

Functional analysis of the *TMPRSS2:ERG* fusion gene in cisplatin-induced cell death

JUNQI WU^{1*}, LINFENG CHI^{2,3*}, ZHANGHUI CHEN^{2,4}, XIANGHONG LU⁵, SUPING XIAO², GUANGLIN ZHANG², JINDAN LUO², GE-MING CHEN² and JUN YANG^{2,6}

¹Clinical Laboratory, Jinhua Hospital of Zhejiang University, Jinhua, Zhejiang 321000;

²Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, Zhejiang University; ³The Clinical Laboratory Center, The Affiliated Stomatology Hospital,

Zhejiang University School of Medicine, Hangzhou, Zhejiang 310003; ⁴Center for Reproductive Medicine,

Zhejiang Academy of Medical Science, Hangzhou, Zhejiang 310013; ⁵Department of Pharmacy,

Lishui People's Hospital, Lishui, Zhejiang 323000; ⁶Department of Public Health,

Hangzhou Normal University School of Medicine, Hangzhou,

Zhejiang 310031, P.R. China

Received March 15, 2015; Accepted January 20, 2016

DOI: 10.3892/mmr.2016.4898

Abstract. The *TMPRSS2:E-twenty-six* (ETS) gene fusion occurs frequently in a high proportion of patients with prostate cancer (PCa) in Western countries, and the aberrant expression of *TMPRSS2: v-ETS avian erythroblastosis virus E26 oncogene homolog (ERG)*, the most common form of the corresponding protein, can regulate cell migration and contribute to tumor invasion and metastasis. However, its association with other cellular events, and in particular, cell death, remain unknown. To examine the function of such fusion genes, an expression plasmid containing the *TMPRSS2:ERG* (T1/E5) sequence (Δ ERG) from a patient sample was constructed and transiently transfected into DU145 cells, which do not express the fusion gene. It was found that the overexpression of Δ ERG significantly inhibited the ability of cisplatin to induce apoptosis in DU145 cells. By contrast, VCaP cells, which do contain *TMPRSS2:ERG*, were sensitized to cisplatin-induced apoptosis through siRNA inhibition of the fusion gene. To elucidate the underlying

mechanism, a stable cell line expressing the Δ ERG gene was constructed. Expression of Δ ERG did not affect cell migration, but did protect cells from DNA damage and apoptosis induced by cisplatin. Furthermore, knockdown of Δ ERG by short interfering RNA resulted in cells regaining their sensitivity to cisplatin. Finally, the gene coding for activating transcription factor 5, which is important for cell survival, may be upregulated by Δ ERG. Taken together, these data point to a new function of the *TMPRSS2:ERG* fusion gene in regulating the apoptotic pathway.

Introduction

Prostate cancer (PCa) is the most common male malignancy, and one of the leading causes of cancer-associated mortality among men in Western countries (1). Extensive studies have been conducted in an effort to understand the genetic mechanisms underlying PCa initiation and progression. Using a systems biology approach, Tomlins *et al* (2) reported that the androgen response gene transmembrane protease, serine 2 (*TMPRSS2*) was fused to the E-twenty-six (ETS) family gene v-ETS avian erythroblastosis virus E26 oncogene homolog (ERG) and ETS variant 1 (ETV1) in certain patients with PCa. Among the various types of fusion genes, the *TMPRSS2:ERG* fusion is the most common, and has been identified in 40-70% of samples from patients with PCa (3).

Functional studies of *TMPRSS2:ERG* have suggested that it is involved in cellular proliferation, differentiation and invasion (4-7), while its association with additional cellular functions, and in particular, apoptosis, remains unknown. Shao *et al* (8) reported that celastrol, a nuclear factor- κ B inhibitor, can suppress *TMPRSS2:ERG*-expressing PCa cell growth by inducing apoptosis, implying a possible anti-apoptotic function for the *TMPRSS2:ERG* fusion gene.

As an effective anticancer drug, cisplatin is widely used in the treatment of many types of solid tumors, including ovarian,

Correspondence to: Dr Ge-Ming Chen, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, Zhejiang University, 79 Qingchun Road, Hangzhou, Zhejiang 310003, P.R. China
E-mail: cgmcs@msn.com

Dr Jun Yang, Department of Public Health, Hangzhou Normal University School of Medicine, 16 Xuelin Street, Hangzhou, Zhejiang 310031, P.R. China
E-mail: gastate@zju.edu.cn

*Contributed equally

Key words: fusion gene, prostate cancer, ERG, *TMPRSS2*, apoptosis

testicular, bladder, cervical, head and neck, and small-cell lung cancer (9,10). Cisplatin is able to interact with DNA, and results in the formation of interstrand and/or intrastrand cross-links (11), with such severe DNA damage considered a major factor in triggering cancer cell apoptosis. However, cisplatin treatment of PCa has been a subject of debate, as while earlier studies suggested a lack of activity, more recent studies using palliation and prostate specific antigen (PSA) endpoints have suggested a greater degree of clinical benefit (12). Therefore, it is of interest to understand why PCa cells have a low response to cisplatin-induced apoptosis, and whether the *TMPRSS2:ERG* fusion gene may serve a role in this response.

In the present study, the role of *TMPRSS2:ERG* in cell death, specifically, apoptosis induced by cisplatin was investigated. The *TMPRSS2:ERG* fusion gene was demonstrated to serve an important role in the regulation of apoptosis induced by cisplatin, and activating transcription factor 5 (*ATF5*) was suggested to be involved in this process.

Materials and methods

Cell culture and treatment. Human embryonic kidney 293 (HEK293) cells and the prostate cancer cell lines DU145 and VCaP cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and the cells were grown in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were plated in 6-well plates and treated at the indicated times or doses with cisplatin (Sigma-Aldrich, St. Louis, MO, USA).

DNA constructs and transfection. cDNA corresponding to the most common truncated ERG (Δ ERG), the *TMPRSS2:ERG* fusion gene (T1/E5), was constructed using pEGP-C1 (Clontech Laboratories, Inc., Mountainview, CA, USA) with *Pst*I and *Bgl*II sites to generate a green fluorescent protein (GFP)- Δ ERG over-expressing vector. Kanamycin resistant colonies were selected and verified by sequencing. Cells were transfected with EugeneHD (Roche Diagnostics, Basel, Switzerland) following the manufacturer's instructions. In brief, 5 μ l EugeneHD was diluted with 50 μ l Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.), mixed and incubated at room temperature for 5 min. Subsequently, 1.5 μ g plasmid DNA was added to the diluted EugeneHD and mixed, followed by incubation for 20 min at room temperature, following which the EugeneHD-DNA mixture was added to the cells.

To establish the stable cell line, the HEK293 cells were transfected as described above. Following selection with 0.8 mg/ml G418 (Gibco, Thermo Fisher Scientific, Inc.) for 14 days, resistant clones were tested for the expression of Δ ERG. The selected stable cell line was designated as Δ 293, and the cells were cultured further in the medium supplemented with 300 μ g/ml G418 for maintenance. Cells transfected with the GFP-empty vector were designated as E293 and were used as a control.

Scratch assay. Cells were seeded at 1x10⁵ cells/well in triplicate in collagen IV coated 6-well plates (Corning Incorporated, Corning, NY, USA). After 24 h, a scratch through the central

axis of the plate was gently made using a pipette tip. The migration of Δ 293 and E293 cells into the scratched region was assessed quantitatively at 12 h following the introduction of the scratch in the monolayer.

Cell proliferation assay. Cells were seeded at 5x10³ cells/well into a 96-well plate. Cell proliferation was examined using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) following the manufacturer's instructions. Absorbance at 450 nm was measured in quintuplicate using a Tecan Infinite M200 plate reader (Tecan Trading AG, Männedorf, Switzerland) at the indicated time points.

Δ ERG knock down. Knockdown of Δ ERG expression was achieved by the transfection of Δ ERG short interfering RNA (siRNA). The Δ ERG siRNA was sequenced by Shanghai GenePharma Co., Ltd., (Shanghai, China), the sequences were as follows: Sense, 5'-CCAGACGUCAACAUCUUGUTT-3' and antisense, 5'-ACAAGAUGUUGACGUCUGGTT-3', which were synthesized by. A nonspecific control strand (UUCUCC GAACGUGUCACGUTT) was used as the negative control (NC).

The siRNA was transfected into the cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions.

Immunoblot analysis. Cells were harvested at the indicated times and washed three times in phosphate-buffered saline (PBS) prior to lysis. Protein concentrations were measured using a Bicinchoninic Acid Protein Assay Reagent kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Equal amounts of the protein (20 μ g) were subjected to 12 or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Inc., Hercules, CA, USA), then transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA), which were incubated first with the primary antibody at 4°C overnight, followed incubation with IR Dye-conjugated secondary antibodies for 1 h at room temperature. The primary antibodies used were as follows: Rabbit polyclonal anti-ERG (1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. sc-376293); rabbit polyclonal anti-ATF5 (1:500; Santa Cruz Biotechnology, Inc.; sc-377168); rabbit monoclonal anti-cleaved-caspase-3 (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA; cat. no. 9661); rabbit polyclonal anti-poly ADP ribose polymerase (PARP-1; 1:1,000; Cell Signaling Technology, Inc.; cat. no. 9542); mouse monoclonal anti- β -actin (1:1,000; Cell Signaling Technology, Inc.; cat. no. 3700); rabbit monoclonal anti-H3 (1:1,000; Cell Signaling Technology, Inc.; cat. no. 4499); and mouse monoclonal anti- γ H2AX (1:3,000; EMD Millipore; cat. no. 05-636). Alexa Fluor 488-conjugated goat anti-mouse (1:5,000; cat. no. A-11001) and goat anti-rabbit (1:5,000; cat. no. A11008) IgG were obtained from Thermo Fisher Scientific, Inc.. The results were observed using an Odyssey Infrared Imaging System (Li-Cor, Inc., Lincoln, NB, USA).

Flow cytometry. Cells were harvested at 48 h post cisplatin treatment. Cells were centrifuged at 300 x g for 5 min at 4°C and washed with PBS three times, then resuspended in 1X Binding Buffer. Subsequently, Annexin V-phycoerythrin

and 7-aminoactinomycin D (EMD Millipore) were added, and cells were incubated at room temperature for 5 min in the dark, following which the apoptotic cells were analyzed by flow cytometry (FC500 MCL; Beckman Coulter, Inc., Brea, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from E293 and Δ 293 cells was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), and 5 μ g of total RNA was reverse-transcribed to cDNA using SuperscriptIII Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). Prior to this, the RNA was treated with DNase I (Sangon Biotech Co., Ltd., Shanghai, China) to remove DNA contamination. The 20 μ l PCR reaction was set up using SYBR Green Premix Ex Taq kit (Takara Bio, Inc., Otsu, Japan) on a 96-well plate, and conducted using a ABI Prism Sequence Detection System 7500 (Applied Biosystems, Inc.; Thermo Fisher Scientific, Inc.) with the following PCR conditions: 95°C for 1 min; 95°C for 10 sec, 60°C for 1 min, repeated for 40 cycles. The primers used were as follows: Sense, 5'-GAGTGGGCGGTGAAAGAA TA-3' and antisense, 5'-AGAAGGATGTCGGCGTTGTA-3' for ERG; sense, 5'-TGCGTGACATAAGGAGAA-3' and antisense, 5'-AAGGAAGGCTGGAAGAGT-3' for β -actin; sense, 5'-TGGAAAGCGTAGACAAGGAGAT-3' and antisense, 5'-AAGGCTCTAGGTGGTCATTCAG-3' for BCL-XL; sense, 5'-CCATCTACTGCCGCAACGAG-3' and antisense, 5'-CTC AGAGCCGCCGAC TTGTT-3' for ATF5; sense, 5'-GGATTG TGGCCTTCTTTGA-3' and antisense, 5'-GTGCCGGTTCAG GTACTCA-3' for BCL-2; sense, 5'-GGACGGCGGTGATGG ACG-3' and antisense, 5'-GAAGCAAAAGGGCCCCTGT-3' for BAX; and sense, 5'-CCGCCACTACCACCACTTGA-3' and antisense, 5'-GGGAACGCAGCGAACCGAAT-3' for BIM. Data analysis was performed using the $2^{-\Delta\Delta C_q}$ method for relative quantification (13), and all samples were normalized to β -actin, which served as an endogenous control.

Chromatin immunoprecipitation (ChIP). ChIP assays were performed using the EZ ChIP kit (EMD Millipore) according to the manufacturer's instructions. Briefly, cross-linked chromatin was sonicated and immunoprecipitated with the indicated antibody. Three independent ChIP analyses were performed to prepare the DNA for future analysis. Immunoprecipitated DNA was purified and quantified by RT-qPCR as described above. The primers were as follows: Sense, 5'-AAAAGACGG ACATCCACCTCG-3' and antisense, 5'-CAAGGGATGGAG AAAAGGACG-3' for ATF5.

Statistical analysis. The data are presented as the mean $\pm$ standard deviation from a minimum of three independent experiments. The differences between groups were assessed using an unpaired two-tailed Student's t-test or one-way analysis of variance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Δ ERG inhibits cisplatin-induced apoptosis in PCa cell lines. The present study evaluated the toxicity of cisplatin on DU145 and VcaP cells. It was observed that these two types of cell

were relatively resistant to the toxic effect of cisplatin as only at concentrations of 50 μ M or higher was significant cell death observed (data not shown). To examine the function of fusion genes in apoptosis, GFP-tagged *TMPRSS2:ERG* (T1/E5) (Δ ERG) was transiently expressed in DU145 cells, which do not express this fusion gene. The cells were then treated with 50 μ M cisplatin for 48 h, and the apoptotic cell population was analyzed by flow cytometry. As shown in Fig. 1A, the Δ ERG protein was expressed in the transfected DU145 cells as indicated by western blotting. Following cisplatin treatment, the apoptotic cell ratio in the control DU145 cells was 30.59%. However, this ratio was reduced to 14.67% in Δ ERG transfected DU145 cells ($P < 0.01$; Fig. 1B). To further investigate the function of Δ ERG in apoptosis, VCaP cells, which harbor the *TMPRSS2:ERG* fusion gene, were transfected with siRNAs to knockdown the expression of the fusion gene (Fig. 1C). Consistent with the idea that the fusion protein inhibits apoptosis, the apoptotic cell ratio increased from 13.59% in control cells to 21.35% in siRNA-transfected cells ($P < 0.05$; Fig. 1D).

Expression of Δ ERG renders HEK293 cells resistant to cisplatin-induced apoptosis. The toxic effects of cisplatin on HEK293 cells were also examined. It was demonstrated that compared with PCa cells, HEK293 cells were more sensitive to the toxic effect of cisplatin, as at a concentration of 1 μ M marked cell death was observed and at 100 μ M, almost all the cells had undergone cell death (data not shown). To determine whether Δ ERG only has the anti-apoptosis function in PCa cells but not in other types of cell, a stable cell line expressing the Δ ERG gene was established using HEK 293 cells (Δ 293 cells), with cells transfected with the empty plasmid serving as controls (E293 cells). GFP fluorescence demonstrated that the majority of the Δ ERG fusion protein was localized within the nucleus, and western blot analysis using a commercial anti-ERG antibody confirmed the expression of Δ ERG (Fig. 2A). The effect of Δ ERG on cell growth was investigated and the results indicated that the expression of the Δ ERG gene in HEK293 cells did not affect cell growth (data not shown). To further examine whether Δ ERG does indeed serve a role in protecting cells from cisplatin-induced apoptosis, Δ 293 cells were treated with 50 μ M cisplatin for 48 h. The cell viability of E293 cells was 10% with cisplatin treatment, while in Δ 293 cells, the viability was ~46%, suggesting that Δ ERG can protect Δ 293 cells from cell death induced by cisplatin treatment (Fig. 2D). Further analysis revealed that the apoptotic cell ratio in E293 cells was 67.26%, while in Δ 293 cells it was 25.89% ($P < 0.01$; Fig. 2E). To verify that apoptosis had indeed occurred, a number of apoptosis-associated proteins, including caspase 3 and PARP-1, were examined by western blotting following cisplatin treatment. As shown in Fig. 2F, caspase 3 and PARP-1 cleavage was apparent in E293 cells following cisplatin treatment, however, was less abundant in Δ 293 cells under the same conditions. To further demonstrate that Δ ERG was responsible for the protective effect, Δ ERG was knocked down in Δ 293 cells using specific siRNA (Fig. 2G), which indicated that loss of Δ ERG can restore the sensitivity of Δ 293 cells to cisplatin treatment (Fig. 2H). Taken together, these data demonstrate that Δ ERG is able to protect cells from apoptosis induced by cisplatin.

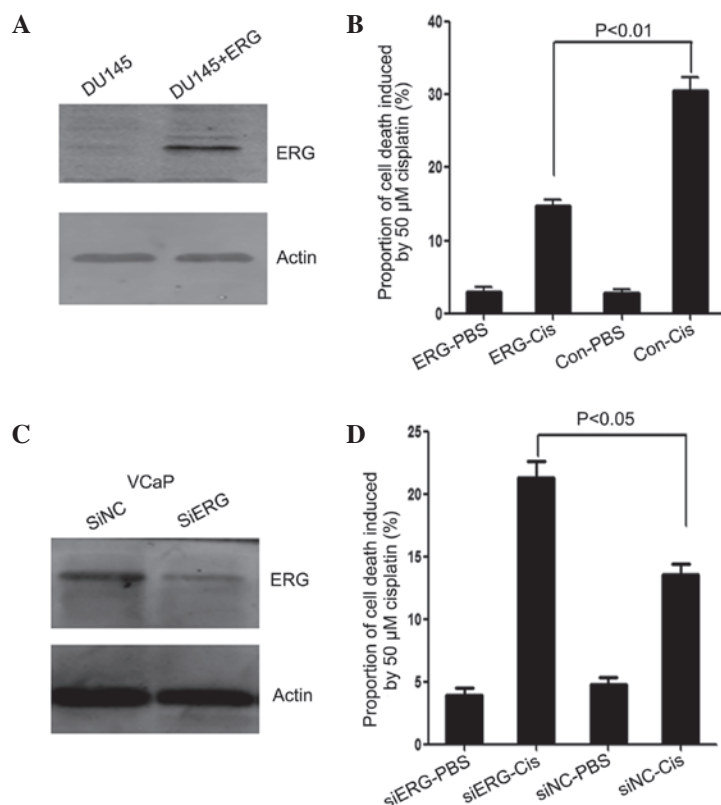


Figure 1. Effects of Δ ERG on cisplatin-induced apoptosis in DU145 and VCaP cells. At 24 h following transfection, cells were treated with 50 μ M cisplatin for 48 h, and apoptosis was detected by 7-aminoactinomycin D and annexin-V phycoerythrin double staining. (A) Western blotting of Δ ERG protein expression in DU145 cells transfected with GFP-vector or GFP- Δ ERG. (B) Quantitative results of cisplatin-induced apoptosis, as measured by flow cytometry analysis in DU145 cells. (C) Western blotting of Δ ERG protein expression in VCaP cells transfected with control or specific siRNA directed against Δ ERG. (D) Quantitative results of cisplatin-induced apoptosis as measured by flow cytometry analysis in VCaP cells. Δ ERG, truncated v-E-twenty-six avian erythroblastosis virus E26 oncogene homolog; GFP, green fluorescent protein; siRNA, short interfering RNA; PBS, phosphate-buffered saline; Cis, cisplatin; siNC, si-negative control; Con, control.

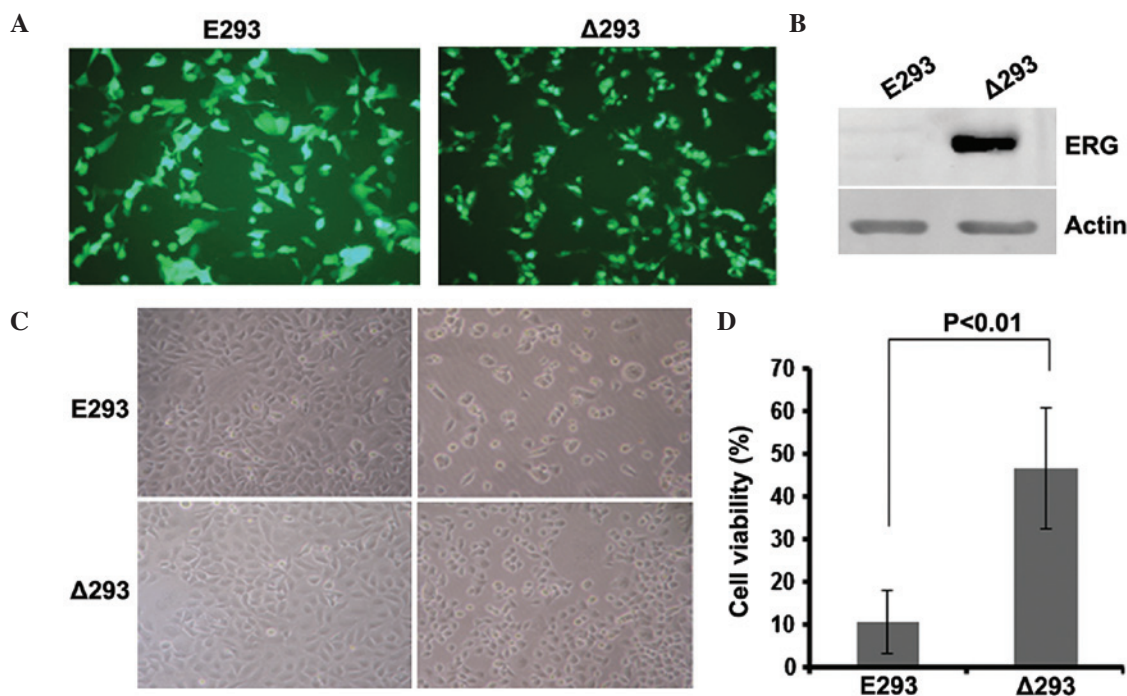


Figure 2. Effects of Δ ERG overexpression on cisplatin-induced apoptosis in HEK293 cells. (A) Fluorescent images of the expression of Δ ERG in E293 or Δ 293 cells (magnification, $\times 200$). (B) Western blotting for Δ ERG expression. (C) Representative phase contrast microscopy images of E293 and Δ 293 cell density and morphological alterations following 50 μ M cisplatin treatment for 48 h (magnification, $\times 200$). (D) Cell viability as measured with a Cell Counting Kit-8 kit. Δ ERG, truncated v-E-twenty-six avian erythroblastosis virus E26 oncogene homolog; E293, p-enhanced green fluorescent vector-transfected HEK293 cells; Δ 293, Δ ERG-transfected HEK293 cells.

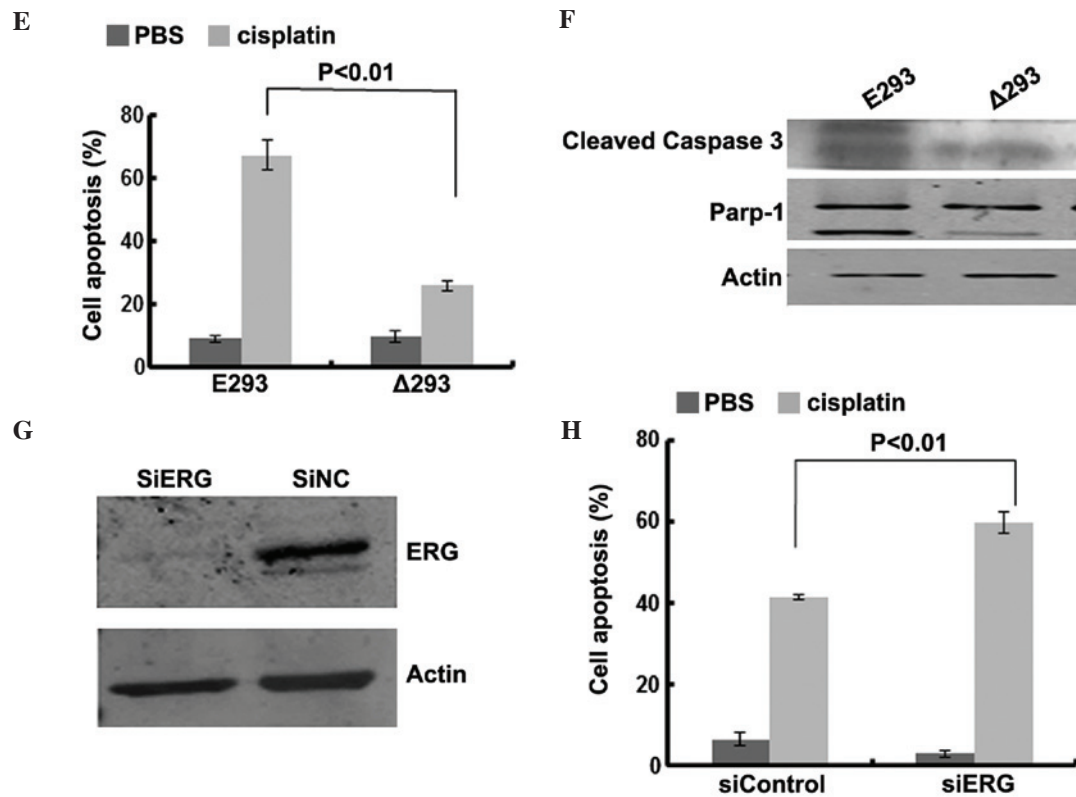


Figure 2. Continued. Effects of Δ ERG overexpression on cisplatin-induced apoptosis in HEK293 cells. (E) Quantitative results of flow cytometry analyses for cell apoptosis using 7-aminoactinomycin D and annexin-V phycoerythrin double staining. Cells were treated with 50 μ M cisplatin for 48 h. (F) Western blotting for cleaved caspase-3 and cleaved PARP-1. E293 and Δ 293 cells were treated with 50 μ M cisplatin for 48 h, total cell lysates were prepared and 10 μ g aliquots of total proteins were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (G) Western blotting was used to measure Δ ERG protein expression in Δ 293 cells transfected with control or specific siRNA against Δ ERG. (H) Quantitative results of flow cytometric analyses for cell apoptosis. Δ ERG, truncated v-E-twenty-six avian erythroblastosis virus E26 oncogene homolog; E293, p-enhanced green fluorescent vector-transfected HEK293 cells; Δ 293, Δ ERG-transfected HEK293 cells; PARP1, poly ADP-ribose polymerase-1; siRNA, short interfering RNA; PBS, phosphate-buffered saline; siNC, si-negative control.

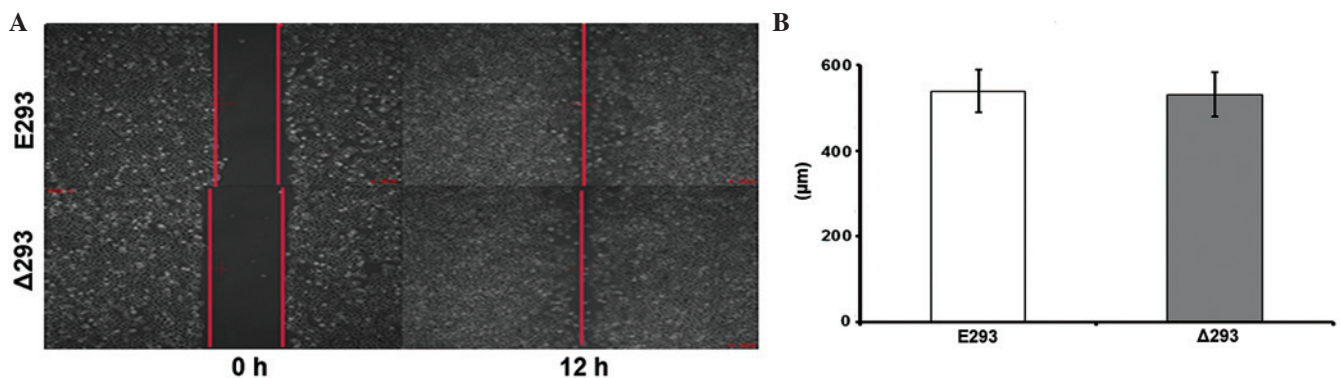


Figure 3. Effects of Δ ERG overexpression on cell migration. E293 and Δ 293 cells were seeded at 1×10^5 cells/well in triplicate in collagen IV coated 6-well plates. After 24 h, a scratch through the central axis of the plate was gently made using a pipette tip. (A) Representative images of a scratch assay measuring cell migration of E293 and Δ 293 cells. (B) Quantitative results for migration analyses. Δ ERG, truncated v-E-twenty-six avian erythroblastosis virus E26 oncogene homolog; E293, p-enhanced green fluorescent vector-transfected HEK293 cells; Δ 293, Δ ERG transfected HEK293 cells.

It has been previously reported that a major function of *TMPRSS2:ERG* is to promote PCa cell migration and invasion (1,14-18). Therefore, the effect of Δ ERG on cell migration was investigated using a scratch assay. Of note, no significant difference was observed in the scratch assay results between the Δ 293 and E293 cells (Fig. 3), suggesting that overexpressing Δ ERG did not affect cell motility, at least in HEK293 cells.

Δ ERG upregulates expression of the anti-apoptotic gene ATF5. Considering the potential anti-apoptotic function of Δ ERG, and given that the protein functions as a transcription factor, whether it was able to influence the expression of key regulators in apoptosis, such as members of the BCL-2 family, was of interest. Therefore, RT-qPCR was conducted to examine the expression of the BCL-2 family members BCL-2, BCL-XL, BIM and BAX in the presence and absence of Δ ERG. Notably,

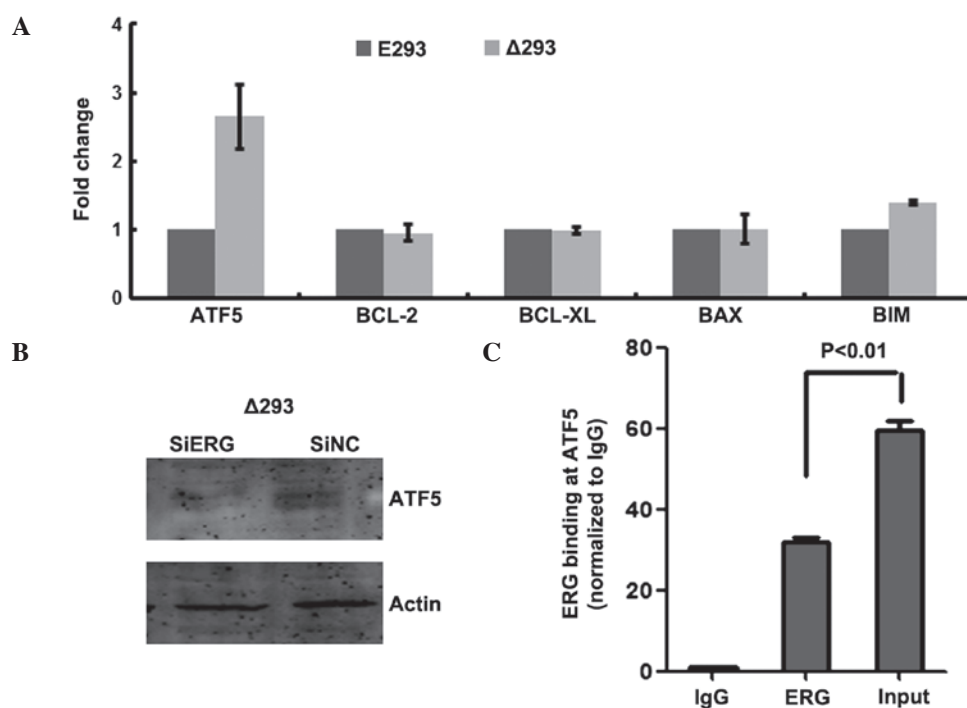


Figure 4. ATF5 expression is upregulated in Δ 293 cells. (A) RT-qPCR detection of apoptosis-associated genes in E293 cells and Δ 293 cells. (B) Δ 293 cells were transfected with control or specific siRNA against Δ ERG, and expression of ATF5 protein was measured by western blotting. (C) Quantitative results of RT-qPCR analyses associated with ChIP assays. ATF5, activating transcription factor 5; Δ 293, truncated v-E-twenty-six avian erythroblastosis virus E26 oncogene homolog-transfected HEK293 cells; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; E293, p-enhanced green fluorescent vector-transfected HEK293 cells; siRNA, short interfering RNA; IgG, immunoglobulin G.

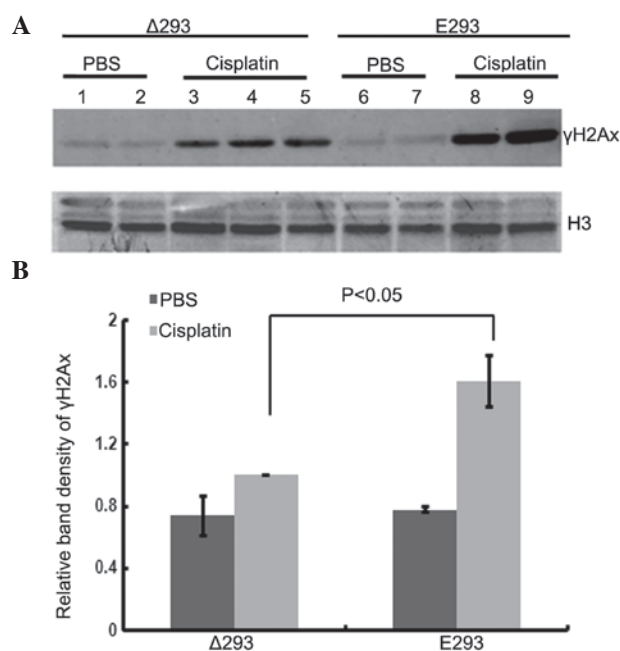


Figure 5. Δ ERG overexpression reduces cisplatin-induced DNA damage in Δ 293 cells. (A) Western blotting detection of γ H2AX protein in E293 and Δ 293 cells following 50 μ M cisplatin treatment for 24 h. Lanes 1 and 2, Δ 293 cells treated with PBS; lanes 3, 4 and 5, Δ 293 cells treated with 50 μ M cisplatin; lanes 6 and 7, E293 cells treated with PBS; and lanes 8 and 9, E293 cells treated with 50 μ M cisplatin. (B) Densitometry analysis of western blotting results. The relative band densities of γ H2AX were normalized to H3, and the value of the bands from Δ 293 cells treated with cisplatin was arbitrarily set as 1. The values were derived from at least three independent experiments. P < 0.05 vs. E293 is indicated. Δ ERG, truncated v-E-twenty-six avian erythroblastosis virus E26 oncogene homolog; Δ 293, Δ ERG-transfected HEK293 cells; E293, p-enhanced green fluorescent vector-transfected HEK293 cells; PBS, phosphate-buffered saline; H3, histone H3.

none of these genes showed differential expression between E293 and Δ 293 cells (Fig. 4A). However, ATF5 (also known as ATFx), a member of the basic zipper protein ATF/cAMP response element-binding protein family which can suppress apoptosis (19), was observed to be up-regulated in Δ 293 cells (Fig. 4A). To further examine the possibility that ATF5 expression was regulated by Δ ERG, the protein levels of ATF5 were examined by western blotting in Δ 293 cells, transfected with either control siRNA or with siRNA designed to knockdown Δ ERG. As shown in Fig. 4B, the ATF5 protein levels were reduced when Δ ERG was knocked down, suggesting that Δ ERG serves a role in the regulation of ATF5 expression. As ERG is a transcription factor, it was then investigated whether Δ ERG is able to bind directly to the ATF5 gene to regulate ATF5 expression. To investigate this, a ChIP assay was performed in Δ 293 cells using a specific anti-ERG antibody, which indicated that Δ ERG was able to bind to the ATF5 promoter region in Δ 293 cells (Fig. 4C).

Expression of Δ ERG protects cells from cisplatin-induced DNA damage. As a classical DNA damage agent, cisplatin is thought to induce apoptosis, at least in part, through the generation of DNA damage. Therefore, the effect of Δ ERG expression on cisplatin-induced DNA damage was assessed. E293 and Δ 293 cells were treated with cisplatin (50 μ M) for 24 h, and the level of γ H2AX, a sensitive indicator for DNA damage, was examined by western blotting. As shown in Fig. 5, cisplatin treatment increased the level of γ H2AX in the Δ 293 and E293 cells. However, the increase was much smaller in the Δ 293 cells (lanes 3, 4 and 5) compared with the E293 cells (lanes 8 and 9), indicating that the expression

of Δ ERG may partially protect cells from cisplatin-induced DNA damage.

Discussion

The potential role of *TMPRSS2:ERG* fusion in prostate tumorigenesis and tumor progression has been under intensive investigation. Enhancing cell invasion and metastasis has been regarded as the major function of this protein, and although overexpression of ERG was observed to induce a neoplastic phenotype in prostate cells in certain studies (5,6), it was thought that the protein may require the cooperation of additional genes, such as phosphatase and tensin homolog or members of the phosphatidylinositol 3-kinase pathway (14,15), to result in transformation. However, its association with apoptosis/cell death, a major mechanism for the majority of cancer chemotherapeutic drugs, was relatively unexplored prior to the current study. Platinum chemotherapy drugs, such as cisplatin and carboplatin, have moderate single-agent activity in PCa (12). However, when combined with a taxane and estramustine, high response rates were observed in several clinical trials (20,21). Considering the high prevalence of *TMPRSS2:ERG* fusion in patients with PCa, its potential role in the relatively low response rate of PCa to cisplatin was explored.

Indeed, PCa cells were observed to be relatively resistant to the cytotoxic effects of cisplatin. For example, only at doses of 50 μ M or above were significant levels of cell death induced by cisplatin in either DU145 or VCaP cells (data not shown). By contrast, significant cell death was not induced in HEK293 cells by cisplatin at concentrations as low as 1 μ M, and high levels of cell death was observed at 100 μ M (data not shown). Therefore, 50 μ M was selected as the test concentration for all subsequent experiments. The response of the PCa cell lines to cisplatin treatment was investigated in DU145 cells, which do not express the fusion gene and VCaP cells, which harbor the fusion gene. This indicated that cisplatin is able to induce up to 30% apoptosis in DU145 cells, while the drug induces ~13% apoptosis in VCaP cells (Fig. 1). However, when Δ ERG was expressed in DU145 cells, the apoptotic cell ratio was reduced to ~15%. In comparison, knocking down the endogenous fusion gene expressed in the VCaP cell line led to an increased apoptotic cell ratio. Thus, this indicates that Δ ERG may influence the response of these cells to cisplatin. To discover whether or not the anti-apoptotic effect of Δ ERG was limited to PCa cells, Δ ERG was introduced into the HEK293 cells. Notably, no significant effect of Δ ERG on HEK293 cell growth or migration was observed (data not shown and Fig. 3). These observations are in contradiction to the report by Tian *et al* (7) in which it was shown that the overexpression of *TMPRSS2:ERG* in PCa cells markedly increased invasion. A possible explanation may be that different types of cells were used, with HEK293 cells originating from the kidney rather than the prostate, and thus may have a different genetic background. Alternatively, an effect of Δ ERG on HEK293 cells was observed with respect to apoptosis. Although cisplatin induced apoptosis in ~70% of the HEK293 cells, which only expressed the GFP-tag, the apoptotic cell ratio was around 30% in the Δ 293 cells that expressed the Δ ERG (Fig. 2). An additional piece of evidence for the anti-apoptotic function of

Δ ERG comes from the observation that knocking down Δ ERG in Δ 293 cells rendered the cells sensitive to cisplatin (Fig. 2). Taken together, these data indicate an anti-apoptotic effect of Δ ERG. Therefore, future treatment planning for patients with PCa may be enhanced by assessing the status of the *TMPRSS2* gene fusion.

Once the anti-apoptotic function of the fusion gene had been determined in the current study, the underlying molecular mechanism was further probed. The BCL-2 family of proteins include both anti-apoptotic (BCL-2, BCL-XL and MCL-1) and apoptotic (BAK, BAX, BID and BIM) members (22). The regulation and balance of the BCL-2 family proteins in a particular cell results in either the inhibition or induction of the apoptotic signaling pathways (23). Therefore, the expression of several BCL-2 family members was examined. Notably, none of the BCL-2 family members examined exhibited differential expression between the E293 and Δ 293 cells (Fig. 4), suggesting that they may not be involved in the anti-apoptotic activity of Δ ERG. However, it was observed that the expression of ATF5, a transcriptional activator with anti-apoptotic activity, was increased in Δ 293 cells. It has been reported that when ATF5 was stably expressed in an IL-3-dependent cell line, apoptosis was suppressed through cytokine deprivation (24). Additionally, overexpression of ATF5 suppressed apoptosis induced by serum withdrawal in HeLa cells (25). By contrast, inhibition of endogenous ATF5 activity by the introduction of a dominant-negative form of ATF5 led to apoptosis in asynchronously growing cells (24,25). To determine whether Δ ERG was involved in the regulation of ATF5 expression, Δ ERG was knocked down in Δ 293 cells, and the protein levels of ATF5 were examined by western blotting. As shown in Fig. 4, knock down Δ ERG resulted in a reduction in ATF5 expression. Subsequently, ChIP analysis was performed, and the results indicated that Δ ERG is able to bind directly to the promoter region of ATF5 (Fig. 4). Based on these observations, Δ ERG is proposed to potentially upregulate the expression of ATF5, which in turn exerts anti-apoptotic activities through the activation of downstream signaling pathways.

Finally, since cisplatin-induced DNA damage is considered to be a major factor in triggering apoptosis, it was examined whether Δ ERG is able to influence cisplatin-induced DNA damage. γ H2AX is regarded to be a sensitive indicator for DNA damage (26). The results of the present study indicated that expression of the γ H2AX protein was significantly reduced in Δ 293 cells, suggesting that overexpression of Δ ERG may protect cells from cisplatin-induced DNA damage (Fig. 5). The role of *TMPRSS2:ERG* in the DNA damage process remains controversial. *TMPRSS2:ERG* has been previously shown to interact with the enzyme PARP1 and the catalytic subunit of DNA protein kinase in a DNA-dependent manner, and overexpression of similar fusion genes induces DNA double-strand breaks (27). In addition, Han *et al* (28) observed that overexpression of *TMPRSS2:ERG* increases PARP1 activity and clonogenic survival (28). Haffner *et al* (29) reported that androgen signaling promotes co-recruitment of the androgen receptor and topoisomerase II beta (TOP2B) to sites of *TMPRSS2:ERG* genomic breakpoints, triggering recombinogenic TOP2B-mediated DNA double-strand breaks (DSBs). Furthermore, androgen stimulation resulted in *de novo* production of *TMPRSS2:ERG* fusion transcripts in a process that required TOP2B and components of

the DSB repair machinery (29). Thus, how Δ ERG may interfere with the DNA damage process remains clear, and the potential link between Δ ERG and DNA damage is of great interest for future studies.

In summary, the current study indicated that the Δ ERG protein is able to inhibit cisplatin-induced DNA damage and cell death, and that the *ATF5* gene may participate in such events.

Acknowledgements

The current study was supported by grants from the National Natural Science Foundation of China (grant nos. 81172692, 81302398, 81502752 and 81373036), Department of Science and Technology, Zhejiang Province (grant no. 2013C14016), the Hangzhou City S&T Committee (grant no. 20130633B50) and the Zhejiang Provincial Natural Science Foundation (grant no. Q16H220001).

References

1. Siegel R, Naishadham D and Jemal A: Cancer statistics for Hispanics/Latinos, 2012. *CA Cancer J Clin* 62: 283-298, 2012.
2. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, *et al*: Recurrent fusion of *TPRSS2* and *ETS* transcription factor genes in prostate cancer. *Science* 310: 644-648, 2005.
3. Falzarano SM and Magi-Galluzzi C: ERG protein expression as a biomarker of prostate cancer. *Biomark Med* 7: 851-865, 2013.
4. Helgeson BE, Tomlins SA, Shah N, Laxman B, Cao Q, Prensner JR, Cao X, Singla N, Montie JE, Varambally S, *et al*: Characterization of *TPRSS2:ETV5* and *SLC45A3:ETV5* gene fusions in prostate cancer. *Cancer Res* 68: 73-80, 2008.
5. Tomlins SA, Laxman B, Varambally S, Cao X, Yu J, Helgeson BE, Cao Q, Prensner JR, Rubin MA, Shah RB, *et al*: Role of the *TPRSS2-ERG* gene fusion in prostate cancer. *Neoplasia* 10: 177-188, 2008.
6. Klezovitch O, Risk M, Coleman I, Lucas JM, Null M, True LD, Nelson PS and Vasioukhin V: A causal role for ERG in neoplastic transformation of prostate epithelium. *Proc Natl Acad Sci USA* 105: 2105-2110, 2008.
7. Tian TV, Tomavo N, Huot L, Flourens A, Bonnelye E, Flajollet S, Hot D, Leroy X, de Launoit Y and Duterque-Coquillaud M: Identification of novel *TPRSS2:ERG* mechanisms in prostate cancer metastasis: Involvement of *MMP9* and *PLXNA2*. *Oncogene* 33: 2204-2214, 2014.
8. Shao L, Zhou Z, Cai Y, Castro P, Dakhov O, Shi P, Bai Y, Ji H, Shen W and Wang J: Celastrol suppresses tumor cell growth through targeting an AR-ERG-NF- κ B pathway in *TPRSS2/ERG* fusion gene expressing prostate cancer. *PLoS One* 8: e58391, 2013.
9. Loehrer PJ and Einhorn LH: Drugs five years later. Cisplatin. *Ann Intern Med* 100: 704-713, 1984.
10. Prestayko AW, D'Aoust JC, Issell BF and Crooke ST: Cisplatin (cis-diamminedichloroplatinum II). *Cancer Treat Rev* 6: 17-39, 1979.
11. Eastman A: The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes. *Pharmacol Ther* 34: 155-166, 1987.
12. Oh WK, Tay MH and Huang J: Is there a role for platinum chemotherapy in the treatment of patients with hormone-refractory prostate cancer? *Cancer* 109: 477-486, 2007.
13. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Δ Delta C(T)) method. *Methods* 25: 402-408, 2001.
14. Carver BS, Tran J, Gopalan A, Chen Z, Shaikh S, Carracedo A, Alimonti A, Nardella C, Varmeh S, Scardino PT, *et al*: Aberrant ERG expression cooperates with loss of PTEN to promote cancer progression in the prostate. *Nat Genet* 41: 619-624, 2009.
15. King JC, Xu J, Wongvipat J, Hieronymus H, Carver BS, Leung DH, Taylor BS, Sander C, Cardiff RD, Couto SS, *et al*: Cooperativity of *TPRSS2-ERG* with PI3-kinase pathway activation in prostate oncogenesis. *Nat Genet* 41: 524-526, 2009.
16. Esgueva R, Perner S, J LaFargue C, Scheble V, Stephan C, Lein M, Fritzsche FR, Dietel M, Kristiansen G and Rubin MA: Prevalence of *TPRSS2-ERG* and *SLC45A3-ERG* gene fusions in a large prostatectomy cohort. *Mod Pathol* 23: 539-546, 2010.
17. Galluzzi L, Aaronson SA, Abrams J, Alnemri ES, Andrews DW, Baehrecke EH, Bazan NG, Blagosklonny MV, Blomgren K, Borner C, *et al*: Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. *Cell Death Differ* 16: 1093-1107, 2009.
18. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, *et al*: Molecular definitions of cell death subroutines: Recommendations of the nomenclature committee on cell death 2012. *Cell Death Differ* 19: 107-120, 2012.
19. Hai TW, Liu F, Coukos WJ and Green MR: Transcription factor ATF cDNA clones: An extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes Dev* 3: 2083-2090, 1989.
20. Papandreou CN, Daliani DD, Thall PF, Tu SM, Wang X, Reyes A, Troncso P and Logothetis CJ: Results of a phase II study with doxorubicin, etoposide, and cisplatin in patients with fully characterized small-cell carcinoma of the prostate. *J Clin Oncol* 20: 3072-3080, 2002.
21. Berry W, Friedland D, Fleagle J, Jackson D, Ilegbodun D, Boehm KA and Asmar L: A phase II study of weekly paclitaxel/estramustine/carboplatin in hormone-refractory prostate cancer. *Clin Genitourin Cancer* 5: 131-137, 2006.
22. Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ and Green DR: The BCL-2 family reunion. *Mol Cell* 37: 299-310, 2010.
23. Harris MH and Thompson CB: The role of the Bcl-2 family in the regulation of outer mitochondrial membrane permeability. *Cell Death Differ* 7: 1182-1191, 2000.
24. Persengiev SP and Green MR: The role of ATF/CREB family members in cell growth, survival and apoptosis. *Apoptosis* 8: 225-228, 2003.
25. Persengiev SP, Devireddy LR and Green MR: Inhibition of apoptosis by ATFx: A novel role for a member of the ATF/CREB family of mammalian bZIP transcription factors. *Genes Dev* 16: 1806-1814, 2002.
26. Zhou C, Li Z, Diao H, Yu Y, Zhu W, Dai Y, Chen FF and Yang J: DNA damage evaluated by gammaH2AX foci formation by a selective group of chemical/physical stressors. *Mutat Res* 604: 8-18, 2006.
27. Brenner JC, Ateeq B, Li Y, Yocum AK, Cao Q, Asangani IA, Patel S, Wang X, Liang H, Yu J, *et al*: Mechanistic rationale for inhibition of poly (ADP-ribose) polymerase in ETS gene fusion-positive prostate cancer. *Cancer Cell* 19: 664-678, 2011.
28. Han S, Brenner JC, Sabolch A, Jackson W, Speers C, Wilder-Romans K, Knudsen KE, Lawrence TS, Chinnaiyan AM and Feng FY: Targeted radiosensitization of ETS fusion-positive prostate cancer through PARP1 inhibition. *Neoplasia* 15: 1207-1217, 2013.
29. Haffner MC, Aryee MJ, Toubaji A, Esopi DM, Albadine R, Gurel B, Isaacs WB, Bova GS, Liu W, Xu J, *et al*: Androgen-induced TOP2B-mediated double-strand breaks and prostate cancer gene rearrangements. *Nat Genet* 42: 668-675, 2010.