Characterization and clinical relevance of ALDH\textsuperscript{bright} populations in prostate cancer

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High ALDH activity has been shown to be associated with tumorigenesis and proposed to represent a functional marker for tumor-initiating cells (TIC) in various tumor types, including prostate cancer (PCa). As previous studies were mainly based on the use of established cell lines, clinical relevance of ALDHbright populations has not been investigated in detail so far in PCa. Here, using a large cohort of multi-stage PCa specimens, we show that ALDHbright populations are present and heterogeneously distributed in PCa tissues. Furthermore, we demonstrate that expression of the ALDH1A1 specific isoform, at both the gene and protein levels, is associated with advanced clinical stage and unfavorable prognosis in hormone-naïve PCa. Our findings highlight the potential importance of ALDH in PCa pathogenesis and suggest that its clinical significance might be specific for patient sub-groups. These results might help to improve stratification and identification of high-risk patients with PCa.
Abstract (240 words)

**PURPOSE:** High aldehyde dehydrogenase (ALDH) has been suggested to selectively mark cells with high tumorigenic potential in established prostate cancer (PCa) cell lines. However, the existence of cells with high ALDH activity (ALDH\textsuperscript{bright}) in primary PCa specimens has not been demonstrated so far. We investigated the presence, phenotype, and clinical significance of ALDH\textsuperscript{bright} populations in clinical PCa specimens.

**EXPERIMENTAL DESIGN:** We used Aldefluor\textsuperscript{™} technology and FACS staining to identify and characterize ALDH\textsuperscript{bright} populations in cells freshly isolated from clinical PCa specimens. Expression of genes encoding ALDH specific isoforms was evaluated by quantitative real-time PCR in normal prostate, benign prostatic hyperplasia (BPH) and PCa tissues. ALDH1A1 specific expression and prognostic significance was assessed by staining two tissue microarrays that included over 500 samples of BPH, prostatic intraepithelial neoplasia (PIN), and multi-stage PCa.

**RESULTS:** ALDH\textsuperscript{bright} cells were detectable in freshly excised PCa specimens (n= 39), and were mainly included within the EpCAM\textsuperscript{(+) and Trop2\textsuperscript{(+) cell populations. Although several ALDH isoforms were expressed to high extents in PCa, only ALDH1A1 gene expression significantly correlated with ALDH activity (p<0.01), and was increased in cancers with high Gleason scores (p=0.03). Most importantly, ALDH1A1 protein was expressed significantly more frequently and at higher levels in advanced-stage as compared to low-stage PCa and BPH. Notably, ALDH1A1 positivity was associated with poor survival (p=0.02) in hormone-naive patients.

**CONCLUSIONS:** Our data indicate that ALDH contributes to the identification of subsets of PCa cells of potentially high clinical relevance.
Introduction

Despite the availability of several therapeutic options, prostate cancer (PCa) remains a leading cause of cancer-related death in men (1). PCa is characterized by remarkable cellular heterogeneity, and includes cells with different phenotypes, proliferative capacities and differentiation states. However, the clinical significance and the prognostic relevance of specific cancer cell subpopulations is still unclear (2).

Aldehyde dehydrogenase (ALDH) enzyme is responsible for the oxidization of cellular aldehydes resulting in the production of retinoic acid (3). Notably, ALDH has been shown to be involved in stem cell protection and differentiation, and high levels of ALDH activity have been found in several stem cell populations (4, 5). Thus, high ALDH activity has been used to select and identify normal hematopoietic stem cells (4, 6) and tumor-initiating cells (TIC) in hematopoietic malignancies (7).

TIC, functionally defined as cells capable of initiating tumors in immunodeficient mice (8), have also been identified in a variety of human solid tumors (8-11). In a number of cancers of diverse histological origin TIC have been reported to exhibit high levels of ALDH activity (5, 12-14).

In the prostate, high ALDH activity has been shown to represent a functional marker for murine normal progenitor/stem cells (15). More recently, cells exhibiting high ALDH activity, referred to as “ALDH<sup>bright</sup>” cells, have been identified in human PCa cell lines and expanded primary PCa cultures (16). ALDH<sup>bright</sup> cells isolated from established PCa cell lines, were found to be associated with increased clonogenicity, invasiveness, as well as high tumorigenic and metastatic capacities (16, 17). However, culture conditions might modulate cell characteristics, potentially favoring selection of specific phenotypes, and PCa cell lines may therefore be inadequate to reflect the biology of human PCa (18-21).
To date, there is no evidence of the existence and functional characterization of ALDH<sup>bright</sup> populations in uncultured clinical PCa specimens. Additionally, it is still unclear whether such cells have clinical and prognostic relevance in PCa.

In this study, we have identified, quantified, and characterized ALDH<sup>bright</sup> populations in cells isolated from freshly excised clinical PCa specimens. Our results indicate that ALDH<sup>bright</sup> subsets are detectable to various extents in all clinical PCa samples. Moreover, expression of several ALDH specific isoforms was increased in cancers as compared to benign samples.

Furthermore, we have assessed localization and clinical relevance of cells expressing ALDH1A1 isoform using two distinct tissue microarrays (TMAs). Our results support an association between ALDH1A1 positivity and poor prognosis in PCa.
Materials and Methods

Clinical specimens
A series of 38 patients with BPH and 71 patients with pT2a-pT3b stage PCa referred for treatment at the Department of Urology of the University Hospital of Basel (Switzerland) from 2008 to 2012 was studied. Patients with BPH underwent conventional transurethral resection (TUR-P), while patients with PCa underwent radical prostatectomy (RP). Written informed consent was obtained from all patients in accordance with the requirements of the local Ethical Committee (EKBB, Ref.Nr.EK: 176/07). Clinical and pathological data of the PCa patients included in the present study are summarized in Table S1.

Isolation of primary cells freshly derived from PCa surgical specimens
Prostate specimens were examined for the presence of cancer tissues by experienced pathologists. PCa samples were chopped, washed, and digested in a mixture containing DMEM, 5 % Knockout Serum Replacement (KO serum, Gibco, Paisley, UK), 1% Pen/Strep and 200IU/ml of type I collagenase (Worthington, Lakewood, NJ). After an overnight incubation at 37°C, digested tissues were washed and centrifuged as previously described, to separate the epithelial and stromal fractions (22). Cell pellets enriched in the epithelial fraction were resuspended in PBS and passed through a 100 μm cell strainer to obtain single cell suspensions. Cells were immediately used for FACS analysis to assess ALDH activity and surface markers expression (see below).

Quantification of gene expression by quantitative Real-Time PCR (qRT-PCR)
A normal human prostate RNA pool was purchased from Clontech (Clontech Laboratories Inc., Mountain View, CA).
Total cellular RNA was extracted from tissues, and DNase treated by using NucleoSpin® RNA II (Macherey-Nagel, Oensingen, Switzerland). RNA was reverse transcribed by using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen). Quantitative real-time PCR assays were performed and specific gene expression was analyzed as previously described (23). **TMPRSS2-ERG** primers and probe sequences were derived from existing literature (24). Primers and probes for **ALDH1A1, ALDH1A3, ALDH3A1, ALDH4A1, ALDH7A1, ALDH9A1**, and **ALDH18A1** specific isoforms were provided by Assays-on-Demand, Gene Expression Products (Applied Biosystems).

**Identification and isolation of cells with high ALDH activity (ALDHbright cells)**

ALDH activity was assessed by using the ALDEFLUOR™ assay system (StemCell Technologies, Grenoble, France). Single cells obtained from PCa specimens were re-suspended in aldefluor buffer and incubated with the ALDH substrate, biodipy-amoacetaldehyde (BAAA). As negative control, an aliquot of the treated cells was also incubated with the ALDH inhibitor, diethylaminobenzaldehyde (DEAB). Following 35-40 minutes of incubation at 37°C, cells were washed and analyzed using a dual laser BD FACS Calibur (BD Biosciences, San Jose, CA). Dead cells were excluded based on propidium iodide (PI) incorporation. Results are presented as percentages of ALDHbright cells or as ratios of mean fluorescence intensities (MFI) as compared to control cells incubated with DEAB.

**Phenotypic characterization of ALDHbright cells**

Following treatment with ALDEFLUOR™ assay system, cells were re-suspended in buffer and incubated with phycoerythrin (PE)-labeled anti-CD44 (BD Biosciences, San José, CA), allophycocyanin (APC)-labeled anti-EpCAM (BD Biosciences), and APC-labeled anti-Trop2 (R&D Systems, Cambridge, UK) antibodies or isotype-matched immunoglobulins, at concentrations recommended by the manufacturers.
Tissue microarrays (TMAs)

The two prostate TMAs used in this study were constructed as previously described (25). The “progression TMA” contained single tissue cores from prostate cancers from all stages (25). The “castration-resistance TMA” has been recently described (26) and addresses the progression from hormone-naive (HN) to lethal castration-resistant (CR) PCa (27). Characteristics and number of samples included in the two TMAs are given in Table 1.

Immunohistochemistry

Immunohistochemical analyses were performed according to standard indirect immunoperoxidase procedures as previously reported (25). Expression of ALDH1A1 in TMAs was assessed using a specific rabbit polyclonal antibody (ab51028, Abcam, Cambridge, UK) at 1/200 (pre-treatment: heat-mediated antigen retrieval at 100°C). Other immunohistochemical procedures are described in detail in the Supplementary Information.

TMA analysis

Staining was visually scored and stratified into four groups: negative (absence of staining), weak (weak but distinct immunoreactivity), moderate (between weak and strong) and strong intensity (apparent even at low magnification: x2.5 objective). As previously described, a histoscore (H-score) was calculated by multiplying the staining intensity (0, 1, 2, or 3) by the percentage of positive cells, leading to an H-score ranging from 0 to 300 (25). A score >0 was considered positive. Stainings were independently evaluated by two experienced members of the team (LB and JG) with excellent correlation between paired measurements.

Statistical analysis

Statistical analyses were performed using GraphPad Prism5.0 (GraphPad Software Inc., La Jolla, CA) and SPSS softwares (IBM, New York, NY). To assess the equality of means, parametric T-test
or non-parametric Mann-Whitney test were used. Correlation of specific expression between two groups was assessed using Pearson or Spearman correlation tests. Fisher and \( \chi^2 \) tests were used to compare ALDH1A1 positivity frequency in different groups of patients. Survival curves were constructed according to Kaplan-Meier and compared using log-rank (Mantel Cox) tests. \( P \)-values lower than 0.05 were considered statistically significant.
Results

Epithelial cells freshly isolated from PCa clinical specimens contain a heterogeneous ALDH<sub>bright</sub> population.

Presence of ALDH<sub>bright</sub> cells was investigated in cells freshly isolated from 39 PCa specimens directly retrieved following surgery. Representative cytograms are shown in Figure 1A. As shown in Figure 1B, ALDH<sub>bright</sub> populations could be detected in all cell preparations obtained from clinical PCa samples. Percentages of ALDH<sub>bright</sub> cells were highly variable and differed from patient to patient (average ±SE=1.56±0.24% bright cells; MFI ratio=257.2±40.87; n=39). When gated on EpCAM<sup>(+)</sup> cells to identify the bulk of the epithelial population, percentages of ALDH<sub>bright</sub> cells were significantly higher and exceeded 15% of EpCAM<sup>(+)</sup> cells in several specimens (average ±SE=9.06±0.97% bright cells; MFI ratio=292.3±32.99; n=31). As shown in Figure 1C, a trend towards a higher percentage of ALDH<sub>bright</sub> cells in high (G8-9) as compared to medium (G7) Gleason score was evident (p=0.09).

Phenotypic characterization of ALDH<sub>bright</sub> cells in PCa clinical specimens

The epithelial cell-specific marker EpCAM has been shown to be highly expressed in a majority of carcinomas including PCa (28), and has also been proposed as TIC marker in a variety of solid tumors (29). Interestingly, the majority of the ALDH<sub>bright</sub> population was also positive for EpCAM (average ±SE=75.47±4.06% of EpCAM<sup>(+)</sup> cells within the ALDH<sub>bright</sub> subset; Figure 2A-B). Expression of Trop2, previously shown to be expressed by epithelial prostate cells (30) and to be overexpressed in human PCa, was also tested (31, 32). Likewise, a high proportion of ALDH<sub>bright</sub> cells was also positive for Trop2 (average ± SE=81.41±4.94% Trop2<sup>(+)</sup> cells within the ALDH<sub>bright</sub> subset, Figure 2B). To gain additional insights into the phenotype of ALDH<sub>bright</sub> cells in PCa, aldefluor-treated PCa cells were co-stained with antibodies recognizing EpCAM and CD44 (n=4). In all cases, ALDH<sub>bright</sub> phenotype was heterogeneous, as illustrated by
two representative staining profiles in Figure 2C. Two populations of EpCAM\textsuperscript{high} cells were present within the ALDH\textsuperscript{bright} population, either positive or negative for CD44. In one particular PCa case, an EpCAM\textsuperscript{(G3)} CD44\textsuperscript{high} subset was detected in the ALDH\textsuperscript{bright} population.

**ALDH specific isoforms are highly expressed in PCa clinical specimens**

We next investigated the expression of selected ALDH specific isoform genes. High expression of all tested ALDH isoforms was found in all PCa tissues (Figure 3A, left panel). Expression of these genes was then comparatively evaluated in PCa, BPH, and normal prostate. ALDH3A1 was the only isoform displaying a significantly lower gene expression in PCa as compared to BPH (Figure 3A). In contrast a trend towards higher expression of ALDH1A1, ALDH4A1, and ALDH9A1 was observed in PCa as compared to BPH and normal tissues (Table S2 and Figure 3A). The ALDH1A3, ALDH7A1, and ALDH18A1 isoforms were expressed at significantly higher levels in PCa as compared to BPH specimens and normal tissues (Table S2 and Figure 3A). The same trends were observed when selected PCa samples found to be positive for the tumor specific TMPRSS2-ERG gene fusion (33) were used (Table S2).

Expression of ALDH isoform genes was then comparatively analyzed in PCa with different Gleason scores. Interestingly, expression of ALDH1A1 was up-regulated in high-grade (G8-9) as compared to low-grade (G5-6) cancers \((p=0.03; \text{Figure 3B})\). The other isoforms did not show any differential gene expression in high-grade as opposed to low-grade cancers (data not shown).

ALDEFLUOR™ reagent is generally thought to act as a substrate for the ALDH1A1 isoform (34). However, in breast cancer, aldefluor-dependent ALDH activity has been attributed to the ALDH1A3 isoform (35). In PCa, ALDH1A1 was the only isoform, whose gene expression correlated with levels of ALDH activity as detected in the same tissue specimens (Figure 3C). These data indicate that ALDH1A1 is the main isoform contributing to measurable ALDH activity in primary PCa.
**Expression and localization of ALDH1A1 protein in prostate tissues**

ALDH1A1 gene expression appears to be higher in tumors of higher grade and correlates with enzymatic activity. To gain insights into its potential clinical relevance, we investigated the expression of ALDH1A1 protein in a series of BPH, PIN, and multi-stage PCa specimens included in two TMAs. Numbers and characteristics of samples represented in the TMAs are given in Table 1.

To investigate ALDH1A1 protein expression and localization, we first used a prostate "progression TMA" comprising 290 evaluable samples including BPH, PIN, early stage PCa (T1a/b), radical prostatectomy specimens (RP), and castration-resistant (CR) PCa samples.

In BPH, ALDH1A1 protein was detectable in 3/65 specimens (4.6%, Table 1). In the few positive BPH cases, ALDH1A1 protein expression was clearly restricted to a few cells in the basal layer of the epithelium (Figure 4A-C). In contrast, ALDH7A1 protein in BPH was uniformly expressed in both basal and luminal cells (Figure S1).

To strengthen these data, additional staining of larger sections from BPH specimens was analyzed. This analysis confirmed that in positive BPH samples, ALDH1A1 protein expression is usually limited to a few epithelial basal cells (Figure S2B). Additionally, ALDH1A1 protein was also detectable in non-epithelial cells, such as histiocytes and peripheral nerve cells (Figure S2C-D).

In PIN and low stage T1a/b PCa samples, ALDH1A1 positivity was detectable in 3/38 (8%) and 2/47 samples (4%), respectively (Table 1). For the few positive PIN samples, ALDH1A1 protein expression was specifically localized in PIN premalignant cells, but not in benign glands (Figure 4D-E).

Consistent with the PIN data, ALDH1A1 positivity was specifically detectable in cancerous cells of RP samples (Figure 4F-G). In CR samples, ALDH1A1 expression showed large variations, ranging from fully negative (Figure 4H), to focally, or diffusely positive tumors.
Interestingly, ALDH1A1 (+) cells were also present in the peri-epithelial stroma (Figure 4D).

Importantly, ALDH1A1 protein expression was more frequent in RP (12/69, 17%) and CR (15/80, 18%) as compared to BPH (p= 0.02 and p= 0.01, respectively) or low-stage samples (p= 0.04 and p= 0.02 respectively, Figure 4A and Table 1).

Using ALDH1A1 scoring, the same trends were observed by comparing RP and CR with BPH (p= 0.01 and p= 0.003 respectively, Figure 4A). However, we did not find significant differences in percentages of ALDH1A1(+) tissues or global score among samples with different Gleason scores or Ki67 levels (data not shown).

**ALDH1A1 expression is a predictor of poor prognosis in hormone-naive patients**

To evaluate the prognostic relevance of ALDH1A1 in PCa, ALDH1A1 expression was examined in the “castration-resistance TMA” which contained hormone-naive (HN) and CR samples with complete follow-up data. Consistent with the results from the “progression TMA”, ALDH1A1 protein was detectable with significantly higher frequency in HN (40/100, 40%) and CR (40/107, 37%) PCa as compared to BPH (Table 1). Score data were also comparably different (HN vs BPH: p= 0.03; CR vs BPH: p= 0.04).

In the HN patient set, the median survival (MS) was 34 months for patients with ALDH1A1 positive tumors and 56 months for patients with ALDH1A1 negative tumors (p=0.02; Figure 5A).

In the CR group however, MS for patients with ALDH1A1 positive or negative tumors was similar (13 months and 10 months, respectively) and no correlation was observed between ALDH1A1 expression and patients overall survival (p= 0.89; Figure 5B).
Discussion

Aldehyde dehydrogenases are detoxification enzymes that catalyze the oxidation of various aldehydes. Their dysfunction is involved in several types of disease including cancer (3). High ALDH activity was shown to characterize hematopoietic stem cells and to select for TIC in various types of tumors (5, 12-14), supporting a link between ALDH expression and carcinogenesis.

High ALDH activity was also proposed to select for highly tumorigenic cells in PCa cell lines (16, 17, 36). To date however, there is no evidence for the existence of ALDH<sup>bright</sup> populations in primary PCa. In the present work, we therefore investigated presence, prevalence, characteristics, and clinical relevance of ALDH<sup>bright</sup> populations in primary PCa.

First, we determined whether ALDH<sup>bright</sup> cell populations were present in cells freshly isolated from clinical PCa specimens collected after surgery. We successfully identified and quantified ALDH<sup>bright</sup> populations among cell suspensions freshly isolated from 39 clinical PCa specimens. To our knowledge, this is the first study reporting the identification of cells with high functional ALDH activity in uncultured PCa specimens. Overall, ALDH<sup>bright</sup> cells could be detected with variable and relatively high frequency in most PCa samples with a trend towards higher percentages in cancers with higher histological grade.

We found that high ALDH activity, in PCa, mainly localized to cells highly positive for the epithelial markers EpCAM and Trop2, suggesting an epithelial origin for these cells. Notably, both these markers have been reported to be highly expressed in PCa (28, 31).

We also tested whether cells with high ALDH activity expressed CD44, a mostly basal cell-specific marker. Interestingly, both CD44-positive and negative phenotypes were observed within the ALDH<sup>bright</sup> population. Our preliminary analysis paves the way towards a thorough phenotypic characterization of ALDH<sup>bright</sup> populations in primary PCa.
The aldefluor™ assay is the sole accurate method to assess functional ALDH activity but is unsuitable to investigate the clinical and prognostic relevance of ALDH[^bright] populations in large cohorts of archived specimens (34).

To date, nineteen ALDH isoforms with different substrate specificities have been identified in the human genome (3). Among these, ALDH1A1 is generally considered to be the main isoform contributing to ALDH activity, as measured by aldefluor™ (34). However, evidence for the contribution of other isoforms in solid tumors has recently been obtained. In fact, ALDH1A3 rather than ALDH1A1 has been identified as the main isoform involved in aldefluor™-measured ALDH activity and a strong predictor of metastasis in breast cancer (35). Moreover, ALDH7A1 has recently been shown to contribute to ALDH activity in a PC3-derived cell line and to be involved in bone metastasis in PCa (37). Thus, it appears that the nature of the isoforms contributing to high ALDH activity, as detected by the aldefluor™ assay, is tissue and cell specific (34). To identify PCa relevant isoforms, we have tested the expression of seven ALDH isoforms, previously shown to be implicated in ALDH activity, as detected by aldefluor™, and in cancer initiation (reviewed in (34)). Except for ALDH3A1, we observed a trend towards higher expression of ALDH isoforms in PCa as compared to BPH and normal samples. In particular, ALDH1A3, ALDH7A1, and ALDH18A1 were expressed to significantly higher extents in PCa as compared to BPH. ALDH1A1 gene was not expressed to significantly higher extents in PCa as compared to BPH (p = 0.12) but we observed a significantly higher gene expression in high-grade as compared to low-grade PCa. Importantly, ALDH1A1 was the sole isoform whose gene expression was correlated with high ALDH activity detected in the same specimens, suggesting its major contribution to functional ALDH activity in PCa.

These results led us to investigate expression of ALDH1A1 at the protein level, in a large cohort of benign and cancerous samples. In the two TMAs analyzed, we found a minor (<5%) proportion of BPH samples with ALDH1A1 positivity restricted to a few cells in the basal layer
of the epithelium, in agreement with a recent study (38). This was confirmed by staining of large tissue sections.

In contrast, ALDH1A1 expression was specifically detected in luminal cancer cells and showed broad variations, ranging from full negativity, to focal, diffuse, or strong positivity. These expression patterns highlight the patient-to-patient heterogeneity common in PCa. ALDH1A1 expression was higher and more frequently found in RP and CR as compared to early stage PCa. We also observed intra-tissue heterogeneity with concomitant presence of negative and positive glands within the same samples. In these cases, ALDH1A1 expression was convincingly restricted to cancerous glands. Interestingly, ALDH1A1 positivity was also found in a subgroup of PIN (8%). While these samples usually contain a mixture of benign and PIN glands, ALDH1A1 expression was restricted to PIN lesions. Since PIN is a precursor for PCa (39), these findings may have implications for early identification of patients at high risk of developing PCa.

Next, we assessed the prognostic relevance of ALDH1A1 expression using a “castration-resistance” TMA. In the hormone-naive cohort (HN), we found a significant correlation between ALDH1A1 positivity and poor patient outcome. These results are consistent with a study by Li and colleagues, who evaluated a smaller and less diversified patient cohort (38). In contrast, in patients with castration-resistant disease, no significant correlation was found between ALDH1A1 positivity and clinical outcome. These data may suggest a prognostic relevance of ALDH activity limited to early phases of PCa.

ALDH1A1+ cells have been proposed to be responsible for tumor re-initiation after castration (40, 41). Our data however, indicate that these cells are not likely associated with prognostic significance in CR PCa, suggesting that ALDH1A1 negative cells might acquire a predominant clinical relevance following cancer progression to androgen independence.

High ALDH activity has previously been shown to identify TIC in solid cancers (5, 12, 13) and established PCa cell lines (16). Based on this background, it would be tempting to speculate
that ALDH\textsuperscript{bright/+} primary PCa cells could possess tumor-initiating capacity. However, a formal demonstration of the TIC nature of these ALDH\textsuperscript{bright} primary PCa cells would require direct testing of their tumorigenicity \textit{in vivo}. These studies are hampered by a lack of appropriate experimental models and the difficulties inherent in the generation of primary cultures and xenografts derived from PCa primary cells (19, 20). Recent successful attempts are based on the implantation of tumor fragments under the renal capsules of immunodeficient mice in the presence of androgen releasing pellets (42). Yet generation of cell suspensions from clinical samples and cell sorting are known to affect viability of prostate cancer cells (42, 43), therefore preventing successful tumor transplantation (reviewed in (19). To the best of our knowledge, tumor transplantation of cells derived from primary human PCa, as selected by the expression of putative TIC markers, has never been reported so far (44). This prevented us from testing the tumorigenicity of ALDH\textsuperscript{bright} primary PCa cells.

Alternatively, considering its expression in rare benign basal cells and in some untransformed interstitial cells, in addition to tumor cells, ALDH1A1 could represent a marker associated with undefined cell differentiation stages, irrespective of a putative role in cancer initiation. Nevertheless, our data clearly document a prognostic relevance of ALDH1A1 expression in untreated PCa. This might have important implications for the identification of patients at high risk for progression to castration-resistant disease.

In summary, we provide novel evidence of cells with high ALDH functional activity in primary PCa. Detection at functional, mRNA and protein levels suggests that ALDH might be involved in PCa outgrowth and progression. However, this involvement might be specific for sub-groups of patients as suggested by the high variability observed between PCa specimens.
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References


Legends to the figures

Figure 1: Identification of ALDH<sup>bright</sup> populations in cells freshly isolated from PCa surgical specimens.

A. Flow cytometry analysis of cells from three representative PCa patients (#1-3). Single cell suspensions were obtained after digestion of PCa tissues. ALDH activity was tested on live cells (left panel) using the aldefluor™ technology. Cells were also tested for EpCam specific expression in order to identify ALDH<sup>bright</sup> cells within the EpCam<sup>(+)</sup> population (right panel).

B. Cells suspensions freshly derived from 39 PCa specimens were assessed for ALDH activity ("All"). For 31 specimens, ALDH activity was also tested in the EpCam<sup>(+)</sup> population ("EpC(+)"). Left panel shows percentages of ALDH<sup>bright</sup> cells. Right panel shows MFI ratios to the DEAB control.

C. Percentage of ALDH<sup>bright</sup> cells in the EpCam<sup>(+)</sup> population in patients with Gleason 7 (G7) as compared to patients with high (G8-9) Gleason score. Four patients with Gleason 5-6 were not included in the analysis.

Figure 2: Characterization of ALDH<sup>bright</sup> populations derived from PCa surgical specimens.

In PCa tissues, ALDH<sup>bright</sup> subsets are mainly comprised within the EpCam<sup>(+)</sup> and the Trop2<sup>(+)</sup> populations. A. Expression of EpCam and Trop2 markers in the ALDH<sup>bright</sup> population derived from one representative patient. B. Percentages of EpCam positive (n=31) and Trop2 positive (n=7) cells in the ALDH<sup>bright</sup> populations from a panel of PCa samples.

C. Left panel: Expression of CD44 or EpCam in cell populations with different levels of ALDH activity (blue: dim, red: intermediate, green: bright). Right panel: Expression of CD44 and EpCam in all cells and gated on ALDH<sup>bright</sup> cells. Gate 1: EpCam<sup>high</sup>CD44<sup>(−)</sup>; Gate 2: EpCam<sup>high</sup>CD44<sup>(−)</sup>; Gate 3: EpCam<sup>low</sup>/CD44<sup>high</sup>; Gate 4: EpCam<sup>(−)</sup>CD44<sup>high</sup>.

Figure 3: Comparative expression of ALDH specific isoform genes in PCa and BPH samples.

A. Expression of genes encoding ALDH1A1, ALDH1A3, ALDH3A1, ALDH4A1, ALDH7A1, ALDH9A1, and ALDH18A1 isosforms in clinical PCa specimens (left panel) and in these clinical PCa specimens (♦) as compared to BPH samples (□) (other panels). Expression levels are reported as relative values as compared to the housekeeping gene GAPDH. P values and number of samples are listed in Table S3. As control, cDNA from a normal prostate pool (N) was tested (▲). Mean values are indicated by horizontal bars. p≤0.05: *; p≤0.01: **; p≤0.001: ***.
B. PCa samples were divided into three groups according to their Gleason grade (Gleason 5 to 9) and expression of ALDH1A1 was compared using a Mann Whitney test. Horizontal lines represent the medians, while outer limits of the whiskers show the minimal and maximal values. p≤0.05: *.

C. Spearman test was used to analyze the correlation between ALDH1A1 (1A1) gene expression and percentages of ALDH<sup>bright</sup> cells (left panel) or MFI ratio to control (right panel) in clinical PCa samples.

**Figure 4: Expression of ALDH1A1 protein in benign and cancerous prostate tissues**

A. ALDH1A1 is expressed more frequently (left panel) and at higher levels (right panel) in RP and CR PCa as compared to BPH and low stage PCa (T1a/b). p≤0.05: *; p≤0.01: **.

B-I. Representative pictures of ALDH1A1 expression in a panel of prostate tissues. B: Negative BPH glands. C. BPH glands exhibiting a few positive basal cells. D. Interstitial stromal cells showing some degree of positivity. E. Positive PIN lesions. F and G. Positive cancer glands (brown arrow) and negative benign glands (right arrow). H. Negative CR cancer. I. CR with diffuse positivity. Brown arrows indicate positive areas enlarged and shown in inserts.

**Magnification:** B, F: 200x. C, D, E, G, H, I: 400x.

**Figure 5: ALDH1A1 expression is predictive of poor prognosis in hormone-naive patients.**

Kaplan-Meier curves of patient overall survival according to ALDH1A1 protein expression. Log rank (Mantel Cox) tests were used to compare ALDH1A1 positive and negative tumors in hormone-naive patients (HN; A) and castration-resistant patients (CR; B). p≤0.05: *.
**Table 1: Immunohistochemical analysis of ALDH1A1 expression on TMAs**

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<th>Positive</th>
<th>Average score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PROGRESSION TMA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPH</td>
<td>54 (100%)</td>
<td>52 (96%)</td>
<td>2 (4%)</td>
<td>0.3</td>
</tr>
<tr>
<td>PIN</td>
<td>38 (100%)</td>
<td>35 (92%)</td>
<td>3 (8%)</td>
<td>8.2</td>
</tr>
<tr>
<td>T1a/b</td>
<td>49 (100%)</td>
<td>47 (96%)</td>
<td>2 (4%)</td>
<td>9.0</td>
</tr>
<tr>
<td>RP (pT2-pT3b)</td>
<td>69 (100%)</td>
<td>57 (83%)</td>
<td>12 (17%)</td>
<td>12.4</td>
</tr>
<tr>
<td>CR</td>
<td>80 (100%)</td>
<td>65 (82%)</td>
<td>15 (18%)</td>
<td>18.3</td>
</tr>
<tr>
<td><strong>CASTRATION-RESISTANCE TMA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPH</td>
<td>11 (100%)</td>
<td>10 (91%)</td>
<td>1 (9%)</td>
<td>1.7</td>
</tr>
<tr>
<td>HN</td>
<td>100 (100%)</td>
<td>60 (60%)</td>
<td>40 (40%)</td>
<td>40.5</td>
</tr>
<tr>
<td>CR</td>
<td>107 (100%)</td>
<td>67 (63%)</td>
<td>40 (37%)</td>
<td>33.7</td>
</tr>
</tbody>
</table>
Figure 1

A

All live cells

PCA patient 1

- DEAB

+ DEAB

SSC

ALDH activity

EpCAM (+)

SSC

ALDH activity

PCA patient 2

SSC

ALDH activity

PCA patient 3

SSC

ALDH activity

B

% of ALDH bright cells

MFI ratio to control

C

% of ALDH bright cells within EpCAM(+) cells

G7

n=20

G8-9

n=7
Figure 2

A

B

C

All cells

ALDH bright

low med bright

PCa 1

PCa 2

SSC

ALDH activity

EpCam

CD44

EpCam

CD44

EpCam

EpCam

CD44

EpCam

CD44
Figure 3

A

B

C

Relative Gene Expression

ALDH3A1

ALDH18A1

ALDH1A3

ALDH9A1

ALDH1A1

ALDH7A1

ALDH4A1

ALDH1A1

% of ALDH bright

MF1 ratio to control

N = BPH, PCa

P < 0.05

P < 0.001

P < 0.006

P < 0.59

n = 20

n = 20

n = 32

n = 11
Clinical Cancer Research

Characterization and clinical relevance of ALDHbright populations in prostate cancer
Clémentine Le Magnen, Lukas Bubendorf, Cyrill A. Rentsch, et al.

Clin Cancer Res Published OnlineFirst August 22, 2013.

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