

Double-stranded RNA-specific adenosine deaminase-knockdown inhibits the proliferation and induces apoptosis of DU145 and PC3 cells by promoting the phosphorylation of H2A.X variant histone

XIEZHAO LI*, RUI ZHU*, YAOJI YUAN, ZHIDUAN CAI,
SIYANG LIANG, JUN BIAN and GUIBIN XU

Department of Urology, The Fifth Affiliated Hospital of Guangzhou Medical University,
Guangzhou, Guangdong 510700, P.R. China

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Abstract. Double-stranded RNA-specific adenosine deaminase (ADAR1) is a member of the adenosine deaminases acting on RNA family that catalyze the adenosine-to-inosine editing of double-stranded RNA substrates. Several studies have reported that ADAR1 is closely associated with numerous malignancies. However, the functional roles of ADAR1 in prostate cancer (PCa) have not been fully elucidated. Thus, the present study aimed to investigate the effects of ADAR1 on PCa. The results demonstrated that ADAR1 was highly expressed in PCa tissues compared with normal tissues. Furthermore, the protein expression level of ADAR1 was significantly increased in castration-resistant PCa (CRPCa) tissues and CRPCa cell lines. Thus, these findings indicated that ADAR1 may act as a tumor promoter for PCa development. Next, the potential effects of ADAR1-knockdown on the proliferation of DU145 and PC3 cells were investigated. ADAR1 was knocked down via small interfering RNA transfection, which was found to exert antitumor effects on DU145 and PC3 cells at 24 and 48 h post transfection. Furthermore, a significant positive association was observed between ADAR1-knockdown and the apoptosis of DU145 and PC3 cells, which increased the phosphorylation of H2A.X variant histone. The results of the present study indicated a positive association between

ADAR1 expression and PCa, which may promote the development of CRPCa. Moreover, ADAR1-knockdown may serve as a tumor suppressor and represent a potential target for the treatment of PCa.

Introduction

Prostate cancer (PCa) is the most frequently diagnosed type of cancer, and the second leading cause of cancer-related mortality in American men (1). Castration-resistant PCa (CRPCa) is the final stage of PCa, for which there is currently a relative lack of treatment options. Therefore, the development of effective and innovative therapies against PCa is necessary.

Adenosine deaminases acting on RNA (ADARs) are enzymes that catalyze the adenosine-to-inosine (A-to-I) editing of double-stranded RNA (dsRNA) substrates (2,3). At present, two different enzymes with ADAR activity, double-stranded RNA-specific adenosine deaminase (ADAR1) and double-stranded RNA-specific editase 1 (ADAR2), have been identified in mammals (3,4). Previous studies have highlighted the dependence of certain types of cancer on ADAR1 expression (5,6). For example, high expression levels of ADAR1 are closely associated with metastasis and poor prognosis in carcinoma (7,8). ADAR1 also functions as a checkpoint that limits antitumor immunity by preventing the sensing of endogenous dsRNA (9). However, a limited number of studies have aimed to characterize the expression patterns of ADAR1 in PCa development.

Therefore, the aim of the present study was to investigate the effect of ADAR1 on PCa. The expression levels of ADAR1 were detected in PCa and CRPCa tissues, as well as CRPCa cell lines. ADAR1 was then knocked down in two stable cell lines, DU145 and PC3. The effects of ADAR1-knockdown on cellular proliferation and apoptosis were subsequently determined, and the interaction between ADAR1 and the phosphorylation of H2A.X variant histone was evaluated. These results suggested ADAR1 as a promising target for anti-cancer therapy in CRPCa.

Correspondence to: Professor Guibin Xu, Department of Urology, The Fifth Affiliated Hospital of Guangzhou Medical University, 621 Gangwan Road, Huangpu, Guangzhou, Guangdong 510700, P.R. China
E-mail: gyxgb@163.com

*Contributed equally

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Materials and methods

Cells lines and, tissue samples. The cell lines, RWPE-1 (cat. no. CRL-11609), LnCap (cat. no. CRL-1740), 22RV1 (cat. no. CRL-2505), DU145 (cat. no. HTB-81) and PC3 (cat. no. CRL-1435) were purchased from the American Type Culture Collection. RWPE-1 cells were maintained in keratinocyte serum-free medium (Thermo Fisher Scientific, Inc.), DU145 cells were maintained in DMEM (Thermo Fisher Scientific, Inc.), and LnCap, 22RV1 and PC3 cells were maintained in RPMI 1640 (Sigma-Aldrich; Merck KGaA). The media were supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), and the cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. DU145 and PC3 cells were transfected with negative control (NC) small interfering (si)RNA and ADAR1-specific siRNAs (Shanghai Gene Pharma Co., Ltd.), and the suppressed expression of ADAR1 was confirmed using western blot analysis.

Preliminary screening of ADAR1 protein expression via immunohistochemistry (IHC) and western blot analysis with an antibody against ADAR1, was conducted on tissues from patients diagnosed with benign prostatic hyperplasia (BPH), PCa and CRPCa at The Fifth Affiliated Hospital of Guangzhou Medical University.

All procedures carried out in studies involving patients were conducted in accordance with the ethical standards of the institutional and/or national research committee, as well as with the 1964 Declaration of Helsinki and its later amendments, or comparable ethical standards. The present study was a retrospective study reviewed and approved by the Ethics Committee, The Fifth Affiliated Hospital, Guangzhou Medical University (Guangzhou, China). Written informed consent was obtained from all patients.

Sample collection. Samples were obtained from patients (28 male patients, including 10 BPH, 10 PCa and 8 CRPCa cases; age, 63-74 years) who underwent surgery at the Department of Urology, the Fifth Affiliated Hospital of Guangzhou Medical University (Guangzhou, China) between March 2019 and September 2020. BPH tissue samples were obtained from transurethral resection of the prostate; PCa and CRPCa tissue samples were obtained from radical prostatectomy. Pathological evaluation of these tissues was confirmed. Patients with CRPCa were selected according to the European Association of Urology guidelines (10). The inclusion criteria were as follows: i) Histologically confirmed diagnosis of BPH, PCa or CRPCa; ii) aged between 60 and 75 years; and iii) willing to donate tissue samples. Patients who had incomplete information or a history of other malignant tumors were excluded. In the present study, patient characteristics (age, prostate volume, preoperative prostate-specific antigen level, Gleason score and clinical stage) were collected retrospectively. The characteristics of the patients are displayed in Table I.

IHC. A standard IHC protocol was used to stain the tissue samples, using a rabbit monoclonal antibody against ADAR1 [Cell Signaling Technology, Inc.; cat. no. 81284] (11). Briefly, paraffin-embedded tissue sections were deparaffinized, rehydrated and subjected to antigen retrieval. Samples were

incubated with anti-ADAR1 diluted with TBS (1:800). All samples were observed under a light microscope (Carl Zeiss AG) and automated image quantification was conducted using ImageJ2x software (National Institutes of Health).

Western blotting. The tissue and cell samples were homogenized in lysis buffer containing: 0.1 mol/l NaCl, 0.01 M Tris-HCl (pH 7.5), 1 mM EDTA and 1 µg/ml aprotinin. After centrifugation at 16,000 x g for 5 min (4°C), total protein content in the supernatants was determined using a Bradford Protein Assay kit (Bio-Rad Laboratories, Inc.). In total, 40-80 µg protein was separated via 8% SDS-PAGE and then transferred to nitrocellulose membranes (12,13). The membranes were blocked for 60 min with blocking buffer [composed of 5% skimmed milk in TBS-Tween-20 (0.25 M Tris-HCl pH 7.5, 0.1% Tween-20 and 0.15 M NaCl)], at 37°C, and subsequently incubated with anti-GAPDH (1:1,000; cat. no. 5174), anti-β-actin (1:1,000; cat. no. 4970), anti-β-tubulin (1:1,000; cat. no. 2128), anti-phosphorylated (p)-H2A.X (1:2,000; cat. no. 2577), anti-H2A.X (1:2,000; cat. no. 7631), anti-cleaved caspase-3 (1:1,000; cat. no. 9661), anti-caspase-3 (1:1,000; CST; cat. no. 9662), anti-cleaved PARP (1:500; cat. no. 5625) and anti-PARP (1:500; cat. no. 9542) antibodies (all CST Biological Reagents Co., Ltd.) overnight at 4°C. The primary antibodies were detected using an HRP-conjugated secondary anti-rabbit IgG antibody (1:2,000; cat. no. 7076; CST Biological Reagents Co., Ltd.) for 1 h at 37°C. Bands were visualized using 3,3'-diaminobenzidine reagent at room temperature for 2 min, and were semi-quantified via scanning with a Molecular Imager (Bio-Rad Laboratories, Inc.). The results were analyzed with ImageJ2x and Origin 8.0 software (National Institutes of Health).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cell lines using the RNeasy mini kit (Qiagen, Inc.) per the manufacturer's protocol, and was quantified through spectrophotometric measurement using a NanoDrop system (Thermo Fisher Scientific, Inc.). qPCR analysis was conducted using the Verso One-Step SYBR qRT-PCR kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Primers were custom-synthesized and obtained from Guangzhou HYY Co. (<http://www.hyymed.com/>) (Table II). The amplification reaction was performed as follows: Initial denaturation for 5 min at 95°C, followed by 30 cycles at 95°C for 10 sec, 60°C for 15 sec and 72°C for 20 sec. β-actin was used as a control for relative quantification. Rotor-Gene Software (Qiagen, Inc.) was used for comparative concentration analysis (14,15). All reactions were carried out in triplicate, and the 2^{-ΔΔCq} method was used to determine the relative quantification of mRNA expression (16).

Transfection. DU145 and PC3 cells were seeded into 6-well plates at a density of 1x10⁴ cells/well, and transfected with negative control siRNA (NC-siRNA sense, 5'-UUCUCC GAACGUGUCACGUTT-3' and antisense, 5'-ACGUGA CACGUUCGGAGAATT-3') or ADAR1-specific siRNA (ADAR1-siRNA-a sense, 5'-GCAUCUGACCCGUGCUAU UTT-3' and antisense, 5'-AAUAGCACGGGUCAGAU CTT-3'; ADAR1-siRNA-b sense, 5'-GCAGCCCAUUUAUCU

Table I. Characteristics of patients with BPH, PCa and CRPCa (n=28).

Characteristic	BPH (n=10)	PCa (n=10)	CRPCa (n=8)
Age (years), median (range)	68.4 (64-73)	67.2 (63-70)	69.5 (67-74)
Prostate volume (cm ³), median (range)	78.2 (65-91)	46.2 (37-53)	46.3 (38-55)
Preoperative prostate-specific antigen (ng/ml), median (range)	3.6 (0.8-10.5)	22.1 (12.6-32.1)	57.6 (42.5-81.4)
Source of tissue	Transurethral resection of the prostate	Radical prostatectomy	Radical prostatectomy
Gleason score, median (range)		7.8 (6-10)	8.8 (7-10)
Clinical stage			
T1		3	1
T2		4	3
T3		3	2
T4		0	2

BPH, benign prostatic hyperplasia; PCa prostate cancer; CRPCa, castration-resistant PCa.

Table II. Primers used for reverse transcription-quantitative PCR analysis.

Gene	Primer	Sequence (5'-3')
Double-stranded RNA-specific adenosine deaminase	Forward	CGCCCTCTTTGACAAGTCCT
	Reverse	GGGATTGTGCCTTCTCCGTTT
β-actin	Forward	TGGCACCAGCACAATGAA
	Reverse	CTAAGTCATAGTCCGCCTAGAAGCA

CAAATT-3' and antisense, 5'-UUUGAGAUAAAUGGGCUG CTT-3'; and ADAR1-siRNA-c sense, 5'-GCUUCAACACUC UGACUAATT-3' and antisense, 5'-UUAGUCAGAGUGUUG AAGCTT-3') (final concentration, 20 nmol/l; all Shanghai GenePharma Co., Ltd.) using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following culture for 48 h at 37°C, transfection efficiency was assessed by western blotting. After transfection, cells were cultured for 24 h and then used for subsequent experimentation.

Cell Counting Kit 8 (CCK-8) assay. Cell viability was measured using the CCK-8 kit (Dojindo Molecular Laboratories, Inc.) following the manufacturer's protocol. Briefly, cells were seeded into a 96-well plate at a density of 8x10³ cells/well, and incubated with CCK-8 reagent at 37°C for 4, 24, 48 and 72 h. The optical density was measured at 450 nm to estimate the number of viable cells.

Apoptosis analysis. Apoptosis was analyzed using an Annexin V-FITC/PI kit (eBioscience; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Cells in each group were seeded into a 6-well plate at a density of 1x10⁵ cells/well, and transfected with NC siRNA and ADAR1-specific siRNAs. After incubation for a further 2 days, the cells were harvested, washed with PBS and resuspended in staining buffer (Annexin V-FITC/PI kit) at a final density of 3x10⁵/ml. Then, apoptosis was analyzed using a FACScan flow cytometer (FACSCalibur; BD Biosciences).

Statistical analysis. Statistical analysis was performed using SPSS software ver. 28.0 (SPSS, Inc.) and graphs were generated using GraphPad Prism 8 (GraphPad Software, Inc.). Statistical results are presented as the mean ± SD of ≥3 independent experiments. Unpaired Student's t-test was used to compare differences between two groups. One-way or two-way ANOVA were used for multiple comparisons, followed by Dunnett's or Tukey's post hoc tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Patient characteristics. Patient characteristics including age, prostate volume, preoperative prostate-specific antigen (PSA), source of tissue, Gleason score and clinical stage are summarized in Table I. In the present study, 28 male patients aged 63-74 were enrolled, including 10 BPH, 10 PCa and 8 CRPCa cases. The mean prostate volume in each group was 78.2 (65-91), 46.2 (37-53) and 46.3 (38-55) cm³, respectively. The mean preoperative PSA was 3.6 (0.8-10.5), 22.1 (12.6-32.1) and 57.6 (42.5-81.4) ng/ml, respectively. Histopathological Gleason score was determined based on the criteria of the World Health Organization. The mean Gleason score of patients with PCa was 7.8 (6-10), and that of patients with CRPCa was 8.8 (7-10). Tumors were staged according to the eighth edition American Joint Committee on Cancer Staging Manual (17). The number of PCa patients at stages T1, T2 and T3 was 3, 4 and 3, respectively. The number of CRPCa patients at stages T1, T2, T3 and T4 was 1, 3, 2 and 2, respectively.

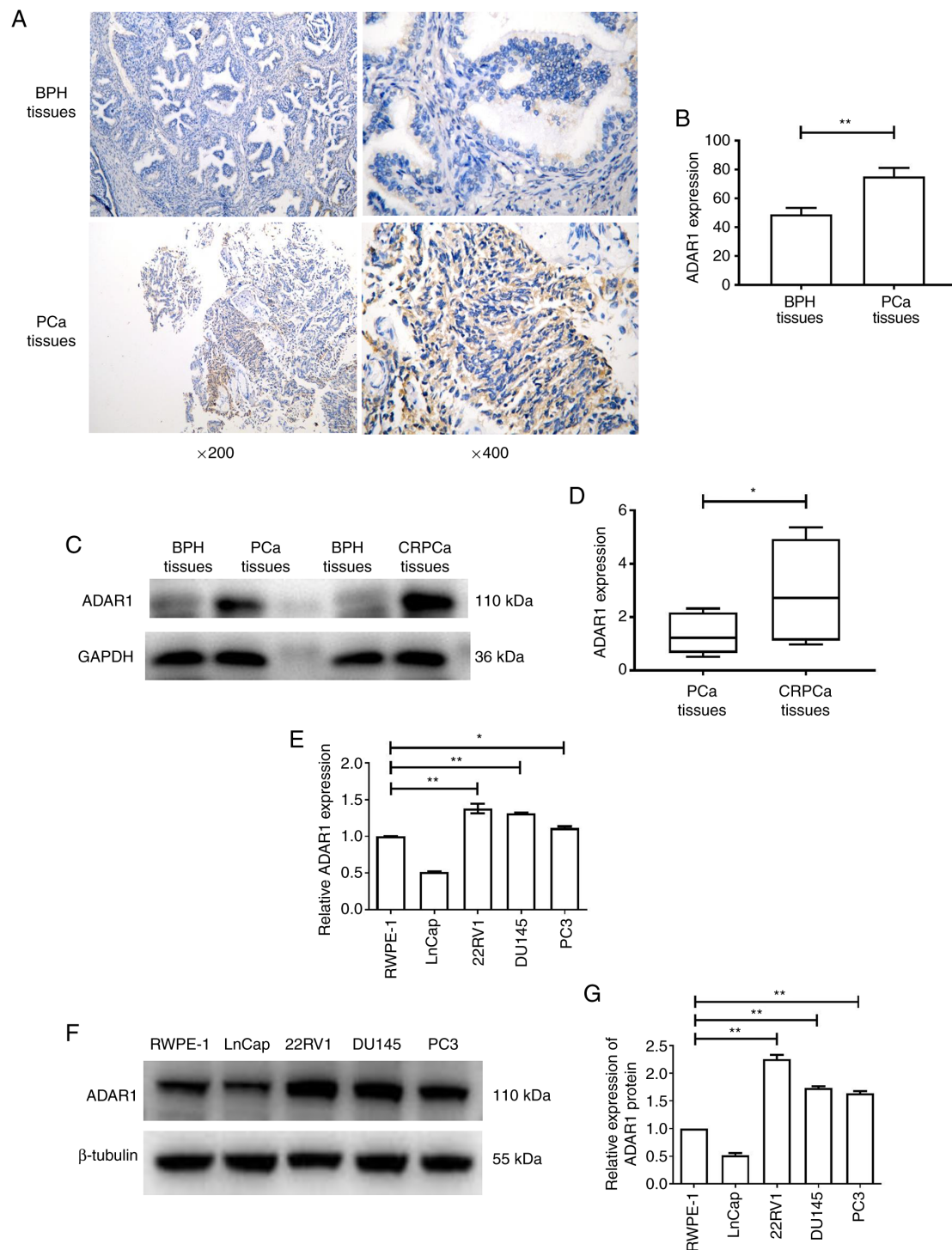


Figure 1. ADAR1 is highly expressed in CRPCa. (A) Representative images and (B) quantification of immunohistochemical staining for ADAR1 in BPH and PCa tissues. (C and D) Western blot analysis of ADAR1 protein expression in different tissue types. (E) Reverse transcription-quantitative PCR and (F and G) western blot analysis of the mRNA and protein expression levels of ADAR1 in different cell lines. * $P < 0.05$ and ** $P < 0.01$. ADAR1, double-stranded RNA-specific adenosine deaminase; BPH, benign prostatic hyperplasia; PCa, prostate cancer; CRPCa, castration-resistant PCa.

ADAR1 is highly expressed in CRPCa. The expression level of ADAR1 was determined using IHC images of PCa tissues. Via IHC staining and automated image quantification, ADAR1 expression was found to be significantly increased in PCa, compared with BPH tissues (Fig. 1A and B). Furthermore, PCa and CRPCa tissues were extracted and probed with an ADAR1 monoclonal antibody. Notably, higher protein expression levels of ADAR1 were detected in

CRPCa tissues compared with PCa tissues (Fig. 1C and D). Furthermore, RT-qPCR and western blot analysis demonstrated that the mRNA and protein expression levels of ADAR1 were decreased in LNCaP cells and significantly increased in the CRPCa cell lines (22RV1, DU145 and PC3) compared with RWPE-1 cells (Fig. 1E-G). These results suggest that ADAR1 is upregulated in CRPCa tissues and cell lines.

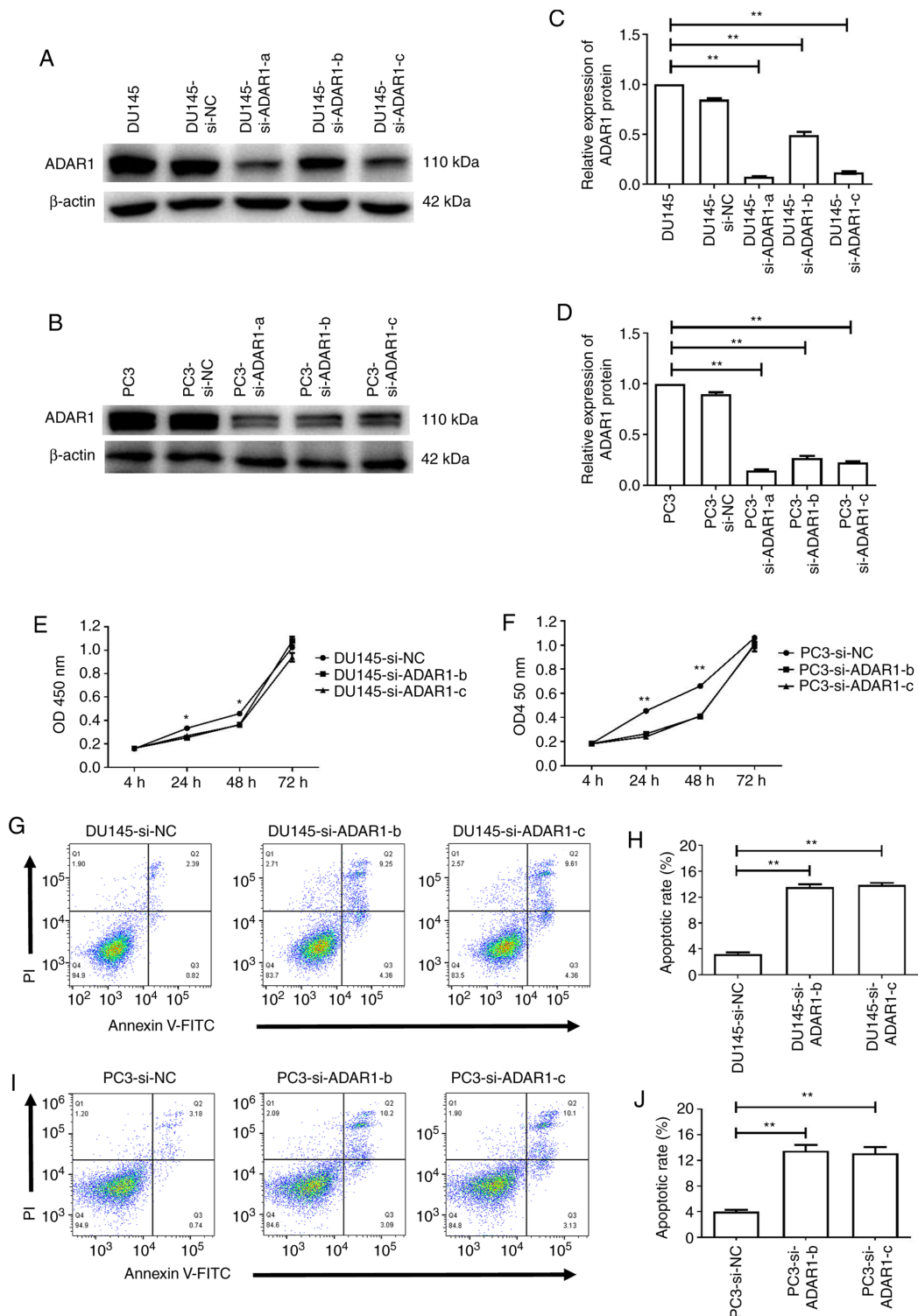


Figure 2. ADAR1-Knockdown inhibits the proliferation and induces the apoptosis of DU145 and PC3 cells. Western blot analysis was used to confirm ADAR1-knockdown in (A and B) DU145 and (C and D) PC3 cells after siRNA transfection. Proliferation of (E) DU145 and (F) PC3 cells was inhibited by ADAR1-knockdown. ADAR1-Knockdown promoted the apoptosis of (G and H) DU145 and (I and J) PC3 cells, which was analysed by flow cytometry using Annexin V-FITC/PI staining. Statistical results are presented as the mean \pm SD of three independent experiments. * $P < 0.05$ and ** $P < 0.01$. ADAR1, double-stranded RNA-specific adenosine deaminase; OD, optical density; si (RNA), small interfering; NC, negative control.

ADAR1-knockdown inhibits the proliferation and induces the apoptosis of DU145 and PC3 cells. DU145 and PC3 cells that are considered to be androgen receptor negative (with high expression levels of ADAR1) were transiently transfected with ADAR1-specific siRNAs. Total protein was isolated and analyzed via western blotting 48 h post-transfection.

Compared with the blank (no siRNA) and mock-transfected cells (DU145 si-NC and PC3 si-NC), the expression level of ADAR1 was markedly suppressed in cells transfected with ADAR1 siRNAs (Fig. 2A-D).

Next, the potential effects of ADAR1-knockdown on the proliferation of DU145 and PC3 cells were assessed. As

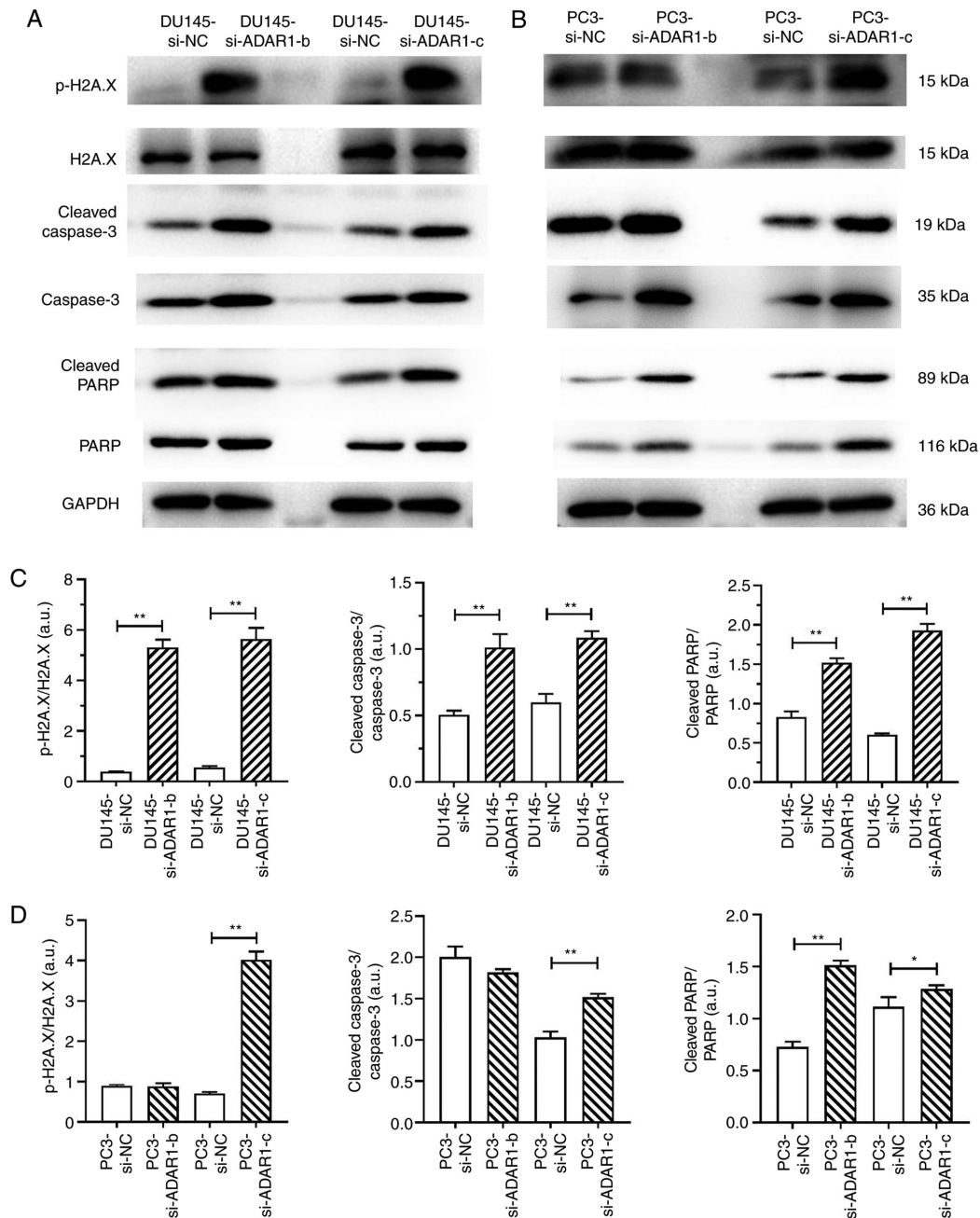


Figure 3. ADAR1-knockdown induces the apoptosis of DU145 and PC3 cells by promoting the phosphorylation of H2AX. Lysates from transfected (A) DU145 and (B) PC3 cells were analyzed via western blotting to detect the expression levels of apoptotic markers (cleaved caspase-3, caspase-3, cleaved PARP and PARP), and the DNA double-strand-break markers, p-H2AX and H2AX. Data are representative of three independently performed experiments. Protein expression levels of p-H2AX, H2AX, cleaved caspase-3, caspase-3, cleaved PARP and PARP in (C) DU145 and (D) PC3 cells. Statistical results are presented as the mean \pm SD of five independent experiments. * $P < 0.05$ and ** $P < 0.01$. ADAR1, double-stranded RNA-specific adenosine deaminase; si (RNA), small interfering; NC, negative control; p-, phosphorylated; H2AX, H2A.X variant histone.

shown in Fig. 2E and F, in both cell lines, ADAR1-knockdown significantly inhibited cellular proliferation compared with the untransfected cells after 24 and 48 h ($P < 0.05$). However, there was no statistically significant difference in cellular proliferation 72 h post-transfection.

Flow cytometry was used to assess whether ADAR1-knockdown was associated with DU145 and PC3 cell apoptosis. The results demonstrated that the apoptotic rate of ADAR1 siRNA-transfected cells was increased compared with that of the untransfected cells (Fig. 2G-J). Thus, these results indicated that knockdown of ADAR1 expression

inhibited the proliferation and induces the apoptosis of DU145 and PC3 cells.

ADAR1-Knockdown induces DU145 and PC3 cell apoptosis by promoting the phosphorylation of H2AX. Next, it was determined whether the phosphorylation of H2AX during ADAR1-knockdown induced the apoptosis of DU145 and PC3 cells. The expression levels of p-H2AX, H2AX, cleaved caspase-3, caspase-3, cleaved PARP and PARP were detected using western blotting (Fig. 3A and B). Results showed that DU145 si-ADAR1 cells and PC3 si-ADAR1-c

cells exhibited markedly increased activation of caspase-3 and cleavage of PARP, which was accompanied by the phosphorylation of H2AX (Fig. 3C and D). Collectively, these data confirm that ADAR1-knockdown inhibits the proliferation and induces the apoptosis of DU145 and PC3 cells by promoting the phosphorylation of H2A.X.

Discussion

RNA editing, also known as RNA modification, was first discovered by Benne *et al* (18) in mitochondrion-encoded mRNA of the trypanosome. Dysregulated RNA editing has been associated with multiple cancer types (19). A-to-I RNA editing is the posttranscriptional deamination of A-to-I in dsRNA, which is catalyzed by enzymes of the ADAR family (20). Currently, three members of the ADAR family have been identified, namely ADAR1 (encoded by ADAR), ADAR2 (encoded by ADARB1) and adenosine deaminase RNA-specific B2 (inactive; encoded by ADARB2) (3,21). ADAR1 was the first identified active A-to-I RNA enzyme that was more ubiquitously expressed in different tissues. While both ADAR1 and ADAR2 serve roles in tumorigenesis, additional editing events regulated by ADAR1 have been observed in some cancer types, along with its abundant expression (22). Thus, ADAR1 activation in cancer is an interesting topic that requires continued in-depth investigation.

The present study revealed that ADAR1 expression was significantly increased in PCa tissues, and that higher ADAR1 protein expression was apparent in CRPCa tissues and cell lines (22RV1, DU145 and PC3). Therefore, these results suggest an important role for ADAR1 in PCa progression. In most tumor types, ADAR1 expression is elevated compared with that in matched normal tissues, indicating that RNA editing may supplement genomic DNA alterations and drive tumorigenesis (6,23,24). These data suggest that the loss of ADAR1 may be associated with antitumor activities.

In the current study, ADAR1 expression was knocked down using siRNA transfection, which was shown to exert antitumor effects on DU145 and PC3 cells 24 and 48 h post-transfection. However, after 72 h in culture, there was no statistically significant difference in cellular proliferation between NC siRNA and ADAR1-specific siRNAs. These data suggest that ADAR1-mediated RNA editing is a complicated process regulated by various factors and signaling pathways, which requires further exploration in the future. It has been revealed that ADAR1 regulates the apoptosis of stressed cells, and that numerous anti-apoptotic genes contain 3' untranslated regions in the dsRNA that are protected by ADAR1 (25,26). In the present study, flow cytometric analysis demonstrated that ADAR1-knockdown significantly induced apoptosis, which indicated that knocking down ADAR1 may act as a potential inhibitor of CRPCa expansion by promoting apoptosis.

The critical role of caspases in regulating apoptosis has been well documented in PCa (27,28). H2AX is a ubiquitous member of the H2A histone family, and the generation of its phosphorylated form has been suggested to affect dsRNA stability and the repair of DNA double-strand breaks (29-31). The present results demonstrated that the expression levels of cleaved caspase-3 and cleaved PARP, and the phosphorylation of H2AX were significantly increased in ADAR1-knockdown cells.

In conclusion, the present results indicated that ADAR1-knockdown inhibited the proliferation and induced the apoptosis of CRPCa cells by promoting the phosphorylation of H2AX. These findings provide a novel insight into the direct anticancer effect of ADAR1 silencing, and support its application as a promising anticancer treatment method in CRPCa.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XL, RZ, GX and JB conceived and designed the study. JB, SL, ZC and YY conducted the experiments and performed data analysis. XL and GX confirm the authenticity of all the raw data. RZ and XL drafted the initial manuscript. XL guided the writing. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was a retrospective study reviewed and approved by the Ethics Committee of The Fifth Affiliated Hospital, Guangzhou Medical University in Guangzhou, China (approval no. GZMU-2019-021), and all patients provided written informed consent for the use of their tissue samples.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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