Research Article

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Epigenomics

LncRNA *HORAS5* promotes taxane resistance in castration-resistant prostate cancer via a BCL2A1-dependent mechanism

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Background: Castration-resistant prostate cancer (CRPC) is an incurable malignancy. Long noncoding RNAs (lncRNAs) play key roles in drug resistance. **Materials & methods:** LncRNA *HORAS5* role in cabazitaxel resistance (i.e., cell-count, IC_{50} and caspase activity) was studied via lentiviral-mediated overexpression and siRNA-based knockdown. Genes expression was analyzed with RNA-sequencing, reverse transcription quantitative PCR (RT-qPCR) and western blot. *HORAS5* expression was queried in clinical database. **Results:** Cabazitaxel increased *HORAS5* expression that upregulated *BCL2A1*, thereby protecting CRPC cells from cabazitaxel-induced apoptosis. *BCL2A1* knockdown decreased cell-count and increased apoptosis in CRPC cells. *HORAS5*-targeting antisense oligonucleotide decreased cabazitaxel IC_{50}. In CRPC clinical samples, *HORAS5* expression increased upon taxane treatment. **Conclusion:** *HORAS5* stimulates the expression of BCL2A1 thereby decreasing apoptosis and enhancing cabazitaxel resistance in CRPC cells.

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Keywords: BCL2A1 • castration-resistant prostate cancer • drug resistance • *HORAS5* • lncRNA

Castration-resistant prostate cancer (CRPC) is an incurable malignancy [1]. It occurs when prostate cancer (PCa) cells acquire mutations and other genetic and epigenetic alterations in the (androgen receptor) AR signalling and in cell survival pathways; hence they stop responding to androgen deprivation therapies [2–4]. At this stage, next-generation hormonal treatments such as enzalutamide and abiraterone and chemotherapy such as docetaxel and cabazitaxel are the only effective treatment options [5–8]. In advanced CRPC stages, normally characterized by high metastatic burden (mCRPC), cancer cells can become resistant to these treatments. At this stage mCRPC patients median survival is very poor (1–2 years) [4], therefore novel therapeutic approaches to increase drug efficacy are urgently needed.

Long noncoding RNAs (lncRNAs) are >200 bp transcripts that lack of protein-coding capacity and the majority are still uncharacterized [9]. Due to their ability to fold into various structures [10], they could have several functions and mechanisms of action inside the cells, some of which were recently described [11–14]. LncRNAs have been investigated in health and disease and some of them were characterized as key players in cancer-associated pathways such as development of metastatic status and drug resistance [15–19]. In the context of drug response, some studies showed that lncRNAs can increase drug sensitivity in specific cancers. However, several lncRNAs have been shown to promote resistance to various agents, such as hormonal therapy or chemotherapy agents [13,17,20,21].

Future Medicine

In a recently published study, we have characterized the lncRNA *HORAS5* (i.e., *linc00161*) in PCa, showing that it was upregulated in patient-derived xenografts (PDXs) models and increased the survival of AR$^+$ CRPC cells [22]. *HORAS5* was already studied in osteosarcoma cells where it showed a proapoptotic oncosuppressive role that determined the increase of cisplatin sensitivity via *miR-645* inhibition and action in the IFIT2 pathway [13]. Despite this evidence, *HORAS5* was then found to increase migration and invasion in hepatocellular carcinoma patients and higher expression of this transcript correlated with poor prognosis [23]. A recent study also showed that *HORAS5* can promote drug resistance in ovarian cancer via acting as a competing endogenous RNA with another miRNA in a different pathway (*linc00161-miR-128*-MAPK1 axis) [21]. All this evidence shows that *HORAS5* modulates drug-associated response in different malignancies and that this transcript can have different functions in different tissues.

Based on this evidence, we have decided to investigate whether *HORAS5* plays a role in the drug response of AR$^-$ and AR$^+$ CRPC cells. For this purpose, we have tested a panel of clinically relevant drugs on CRPC cells expressing different levels of this transcript [22]. We have shown that cabazitaxel was the only drug tested that induced a significant, concentration-dependent increase in *HORAS5* expression. Hence, we have decided to further investigate the role of *HORAS5* in cabazitaxel response. Our results suggest that *HORAS5* activates a BCL2A1-dependent mechanism of action, thereby inducing cabazitaxel resistance in CRPC cells.

Materials & methods

Cell culture

Human PCa LNCaP, DU145 and 22rv1 cell lines were purchased from the American-Type Culture Collection (ATCC, ON, Canada). In particular, we have selected LNCaP cells, which express a mutated *AR* gene and grow in castrate testosterone concentrations [24] and in DU145 cells, which are AR$^-$ and a model of anaplastic CRPC [25,26]. LNCaP cells were cultured in RPMI-1640 ATCC modification from GIBCO (cat# A1049101) and DU145 and 22rv1 cells (DU145-NC, DU145-OE, 22rv1-NC and 22rv1-OE; see below and in Supplementary Figure 4) in RPMI-1640 from GIBCO (cat# 21875034), supplemented with 10% of fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin). All the cell lines were cultured according to the protocols available from the ATCC website. Cells were cultured at 37°C in a 5% CO_2-humidified incubator.

HORAS5 overexpression with lentiviral stable transduction

DU145 cells that express *HORAS5* at undetectable levels were stably transfected with a lentivirus-derived particle that induced *HORAS5* overexpression (Genecopoeia, MD, USA, Cat# LPP-GS266B-Lv105-050). 7.0×10^4 AR$^-$ Du145 and AR$^+$ 22rv1 cells were first seeded in 24-well plates and were incubated overnight. The following day, old media was replaced by media supplemented with polybrene (aka Hexadimethrine Bromide, Sigma Aldrich, Gillingham, UK, Cat# H9268) at a final concentration of 8 μg/ml to increase transduction efficiency [27]. After that, 5 μl of the purified human *linc00161* (*HORAS5*) lentiviral particles (Titer: 1.37 × 108 TU/ml where 1 TU = 100 copies of viral genomic RNA) were added to the wells and incubated overnight. The following day, the wells were washed with RPMI-1640 containing 10% FBS three-times and were subsequently left for 2 days to reach confluence. 48- to 72 h post-transduction, cells were split one-in-two into six-well plates and were allowed to adhere for 5–6 h prior to antibiotic selection using puromycin (Gibco, Loughborough, UK, Cat# A1113803). Selection lasted for 2 weeks with media change every 3–4 days. To achieve high copy number, 3 μg/ml of puromycin was selected. These cells were called Du145-OE and 22rv1-OE, respectively, and were passaged and frozen for long-term storage in liquid nitrogen. All overexpression experiments were normalized to cells transduced with the empty vector (DU145-NC and 22rv1-NC, respectively) as lentiviral vectors did not induce phenotypic changes.

Total & subcellular-fractionated RNA extraction

Total RNA was extracted using the RNeasy plus mini Kit (Qiagen, Manchester, UK) from cultured cells according to the manufacturer's protocol.

Nuclear- and cytoplasmic-fractionated RNA extraction was performed on cultured cells using the PARIS™ kit (Ambion, Loughborough, UK) following the manufacturer's protocol. DNAse digestion of the fractionated RNA was performed using the TURBO DNA-free™ Kit (Ambion, Cat# AM1907).

Reverse-transcription & quantitative PCR

Upon extraction, 1 μg of total RNA was reverse-transcribed using high capacity cDNA reverse transcription kit (Applied Biosystems, Loughborough, UK) following the kit instructions. The cDNA obtained was diluted tentimes prior the quantitative PCR (qPCR). TaqMan assays (Applied Biosystems) were used for the qPCR to assess gene expression as per the manufacturer's protocol. The TaqMan assays used were *LINC00161* (Hs00863167_g1) and *BCL2A1* (Hs00187845_m1). *HPRT1* (Hs02800695_m1) was used as housekeeping control in all the reverse transcription (RT)-qPCR experiments. For subcellular localization of RT-qPCR experiments, the probes *MALAT1* (Hs00273907_s1) and *GAPDH* (Hs02786624_g1) were also used as nuclear and cytoplasmic control respectively.

siRNA-mediated gene knockdown

Gene knockdown (KD) experiments were performed using the reverse transfection method [28]. Cells were seeded in six-well or 96-well plates with the lipid:siRNA mixture prepared using the RNAiMAX reagent (Invitrogen, Loughborough, UK) according to the manufacturer's protocol. Final siRNA treatment dosages were 2 nM concentrated. All duplexes were purchased from Integrated DNA Technologies (IDT) (Leuven, Belgium): anti-*HORAS5* (aka *Linc00161*) DsiRNA hs.Ri.LINC00161.13.2, anti-BCL2A1 DsiRNAs hs.Ri.BCL2A1.13.1 and hs.Ri.BCL2A1.13.2 and nontargeting negative control (scramble) DS NC1. After 48 or 72 h post-transfection, treated cells were harvested for extracting total RNA and/or total protein.

Drug treatments

5 mg of cabazitaxel (Jevtana, Selleckchem, Ely, UK, cat#S3022) was resuspended in dimethyl sulfoxide (DMSO) in order to obtain a final stock solution of 5 mM. 5 mg of enzalutamide (MDV3100, Selleckchem, cat#S1250) was resuspended in DMSO in order to obtain a final stock solution of 1 mM. Both drugs were stored at –80°C. Carboplatin was resuspended in water to obtain a stock solution of 10 mg/ml and stored at –20°C. All drugs were thawed at room temperature and diluted in cell culture media to treat the cells at different concentrations, according to the experiments:

- Cabazitaxel was used to treat LNCaP and DU145 cells for 24–48–72 h at (5–50) nM in gene expression experiments, caspase assays, RNA sequencing and at (000005–0.0005–0.05–0.5–5–50–100) nM in the Trypan blue-based cell counting and IC_{50} calculation experiments. 22rv1 were treated for 48–72 h with 5–50 nM of cabazitaxel in gene expression experiments and Trypan blue-based cell counting;
- Enzalutamide was used to treat LNCaP (1–10) μM and 22rv1 (10–100) μM cells for 72 h at in gene expression experiments;
- Carboplatin was used to treat DU145 cells for 72 h at (1–10) μM in gene expression experiments. All concentrations selected were clinically achievable.

All the drugs concentrations have been selected based on the IC_{50} and concentrations used in the literature for CRPC cells, following a $\log_{10}$ criteria, in order to select a wide range of concentrations for our analyses [29–32].

Trypan blue-based cell counting & IC_{50} calculation

Cell proliferation was assessed via Trypan blue-based cell counting; in this contest cell metabolic assays can give altered results due to taxane interference with mitochondria metabolism [33].

2×10^5 DU145-NC and -OE (and 22rv1-NC and -OE, Supplementary Figure 4E) cells were seeded in a six-well plate and treated with DMSO/cabazitaxel in the concentrations specified above (see drug treatments).

2.5×10^5 LNCaP cells and 5×10^5 DU145-OE cells were seeded in a six-well plate and reverse transfected with 2 nM of control siRNA and either anti-*HORAS5* siRNA or anti-*BCL2A1* siRNAs, respectively. At day two post-transfection, the cells were treated with DMSO/cabazitaxel in the concentrations specified above (see drug treatments).

For all the cells, at day 3 (LNCaP) and 2 (DU145-NC/-OE and 22rv1-NC/-OE) after the drug treatment, trypan blue-based cell counting was performed and the IC_{50} was calculated by nonlinear regression analysis (variable-slope inhibitor fitting), after normalization to untreated (DMSO) cells.

Caspase activity assay

10^4 DU145-NC and -OE cells were seeded in a white, flat-bottom 96-well plate and treated with DMSO/5 nM of cabazitaxel.

1.25×10^4 LNCaP cells and 2.5×10^4 DU145-OE cells were seeded in a white, flat-bottom 96-well plate and reverse transfected with 2 nm of control siRNA and either anti-*HORAS5* siRNA or anti-BCL2A1 siRNAs, respectively. At day 2 post-transfection, the cells were treated with DMSO/5 nM/50 nM of cabazitaxel.

At day 3 (LNCaP) and 2 (DU145-NC and -OE) after the drug treatment, Caspase-Glo reagent (Promega, Southampton, UK) was added to the cells and total luminescence was quantified, following the manufacturer's protocol. Results were normalized to the cell count at the respective treatment concentration and time of treatment.

RNA sequencing & differential expression analysis

Total RNA samples were isolated from DU145-NC/OE cells untreated (DMSO) versus treated for 48 h with 5 nM of cabazitaxel. Next-generation sequencing (NGS) based on the Ion Torrent Semiconductor technology (Thermo Fisher Scientific, Loughborough, UK) and bioinformatics analysis were carried out by the Institute of Pathology of the University Hospital Basel, Switzerland. The resulting dataset was further analyzed to determine the protein-coding genes upregulated when *HORAS5* is overexpressed (DU145-OE) versus negative control (DU145-NC) upon cabazitaxel treatment (cabazitaxel-driven genes in Figure 4A). The expression threshold was set as log2 fold-change >2 and $p < 0.01$ for the cells overexpressing *HORAS5*. The 87 genes were then filtered for DU145-NC p-value and sorted in descending order (top 25 genes in Figure 4A). The final shortlist was obtained by ranking the top three genes based on literature evidence on cancer and taxane resistance.

Protein extraction & western blot analysis

Cell lysates were obtained using 15–50–100 μl of radioimmunoprecipitation assay (RIPA) buffer (Tris pH 8.0 [Sigma Aldrich]; NaCl [Sigma Aldrich]; EDTA [Sigma Aldrich]; Igepal [Sigma Aldrich]; SDS [Sigma Aldrich], NaF [Sigma Aldrich]; NaVO3 [Sigma Aldrich]) according to the number of cells used. Proteins were quantified with the Peierce BCA assay (Thermo Fisher Scientific) as per the manufacturer's protocol. 15 mg of proteins were resolved via gel electrophoresis on reducing SDS-polyacrylamide gels (Tricine 10–20%, Thermo Fisher Scientific) run at 110 V for 2 h. Protein transfer was performed using nitrocellulose membrane at 300 mA for 2.5 h. The membranes were blocked in 8% skimmed milk dissolved in tris-buffered saline (TBS; Sigma Aldrich) containing 0.1% tween-20 (TBS-T) at room temperature for 1 h. After 1 h, the blots were incubated overnight at 4°C with protein-specific primary antibodies dissolved in 5% BSA diluted in TBS-T for anti-BCL2A1 (Cell Signalling Technology, Leiden, The Netherlands, A1/Bfl-1 (D1A1C) Rabbit mAb, Cat# 14093) and 5% milk diluted in TBS-T for anti-GAPDH (Sigma Aldrich, cat# G9545). After the overnight incubation, protein blots were washed three-times with TBS-T for 10 min. Blots were then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Thermo Fisher Scientific, cat#31460) dissolved in 8% milk diluted in TBS-T at room temperature for 1 h. After the incubation, blots were washed four-times in TBS-T for 10 min each.

After washing, enhanced chemiluminescence (ECL) western blotting substrate kit was used (Millipore, Watford, UK) to visualize blot chemiluminescence, using Syngene Gbox with GeneTools software (Syngene, Bangalore, India).

CBioPortal analysis of clinical samples

To assess the clinical relevance of *HORAS5* expression in PCa samples, CBioPortal (www.cbioportal.org) was queried using a publicly available Agilent microarray dataset [34]. This dataset consisted of a single study with 63 patients, of which 15 patients did not undergo chemotherapy treatment and ten were treated with taxane only. We compared none versus taxane only treatment.

Antisense oligonucleotides

1.5×10^5 LNCaP and 1.5×10^5 DU145-NC cells were seeded in six-well plates and after 24 h, the cells were transfected with 2 nM of a negative control antisense oligonucleotides (ASOs) (Eurofins Genomics, Wolverhampton, UK, ASO-NC: C*C*T *T*C*C *C*T*G *A*A*G *G*T*T *C*C*T *C*C) and *HORAS5*-ASO3 (Eurofins Genomics, *HORAS5* V3*: G*G*C *T*G*C *T*G*C *A*T*G *T*C*T *A*C*A *G*T) preselected as the most effective of eight tested ASO sequences for *HORAS5* KD (Figure 6A), using the RNAiMAX reagent (Invitrogen, Loughborough, UK), according to the manufacturer's protocol. At day 2 post ASO treatment, the cells were treated with DMSO/cabazitaxel at concentrations specified above (see drug treatments). At day 3 (LNCap) or day 2 (DU145-NC) postdrug treatment, the cells were counted using the tripan blue-based method.

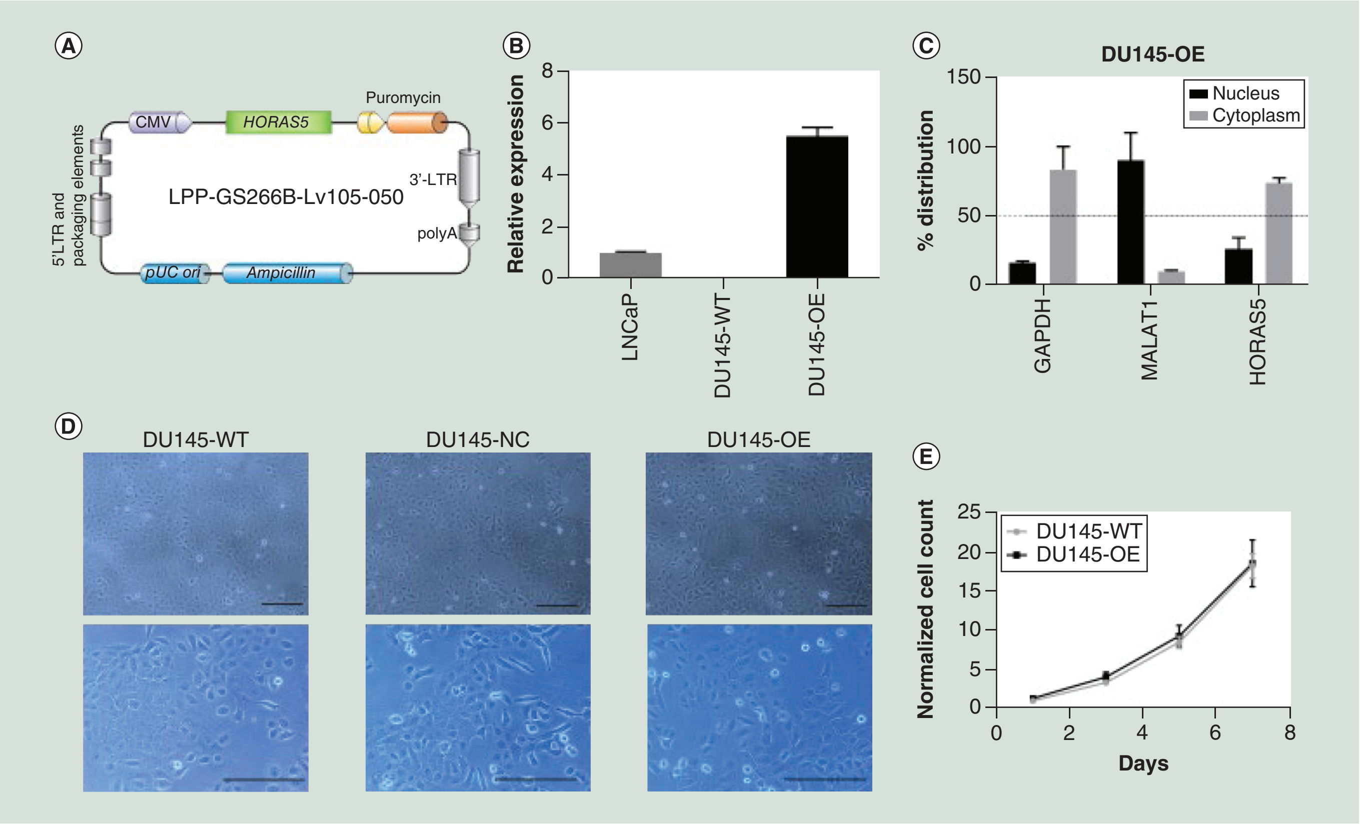

Figure 1. ***HORAS5*** **overexpression maintains its cytoplasmic location and does not affect cell proliferation and morphology. (A)** Schematic representation of the vector LPP-GS266B-Lv105-050 used to induce the expression of *HORAS5* in the transduced cells (DU145). The plasmid contains the long teminal repeat packaging elements, the citomegalovirus strong promoter, the pUC Ori bacteria-start replication site and the antibiotic resistance cassettes to select the cells transduced and containing *HORAS5*. **(B)** qPCR expression of *HORAS5* (aka *linc00161*) in AR$^+$ PCa cells with endogenous expression of the lncRNA (LNCaP) and AR$^-$ PCa cells with lentivector-induced expression (DU145-OE). **(C)** qPCR expression of *HORAS5* in the nuclear and cytoplasmic subcellular fractions of DU145-OE cells. *GAPDH* and *MALAT1* are used as controls for cytoplasmic and nuclear fractions, respectively. *HPRT1* was used as housekeeping gene for data normalization in all the qPCR experiments. **(D & E)** Pictures of DU145-WT, DU145-NC and DU145-OE cells` morphology **(D)** and MTS proliferation curves of DU145-OE versus DU145-WT **(E)**. The size bars in figure **(D)** represent 100 μm. Results expressed as means ± SD from two independent replicates. Two-way ANOVA with Sidak's post-test was performed for statistical comparison in **(E)**.
AR: Androgen receptor; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PCa: Prostate cancer; qPCR: Quantitative PCR; SD: Standard deviation.

Statistical analysis

All data were obtained from at least two or three independent experiments and analyzed using GraphPad Prism 7 software. Values are presented as mean ± standard deviation (SD). Significant differences between the groups were calculated using linear trend test, Student's t-test, one-way ANOVA with Tukey's multiple comparison post-test and two-way ANOVA with Sidak's multiple comparison post-test and nonlinear fit (log inhibitor vs normalized response-variable slope) IC_{50} analysis. A $p < 0.05$ was set as threshold for statistical significance. Outlier test was carried out to remove the extreme experimental replicate for the IC_{50} calculation.

Results

HORAS5 overexpression & sub cellular localization

Based on previously published data on *HORAS5* expression in a panel of PCa cells [22], we selected LNCaP (AR$^+$) as cell line expressing this lncRNA and DU145 (AR$^-$) as cell line with undetectable levels of *HORAS5* [22]. To investigate the effects of *HORAS5* in AR$^-$ PCa cells, *HORAS5* expression was artificially induced via DU145 cells' stable transduction with lentiviral particles containing *HORAS5* gene, expressed under the strong CMV promoter (Figure 1A). This transduction induced elevated *HORAS5* expression, which was stable for at least 20 passages (Figure 1B). We called these cells DU145-OE and the corresponding cells transfected with the empty vector

Table 1. Treatment selection based on clinical and *in vitro* evidence.

Cancer Type	Clinical treatment used	*In vitro* evidence	Cell line miming cancer	Treatment selected	Ref.
AR$^+$ CRPC	Hormonal (abiraterone, enzalutamide)	Active in AR$^+$ CRPC cells	LNCAP	Enzalutamide	[5,27,29]
	Chemotherapy (docetaxel, cabazitaxel)	Active in both AR$^+$ and AR$^-$ CRPC cells		Cabazitaxel	[30,31]
AR$^-$ CRPC	Chemotherapy (docetaxel, cabazitaxel, platinum agents)	Active in both AR$^+$ and AR$^-$ CRPC cells	DU145	Cabazitaxel	[30,31]
		Platinum agents active in AR$^-$ CRPC cells		Carboplatin	[32,33]

AR: Androgen receptor; CRPC: Castration-resistant prostate cancer.

were called DU145-NC. Additionally, we have confirmed *HORAS5* overexpression in AR$^+$ 22RV1 cells, which express undetectable endogenous levels of *HORAS5* (Supplementary Figure 4A). Our previous studies showed that *HORAS5* is located in the cytoplasm of LNCaP cells [22]. Our results in DU15-OE cells indicate that the artificial expression of this lncRNA preserved its cytoplasmic location (Figure 1C). Our analyses in 22rv1-OE also confirmed *HORAS5* cytoplasmic location (Supplementary Figure 4B). Additionally, we observed no morphological changes induced by *HORAS5* overexpression in DU145-OE versus DU145-NC and DU145-WT (Figure 1D) and *HORAS5* overexpression did not influence cell proliferation in untreated cells (Figure 1E).

HORAS5 expression is induced by cabazitaxel in a concentration- & time-dependent manner

Since our previous studies have shown *HORAS5* upregulation in CRPC versus hormone sensitive PDXs [22] and other studies suggested that *HORAS5* modulates drug response [13,21], we sought to investigate whether exposure to clinically employed drugs could affect the expression of *HORAS5*. To this aim, we selected three drugs, which are representative of the most commonly used treatments in CRPC patients: the AR inhibitor enzalutamide, which is used in AR$^+$ CRPC [5,35,36]; the microtubule inhibitor cabazitaxel, which is active against both AR$^+$ and AR$^-$ CRPCs [37,38]; the platinum agent carboplatin, which displayed some activity in AR$^-$ CRPCs (Table 1) [39,40]. In line with clinical indications, enzalutamide was used in LNCaP and 22rv1-OE cells, carboplatin in DU145-OE cells only and cabazitaxel in all the cell lines with doses ranging around the IC$_{50}$ and concentrations found in the literature for CRPC cells, on a log$_{10}$ basis [29–32]. Based on our criteria ($p < 0.05$ and $R^2 > 0.5$) enzalutamide did not determine a significant change in *HORAS5* expression in LNCaP ($p = 0.2451$, $R^2 = 0.06073$) (Figure 2A) and 22rv1-OE cells ($p = 0.0029$, $R^2 = 0.3195$) (Supplementary Figure 4C) at the clinically achievable concentrations used [41]. Similarly, no effect was observed for carboplatin ($p = 0.0061$, $R^2 = 0.1657$) (Figure 2B) [42,43]. Cabazitaxel induced a concentration-dependent increase of *HORAS5* expression in LNCaP ($p < 0.0001$, $R^2 = 0.6513$) (Figure 2C), DU145-OE ($p < 0.0001$, $R^2 = 0.7563$) (Figure 2D) and 22rv1-OE cells ($p < 0.0001$, $R^2 = 0.7185$) (Supplementary Figure 4D). All the concentrations of cabazitaxel used in these experiments are clinically achievable [44]. Overall, just cabazitaxel treatment determined a linear concentration-dependent increase in the expression of *HORAS5* ($R^2 > 0.5$ linear trend test). A time-course experiment revealed that cabazitaxel-induced *HORAS5* upregulation is time-dependent: we did not observe any significant transcript upregulation up to 24 h after treatment. However, *HORAS5* was significantly upregulated in DU145 (48 h, 72 h) and in LNCaP (72 h) cells (Figure 2E & F). For this reason, we chose 72 h for LNCaP and 48 h for DU145-OE as time points for further experiments.

HORAS5 modulation affects the proliferation & survival of PCa cells exposed to cabazitaxel

So far, we have shown that cabazitaxel induces a dose- and time-dependent increase in *HORAS5* expression in both AR$^+$ and AR$^-$ CRPC cells. We therefore sought to investigate the functional significance of *HORAS5* in CRPC cells exposed to cabazitaxel. We optimized the silencing procedure and obtained a KD of 77%, at day 5 after transfection (Supplementary Figure 1A). This time point results from 2 days of silencing + 3 additional days of cabazitaxel treatment. We then hypothesized that *HORAS5* silencing and overexpression can affect CRPC cell proliferation. We analyzed the effect of *HORAS5* modulation on cabazitaxel anticancer activity. Our data showed that cabazitaxel induced a dose-dependent growth inhibition (Figure 3A & B). This inhibition decreased in DU145-OE compared with DU145-NC (Figure 3A) with a significant increase of cabazitaxel IC$_{50}$ of 9.8-times (from 3.11 ± 1.48 nM to 30.55 ± 3.9 nM, Supplementary Figure 1B) in the cells that overexpress *HORAS5* ($p = 0.0114$) (Figure 3C). *Vice versa*, cabazitaxel-dependent growth inhibition increased in LNCaP cells upon *HORAS5* KD (Figure 3B). Our calculation showed that *HORAS5* silencing caused dramatic decrease of cabazitaxel IC$_{50}$ in this cell line (from 20.80 ± 0.74 nM to 2.59 ± 0.77 nM, $p = 0.0033$) (Figure 3D, Supplementary Figure

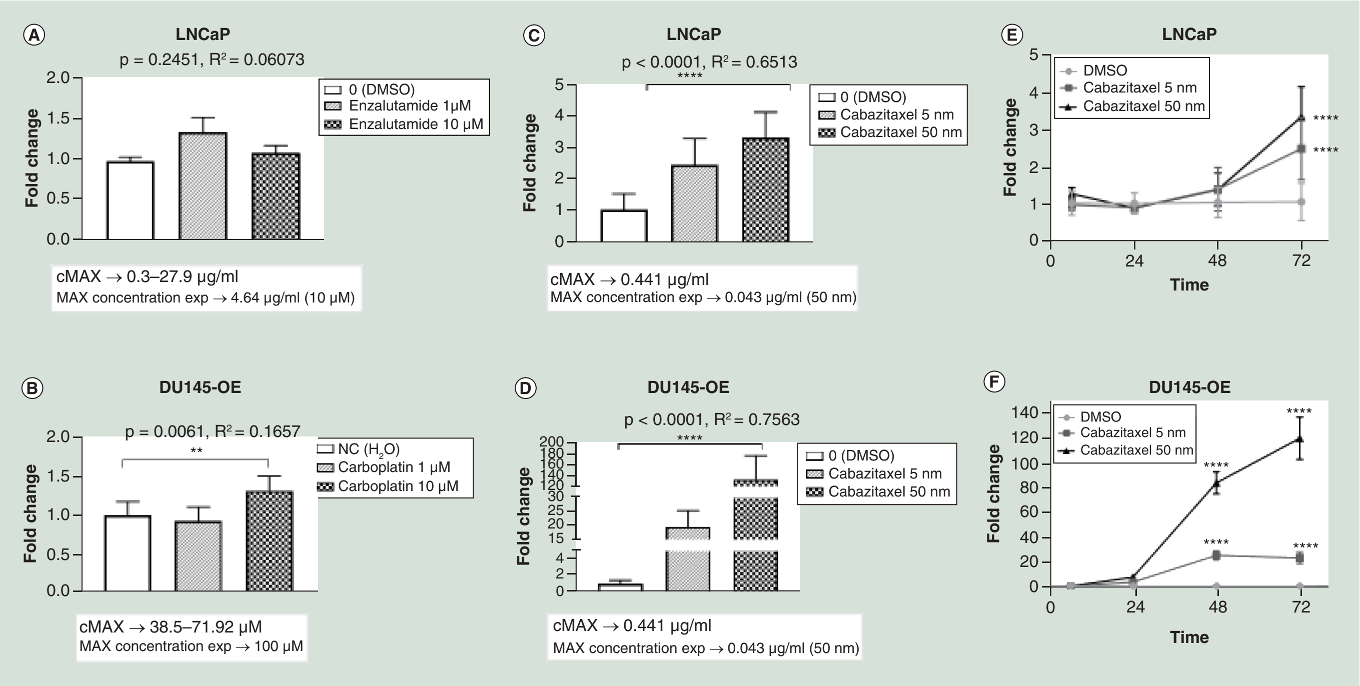

Figure 2. Effect of commonly used drugs in the clinics on *HORAS5* expression and selection of cabazitaxel. (A–D) qPCR expression of *HORAS5* and reported *cMAX* and experimental concentrations upon treatment with enzalutamide (LNCAP, **(A)**, carboplatin (DU145-OE, **(B)** and cabazitaxel (LNCAP) **[C]**; DU145-OE, **[D]**) drugs on LNCaP AR$^+$ and DU145 AR$^-$ PCa cell lines for 72 h. Results expressed as means ± SD from two independent replicates. One-way ANOVA with Tukey's post-test was performed for statistical comparison**p = 0.0061, ****p < 0.0001. **(E & F)** Expression of *HORAS5* at different concentrations and different time points of cabazitaxel treatment in LNCAP **(E)** and DU145-OE **(F)**. Results expressed as means ± SD from two independent replicates. Two-way ANOVA with Sidak's post-test was performed for statistical comparison ****p < 0.0001.
DMSO: Dimethyl sulfoxide; PCa: Prostate cancer; qPCR: Quantitative PCR; SD: Standard deviation.

1B). These results demonstrate that *HORAS5* promotes the survival of both AR$^+$ and AR$^-$ CRPC cells exposed to cabazitaxel. In order to investigate the role of the apoptotic pathway in this phenomenon, we measured caspase 3/7 activity in LNCaP and DU145 cells exposed to cabazitaxel. Our data showed that *HORAS5* overexpression significantly reduced caspase 3/7 activation in response to cabazitaxel exposure (Figure 3E). In keeping with this result, *HORAS5* KD increased caspase 3/7 activation in LNCaP cells exposed to cabazitaxel (Figure 3F). Overall, these findings indicated that *HORAS5* promoted cabazitaxel resistance in both AR$^+$ and AR$^-$ PCa cells via increase of cell proliferation and inhibition of caspase-mediated apoptosis.

BCL2A1 expression is induced by cabazitaxel & increased with *HORAS5* expression

To evaluate the transcriptomic profile of CRPC cells exposed to cabazitaxel and the effects of *HORAS5* modulation, we performed an NGS transcriptome analysis (Supplementary Figure 2A–E) using the following samples: DU145-NC, untreated; DU145-NC, exposed to cabazitaxel; DU145-OE, untreated; DU145-OE, exposed to cabazitaxel. Based on this analysis, 87 genes were significantly upregulated (fold change (FC) ≥2, p < 0.01) in DU145-OE treated with cabazitaxel versus untreated cells (Figure 4A). Notably, these genes were not significantly upregulated in DU145-NC exposed to cabazitaxel (Figure 4A). We then ranked these 87 genes according to the DU145-NC p-value and shortlisted the top 25 (Figure 4A, Supplementary Table 1). Three of these 25 genes (*SOX9*, *CCL20*, *BCL2A1*) have been previously implicated in cancer and drug resistance (Figure 4 [45–49]A). Hence, we sought to validate our transcriptome results by measuring the expression of *SOX9*, *CCL20* and *BCL2A1* via RT-qPCR. No significant differences in *SOX9* expression were found (Figure 4B). *CCL20* and *BCL2A1* were both significantly upregulated in the *HORAS5* expressing cells versus negative control upon cabazitaxel exposure. This differential expression pattern was particularly significant for *BCL2A1* (Figure 4C & D), which is also a well described antiapoptotic gene [48,50,51]. For this reason, we decided to investigate the role of BCL2A1 in *HORAS5*-dependent cabazitaxel resistance. First, we confirmed that BCL2A1 protein expression was induced by cabazitaxel treatment (Figure 4E). According to our

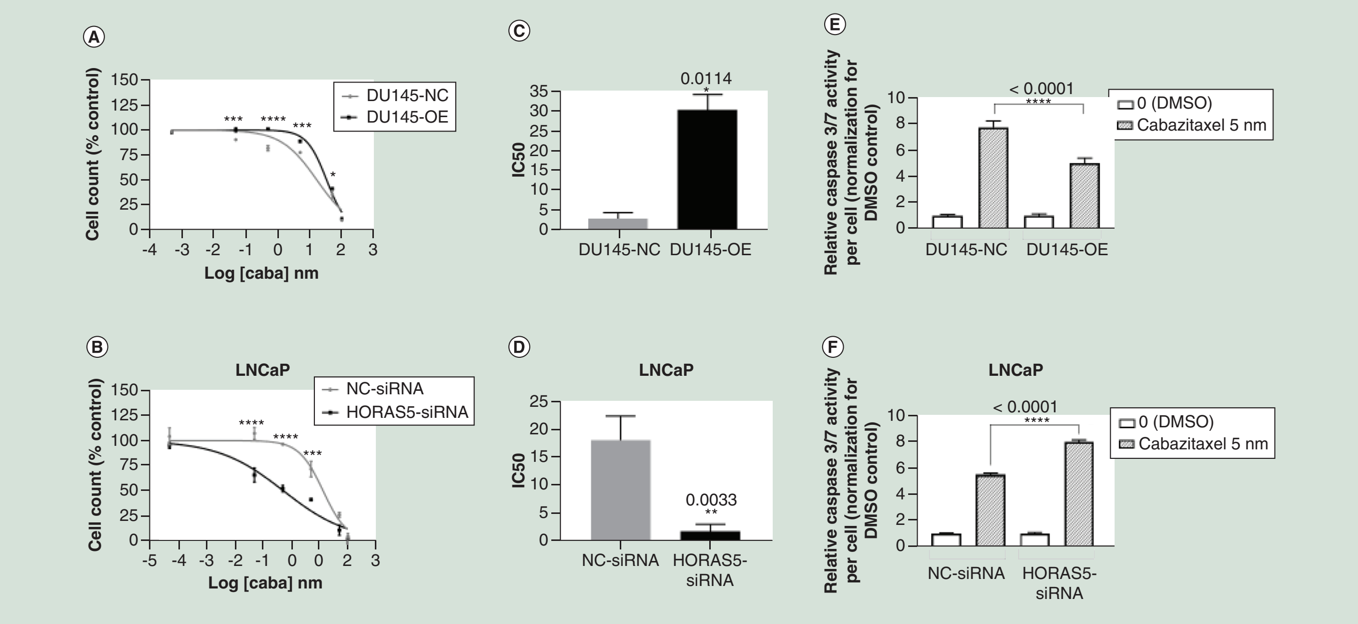

Figure 3. ***HORAS5* silencing and overexpression affect prostate cancer cells proliferation and survival under cabazitaxel exposure. (A–D)** DU145 **(A)** and LNCaP **(B)** cell count upon 48 h (DU145) and 72 h (LNCaP) of cabazitaxel treatment, with *HORAS5* overexpression and silencing respectively, compared with the respective negative controls, and resulting IC_{50}s (**[C]**, DU145; **[D]**, LNCaP); cell count is expressed as nonlinear fit curves of the cell number in percentage normalized to the untreated (DMSO) control. Two-way ANOVA with Sidak's post-test was performed for statistical comparison $*p = 0.0230$, $***p < 0.0005$, $****p < 0.0001$ and nonlinear fit was used to calculate the IC_{50}s. Student's t-test was used in **(C & D)**. **(E & F)** Caspase 3/7 activity normalized to relative cells number 48 h after cabazitaxel treatment (DU145) and 48 h post-transfection of *HORAS5*-siRNA + 72 h after cabazitaxel treatment (LNCaP). One-way ANOVA with Tukey's post-test was used in **(E & F)**. for statistical comparison $****p < 0.0001$. Results expressed as means ± SD from three independent replicates. DMSO: Dimethyl sulfoxide; SD: Standard deviation.

results *BCL2A1* seemed to be the most consistently upregulated gene in cabazitaxel-treated cells that overexpress *HORAS5*. Therefore, we investigated whether *BCL2A1* KD could rescue the drug-resistant phenotype induced by *HORAS5* overexpression.

Knockdown of *BCL2A1* decreases cell proliferation & increases apoptosis in CRPC cells exposed to cabazitaxel treatment

Based on our transcriptomic analysis, we hypothesized that BCL2A1 mediates the drug-resistance phenotype induced by *HORAS5*. To test this hypothesis, we optimized the KD procedure using two different siRNAs directed against both variants of *BCL2A1* mRNA. Our results show that siRNA1 and siRNA2 determined a reduction in *BCL2A1* mRNA of 84 and 95% respectively (Figure 5A). Our results also showed that both siRNAs reduced the expression of BCL2A1 protein (Figure 5B). These two siRNAs were used to transfect DU145-OE cells in order to investigate whether *BCL2A1* KD could revert the cabazitaxel resistance phenotype induced by *HORAS5* overexpression. Our data showed that *BCL2A1* KD significantly reduced DU145-OE resistance to cabazitaxel (Figure 5C). We also tested whether BCL2A1 effect on cabazitaxel response was a consequence of its antiapoptotic activity. As we showed in Figure 5D, *BCL2A1* KD determined a small increase in apoptosis in untreated cells (Figure 5D). However, *BCL2A1* KD caused a highly significant increase in apoptosis when the cells are exposed to both 5 nM ($1.98 \leq FC \leq 2.21$, $p < 0.0001$) and 50 nM ($3.06 \leq FC \leq 4.36$, $p < 0.0001$) cabazitaxel. These findings show that BCL2A1, which is upregulated upon *HORAS5* expression, enhances cabazitaxel resistance by inhibiting the apoptosis response.

Translational research: ASO directed against *HORAS5* can decrease cabazitaxel IC_{50} & implications of *HORAS5* expression for the clinics

Since *HORAS5* modulation via lentiviral-driven overexpression and siRNA-mediated KD affected cabazitaxel resistance, we investigated *HORAS5* expression in clinical PCa samples from a published study [34], accessed via

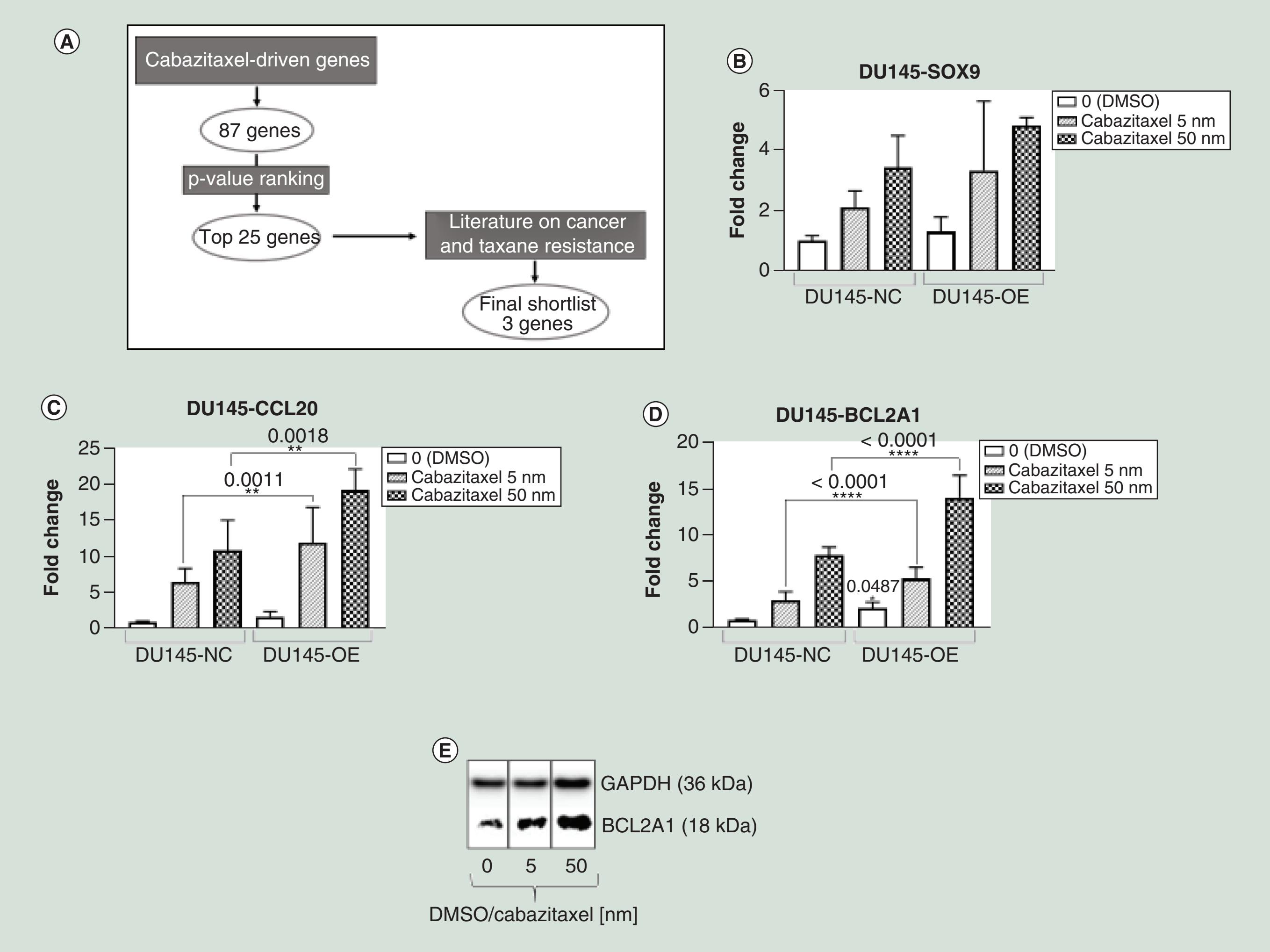

Figure 4. *BCL2A1* is the most consistently upregulated gene in prostate cancer cells with *HORAS5* overexpression and is induced by cabazitaxel. (A) Flow chart showing the method used to shortlist cabazitaxel-driven genes in PCa cells that overexpress *HORAS5* (DU145-OE vs DU145-NC) from RNA sequencing. **(B–D)** qPCR validation of the three shortlisted genes: SOX9, CCL20 and BCL2A1. **(E)** BCL2A1 expression is significantly increased at the protein level upon cabazitaxel treatment in the cells that overexpress *HORAS5* (DU145-OE). Results expressed as means ± SD from four independent experiments in **(B–D)**. One-way ANOVA with Tukey's post-test was used in for statistical comparison $*p = 0.049$, $**p < 0.002$, $****p < 0.0001$.
DMSO: Dimethyl sulfoxide; PCa: Prostate cancer; SD: Standard deviation.

cBioPortal. *HORAS5* expression was significantly higher ($p = 0.0086$) in metastatic samples from patients treated with taxanes than in untreated patients (Figure 6A). Given this evidence, we analyzed if *HORAS5* could effectively be silenced using ASOs. Since ASOs are currently used in clinical trials [52–54], we thought that this set of experiments could highlight the therapeutic potential of targeting lncRNAs like *HORAS5* in the clinical setting. For this set of experiments, we used LNCaP cells, which express endogenous detectable levels of *HORAS5*. We tested eight different ASOs and found that ASO3 was the most effective inhibitor of *HORAS5* expression (78.2%) (Figure 6B, Supplementary Figure 3). Hence, we tested ASO3 in combination with cabazitaxel to analyze if this *HORAS5*-targeting ASO influenced cabazitaxel IC_{50}. Our results showed that the *HORAS5*-targeting ASO determined a decrease in the cell count upon cabazitaxel treatment and therefore a significant decrease in the IC_{50} (FC = 6.55, $p = 0.0034$) (Figure 6C & D). To rule out off-target effects, we have tested ASO3 in DU145-NC cells, which express undetectable levels of *HORAS5*. ASO3 does not affect *HORAS5* expression (Supplementary Figure 5A) neither cabazitaxel effect on cell count of DU145-NC (Supplementary Figure 5B). This evidence showed that ASO-directed *HORAS5* KD decreased cabazitaxel resistance in LNCaP cells and that its role *in vivo* should be

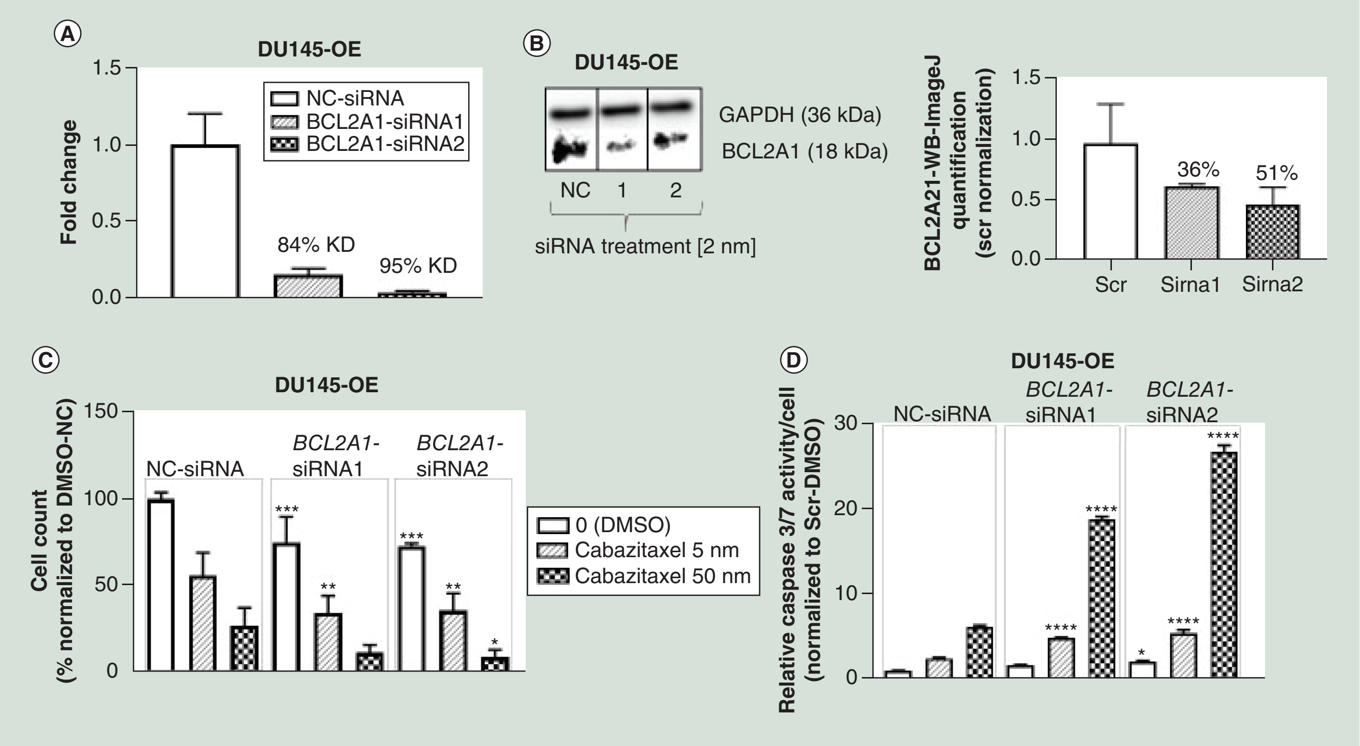

Figure 5. ***BCL2A1*** **silencing decreases cell proliferation and increases apoptosis in castration-resistant prostate cancer cells exposed to cabazitaxel. (A & B)** *BCL2A1* expression is significantly reduced at the mRNA **(A)** and protein **(B)** level upon knockdown (GAPDH was used as western blot loading control). **(C)** Cell count upon cabazitaxel treatment, with *BCL2A1 KD* in cells that overexpress *HORAS5* (DU145-OE); cell count is expressed as percentage normalized to the untreated (DMSO) control. **(D)** Caspase 3/7 activity normalized to relative cell number in DU145-OE cells upon 48 h BCL2A1 KD + 48 h of cabazitaxel treatment. Results expressed as means ± SD from three independent replicates. One-way ANOVA with Tukey's post-test was used in for statistical comparison $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. western blots are visualized using Syngene Genesys software.
DMSO: Dimethyl sulfoxide; KD: Knockdown; SD: Standard deviation; WB: western blot.

further evaluated. Overall, these findings suggested that *HORAS5* could have a clinical relevance and paved the way to *HORAS5* targeting in combination with taxanes to increase drug response in CRPC patients.

Discussion

CRPC is an incurable malignancy [4], therefore there is an urgent need to find novel diagnostic and prognostic biomarkers and effective therapeutic targets. Recent findings showed that lncRNAs can have a determinant role in drug response pathways in some cancers [13,17–21]. Therefore, we hypothesized that lncRNAs may act as promoters of CRPC aggressive phenotypes.

Our previous work showed that the lncRNA *HORAS5* (i.e., linc00161) was upregulated in CRPC PDXs and increased the survival of AR^{+} CRPC cells [22], while a previous study showed that *HORAS5* decreased the survival of cisplatin-treated osteosarcoma cells [13]. Based on other studies showing the multifaceted roles of lncRNAs in different cancers [13,21,55–57], we investigated if this particular lncRNA was involved in drug response in CRPC, using a panel of clinically used drugs at achievable concentrations [41–44] (see drug treatments section in Materials & methods). In this study, we have shown for the first time a specific cabazitaxel-driven induction of *HORAS5* expression, using our new model of CRPC cells with lentiviral-induced *HORAS5* overexpression (Figure 7). Cabazitaxel is clinically approved for the treatment of advanced CRPCs that acquired resistance to docetaxel [8,37,38,58]. So far, there is no evidence implicating lncRNAs in cabazitaxel response.

Since we had already characterized *HORAS5* in untreated AR^{+} cells and targeted it with siRNAs [22], we wanted to investigate if *HORAS5* overexpression changed or maintained the endogenous subcellular localization. While cytoplasmic molecules are easier to target, nuclear membrane-crossing carriers are needed for nuclear targets. LncRNA subcellular localization can also be predictive of its putative mechanism of action. Indeed, lncRNAs are

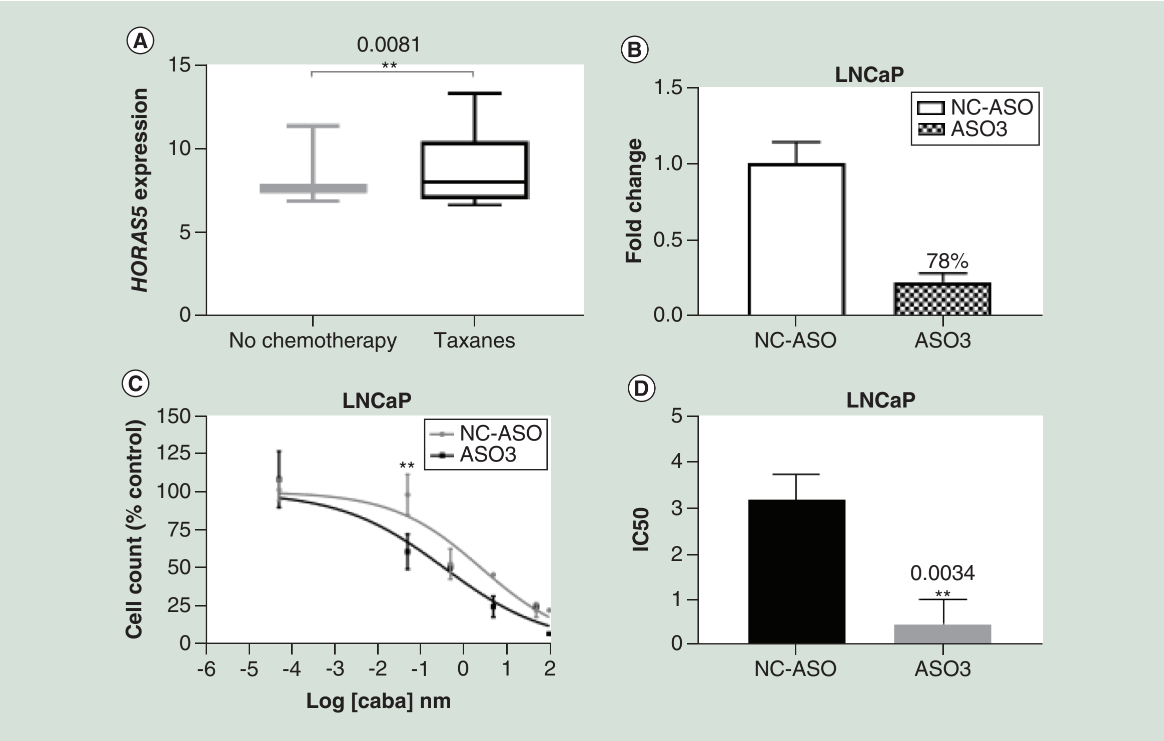

Figure 6. Clinical relevance of *HORAS5*: antisense oligonucleotide-mediated inhibition to decrease cabazitaxel resistance and expression in clinical samples. (A) *HORAS5* expression from patients' metastatic samples treated with taxanes-only compared with untreated. **(B)** *HORAS5* expression was significantly reduced upon treatment with our specifically designed ASO (ASO3) **(A)**. **(C & D)** *HORAS5* KD mediated by ASO3 (48 h) induced a decrease in the cell count upon cabazitaxel treatment (72 h) **(C)** and a reduction in the IC_{50} **(D)**. Cell count is expressed as nonlinear fit curves of the cell number in percentage, normalized to the untreated (DMSO) control. Results expressed as means ± SD from three independent replicates. Two-way ANOVA with Sidak's post-test was performed for statistical comparison $^{**}p = 0.0056$. Student's *t*-test was used for statistical comparison in **(A)** and **(D)** $^{**}p = 0.0081$ and $p = 0.0034$.
DMSO: Dimethyl sulfoxide; KD: Knockdown; SD: Standard deviation.

often found to interact with miRNAs as competing endogenous RNAs when they act in the cytoplasm. Other studies have shown that lncRNAs can act also in the nucleus via interaction with protein complexes such as epigenetic and splicing factors [59–61]. Since our data indicated that *HORAS5* overexpression promoted a mainly cytoplasmic localization pattern, we hypothesized that it could be effectively targeted and that it could interact with cytoplasmic molecules and complexes [62,63].

Our data show that *HORAS5* overexpression increases cabazitaxel resistance via regulation of apoptotic signals. This is consistent with previous studies implicating lncRNAs as regulators of cell apoptosis [64,65] and as mediators of drug resistance [66,67]. In AR^{+} PCa cell, *HORAS5* acts mainly by stabilizing the *AR* mRNA, thereby activating AR-target genes, such as the oncogenic KIAA0101 [22]. Since the AR pathway is a main mediator of taxane resistance [68–70], this mechanism of action explains the increased cabazitaxel sensitivity of LNCaP cells upon *HORAS5* KD. However, our data on DU145 cells indicate that *HORAS5* could mediate taxane-resistance via AR-independent mechanisms. Hence, we decided to investigate additional *HORAS5* mechanisms of action in AR^{-} cells. From NGS transcriptome analysis, *BCL2A1* resulted as the most differentially *HORAS5*-upregulated gene upon cabazitaxel treatment. *BCL2A1* encodes for an antiapoptotic factor [48,50,51] already described to participate in drug resistance phenotypes [48,49,71]. Currently there are no studies which implicate BCL2A1 in cabazitaxel response. Hence, we evaluated the effect of *BCL2A1* KD on the drug-resistant phenotype induced by *HORAS5* overexpression. Our system efficiently silenced *BCL2A1* in untreated PCa cells (KD efficiency 90%). However, in these conditions we did not observe a striking cell count reduction. This could be attributed to the fact that BCL2A1 levels are relatively low in untreated PCa cells. However, in the presence of cabazitaxel, BCL2A1 levels are highly increased. In these conditions, we observed an additive effect between BCL2A1 KD and cabazitaxel treatment. Taken together, our data indicate that BCL2A1 facilitates the drug-resistance phenotype induced by

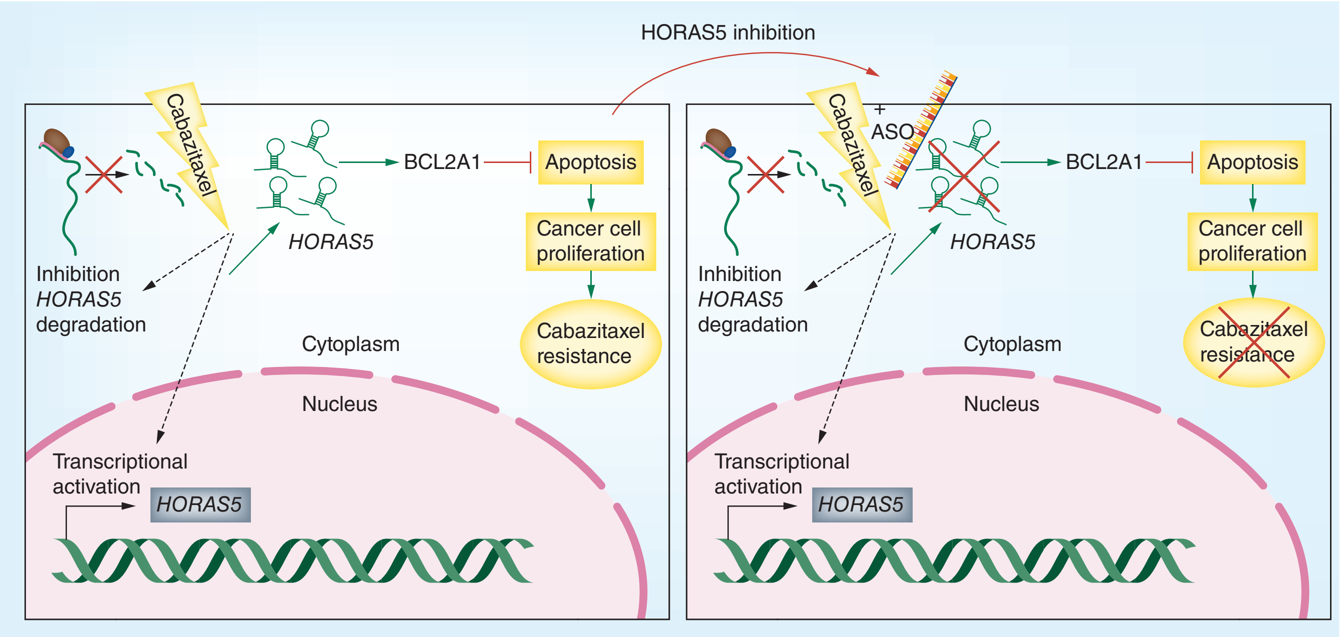

Figure 7. Diagram summarizing our findings. Castration-resistant prostate cancer cells treated with cabazitaxel show upregulation of *HORAS5* that could happen via *HORAS5* gene transcriptional activation or degradation inibhition. *HORAS5* upregulation increases BCL2A1 expression and this decreases apoptosis and increases cell proliferation. In response to cabazitaxel treatment, this pathway enhances drug resistance. When *HORAS5* is inhibited via a specifically designed ASO, *HORAS5* levels highly decrease causing a decrease in cabazitaxel resistance.

HORAS5 by promoting AR$^-$ CRPC cell survival (Figure 7). Although the specific mechanism by which *HORAS5* and *BCL2A1* interact needs to be clarified by further studies, we suggest that *HORAS5* could sequester one or more miRNAs which inhibit *BCL2A1* translation. For example, mir-128 has been shown to bind *HORAS5* in ovarian cancer cells [21]. Interestingly, *BCL2A1* is one of the predicted targets of this miRNA. Moreover, in line with our previous findings on *HORAS5* mechanism of action in AR+ LNCaP cells [22], we also suggest that *HORAS5* could directly interact with *BCL2A1*, thereby stabilizing and protecting its mRNA. In keeping with this hypothesis, our *in silico* predictions suggested that *HORAS5* and *BCL2A1* have common miRNA-binding sites. Finally, we would like to point out that the BCL2A1-dependent mechanism of action characterized in this study is likely one of several ways by which *HORAS5* mediates taxane resistance in AR$^-$ cells. Our transcriptomic data identified also other genes potentially regulated by *HORAS5*, such as the top 25 genes reported in Supplementary Table 1. This is in line with the pleiotropic mechanism of action displayed by other lncRNAs [72].

We have also shown that *HORAS5* overexpression in untreated AR$^-$ PCa cells does not affect cellular features such as cell shape and proliferation. This observation seems to be in contrast with our previous study, which showed that *HORAS5* KD decreases AR$^+$ PCa growth and survival [22]. This discrepancy could be explained by the different gene modulating approaches employed (transient silencing vs stable overexpression) and by the fact that *HORAS5* seems to act via different mechanisms in AR$^+$ and AR$^-$ PCa cells: activation of the AR-pathway in the former, upregulation of BCL2A1 and other antiapoptotic genes in the latter (particularly in response to proapoptotic stimuli).

Conclusion

Our findings highlight the potential of *HORAS5* in translational studies. The clinical relevance of *HORAS5* was assessed by our analyses on database's available data [34]. Indeed, we have shown that *HORAS5* is significantly upregulated in PCa metastatic samples from patients treated with taxanes compared with taxane-untreated patients. Moreover, the possibility to detect *HORAS5* in biological fluids (i.e., urine and blood) could pave the way for the use of this lncRNA as noninvasive biomarker for treatment response as well as novel therapeutic target for CRPC. In this context, we designed a specific antisense molecule (ASO3) capable of effectively inhibiting *HORAS5* expression *in vitro,* thereby reducing cabazitaxel resistance in CRPC cells (Figure 7). We would like to point out that our silencing experiments reduced the chance of off-target effects in many ways: both IDT siRNAs and ASOs were designed to uniquely match *HORAS5* sequence; we used appropriate negative controls at each step; we used two different silencing methods, obtaining very similar results. Hence, we are confident that the phenotypic effects observed in this study are uniquely attributable to the silencing of *HORAS5*.

Since ASOs have been successfully employed in clinical trials [52–54], the use of *HORAS5* ASOs in combination with cabazitaxel could increase CRPC patients' drug sensitivity and their survival. Therefore, our findings could bring novel insights in the fields of personalized medicine and innovative diagnostic strategies.

Future perspective

HORAS5 is a lncRNA upregulated in different cancers, including CRPC. It has been recently shown that *HORAS5* promotes drug resistance in ovarian cancer. Our work revealed the role of *HORAS5* in CRPC drug resistance and identified a mechanism of action by which *HORAS5* inhibits cabazitaxel-induced apoptosis. In light of this evidence, we proposed a novel approach (ASO-mediated gene silencing) to inhibit this oncogenic lnRNA. This approach could be used *in vivo* in future studies and in clinical trials. In the next steps of this work, we plan to test *HORAS5*-targeting ASOs using *in vivo* cancer models. We also plan to further investigate *HORAS5* expression in clinical samples, especially in biological fluids in order to clarify *HORAS5* potential as a noninvasive biomarker for cancer diagnosis and treatment response monitoring. With this future perspective, we are planning to collect urine and plasma samples from PCa patients, before and after treatment with cabazitaxel and to treat CRPC animal models with cabazitaxel and *HORAS5*-ASO combination treatments. Moreover, further mechanistic studies on *HORAS5* action in this context will give more insights on the interaction of *HORAS5* with BCL2A1 and other molecules, paving the way for the discovery of novel molecular pathways and additional targets to reduce cabazitaxel resistance and increase patient survival.

Summary points

- Since *HORAS5* was upregulated in castration-resistant prostate cancer (CRPC) patient-derived xenografts according to our previous publication, we evaluated if *HORAS5* was involved in treatment resistance.
- We showed that *HORAS5* expression was highly increased upon cabazitaxel treatment in both AR^+ and AR^- CRPC cells.
- *HORAS5* knockdown and overexpression affected cabazitaxel response, suggesting that *HORAS5* promoted resistance to this drug by increasing cells proliferation and inhibiting caspase-mediated apoptosis.
- We found that *HORAS5* upregulated the antiapoptotic factor BCL2A1.
- BCL2A1 knockdown reverted cabazitaxel resistance phenotype promoted by *HORAS5* overexpression in AR^- CRPC cells. This finding confirmed that *HORAS5* promoted cabazitaxel resistance by upregulating BCL2A1, thereby inhibiting drug-induced apoptosis.
- *HORAS5* was found upregulated in taxane-treated clinical samples; this finding emphasizes the translational potential of inhibiting *HORAS5*.
- Due to these findings, *HORAS5* expression was successfully inhibited in CRPC cells, using antisense oligonucleotides that can be used in future clinical studies. One of our *HORAS5* targeting antisense oligonucleotides decreased cabazitaxel IC_{50} showing reduction of *HORAS5*-induced drug resistance.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/epi-2019-0316

Financial & competing interest disclosure

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No writing assistance was utilized in the production of this manuscript.

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