosed as ESCC. According to strict entry criteria, 144 patients who
117 underwent esophagectomy followed by adjuvant chemotherapy and 110
118 patients who underwent neoadjuvant chemotherapy followed by
119 esophagectomy were enrolled in this study. The detailed clinicopathological
120 characteristic of patients were listed in supplementary information. The study
121 was approved by the Ethics and the Academic Committees of Peking
122 University Cancer Hospital (Beijing, China) and informed verbal consent was
123 obtained from all patients.
124
125 *2.2 Chemotherapy and surgery methods*
126 One hundred and forty-four patients underwent esophagectomy first. After
127 4-6 weeks, they were treated with adjuvant chemotherapy including
128 platinum-based double drug regimen, mainly of which are the paclitaxel and
129 cisplatin at the proportion of 95%. One hundred and ten patients were treated
130 by neoadjuvant chemotherapy including platinum-based double drug

combination, mainly the paclitaxel and cisplatin with the proportion of 95%. The curative effects of the treatment were evaluated by enhanced chest computed tomography (CT) and esophagography. Approximately 1-4 cycles of neoadjuvant chemotherapy were administered before surgery. Surgery was carried out 3-5 wk. after neoadjuvant chemotherapy. Chemotherapy regimen was as follows: On day 1, paclitaxel at a dose of 175 mg/m^2 of body surface area was administered intravenously. On day 1-3, cisplatin at a dose of 25 mg/m^2 of body surface area was administered intravenously, a single course of treatment lasted 21 days.

2.3 Tumor regression grade assessment (TRG)

H&E staining results of all the enrolled subjects were reviewed by two experienced pathologists who were blinded to the clinical information and associated issues. Tumors were graded by TRG which was a four-point scale based on the histological tumor response assessment[23]. This assessment was described as: grade I, no residual tumor cells; grade II, nearly complete response with <10% vital residual tumor cells (VRTCs); grade III, 10-50% VRTCs; and grade IV, >50% VRTCs.

2.4 Follow-up

Follow-up evaluation consisted of outpatient interviews at 3-month intervals for 2 years, then at 6-month intervals for 3 years, and finally at 12-month intervals until death. Outpatient follow-up visits included recording of symptoms and findings of body examinations such as CT, upper esophagography, ultrasound, and gastroscopy, if necessary. After 2010, some subjects underwent positron emission tomography-computed tomography (PET-CT) examinations. Overall survival (OS) was measured from surgery

158 date until death or the last follow-up. The latest follow-up was June 1st, 2017
159 at the rate of 93%.
160
161 *2.5 Immunohistochemistry (IHC)*
162 Specimens of the included patients were retrieved from department of
163 pathology, Peking University Cancer Hospital. After routine deparaffinization
164 and hydration, tissue sections were treated with 3% hydrogen peroxide and
165 then heated in citrate solution for antigen retrieval. After antigen retrieval, the
166 sections were incubated with 10% normal goat serum to block any nonspecific
167 reaction. Then, the sections were incubated with rabbit monoclonal anti-ATR
168 (phospho S428) antibody (Abcam, ab178407, at 1:500) or mouse monoclonal
169 anti-ATM antibody (Abcam, ab78, at 1:1000) overnight at 4°C. Dako REAL
170 EnVision Detection System, Peroxisase/DAB, Rabbit/Mouse (K5007), was
171 used as the secondary antibody and for staining. The immunohistochemical
172 signals were scored by two independent pathologists. To evaluate ATR-pS428
173 and ATM expressions, immunohistochemical staining was classified into the
174 following four groups according to intensity. The staining intensity was
175 categorized by relative intensity as follows: 0, negative; 1, weak; 2, moderate;
176 and 3, strong.
177
178 *2.6 Cell lines and cell culture*
179 Human ESCC cell lines KYSE450, KYSE150, KYSE70, KYSE180, and
180 KYSE 510 were purchased from the Japanese Collection of Research
181 Biosources cell bank (Osaka, Japan). Identities of the cell lines were confirmed
182 by standard STR analysis matched with the American Tissue Culture
183 Collection (ATCC) and Deutsche Sammlung von Mikroorganismen und
184 Zellkulturen GmbH (DSMZ). All cells were passaged for less than 1 year
185 before use and cultured in RPMI-1640 medium (Hyclone; GE Healthcare,

Logan, UT, USA) with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin solution at 37°C in a humidified atmosphere containing 5% CO_2. ATM-deficient ESCC cells were established in KYSE450, KYSE70, KYSE180 and KYSE150 by CRISPR.

2.7 CRISPR/cas9 plasmids and virus infection

Establishment of ATM-deficient ESCC cells by lenti-CRISPR/CAS9 vectors was associated with the following sequences: GTTTCAGGATCTCG AATCAGG/CAAGGAAAATATTTGAATTGG. 5×10^6 cells were seeded in a 10cm dish overnight at 37°C and virus in the presence of polybrene (8μg/ml, Sigma, Japan) were added to KYSE cell lines. For selection, using puromycin (2 μg/ml, Beyotime Biotechnology, China) to treat cells for 3 weeks to eliminate uninfected cells.

We used PCR and DNA-seq for validation of ATM knockdown. Total DNA from KYSE70, KYSE450 and their ATM knockout cells was extracted with TIANamp Genomic DNA Kit (TIANGEN, DP304; China). The sequences of the PCR primers were as follows: ATM forward, 5'- CTGCTTATCTGCTGCCGT-3' and reverse, 5'- GTTTGCCACTCCTGTCC-3'; GAPDH forward, 5'-GTTTGCCACTCCTGTCC-3'; and reverse, 5'-GGCATGGACTGTGGTCA TGAG-3'. Then, we authorized Microread Gene Company (Beijing, China) for DNA-seq.

2.8 Regents

Cisplatin and ATR inhibitor VE-822 were purchased form Sigma (479306) and Selleck (S7102), respectively. Cisplatin were dissolved in normal saline as 1 mg/ml and VE-822 was dissolved in DMSO as 10mM.

2.9 Cisplatin modified DNA accumulation test

Cisplatin modified DNA accumulation was examined using flow cytometry (BD, Biosciences) by anti-cisplatin modified DNA antibody (Abcam, ab103261). Cells in culture were treated with cisplatin, VE-822, or combination of these two drugs, with un-treated cells as control. Cells were fixed with 70% ehtanol for 30 min at 4°C and permeabilized with 0.3% TritonX-100 in PBS. The cells were incubated with the primary antibody (1:200) for 18 hours at 4°C. A rabbit anti-rat IgG/Alexa Fluor 488 (Bioss, 1/100) was used as the secondary antibody. An isotype control used Rat IgG2a kappa monoclonal (Abcam, ab18450), simultaneously.

2.10 Western blotting

The proteins were extracted by using RIPA lysis buffer and separated by 10% SDS-PAGE with 30μg protein per lane and transferred onto a polyvinylidene uoride membrane, followed by western blot analysis. The membrane was blocked using 5% bovine serum albinum at room temperature for 1 h. It was then immunoreacted with, ATM, p-ATM (S1981), ATR, p-ATR (Ser428), Chk1, p-Chk1, Chk2, p-Chk2, p53, p21, p-STAT3, STAT3, p-AKT, AKT, caspase-3, p-histone H2A.X and PARP were acquired from Cell Signaling Technology(America). GAPDH (ZSGB-BIO, China) were also purchased. Goat anti-rabbit or anti-mouse polyclonal IgG was used as a secondary antibody.

2.11 Cell proliferation assay (CCK-8 assay)

Five thousand cells per well were plated in 96-well plates overnight at 37°C and treated with gradient dilution of cisplatin (0.125, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 μg/ml) or VE-822 (0.125, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 μM), or combination of cisplatin (0.125, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 μg/ml) and VE-822 (2 μM). After 48h of incubation, 10μl CCK-8 reagent (Dojindo

Molecular Technologies Inc., Kumamoto, Japan) was added to each well about 2h at 37°C. Then, the absorbance of each well was examined at 450 nm by the Microplate reader (iMark, Bio-rad, USA).

2.12 Cell cycle analysis

Cells at a density of $1*10^6$ per well were plated in 6-well plates overnight at 37°C and treated with cisplatin, or VE-822 or combination for 24h until cells were harvested. Cells at a density of $1*10^6$ were collected and fixed with 70% cold ethanol overnight at 4°C. After fixation, cells were washed 3 times in PBS. Then, the PI-staining solution with RNase A (BD Biosciences) was added about 30 min in room temperature to stain samples and were run on the FACScan cytometry (BD Biosciences, America), and data were analyzed using FlowJo software (Tree Star).

2.13 Apoptosis analysis in vitro

Cells at a density of $1*10^6$ per well were plated in 6-well plates overnight at 37°C and treated with cisplatin, or VE-822 or combination for 8h until cells were harvested. Cells were washed 3 times in PBS and incubated in trypsin (without EDTA) at 37°C for 10min. Cells were rinsed 3 times in PBS and re-suspended in binding buffer (Dojindo, Japan). Annexin V-FITC antibody (Dojindo, Japan, 5 μl) and PI (Dojindo, Japan, 5 μl) were added in cells (1×10^5cells/100μl) and incubated for 15 min at room temperature in the dark. Then, the samples were analyzed by flow cytometry (BD Biosciences, America) within 1 h.

2.14 Immunofluorescence（γ-H2AX and p53 foci formation）

Before the assay, the cells treated with 2μg/ml cisplatin, 2μM VE-822 and the combination of these two drugs for 24h. The coverslips were rinsed 3 times

in PBS, fixed in 3.7% paraformaldehyde for 15min, permeabilized with PBST (0.5% Triton X-100 in PBS) for 5min and blocked specimen in blocking buffer (5% normal serum in PBST) for 60min at room temperature. After washing 3 times in PBS, the coverslips were incubated with phospho-Histone H2A.X (Ser139) primary antibody (#9718, Cell Signaling Technology; America) at a dilution of 1:200 and p53 primary antibody at a dilution of 1:2000 (#2524, Cell Signaling Technology; America) overnight at 4°C. Then, the coverslips were washed 3 times in PBS and incubated with the appropriate fluorophore-conjugated secondary antibody: TRITC for γ-H2A.X (tetramethylrhodamine goat anti-rabbit IgG, Invitrogen, American) and FITC for p53 (fluorescein goat-mouse IgG, Invitrogen, American) for 1h at room temperature in the dark. Finally, the cells were counterstained with DAPI (300 nmol/l; Invitrogen, American). Immunofluorescence was visualized by Zeiss scanning microscope(Germany).

2.15 RNA-seq library construction and sequencing

Total RNA from KYSE450 and KYSE450 treated with cisplatin (1μg/ml) and VE-822(1μM) (named KYSE450.1) with three replications was isolated using Trizol for the construction of a RNA-seq library and sequencing. The construction of RNA-seq library was performed using the KAPA Stranded mRNA-Seq Kit (Illumina® platform) (product codes KK8420 and KK8421, Boston, Massachusetts, United States) following the manufacturer's instructions. Briefly, mRNA was extracted and purified from total RNA ,then fragmented and primed for cDNA synthesis. Double-stranded cDNAs were synthesized and then purified with 1.8× Agencourt AMPure XP beads (Beckman Coulter, Beverly, USA) followed by the end 2nd Strand Synthesis. After A-Tailing, Illumina adapter oligonucleotides were ligated to cDNA fragments, and the 1X SPRI® cleanup was performed. Suitable cDNA

fragments were selected as templates for PCR amplification using the KAPA Library Amplification Primer Mix and KAPA HiFi Hot Start Ready Mix. Products were purified with the AMPure XP bead system and quantified using a Bioanalyzer (Agilent high sensitivity chip). Finally, RNA-seq libraries were sequenced using an Illumina HiSeq at Beijing Microread Genetics Co Ltd (Beijing, China). Raw data were processed with Fastp using recommended parameters.The filtered reads were mapped to Hg19 by hista2 . The bam files were processed with samtools. Feature Counts was used to calculate gene expression. A list of differential expression genes (DEGs) was identified using the R packages" EdgeR". P-value of 0.05 and |log2(foldchange)|>2 were set as the threshold for significantly differential expression by default. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed genes were implemented with KOBAS3.0. GO classification was done by the R packages "TopGO".To further validate the RNAseq results, we selected 10 DEGs of our interests to examine their expression in both samples by using qRT-PCR. The gene-specific primers for these twenty genes are listed in Table S1.

2.16 Primary ESCC xenograft models

BALB/c nude mice were raised under the care of the Laboratory Animal Unit of First Affiliated Hospital of PLA General Hospital, China. KYSE70, KYSE70 ATM (-), KYSE450, and KYSE450 ATM (-) (3×10^6) cells suspending in 200 μl PBS were injected into the right groin of 8 weeks old female Balb/C nude mice. All mice were housed and raised under specific pathogen-free conditions. The sizes of tumors and body weight of each mouse were measured every 3 days. Tumor volumes were calculated using the following formula: tumor volume = [(length) × (width) × (width)] / 2. When the tumor volume reached about 200 mm^3, the mice were divided into two groups

326 randomly (6 mice per group). First group of mice were given 5mg/kg cisplatin
327 on days1, 4, 7, 10 via intravenous injection. The second group were given 60
328 mg/kg VE-822 on days 1, 3, 5 via oral gavage. The third group were given both
329 of cisplatin and VE-822, the last group were given PBS as control. At the end
330 of the measurement period, excised tumors were measured by a slide caliper
331 for volume and weighted by an electronic analytical balance. All experiments
332 were done in accordance with institutional standard guidelines of Peking
333 University Cancer Hospital and Unit of First Affiliated Hospital of PLA General
334 Hospital for animal experiments.
335
336 *2.17 Statistical analysis*
337 SPSS software (version 24.0; IBM SPSS, Armonk, NY, USA) was used to
338 perform the statistical analysis. The relationships between ATR-pSer428 and
339 ATM expression and clinicopathologic characteristics were tested using
340 Chi-square test. Survival curves were plotted by Kaplan-Meier method and
341 compared by log rank test. The association between gene expression and
342 TRG were evaluated using a χ^2 test. All in vitro experiments were performed at
343 least 3 times with triplicates. Comparisons between groups for statistical
344 significance were performed with a 2-tailed unpaired Student's t test. Bars and
345 error bars on the graphs as well as data in the text represent the mean ± SD. P
346 < 0.05 was considered statistically significant.
347
348 **3. Results**
349 ***3.1 ATM protein was deficient in 21.5% ESCC patients***
350 Many studies have reported that various tumors had ATM deficiency with
351 different degrees. Therefore, we examined the expression rate of ATM through
352 IHC in 144 ESCC patients' samples without preoperative treatment(Fig 1a).
353 The result showed that 21.5%(31/144) of cases were ATM expression

negative. ATM expression was not associated with the clinicopathologic factors such as age, gender, pathologic stage and tumor location as well as overall survival (Fig S1, Table S2).

3.2 ATR activation was associated with efficacy of neoadjuvant chemotherapy

ATR-pSer428 is the active form of ATR protein. ATR-pSer428 expression was examined in a cohort of 110 ESCC patients underwent neoadjuvant chemotherapy. Then, we evaluated the relationship between the activation of ATR (substituted by the expression of ATR-pSer428) in resected specimens and overall survival or TRG. The expression of ATR-pSer428 in ESCC mainly occur in nucleus. Among the 110 subjects in the study, 25.5% (28/110) cases were ATR-pSer428 negative, whereas 74.5%(82/110) cases were ATR-pSer428 positive. Then, we divided the patients with ATR-pSer428 expression into subgroups according to ATR-pSer428 expression intensity, which was graded 0, 1, 2, or 3 (Fig 1b). We found that 25.5% (28/110), 17.3% (19/110), 32.7% (26/110), and 24.5% (27/110) of patients displayed grade 0, 1, 2 and 3, respectively. Kaplan-Meier analysis of the 110 subjects showed that more than half of the subjects without ATR-pSer428 expression survived until the follow-up endpoint (144 months), whereas the median survival time for ESCC patients with ATR-pSer428 expression was only 32 months ($P < 0.05$). The median survival time of patients with grade 1, 2, and 3 were 38, 28 and 28 months, respectively. More than half of the subjects with grade 0 expression survived until the follow-up endpoint (144 months) ($P < 0.05$) (Table 1, Fig 1c). We further defined grades 0 and 1 as low-level expression and grades 2 and 3 as high-level expression. Thus, 42.7% (47/110) of patients had low-level expression of ATR-pSer428, and 57.2% (63/110) of patients had high-level expression of ATR-pSer428. Half of the patients with low-level expression of

ATR-pSer428 survived until the follow-up endpoint (144 months). The median survival time of the patients with high-level expression of ATR-pSer428 was 28 months ($P < 0.05$) (Table 1, Fig 1c).

TRGs is currently the standard pathological indicators of neoadjuvant chemotherapy responsiveness. In this study, we found that ATR-pSer428 expression was significantly associated with TRGs. ATR-pSer428 expression in subjects with TRGs 2/3/4 was higher than that in subjects with TRGs 1 ($P < 0.001$) (Table 2). That is, patients with ATR activation after chemotherapy display an unfavorable response to chemotherapy compared with those without ATR activation, indicating that ATR-pSer428 expression can be used to determine chemotherapy sensitivity.

3.3 DDR signaling pathway was activated in ESCC cell lines and was inhibited by VE-822, which also inhibited cell proliferation

To assess the baseline of ATM/ATR signaling pathway activation, we performed western blotting to examine the expression of phosphorylated ATM, ATR, CHK1, and CHK2, which serve as surrogate markers for ATR and ATM pathway activation, and the expression of the downstream target p53 in KYSE70, KYSE150, KYSE180, KYSE450, and KYSE510 cell lines. The results showed that p-ATM, p-ATR, p-CHK1, p-CHK2 and p53 were endogenously activated in all ESCC cell lines (Fig 2a and Fig S2). Based on these results, we inferred that ATM/ATR signaling pathway activation is essential for overcoming replication stress and sustaining tumor cell genomic stability. In these five cell lines, the baseline ATR activation was stronger in KYSE450 and KYSE70 than other cell lines.Then, we used CCK-8 assay to investigate the effect of VE-822 on cell viability. Forty-eight hours of treatment with VE-822 robustly inhibited ESCC cell viability. The half maximal inhibitory concentration (IC50) were 3.982, 11.870, 2.606, 6.922 and 9.387 in KYSE450,

KYSE150, KYSE510, KYSE180 and KYSE70 cells, respectively (Fig 2b). We chose two ESCC cell lines, namely, KYSE70 and KYSE450, for the ATR inhibition experiments. We found that p-ATR and p-CHK1 activation was significantly inhibited by the ATR inhibitor VE-822. We also found that DNA damage remarkably accumulated and γ-H2AX fluorescence intensity amplified, as detected by Western Blotting (Fig 2c). In addition, VE-822 at least partially inhibited cancer cell proliferation through Stat-Akt signaling pathway inhibition (Fig 2d).

3.4 VE-822 promoted cell apoptosis and induced cell cycle arrest in ESCC cells

ATR activation is a key step in DDR initiation, as ATR-pSer428 induces activation of DNA damage checkpoint signaling, which induces cell cycle arrest. ATR plays an important role in the G2-M phase transition. Consistent with this finding, we found that the cell cycle was arrested in G1 phase in a concentration-dependent manner after the cells being exposed to increasing concentrations of VE-822 (1.0 μM, 2.0 μM, and 4.0 μM) for 24 h. As Fig 2e shown, VE-822 increased the fraction of cells in G1 phase to 68.48% (compared with baseline 52.62%) in KYSE70 cells and 42.22% (compared with baseline 37.48%) in KYSE450 cells when administered at a dose of 4.0 μM. VE-822-induced cell cycle arrest also led to the downregulation of p21 expression (Fig 2f).

To determine the effect of VE-822 on cell apoptosis, we examined the ratio of apoptotic cells to live cells using flow cytometry. VE-822 induced a significant concentration-dependent increase in cell apoptosis in both KYSE70 and KYSE450 cells after 8 h of treatment (Fig 2g). We also examined the expression of caspase-3, cleaved PARP by western blotting, as the indicated

proteins are biomarkers for cell apoptosis. The results showed that caspase-3 and cleaved PARP expression was increased by exposure to VE-822 (Fig 2g).

3.5 VE-822 sensitized ESCC cells, especially ATM-deficient cells, to cisplatin in vitro and in vivo

Cisplatin is commonly used as a first-line chemotherapy for patients with ESCC. However, chemoresistance inevitably occurs spontaneously or develops during treatment. Previous studies have demonstrated that ATR kinase inhibitors could enhance the sensitivity of cancer cells to DNA damaging agents, such as cisplatin, in solid-tumor models both in vitro and in vivo. To validate the hypothesis that ATR inhibition enhances the efficacy of cisplatin in ESCC, we performed CCK-8 assay to evaluate the viability of cells exposed to cisplatin, VE-822 or the combination of cisplatin and VE-822 and to determine the effect of these treatments on tumor growth in ESCC mouse xenograft models. The combination of ATR inhibition and cisplatin synergistically inhibited cell viability in KYSE 450 and KYSE70 cells. The combination index(CI) values were calculated according to the Chou-Talalay[24] median-effect principle. As shown in the figure 3a, there is significant synergistic effect between VE-822 and cisplatin. Then, to investigate whether ATM deficiency affect the efficacy of VE-822, we knockout ATM expression by using CRISPR and established stable cell strain by screening (Fig 3b). And we found that IC50 of VE-822 was decreased significantly in ATM (-) cells (Fig 3c) and more striking synergistic inhibition combination treatment was observed in ATM-deficient cells than in cells expressing ATM (Fig 3d). Then, we treated the mouse xenograft models with cisplatin (5 mg/kg) on days 1, 3, and 5; VE-822 (60 mg/kg) on days 1, 2, and 3; or combination therapy, with PBS as control. The growth of tumors treated with combination therapy was significantly slower than that of tumors treated with

cisplatin or VE-822 alone. On day 14, the TGI of KYSE70 xenograft tumor were 28.8%、25.9% and 73.3% for cisplatin-alone group, VE-822-alone group and combination group respectively. And on day 16, the TGI of KYSE450 xenograft tumor were 58.7%, 30.6% and 83.2%, respectively. However, the TGI of ATM-deficient KYSE450 xenograft tumor were 38.0%, 48.1% and 85.2% respectively (Fig 3e). The result indicated that ATM-deficiency magnified the effect of VE-822 in KYSE450 cell line. However, body weight loss in mice treated with combination therapy was not significantly greater than that in mice treated with monotherapy (Fig 3f). Furthermore, p-CHK1, p-AKT activation was inhibited, and cleaved-PARP expression was increased as detected by Western Blotting (Fig 3g). The accumulation of cells in S phase was greater in ATM-deficient KYSE450 and KYSE70 cells than control cells treated with VE-822 (Fig 4a). VE-822 reinforced the effects of cisplatin to induce cell apoptosis, especially for ATM-deficient cells. As Fig 4b shown, the combination of VE-822 and cisplatin caused a significant increase in cell apoptosis in both ATM-deficient KYSE450 and KYSE70 cells compared with control cells. Accordingly, caspase-3 and cleaved-PARP expression remarkably increased (Fig 4c). Then, we observed that VE-822 induced accumulated DNA damage and amplified γ-H2AX and p53 fluorescence intensity in wild-type cells (Figure 5a and 5b) and ATM-deficiency cells (Fig 5c and 5d). These results suggested that the combination of VE-822 and cisplatin has therapeutic potential, especially in ATM-deficiency cells.

3.6 ATR inhibition along with cisplatin induces DNA-cisplatin adduct accumulation, especially in ATM (-) ESCC cells

Cisplatin cross-links with DNA to form cisplatin-DNA adducts, causing cells to experience replication stress and undergo apoptosis; however, cells have an endogenous repair mechanism, known as the DDR, to conquer this type of

stress. Inhibitors targeting the DDR can theoretically block the DDR and enhance cisplatin-DNA adduct formation. To explore the mechanism by which VE-822 sensitizes ESCC cells to cisplatin, we used an anti-cisplatin-modified DNA antibody to detect cisplatin-modified DNA accumulation in cells treated with cisplatin, VE-822 or the combination of the two drugs. The results showed that VE-822 combined with cisplatin induced greater accumulation of modified DNA than either agent alone, especially in ATM-deficient ESCC cells (Figure 6). In conclusion, VE-822 sensitized ESCC cells to cisplatin by increasing the DNA damage induced by cisplatin and inducing the enrichment of cisplatin in cells.

4. Discussion

Chemoresistance to platinum-based chemotherapy has been a huge challenge with respect to achieving optimal treatment outcomes in ESCC. DNA damage induced by cisplatin-DNA adducts causes DNA replication stress and triggers the DDR, which is considered an important mechanism for the development of chemoresistance [6]. Previous studies have shown that DNA replication stress induced by genotoxic agents, such as cisplatin, allows inhibitors targeting the DDR pathway to serve as an effective therapy for ESCC. ATM and ATR inhibitors have been entered into clinical trials pertaining to some types of solid cancers. However, whether DDR inhibitors can be used in ESCC treatment is not known. In our study, we found that endogenous DNA replication stress occurs in ESCC cells. p-ATM and p-ATR were endogenously expressed in ESCC cells, and their activation was significantly enhanced upon exposure to cisplatin. This finding serves as an important clue indicating that DDR pathway inhibitors can be used in ESCC treatment.

Interestingly, we found that ATR activation (represented by ATR-pSer428 expression) was associated with a pathologic response to chemotherapy and

poorer long-term outcomes in patients who underwent neoadjuvant chemotherapy, indicating that p-ATR expression may be used as a biomarker for chemoresistance in ESCC. Therefore, we hypothesized that the combination of ATR inhibition and platinum is an effective therapy for ESCC, which relies on ATR signaling to facilitate DNA repair. We found that ESCC cells were sensitized to cisplatin upon exposure to an ATR inhibitor in vitro and in vivo, a finding supported by the data pertaining to cell viability. Additionally, we demonstrated that STAT3 may play a critical role in VE-822-mediated effects, as p-STAT3 expression was remarkably inhibited in KYSE450 and KYSE70 cells treated with the combination of cisplatin and VE-822. In previous study, it has been shown that STAT3 was essential for efficient repair of damaged DNA, which was suppressed when DNA damage response was inhibited[25-27]. To further learn the effect of combination of VE-822 and cisplatin on the transcriptional profiling, we performed RNA-seq in KYSE450 exposure to combination of VE-822 and cisplatin. DEG analysis identified 1163 genes significantly altered in cells bearing treatment, with 555 genes up-regulated and 608 genes down-regulated(Fig S3a,b). These genes were then classified based upon their signaling pathways and biological functions using KEGG analysis(Fig S3c,d) and GO analysis(Fig S3e,f). We found that treatment with the combination of VE-822 and cisplatin caused a wide of pathways changes including Hippo pathway, MAPK pathway, JAK-STAT pathway, PI3K-AKT pathway and platinum resistance pathway, etc. To validate the RNA-seq results, we selected 10 DEGs to examine their expression by RT-PCR. These 10 genes, were enriched into drug resistance related signaling pathways, including p53 pathway, apoptosis, platinum drug resistance, PI3K-Akt signaling pathway[28-31] and JAK-STAT signaling pathway[32]. The expression patterns of selected DEGs in the RNA-seq and RT-PCR were highly similar(Fig S4). It indicated that our RNA-seq results was

549 reliable, and on the other hand, it implied that the dysregulated signaling
550 pathway may be the key to explore the mechanism underlying the
551 sensitization.

552 Moreover, we demonstrated that the combination of cisplatin and VE-822
553 was effective in vivo in KYSE450 and KYSE70 tumor xenograft models but did
554 not affect mouse body weights, suggesting that ATR inhibition is well tolerated.
555 Multiple in vivo studies have established that ATR inhibitors do not exacerbate
556 the toxic effects of multiple genotoxic agents but still synergize with genotoxic
557 therapies.

558 Given that DDR initiation depends on ATM and ATR pathway activation,
559 the presence of ATM may influence the effects of ATR inhibitors. Interestingly,
560 ATM deficiency was detected in 21.5% of patients with ESCC, which
561 motivated us to hypothesize that using an ATR inhibitor in patients with ATM
562 deficiency will lead to synthetic lethality and that patients with ATM deficiency
563 will benefit more from ATR inhibitor treatment. We obtained ATM-deficient
564 ESCC cells by knocking down ATM expression with the CRISPR method. In
565 ATM-deficient KYSE450 and KYSE70 cells, the combination of cisplatin and
566 VE-822 induced cell cycle aberrations. Specifically, more cells accumulated at
567 S phase. This was not the case in wild-type KYSE cells. Both cell lines
568 exhibited a greater apoptotic response following treatment with the
569 combination of VE-822 and cisplatin, as demonstrated by the data showing
570 that caspase-3 expression and PARP cleavage were increased in combination
571 therapy-treated cells. Similar results were observed in the VE-822-alone group.
572 Thus, ATM deficiency may be a predictive biomarker for tumor responses to
573 ATR inhibitor monotherapy and combination therapies. Additionally, previous
574 preclinical studies have identified a series of tumor-specific alterations that
575 affect sensitivity to ATR inhibition. These include defects in the ATM and p53
576 pathways. However, the usefulness of ATM deficiency as a biomarker has not

been extensively examined in ESCC; thus, additional works regarding this issue are required.

Exposure to cisplatin with ATR inhibitor resulted in an increase in cisplatin-DNA adducts, especially in cells with ATM deficiency. This finding indicates that suppressing ATR-Chk1 signaling with VE-822 enhances cisplatin activity by enabling the drug to form DNA adducts. Therefore, VE-822 may increase cisplatin-DNA accumulation by hindering DNA damage repair induced by cisplatin-DNA. On the other hand, in the previous study, the cells treated with VE-822 along with cisplatin had decreased expression of p-glycoprotein. It inferred that VE-822 inhibited the expression of p-glycoprotein to prevent cisplatin efflux, then increasing its concentration[33].

In conclusion, our study revealed that endogenous DDR signaling activation plays a critical role in mediating the cisplatin resistance of ESCC. We determined that the ATR inhibitor VE-822 may be an attractive treatment for ESCC, especially when used in combination with cisplatin, as VE-822 induces significant sensitization to cisplatin. Specifically, VE-822 synergizes with cisplatin in ATM-deficient models of ESCC. Additional works are warranted to explore the possibility that ATM deficiency can serve as a biomarker enabling the prospective identification of patients with ESCC who will benefit most from the combination of ATR inhibition and cisplatin.

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6. Conflicts of Interest

No potential conflicts of interest were disclosed.

7. Author Contribution

Conceived and designed the experiments: Keneng Chen, Luyan Shen. Performed the experiments: Qi Shi, Luyan Shen. Analyzed the data: Luyan Shen, Qi Shi, Bin Dong. Wrote the paper: Luyan Shen, Qi Shi. Patients care, cases provision and data collection: Keneng Chen, Wanpu Yan, Liang Dai, Xiaozheng Kang, Yongbo Yang, Hongchao Xiong, Zhen Liang.

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Figure 1. ATM deficiency occurred in ESCC and p-ATR(Ser428) expression was significantly associated with the overall survival of patients underwent neoadjuvant chemotherapy.

(a). ATM-positive and ATM-negative case.

(b). p-ATR(Ser428) expression pattern by the intensity of positive tumor cells. 0, Negative; grade 1, weak; grade 2, moderate; grade 3, strong.

(c). The association between p-ATR expression and the survival of patients with ESCC underwent neoadjuvant chemotherapy. The Kaplan-Meier survival curve showed that the median survival time (MST) of patients with p-ATR positive expression was significantly shorter than that of patients with negative expression; in the further analysis, with the increase of positive expression cell proportion, the MST of patients was extending; when the p-ATR expression pattern was classified into high expression group and low expression group according to the proportion of positive cells, the MST of high group was significantly shorter than that of low group.

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Figure 2. VE-822 inhibited cell growth, induced cell cycle arrest and cell apoptosis in ESCC cells.

(a). The basal level of ATM-CHK2 and ATR-CHK1 activation examined using Western Blotting in a panel of ESCC cell lines, including KYSE70, KYSE150, KYSE450, and KYSE510, which indicated that baseline ATM-CHK2 and ATR-CHK1 activation was essential for cell survival. The densitometry quantification was presented in Fig S2.

(b). KYSE70, KYSE150, KYSE450 and KYSE510 were seed in 96-well plate, and then treated for 48h with gradually increasing concentration of VE-822. The cell viability was assessed using CCK8 staining. Results was presented as the mean percentage of viable cells (Mean±SD), averaged from 3 independent experiments, each with 4 replicates per condition.

(c). The p-ATR and p-CHK1 expression were inhibited and DNA damage accumulation was induced by treatment with VE-822 in a dose-dependent manner as measured by Western Blotting.

(d). VE-822 treatment inhibited STAT3-AKT pathway activation in both KYSE70 and KYSE450.

(e). The effect of VE-822 on cell cycle progression was assessed by flow cytometry using PI/RNase staining. Exactly, VE-822 treatment for 24h significantly increased the proportion of cells in G1 phase, decreased proportion of G2 phase in KYSE70 and KYSE450.

(f). Several cell cycle and apoptosis markers were analyzed using Western Blotting. VE-822 treatment significantly augmented the DNA damage presented by γ-H2AX; VE-822 treatment down-regulated p21 expression for cells to pass the G1/S checkpoint and progress into G2 phase and up-regulated the expression of caspase-3.

(g). VE-822 affected the cancer cell apoptosis. To further investigate whether VE-822 affect cellular apoptosis, cells was stained by Annexin V/PE and underwent flow cytometry analysis after incubated with VE-822 for 8h. The apoptosis rate for VE-822-treated cells was significantly higher than control

Figure 3. VE-822 sensitizes ESCC cells to cisplatin and synergies strongly with cisplatin in ATM-deficient cells.

(a). CI value was calculated according to the Chou-Talalay median effect principle. We observed shift in cisplatin sensitivity in either KYSE450 or KYSE70 cell line. The CI value ≤ 0.9 stands for the synergistic effect.

(b). Western Blotting analysis confirmed that ATM expression was knocked out well both in KYSE450 and KYSE70.

(c). Cells were treated with select doses of VE-822 (as indicated) for 48 hours and viability was assessed using CCK8 assay. In the ATM-deficient cells, the IC50 of VE-822 was smaller than control cells, especially for relatively insensitive cell line KYSE70.

(d). There was more significant synergistic effect in ATM-deficient cells than control cells for both of KYSE450 and KYSE70 as presented by lower CI value.

(e). VE-822 potentiated cisplatin efficacy in ESCC xenografts, and the combination causes rapid regression. Nude mice bearing KYSE70 or KYSE450 or ATM-deficient KYSE70 or ATM-deficient KYSE450 were treated with 5mg/kg cisplatin on days1, 4, 7, 10 via intravenous injection or 60 mg/kg VE-822 on days 1, 3, 5 via oral gavage or combination of these two reagents. Tumor growth curves indicated that combination of VE-822 and cisplatin slowed down the tumor growth significantly compared to the single drug, especially for relative insensitive KYSE70.

(f). Although combination of two drugs, the mice did not suffer more weight loss than those in single drug group. In the ATM-deficient xenografts, the gap the weight loss curve between combination group and single group was narrower.

(g). The inhibition of p-CHK1, p-AKT activation were more obvious in ATM-deficiency cells and cleaved PARP was increased as measured by Western Blotting.

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Figure 4. In the ATM-deficient cells, VE-822 alone or combination with cisplatin induced more severe DNA damage and cell apoptosis than control cell, as well as induced cell cycle arrest.

(a). Cells were treated with indicated concentration of VE-822, cisplatin, or combination of VE-822 and cisplatin for 24h and underwent flow cytometry analysis by staining with PI/RNase staining for assess the cell cycle progression. Cell cycle was arrested in phase S for ATM-deficient cells by VE-822 treatment.

(b). Cells were treated with indicated concentration of VE-822(2μM), cisplatin(2μg/ml), or combination of VE-822(2μM) and cisplatin(2μg/ml) for 8h, and then underwent flow cytometry analysis by staining with Annexin V/PE. we observed dramatic cell apoptosis in ATM-deficient cells either with VE-822 or combination treatment.

(c). Expression of several markers which reflects the cell proliferation or cell apoptosis or cell cycle progression or DNA damage was examined using western blotting. Caspase-3 and cleaved-PARP expression remarkably increased in cells exposure to combination of VE-822 and cisplatin. In the ATM-deficient cell, VE-822 combination with cisplatin more dramatic changes in the expression of p-AKT, p21, and γ-H2AX than control cells.

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Figure 5. Combination treatment enhanced the DNA damage effect presented by examining fluorescence intensity of γ-H2A.X and p53 in ESCC cells.

Cells treated with cisplatin, VE-822, or combination of two drugs for 24 h were fixed and co-labeled with anti-γ H2AX and anti-p53 antibodies. The γ-H2A.X and p53 foci were analyzed by immunofluorescence microscopy. combination treatment resulted in accumulation of DNA damage presented by stronger and more fluorescence staining of γ-H2AX and p53 (a,b). The enhancement of the DNA damage effect was stronger in ATM-deficiency cells (c,d).

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Figure 6. Combination of VE-822 and cisplatin increased cisplatin-DNA accumulation. (a) Cells were treated with cisplatin, VE-822, or combination of two drugs for 24 h. Anti-cisplatin modified DNA antibody was used to specifically bind to Cisplatin-DNA，and the positive signal was examined by using flow cytometry. The combination of VE-822 and cisplatin induced augmentation of cisplatin-DNA adduct.

(b) The percentage of cisplatin modified DNA positive cells were measured by flow cytometry and are represented as mean ± SD of three replications.

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Table 1. Association between ATR-pSer428 expression and Median survival time(months)/5-y survival rate in the study cohort (n=110)

Item	No. (%)	Median survival time,□months (95%CI)	5-y survival□rate (%)	*P* value
Negative VS Positive				0.0152
ATR-pSer428 non-expression	28(25.6)	84(66-102)	62.2	
ATR-pSer428 expression	82(74.5)	60(47-74)	17.1	
Expression Grades				0.0335
ATR-pSer428 non-expression	28(25.5)	84(66-102)	62.2	
ATR-pSer428 expression Grade1	19(17.2)	65(42-88)	42.6	
ATR-pSer428 expression Grade2	36(32.7)	48(35-60)	34.2	
ATR-pSer428 expression Grade3	27(24.5)	49(28-70)	30.1	
Low vs. High				0.0109
ATR-pSer428 low expression	47(42.7)	76(62-91)	54.7	
ATR-pSer428 high expression	63(57.3)	53(38-69)	30.2	

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Table 2. Association between ATR-pSer428 expression and TRG in the study cohort (n=110)

Item	No. (%)		*P* value
	TRG1	TRG2/3/4	
ATR-pSer428 expression			<0.001
non-expression	10(83.3)	18(18.4)	
expression	2(16.7)	80(81.6)	
ATR-pSer428 expression			<0.001
low expression	11(91.7)	36(36.7)	
high expression	1(8.3)	62(63.3)	

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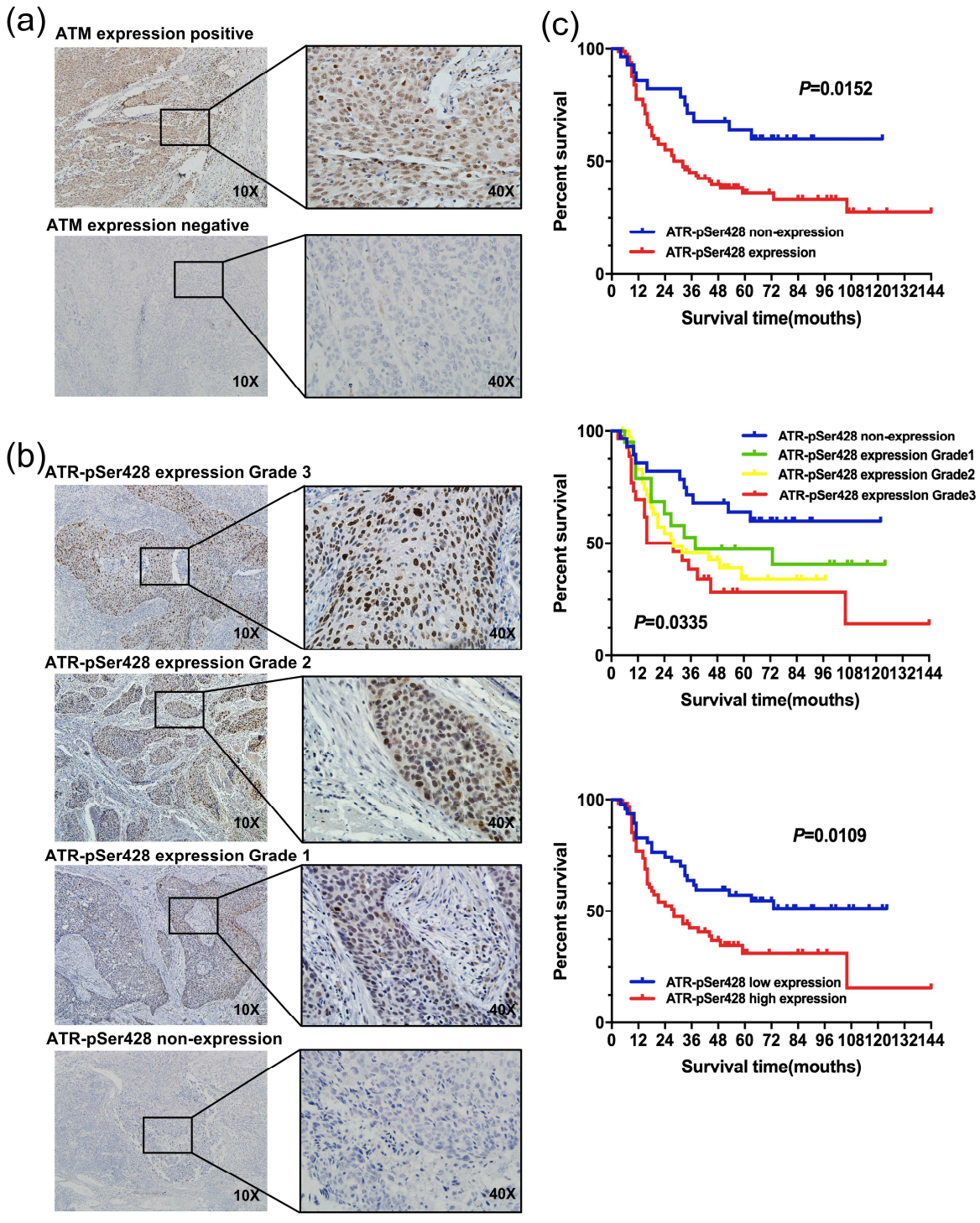
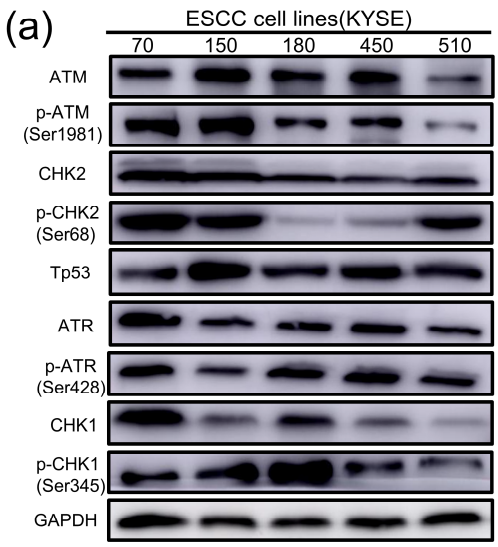
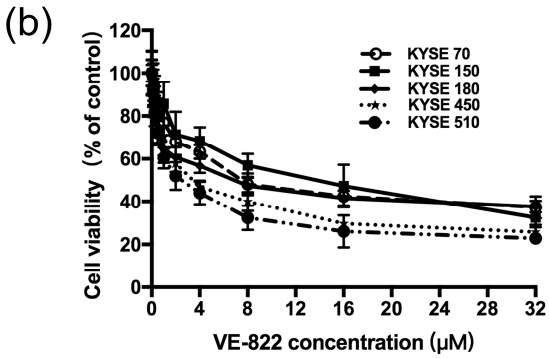
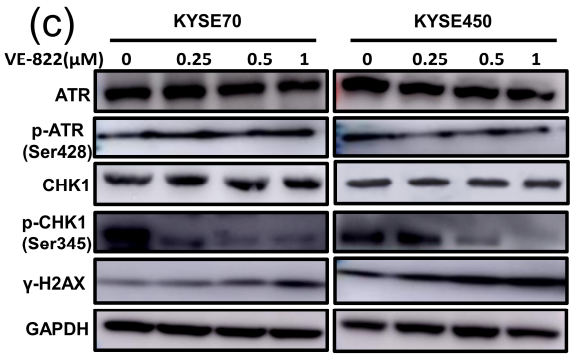
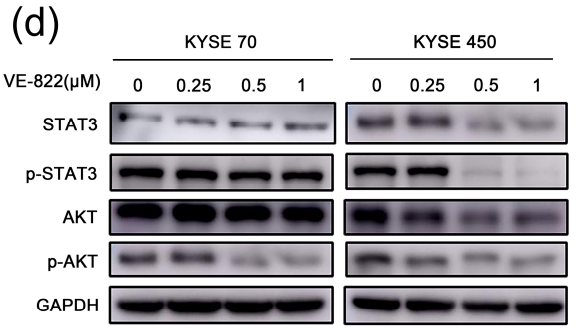

(a)
ATM expression positive
10X
40X
ATM expression negative
10X
40X
(b)
ATR-pSer428 expression Grade 3
10X
40X
ATR-pSer428 expression Grade 2
10X
40X
ATR-pSer428 expression Grade 1
10X
40X
ATR-pSer428 non-expression
10X
40X
(c)
Percent survival
100
50
0
P=0.0152
ATR-pSer428 non-expression
ATR-pSer428 expression
0 12 24 36 48 60 72 84 96 108120132144
Survival time(mouths)
Percent survival
100
50
0
ATR-pSer428 non-expression
ATR-pSer428 expression Grade1
ATR-pSer428 expression Grade2
ATR-pSer428 expression Grade3
P=0.0335
0 12 24 36 48 60 72 84 96 108120132144
Survival time(mouths)
Percent survival
100
50
0
P=0.0109
ATR-pSer428 low expression
ATR-pSer428 high expression
0 12 24 36 48 60 72 84 96 108120132144
Survival time(mouths)

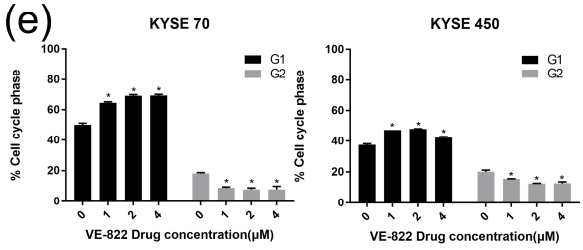




Item	IC50(VE-822)	95%CI
KYSE70	9.387	7.996 to 11.17
KYSE150	11.87	9.88 to 14.53
KYSE180	6.922	5.945 to 8.147
KYSE450	3.982	3.572 to 4.448
KYSE510	2.606	2.268 to 3.001

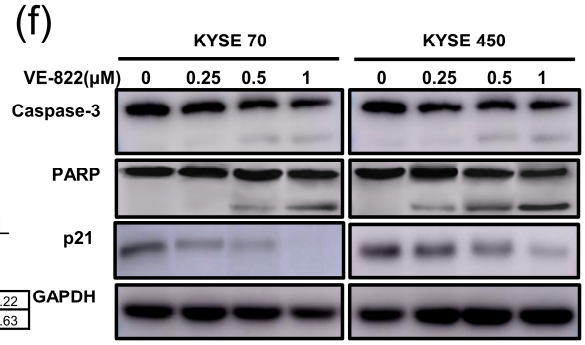


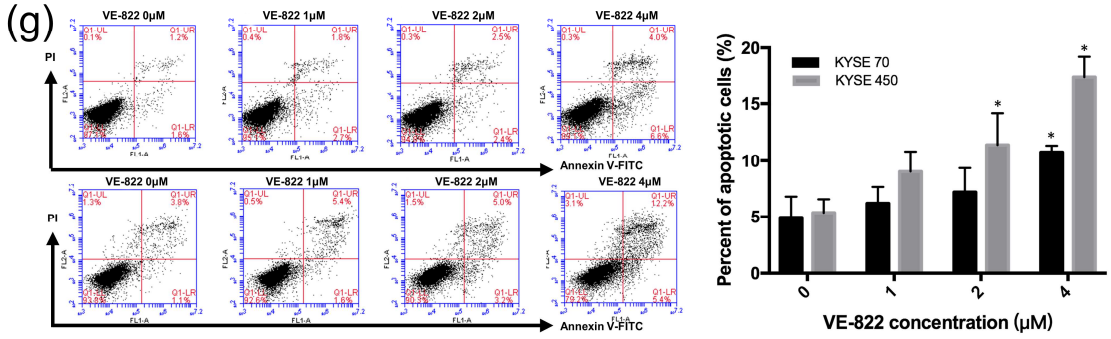




G1(%)	52.62	64.64	69.26	69.48
G2(%)	18.16	8.83	7.88	15.59

G1(%)	37.48	47.29	47.98	42.22
G2(%)	20.10	15.57	12.50	12.63





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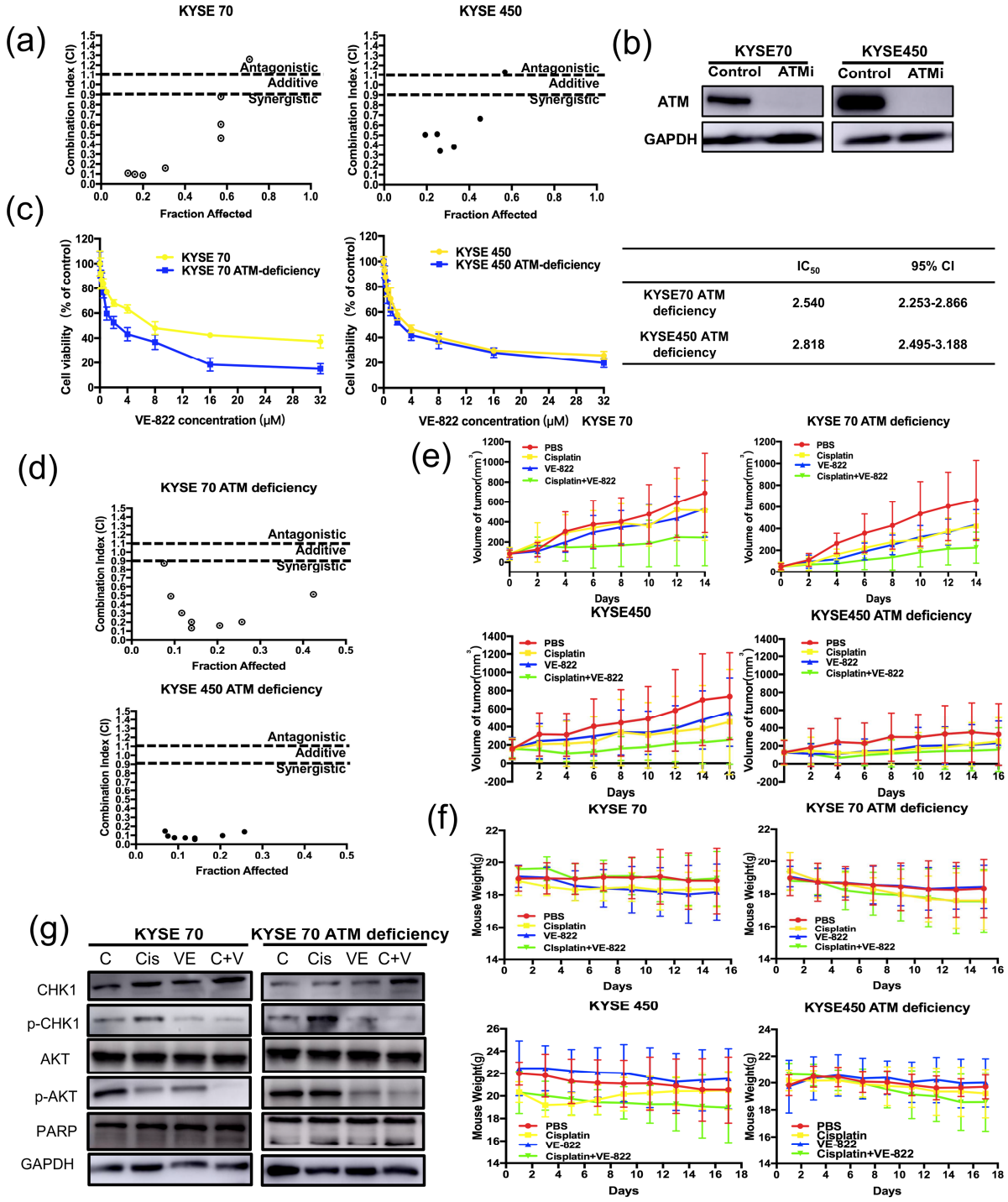
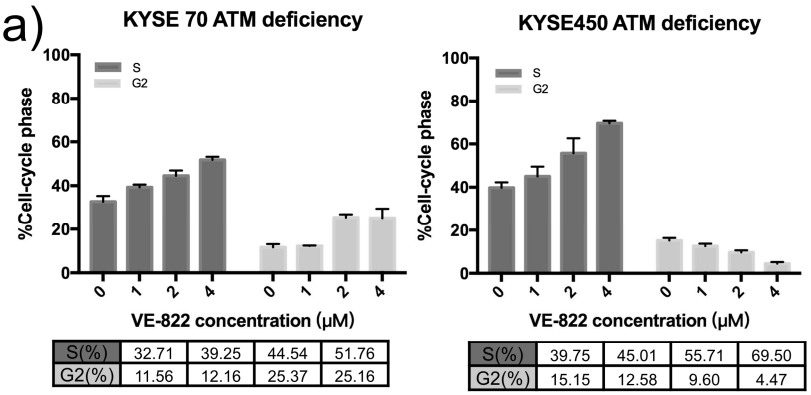


	IC_{50}	95% CI
KYSE70 ATM deficiency	2.540	2.253-2.866
KYSE450 ATM deficiency	2.818	2.495-3.188

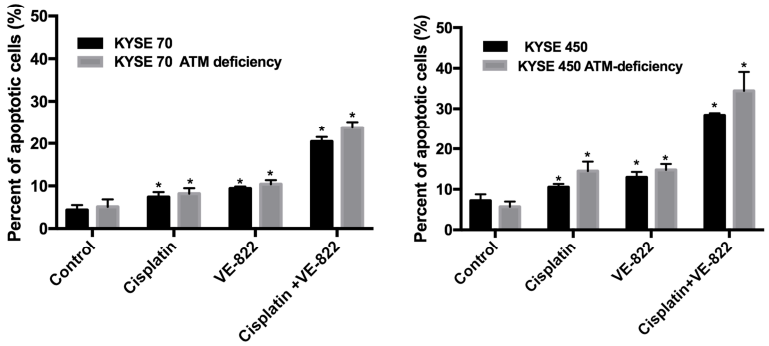
(a)



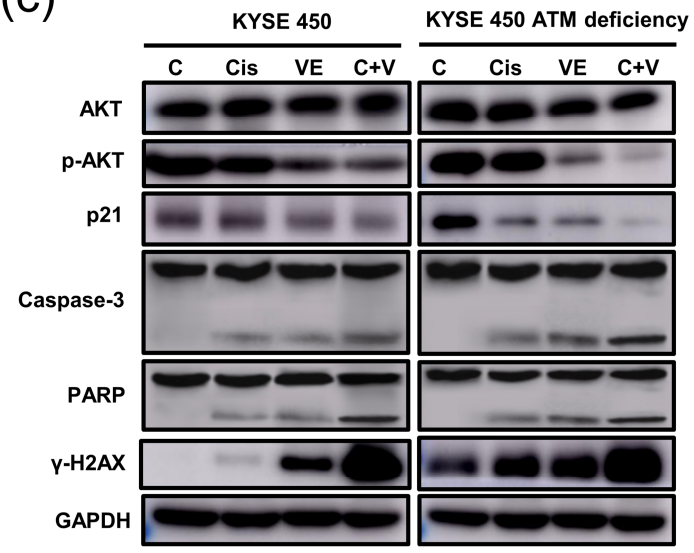
S(%)	32.71	39.25	44.54	51.76
G2(%)	11.56	12.16	25.37	25.16

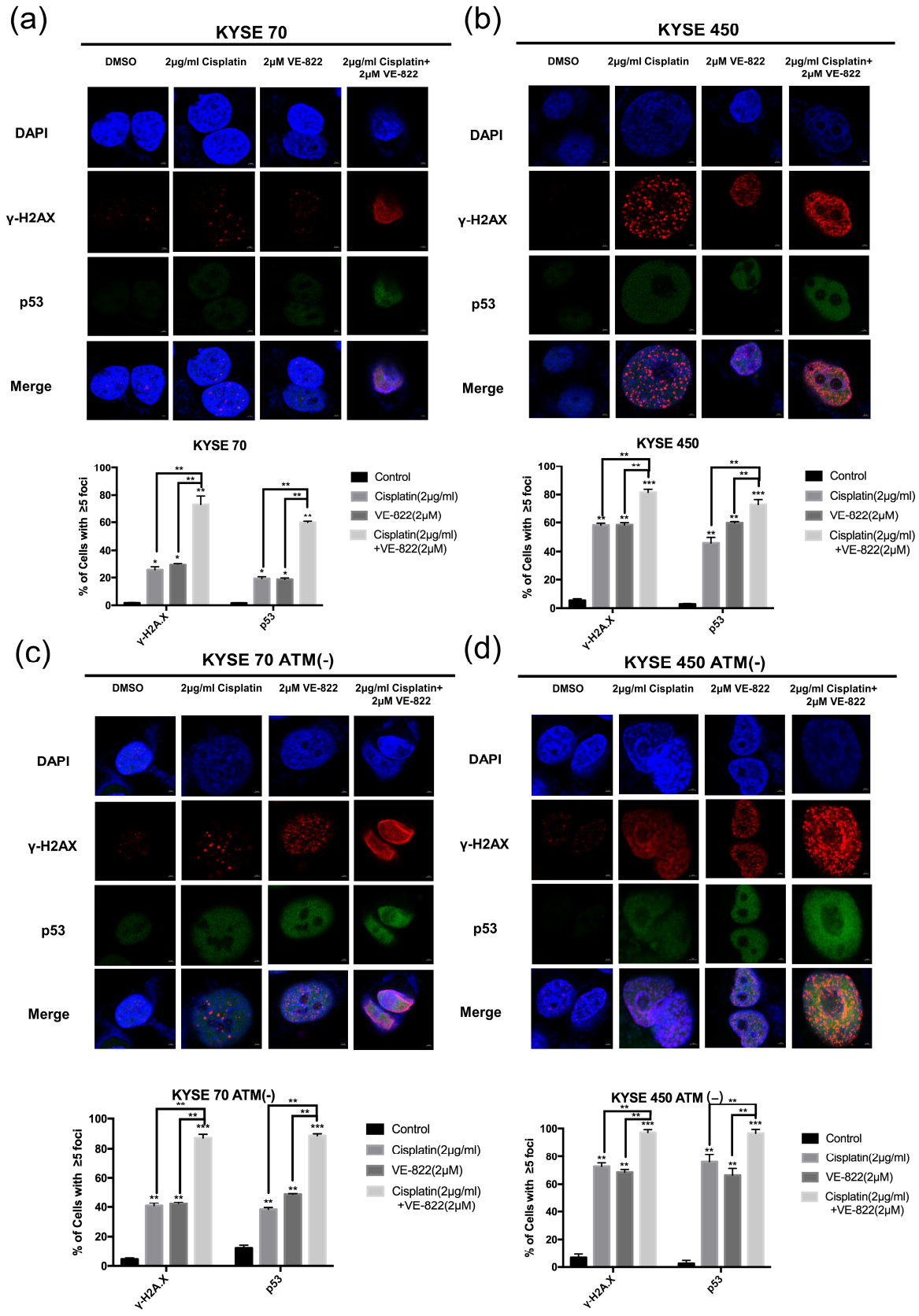
S(%)	39.75	45.01	55.71	69.50
G2(%)	15.15	12.58	9.60	4.47

(b)



(c)




(a)
KYSE 70
DMSO
2μg/ml Cisplatin
2μM VE-822
2μg/ml Cisplatin+ 2μM VE-822
DAPI
γ-H2AX
p53
Merge
KYSE 70
% of Cells with ≥5 foci
γ-H2A.X
p53
Control
Cisplatin(2μg/ml)
VE-822(2μM)
Cisplatin(2μg/ml) +VE-822(2μM)
(b)
KYSE 450
KYSE 450
(c)
KYSE 70 ATM(-)
KYSE 70 ATM(-)
(d)
KYSE 450 ATM(-)
KYSE 450 ATM (–)

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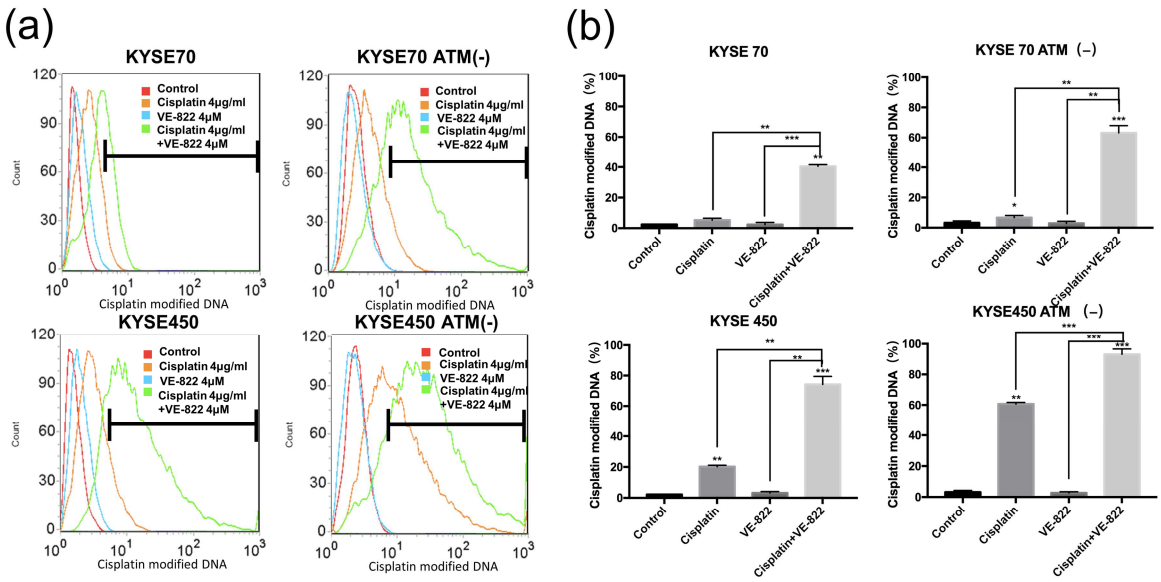

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ACCEPTED MANUSCRIPT

Highlights:

- ATR activation was associated with efficiency of neoadjuvant chemotherapy.
- VE-822 promoted cell apoptosis and induced cell cycle arrest in ESCC cells.
- VE-822 sensitized ESCC cells to cisplatin, in vitro and in vivo.
- VE-822 along with cisplatin induces DNA-cisplatin adduct accumulation.

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