Published OnlineFirst September 23, 2016; DOI: 10.1158/1078-0432.CCR-16-0718

Cancer Therapy: Preclinical

Clinical Cancer Research

BIRC6 Targeting as Potential Therapy for Advanced, Enzalutamide-Resistant Prostate Cancer

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Abstract

Purpose: Enzalutamide resistance has emerged as a major problem in the management of castration-resistant prostate cancer (CRPC). Research on therapy resistance of CRPCs has primarily focused on the androgen receptor pathway. In contrast, there is limited information on antiapoptotic mechanisms that may facilitate the treatment resistance. The inhibitor of apoptosis proteins (IAP) family is well recognized for its role in promoting treatment resistance of cancers by inhibiting drug-induced apoptosis. Here, we examined whether BIRC6, an IAP family member, has a role in enzalutamide resistance of CRPCs and could provide a therapeutic target for enzalutamide-resistant CRPC.

Experimental Design: Use of enzalutamide-resistant CRPC models: (i) the transplantable, first high-fidelity LTL-313BR patient-derived enzalutamide-resistant CRPC tissue xenograft line showing primary enzalutamide resistance, (ii) MR42D and MR49F CRPC cells/xenografts showing acquired enzalutamide resistance. Specific BIRC6 downregulation in these models was produced using a *BIRC6*-targeting antisense oligonucleotide (ASO-6w2). Gene expression was determined by qRT-PCR and gene expression profiling. Molecular pathways associated with growth inhibition were assessed via gene enrichment analysis.

Results: Of eight IAPs examined, BIRC6 was the only one showing elevated expression in both enzalutamide-resistant CRPC models. Treatment with ASO-6w2 markedly suppressed growth of LTL-313BR xenografts and increased tumor apoptosis without inducing major host toxicity. Pathway enrichment analysis indicated that GPCR and matrisome signaling were the most significantly altered pathways. Furthermore, ASO-6w2 inhibited expression of prosurvival genes that were upregulated in the LTL-313BR line.

Conclusions: *BIRC6* targeting inhibited the growth of enzalutamide-resistant CRPC models and may represent a new option for clinical treatment of advanced, enzalutamide-resistant prostate cancer. *Clin Cancer Res; 23(6); 1542–51. ©2016 AACR.*

Introduction

Castration-resistant prostate cancer (CRPC) presents a major challenge in the clinical management of advanced prostate cancer. As most forms of CRPC are still dependent on the androgen receptor (AR) for survival, the advent of new, powerful second-generation AR antagonists, such as enzalutamide, has been beneficial for patients with metastatic CRPC (1). Enzalutamide significantly improves patient survival and has been approved for treating CRPC in post-docetaxel (2012) and pre-docetaxel settings (2014). However, treatment with enzalutamide is not curative, and enzalutamide resistance in the clinic has been noted (1, 2). One fourth of patients showed primary resistance to enzalutamide (i.e., the presence of enzalutamide resistance in patients who never had been exposed to the drug) and progressed in 3 months, whereas all the remaining patients eventually progressed by 24 months in spite of an initial positive response (3, 4). Major mechanisms of acquired resistance to such AR inhibitors have been reported, including restored AR signaling, AR bypass signaling, and complete AR independence (5). However, other mechanisms that are not directly related to AR signaling, such as blockage of apoptosis, could also contribute to resistance of prostate cancers to increasingly powerful AR inhibitors. So far, not much attention has been given to this possibility.

The inhibitors of apoptosis proteins (IAP) are a family of proteins that serve as endogenous inhibitors of programmed cell death by regulating the activity of caspases, the executors of apoptosis. The IAP family is characterized by the presence of baculovirus IAP repeat (BIR) domains, which bind and inhibit caspases. There are eight IAP members, namely BIRC1 (NAIP), BIRC2 (cIAP1), BIRC3 (cIAP2), BIRC4 (XIAP), BIRC5 (survivin), BIRC6 (Apollon/BRUCE), BIRC7 (ML-IAP/LIVIN), and BIRC8 (ILP-2). Some IAPs, such as cIAP1/2, XIAP, and survivin, are well known to enhance survival and treatment resistance of various types of cancer (6). However, to date, no studies have reported a role for the IAP family in enzalutamide resistance of CRPC.

There is increasing evidence that BIRC6, a lesser studied member of the IAP family, is also involved in promoting treatment resistance of a variety of cancers. BIRC6 is a large protein (528 kDa) with pleiotropic functions, including

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-16-0718

Published OnlineFirst September 23, 2016; DOI: 10.1158/1078-0432.CCR-16-0718

Translational Relevance

Enzalutamide, a second-generation androgen receptor antagonist, is used in first-line therapy of metastatic, castration-resistant prostate cancer (CRPC). However, resistance to enzalutamide quickly emerges in patients in the form of primary or acquired resistance. Whereas research on therapy resistance of CRPCs has primarily focused on the AR pathway, there is limited information on antiapoptotic mechanisms that may underlie their treatment resistance. Here, we investigated a role for the inhibitor of apoptosis proteins (IAP) family in enzalutamide resistance. We found that the IAP, BIRC6, has a key role in the survival of CRPCs with primary or acquired enzalutamide resistance. Using a high-fidelity, enzalutamide-resistant, patient-derived CRPC tissue xenograft model, we showed that a *BIRC6*-targeting antisense oligonucleotide (ASO-6w2) effectively suppressed enzalutamide-resistant CRPC growth without inducing major host toxicity. These results provide a rationale for *BIRC6* targeting in therapy of patients with enzalutamide-resistant CRPC.

inhibition of apoptosis, cytoprotection and regulation of cytokinesis (7), and mitosis (8). Its activity is upregulated in many cancers, including prostate cancer, particularly CRPC (9), associated with poor patient prognosis (10–14), and shown to enhance chemoresistance (12, 13, 15–19). In a previous study, we developed an antisense oligonucleotide (ASO), ASO-6w2, that specifically targets synthesis of BIRC6 and to a lesser extent cIAP1 (BIRC2), another IAP family member that is upregulated in prostate cancer (19). *BIRC6* targeting by ASO-6w2 was found to markedly inhibit the proliferation of CRPC cells *in vitro* and *in vivo* through the induction of cell-cycle arrest, apoptosis, and suppression of NFκB signaling (19). The results suggest that BIRC6 plays an important role in promoting survival of castration-resistant malignancies.

Development of effective prostate cancer therapeutics has been hampered by a lack of clinically relevant experimental models of the disease. Traditional xenograft models based on human prostate cancer cell lines lack the tumor heterogeneity and the 3-dimensional architecture of the original cancer specimens from which the cell lines were derived. To overcome these deficiencies, we developed transplantable patient-derived xenograft (PDX) lines of prostate cancer tissues at the Living Tumor Laboratory (LTL; www.livingtumorlab.com). These PDX lines, developed via implantation of patients' cancer tissue specimens into NOD/SCID mice at the well-vascularized subrenal capsule graft site, retain the tumor heterogeneity and molecular characteristics of the original cancers. As such, these "high fidelity" PDXs represent highly accurate preclinical model systems for therapeutic target identification and drug efficacy testing (20, 21).

In the current study, we established that the transplantable patient-derived CRPC tissue xenograft line, LTL-313BR (21), is enzalutamide resistant and provides, together with its enzalutamide-sensitive, hormone-naïve parent line, LTL-313B, a novel *in vivo* model for studying the development of enzalutamide-resistant CRPC, as well as a role of IAPs in that process. Using the LTL-313B/LTL-313BR xenograft model and xenografts based on cultured, enzalutamide-sensitive, and enzalutamide-resistant prostate cancer cell lines, we found that of the IAP family, BIRC6 was the top upregulated IAP member in both enzalutamide-resistant systems. We then investigated whether BIRC6 has a prosurvival role in enzalutamide-resistant cells and provides a potential target for therapy of enzalutamide-resistant CRPC.

Materials and Methods

Materials

Chemicals, solvents, and solutions were obtained from Sigma-Aldrich, unless otherwise indicated. Six- to 8-week-old NOD/SCID IL2 receptor gamma chain null (NSG) mice were bred by the BC Cancer Research Centre Animal Resource Centre, Vancouver, Canada. ASOs 6w2 and scramble control (Scrb) with full phosphorothioate-modified backbone were purchased from Eurofins MWG Operon. ASO-6w2 has perfect complementary matches to BIRC6 mRNA sections and contains 1 base mismatch to BIRC2 (cIAP1) mRNA. The DNA sequences of ASO-6w2 and Scrb have been reported (22). Anti-BIRC6 (NB110-40730, Novus Biologicals) was used for immunohistochemical staining and Western blotting. Anti-cleaved caspase-3 (#9664; Cell Signaling Technology) was used for immunohistochemical staining.

IHC

Staining and scoring of BIRC6 protein was performed as previously reported (22). For cleaved caspase-3 staining, images of 3 to 5 representative fields at ×400 magnification were taken per tumor and cells counted to determine the number of positively stained cells per field.

Cell culture

Human enzalutamide-sensitive, castration-resistant LNCaP-V16D prostate cancer cells and enzalutamide-resistant, castration-resistant MR49F and MR42D prostate cancer cells (obtained from Dr. Amina Zoubeidi, Vancouver Prostate Centre, Vancouver, Canada) were maintained in RPMI1640/5% FBS medium, supplemented with 10 μmol/L enzalutamide for the enzalutamide-resistant cells. MR49F cells were authenticated using short tandem repeat profile analysis at the Genetics Resources Core Facility at John Hopkins (Baltimore, MD; ref. 23). V16D and MR42D cells were authenticated by whole-genome and whole-transcriptome sequencing (Illumina Genome Analyzer IIx, 2012; ref. 24).

Western blotting

Western blotting of BIRC6 and actin was performed as described previously (22).

Xenografts

The transplantable, hormone-naïve, patient-derived prostate cancer tissue xenograft line, LTL-313B, was maintained in male NSG mice, using serial subrenal capsule transplantations as described previously (21, 25). Its castration-resistant subline, LTL-313BR, was maintained in castrated mice. The LTL-313BR line was derived from the hormone-naïve LTL-313B line by castration of LTL-313B tumor-bearing NSG mice and propagation of tumors recurring after relapse of the LTL-313B tumors (Fig. 1). The LTL-313BR line is AR positive, PSA positive, has a PTEN copy deletion, and contains the TMPRSS2-ERG fusion (21). The original cancer specimen had been obtained with the patient's signed consent following a protocol approved by the Clinical Research Ethics Board of the University of British Columbia and the BC

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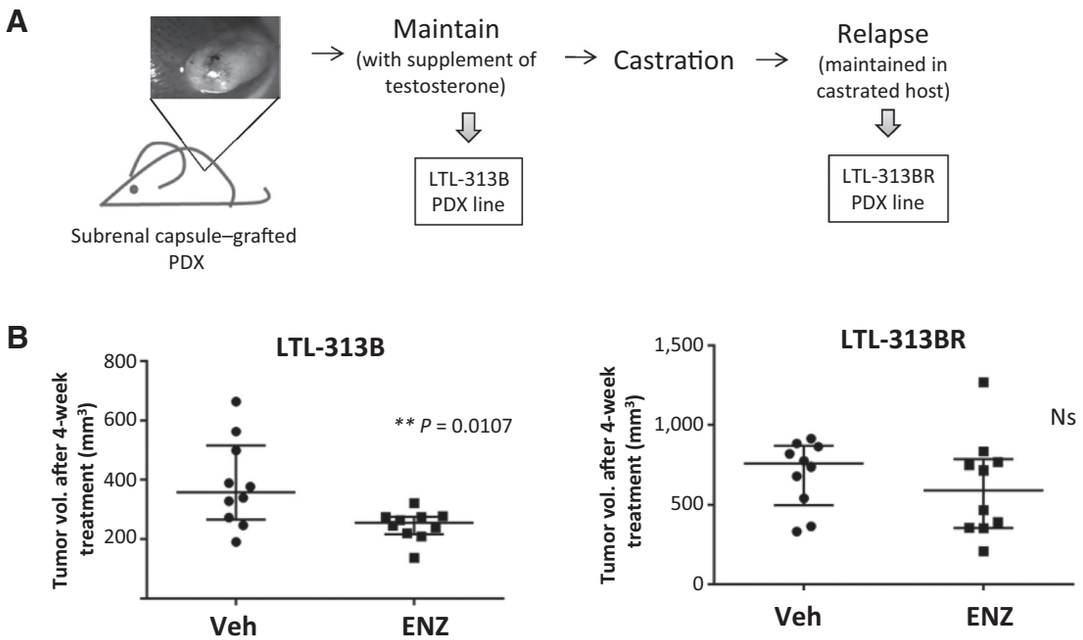

Figure 1.

A PDX model for enzalutamide-resistant CRPC. **A,** Development of patient-derived LTL-313B and LTL-313BR tumor tissue xenograft lines by serial subrenal capsule transplantation. The castration-resistant LTL-313BR subline was derived from the hormone-naïve LTL-313B line. **B,** LTL-313BR showed primary enzalutamide resistance. NS, not significant. Mice bearing LTL-313B or LTL-313BR tumors under the renal capsules were treated for 4 weeks with 10 mg/kg enzalutamide or vehicle (Veh). Tumor volume was measured at the end of the treatment. The LTL-313BR subline was resistant to enzalutamide treatment without prior exposure to the drug, whereas the parental LTL-313B remained sensitive.

Cancer Agency (21). Cell line–based xenografts V16D (enzalutamide-sensitive CRPC), MR42D, and MR49F (enzalutamide-resistant CRPC) were generated and maintained as described previously (24, 26).

Treatments with enzalutamide and ASOs

Treatment with enzalutamide. Mice bearing subrenal capsule–grafted LTL-313B or LTL-313BR tissues were randomized for treatment when the volumes of the grafts reached approximately 250 mm^3. Mice were treated with enzalutamide (10 mg/kg) or vehicle for 4 weeks ($n = 10$; 5 days on and 2 days off). Tumor volumes were measured at the end of the treatments.

Treatments with ASOs. Mice bearing LTL-313BR tumors were randomized into Scrb or ASO-6w2 groups ($n = 30$) for a 21-day treatment. A 30 mg/kg loading dose on day 1 was followed by a daily maintenance intraperitoneal dose of 15 mg/kg. Tumors were harvested 1 week after the end of the treatment for immunohistochemical analysis or RNA extraction. Serum PSA levels were determined using a Cobas Total-PSA Kit and Cobas e411 Analyzer (Roche Diagnostics).

RNA extraction and qRT-PCR

Total RNA was isolated and qRT-PCR was performed as described previously (27). The primer sequences used are presented in Supplementary Table S1. All qRT-PCR primers are human specific and do not crossreact with mouse transcripts as confirmed by NCBI BLAST search.

Gene expression profiling and RNA sequencing

Gene expression profiling of Scrb- and ASO-6w2–treated LTL-313BR xenografts was performed using four replicates. The quality of the RNA samples was checked with the Agilent 2100 Bioanalyzer and NanoDrop ND-2000 UV-VIS spectrophotometer. Only samples with A260/280 optical density (OD) values between 1.8 and 2.0, an A260/A230 OD value of 2.0, and RNA Integrity Number (RIN) $\geq$8.0 were used for one-color labeling using Agilent's One-Colour Microarray-Based Gene Expression Analysis Low Input Quick Amp Labelling v6.0 (Agilent Technologies). Total RNA (100 ng) was used to generate cyanine-3–labeled cRNA. cRNAs were hybridized on Agilent SurePrint G3 Human GE 8 × 60K Microarray (AMDID 028004). Arrays were scanned with an Agilent DNA Microarray Scanner at a 3-μm scan resolution, and data were processed with Agilent Feature Extraction 11.0.1.1. Processed signals were quantile normalized using Agilent GeneSpring 12.0. The data have been deposited in NCBI's Gene Expression Omnibus (GEO; ref. 28) and are accessible through GEO Series accession number GSE77516 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE77516). Transcriptome sequencing [RNA sequencing (RNA-seq)] of PDX models (LTL-313B and LTL-313BR) and cell line–based xenograft models (V16D, MR42D, and MR49F) was performed as described previously (21, 23).

Pathway enrichment analysis

Top 1,000 significantly differentially expressed genes with log2 fold change >1.5, identified in gene expression profiling of ASO-6w2–treated ($n = 4$) versus Scrb-treated xenografts

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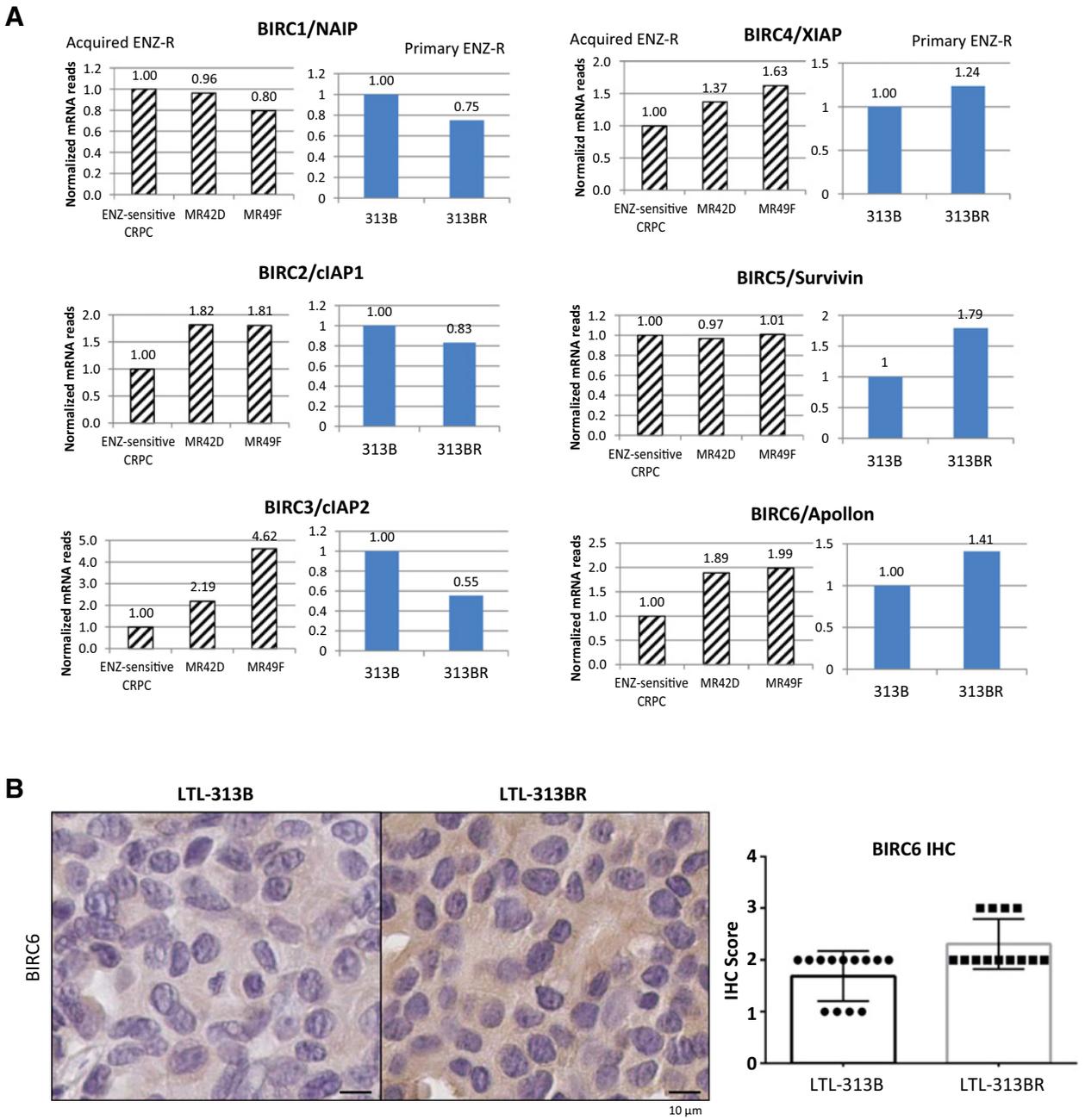

Figure 2.

Level of IAP member, BIRC6, is elevated in models showing acquired and primary enzalutamide resistance. **A,** The mRNA expressions of the IAP family members were determined in PDX models (LTL-313BR vs. LTL-313B) and LNCaP cell line–based xenografts models (enzalutamide-sensitive CRPC V16D vs. enzalutamide-resistant MR42D and MR49F) by transcriptomic sequencing. MR42D and MR49F are enzalutamide-resistant sublines developed after *in vivo* exposure to enzalutamide of parental V16D xenografts, that is, showing acquired enzalutamide resistance. **B,** BIRC6 protein expression determined by IHC in LTL-313B and LTL-313BR xenografts. Scale bar, 10 μm. Magnification, ×400. IHC scores 0, 1, 2, and 3 refer to negative, weak, mild, and strong staining intensities, respectively. ENZ-R, enzalutamide resistant.

($n = 4$), were analyzed for gene set enrichment against gene sets of pathways present in the Molecular Signature Database (MSigDB) v5.0 (29). A Fisher exact test–based gene set enrichment analysis was used. A cut-off threshold of FDR ≤ 0.05 was used to obtain significantly enriched pathways.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad). The Student t test was used unless otherwise indicated. Results with a $P < 0.05$ were considered significant.

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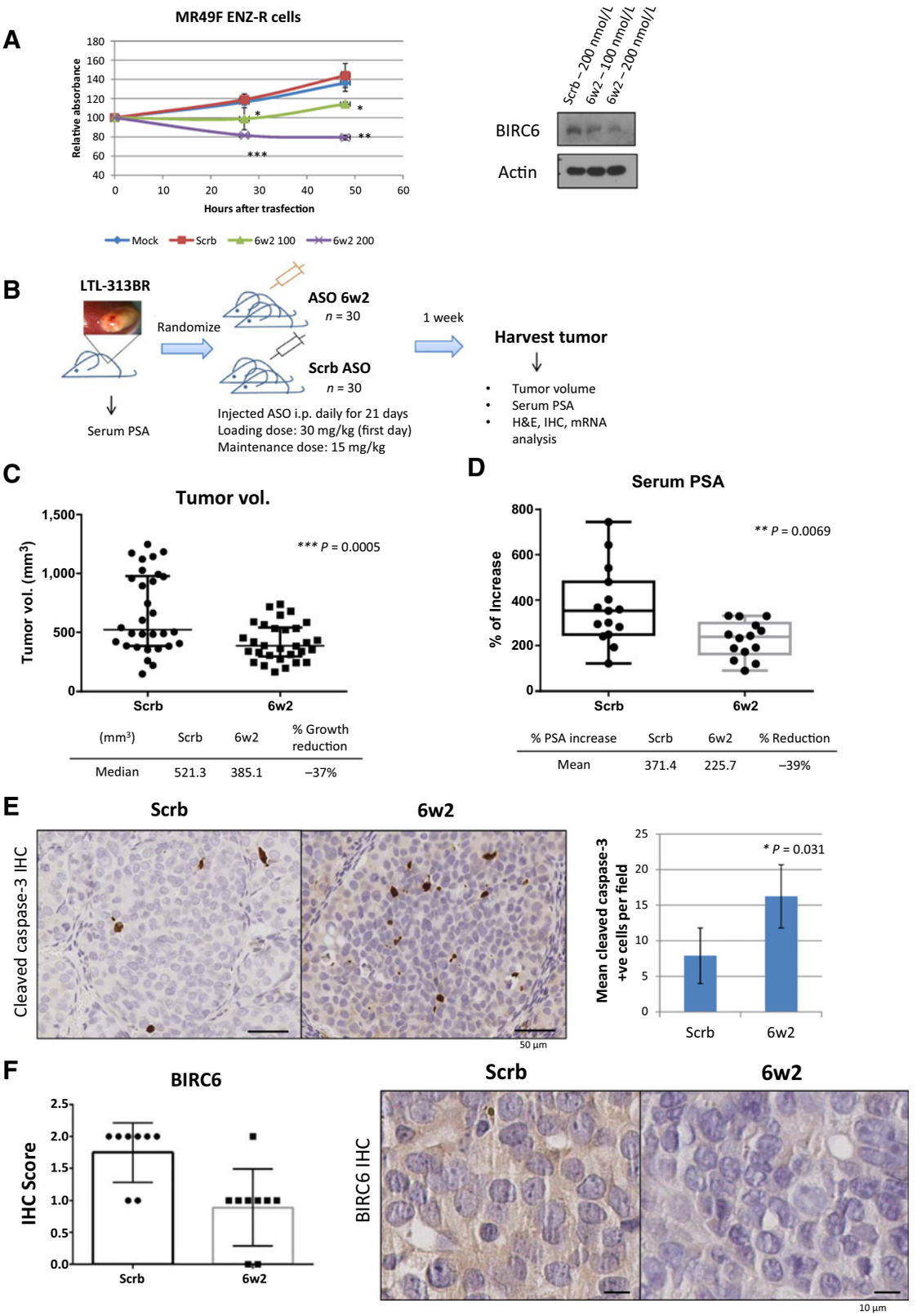

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Results

A patient-derived prostate cancer tissue xenograft model for studying the development of enzalutamide-resistant CRPC

In search of a clinically relevant *in vivo* model for studying the development of enzalutamide-resistant CRPC, we tested a number of our transplantable, patient-derived prostate cancer tissue xenograft lines for enzalutamide sensitivity. Included were the LTL-313B line, a hormone-naïve prostatic adenocarcinoma PDX line, and its CRPC subline, LTL-313BR, developed from the LTL-313B line via host castration and propagation of recurrent tumors (Fig. 1A; ref. 21). Groups of randomized mice, bearing tumors of these lines under the renal capsules, were treated for 4 weeks with enzalutamide (10 mg/kg) or vehicle. Whereas the growth of the LTL-313B parental line during the 4-week period was inhibited by enzalutamide (as compared with the control), the growth of the LTL-313BR CRPC subline was not (Fig. 1B). As such, the enzalutamide-sensitive, hormone-naïve LTL-313B line and its enzalutamide-resistant, castration-resistant LTL-313BR subline provide a PDX model for studying the development of enzalutamide-resistant CRPC. It may be noted that the enzalutamide insensitivity of the LTL-313BR line did not result from preexposure to enzalutamide, indicating that this line harbors primary resistance to enzalutamide.

BIRC6 is the highest upregulated IAP in enzalutamide-resistant CRPC

A study in a role for IAPs in the development of enzalutamide-resistant prostate cancer was initiated by determining their relative mRNA expressions in enzalutamide-sensitive versus enzalutamide-resistant xenografts. To this end, we used the enzalutamide-resistant LTL-313BR xenograft line, in combination with the enzalutamide-sensitive, hormone-naïve LTL-313B parent line, as a model for development of primary enzalutamide resistance. In addition, we used a model of acquired enzalutamide resistance consisting of xenografts of cultured enzalutamide-resistant, castration-resistant MR42D and MR49F prostate cancer cells versus enzalutamide-sensitive, castration-resistant LNCaP-V16D parental prostate cancer cells (24). The relative mRNA levels in the xenografts of all IAP family members were determined by transcriptomic sequencing.

As shown in Fig. 2A, the transcript levels of cIAP1, cIAP2, XIAP, and BIRC6 were upregulated in the enzalutamide-resistant MR42D and MR49F CRPC cell-based xenografts relative to the parental enzalutamide-sensitive V16D line. However, transcripts in the enzalutamide-resistant LTL-313BR cancer tissue xenografts, relative to the enzalutamide-sensitive LTL-313B xenografts, were only elevated in the case of survivin, BIRC6, and marginally for XIAP; the BIRC6 protein upregulation in the LTL-313BR xenografts was confirmed by immunohistochemical analysis (Fig. 2B). BIRC6 was among the top upregulated IAP members in both enzalutamide-resistant models. Taken together, the results suggest that BIRC6 may be functionally important in promoting enzalutamide resistance whether or not it is a primary or an acquired resistance.

BIRC6-targeting ASO suppresses growth of enzalutamide-resistant CRPC *in vitro* and *in vivo*

The effect of targeting *BIRC6* by ASO-6w2 on enzalutamide-resistant CRPC growth was first examined *in vitro* using the enzalutamide-resistant cell line, MR49F (growing *in vitro* in the continuous presence of enzalutamide). The MR49F cell line expresses functional AR and PSA, thus resembling clinical enzalutamide-resistant cases; the MR42D line expresses AR but not PSA (24). As shown in Fig. 3A, downregulation of BIRC6 by ASO-6w2 was confirmed by Western blotting and qRT-PCR (Supplementary Fig. S1). Treatment of MR49F cells with 100 nmol/L ASO-6w2 resulted in growth suppression, while treatment with 200 nmol/L ASO-6w2 led to marked growth suppression with loss of cells (Fig. 3A). A significant growth-inhibitory effect was also observed in MR42D cultures (Supplementary Fig. S2). On the other hand, silencing of XIAP by siRNA also resulted in significant reduction of MR49F cell replication at 96 hours after transfection (Supplementary Fig. S3).

The anticancer activity of ASO-6w2 was then studied *in vivo* using the LTL-313BR patient-derived CRPC tissue xenograft line, as it has greater clinical relevance and precision in predicting patients' responses than cell line–based xenografts. Mice bearing LTL-313BR tumors under the renal capsules were treated with Scrb ASO or ASO-6w2 daily for 21 days, and tumors were harvested 1 week after the end of the treatment (Fig. 3B). As shown in Fig. 3C, the treatment with ASO-6w2 led to marked inhibition of tumor growth, with a 37% reduction ($P < 0.001$) compared with the control group. As well, a substantially lower increase in serum PSA levels was observed, with a 39% reduction ($P < 0.01$; Fig. 3D) in the ASO-6w2–treated group. Individual mice PSA levels before and after treatment are shown in Supplementary Fig. S4. The antitumor effect of ASO-6w2 was associated with a significant increase in tumor apoptosis (Fig. 3E), but no major host toxicity was observed (Supplementary Fig. S5). Hoechst 33258 staining of tumors was performed and confirmed that the tumor cells assessed for cleaved caspase-3 IHC are of human origin (Supplementary Fig. S6). The inhibition of BIRC6 expression by ASO-6w2 in LTL-313BR xenografts was validated by IHC (Fig. 3F). In addition, the amounts of serum PSA per unit tumor volume per mouse in Scrb- and 6w2-treated groups were examined. The two treatment groups do not show significant difference in PSA

Figure 3.

BIRC6-targeting ASO-6w2 suppressed growth of enzalutamide-resistant LTL-313BR xenografts and induced apoptosis. **A,** *BIRC6*-targeting ASO (ASO-6w2) effectively suppressed proliferation of enzalutamide-resistant MR49F cells *in vitro* in a dose-dependent manner. Decreased BIRC6 protein expression after ASO-6w2 treatment was confirmed by Western blotting. **B,** *In vivo* therapeutic potential of ASO-6w2 was examined in the enzalutamide-resistant PDX model LTL-313BR. Groups of mice bearing LTL-313BR xenografts were treated with Scrb ASO or ASO-6w2 at 30 mg/kg on the first day, followed by 15 mg/kg for 20 days. Tumors were harvested and sera obtained for analysis 1 week after the end of the treatment. **C,** The ASO-6w2–treated group showed significantly smaller tumor volumes than the Scrb-treated group. **D,** The increase in average serum PSA levels of the ASO-6w2–treated mice was also significantly lower than that of the Scrb control. Whisker, median/mean ± interquartile range. **E,** Representative images of cells stained by IHC for cleaved caspase-3. The ASO-6w2–treated group showed a significant increase in apoptosis, with a 2-fold increase in the number of cleaved caspase-3–positive cells. The numbers of positively stained cells were quantified in 3 to 5 fields per sample (magnification, ×400). Scale bar, 50 μm. Error bars, mean ± SD. **F,** Suppression of BIRC6 expression by ASO-6w2 was confirmed by immunohistochemical staining. Scale bar, 10 μm. ENZ-R, enzalutamide resistant.

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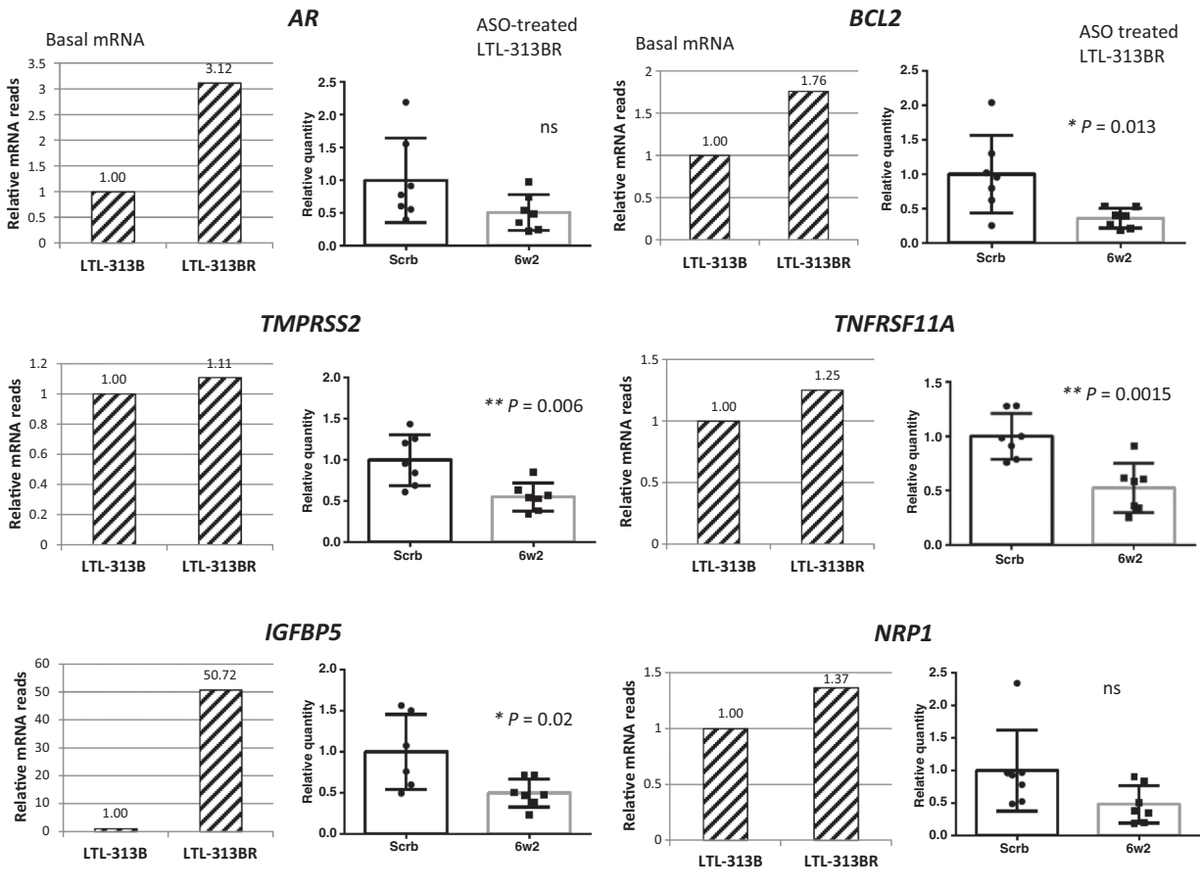

Figure 4.

ASO-6w2 suppressed the expression of prosurvival genes upregulated in enzalutamide-resistant tumors. The LTL-313BR enzalutamide-resistant CRPC xenograft line showed upregulated expression of prosurvival genes in *AR, TMPRSS2* (AR pathway), *IGFBP5* (IGF signaling), *BCL2, TNFRSF11A*, and *NRP1* (NFκB pathway) compared with the parental, enzalutamide-sensitive LTL-313B line. Results are presented as mRNA reads relative to those of the LTL-313B line from transcriptomic sequencing expression data shown in stripped histograms. The expression of these genes in LTL-313BR were shown to be reduced upon treatment with ASO-6w2, including significant downregulations of *TMPRSS2, IGFBP5, BCL2, TNFRSF11A*, and a reduced expression trend for *AR* and *NRP1*. Relative mRNA expressions were determined by qRT-PCR; $n = 7$ per group. Error bars, mean ± SD. ns, not significant.

production per unit tumor, indicating that there is no significant change in the differentiation status of ASO-6w2–treated tumors (Supplementary Fig. S7). Taken together, the results show that ASO-6w2, as a single agent, can significantly inhibit the growth of enzalutamide-resistant CRPCs.

BIRC6 targeting suppresses prosurvival pathways that are upregulated in the enzalutamide-resistant LTL-313BR CRPC tissue xenograft model

In comparing the expressions of genes of the enzalutamide-sensitive LTL-313B line with those of the enzalutamide-resistant LTL-313BR subline using RNA-seq, we noticed that in the LTL-313BR line, genes were upregulated in prosurvival pathways, that is, *AR* (AR pathway), *IGFBP5* (IGF signaling) and *BCL2*, and *TNFRSF11A* and *NRP1* (NFκB pathway). Using qRT-PCR, we determined whether the expression of these genes in LTL-313BR xenografts was affected by treatment with ASO-6w2. As shown in Fig. 4, the treatment with ASO-6w2 led to a downward trend in *AR* and *NRP1* gene expression and to significant downregulations of *TMPRSS2* (−44%, $P < 0.01$), *IGFBP5* (−50%, $P < 0.05$), *BCL2* (−64%, $P < 0.05$), and *TNFRSF11A* (−48%, $P < 0.05$).

Multiple pathways involved in ASO-6w2–induced growth inhibition of enzalutamide-resistant LTL-313BR xenografts

To investigate the pathways involved in the inhibitory effect of ASO-6w2 on LTL-313BR xenograft growth, differential gene expression profiling was performed. Genes (253) were selected from the top 1,000 significantly differentially expressed genes with fold changes >1.5. These genes were subjected to pathway enrichment analysis using MSigDB. As shown in Table 1, G-protein–coupled receptor (GPCR) signaling and matrisome (extracellular matrix signaling) are among the top enriched canonical pathways. There was a general reduction in GPCR activation for ASO-6w2–treated tumors, including a reduction in the expression of *F2R*, an upstream regulator of oncogenic pathways (Supplementary Table S2). On the other hand, treatment with ASO-6w2 induced deregulation in matrisome (extracellular matrix or extracellular matrix–associated protein) pathways, collectively leading to reduced cell proliferation/

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Table 1. Top enriched gene sets in MSigDB all canonical pathways and GO biological process in ASO-6w2 versus Scrb-treated LTL-313BR tumors

Functions	ASO-6w2 vs. Scrb - Top enriched gene sets	Up/down	FDR
GPCR signaling	Reactome signaling by GPCR	↓	8.06E−19
	Reactome GPCR ligand binding	↓	3.63E−14
	Reactome G alpha I signalling events	↓	4.96E−11
Matrisome (extracellular matrix)	Naba matrisome	↑↓	1.33E−15
	Naba matrisome associated	↑↓	3.08E−13
	Receptor activity	↓	7.00E−11
Transmembrane transport	Establishment of localization	↑	3.08E−16
	Transport	↑	1.35E−15
	Reactome transmembrane transport of small molecules	↑	2.07E−12
	Reactome SLC-mediated transmembrane transport	↑	1.34E−08
Response to external stimulus	Response to external stimulus	↑	4.10E−15
	Response to wounding	↑	3.05E−13
	Response to stress	↑	1.23E−10

NOTE: Analysis was based on gene expression profiling in ASO-6w2- and Scrb-treated LTL-313BR xenografts ($n = 4$ per group; fold change > 1.5).

migration, as well as increased apoptosis (Supplementary Table S3). In addition, treatment with ASO-6w2 led to significant upregulation of (i) genes responding to external stimulus, stress, and wounding; and (ii) membrane transporter genes, including genes encoding transporters of cations, anions, amino acids, and water (Table 1).

Taken together, the data indicate that the antitumor activity of *BIRC6*-targeting ASO-6w2 involves the suppression of multiple pathways, including those mediating mitogenesis, cell proliferation, and tissue invasion.

Discussion

Enzalutamide is currently widely used in CRPC therapy. The development of enzalutamide resistance is therefore a major setback in the clinical management of late-stage prostate cancer and novel therapeutic targets, and more effective regimens are urgently needed (1, 2). Using two distinct enzalutamide-resistant CRPC models, the current study has demonstrated that BIRC6, a member of the IAP family, plays a key prosurvival role in the development of enzalutamide resistance of CRPCs. Thus, BIRC6 expression was found to be elevated in enzalutamide-resistant CRPC cells relative to enzalutamide-sensitive parental cells (Fig. 2), and importantly, enzalutamide-resistant CRPC cell proliferation and xenograft growth were markedly inhibited by specific, ASO-induced downregulation of BIRC6 (Fig. 3). Furthermore, treatment of CRPC tumor-bearing mice with *BIRC6*-targeting ASO-6w2 did not lead to major host toxicity (Supplementary Fig. S5). Taken together, the results indicate that *BIRC6* targeting is a promising new strategy for therapy of CRPCs harboring enzalutamide resistance. It is likely that the efficacy of *BIRC6*-targeting ASOs can be further enhanced by incorporating 2′-methoxyethyl modifications and constrained ethyl chemistry (Gen 2.5) in their backbone.

For the validation of *BIRC6* as a potential therapeutic target for enzalutamide-resistant CRPC, the current study made use of the transplantable, enzalutamide-resistant LTL-313BR patient-derived CRPC tissue xenograft line that was developed in our laboratory (Fig. 1; ref. 21). Use of patient-derived cancer tissue xenograft lines, as distinct from cell line–based xenografts, is increasingly required in cancer research, as there is a widespread push for "high-fidelity" cancer models showing a closer link to the patients (30). Use of PDX models instead of cell line–based models has also been advocated for studies of enzalutamide resistance (31). So far, studies of enzalutamide resistance have employed cell line–based models (32–35), and to our knowledge, this is the first report using a PDX CRPC tissue model for studying enzalutamide-resistant CRPC. The enzalutamide-resistant LTL-313BR line is AR positive, PSA positive, has a PTEN copy deletion, and contains the TMPRSS2-ERG fusion (21), characteristics which reflect major features of CRPCs in the clinic. The LTL-313BR line was derived from the hormone-naïve LTL-313B line by castration of LTL-313B tumor-bearing NSG mice and propagation of tumors recurring after relapse of the LTL-313B tumors (21). The LTL-313BR line shows high resistance to bicalutamide (unpublished data), anti-*AR* antisense oligonucleotides (23), as well as primary resistance to enzalutamide. These features resemble those of a subset of CRPCs showing high, primary enzalutamide resistance. Thus, as observed in a recent enzalutamide phase II trial, 37% of patients in a 60-patient bone mCRPC cohort exhibited primary enzalutamide resistance (2). The finding that ASO-6w2 as a single agent markedly inhibited LTL-313BR xenograft growth (Fig. 3C) suggests that BIRC6 is a promising therapeutic target for CRPC patients showing primary enzalutamide resistance. Furthermore, the growth-inhibitory effect of ASO-6w2 was also observed in the case of acquired enzalutamide resistance exhibited by MR49F cells (Fig. 3A). This suggests that BIRC6 plays a fundamental role in promoting the survival of enzalutamide-resistant CRPCs, showing either primary or acquired enzalutamide resistance.

Treatment of LTL-313BR xenografts with ASO-6w2 led to gene expression alterations in diverse biological signaling pathways. As indicated by pathway enrichment analysis, GPCR signaling at the plasma membrane and matrisome signaling at the extracellular matrix were the major pathways that were deregulated by treatment with ASO-6w2 (Table 1). Their deregulation would collectively lead to the suppression of cancer cell proliferation. It is of particular interest that the treatment with ASO-6w2 led to inhibition of *F2R* and *PDGFC* genes, which encode proteins of the plasma membrane and extracellular matrix (Supplementary Tables S2 and S3). Inhibition of the expression of these genes can be expected to lead to effective blockage of downstream activities of PI3K, MAPK, JNK, and multiple kinase cascades and reduction of potential cross-talks of pathways. This would lead to growth inhibition. As the PI3K/Akt pathway is a prominent AR-independent pathway promoting resistance to androgen deprivation and anti-AR treatment (36, 37), ASO-6w2 may be effective in suppressing the growth of enzalutamide-resistant cancers driven by this pathway. Furthermore, treatment with ASO-6w2

Published OnlineFirst September 23, 2016; DOI: 10.1158/1078-0432.CCR-16-0718

resulted in the downregulation of prosurvival genes that showed elevated expression in the LTL-313BR xenograft line (Fig. 4). As ASO-6w2 was found to impede NFκB transactivation (22), it may inhibit AR signaling via suppression of NFκB (38).

It is not clear how *BIRC6* targeting can induce the above gene expression changes. One could speculate that BIRC6 protein may regulate the expression of relevant transcription factors and/or their upstream regulators with its chimeric E2/E3 UBC domain. Also, BIRC6 may, in a non-IAP function, facilitate interaction of certain regulatory proteins by acting as a scaffold structure (39); downregulation of BIRC6 would then lead to disruption of that interaction and changes in the expression of genes. Further mechanistic studies are needed to elucidate how *BIRC6* targeting can lead to growth inhibition of enzalutamide-resistant CRPCs.

Using the transplantable LTL-313BR xenograft line, a first PDX cancer tissue model for enzalutamide-resistant CRPCs, we have shown that BIRC6 plays an important prosurvival role in CRPCs exhibiting enzalutamide resistance and that growth of enzalutamide-resistant CRPCs can be inhibited by downregulation of BIRC6 without inducing major host toxicity. *BIRC6* targeting may hence represent a new option for the clinical treatment of advanced, enzalutamide-resistant prostate cancer.

Disclosure of Potential Conflicts of Interest

I.S.U. Luk is listed as a co-inventor on a patent on the use the anti-BIRC6 antisense oligonucleotide in the treatment of cancers. M.E. Gleave is listed as a co-inventor on a patent, which is owned by the University of British Columbia, on antisense oligos targeting BIRC-6. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.W. Wang, F. Zhang, D. Lin, R. Wu, Y.Z. Wang
Study supervision: Y.Z. Wang
Other (development of enzalutamide-resistant cells): A. Zoubeidi

Grant Support

This work was supported by grants from the Canadian Institutes of Health Research (#102604-1, #119991-1, and #123449-1; to Y.Z. Wang), Centres of Excellence for Commercialization and Research (to M.E. Gleave), Prostate Cancer Canada (to C.C. Collins and Y.Z. Wang), Terry Fox Research Institute (#116129-1; to Y.Z. Wang), BC Cancer Foundation (to Y.Z. Wang), and Prostate Cancer Canada Personnel Award (#GS2015-03; to I.S.U. Luk).

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Received March 19, 2016; revised August 11, 2016; accepted September 5, 2016; published OnlineFirst September 23, 2016.

References

1. Claessens F, Helsen C, Prekovic S, Van den Broeck T, Spans L, Van Poppel H, et al. Emerging mechanisms of enzalutamide resistance in prostate cancer. Nat Rev Urol 2014;11:712–6.
2. Efstathiou E, Titus M, Wen S, Hoang A, Karlou M, Ashe R, et al. Molecular characterization of enzalutamide-treated bone metastatic castration-resistant prostate cancer. Eur Urol 2015;67:53–60.
3. Scher HI, Fizazi K, Saad F, Taplin ME, Sternberg CN, Miller K, et al. Increased survival with enzalutamide in prostate cancer after chemotherapy. N Engl J Med 2012;367:1187–97.
4. Chandrasekar T, Yang JC, Gao AC, Evans CP. Targeting molecular resistance in castration-resistant prostate cancer. BMC Med 2015;13:206.
5. Watson PA, Arora VK, Sawyers CL. Emerging mechanisms of resistance to androgen receptor inhibitors in prostate cancer. Nat Rev Cancer 2015;15:701–11.
6. Fulda S, Vucic D. Targeting IAP proteins for therapeutic intervention in cancer. Nat Rev Drug Discov 2012;11:109–24.
7. Pohl C, Jentsch S. Final stages of cytokinesis and midbody ring formation are controlled by BRUCE. Cell 2008;132:832–45.
8. Kikuchi R, Ohata H, Ohoka N, Kawabata A, Naito M. APOLLON protein promotes early mitotic CYCLIN A degradation independent of the spindle assembly checkpoint. J Biol Chem 2014;289:3457–67.
9. Low CG, Luk IS, Lin D, Fazli L, Yang K, Xu Y, et al. BIRC6 protein, an inhibitor of apoptosis: role in survival of human prostate cancer cells. PLoS One 2013;8:e55837.
10. Sung KW, Choi J, Hwang YK, Lee SJ, Kim HJ, Lee SH, et al. Overexpression of Apollon, an antiapoptotic protein, is associated with poor prognosis in childhood de novo acute myeloid leukemia. Clin Cancer Res 2007;13:5109–14.
11. Wang L, Chen YJ, Hou J, Wang YY, Tang WQ, Shen XZ, et al. Expression and clinical significance of BIRC6 in human epithelial ovarian cancer. Tumour Biol 2014;35:4891–6.
12. Tang W, Xue R, Weng S, Wu J, Fang Y, Wang Y, et al. BIRC6 promotes hepatocellular carcinogenesis: interaction of BIRC6 with p53 facilitating p53 degradation. Int J Cancer 2015;136:E475–87.
13. Dong X, Lin D, Low C, Vucic EA, English JC, Yee J, et al. Elevated expression of BIRC6 protein in non-small-cell lung cancers is associated with cancer recurrence and chemoresistance. J Thorac Oncol 2013;8:161–70.
14. Hu T, Weng S, Tang W, Xue R, Chen S, Cai G, et al. Overexpression of BIRC6 is a predictor of prognosis for colorectal cancer. PLoS One 2015;10:e0125281.
15. Chen Z, Naito M, Hori S, Mashima T, Yamori T, Tsuruo T. A human IAP-family gene, apollon, expressed in human brain cancer cells. Biochem Biophys Res Commun 1999;264:847–54.
16. Zhang S, Tang W, Weng S, Liu X, Rao B, Gu J, et al. Apollon modulates chemosensitivity in human esophageal squamous cell carcinoma. Oncotarget 2014;5:7183–97.
17. Van Houdt WJ, Emmink BL, Pham TV, Piersma SR, Verheem A, Vries RG, et al. Comparative proteomics of colon cancer stem cells and differentiated tumor cells identifies BIRC6 as a potential therapeutic target. Mol Cell Proteomics 2011;10:M111.011353.
18. Chu L, Gu J, Sun L, Qian Q, Qian C, Liu X. Oncolytic adenovirus-mediated shRNA against Apollon inhibits tumor cell growth and enhances antitumor effect of 5-fluorouracil. Gene Ther 2008;15:484–94.
19. Tassi E, Zanon M, Vegetti C, Molla A, Bersani I, Perotti V, et al. Role of Apollon in human melanoma resistance to antitumor agents that activate the intrinsic or the extrinsic apoptosis pathways. Clin Cancer Res 2012;18:3316–27.
20. Eirew P, Steif A, Khattra J, Ha G, Yap D, Farahani H, et al. Dynamics of genomic clones in breast cancer patient xenografts at single-cell resolution. Nature 2015;518:422–6.
21. Lin D, Wyatt AW, Xue H, Wang Y, Dong X, Haegert A, et al. High fidelity patient-derived xenografts for accelerating prostate cancer discovery and drug development. Cancer Res 2014;74:1272–83.

22. Luk SU, Xue H, Cheng H, Lin D, Gout PW, Fazli L, et al. The BIRC6 gene as a novel target for therapy of prostate cancer: dual targeting of inhibitors of apoptosis. Oncotarget 2014;5:6896–908.
23. Yamamoto Y, Loriot Y, Beraldi E, Zhang F, Wyatt AW, Nakouzi NA, et al. Generation 2.5 antisense oligonucleotides targeting the androgen receptor and its splice variants suppress enzalutamide-resistant prostate cancer cell growth. Clin Cancer Res 2015;21:1675–87.
24. Bishop JL, Sio A, Angeles A, Roberts ME, Azad AA, Chi KN, et al. PD-L1 is highly expressed in Enzalutamide resistant prostate cancer. Oncotarget 2015;6:234–42.
25. Wang Y, Xue H, Cutz JC, Bayani J, Mawji NR, Chen WG, et al. An orthotopic metastatic prostate cancer model in SCID mice via grafting of a transplantable human prostate tumor line. Lab Invest 2005;85:1392–404.
26. Kuruma H, Matsumoto H, Shiota M, Bishop J, Lamoureux F, Thomas C, et al. A novel antiandrogen, compound 30, suppresses castration-resistant and MDV3100-resistant prostate cancer growth in vitro and in vivo. Mol Cancer Ther 2013;12:567–76.
27. Chiang YT, Wang K, Fazli L, Qi RZ, Gleave ME, Collins CC, et al. GATA2 as a potential metastasis-driving gene in prostate cancer. Oncotarget 2014; 5:451–61.
28. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 2002;30:207–10.
29. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545–50.
30. Ledford H. US cancer institute to overhaul tumour cell lines. Nature 2016;530:391.
31. Buonerba C, Di Lorenzo G. Is *in vitro*-acquired resistance to enzalutamide a useful model? Future Oncol 2014;10:2551–3.
32. Liu C, Lou W, Zhu Y, Yang JC, Nadiminty N, Gaikwad NW, et al. Intracrine androgens and AKR1C3 activation confer resistance to enzalutamide in prostate cancer. Cancer Res 2015;75:1413–22.
33. Nguyen HG, Yang JC, Kung HJ, Shi XB, Tilki D, Lara PN Jr, et al. Targeting autophagy overcomes enzalutamide resistance in castration-resistant prostate cancer cells and improves therapeutic response in a xenograft model. Oncogene 2014;33:4521–30.
34. Toren P, Kim S, Cordonnier T, Crafter C, Davies BR, Fazli L, et al. Combination AZD5363 with enzalutamide significantly delays enzalutamide-resistant prostate cancer in preclinical models. Eur Urol 2015;67:986–90.
35. Kato M, Banuelos CA, Imamura Y, Leung JK, Caley DP, Wang J, et al. Cotargeting androgen receptor splice variants and mTOR signaling pathway for the treatment of castration-resistant prostate cancer. Clin Cancer Res 2016;22:2744–54.
36. Carver BS, Chapinski C, Wongvipat J, Hieronymus H, Chen Y, Chandarlapaty S, et al. Reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient prostate cancer. Cancer Cell 2011; 19:575–86.
37. Mulholland DJ, Tran LM, Li Y, Cai H, Morim A, Wang S, et al. Cell autonomous role of PTEN in regulating castration-resistant prostate cancer growth. Cancer Cell 2011;19:792–804.
38. Zhang L, Altuwaijri S, Deng F, Chen L, Lal P, Bhanot UK, et al. NF-kappaB regulates androgen receptor expression and prostate cancer growth. Am J Pathol 2009;175:489–99.
39. Ge C, Che L, Ren J, Pandita RK, Lu J, Li K, et al. BRUCE regulates DNA double-strand break response by promoting USP8 deubiquitination of BRIT1. Proc Natl Acad Sci U S A 2015;112:E1210–9.

Published OnlineFirst September 23, 2016; DOI: 10.1158/1078-0432.CCR-16-0718

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Clin Cancer Res 2017;23:1542-1551. Published OnlineFirst September 23, 2016.