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ORIGINAL ARTICLE – CANCER RESEARCH

IL-6 signaling contributes to radioresistance of prostate cancer through key DNA repair-associated molecules ATM, ATR, and BRCA 1/2

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Abstract

Purpose To study an association between IL-6 signaling and resistance to radiotherapy of prostate cancer (PCa) and explore the molecular mechanisms involved.

Methods IL-6 expressing C4-2 and CWR22Rv1 (C4-2IL-6/CWRIL-6) and vector control (C4-2vec/CWRvec) cell lines were developed. Radiation-sensitivities of these cells were compared in clonogenic assay, Comet assay, and γH2AX staining. In xenograft animal studies, radiation-sensitivity of C4-2IL-6 cell-derived tumors vs. C4-2vec cell-derived tumors was investigated. To reveal IL-6 downstream molecules involved in DNA repair after radiation, qPCR and Western blot analyses as well as immunofluorescence staining were performed. Transcriptional control of IL-6 on ATM and ATR molecules was also investigated.

Results We found C4-2IL-6 and CWRIL-6 cells survived better than their vector control cells after irradiation, and animal studies confirmed such in vitro results. We discovered that DNA repair-related molecules such as ATM, ATR, BRCA1, and BRCA2 were significantly upregulated in irradiated IL-6 expressing cells compared with vector control cells. We further defined that IL-6 signaling regulated cellular expressions of ATM and ATR at the transcriptional level through the activation of Stat3 signaling pathway.

Conclusions IL-6 leads to PCa resistance to radiation through upregulation of DNA repair associated molecules ATM, ATR, BRCA1, and BRCA2.

Keywords Prostate cancer · IL-6/Stat3 · Radioresistance · ATM/ATR · BRCA1/2

Introduction

Radiotherapy (RT) remains a major curative treatment modality for localized prostate cancer (PCa) (Hayden et al. 2010). Excluding patients who are recommended for expectant observation, approximately 50% of newly diagnosed PCa will receive RT and the other 50% will receive surgery. Fractionated external-beam radiotherapy (EBRT) and/or brachytherapy (BT) are effective treatments for localized PCa (Vanneste et al. 2016), while RT in combination with a short-course (intermediate-risk PCa) or a long-course (high-risk PCa) of androgen deprivation therapy (ADT) has been shown to improve survival for PCa with clinical/pathologic adverse features (higher Gleason score, higher PSA and/or larger tumors/T-stage) (Bonkhoff 2012). Despite overall success, approximately 20–30% of PCa patients with such adverse features will develop local/regional or distant metastasis due to RT resistance. At this stage, the cure is no longer

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possible despite subsequent salvage and/or systemic therapies. Research in the understanding of mechanisms leading to radioresistance will have significant clinical relevance and impact.

It is known that the serum IL-6 level is increased in PCa patients (Culig and Puhr 2012), and serum IL-6 correlates negatively with patient survival (Nguyen et al. 2014). In addition, increased IL-6 expression in PCa tissue has been reported. Giri et al. (2001) showed an 18-fold increase in IL-6 level in clinically localized PCa tumors when compared with normal prostate tissue. It was also reported that IL-6 receptor levels were increased in PCa tissues compared with normal tissues.

Several previous reports have implicated the role of IL-6 signaling in the development of radioresistance in different types of cancers. For example, it has been reported that IL-6 signaling is important in determining the radiation response of liver tumor cells by enhancing the recruitment of myeloid-derived suppressor cells (MDSCs) (Chen et al. 2012). It has also been suggested that IL-6 signaling is involved in radiation responses by regulating the reactive oxygen species (ROS) in PCa cells (Wu et al. 2013). Zang et al. (2017) showed that IL-6/STAT3/TWIST signaling-induced epithelial mesenchymal transition (EMT) contributed to the development of radioresistance in esophageal squamous carcinoma.

Our laboratory has previously observed that IL-6 signaling promoted DNA repair in CD133+ lung cancer stem cells after radiation (Chen et al. 2015b). However, it remains unknown if IL-6 regulates the expression of DNA repair-associated molecules to affect the radiation response in PCa cells. In this study, we examined the role of IL-6 signaling in modulating the expression of DNA repair-associated molecules in the development of radioresistance in PCa cells.

Materials and methods

Gene database analyses

We obtained data from The Cancer Genome Atlas (TCGA) through web access at https://cancergenome.nih.gov and analyzed IL-6 expression of PCa.

Cell culture

Two established PCa cell lines (C4-2 and CWR22Rv1) were obtained from Dr. YiFen Lee's laboratory at the University of Rochester Medical Center. Cells were cultured in RPMI 1640 containing 10% charcoal stripped (CS)-FBS and maintained in a humidified 5% CO_2 environment at 37 °C. In studies using inhibitors, we used JAK inhibitor 1 (5 μM) (CAS457081-03-7, Calbiochem, San Diego, CA, USA), Stattic (10 μM) (CAS19983-44-9, Calbiochem), LY294002 (5 μM) (440202, Sigma, St Louis, MO, USA), U0126 (10 μM) (9903, Cell Signaling Technology, Danvers, MA, USA), VX745 (10 μM) (SML1638, Sigma), Bay11-7082 (5 μM) (B5556, Sigma), rapamycin (100 nM) (R8781, Sigma), and thalidomide (100 μg/ml) (T144, Sigma) that inhibit JAK, Stat3, PI3K/Akt, MEK/Erk, MAPK, NFκB, mTOR, and TNFα pathways, respectively.

Development of IL-6 expressing and vector control cells by lentiviral transduction

To incorporate IL-6 cDNA or vector (vec) control plasmids into C4-2 and CWR22Rv1 cells, lentiviral construct carrying either empty vector or IL-6 cDNA (pLenti-II vector, Applied Biological Materials Inc, Canada) was transfected into 293T cells with a mixture of pLent-II-IL-6cDNA, psPAX2 (virus-packaging plasmid), and pMD_2G (envelope plasmid) (4:3:2 ratio) using PolyFect Transfection reagent (Qiagen, Valencia, CA, USA). After C4-2 and CWR22Rv1 cells were virally infected overnight, the culture media containing the virus was replaced with normal culture media and maintained under normal cell culture conditions. After sub-culturing cells, the IL-6 incorporated cells were selected by culturing cells in the presence of 2 μg/ml puromycin (540411, Millipore, Billerica, MA, USA) and then maintained in media containing 0.1 μg/ml puromycin.

Clonogenic assay

Cells were exposed to different doses (0, 1, 2, 4, 6, and 8 Gy) of radiation using a Cs-137 source with a dose rate of 180 cGy/min to 205 cGy/min. After radiation treatment, clonogenic assay was performed as previously described (Franken et al. 2006). Cells were seeded in culture dishes with appropriate dilutions to form colonies after 7–9 days of incubation. Colonies were fixed with methanol, stained with crystal violet (0.5% w/v), and counted under the microscope. Colonies consisting of at least 50 cells were counted and the surviving fraction was calculated after adjusting for the plating efficiency.

Comet assay

Cells were irradiated (4 Gy) and used in the assay following the procedure of Singh et al. (1988) with some modifications. Briefly, cells were embedded in low-melting-point agarose in lysis buffer (10 mM Tris–HCl/pH 10, 2.5 M NaCl, 100 mM EDTA, 10% DMSO, 1% Triton X-100). The unwinding step was performed for 20 min at 4 °C in unwinding/electrophoresis buffer (300 mM NaOH, 1 mM EDTA). Electrophoresis was performed at 4 °C for 20 min in unwinding/electrophoresis buffer at an electric field strength

of 25 V and a current of 300 mA on combslide™ (4252-040-K, Trevigen, Gaithersburg, MD, USA). The slides were then neutralized with a neutralizing buffer (0.4 M Tris–HCl/pH 7.5) for 20 min, rinsed with distilled water, air-dried, and stained with 30 µg/ml ethidium bromide. Images were recorded using a fluorescent microscope (Axioskop 40, Zeiss, Germany).

In vivo mouse studies

C4-2IL-6 and C4-2vec cells (1×10^6/site) were subcutaneously injected into flanks of castrated 8-week-old male nude mice (NCI) ($n = 10$/group). Tumor volumes (V), calculated by $V = (L \times S^2)/2$ formula (L and S represent long and short axis of tumors, respectively) were recorded twice a week. When tumor volumes reached about 200–250 mm^3, mice in two groups were randomly divided into two sub-groups: one sub-group of mice was irradiated (2 Gy × 5 days, total body irradiation) while the other sub-group of mice was non-irradiated. Tumor volumes were measured twice a week for 3 weeks. At the end of experiments, mice in all groups were killed and tumor tissues were obtained and processed for staining. All animal studies were performed under the supervision and guidelines of the University of Rochester Medical Center's Animal Care and Use Committee.

Histology and immunohistochemistry

Mouse tissues obtained were fixed in 10% (v/v) formaldehyde in PBS, embedded in paraffin, and cut into 5-µm sections. Tissue sections were deparaffinized in xylene solution, rehydrated, and immunostaining was performed. Antibodies of IL-6 (ab9324, Abcam, Cambridge, MA, USA) and Ki67 (ab15580, Abcam) were applied in staining. For staining of human patient tissues, eight tissues of PCa human tumor tissues were performed using IL-6 Ab (ab6672, Abcam).

Immunofluoresence (IF) staining

C4-2IL-6/vec and CWRIL-6/vec cell sets (1×10^3) were mounted on chamber slides, irradiated (6 Gy vs. non-irradiated cells as control), and stained with appropriate primary antibodies. Antibodies of ATM (GTX70107, GeneTex, Irvine, CA, USA), ATR (GTX70109, GeneTex), phosphorylated ATM (Ser 1981), (GTX30636, GeneTex) and phosphorylated ATR (Thr1989, GTX128145, GeneTex), BRCA1 (MAB22101, R&D, Minneapolis, MN, USA), and BRCA2 (MAB2476, R&D) and γH2AX antibody (05-636, Upstate Biochemical, Syracuse, NY, USA) were used. After reaction with Alexa flour ® 488 anti-goat secondary antibody (A11034, Life Technologies, Grand Island, NY, USA), images were recorded using a fluorescent microscope (Zeiss, Germany). Triplicate experiments were performed.

RNA extraction and quantitative real-time PCR (qPCR) analysis

Total RNAs were isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). One µg of total RNA was subjected to reverse transcription using Superscript III transcriptase (Invitrogen). qPCR was conducted using the appropriate primers (sequence is provided in Supplementary Table 2) and a Bio-Rad CFX96 system with SYBR green to determine the mRNA expression levels of genes of interest. Expression levels were normalized to GAPDH level. Triplicate experiments were performed.

Western blot analysis

To obtain total cell extracts, cells were lysed in RIPA buffer. For isolation of cytoplasmic and nuclear extracts, cells were harvested and incubated in buffer A containing 10 mM HEPES, pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, 1% NP40, 5 mM EDTA, and protease inhibitor for 20 min and centrifuged (500g, 5 min). The resulting supernatants were designated as cytosolic fraction. The nuclear pellet was re-suspended in the same buffer A, supplemented with 500 mM NaCl, 25% glycerol, and kept on ice for 30 min. Samples were centrifuged (12,000g, 5 min), and the supernatant obtained was used as nuclear extracts. Proteins (20 µg for total extracts and 10 µg for cytosolic/nuclear extracts) were separated on 8–10% SDS/PAGE gel and then transferred onto PVDF membranes (Millipore). After blocking procedure, membranes were incubated with primary antibodies, HRP-conjugated secondary antibodies, and visualized in Imager (Bio-Rad) using ECL system (Thermo Fisher Scientific, Rochester, NY, USA). Antibodies of GAPDH and cyclin D1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and same antibodies of ATM, p-ATM, ATR, p-ATR, BRCA-1, BRCA-2 used in IF staining were used. For γH2AX detection, antibody from Upstate Biotechnology (Lake Placid, NY, USA) was applied. Triplicate experiments were performed.

Preparation of ATM- and ATR-luciferase plasmids

ATM luciferase and ATR luciferase were constructed according to Zheng et al. (2015). The human genome was extracted from C4-2 cells. ATM promoter fragment from −860 to +53 bp was amplified by PCR using the primers (F: 5′-TATGGTACCCGTATTGCGTGGA-GGATGGAG-3′; R: 5′-TTACTCGAGCAGCGACTTAGCGTTTGCGG-3), and ATR promoter fragment from −504 to +115 bp was amplified by PCR using the primers (F: 5′-TTAGGTACC GTCCTCAACGAAACCTAACAGT-3′; R: 5′-TTACTC GAGACTAGTCAAC-CACGCCAACG-3′) using the PCR condition as follows: pre-degeneration for 5 min at 94 °C,

denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and extension for 2 min at 72 °C. PCR reaction was carried out for 35 cycles. The PCR product of ATM and ATR, and pGL3-basic vehicle plasmids were digested with restriction enzyme KpnI and XhoI at 37 °C for 1 h. These fragments of PCR product and pGL3-basic vehicle plasmids were mixed with 2 µL T4 ligase buffer and 1 µL T4 DNA ligase, incubated at 16 °C for 24 h, and then transformed into competent *E. coli*. A single colony was picked and cultured in Lysogeny broth (LB), which contains ampicillin. These plasmids were extracted and sequenced.

Luciferase assay

Cells in 24-well plates were transfected with 2 µg/ml ATM or ATR reporter plasmids and 0.02 µg/ml phRL-cytomegalovirus *Renilla* luciferase plasmids (used as control for normalizing transfection efficiencies) using Polyfect (Qiagen, Valencia, CA, USA). When necessary, varied concentrations of recombinant IL-6 (rhIL-6) were added into culture. Twenty-four to 48 h later, luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to manufacturer's instructions. Luciferase activity was measured using the GloMax® 20/20 luminometer (Promega). For data analyses, the experimental reporter was normalized to the level of constitutive reporter to adjust for the differences in transfection efficiency. Triplicate experiments were performed.

Statistics

The data values were presented as the mean ± SEM. Differences in mean values between two groups were analyzed by two-tailed Student's *t* test. When comparing more than two groups, two-way ANOVA test was used. $p \leq 0.05$ was considered statistically significant.

Results

IL-6 expression in prostate tumor tissues

We analyzed data of IL-6 expression in prostate tumors of various Gleason scores from the Cancer Genome Atlas (TCGA) dataset (https://cancergenome.nih.gov) (Fig. 1a). The data showed a significant increase of IL-6 expression in Gleason 10 tumors when compared with normal prostate tissue and with Gleason 9 tumors. We also stained IL-6 expression in selected PCa tumor tissues from patients with different T stages (Table S1). We noted a much stronger expression of IL-6 in T3/T4 tumors than in earlier stage tumors (Fig. 1b).

Incorporation of IL-6 into C4-2 and CWR22Rv1 cells increased cancer cell survival after radiation exposure

We sought to explore whether intracellular IL-6 level might affect the efficacy of radiation treatments. We developed IL-6 expressing C4-2 and CWR22Rv1 cells and control cells by incorporating IL-6 cDNA (vs. empty vector as control) into C4-2 and CWR22Rv1 PCa cells via lentiviral transduction. Increased IL-6 levels in the IL-6 expressing C4-2 (C4-2IL-6) and CWR22Rv1 (CWRIL-6) cells compared to vector control (C4-2vec and CWRvec) cells were confirmed in qPCR and Western blot analyses (Fig. 2a). We tested the survival of these IL-6 incorporated and control cells after radiation in clonogenic assay and found that IL-6 expressing cells exhibited higher survival than control cells (Fig. 2b), suggesting the IL-6 role in contributing to higher survival of cells after radiation.

Confirming IL-6 role in DNA damage repair in Comet assay and γH2AX staining

To confirm the role of IL-6 in contributing to the higher survival of cells after irradiation, radiation-induced DNA damages in C4-2IL-6/vec and CWRIL-6/vec cell sets were analyzed by Comet assay, a sensitive and rapid technique for quantifying DNA damage and repair (Figueroa-Gonzalez and Perez-Plasencia 2017). Right after irradiation (0 min), both IL-6 expressing (C4-2IL-6 and CWRIL-6) and control (C4-2vec and CWRvec) cells showed the same extent of DNA damage, but differences in DNA recovery (indicated by shortened DNA tail) were observed between IL-6 expressing cells and control cells at 30 min after irradiation (Fig. 2c).

Immunofluorescence (IF) staining of H2AX was conducted in IL-6 expressing and control cell sets by staining cells with phosphorylated H2AX (γH2AX) antibody (Bonner et al. 2008; Ji et al. 2017). We observed diminished γH2AX staining in IL-6 expressing cells compared to vector control cells at indicated time points after radiation (Fig. 2d), suggesting higher recovery from DNA damage of IL-6 expressing cells than vector control cells. Consistently, we observed lower γH2AX levels in the nuclear compartment of IL-6 expressing cells than in control cells in Western blot analyses (Fig. 2e).

In vivo study confirming the role of IL-6 in developing radioresistance of prostate tumor

To confirm the in vitro observation of the role of IL-6 in contributing to radioresistance of PCa cells, we performed mouse studies of human PCa tumor xenografts. C4-2IL-6 or C4-2vec cells were subcutaneously implanted into

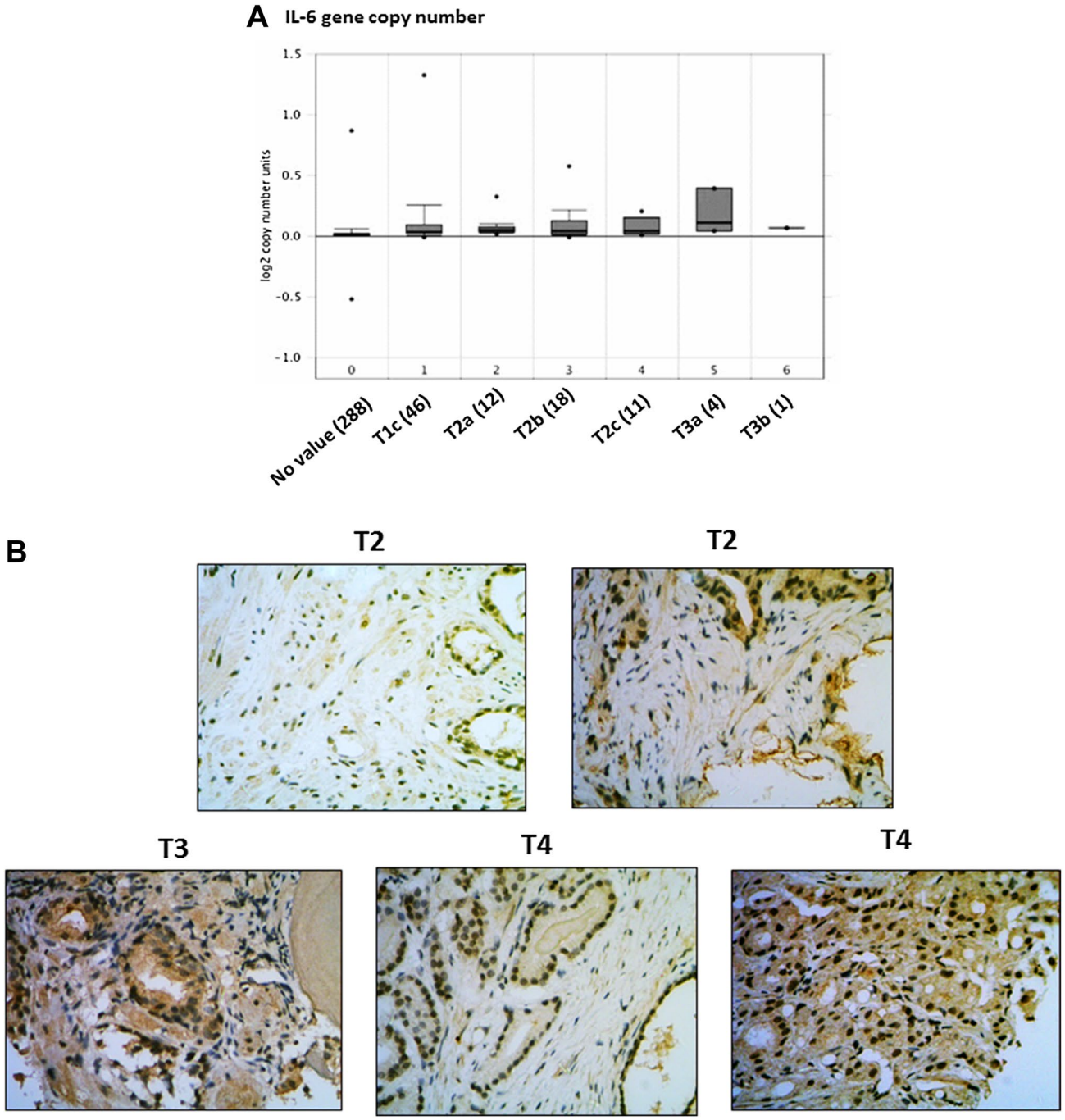


Fig. 1 **a** Cancer genome atlas (TCGA) dataset showing IL-6 gene expression in prostate tumors of various stages. **b** IHC stain of IL-6 expression in prostate patient tumors of different stages. Tumor tissues were obtained from Ningbo Hospital in China (patient information in Table S1). Tissues were stained with IL-6 Ab (magnification, ×20)

flanks of castrated athymic nude mice. Figure 3a shows the immunohistochemical (IHC) IL-6 staining in tumor tissues, confirming the difference in IL-6 level between C4-2vec and C4-2IL-6 cell-derived tumors. When tumors reached the size of 200–250 mm^3, mice were treated with radiation (2 Gy × 5 consecutive days). Non-irradiated mice were used as controls. After irradiation, tumor growth was monitored twice a week for 3 weeks. We observed that radiation treatment significantly delayed growth of C4-2vec cell-derived tumors when compared with non-irradiated tumors (Fig. 3b, black lines). On the contrary, irradiation had no significant effects on retarding the tumor growth of C4-2IL-6 cell-derived xenografts (Fig. 3b, red lines). The in vivo xenograft growth data further support

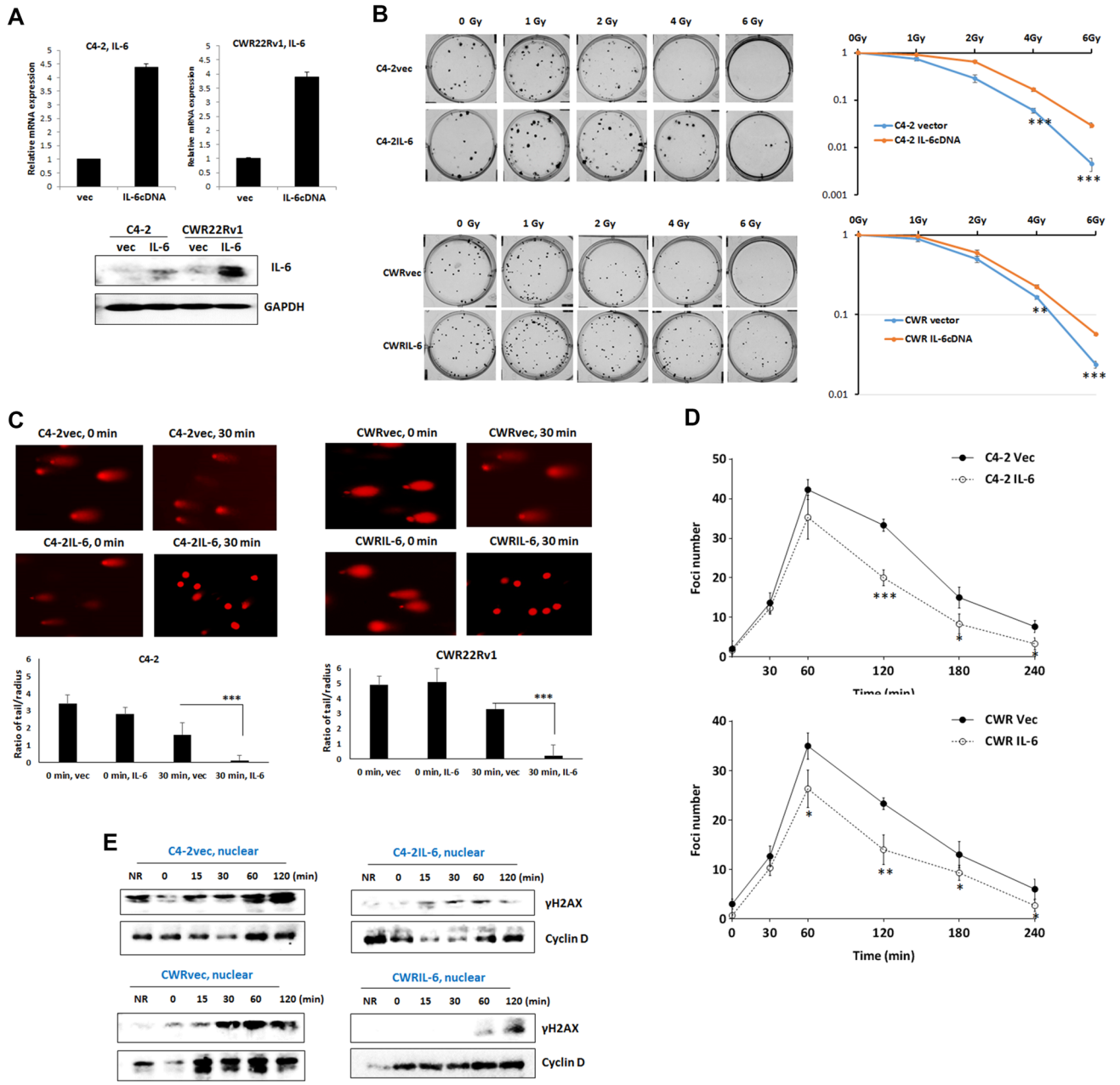
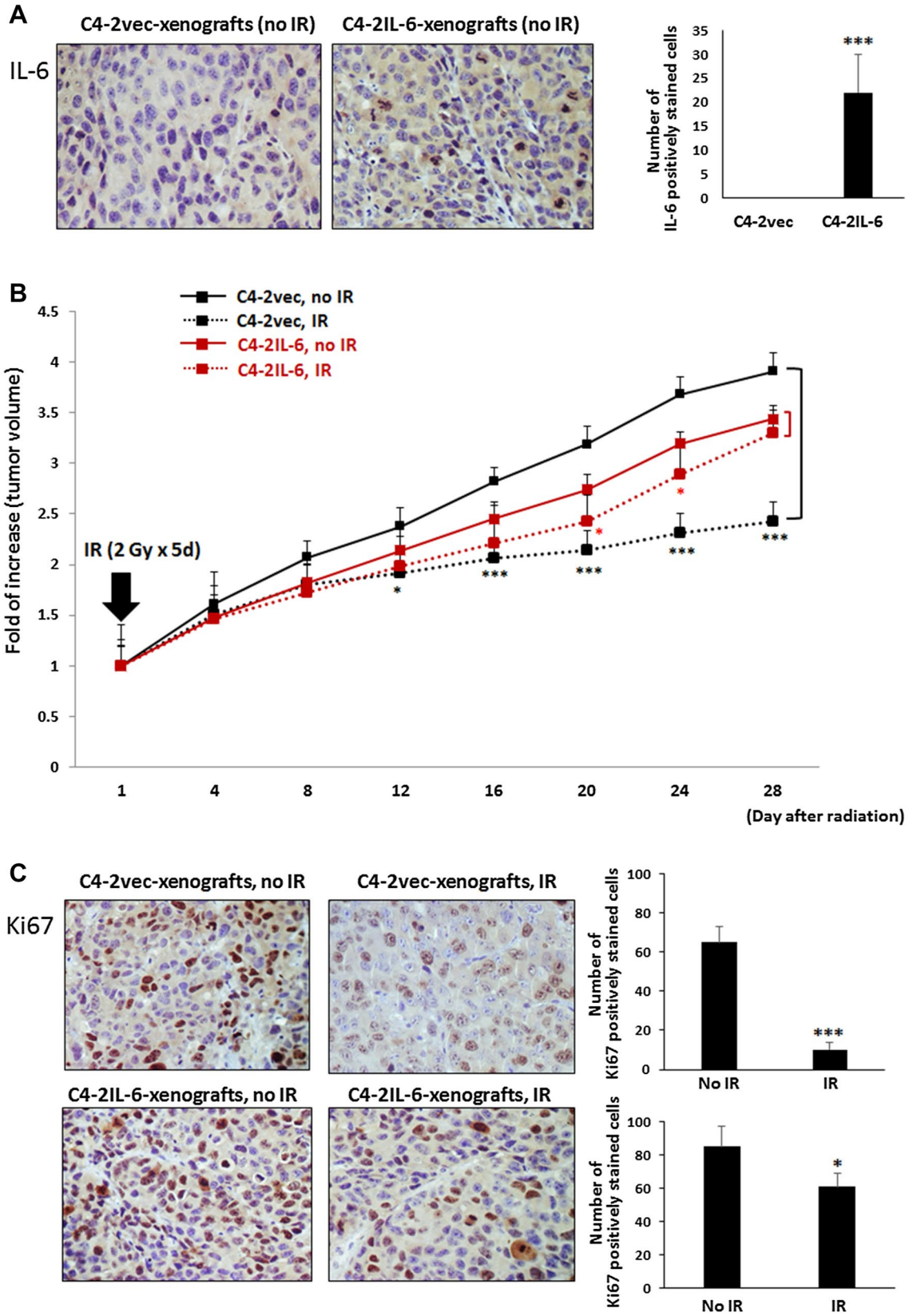


Fig. 2 IL-6 expressing cells are more resistant to radiation-induced DNA damage. **a** IL-6 expression by qPCR (m-RNA) (upper panel) and Western blot (protein) analyses (lower panel). Results confirm that C4-2IL-6 and CWRIL-6 cells contain much higher levels of IL-6 than respective vec cells. **b** Clonogenic assay testing the survival of C4-2IL-6/vec and CWRIL-6/vec cells after irradiation. **c** Comet assay. C4-2IL-6/vec and CWRIL-6/vec cells were irradiated (4 Gy) and DNA damages in these cell sets at 0 and 30 min after radiation were analyzed in Comet assay. Quantification of data is shown to the left of cell photos. **d** Graphs showing γH2AX loci number obtained from immunofluorescence (IF) stains. C4-2IL-6/vec and CWRIL-6/vec cells were irradiated (4 Gy); cells were fixed at indicated time points after radiation and stained with γH2AX Ab. **e** Western blot results analyzing γH2AX levels in nuclear compartment of cells. Cells were irradiated similarly and expressions of γH2AX in nuclear compartments at indicated time were analyzed. $*p<0.05$, $**p<0.01$, $***p<0.001$

Fig. 3 In vivo xenograft tumor investigations. **a** IL-6 IHC staining of tumor tissues. PCa xenograft tumor tissues were obtained at killing of mice; tissues processed and stained with IL-6 Ab (magnification, ×40). Quantitation of positively stained cells is shown on the right. Error bars and significance values in IHC staining were obtained by counting positively stained cells in one randomly chosen area of slides of three different slides. **b** Tumor growth analysis after ▶ radiation treatment. C4-2IL-6 and C4-2vec cell-derived subcutaneous xenografts were developed in athymic nude mice. After tumors developed, mice were irradiated and tumor growth after radiation was monitored. **c** Ki67 IHC staining of tumor tissues (magnification, ×40). $*p<0.05$, $***p<0.001$


A
C4-2vec-xenografts (no IR)
C4-2IL-6-xenografts (no IR)
IL-6
Number of IL-6 positively stained cells
***
C4-2vec
C4-2IL-6
B
C4-2vec, no IR
C4-2vec, IR
C4-2IL-6, no IR
C4-2IL-6, IR
Fold of increase (tumor volume)
IR (2 Gy x 5d)
(Day after radiation)
C
C4-2vec-xenografts, no IR
C4-2vec-xenografts, IR
C4-2IL-6-xenografts, no IR
C4-2IL-6-xenografts, IR
Ki67
Number of Ki67 positively stained cells
No IR
IR

the role of IL-6 signaling in radiation resistance development in PCa.

Next, we excised the tumors and stained them with Ab of the proliferation marker, Ki67. We noted a significantly lower number of Ki67 positively stained cells in irradiated tumors of C4-2vec cell-derived xenografts than in non-irradiated tumors. On the contrary, for C4-2IL-6 cell-derived xenografts, similar numbers of Ki67 positively stained cells were observed with or without radiation treatments (Fig. 3c). Taken together, the in vivo study results corroborated the in vitro findings in supporting the role of IL-6 in contributing to radioresistance of PCa cells.

IL-6 signaling upregulated DNA repair-associated molecules in PCa cells after radiation exposure

As the DNA repair pathway is critical in repairing radiation-induced DNA damage, we investigated whether the IL-6 effect on radioresistance development is through modulation of DNA repair pathway-associated molecules. We analyzed expressions of a panel of molecules associated with DNA repair/cell survival in IL-6cDNA/vec PCa cell sets after 4 Gy irradiation, with non-irradiated cells as control. The candidate DNA repair molecules include Ataxia Telangiectasia Mutated (ATM) (Shiloh and Ziv 2013), ATM- and RAD3-related (ATR) (Flynn and Zou 2011), checkpoint (CHK1/2) (Smith et al. 2010), BRCA1 (Lohse et al. 2015), BRCA2 (Lohse et al. 2015), DNA-dependent protein kinase (DNA-PK) (Yoon et al. 2012), p53 (Williams and Schumacher 2016), p21 (CDKN1A) (Cazzalini et al. 2010), NFκB (Volcic et al. 2012), TNFα, survivin (Vequaud et al. 2016), bcl-2 (Leisching et al. 2015), and bcl-xL (Zhao et al. 2004). Primer sequences of these molecules are shown in Table S2. At baseline (no radiation) condition, we detected IL-6 modulation of some DNA repair/cell survival-related molecules in C4-2IL-6/vec and CWRIL-6/vec cell sets (Fig. 4a). However, we observed different IL-6 modulation profiles in these cell sets after radiation treatment (Fig. 4b), suggesting that the IL-6 action mechanism on these molecules is different in the resting state of cells and when radiation was given. We observed significant upregulation of ATM, ATR, and BRCA1/2 in IL-6 expressing C4-2IL-6 and CWRIL-6 cells compared to respective vec control cells (Fig. 4b). The upregulation of protein of these molecules in IL-6 expressing PCa cells compared to non-irradiated cells after radiation was also demonstrated in Western blot analyses (Fig. 4c). As the molecules that showed notable differences after radiation treatment were thought to be more important in the DNA repair process, we selected these molecules and used them in further investigations.

It was reported that ATM and ATR were shuttled into the nuclear compartment to activate the DNA repair process (Cheng et al. 2018; Kidiyoor et al. 2016). However, conflicting reports exist regarding the nuclear localization of BRCA1 and BRCA2 (Feng et al. 2004; Henderson 2005; Yano et al. 2000). We investigated the expression of ATM, ATR, phosphorylated ATM (p-ATM), p-ATR, and BRCA1/2 molecules in cytosolic and nuclear compartments of C4-2IL-6/vec and CWRIL-6/vec cell sets at different time points (0, 15 min, 30 min, 1 h, and 2 h) after irradiation. We detected higher levels of all investigated molecules in C4-2IL-6 and CWRIL-6 cells than vector control cells after irradiation and detected ATM, p-ATM, BRCA 1/2 molecules primarily located in the nucleus, while ATR and p-ATR were in both cytosolic and nuclear compartments (Fig. 5).

IF staining was also performed to further confirm upregulation of ATM, ATR, and BRCA1/2 molecules in IL-6 expressing PCa cells and vec control cells after radiation. At 0, 30 min, and 1 h after radiation, we observed stronger signals of all these molecules in irradiated IL-6 expressing cells than vec cells (Figure S1), confirming higher expressions of these four molecules in IL-6 expressing cells than vec control cells after radiation.

IL-6 mediated upregulation of ATM and ATR at the transcriptional level

While our data showed that IL-6 upregulated the key DNA repair molecules, we were interested in knowing if IL-6 mediates the upregulation of ATM and ATR molecules at the transcriptional level. We chose these two molecules because they showed more significant differences in expression in IL-6cDNA/vec cell sets (Figs. 4d, 5).

We constructed luciferase plasmids containing the promoter region of ATM and ATR according to Zheng et al. (2015) (Fig. 6a). We first tested basal ATM- and ATR-luciferase activities in IL-6cDNA/vec cell sets. We observed significantly higher luciferase activities in C4-2IL-6 and CWRIL-6 cells than respective vector control cells (Fig. 6b). We then tested luciferase activities in parental C4-2 and CWR22Rv1 cells with the addition of human recombinant IL-6 (rhIL-6). We detected increases in ATM- and ATR-luciferase activities upon the addition of exogenous IL-6 in a dose-dependent manner (Fig. 6c). These results strongly support the idea that IL-6 mediates upregulation of ATM and ATR at the transcriptional level.

We then explored the IL-6 downstream signaling in mediating the ATM/ATR upregulation of PCa cells after irradiation. We applied inhibitors of several IL-6 downstream signaling pathways, including Janus kinase (JAK) 1/2 (JAK inhibitor 1) (Bellucci et al. 2015; Ikeda et al. 2016), signal transducers, activators of transcription factors (Stat) 3 (Stattic) (Fujita et al. 2015; Marzec et al. 2008), nuclear factor κB (NFκB) (Bay 11-7082) (Gowrishankar et al. 2015), mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) (U0126) (Chen

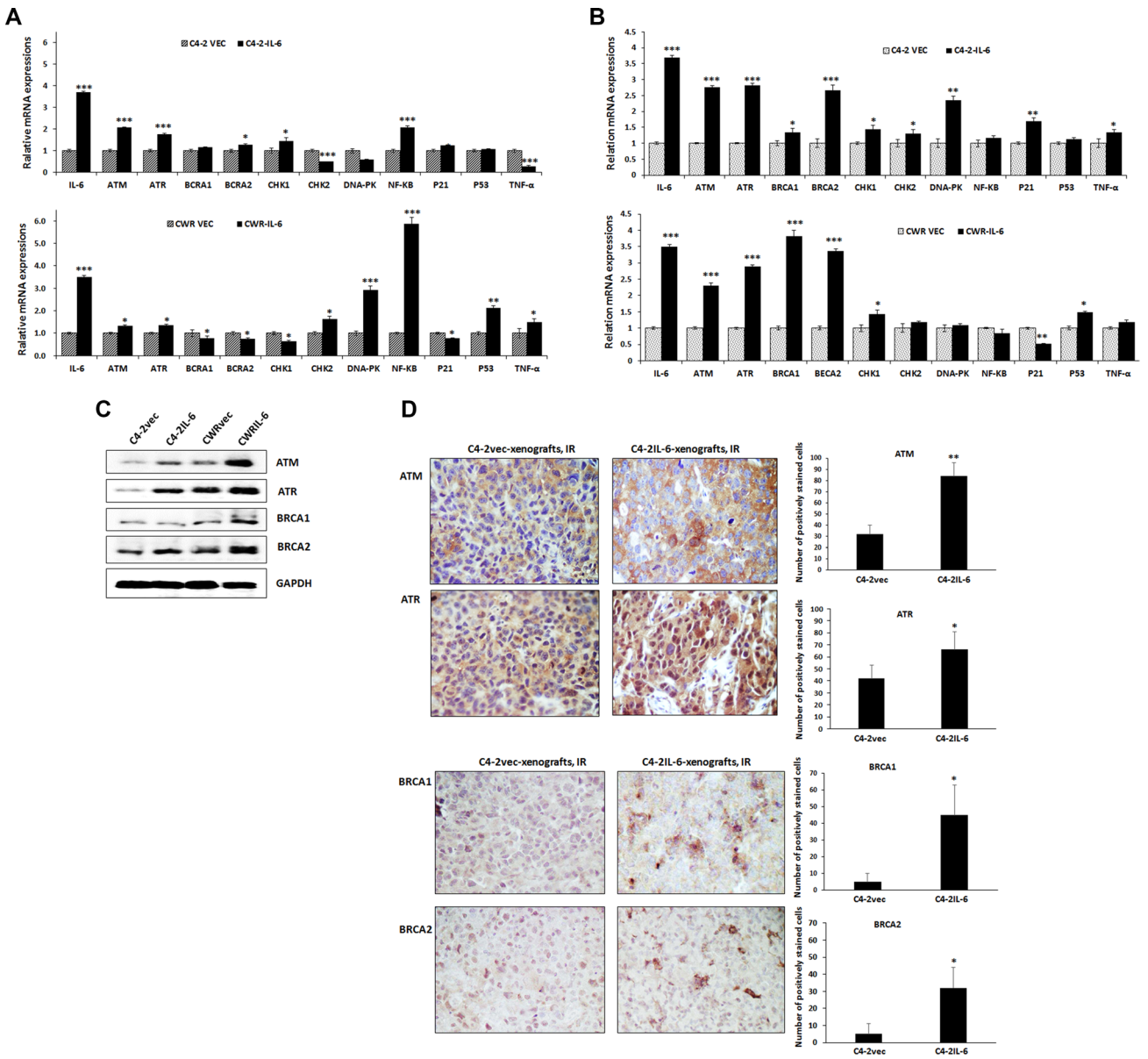


Fig. 4 IL-6 incorporated C4-2 and CWR cells showed higher expressions of 4 key DNA repair molecules upon irradiation than control cells. qPCR results of analyzing mRNA expressions of DNA repair-associated molecules in C4-2IL-6/vec and CWRIL-6/vec cells, without radiation (**a**) and with radiation (4 Gy) (**b**). **c** Western blot analyses results analyzing expressions of the DNA repair-associated molecules in irradiated (4 Gy) C4-2IL-6/vec and CWRIL-6/vec cells. **d** IHC staining of tumor tissues of C4-2IL-6/vec xenografts with antibodies of ATM, ATR, BRCA1, and BRCA2. Quantitation of positively stained cells is shown on the right. Error bars and significance values in IHC staining were obtained by counting positively stained cells in one randomly chosen area of slides with three different stains. Magnification, ×40. $*p<0.05$, $**p<0.01$, $***p<0.001$

et al. 2015a; Yamamoto et al. 2009), phosphatidylinositol 3-kinase (PI3K)/Akt (LY294002) (Lastwika et al. 2016; Xu et al. 2014), and mitogen-activated protein kinase (MAPK) (VX745) (Noh et al. 2015), mechanistic target of rapamycin (mTOR) (Rapamycin), and tumor necrosis factor (TNF) α (thalidomide) into the C4-2IL-6 and CWR22IL-6 cell cultures during radiation treatment procedure and analyzed ATM/ATR levels. We discovered that the inhibition of the Stat3 signaling pathway significantly reduced the ATM and ATR levels in C4-2IL-6 and CWRIL-6 cells (Fig. 6d), suggesting that Stat3 signaling is the critical IL-6 downstream signaling that mediates transcriptional upregulation of ATM/ATR molecules. We next tested whether adding the inhibitor of Stat3 signaling pathway blocks the transcription of ATM and ATR molecules. As shown in Fig. 6e, we detected a significant decrease in transcriptional activities when the inhibitor of Stat3 signaling was added to the cell culture, confirming the Stat3 signaling as the critical IL-6

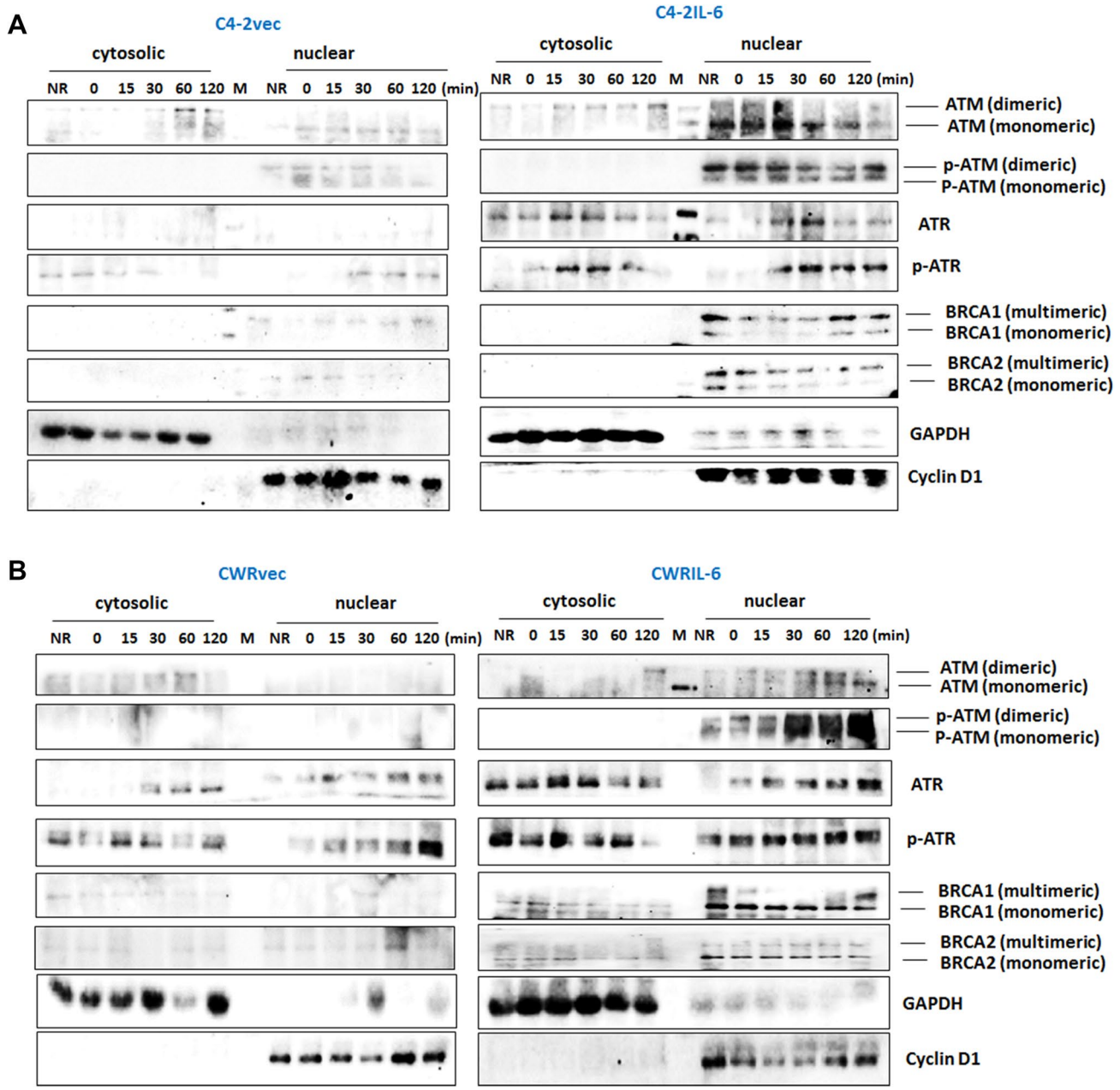


Fig. 5 IL-6 incorporated C4-2 and CWR cells showed higher nuclear expressions of 4 key DNA repair molecules upon irradiation than control cells. Western blot analysis analyzing the expression of ATM, p-ATM, ATR, p-ATR, BRCA1, and BRCA2 in cytosolic and nuclear fractions of irradiated **a** C4-2IL-6/vec and **b** CWRIL-6/vec cells. Nuclear and cytosolic extracts were obtained at indicated time points after radiation (4 Gy). Left panel shows C4-2IL-6/vec cell set data and right panel shows CWRIL-6/vec cell set data

downstream signaling pathways that promoted the transcription of ATM/ATR.

Discussion

Through both in vitro and in vivo investigations, we discovered a link between the IL-6 signaling and radioresistance of PCa through mediating the DNA repair pathways.

IL-6 effects on developing radioresistance in cancer cells may be explained in several ways. First, IL-6 effects can be explained via controlling radiation-induced reactive oxygen species (ROS). Wu et al. (2013) showed that IL-6 expression was positively linked to radiation-induced ROS and oxidative DNA damage in PCa. Tamari et al. (2017) suggested the mechanism of IL-6 mediated-acquisition of radioresistance through the control of ROS in glioma tumors. In addition, Matsuoka et al. (2016) showed that IL-6 controls

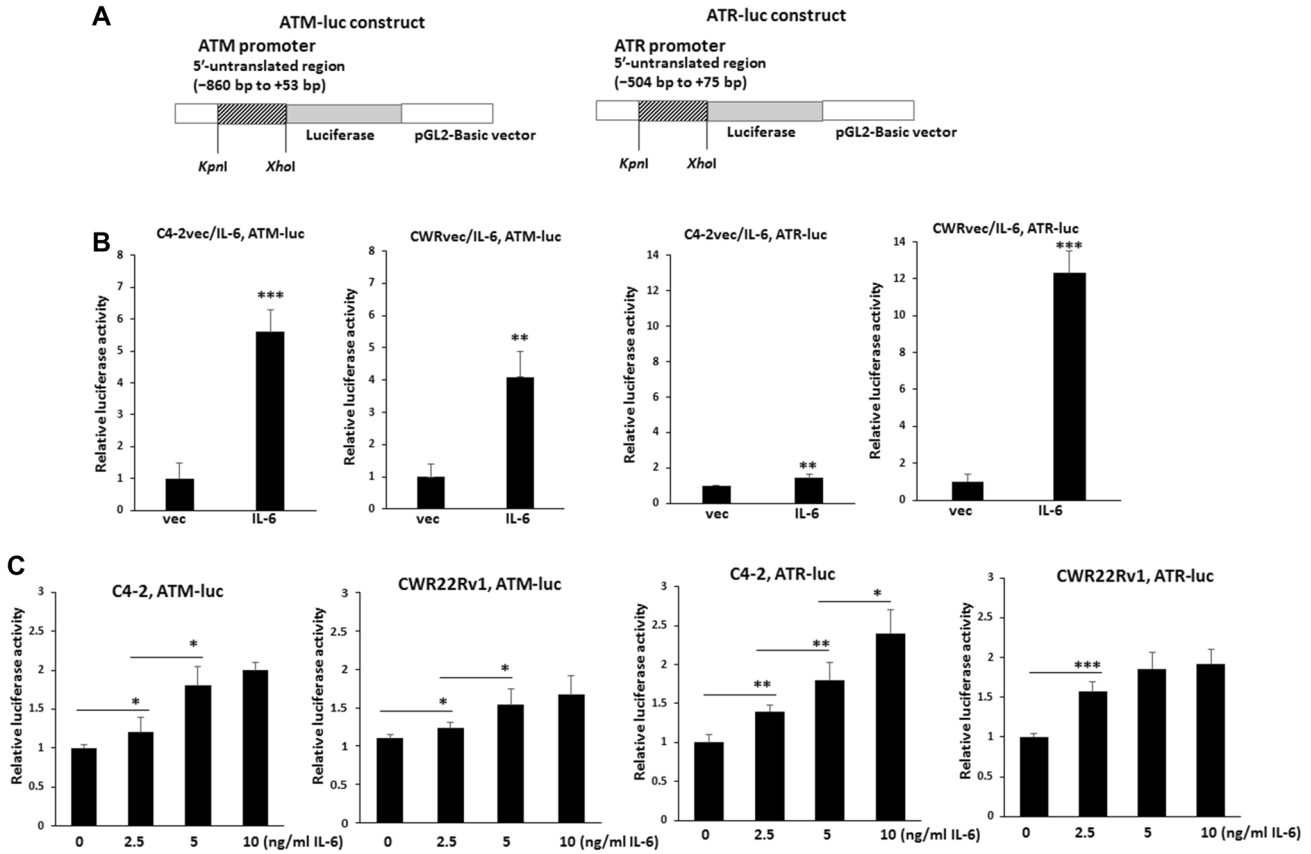


Fig. 6 IL-6 regulation of ATM and ATR expression at the transcriptional level. **a** Design of ATM- and ATR-luciferase constructs. **b** ATM- and ATR-luciferase assay in C4-2IL-6/vec and CWRIL-6/vec cell sets. **c** ATM- and ATR-luciferase assay in C4-2 and CWR22Rv1 cells, with the addition of varied amounts of rhIL-6. **d** ATM and ATR mRNA levels in C4-2IL-6 and CWRIL-6 cells after incubation with inhibitors of various signaling pathways. ATM or ATR expressions (mRNA level) in irradiated C4-2IL-6 and CWRIL-6 cells, in the presence of inhibitor of JAK (JAK inhibitor 1, 5 μM), Sta3 (Stattic, 5 μM), PI3 K/Akt (LY294002, 5 μM), MEK/Erk (U0126, 10 μM), MAPK (VX745, 10 μM), NFκB (Bay-7082, 5 μM), mTOR (rapamycin, 100 nM), and TNFα (thalidomide, 100 μg/ml) signaling pathways were analyzed (vehicle was used as control) in qPCR analyses. Inhibitors were added 30 min before irradiation (4 Gy). **e** Luciferase assay in C4-2IL-6 and CWRIL-6 cells in the presence of inhibitor of Stat3 signaling pathway (Stattic, 5 μM). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

resistance to radiation by suppressing oxidative stress via the nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant pathway in oral squamous cell carcinoma. The role of IL-6 in developing radioresistance can also be explained by increasing the myeloid-derived suppressor cell (MDSC) numbers as radiation induced-MDSC recruitment to tumors has been suggested (Wu et al. 2013), and effects of irradiation-induced IL-6 on increasing MDSCs in hepatocarcinoma (Chen et al. 2012) have been reported.

In animal studies, we observed retarded tumor growth of IL-6 incorporated tumors compared with control cell-derived tumors (Fig. 3). Although the IL-6 role in PCa progression, increasing metastasis, and therapy-resistance has been appreciated, whether IL-6 promotes PCa growth is controversial. Lin et al. (2001) showed IL-6 induced proliferation of PCa cells, but Culig et al. (2002) suggested that IL-6-induced Stat3 phosphorylation may result in in vitro and in vivo growth arrest of PCa. As IL-6 is known to be the mediator of neuroendocrine differentiation in PCa (Chang et al. 2014), it is possible that IL-6 signaling may trigger growth inhibition through this process. In addition, Sanford and DeWille (2005) reported that the IL-6 downstream CCAAT-enhancer-binding proteins (CEBP) delta mediates growth arrest of PCa cells. So, our observation of retarded tumor growth of IL-6 expressing tumors compared with non-IL-6 expressing tumors may not be surprising.

ATM is one of the central kinases involved in the cellular DNA damage response (DDR). ATM forms homodimers or higher order multimers, which dissociate into active monomers following rapid intermolecular auto phosphorylation of serine 1981 upon ATM activation (Weber and Ryan 2015). We also observed both dimeric and monomeric forms of ATM in cytosolic and nuclear compartments of PCa cells, respectively (Fig. 5). More importantly, we observed higher levels of monomeric ATM in C4-2IL-6 and CWR22IL-6 cells than vec control cells after radiation, indicating that

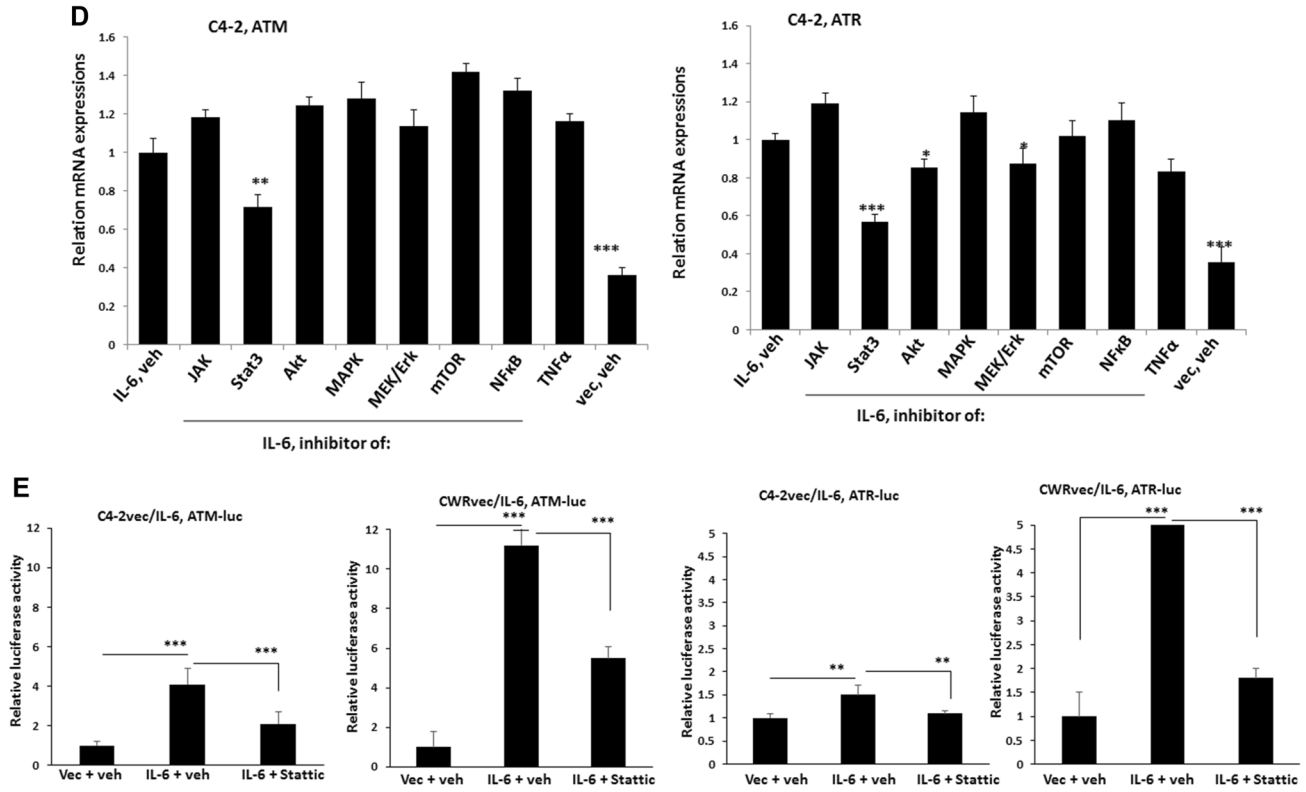


Fig. 6 (continued)

IL-6 signaling promoted not only ATM level increase, but also the ATM activation.

ATR, another central kinase involved in the DDR, is activated by single-stranded DNA structures which may arise at resected DNA DSBs or stalled replication forks (Weber and Ryan 2015). Higher levels of ATR at both higher cytosolic and nuclear compartments were also observed in IL-6 expressing cells than in vector cells after irradiation (Fig. 5). The presence of ATR in the cytoplasm and organelles has been shown previously, suggesting the role of this molecule in DNA damage-independent pathways (Kidiyoor et al. 2016). Thus, the detection of abundant ATR in the cytoplasm in our Western blot analyses was not surprising.

Several molecules, such as CHK1, CHK2, BRCA1, and BRCA2 are known to be the downstream signals of ATM and ATR to activate p53 (Principles of Cancer Genetics. Second Edition 2016). While we did not observe IL-6 upregulation of CHK1/2, we did observe increased expressions of BRCA1 and BRCA2 in PCa cells containing IL-6 cDNA compared to vec cells. Similar to ATM, BRCA1/2 molecules were also reported to be converted from inactive dimeric form to active monomeric form, and nuclear localization of BRCA1 was thought to be essential for DNA repair signaling (Henderson 2005). It has been suggested that the BRCA1-associated RING domain protein 1 (BARD1) molecule forms heterodimeric form with BRCA1 and helps shuttling into nucleus (Henderson 2005). On the other hand, Feng et al. (2004) suggested that nuclear export of BRCA1 is important for DNA repair process. In this report, we detected BRCA1 in both cytosolic and nuclear compartments after irradiation. Further investigation of the nuclear trafficking of these proteins will be informative.

Our data suggest that targeting IL-6 signaling or ATM, ATR, and BRCA1/2 can increase the radiation sensitivity of PCa cells. However, given the complexity of the physiological activities of IL-6 in producing both pro- and anti-inflammatory effects in the immune system (Scheller et al. 2011), applying more selective inhibitors of ATM, ATR, or BRCA1/2 may be a better option. Coincidentally, highly selective small molecule inhibitors of ATM and ATR are currently in preclinical and clinical development to increase radiation-sensitivity (Manic et al. 2015), while inhibitors of BRCA1/2 for preclinical and clinical use are not available yet. Another option would be to apply the inhibitor of IL-6 downstream signaling Stat3, as we observed that inhibition of Stat3 signaling reduced the transcription of ATM and ATR after irradiation. Several Stat3 inhibitors or Stat3 antisense or decoy oligonucleotides have been in clinical trials (Johnson et al. 2018). Some of these compounds, such as OPB31121, showed promising antitumor activity without much toxicity in a clinical trial (Oh et al. 2015). Analyzing tumor

samples or circulating cancer cells in PCa patients receiving radiotherapy for the expression of IL-6, ATM, ATR, and BRDA1/2 will be useful to further confirm our novel discovery, but this is beyond the scope of this pre-clinical investigation.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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