Aldehyde dehydrogenase is regulated by β-catenin/TCF and promotes radioresistance in prostate cancer progenitor cells

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Abstract

Radiotherapy is a curative treatment option in prostate cancer. Nevertheless, patients with high-risk prostate cancer are prone to relapse. Identification of the predictive biomarkers and molecular mechanisms of radioresistance bears promise to improve cancer therapies. In this study, we show that aldehyde dehydrogenase (ALDH) activity is indicative of radioresistant prostate progenitor cells with an enhanced DNA repair capacity and activation of epithelial-mesenchymal transition (EMT). Gene expression profiling of prostate cancer cells, their radioresistant derivatives, ALDH⁺ and ALDH⁻ cell populations revealed the mechanisms, which link tumor progenitors to radioresistance, including activation of WNT/β-catenin signaling pathway. We found that expression of ALDH₁A₁ gene is regulated by WNT signaling pathway and co-occurs with expression of β-catenin in prostate tumor specimens. Inhibition of WNT pathway led to a decrease in ALDH⁺ tumor progenitor population and to radiosensitization of cancer cells. Taken together, our results indicate that ALDH⁺ cells contribute to tumor radioresistance and their molecular targeting may enhance the effectiveness of radiotherapy.

Precis

Results indicate that expression of a stem cell marker contributes to radioresistance in prostate cancer stem-like cells, suggesting a strategy to enhance the effectiveness of radiotherapy in prostate cancer.
Introduction

Prostate cancer is the most commonly diagnosed noncutaneous neoplasm in men worldwide (1). Most of prostate cancer patients are diagnosed at an early stage and may be cured with surgery or radiotherapy, with or without androgen deprivation (1-3). Depending on tumor stage, from 55 to more than 90% of all prostate cancers can be permanently controlled by radiotherapy (3-5). This supports that radiotherapy has significant potential to inactivate tumor-initiating, or cancer stem cells (CSCs) which can potentially provoke tumor relapse. Nevertheless, some patients with high-risk prostate cancer develop local relapse after radiation therapy. The most obvious explanation for radiotherapy failure is related to the CSC population, which was not completely sterilized during the treatment and caused tumor recurrence (6, 7).

The proportion of CSCs in the bulk tumor cell population has a high intratumoral variability. Few studies have correlated the density of CSC markers with poor clinical outcome of head and neck squamous cell carcinomas (HNSCC), glioma, cervical squamous cell carcinoma and rectal carcinoma patients (8, 9). Despite the expression of CSC markers varies considerably between tumors of the same entity, the current findings suggest that estimation of the number of CSCs in pre-therapeutic biopsies is of high importance for prediction of tumor radiocurability and selection of the optimal therapy (6). However, the clinical and preclinical studies analyzing the predictive value of CSC biomarkers on prostate cancer radiocurability are missing so far.

Aside from the impact of CSC density on tumor radiocurability, recent experimental reports suggest a number of different intrinsic and extrinsic adaptations that confer tumor radioresistance and which also occur in CSC populations, including senescence, increased DNA-repair capability, intracellular scavenging of reactive oxygen species (ROS), and activation of cell survival pathways (8). All these mechanisms are dynamic in nature due to the plasticity of CSC populations and multiplicity of the microenvironmental conditions that
can differently contribute to the CSC response to irradiation through the course of therapy (8, 9).

Identification of predictive biomarkers for individualized radiotherapy and characterization of the molecular mechanisms by which prostate tumor initiating cells can survive treatment may ultimately lead to more efficient cancer treatment. In this study we show that aldehyde dehydrogenase (ALDH) activity is indicative of prostate tumor progenitors with increased radioresistance, improved DNA double strand break repair and activation of signaling pathways, which promote epithelial-mesenchymal transition (EMT) and migration.

Radioresistant derivatives of established prostate cancer cell lines share many properties with tumor progenitor cells including an enhanced expression of CSC markers (CD133, CXCR4, ABCG2, OCT4, NANOG), high aldehyde dehydrogenase (ALDH) activity, induction of EMT and an increase in DNA repair capacity. mRNA expression analysis of the prostate cancer cells, their radioresistant derivatives and ALDH+ tumor progenitor population revealed common signaling pathways, which link CSCs to radioresistant cancer cells and include WNT/β-catenin, G protein-coupled receptor, TGFβ and Integrin signaling pathways. This is, to our knowledge, the first study showing that expression of ALDH1A1 gene is directly regulated by β-catenin/TCF transcriptional complex. Consistent with these results, inhibition of the WNT/β-catenin signaling pathway by the tankyrase inhibitor XAV939 or siRNA-mediated knockdown of β-catenin expression led to a decrease in the ALDH+ cell population and radiosensitization of prostate cancer cells.

Taken together, our data suggest that ALDH+ cell population contributes to prostate tumor radioresistance and that targeting this cell population may be beneficial in prostate cancer treatment.
Material and methods

Cell lines and culture condition

Prostate cancer cell lines DU145, PC3 and LNCaP were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured according to the manufacturers recommendations in a humidified 37°C incubator supplemented with 5% CO₂. DU145 and PC3 cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) medium (Sigma-Aldrich) and LNCaP cells in RPMI-1640 medium (Sigma-Aldrich) containing 10% fetal bovine serum (FBS, PAA Laboratories) and 1% L-glutamine (Sigma-Aldrich). All cell lines were genotyped using microsatellite polymorphism analysis and tested for mycoplasma directly prior to experimentation.

Human tissue samples and preparation of cell suspension

Clinical material was collected at Massachusetts tissue bank, Department of Urology of Medical Faculty at Technical University Dresden, and Kyiv National Cancer Institute with informed consent and approval from the local ethics committees (Office of the Vice Provost for Research, Human Subjects Research 55 Lake Ave North Worcester, Massachusetts 01655, FWA #00004009, docket #H-11731; Institutional review board of the Medical Faculty at the Technical University of Dresden, EK195092004 and EK194092004; Ethics Committee of Kyiv National Cancer Institute, 33-34, Lomonosova str, 02166 Kyiv, Ukraine, protocol no. 44). Primary tumor tissues used in the study are described in Table S1.

Animals and in vivo tumorigenicity assay

The animal facilities and the experiments were approved according to the institutional guidelines and the German animal welfare regulations (protocol number 24-9168.11-1/2010-21). The experiments were performed using 8 to 12-week-old male NMRI (nu/nu) mice obtained from the animal breeding facility (Experimental Centre, Medical Faculty, Technical University Dresden). To immnosupress the nude mice further, the animals underwent total
body irradiation 1-3 days before tumor transplantation with 4 Gy (200 kV X-rays, 0.5 mm Cu filter, 1 Gy/min). Mice were examined once a week and relative tumor volume (mm³) based on caliper measurements was calculated as \((\text{length} \times \text{width} \times \text{height})/2\). At tumor volume of 100 mm³ mice were counted as tumor bearing to calculate tumor uptake and Kaplan-Meier plots were analyzed using the log rank test (Graph Prism 5). The mice were observed for 100 days for tumor appearance and development. Xenograft tumors were excised, fixed in 4% para-formaldehyde overnight and kept in 30% sucrose before embedding in TissueTek O.C.T compound (Sakura Finetek). Tissue sectioning was performed using Microm HM 560, Cryo-Star Cryostat. Xenograft tumors were histopathologically and immunohistochemically examined by a pathologist following diagnostic protocols of the Department of Pathology, University Hospital Dresden. For flow cytometry analysis the single cell suspension was obtained after 1h incubation at 37°C in DMEM containing collagenase Type 3 (Worthington) and mechanical dissociation using the Gentle MACS Dissociator (Miltenyi Biotec). Samples for western blot analysis were flash frozen in liquid nitrogen. Analysis of the tumor initiating cell frequency was performed using the web-based ELDA (Extreme Limiting Dilution Analysis) statistical software, which uses the frequency of tumor positive and negative animals at each transplant dose to determine the frequency of tumor initiating cells within the injected cell populations (9).

**Microarray analysis of the prostate cancer cell lines**

Gene expression profiling of the DU145, DU145-RR, ALDH⁺ DU145, ALDH⁻ DU145, ALDH⁺ DU145-RR, ALDH⁻ DU145-RR cells was performed using SurePrint G3 Human Gene Expression 8x60K v2 Microarray Kit (Design ID 039494, Agilent Technologies) according to manufacturer's recommendations. Total RNA was isolated from cell pellets using the RNeasy kit (Qiagen, Valencia, CA, USA). Sample preparation for analysis was carried out according to the protocol detailed by Agilent Technologies (Santa Clara, CA, USA). Briefly, first and second cDNA strands were synthesized; double stranded
cDNA was in vitro transcribed using the Low Input Quick Amp Labeling Kit; and the resulting cRNA was purified and hybridized to oligonucleotide arrays (SurePrint G3 Human Gene Expression 8x60K v2 Microarray, Design ID 039494) representing about 60,000 features, including 27,958 Entrez Gene RNAs and 7,419 lincRNAs. Arrays were processed using standard Agilent protocols. Probe values from image files were obtained using Agilent Feature Extraction Software. The dataset was normalized using GeneSpring software, and the list of differentially regulated genes with fold change > 2 and p-value < 0.05 was further analyzed using web-based Panther Pathway Analysis tool. Data deposition: all data is MIAME compliant and that the raw data has been deposited in the Gene Expression Omnibus (GEO) database, accession no GSE53902.

**Statistical analysis**

The results of colony formation assays, microscopy image analysis, transwell migration assay, luciferase reporter assay, flow cytometry analysis, cell proliferation assays, and in vivo tumorigenicity assays were analyzed by paired t-test. A p value of < 0.05 was regarded as statistically significant.

**Results**

*Prostate cancer cells with a high ALDH activity have stem cell-like properties*

Previous studies have shown the existence of cancer progenitor populations in prostate tumors and established cell lines. These progenitor cells have been characterized by the expression of the surface markers CD44, CD133, integrin α2β1, and high ALDH activity (10-13). The inhibition of PI3K activity by the chemical inhibitor NVP-BEZ235 led to a significant decrease in the population of prostate tumor initiating cells in DU145 prostate xenograft tumors. In contrast, the use of the cytotoxic drug Taxotere resulted in a decrease in proliferating tumor cells, but led to an overall increase in the population of cancer progenitor...
cells in vivo (12). To identify genes, for which expression levels correlate with the dynamics of CD44+/CD133+ progenitor cell population in DU145 xenograft tumors, we performed a whole genome gene expression profiling of the xenograft tumors from mice, which received Taxotere (10 mg/kg, intravenously) once per week for 4 weeks, the dual PI3K/mTOR inhibitor NVP-BEZ235 (12.5 mg/kg, per orally) once per day for 4 weeks, or were treated with vehicle, as described earlier (12), Fig. 1A.

Comparative analysis of gene expression profiles has shown that transcription of the different ALDH isoforms including ALDH1A1, ALDH3A1, ALDH3B1, ALDH6A1 correlates with the percentage of CD44+/CD133+ cancer progenitors in xenograft tumors as well as with expression of other stem cell and developmental genes including WNT5A, WNT9A, DLL1, FGFR2, KIT, KITLG, CTNNA1, EGF, IGFBP2 and IGFBP3 (Fig. 1A).

Analysis of the Oncomine database and immunostaining of tumor specimens revealed that expression of the ALDH isoforms, such as ALDH1A1 and ALDH6A1 was significantly increased in primary prostate carcinoma tissues as compared to the normal prostate gland (Fig. 1B, Fig. S1A). Strikingly, a gene signature, which includes expression of ALDH1A1, ALDH3B1, ALDH6A1 CD44 and PROM1 (CD133) correlated with reduced disease-free survival in patients with prostate cancer. Data are based on the Taylor study (14) (Fig. S1B).

To analyze if the cells with a high ALDH activity isolated from prostate cancer cell lines have functional characteristics of tumor progenitor cells, we examined their clonogenic, spherogenic and tumorigenic properties. Prostate cancer cells which have a high level of ALDH activity can be enriched from prostate cancer cell lines by growing them under serum-free prostatosphere-forming conditions (Fig. S2A). We and others have shown that prostate cell populations expanded under these conditions have increased clonogenic potential in vitro and tumorigenic potential in vivo (13, 15). ALDH+ cell populations prospectively isolated by fluorescence-activated cell sorting (FACS) from DU145 and PC3 cells showed an increase in their colony and sphere forming ability over ALDH- cells (Fig. S2BCD). To determine the
frequency of tumor-initiating cells within ALDH⁺ and ALDH⁻ prostate cancer cell populations, we used an in vivo limiting dilution analysis. The highly purified ALDH⁺ and ALDH⁻ DU145 cells at varying cell numbers (100, 1000 and 3000 cells) were injected subcutaneously (s.c.) into athymic immunodeficient NMRI nu/nu mice. Animals alive and tumor-free at the end of the observation period (100 days) were defined as long-term survivors. The ALDH⁺ population showed a significantly higher tumor uptake rate and a higher frequency of tumor initiating cells than ALDH⁻ cell population (1/109 (at 95% confidence interval: 1/349 to 1/35 cells) and 1/548 (at 95% confidence interval: 1/1285 to 1/234 cells), respectively; p-value < 0.03), Fig. 1C.

Next we analyzed if ALDH⁺ and ALDH⁻ cells are capable of recapitulating the populations of the original cell lines. DU145 cell line consisted of 8.3 % of ALDH⁺ cells and 91.7 % of ALDH⁻ cells was sorted into ALDH⁺ and ALDH⁻ populations which were maintained in standard tissue culture conditions. The cells were cultured for a period of 14 days and then analyzed by flow cytometry. We found that prolonged culture resulted in the reversion of both populations to a bimodal profile. The ALDH⁺ population gave rise to the cell population which included 22.2% ALDH⁺ cells and 77.8% ALDH⁻ cells. These cell frequencies are closer to the cell population ratio in the original unsorted cell population compared to the cell population generated by ALDH⁻ cells (1.8% ALDH⁺ cells and 98.2% ALDH⁻ cells). Similar results were obtained when the same experiment was performed with PC3 cells (Fig. S2E). Cell viability assay revealed no statistical difference between growth and viability of ALDH⁺ and ALDH⁻ cells (Fig. S2F). To determine if ALDH⁺ and ALDH⁻ cells exhibit distinct differentiation potential in vivo, we analyzed the expression of prostate lineage markers in xenograft tumors derived from FACS-purified ALDH⁺ or ALDH⁻ cells. The xenograft tumors originated from both, ALDH⁺ and ALDH⁻ cell subsets included a CK5⁺ population of basal epithelial cells and CK18⁺ population of luminal epithelial cells.
However, ALDH$^-$ cells were less efficient to generate CK5$^+$ cell population in vivo as compared to ALDH$^+$ cells (Fig. S2G).

**Dynamics of the stem cell marker expression in prostate cancer cells in response to irradiation**

Recent findings suggest that X-ray irradiation leads to enrichment of the CSCs population (16, 17). To analyze the radiation-induced changes in the number of progenitor cells, we treated established prostate cancer cell lines and primary prostate tumor cells with fractions of 2 Gy or 4 Gy of X-ray irradiation and assessed the expression of CSC markers by flow cytometry, Western blotting and immunohistochemistry (IHC) seven days after irradiation. We have found that irradiation led to a dose-dependent increase in the fraction of DU145 and LNCaP cells with a high ALDH activity, a high expression of CSC markers, such as CD133, CXCR4, ABCG2 as well as to upregulation of AKT phosphorylation at Ser 473 and increase of expression of the transcription regulators NANOG and β-catenin (Fig. 2ABC, Fig. S3A). We found that CD133$^+$/CD44$^+$ cell population was increased in 22Rv1 prostate cancer cells about 20- fold after fractionated irradiation with 7x2 Gy of X-ray. In contrast to the prostate cancer cells, population of CD133$^+$/CD44$^+$ cells was decreased in normal prostate epithelial cells RWPE-1 upon fractionated irradiation (Fig. S3B).

Further we assessed the enrichment of tumor progenitors in primary prostate tumor specimens. In this study we used single cell suspensions obtained from three freshly isolated human prostate cancer specimens. The cells were plated and irradiated with 2 Gy of X-ray the following day and three days later. Four days after irradiation, we assessed the number of ALDH1A1$^+$, ALDH1A3$^+$ and ALDH1A1$^+$/ALDH1A3$^+$ cells by fluorescence microscopy and found that irradiation led to significant enrichment of these cell populations in the tumor specimens (Fig. 2DE, Fig. S3C).
ALDH⁺ prostate progenitor cells are radioresistant

Several findings suggest that CSCs might be less sensitive to irradiation than non-CSCs in in vitro assays (8). However, these reports still remain controversial (9). We employed clonogenic and spherogenic radiobiological cell survival assays in order to analyze the relative radioresistance of ALDH⁺ and ALDH⁻ cell populations. FACS purified ALDH⁺ and ALDH⁻ cells were plated as single cell suspension, irradiated with a single dose of 2 Gy, 4 Gy and 6 Gy of X-ray and cultured under colony and sphere forming conditions. Both, clonogenic and spherogenic survival assays demonstrated a significantly higher radioresistance of DU145 and PC3 ALDH⁺ cells as compared to ALDH⁻ cells (Fig. 3AB). Remarkably, not only the number, but also the size of the colonies formed by ALDH⁺ cells after X-ray treatment was increased as compared to the colonies derived from ALDH⁻ cells, showing both a higher survival rate and a higher proliferative capacity of ALDH⁺ cells after irradiation in comparison to ALDH⁻ cells (Fig. S4A).

Finally, we compared the relative tumorigenicity of DU145 ALDH⁺ and ALDH⁻ cells in mice after in vitro irradiation. The cells were purified by FACS irradiated with 0 Gy or 4 Gy of X-ray, embedded in matrigel and injected subcutaneously into NMRI nu/nu mice (Fig. 3C). Animals alive and tumor-free at the end of the observation period (100 days) were defined as long-term survivors. In contrast to the ALDH⁺ cells, which tumor take was not significantly impaired by irradiation, ALDH⁻ cells had a significantly lower tumor take after irradiation than non-irradiated ALDH⁺ cells (Fig. 3D). In addition, the difference between relative tumor growth rate of the irradiated ALDH⁺ cells and ALDH⁻ cells was significantly higher than for their non-irradiated counterparts (, Fig. S4BC). It is noteworthy that chemical inhibition of ALDH enzymatic activity with diethylaminobenzaldehyde (DEAB) or galiellalactone resulted in significant increase in cell radiosensitivity in vitro (Fig. 3E). Taken together, our results indicate that prostate cancer progenitor cells with a high ALDH activity harbor more radioresistant cells than ALDH⁻ cell population.
The emergence of radioresistance is associated with a gain of cancer stem cell phenotype

To obtain the radioresistant cell sublines, the established prostate cancer cell lines were treated with multiple fractions of 4 Gy of X-ray irradiation until a total dose of more than 56 Gy was reached (Fig. 4A). The surviving cells showed enhanced radioresistant properties, as it was evaluated in a colony assay. The established radioresistant cell lines maintain their radioresistant status for a few months (Fig. 4B). The radioresistant (RR) sublines showed enhanced ALDH activity and elevated expression of stem cell markers such as CD133, ABCG2, CXCR4 and CD44 (Fig. 4C, Fig. S5AB).

The radioresistant LNCaP-RR cells maintain their radioresistant properties in vivo and have a significantly increased relative tumor growth rate and tumor uptake after in vitro irradiation with 4 Gy of X-rays compared to parental, more radiosensitive LNCaP cells. In contrast, LNCaP and LNCaP-RR cells which were not irradiated before injection did not differ in their tumorigenic properties (Fig. 4DE, Fig. S5CD). Consistently with the in vitro results, analysis of the xenograft tumors formed by radioresistant tumor cells showed an elevated ALDH activity, increased expression of other stem cell markers such as CD133, NANOG and BMI1 compared to the xenograft tumors formed by parental LNCaP and DU145 cells (Fig. S5EFG).

The mechanisms of prostate cancer cell radioresistance

A number of different intrinsic and extrinsic adaptations might confer high cellular radioresistance, including activation of the radiation-induced DNA damage response and enhanced DNA repair capability, increased intracellular defense against reactive oxygen species (ROS), and activation of survival pathways, including PI3K/AKT signaling (7, 8).
Cellular response to DNA damage is coordinated primarily by two signaling cascades, the ATM-Chk2 and ATR-Chk1 pathways, which are activated by double-strand DNA breaks (DSBs) and single-strand DNA breaks (SSBs), respectively. After DNA damage by ionizing irradiation, Chk1/2 becomes phosphorylated by ATR/ATM and arrests cell proliferation to allow DNA reparation. Recent studies have also implicated PI3K/AKT signaling pathway in modulation of DNA damage responses. AKT is activated by DSBs in a DNA-protein kinase (DNA-PK) or ATM/ATR-dependent manner and can contribute to tumor radioresistance by enhancing DNA repair through non-homologous end joining (NHEJ) and enhanced cell survival (8, 18). We found that radioresistant prostate cancer cell sublines DU145-RR, PC3-RR and LNCaP-RR have an increased baseline phosphorylation of Chk2 (Thr68) and AKT (Ser473) suggesting potential molecular mechanisms underlying acquired radioresistance of these tumor cells (Fig. 5AB).

Another molecular determinant of a high radioresistance of CSC population is an intracellular defense against reactive oxygen species (ROS). Excessive ROS production after irradiation can lead to cell death via damage of critical macromolecules including DNA, lipids and proteins (8). The radioresistant prostate cancer cell sublines DU145-RR and LNCaP-RR have significantly lower baseline level of intracellular ROS as compared to parental cells that together with activated DNA repair can contribute to their higher radioresistance (Fig. 5C, Fig. S6A). Chemical inhibition of ALDH enzymatic activity with DEAB had an additive effect with radiation to increase level of intracellular ROS in prostate cancer cells (Fig. 5D).

Microscopy analysis of the FACS-purified ALDH+ and ALDH- cell populations suggests that ATM-Chk2 signaling pathway is also highly activated in cells with a high ALDH activity (Fig. 5E, Fig. S6B). Furthermore, analysis of the xenograft tumors formed by ALDH+ or ALDH- cells has shown that ALDH+-derived tumors are enriched for ALDH1A1 cells, have activated PI3K/AKT signaling and a relatively low level of ROS production (Fig. S6CD).
One of the best-characterized chromatin modification events in response to DNA DSB is histone H2A.X phosphorylation by ATM or ATR protein kinase. The phosphorylated form of H2A.X (γ-H2A.X) forms nuclear foci on the sites of DNA damage (19). Residual γ-H2A.X foci measured at 24 h after irradiation can be used as an indicator of lethal DNA lesions. Analysis of the residual γ-H2A.X foci in the parental and radioresistant LNCaP and DU145 cells 24h after irradiation showed that RR cells have a significantly fewer number of foci as compared to the parental cell lines suggesting that RR cells have more efficient DSB repair after irradiation (Figure 5F).

It is noteworthy that ALDH⁺ cell population from DU145 and PC3 cells had significantly fewer number of γ-H2A.X foci at an early time point of 1 h after irradiation and significantly less residual γ-H2AX foci 24 hours after irradiation than ALDH⁻ cells. The results of this experiment demonstrated that a lower number of the residual γ-H2A.X foci in ALDH⁺ cells can be attributed to both, lower level of DNA double-strand breaks and more efficient DNA repair (Fig. 5G).

A number of recent reports suggest that hypoxic niches directly protect CSCs by lack of oxygen and support CSC maintenance as well as expansion at least in part through the activation of hypoxia inducible factor (HIF) signaling pathway (20, 21). Expression of the stem cell markers ALDH1A1, CD44 and PROM1 (CD133) has a tendency toward co-occurrence with HIF-1α expression in prostate tumor specimens (Fig. S6E). Moreover, immunohistochemical analysis revealed an overlap in ALDH1A1 and HIF-1α expression in primary human prostate tumors, suggesting that a subset of ALDH⁺ cells might be radioresistant in vivo due to their protection from radiation damage by hypoxia (Fig. 5H, Fig. S6FG).

*Comparative gene expression profiles of prostate cancer progenitor and radioresistant cells*
By employing a global gene expression profiling we performed a more comprehensive analysis of the molecular pathways which are similarly activated in DU145 ALDH+ and DU145-RR cells. We performed pathway enrichment analysis using PANTHER pathway tool to highlight shared intracellular signaling mechanisms and found that 36% (9 of 25) of the molecular pathways overrepresented in DU145 ALDH+ cell population comprise 47% (9 of 19) of the pathways are also overrepresented in DU145-RR cells (Fig. S7A). The signaling axes which are commonly overrepresented in ALDH+ and DU145-RR cells include already known mechanisms regulating CSC population, such as WNT/cadherin, heterotrimeric G-protein, TGF-β and integrin signaling pathways, angiogenesis and inflammation, but also signaling axes that were not previously known to be associated with CSC and radioresistance, such as Alzheimer disease-presenilin pathway, Huntington disease and Oxytocin receptor mediated signaling (Fig. S7B).

**WNT/β-catenin signaling plays a role in the maintenance of radioresistant ALDH+ prostate cancer cells**

A number of studies indicate that WNT signaling plays a central role in the maintenance of the CSC populations of various tumor types (22). Activation of the canonical WNT signaling pathway is associated with the stabilization and accumulation of nuclear β-catenin, which interact with T-cell factor/lymphoid enhancing factor-1 (TCF/LEF) transcription factors to promote gene expression. Numerous experiments have demonstrated an interplay of cadherin-mediated cell adhesion and WNT signaling in regulation of EMT, therapy resistance and metastasis development (22-24). Western blot analysis of the FACS-purified ALDH+ and ALDH- cells showed a high level of β-catenin expression in ALDH+ cell population (Fig. 6A). In addition, analysis of the intracellular distribution of β-catenin in ALDH+ and ALDH- cell subsets showed that ALDH+ cells have an increased nuclear β-catenin level that indicates the activation of WNT signaling pathway (Fig. 6B). Consistently
with the results of western blot analysis and immunofluorescent staining, the chemical inhibition of WNT pathway by tankyrase inhibitor XAV939, which stimulates β-catenin degradation led to significant inhibition of the ALDH⁺ population in LNCaP and DU145 cells (Fig. 6C). Moreover, WNT inhibition by XAV939 had a radiosensitization effect for LNCaP and DU145 prostate cancer cells (Fig. 6D). Knockdown of β-catenin mediated by siRNA resulted in a significant increase in PC3 prostate cancer cells radiosensitivity as it was demonstrated by a clonogenic cell survival assay (6E).

WNT and TGFβ signaling, which are overrepresented in both radioresistant prostate cancer cells and ALDH⁺ tumor progenitor population are potent inducers of EMT, which plays a critical role in the maintenance and generation of CSCs (26-28). Moreover, recent studies have shown that CSCs population surviving chemo- and radiotherapy have the phenotypic hallmarks of EMT (29-31). Emergence of radioresistance resulted in reversion from the epithelioid shape of the parental DU145 and LNCaP cells to the stellate shape observed during EMT (Fig. S8A). Analysis of the expression of EMT markers in the radioresistant and parental prostate cancer cell lines showed that acquired radioresistance is associated with the loss of E-cadherin expression and increased expression of β-catenin (Fig. 6F). Western blot analysis showed an induction of β-catenin expression after irradiation (Fig. S8B). The results of the wound-healing and Boyden chamber-based assays demonstrated a high migratory potential of the radioresistance prostate cancer cells (Fig. 6G, Fig. S8C). Finally, we analyzed the migratory potential of ALDH⁺ and ALDH⁻ populations isolated from DU145 and LNCaP cell lines and found that ALDH⁺ cells have a higher migratory properties as compared to ALDH⁻ cell population (Fig. 6H, Fig S8D).

Activation of WNT/β-catenin signaling pathway by glycogen synthase kinase (GSK-3) inhibitor LiCl or stimulation of β-catenin degradation by XAV939 led to an increase or downregulation of ALDH1A1 protein expression, respectively (Fig. S9A). Knockdown of β-catenin mediated by siRNA transfection strongly decreased protein expression of ALDH1A1.
A correlation between the protein expression level of ALDH1A1 and β-catenin in prostate cancer cells suggested that WNT pathway could regulate ALDH1A1 level directly through β-catenin/TCF-dependent transcription or indirectly via different β-catenin/TCF target genes. Analysis of the ALDH1A1 gene promoter revealed two core β-catenin/TCF binding sites 5'-A/T A/T CAAAG-3' (Fig. 7B). To determine if β-catenin/TCF transcriptional complex can bind to the ALDH1A1 promoter, a chromatin Immunoprecipitation assay (ChIP) was performed. The cross-linked DNA-protein complexes were immunoprecipitated either with anti-β-catenin or with anti-TCF4 antibodies. PCR amplification was performed using primers flanking two putative β-catenin/TCF binding sites named ALDH1A1(i) and ALDH1A1(ii) (Fig. 7B). The results of ChIP analysis showed that ALDH1A1 promoter DNA was precipitated with both, anti-β-catenin or with anti-TCF4 antibodies suggesting a direct regulation of ALDH1A1 expression by β-catenin/TCF transcriptional complex (Fig. 7C). To analyze the transcriptional regulation of ALDH1A1, we used reporter system where luciferase gene expression is regulated by an endogenous ALDH1A1 gene promoter. The results of luciferase reporter assay showed that XAV939 or siRNA-mediated downregulation of the endogenous β-catenin, or overexpression of wild type β-catenin led to a significant decrease or upregulation of ALDH1A1 transcription, respectively (Fig. 7D, Fig S9BCD). Moreover, expression of ALDH1A1 and ALDH6A1 genes has a tendency toward co-occurrence with CTNNB1 (β-catenin) in prostate tumor specimens (Fig. 7E). Immunohistochemical analysis showed an overlap in ALDH1A1 and β-catenin – positive cell populations in primary prostate tumor cells (Fig. 7F). This data suggest overlapping signaling mechanisms which govern tumor initiating properties, EMT and radioresistance (Fig. 7G).

Discussion

Radiation therapy is one of the mainstays of curative treatment of clinically localized prostate carcinoma. However, one of the main obstacles to external beam radiotherapy is a
limited dose which can be delivered to the tumor due to normal tissue tolerance (32). From a clinical point of view, a patient suffering from cancer can be only cured if all CSCs are eradicated (6). Recent study has shown that the most lethal metastatic prostate cancer can potentially arise from a single CSC (33). Therefore, efficient anti-cancer therapy is expected to target all CSCs. The CSC concept has been elevated to a higher level of clinical significance by a large number of in vitro investigations showing differences in radiosensitivity of putative CSC and non-CSC populations. Possible relative radioresistance of CSCs has been explained by their quiescent status, activated DNA checkpoints, high free radical scavenger level, activation of the anti-apoptotic signaling pathways, and by extrinsic contribution of the tumor microenvironment (8).

Currently, tumor response in many of clinical studies is determined by volume-related endpoints, such as tumor regression or growth delay, that may not always correlate with local tumor control (34, 35). The relative frequency of CSC is highly variable between different tumor types, and even among tumors of the same clinical entity, ranging from a very small population (<1%) as in acute myeloid leukemia or pancreatic cancer, up to 20%-40% as in some brain, bladder, colorectal cancer and melanoma. Therefore, therapies that eradicate tumor initiating cells may not always result in tumor shrinkage (36). On the other hand, common anti-cancer therapies, including chemotherapy and radiotherapy, eradicate the bulk of tumor cells and lead to decrease in tumor volume, but not always targeting the entire CSC population (34, 35, 37, 38). Prediction of the probability of successful treatment is of high importance for the individualization of prostate cancer treatment. The current prostate cancer risk stratification system, which is based on prostate specific antigen (PSA), T stage and Gleason score and used for treatment decision-making, is of great clinical value, yet not perfect and there remains a lack of reliable indicators of patients’ response to radiotherapy (39, 40).
Retrospective clinical studies for the different types of cancer have shown that analysis of CSC-specific markers in pre-therapeutic biopsies might be an important tool for prediction of radiotherapy outcome and appropriate treatment selection (9). Despite a few shortcomings of these studies, including a low number of the patients that hamper multivariate analysis and the usage of volume-related endpoint parameters such as tumor regression or growth delay as well as the absence of the functional characterization of the marker positive cells, these studies paved an avenue for future biomarker development. Recent analysis of the presence and clinical significance of ALDH\textsuperscript{+} populations in prostate cancer specimens demonstrated that ALDH1 expression was significantly increased in cancers in advanced-stage with high Gleason scores, and was associated with poor survival in hormone-naïve patients. This clinical study used a large cohort of multistage prostate cancer specimens and demonstrated that analysis of ALDH1A1 expression might help to improve risk stratification in patients with prostate cancer (41).

In the current study, we found that prostate cancer progenitor cells with high aldehyde dehydrogenase activity (ALDH\textsuperscript{+} cells) are more radioresistant than ALDH\textsuperscript{-} cells, and inhibition of ALDH activity radiosensitizes prostate cancer cells. Our data demonstrated that resistance to radiotherapy of ALDH\textsuperscript{+} cells might be conferred by more efficient mechanisms of DNA repair and ROS scavenging as well as activation of the cell survival pathways, such as WNT/β-catenin, PI3K/AKT, and Integrin signaling pathways. Primary human prostate tumors consist of ALDH1A1\textsuperscript{+} cells with a high level of expression of HIF-1α, which has been suggested as a master regulator of cancer stem cells and endogenous marker of tumor hypoxia and associated radioresistance (20, 42-44). Importantly, irradiated ALDH\textsuperscript{+} cells maintain their tumorigenic properties \textit{in vivo} suggesting that ALDH\textsuperscript{+} prostate cancer cells are a cell population that may be able to regrow after radiotherapy.

Our results are in agreement with a recent study on breast cancer cells from Duru et al. who showed that HER2\textsuperscript{+} cells isolated from HER2 negative breast cancer cells are...
radioresistant and ALDH positive (45). Another study of Wang et al. demonstrated that ALDH inhibition by disulfiram (DSF) effectively depleted pre-existing and radiation-induced breast cancer stem cells (46). A notable study by Mao et al., demonstrated that highly radioresistant mesenchymal glioma stem cells are maintained by activated glycolytic metabolism involving ALDH1A3, and inhibition of ALDH1 reverses the radioresistant phenotype of CSCs (47). This data and our results are in agreement with recently published study from Pate et al. showing that WNT signaling drives a glycolytic metabolism and stimulates HIF-1α stabilization in colon cancer (48).

We found that chemical and genetic inhibition of the WNT/β-catenin signaling pathway inhibits ALDH⁺ cell population and radiosensitizes prostate cancer cells. In the present study, we identified a new link between tumor radioresistance and tumorigenicity. We report for the first time that WNT pathway directly regulates ALDH1A1 level through β-catenin/TCF-dependent transcription, and expression of ALDH1A1 co-occurs with expression of β-catenin in human prostate tumors. Recent clinical studies of WNT targeting compounds for the treatment of various solid tumors including OMP-54F28 (NCT01608867, NCT02069145, NCT02092363, NCT02050178), OMP-18R5 (NCT01345201), PRI-724 (NCT01606579, NCT01302405) and LGK974 (NCT01351103) are paving the way to improve the effectiveness of cancer treatment by combination of radiotherapy with ALDH⁺ cell-targeting treatment.

Consistent with our results for a high relative radioresistance of ALDH⁺ cells, we found that ALDH⁺ cell population is enriched within prostate cancer cells with acquired radioresistance (RR). Whole genome gene expression profiling revealed a few common signaling pathways that link ALDH⁺ progenitors and RR cells and include WNT/β-catenin, G protein-coupled receptor, TGFβ and integrin signaling pathways. Our data suggest that emergence of radioresistance by prostate cancer cells is associated with gaining of EMT features and migratory behavior, and that both ALDH⁺ cell population and RR prostate cancer
cells have a high migratory potential. This finding is in agreement with a number of recent studies suggesting that gain of the EMT features contribute to radioresistance (47, 49, 50). Thus, targeting ALDH might provide a new approach to improve the efficacy of prostate cancer radiotherapy.

Taken together, our results indicate that ALDH+ tumor initiating cells can potentially contribute to prostate tumor radioresistance, and therapies that specifically target this cell population might be a biologically-driven strategy to enhance the efficacy of radiotherapy. Further functional radiobiological assays and analysis of the expression level of ALDH1 in a retrospective cohort of patients who were treated with radiotherapy may lead to the development of novel predictive biomarker for radiation response in prostate cancer.
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References


Figure legends

Figure 1. Expression of ALDH genes correlates with survival of cancer progenitors in xenograft tumors and is upregulated in prostate carcinoma tissues. A, Analysis of CD133+/CD44+ cancer progenitor population and gene expression in xenograft tumors treated with PI3K inhibitor NVP-BEZ235. B, Representative images of immunohistochemical analysis showing an expression of ALDH1A1 in prostate tumors and normal adjacent tissues. Three formalin-fixed, paraffin-embedded prostate tumor specimens were analyzed. Scale bar, 50μm. *, p < 0.05. C, ALDH+ cells possess higher tumorigenic properties compared to ALDH- cells. Limiting dilution assay calculations were performed using the ELDA software.

Figure 2. X-ray irradiation leads to an increase in the progenitor population in prostate cancer cell lines. A, Cells were irradiated with the dose indicated, cultured for 7 days and analyzed by flow cytometry or Western blotting. B, Irradiation leads to the upregulation of AKT phosphorylation at Ser473 and to expression of the transcription regulators NANOG and β-catenin. C, Flow cytometry analysis of CSC marker expression shows a dose-dependent increase in the number of DU145 and LNCaP cells having high ALDH activity and expressing CD133 and ABCG2. Error bars represent S.E.M; *, p < 0.05; #, p < 0.05. D, Freshly isolated human prostate cancer specimens were subjected to enzymatic dissociation and single cell suspension was plated on 96-well clear bottom collagen I coated plates at 1000 cells/well and irradiated with 2 Gy of X-ray the following day and three days later. E, Four days after irradiation, the number of ALDH1A1+, ALDH1A3+ and ALDH1A1+/ALDH1A3+ cells was assessed by fluorescent microscopy. For quantification, 200 to 1000 cells per condition in at least seven randomly selected fields were counted. Error bars represent S.E.M; Scale bar, 30μm; *, p < 0.05.

Figure 3. ALDH+ cancer progenitor cells are resistant to X-ray irradiation. A, Susceptibility of ALDH+ and ALDH+ FACS purified PC3 and DU145 prostate cancer cells to
X-ray irradiation was evaluated by clonogenic cell survival assay; Error bars represent S.D.*, p < 0.05. **B**, Relative radioresistance of ALDH+ and ALDH- FACS purified PC3 and DU145 prostate cancer cells was evaluated by spherogenic cell survival assay; Error bars represent S.D.*, p < 0.05. **C**, Schematic representation of the setup of *in vivo* study. **D**, Tumor uptake of ALDH+ and ALDH- DU145 cells irradiated with 4 Gy of X-ray before injection. **E**, Inhibition of ALDH enzymatic activity with 25 μmol/L of diethylaminobenzaldehyde (DEAB) or 1 μmol/L of galiellalactone resulted in significant increase in DU145 cells radiosensitivity. Error bars represent S.E.M.; * and # - p < 0.05.

**Figure 4. Characterization of the properties of prostate cancer cells with acquired radioresistance.** **A**, The established prostate cancer cell lines DU145 and LNCaP were treated with multiple fractions of 4 Gy of X-ray irradiation until a total dose of more than 56 Gy was reached. **B**, Cells survived fractionated irradiation with >14x4 Gy of X-rays (radioresistant cell subline, RR) showed enhanced radioresistant properties in a colony assay as compared to the parental cells (P). The cells were analyzed four weeks (4W) and three months (3M) after the last irradiation. **C**, The DU145-RR and LNCaP-RR sublines showed enhanced ALDH activity and elevated expression of stem cell markers, such as CXCR4+, ABCG2+, and CD133+ as compared to the parental cells. The radioresistant cells were analyzed 4 weeks after the last irradiation. Error bars represent S. D. *, p < 0.05. **D**, The radioresistant and parental cells were irradiated with 0 Gy or 4 Gy of X-rays, embedded in matrigel and injected s.c. into NMRI nu/nu mice (10^6 cells per injection site). **E**, Relative tumor growth of LNCaP-RR and LNCaP cells. Error bars represent S.E.M; *, p < 0.05.

**Figure 5. The mechanisms of prostate cancer cell radioresistance.** **A**, Radioresistant (RR) prostate cancer cells have an increased level of Chk2 phosphorylation (Thr68). **B**, Phosphorylation level of AKT (Ser473) is increased in radioresistant cells. **C**, Radioresistant prostate cancer cells have low intracellular level of ROS. RR cells were analyzed 4 weeks after the last irradiation. Error bars represent S.E.M; *, p < 0.05. **D**,
Chemical inhibition of ALDH enzymatic activity with 25 μmol/L of diethylaminobenzaldehyde (DEAB) had an additive effect with radiation to increase level of intracellular ROS in prostate cancer cells, IR- irradiation with 4Gy of X-rays; *, p < 0.05.  

**E**, Immunofluorescence analysis of Chk2 phosphorylation (Thr68) in FACS sorted ALDH⁺ and ALDH⁻ cells without irradiation (NI) and 30 min after irradiation with 4 Gy of X-ray. Scale bars, 30 μm.  

**F**, Analysis of the residual γ-H2A.X foci in the parental and radioresistant LNCaP and DU145 cells 24h after irradiation. Error bars represent S.E.M; *, p < 0.05.  

**G**, A number of γ - H2A.X foci in ALDH⁻ and ALDH⁺ cells 1h and 24 h after irradiation. Error bars represent S.E.M.; Scale bars, 30 μm; *, p < 0.05.  

**H**, Representative image of immunofluorescent staining of ALDH1A1 and HIF1α in prostate tumors. Three formalin-fixed, paraffin-embedded prostate tumor specimens were analyzed. Scale bars, 100 μm. The Pearson's correlation coefficient (r) and Mander’s correlation coefficients (M) were analyzed using Fiji software.  

**Figure 6. Activation of WNT signaling pathway in ALDH⁺ cell population and radioresistant prostate cancer cells.**  

**A**, Western blot analysis of the FACS-purified ALDH⁺ and ALDH⁻ cells showed a high level of β-catenin expression in ALDH⁺ cell population.  

**B**, Immunofluorescence analysis of the intracellular distribution of β-catenin in ALDH⁺ and ALDH⁻ cell subsets. Error bars represent S.E.M; Scale bars, 30 μm; *, p < 0.05.  

**C**, The chemical inhibition of WNT pathway by XAV939 antagonist led to significant inhibition of the ALDH⁺ population in the prostate cancer cells. The cells were serum starved in DMEM with 1% FBS for 24 h followed by treatment with XAV939 antagonist at concentration 1μmol/L for 3 days. Error bars represent S.E.M; Scale bars, 30 μm; *, p < 0.05.  

**D**, The inhibition of WNT signaling pathway with XAV939 inhibitor at concentration 1μmol/L resulted in significant increase in radiosensitivity of LNCaP and DU145 cells. Error bars represent S.E.M; ***, p < 0.01.  

**E**, Knockdown of β-catenin mediated by siRNA led to a significant prostate cancer cells radiosensitization. Error bars represent S.E.M; *, p < 0.05.
Level of E-cadherin and β-catenin expression in the radioresistant (RR) and parental DU145 and LNCaP cells. G, The transwell migration assay showed that radioresistant cells have an increased migratory potential relative to the parental cells. Radioresistant cells were used 4 weeks after the last irradiation. Error bars represent S.D.; *, p < 0.05. H, ALDH+ and ALDH- cells isolated by FACS from DU145 cells were analyzed for their migratory properties in a transwell migration assay. Error bars represent S.E.M; *, p < 0.05.

Figure 7. WNT/β-catenin dependent regulation of the ALDH1A1 protein expression. A, Knockdown of β-catenin mediated by siRNA transfection strongly decreased protein expression of ALDH1A1. The densitometry of western blot bands was performed using ImageJ software based on the analysis of two (LNCaP) or four (PC3) biological replicates of western blotting. O.D: optical density, A.U: arbitrary units. B, Two core β-catenin/TCF binding sites 5’-A/T A/T CAAAG-3’ within the ALDH1A1 gene promoter. C, The results of ChIP analysis showed that ALDH1A1 promoter binds to β-catenin/TCF4 transcription complex. D, The results of luciferase reporter assay where luciferase gene expression is regulated by an endogenous ALDH1A1 gene promoter showed that siRNA-mediated downregulation of the endogenous β-catenin significantly decreases ALDH1A1 transcription. Error bars represent S. D. *, p < 0.05. E, Expression of ALDH1A1 and ALDH6A1 has a tendency toward co-occurrence with expression of CTNNB1 (β-catenin) in prostate tumor specimens. Data are based on the Taylor study (14). Data was analyzed using cBioPortal for Cancer Genomics; p-values <0.05, as derived via Fisher’s Exact test are outlined in red, p-values are not adjusted for FDR. F, Representative images of immunohistochemical analysis showing expression of ALDH1A1 and β-catenin in primary prostate tumor cells. Error bars represent S.E.M. Scale bars, 50μm. G, Model depicting the proposed mechanism of regulation of cell radioresistance by WNT/β-catenin/ALDH1A1 signaling pathway. Activation of the WNT signaling pathway leads to an increase of β-catenin/TCF4 dependent transcription of ALDH1A1 gene. ALDH is required to maintain ROS.
level sufficiently low to prevent irradiation-induced oxidative DNA damage. ALDH activity is indicative of radioresistant prostate progenitor cells with activation of EMT and high migratory potential.
Figure 1

A

Relative signal strength
Vehicle BEZ235 Taxotere
ALDH1A1
ALDH5A1
ALDH3A1
ALDH5A1

B

Normal prostate
Prostate tumor
ALDH1A1 DAPI
ALDH1A1 DAPI

C

Stem cell frequency (1/m)

ALDH+ ALDH-

548
109

p-value: 0.0288

CD133+CD44+ cells, % of total tumor population

Vehicle BEZ235 Taxotere

Tumor tissue Normal tissue

Signal intensity, a.u.

T435 T522 T695

Tumor free survival, %

ALDH-1000 cells
ALDH-3000 cells
ALDH+ 1000 cells
ALDH+ 3000 cells

p<0.05
p=0.0006

*p-value: 0.0006

Figure 3

A. Colonies (2D)

PC3

Surviving fraction

0Gy  4Gy

DU145

ALDH+

ALDH-

Surviving fraction

0Gy  4Gy

ALDH+

ALDH-

E. Surviving fraction

Control  DEAB, 25uM  Gallicelastone, 1uM

Surviving fraction

0  2  4  6  8

Surviving fraction

0  2  4  6  8

Surviving fraction

0  2  4  6  8

Surviving fraction

0  2  4  6  8

B. Spheres (3D)

PC3  0Gy  4Gy

DU145  0Gy  4Gy

Surviving fraction

Surviving fraction

Surviving fraction

Surviving fraction

C.

D.

Tumor-free survival

ALDH+ vs. ALDH+ irradiated 0.142

ALDH- vs. ALDH- irradiated 0.026

ALDH+ vs. ALDH- 0.216
Figure 4

A. Tumor cells are exposed to radiation doses of >14 Gy (radioresistant, RR) or 0 Gy (parental, P). The cells are then analyzed for CSC markers and plated.

B. Survival fraction of DU145 and LNCaP cells exposed to different radiation doses for 4 weeks (RR 4W) and 3 months (RR 3M).

C. ALDH, CD133, ABCG2, and CXCR4 expression levels in DU145 and LNCaP cells with radioresistant (RR) or parental (P) status.

D. Effects of 4 Gy radiation on RR and P cells. Relative tumor volume over time for LNCaP, LNCaP + IR (4 Gy), LNCaP RR, and LNCaP RR + IR (4 Gy) groups.
Figure 6

(A) PC3 and DU145 cells were analyzed for β-catenin and GAPDH expression in ALDH+ and ALDH- populations.

(B) Cells with nuclear β-catenin were stained with DAPI. ALDH+ and ALDH- populations were compared.

(C) ALDH+ population was analyzed in DU145 and LNCaP cells.

(D) Survival fraction of DU145 and LNCaP cells treated with different concentrations of radiation.

(E) Survival fraction of Scrambled siRNA, siRNA#1 CTNNB1, and siRNA#2 CTNNB1 in GAPDH treatment.

(F) E-cadherin, β-catenin, and β-actin expression in DU145 and LNCaP cells.

(G) Migration of DU145 and LNCaP cells after 24h.

(H) ALDH- and ALDH+ populations were compared for migrating cells.
Figure 7

A

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- β-catenin
- ALDH1A1
- GAPDH

B

- CTTTGA
- ALDH1A1 (i)
- ALDH1A1 (ii)

C

- Input
- Anti-TCF4
- Anti-β-catenin
- Control IgG

D

- Luc intensity, AU

- Scr RNA
- siRNA H1
- siRNA H2

E

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- Tendency towards mutual exclusivity (0 < Odds Ratio < 0.1)
- No association (0.5 < Odds Ratio < 2)
- Tendency toward co-occurrence (2 < Odds Ratio < 10)

F

- NCIT1
- NCIT2

- β-catenin
- ALDH1A1
- DAPI

- β-catenin
- ALDH1A1
- DAPI

G

- +Wnt
- Degraded β-catenin
- ALDH1A1
- Stemness, Radioresistance, EMT
- ROS
- CTTTGA/TA/T
- TGF
Aldehyde dehydrogenase is regulated by Beta-catenin/TCF and promotes radioresistance in prostate cancer progenitor cells

Monica Cojoc, Claudia Peitzsch, Ina Kurth, et al.

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