Androgen deprivation-induced OPHN1 amplification promotes castration-resistant prostate cancer

JUNJIANG LIU[1*], YUNXIA ZHANG[2*], SHOUBIN LI[1], FUZHEN SUN[1], GANG WANG[1], DONG WEI[1], TAO YANG[1] and SHOUYI GU[1]

Departments of [1]Urology and [2]Obstetrics and Gynecology, Hebei General Hospital, Shijiazhuang, Hebei 050051, P.R. China

Received June 8, 2021; Accepted September 27, 2021

DOI: 10.3892/or.2021.8214

Abstract. Androgen deprivation therapy (ADT) is used to treat prostate cancer (PCa). However, ADT may increase the expression of androgen receptor (*AR*) through the amplification of chromosome X. The gene oligophrenin 1 (*OPHN1*) is located in the same region as the *AR* gene, which could be amplified by ADT. Thus, the role of *OPHN1* in PCa pathology was investigated. The expression status of *OPHN1* in PCa was searched in The Cancer Genome Atlas (TCGA) database. Androgen-sensitive cells LNCaP and 22RV1 were cultured under ADT conditions, and then the expression of *OPHN1* was evaluated by northern blotting. The expression of *OPHN1* was enhanced or knocked down in LNCaP and 22RV1 cells by transfection. Subsequently, the LNCaP and 22RV1 cells were cultured under ADT, and the viability rate, apoptosis, and migration of cells were assessed by MTT, flow cytometry, and Transwell assay respectively. The expression of *OPHN1* was also enhanced or knocked down in androgen-insensitive PC3 cells, and then the effects of OPHN1 on the viability, apoptosis, and migration of PC3 cells were assessed. A mouse xenograft model was created by injecting LNCaP cells with *OPHN1* overexpression subcutaneously, and the tumor growth rates were monitored. In TCGA database, amplification of the *OPHN1* gene was observed in the PCa tumors. ADT increased the expression of *OPHN1* in LNCaP and 22RV1 cells ($P<0.05$). *OPHN1* could promote resistance of LNCaP and 22RV1 cells to ADT by promoting cell survival and preventing their apoptosis ($P<0.05$). In addition, *OPHN1* contributed to cell viability ($P<0.05$) and enhanced the migration ability in LNCaP, 22RV1 and PC3 cells ($P<0.05$). In the mouse model, the PCa xenograft with *OPHN1* overexpression had a higher growth rate and was more resistant to the ADT condition ($P<0.05$). In summary, ADT induced the overexpression of *OPHN1* in PCa, which facilitated PCa cell survival and promoted PCa progression.

Introduction

Prostate cancer (PCa) is the most common cancer type in males and the second-leading cause of cancer-related mortality in the United States, with an estimated 248,530 new cases and 34,130 cancer-related deaths in 2018 (1). Between 20 and 40% of clinically localized PCa patients could have a recurrence following the initial therapy (radiotherapy or prostatectomy) (2) and will then have to receive androgen deprivation therapy (ADT). Unfortunately, castration-resistant PCa (CRPC) would develop in all patients following 12-18 months of ADT, with disease progression (3). In pathology, androgen receptor (*AR*) gene amplification was identified in 30 to 50% of CRPC patients, resulting in the overexpression of the AR protein (4,5). Clinical research has revealed that *AR* amplification is clearly related to poor prognosis, with lower overall survival and progression-free survival (6,7).

In 2012, Muller *et al* (8) reported the passenger genomic alteration in multiple solid cancers. They revealed the co-deletion of genes with certain key tumor suppressive functions located in the same regions of chromosomes, such as *TP53*. Conversely, it was suggested that there could also exist a co-amplification of genes with certain key tumor driver genes. Furthermore, these co-amplified genes could participate in the bio-behavior of cancer cells. Thus, The Cancer Genome Atlas (TCGA) database was searched and the amplification of AR copies was identified in the majority of PCa tumors. In addition, the gene oligophrenin 1 (*OPHN1*) was identified, which is located in Xq12, the same region of the AR gene, and is amplified in most PCa cases, associated with the *AR* gene (9).

OPHN1 is considered a Rho-GTPase-activating protein (Rho-GAP) involved in the regulation of the G-protein cycle. The aberrant expression of this gene, including nonsense, frameshift, missense variants, and chromosomal deletions, is responsible for X-linked mental retardation, which is associated with cerebellar hypoplasia and distinctive facial features (9-11). In addition, the positive expression of *OPHN1*

Correspondence to: Dr Junjiang Liu, Department of Urology, Hebei General Hospital, 348 West Heping Road, Shijiazhuang, Hebei 050051, P.R. China
E-mail: junjiangliu67@gmail.com

*Contributed equally

Key words: gene oligophrenin 1, prostate cancer, androgen deprivation therapy, resistance

has been identified in PCa by Goto *et al*, and the overexpression of OPHN1 has been revealed to be related to the high Gleason score and poor prognosis of PCa (12). Du *et al* reported that *OPHN1* is related to the prognosis of CRPC (13). Therefore, it was hypothesized that *OPHN1* is amplified in PCa and that the overexpression of *OPHN1* could contribute to PCa progression. In the present study, the potential effects of *OPHN1* on the pathology of CRPC development were investigated. The results revealed that the expression of *OPHN1* could enhance PCa cell resistance to ADT. Additionally, OPHN1 could promote PCa cell proliferation, migration, and resistance to apoptosis.

Materials and methods

Genomic data and analysis. TCGA is a landmark cancer genomics program that provides genomic, epigenomic, transcriptomic, and proteomic data spanning 33 types of cancer, including PCa (14,15). TCGA database was employed to evaluate the expression of both *AR* and *OPHN1* in PCa, and 4 datasets of Pca were recruited: A dataset of metastatic prostate adenocarcinoma (MCTP; 61 samples) (16), another dataset of metastatic prostate adenocarcinoma (SU2C/PCF Dream Team; 444 samples) (17), a dataset of metastatic castration-sensitive PCa (MSK; 424 samples, CRPC) (18), and a dataset of the metastatic PCa project (provisional; 75 samples). However, in these four datasets, there were 965 cases that provided the genomic data of PCa tumors, which were involved for analysis in the present study. Clinical information (data including age, stage, therapy, and clinical variables) was downloaded from TCGA database. All copy number variation (CNV) data of genes (*AR* and *OPHN1*) of PCa were obtained and analyzed from TCGA Data Portal: cBioPortal (https://www.cbioportal.org/). Normally, a copy number of more than 1 of each gene was considered amplification.

Cell cultures. The LNCaP (CRL-1740), 22RV1 (CRL-2505), PC3 (CRL-1435) and 293T (CRL-3216) cell lines were purchased from the American Type Culture Collection (ATCC), and maintained in RPMI-1640 medium (ATCC), or Dulbecco's modified Eagle's medium (DMEM; HyClone; Cytiva). This was supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (10 mg/ml; both from Invitrogen; Thermo Fisher Scientific Inc.) and maintained at 37˚C and 5% CO_2. FBS was treated with dextran-coated charcoal (final concentration 0.25%; Sigma-Aldrich; Merck KGaA) to remove any hormonal effects. Bicalutamide (AR antagonist) was purchased from Sigma-Aldrich; Merck KGaA (product no. B9061). Cells were treated with 1 μM bicalutamide in DMSO (19,20).

Northern blot analysis. Following treatment of PCa cells with bicalutamide, total RNA was extracted using an RNeasy kit (Qiagen, Inc.) for northern blot analysis. Equal amounts of the sample (10 μg) were loaded and separated on 1% agarose-formaldehyde gels by electrophoresis and then transferred to the membranes. The probes were labeled with P^{32}-*OPHN1* by random labeling and hybridized overnight at 42˚C for 16 h. The internal control used was an *18S rRNA* probe. The blots were visualized using Kodak XAR film. The probe of *OPHN1* was 5'-TCTTAGGCGGATGCAGTCAA-3', the probe of *AR* was 5'-TTGGAGCATCTGAGTCCAGG-3', and the probe of *18S* was 5'-TCGGAACTGAGGCCATGATT-3', to generate DNA against the target gene by PCR.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from PCa cells using an RNeasy kit (Qiagen, Inc.), following the manufacturer's protocol, and was then reverse-transcribed into cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR (SYBR™ Green; cat. no. 4309155; Thermo Fisher Scientific, Inc.) was subsequently performed using an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The cycling conditions for the reaction were as follows: An initial hold for 10 min at 95˚C; then 40 cycles of 15 sec at 95˚C denaturation, 30 sec at an annealing temperature of 60˚C and a 30-sec extension at 72˚C. The following primer pairs were used for the qPCR: *GAPDH* forward, 5'-AATGGACAACTGGTCGTGGAC-3' and reverse, 5'-CCCTCCAGGGGATCTGTTTG-3'; *AR* forward, 5'-CCAGGGACCATGTTTTGCC-3' and reverse, 5'-CGAAGACGACAAGATGGACAA-3'; and *OPHN1* forward, 5'-TGGAGACACTCTGACTGATGAT-3' and reverse, 5'-TACCTCGTTGAGCAATTCAGC-3'. Gene expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the internal reference gene *GAPDH* (21). Each experiment was performed in triplicate.

Mouse xenograft models and treatments. All animal care procedures and experiments were conducted in accordance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines (22) and approved by Ethics Committee of Animal Experiments of the Hebei General Hospital (approval no. 20200147), Shijiazhuang, China). The experiments were conducted by the Department of Urology of Hebei General Hospital. BALB/c NU/NU nude mice (male; 8 weeks old; body weight 20-30 g; 40 mice in total) were purchased from Hebei Medical University and housed in the Experimental Animal Facility (five mice per cage) of the hospital under standard laboratory conditions (18-23˚C; 40-60% humidity; 12-h light/dark cycle) with unlimited access to food and water. Subcutaneous injections of $1x10^6$ cells (regular LNCaP or LNCaP-transfected with *OPHN1* recombinant lentiviral vectors) suspended in 100 μl Matrigel (BD Biosciences) were administered in the flank of each mouse (at 8 weeks old). Every two days, each tumor volume was measured with a dial caliper, while the body-weights of the mice were measured. The tumor volumes were determined by the following formula: length x $width^2$ x0.5.

The mice (20/group) were randomly assigned into the following treatment groups: i) Mice injected with regular LNCaP cells transfected with empty vectors; and ii) mice injected with LNCaP cells transfected with *OPHN1* recombinant lentiviral vectors. Subsequently, the tumor volumes of both groups were monitored until they exceeded 500 mm^3. Thereafter, the mice were castrated by surgery (anesthetized using 87.5 mg/kg ketamine and 12.5 mg/kg xylazine by intraperitoneal injection), and the tumor volumes continued to be monitored. At the end of the experiment, the mice were euthanized via CO_2 inhalation (50% of the chamber volume/min),

and the tumor tissues were collected and weighed. In addition, the maximum tumor diameters observed were 12.43x9.68 mm during the experiments.

Cell viability assay. The PCa cells (LNCaP, 22RV1, and PC3) were seeded in 96-well plates (5,000 cells/well). The LNCaP and 22RV1 cells were maintained with/without bicalutamide at a concentration of 1 μM for 72 h at 37˚C, while the PC3 cells were maintained under regular conditions for 72 h at 37˚C. Subsequently, cell viability was determined via an MTT assay (Beyotime Institute of Biotechnology), following the manufacturer's instructions. The formazan crystals were dissolved in DMSO and the absorbance was measured at 440 nm with a multimode plate reader.

Cell apoptosis assay and caspase-3/8 activity assay. The LNCaP and 22RV1 cells received different pre-treatments with: i) ADT by bicalutamide (1 μM); ii) transfection of *OPHN1* recombinant lentiviral vectors; iii) ADT + transfection of *OPHN1* recombinant lentiviral vectors; or iv) ADT + transfection of *OPHN1* siRNA for 72 h, while the PC3 cells received pre-treatments with transfection of *OPHN1* siRNA for 72 h. Subsequently, the cells were collected and resuspended in 500 μl binding buffer. A total of 5 μl Annexin V-FITC (Beyotime Institute of Biotechnology) was added into the cell suspension and incubated for 10 min at room temperature in the dark. Next, 10 μl of propidium iodide (PI; Beyotime Institute of Biotechnology) was added to the cell suspension, which was then incubated for 15 min on ice or at room temperature in the dark. Following incubation, the cell suspensions were loaded onto a FACScan flow cytometer (BD Biosciences) to evaluate cell apoptosis. The number of early apoptotic (Annexin V-positive) and late apoptotic (Annexin V- and PI-positive) cells indicates the total percentage of gated cells. The results were analyzed using FlowJo software (version 10.6; BD Biosciences) to determine the apoptotic rate. The Caspase-3/8 activity was assayed using the Caspase-3 Assay kit and the Caspase-8 Assay kit (cat. no. C1168S and cat. no. C1152, respectively; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The total protein lysates were collected from cells using a cell lysis buffer (cat. no. P0013; Beyotime Institute of Biotechnology). Then, a reaction buffer (85 μl), combined with Leu-Glu-His-Asp-p-nitroanilide (5 μl; cat. no. P9728; Beyotime Institute of Biotechnology), was added to each sample and incubated at 37˚C for 2 h. The absorbance was measured using a multiplate reader at 450 nm.

Cancer invasion assay. The LNCaP, 22RV1 and PC3 cells received different pre-treatments (transfection of *OPHN1* recombinant lentiviral vectors or transfection of OPHN1 siRNA). A 24-well Transwell chamber plate (pore size 5.0 μm; Invitrogen; Thermo Fisher Scientific, Inc.) was used to evaluate the invasion ability of cancer cells. The upper chamber was precoated with 100 μg Matrigel for 30 min at 37˚C, and then the cells (200 μl) were added to the upper chamber at a concentration of $1x10^5$ cells/ml, while a 500-μl RPMI-1640 culture medium containing 10% FBS was added into the lower chamber. The cells (LNCaP, 22RV1 and PC3) were maintained in the chamber system for 24 h at 37˚C. Subsequently, the filter was collected, fixed (2.5% glutaraldehyde, 10 min, room temperature), and stained with a 0.1% crystal violet for 20 min at room temperature. The filter was observed under a light microscope at a magnification of x100, and the number of cells that passed through the filter in five random fields was counted as the invasion ability.

Western blotting. The cell pellets were collected, and a radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich; Merck KGaA) containing protease inhibitors was used to extract proteins from the cell pellets. The protein levels were determined by bicinchoninic acid (BCA) method. Equal amounts of protein were loaded on 8-10% sodium dodecyl sulfate (SDS)-polyacrylamide gels at 20 μg/lane and were then separated for electrophoresis. Thereafter, the protein on the gels was transferred to nitrocellulose membranes. Next, following washing of the membranes using Tris-Cl-buffered saline (TBST, 0.1% Tween-20) and blocking with 5% BSA for 1 h at room temperature, the membranes were incubated overnight with primary antibodies at 4˚C. Subsequently, the membranes were washed and incubated with secondary antibodies: Horseradish peroxidase (HRP)-conjugated anti-mouse (1:5,000; cat. no. sc-2357; Santa Cruz Biotechnology, Inc.) or anti-rabbit (1:5,000; product no. 7074s, Cell Signaling Technology, Inc.) at room temperature for 1 h. The membranes were then stained using an enhanced chemiluminescence (ECL) detection kit (Pierce; Thermo Fisher Scientific, Inc.). The signals were detected via a chemiluminescence detection system (Bio-Rad Laboratories, Inc.). The following primary antibodies were used: rabbit polyclonal OPHN1 (1:3,000; product code ab229655; Abcam) and mouse monoclonal GAPDH (1:3,000; cat. no. sc-32233; Santa Cruz Biotechnology, Inc.). GAPDH was used as the loading control. The protein expression levels were quantified with ImageJ software (version 1.8.0; National Institutes of Health).

Plasmid construction and transfection. Human *OPHN1* cDNA (Origene Technologies, Inc.) was sequenced and subcloned into the PGL3-basic vector (OriGene Technologies, Inc.) with GFP in accordance with the manufacturer's protocol. The 293T cells were then transfected with a 4 μg lentiviral vector (2nd generation, lentiviral plasmid: packaging vector: envelope at 4:3:1) and pCMV-*OPHN1*-plasmid constructs using a Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) transfection reagent for 48 h in accordance with the manufacturer's protocol. Subsequently, 12-h post-transfection, the medium was replaced with DMEM supplemented with 5% FCS and incubated at 5% CO_2 for 48 h prior to the collection of viral supernatant. The conditioned medium was centrifuged at 1,500 rpm for 5 min at 4˚C and then passed through a filter pore (pore size, 0.45 μm; EMD Millipore). The OPHN1 recombinant lentiviral vectors were harvested from the cell supernatant for further experiments. Subsequently, lentiviral vectors were then added with a multiplicity of infection (MOI) of 30. The target cells were incubated at 37˚C with the vectors, and were used for further experiments after 72 h. Western blotting was performed to verify the interference efficiency.

siRNA transfection. Cells (LNCaP, 22RV1 and PC3) were seeded ($1x10^5$ cells/well) into 12-well culture plates and

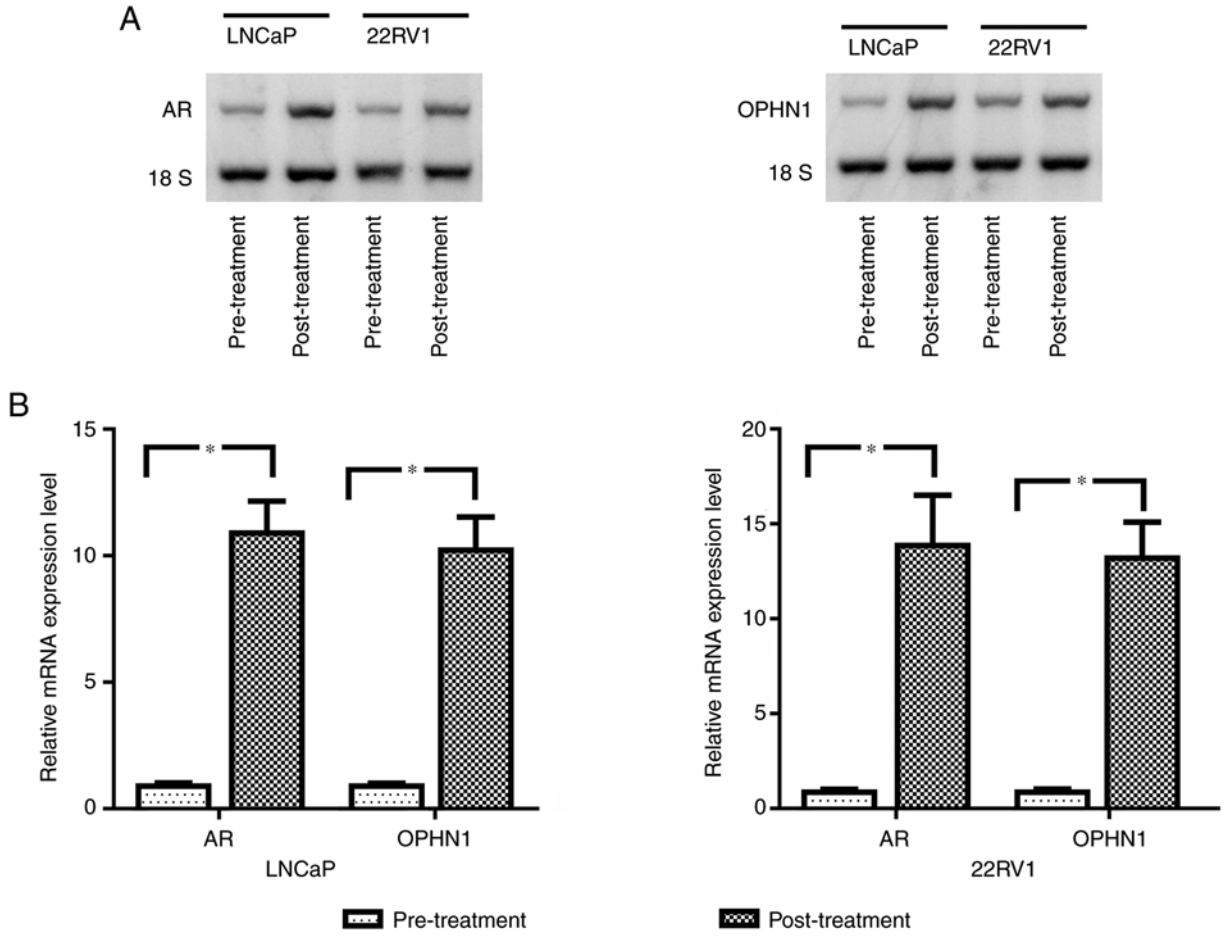

Figure 1. Expression levels of *AR* and *OPHN1* in bicalutamide-treated PCa cells. Both LNCaP and 22RV1 cells were treated with 1 μM bicalutamide for one week, while the same passages of cells treated with DMSO were considered as controls. Subsequently, the cells were collected and total RNA was extracted for analysis. (A) Northern blot analysis of the expression of *AR* and *OPHN1* in LNCaP and 22RV1 cells, as well as the data, revealed that treatment with bicalutamide clearly increased both *AR* and *OPHN1* level compared with that in parental cells. (B) Relative expression levels of *AR* and *OPHN1* mRNA as determined by reverse transcription-quantitative PCR. The *OPHN1* mRNA level was ~10 times higher in LNCaP cells and ~14 times higher in 22RV1 cells than in controls (parental LNCaP and 22RV1). Data are presented as the mean ± SEM of three replicates (*P<0.05 vs. the control, determined by Student's t-test). *OPHN1*, oligophrenin 1; *AR*, androgen receptor; PCa, prostate cancer.

transfected with 40 nM *OPHN1* siRNA (5'-GAGCUCACA CAGGAUUUCCUCCCAU-3'; MyBioSource, Inc.) (12) or scrambled negative control siRNA (5'-UUCUUCGAACGU GUCACGUTT-3'; Santa Cruz Biotechnology, Inc.) using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) for 24 h at 37˚C. Following 24 h of transfection, the transfection efficiency in each type of PCa cell was validated by western blotting, and then the cells were used for subsequent experiments.

Statistical analysis. The data were expressed as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA), repeated measures of two-way ANOVA, followed by Tukey's post hoc test or Student's t-test was used for statistical analysis in the R environment (version 3.6; r-project.org). P<0.05 was considered to indicate a statistically significant difference.

Results

Amplification of OPHN1 in PCa tumors. TCGA database was searched. A total of 4 PCa datasets were included, with a total of 965 cases for reanalysis. The details of the clinicopathological characterization of PCa cohorts used in the present study is presented in Table SI. *AR* amplifications were identified in 26.53% (256 cases) of the selected cases. Both the *AR* and *OPHN1* genes are located in the same region on chromosome X, and *OPHN1* amplifications were identified in 18.96% (183 cases) of the selected cases. The co-amplification of *AR* with *OPHN1* was revealed in 18.13% of the selected samples, with a significant positive association (P<0.001), which may be a phenomenon in PCa due to the amplification of a segment of chromosome X.

OPHN1 promotes resistance to ADT in PCa cells. A previous study revealed that the treatment of bicalutamide could create an androgen deprivation condition, induce the amplification of *AR*, and result in CRPC development (23). Therefore, in the present study, to investigate whether ADT could lead to the amplification of *OPHN1*, bicalutamide was used to treat LNCaP and 22RV1 cells (both are androgen-sensitive PCa cell lines) for 4 weeks. The data revealed that bicalutamide treatment induced *AR* amplification as well as a copy of *OPHN1* as revealed by Northern blotting and RT-qPCR (Fig. 1).

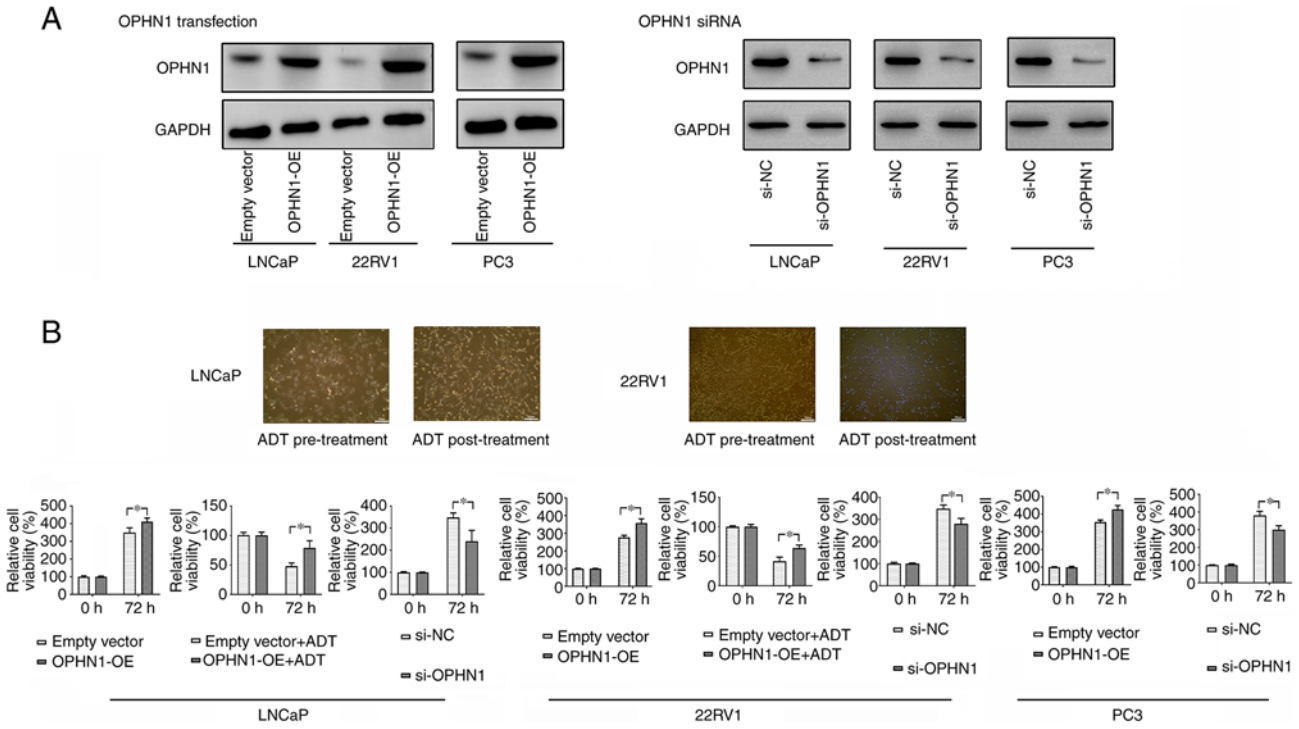

Figure 2. Expression of *OPHN1* promotes PCa cell resistance to ADT. (A) The transfection of *OPHN1* recombinant lentiviral vectors increased OPHN1 expression (OPHN1 over expression, OPHN1-OE) in the LNCaP, 22RV1, and PC3 cells, while the knockdown of *OPHN1* by siRNA (si-OPHN1) inhibited OPHN1 expression in the LNCaP, 22RV1, and PC3 cells (representative images are shown), as determined by western blotting. (B) Effects of OPHN1 on the cell viability of PCa cells. The expression of *OPHN1* was overexpressed or knocked down in LNCaP, 22RV1, and PC3 cells, which were then cultured *in vitro* under various conditions for 72 h. Thereafter, cell viability rates (%) were determined using an MTT assay. The treatment groups were compared with the controls. The data revealed that the enhanced expression of *OPHN1* promoted the viability of LNCaP, 22RV1 and PC3 cells, while blocking the expression of *OPHN1* by siRNA decreased the viability of LNCaP, 22RV1 and PC3 cells. In addition, under ADT (bicalutamide 1 μM) conditions, the overexpression of *OPHN1* contributed to the survival of cells of LNCaP and 22RV1. Data are presented as the mean ± SEM of three replicates. *P<0.05 vs. the control, by Student's t-test. *OPHN1*, oligophrenin 1; PCa, prostate cancer; ADT, androgen deprivation therapy; si-NC, siRNA negative control.

To investigate the mechanism(s) by which *OPHN1* regulates ADT resistance, the expression of *OPHN1* in PCa cell lines (LNCaP, 22RV1, and PC3) was overexpressed or knocked down (Fig. 2A). Subsequently, the LNCaP and 22RV1 cells were cultured in an androgen-depleted medium containing bicalutamide to simulate ADT. Bicalutamide treatment led to the cell death of regular LNCaP and 22RV1. By contrast, less cell death was observed in cells with enhanced *OPHN1* expression. In addition, our results indicated that the expression of *OPHN1* could promote the cell viability rate in androgen-dependent cell lines LNCaP and 22RV1 with or without the pressure of bicalutamide (Fig. 2B). Therefore, the data may indicate that OPHN1 promoted PCa cell survival under androgen-depleted conditions. More interestingly, following overexpression of *OPHN1* by transfection in PC3 cells, an androgen-insensitive cell line, it was revealed that overexpression of *OPHN1* could also promote the viability of PC3 cells. Conversely, blocking the expression of *OPHN1* clearly inhibited the viability of LNCaP, 22RV1 and PC3 cells (Fig. 2B).

Expression of OPHN1 promotes PCa cell invasion and inhibits cell apoptosis. Using Transwell and apoptotic assays, the effect of OPHN1 on PCa cell invasion and apoptosis was investigated. The Transwell invasion assay indicated that the invasion ability of PCa cells was significantly increased via the overexpression of *OPHN1* compared with that of the control group. However, the knockdown of the expression of *OPHN1* in PCa cells significantly inhibited cell invasion ability in all three (LNCaP, 22RV1 and PC3) PCa cell lines (Fig. 3). Consistently, the apoptotic assay revealed that the expression of *OPHN1* facilitated LNCaP and 22RV1 cell resistance to bicalutamide, with a lower apoptotic rate (Fig. 4A) and caspase-3/8 activities (Fig. 4B), while the knockdown of the expression of *OPHN1* increased the apoptotic rate and caspase-3/8 activities of LNCaP and 22RV1 under the pressure of bicalutamide. Additionally, in PC3 cells, knockdown of the expression of *OPHN1* increased the apoptosis rate and caspase-3/8 activities (Fig. 4). Therefore, it was concluded that the upregulation of *OPHN1* promoted the invasion and inhibited the apoptosis of PCa cells *in vitro*.

Expression of OPHN1 promotes castration resistance in vivo. The effects of *OPHN1* expression on tumor growth were examined. Xenografts were created by injecting overexpressed *OPHN1* in LNCaP cells in mice, and the data revealed that *OPHN1*-overexpressing tumors had a growth rate higher than that of the empty vector controls. In addition, mice were castrated when the tumor size exceeded 500 cm^3, and our data demonstrated that the expression of *OPHN1* was more resistant to castration with slower tumor size shrinkage (Fig. 5 and representative tumor image in Fig. S1). Therefore, our data demonstrated that *OPHN1* overexpression conferred resistance to androgen deprivation *in vivo*.

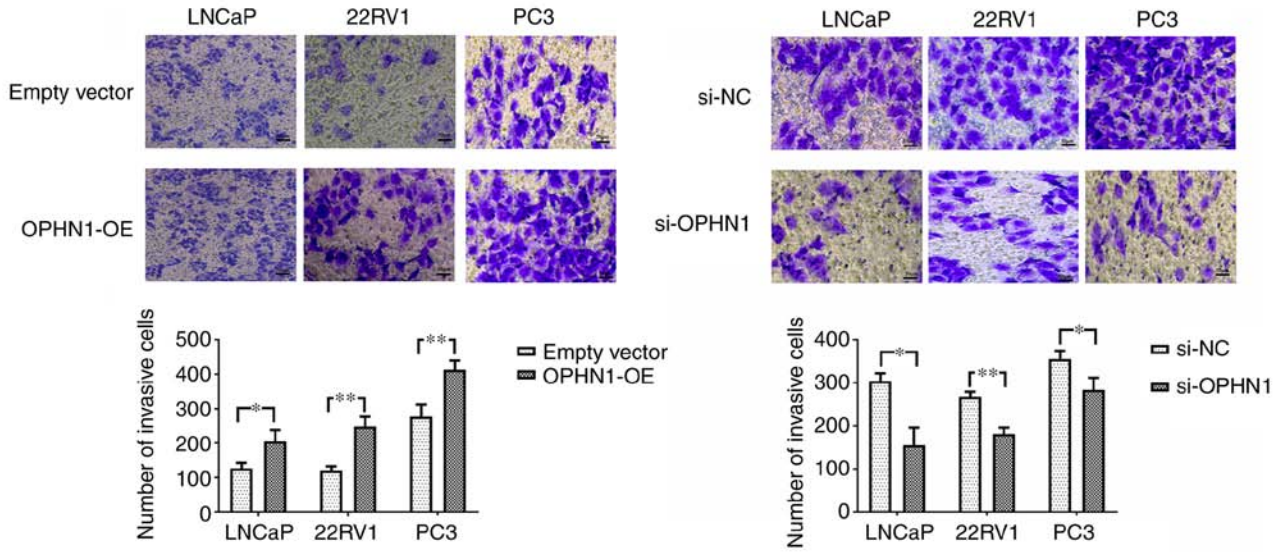

Figure 3. Expression of *OPHN1* promotes the invasion capacity of PCa. The expression of *OPHN1* was enhanced by the transfection of recombinant lentiviral vectors (OPHN1-OE) or blocked by the transfection of siRNA (si-OPHN1). Subsequently, the cells (LNCaP, 22RV1 and PC3) were loaded into the Transwell chambers to evaluate the invasion ability of cells. The number of cells that passed through the filter in five random fields was counted as the invasion ability. The data revealed that the overexpression of *OPHN1* significantly promoted the invasion capacity of the LNCaP, 22RV1 and PC3 cells, with more cells passing through the filters, whereas knockdown *OPHN1* inhibited the invasion capacity of LNCaP, 22RV1 and PC3 cells by decreasing the cell numbers that passed through the filters. Scale bar, 50 μm. The results are expressed as the mean ± SEM for three replicate determinations. *P<0.05 and **P<0.01, by Student's t-test. *OPHN1*, oligophrenin 1; PCa, prostate cancer; si-NC, siRNA negative control.

Discussion

In the present study, our results revealed that ADT could induce the amplification of *OPHN1*, which could then contribute to the survival of PCa cells in an ADT environment. Additionally, our results revealed that the expression of *OPHN1* could promote the viability and invasion of PCa cells, which could lead to PCa progression.

Gene amplification is normally considered an outcome of an increase in the copy number of a restricted region of a chromosome arm, and this amplified region is called an 'amplicon.' Furthermore, gene amplification, particularly oncogenes, is frequently observed in various solid cancers through amplicons and is expected to contribute to tumor evolution by altering gene expression, which is likely to reflect the numerous routes taken by individual tumors to escape normal protective mechanisms (24). In fact, previous studies (25-27) have demonstrated that multiple genes co-amplify with oncogenes in amplicons and cancers. Additionally, these co-amplification genes, particularly genes located in the same region of the chromosome with the oncogenes, may contribute to cancer progression. For example, in the 17q12-q21 amplicon, *ERBB2* amplification was observed in gastric (28), breast (25), and esophageal cancers (28). *ERBB2* is important in tumorigenesis as an oncogene by promoting cancer cell proliferation and survival signaling pathways. Growth factor receptor-bound protein 7 (*GRB7*) (29), retinoic acid receptor alpha (*RAR*) (30), and steroidogenic acute regulatory protein (StAR)-related lipid transfer domain containing 3 (*STARD3*) (31) are all frequently observed as co-amplified genes with *ERBB2*. *GRB7* can bind with receptor tyrosine kinase to mediate downstream signal transduction, and the activation of *GRB7* may induce progression in multiple types of cancers. In addition, *RAR* and *STARD3* may both contribute to cancer progression (32). However, the mechanism of the increase in copy number in an amplicon remains unknown. At present, there have been some hypotheses concerning the potential mechanism of an amplicon, including double rolling-circle replication, extra replication and recombination, replication fork stalling and template switching, and the breakage-fusion-bridge cycle. Nevertheless, more research is clearly required to explore the biochemical mechanism of amplicons, particularly in carcinogenesis (33).

TCGA is a landmark cancer genomics platform that provides genomic, epigenomic, transcriptomic, and proteomic data spanning 33 types of cancer, including prostate cancer. Therefore, TCGA database was accessed, multiple datasets of PCa were downloaded and reanalysis was performed. In our study, by analyzing the gene CNV in PCa in TCGA, the increased CNV of *AR* was identified in 26.53% of all these cases. Thus, it was suggested that *AR* could play a key role in PCa pathology. In fact, *AR* is one of the most investigated genes in PCa research. Increased *AR* gene copy numbers were observed in ~80% of CRPCs, 30% of which had high levels of amplification (34). The role of *AR* in CRPC pathology has been demonstrated for numerous years. The majority of studies suggest that *AR* remains active through multiple pathways in CRPC despite systemic castration (35-37). For example, various *AR* mutations are observed in CRPC, and these mutations could lead to decreased specificity of AR-ligand interaction, and allow AR activation by alternative steroidal molecules, such as, corticosteroids, and progesterone (38,39). *AR* splice variants are another phenomenon observed in CRPC. Researchers have disclosed that one of the most studied variants, *AR-V7* may activate the AR pathway with a ligand-independent manner, in response to ADT, and then contribute to castration resistance (40). In addition, *AR* amplification in CRPC led to higher levels of AR protein, which then contributed to maintain the activation of the AR signaling

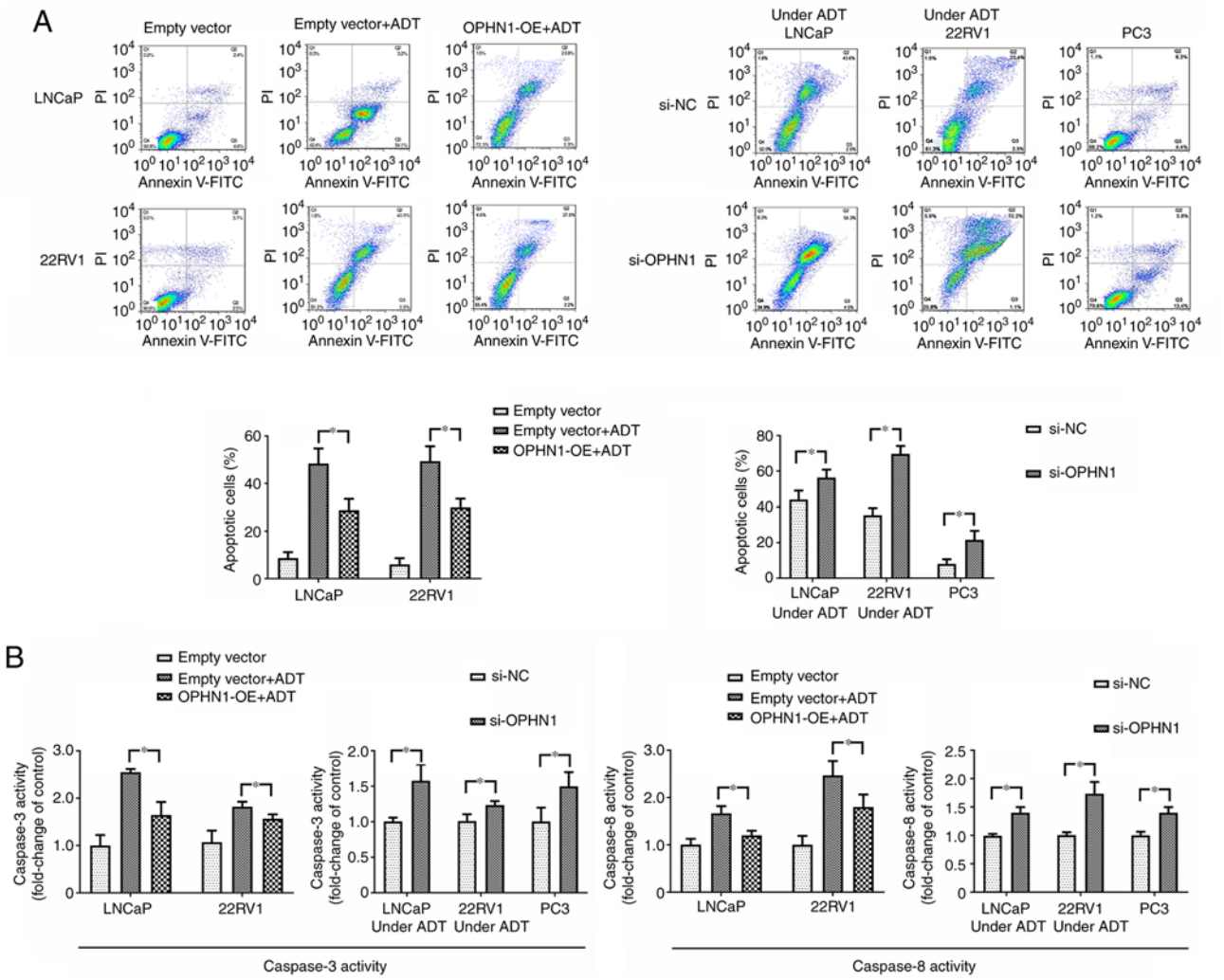

Figure 4. Expression of *OPHN1* prevents cell apoptosis *in vitro*. The expression of *OPHN1* in PCa cells (LNCaP, 22RV1 and PC3) was overexpressed by the transfection of recombinant lentiviral vectors (OPHN1-OE) or blocked by the transfection of siRNA (si-OPHN1). Then the cells were cultured *in vitro* under various conditions for 72 h. (A) Different types of PCa cells (LNCaP and 22RV1) with *OPHN1* overexpression or knockdown were treated with ADT for 72 h, and the PC3 cells with the *OPHN1* knockdown by siRNA continued to be cultured for 72 h. Following treatment, all the PCa cells were collected, stained with Annexin V and PI, and analyzed by FACS. The number of early apoptotic (Annexin V-positive) and late apoptotic (Annexin V- and PI-positive) cells indicates the total percentage of gated cells. Representative images and relative quantifications are shown. Compared with the cells without ADT, both the LNCaP and 22RV1 cells under ADT conditions had a higher percentage of apoptosis. However, overexpression of *OPHN1* alleviated the pro-apoptotic effect of ADT in both LNCaP and 22RV1 cells. Under ADT conditions, both LNCaP and 22RV1 cells with *OPHN1* overexpression displayed a lower percentage of apoptosis than the LNCaP and 22RV1 cells transfected with empty vectors. In addition, under ADT conditions, the knockdown of the expression of *OPHN1* in LNCaP and 22RV1 cells increased the apoptotic rates compared with the cells that were transfected with negative control RNA. In addition, in PC3 cells, the knockdown of the expression of *OPHN1* promoted apoptosis and displayed a higher percentage of apoptosis than cells transfected with negative control RNA. (B) The data on the activities of caspase-3 and caspase-8 were consistent with the apoptotic assay of flow cytometry. ADT promoted the activities of both caspase-3 and caspase-8 in both LNCaP and 22RV1 cells compared with the cells without ADT, which could be alleviated by the overexpression of *OPHN1*. Furthermore, under ADT conditions, blocking the expression of *OPHN1* increased caspase-3 and caspase-8 activities in LNCaP and 22RV1 cells. Blocking the expression of *OPHN1* in PC3 cells promoted the activities of both caspase-3 and caspase-8. All the experiments were performed in triplicate, and the data are presented as the mean ± SEM. *P<0.05 and, by one-way ANOVA followed by Tukey's post hoc test, or by Student's t-test. *OPHN1*, oligophrenin 1; PCa, prostate cancer; ADT, androgen deprivation therapy; si-NC, siRNA negative control.

pathway at lower levels of androgens (41). In the present study, it was revealed that amplification of *OPHN1* may provide an additional mechanistic explanation for the development of CRPC. An increase of *OPHN1* CNV was identified in 18.96% of all the involved cases of the present study. In addition, it was demonstrated that the gene *OPHN1* was co-amplified with *AR*, and the increased CNV of *OPHN1* and *AR* were positively associated. Thus, it was hypothesized that a fragment of the chromosome X is amplified in PCa, particularly in the PCa under ADT, which then results in increased CNV of both *AR* and *OPHN1* since they are located in the same region of chromosome X. In fact, CNV amplification is one of the major causes of gene overexpression in cancer, and the increased CNV of *OPHN1* could result in the overexpression of OPHN1 in PCa tumors, as demonstrated in the *in vitro* experiments of the present study. Additionally, Visakorpi *et al* suggested that ADT could result in a gain of chromosome X, which in turn increases the *AR* gene expression level (4). Zhang *et al* revealed that ADT leads to X-chromosome polysomy (42). *OPHN1* is also located in chromosome X, which could be amplified.

Our results revealed that the expression of *OPHN1* may promote cancer survival and invasion. Thus, the expression

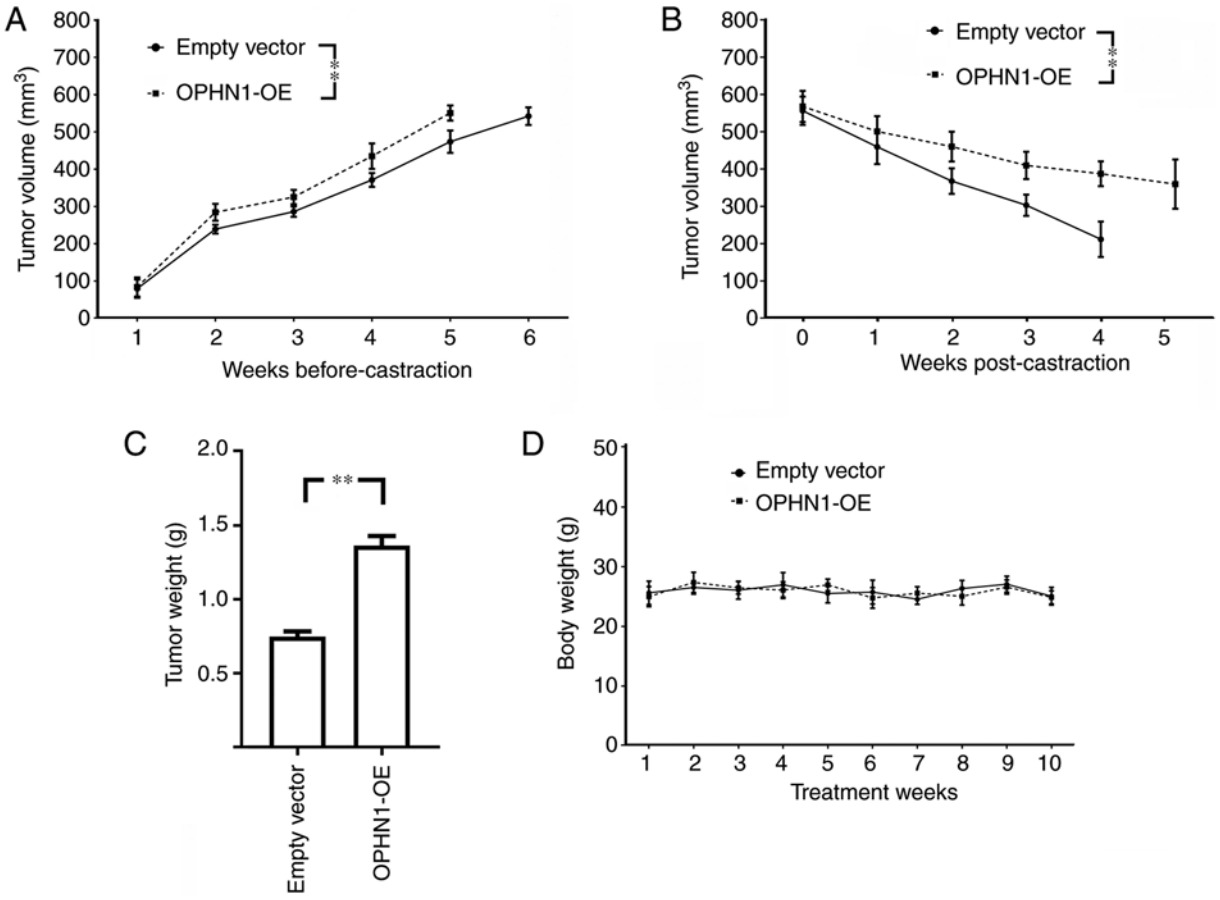

Figure 5. Effects of OPHN1 on tumor xenograft growth in mice. LNCaP ($1x10^6$ cells) transfected with recombinant lentiviral vectors (*OPHN1* overexpression, OPHN1-OE) were injected into mice subcutaneously (20 mice/group), and then the tumor growth was monitored, while the LNCaP cells transfected with empty vectors were injected as controls. (A) The tumor volumes of both groups were monitored until they exceeded 500 mm^3, as revealed with growth curves for xenografts in each group. The data revealed that the tumors of OPHN1-OE LNCaP cells had a higher rate of tumor growth ($F=14.81$; $P<0.001$), which exceeded 500 mm^3 at ~5 weeks, whereas the tumors in the control group exceeded 500 mm^3 at ~6 weeks. Thereafter, the mice were castrated by surgery, and the tumor volumes continued to be monitored. (B) The data revealed that the mouse xenografts of OPHN1-OE LNCaP exhibited a significantly lower decrease rate in tumor volume than the control group ($F=35.34$; $P<0.001$). (C) In addition, the mouse xenografts of OPHN1-OE LNCaP exhibited a significantly higher tumor weight than the control group. (D) There was no difference in body weight between the two groups of mice ($F=0.519$; $P=0.861$). The mouse xenograft experiments were performed as described in the Materials and methods section. The data are presented as the mean ± SEM. $^{**}P<0.01$, by two-way ANOVA followed by Tukey's post hoc test, or by Student's t-test. *OPHN1*, oligophrenin 1.

of *OPHN1* could promote PCa progression, which is consistent with certain other studies on PCa (12,43). In addition, in gastric cancer, Dicken *et al* revealed that the expression of *OPHN1* was related to lymphovascular invasion (44). Furthermore, it is known that OPHN1 normally regulates the Rho-GAP pathway in cells, including RhoA, Cdc42, and Rac1. RhoA is involved in PCa tumorigenesis and progression, and Chen *et al* demonstrated that the activation of *RhoA* and *Rac1* promoted PCa migration and invasion (45). Another previous study revealed that a high expression of *RhoA* had a poor prognosis for PCa, with poor tumor differentiation and higher prostate-specific antigen (PSA) relapse (46). Additionally, it was previously revealed that by activating the Rho-Gap pathway, CDC42, could facilitate cancer cell resistance to the inhibitor of the PI3K/Akt/mTOR pathway (47). The activation of *CDC42* could also promote cell motility, proliferation, and resistance to apoptosis, all of which would result in PCa invasion and metastasis (48). Conversely, blocking *Rac1*/*Cdc42* inhibited PCa tumor growth in mice models by downregulating the PI3K/Akt/mTOR and PAK signaling pathway in PCa cells (49). In addition, the activation of Rac1 could directly contribute to CRPC development activation, and Rac1 is closely related to androgen-independent cell proliferation (50). Both Lyons *et al* (51) and Chen *et al* (52) revealed that androgen deprivation increased the expression of *Rac1*. *Rac1* induced *AR*-dependent gene expression (51), while blocking Rac1 enhanced the efficacy of enzalutamide in enzalutamide-resistant xenograft tumors (52). In addition, a previous study has revealed that blocking Rac1 or *CDC42* may inhibit tumor growth and progression in PCa (49). Zins *et al* indicated that blocking Rac1/Cdc42 GTPase by small molecule inhibitor could suppress growth of primary human PCa xenografts in mice (49). Therefore, in addition to the AR pathway, the OPHN1-Rho-GAP pathway may contribute to CRPC pathology (51). Additionally, the present study had certain limitations. The increased expression of *OPHN1* at the protein level needs to be demonstrated in the human PCa tumors. Further investigations are required to determine whether blocking *OPHN1* could inhibit tumor growth in a mouse model, although it was observed that expression of *OPHN1* promoted PCa progression, and blocking *OPHN1* expression inhibited viability and invasion of PCa cells *in vitro*.

Additionally, further research is required to comprehend the potential pathway regulated by OPHN1 in PCa.

In summary, in prostate cancer, ADT induced the amplification of *OPHN1*, which contributed to the development of CRPC. The overexpression of *OPHN1* facilitated PCa survival under ADT by contributing to PCa viability, invasion, and progression. Therefore, targeting OPHN1 could be used to reverse endocrine therapy resistance in CRPC.

Acknowledgements

Not applicable.

Funding

No funding was received.

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

JL and YZ designed and performed experiments, analyzed the data and wrote the manuscript. SL and FS analyzed the data and performed the experiments. GW and DW performed the experiments. TY and SG contributed to study design. JL and YZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal care procedures and experiments were conducted in accordance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines and approved by Ethics Committee of Animal Experiments of the Hebei General Hospital (Shijiazhuang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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