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Prediction of Radioresistant Prostate Cancer Based on Differentially Expressed Proteins

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Keywords
Prostate cancer · Radioresistance · Protein expression · Androgen receptor · Aldo-keto reductase family 1 member C3

Abstract
Introduction: Although relapses after radiotherapy are common in prostate cancer (PCA) patients, those with a high risk for radioresistance cannot be identified prior to treatment yet. Therefore, this proof-of-concept study was performed to compare protein expression profiles of patients with radio-recurrent PCA to patients treated with primary radical prostatectomy separated by Gleason risk groups. We hypothesized that radio-recurrent PCA have a similar protein expression as high-risk Gleason PCA. ***Methods:*** Patient cohorts consisted of (i) 31 patients treated with salvage prostatectomy for locally recurrent PCA after primary radiotherapy and (ii) 94 patients treated with primary prostatectomy split into a Gleason high-risk (≥4 + 3; $n = 42$ [44.7%]) versus a low-risk group (≤3 + 4; $n = 52$ [55.3%]). Immunohistochemistry was performed using 15 antibodies with known association to radioresistance in PCA in vitro. ELISA was used for validation of selected markers in serum. ***Results:*** Androgen receptor (AR) was overexpressed in most radio-recurrent PCA (89.7%) and in most primary high-risk Gleason PCA (87.8%; $p = 0.851$), while only 67.3% of the low-risk group showed an expression ($p = 0.017$). Considering the highest Gleason pattern in primary PCA, aldo-keto reductase family 1 member C3 (AKR1C3) was most similarly expressed by patients with radio-recurrent PCA and patients with Gleason patterns 4 and 5 ($p = 0.827$ and $p = 0.893$) compared to Gleason pattern 3 ($p = 0.20$). These findings were supported by ELISA. ***Conclusion:*** This is the first study to evaluate protein markers in order to predict radioresistance in PCA. Our results point to AR and AKR1C3 as the most promising markers that might help stratify patients for radiotherapy.

Introduction

Localized prostate cancer (PCA) can be treated surgically with radical prostatectomy, external beam radiation therapy (EBRT) based on intensity-modulated radiation therapy (IMRT), and image-guided radiation therapy (IGRT), brachytherapy, or a combination of both according to current guidelines [1]. Based on the Surveillance, Epidemiology, and End Results (SEER) database, patients

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with localized PCA were treated with radiotherapy in 38%, with radical prostatectomy in 38%, and with an expectant management in 24% in the USA between 2004 and 2013 [2]. Important for the long-term oncological outcome is the prevention of biochemical recurrence (BCR). Efforts have been made to reduce BCR after radiotherapy by implementing IMRT, IGRT, and dose-escalation strategies [3–6]. However, the locally applicable radiation dose is limited to ~80 Gy due to toxicity [7]. Local recurrence following curative intended radiotherapy of localized PCA is frequent. Within 10 years post-radiotherapy, about 40–60% of patients develop BCR, while the long-term results of more recent studies like the CHHiP trial are pending [5, 8]. Reasons for these local relapses are still unknown and might be due to radioresistant PCA cell clones or inadequately performed radiotherapy [9]. Although relapses after radiotherapy are common, there are no clinical models or markers which are feasible for daily practice to select patients precisely at high risk for radioresistance. So far, only in vitro models were investigated to identify markers associated with radioresistance.

The aim of this proof-of-concept study was to evaluate different patterns of protein expression, which have previously been described in cell culture and xenograft models [10–26]. Expression levels of human radioresistant PCA were compared with Gleason scores of primary PCA specimens in order to identify biomarkers indicating radioresistance. Our hypothesis was a high concordance in the expression profiles between the group of radio-recurrent PCA patients and patients from the group of primary PCA with a high-grade Gleason score (high risk) (see online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000509447) as Gleason score >7 has been shown to be significantly related to poor biochemical relapse-free survival, local relapse, and overall mortality [27].

Patients and Methods

Patients

We evaluated patients with localized PCA who had undergone retropubic open salvage radical prostatectomy (SRP) with pelvic lymphadenectomy at the University Hospital of Cologne due to local recurrence after primary radiotherapy. We included 31 consecutive patients between December 2015 and July 2017. The previous radiotherapy was performed outside of the University Hospital. Patients with further adjuvant therapy (i.e., antiandrogen therapy) were not included. Recurrence was confirmed either by MRI-guided fusion biopsy or by transperineal saturation biopsy prior to surgery. Distant metastases were ruled out using ^{68}GaPSMA-PET-CT. Localization of recurrence within the prostate was determined by means of mpMRI and/or PSMA-PET-CT. The control group was treated by primary radical prostatectomy (PRP) and extended pelvic lymphadenectomy between 2012 and 2013. We included 94 consecutive patients. All PCA specimens were reevaluated by 2 experienced uro-pathologists (MW and SS). TNM classification and the Gleason scores (except after radiotherapy) were assigned according to the updated ISUP consensus from 2014 [28, 29]. Gleason risk groups were defined as low risk according to Gleason groups 1–2 (Gleason score ≤3 + 4) and high risk for Gleason groups 3–5 (Gleason score ≥4 + 3) [28]. All experimental protocols in the study were approved by the local ethical committee of the University Hospital of Cologne (ethical approval number 17-426) and performed according to the Declaration of Helsinki.

Immunohistochemistry

Tissue microarrays of the prostatectomy specimens were constructed with 6 cores from different tumor areas for PRP and 3 cores for SRP. Immunohistochemistry was performed using 15 antibodies with known association to radioresistance in PCA in vitro on an automated staining system. Primary and secondary antibodies are listed in online suppl. Table 1.

The prerequisite for inclusion in the analysis was a homogenous staining intensity. Semiquantitative assessment was applied for cytoplasmic staining, considering 0 as negative (<25%), 1 as weak (25–50%), 2 as moderate (50–70%), and 3 as strong (>75%). Nuclear staining was distinguished as negative = 0 or positive = 1. In general, the highest staining intensity was reported. Evaluation of tissue staining was performed by 2 experienced pathologists (SS and MW) who were blinded concerning prior treatment. If scoring discrepancies occurred, the cases were discussed for consensus. In a second analysis, the SRP group was compared to the PRP group only considering the highest Gleason pattern. More detailed information is available in online suppl. 1.)

ELISA in Serum Samples

For ELISA, serum samples of patients immediately prior to PRP (low risk, $n = 11$; high risk, $n = 24$) and SRP ($n = 24$), and those of controls ($n = 7$) without malignant disease were analyzed. More detailed information is available in online suppl. 1.

Statistical Analysis

Values were expressed as median plus interquartile range. Continuous data were compared using the 2-tailed Mann-Whitney *U* test, while χ^2 test was used to compare categorical parameters. One-way ANOVA and Bonferroni correction were used to analyze the different ELISA groups.

Kruskal-Wallis test was used for multiple group comparison in order to identify radio-recurrent PCA patients with similar protein expression as patients in the subgroup of primary Gleason high-risk PCA. The test statistics were adjusted for ties. Dunn-Bonferroni test was used as a post hoc test to analyze all pairs of each analysis to detect intergroup differences. Cohen's *d* was used to calculate the intergroup effect size [30]. Correlations of ≤0.10, 0.20, and ≥0.30 were considered as relatively small, typical, and relatively large, respectively [31]. Values <0.1 indicate group equalities, and values >0.3 indicate differences between the groups. All tests were 2-sided. A p value of <0.05 was considered significant. Statistical calculations were performed using SPSS 24.0 (SPSS) and Prism 7 (GraphPad Software).

Table 1. Patient characteristics of the group of radioresistant patients (left column) and the control group treated with primary prostatectomy (middle column)

Characteristic	Radio-recurrent, *n* (%)		PRP, *n* (%)	*p* value
	before RT	SRP		
Patients, *n*	31		94	
Median age at initial diagnosis, years	62 (46–75)	–	68.0 (49–90)	0.125
Median iPSA, ng/μL	8.5 (2.0–22.9)	–	7.8 (1.7–68.2)	0.702
	–	5.8 (0.8–17.6)		**0.002**
ISUP grade group, Gleason score				
1 (3+3)	13 (41.9)	–	17 (18.1)	**0.033**
2 (3+4)	9 (29.0)	–	36 (38.3)	
3 (4+3)	7 (22.6)	–	18 (19.1)	
4 (8)	2 (6.5)	–	14 (14.9)	
5 (9–10)	0 (0)	–	9 (9.6)	
Gleason risk group				
Low risk (Gl. ≤3+4)	22 (71.0)	–	52 (55.3)	**<0.001**
High risk (Gl. ≥4+3)	9 (29.0)	–	42 (44.7)	
pT				
≤2b	–	5 (16.1)	7 (7.4)	0.155
≥2c	–	26 (83.9)	87 (92.6)	
pN[a]				
0	–	15 (71.4)	75 (81.5)	0.301
1	–	6 (28.6)	17 (18.5)	
pL				
0	–	24 (77.4)	86 (91.5)	**0.037**
1	–	7 (22.6)	8 (8.5)	
pV				
0	–	28 (90.3)	92 (97.9)	0.063
1	–	3 (9.7)	2 (2.1)	
PNI				
0	–	5 (16.1)	40 (42.6)	0.008
1	–	26 (83.9)	54 (57.4)	
R				
0	–	25 (80.6)	68 (72.3)	0.343
1	–	6 (19.4)	26 (27.7)	

SD, standard deviation; iPSA, initial prostate-specific antigen; ISUP, International Society of Urological Pathology; pT, primary tumor; pN, regional lymph node; pL, invasion into lymphatic vessels; pV, invasion into vein; PNI, perineural invasion; R, residual tumor; SRP, salvage radical prostatectomy; PRP, primary radical prostatectomy. Significant *p* values are highlighted in bold. [a] The remainder did not undergo LAD.

Results

Patient Characteristics

We compared a collective of patients with local recurrence of PCA who had undergone SRP following primary radiation therapy and had not received any adjuvant androgen deprivation therapy (n = 31), to PCA patients treated primarily with radical prostatectomy (n = 94) as a control group (Table 1). The patients of the radiation therapy collective had received treatment for PCA between 2003 and 2015: 15 patients had undergone three-dimensional (3D) conformal, CT-guided EBRT (median dose: 75 Gy; range: 74–78 Gy), 9 patients had received EBRT plus temporary brachytherapy (median dose: 88 Gy; range: 83–92 Gy), 7 patients were treated by seed implantation (median dose: 142 Gy; range: 136–151 Gy). A

Table 2. Comparison of different staining intensities between the groups of primary PCA, subdivided by Gleason scoring into low- and high-risk groups, and radioresistant PCA using the Kruskal-Wallis test

Expression	Group	Gleason risk group	TMA *n* (evaluable patients)	Staining intensity				*p* value
				0 % (*n*)	1 % (*n*)	2 % (*n*)	3 % (*n*)	
AKR1C3	Primary PCA	Low risk	47	68.1 (32)	–	–	31.9 (15)	
		High risk	34	50.0 (17)	–	–	50.0 (17)	0.230
	Radio-recurrent PCA		31	54.8 (17)	–	–	45.2 (14)	
ALDOA	Primary PCA	Low risk	50	44.0 (22)	–	54.0 (27)	2.0 (1)	
		High risk	39	43.6 (17)	–	35.9 (14)	20.5 (8)	**0.001**
	Radio-recurrent PCA		29	20.7 (6)	–	34.5 (10)	44.8 (13)	
AR	Primary PCA	Low risk	52	32.7 (17)	–	–	67.3 (35)	
		High risk	41	12.2 (5)	–	–	87.8 (36)	**0.016**
	Radio-recurrent PCA		29	10.3 (3)	–	–	89.7 (26)	
BCL2	Primary PCA	Low risk	52	94.2 (49)	–	–	5.8 (3)	
		High risk	41	85.4 (35)	–	–	14.6 (6)	0.295
	Radio-recurrent PCA		30	93.3 (28)	–	–	6.7 (2)	
BIRC5	Primary PCA	Low risk	52	11.5 (6)	–	–	88.5 (46)	
		High risk	41	4.9 (2)	–	–	95.1 (39)	0.312
	Radio-recurrent PCA		29	3.4 (1)	–	–	96.6 (28)	
CAV-1	Primary PCA	Low risk	51	39.2 (20)	–	–	60.8 (31)	
		High risk	38	44.7 (17)	–	–	55.3 (21)	**0.003**
	Radio-recurrent PCA		31	12.9 (4)	–	77.4 (24)	9.7 (3)	
CLU	Primary PCA	Low risk	52	55.8 (29)	–	30.8 (16)	13.5 (7)	
		High risk	41	43.9 (18)	–	31.7 (13)	24.4 (10)	0.092
	Radio-recurrent PCA		30	26.7 (8)	–	56.7 (17)	16.7 (5)	
CXCR4	Primary PCA	Low risk	50	54.0 (27)	–	–	46.0 (23)	
		High risk	39	43.6 (17)	–	–	56.4 (22)	**0.012**
	Radio-recurrent PCA		30	20.0 (6)	–	–	80.0 (24)	
CyclinD1	Primary PCA	Low risk	51	9.8 (5)	–	–	90.2 (46)	
		High risk	41	9.8 (4)	–	–	90.2 (37)	0.876
	Radio-recurrent PCA		30	6.7 (2)	–		93.3 (28)	
DAB2IP	Primary PCA	Low risk	50	6.0 (3)	10.0 (5)	34.0 (17)	50.0 (25)	
		High risk	38	0	7.9 (3)	42.1 (16)	50.0 (19)	**0.029**
	Radio-recurrent PCA		29	0	0	24.1 (7)	75.9 (22)	
HBP1	Primary PCA	Low risk	52	71.2 (37)	–	–	28.8 (15)	
		High risk	41	56.1 (23)	–	–	43.9 (18)	0.101
	Radio-recurrent PCA		29	48.3 (14)	–	–	51.7 (15)	

Table 2 (continued)

Expression	Group	Gleason risk group	TMA *n* (evaluable patients)	Staining intensity				*p* value
				0 % (*n*)	1 % (*n*)	2 % (*n*)	3 % (*n*)	
HIF1a	Primary PCA	Low risk	48	50.0 (24)	–	–	50.0 (24)	
		High risk	38	47.4 (18)	–	–	52.6 (20)	0.470
	Radio-recurrent PCA		28	35.7 (10)	–	–	64.3 (18)	
LDH5	Primary PCA	Low risk	48	12.5 (6)	–	–	87.5 (42)	
		High risk	32	3.1 (1)	–	–	96.9 (31)	0.316
	Radio-recurrent PCA		31	12.9 (4)	–	–	87.1 (27)	
PIK3R1	Primary PCA	Low risk	47	85.1 (40)	–	–	14.9 (7)	
		High risk	33	84.8 (28)	–	–	15.2 (5)	0.684
	Radio-recurrent PCA		32	84.8 (28)	–	–	15.2 (5)	
PTEN	Primary PCA	Low risk	50	30.0 (15)	8.0 (4)	18.0 (9)	44.0 (22)	
		High risk						0.46
	Radio-recurrent PCA		29	31.0 (9)	3.4 (1)	6.9 (2)	58.6 (17)	

PCA, prostate cancer; TMA, tissue microarray; AKR1C3, aldo-keto reductase family 1 member C3; AR, androgen receptor. Significant *p* values are highlighted in bold.

median time period of 43 months (range 12–167 months) had passed after completion of radiotherapy (or the date of seed implantation) and the diagnosis of PCA recurrence.

Initial PSA levels did not differ significantly (prior radiotherapy 8.5 ng/μL vs. prior PRP 7.8 ng/μL; $p = 0.702$), whereas the median PSA prior to SRP was significantly lower than that prior to PRP (5.8 vs. 7.8 ng/μL; $p = 0.002$). Comparing Gleason scores of primary needle biopsies of the SRP patients prior to radiotherapy with prostate specimens in PRP patients showed more advanced stages in the PRP group ($p = 0.033$). Further tumor characteristics analyzed on the prostatectomy specimen regarding pT status, pN status, and R status showed mainly similar findings in the PRP and SRP groups (Table 1).

Immunohistochemical Analysis

First, staining characteristics between radio-recurrent PCA and primary PCA separated by Gleason risk groups were compared (Table 2). Kruskal-Wallis test showed significantly different staining intensities for ALDOA ($p = 0.001$), androgen receptor (AR) ($p = 0.016$), CAV-1 ($p = 0.003$), CXCR4 ($p = 0.012$), and DAB2IP ($p = 0.029$). However, the pairwise ratio for each significantly differentially expressed protein only showed a very small effect size for AR when comparing the PRP high-risk group and the group of radio-recurrent PCA (Cohen's *d*: 0.05). This indicates similarities between both groups, while the low-risk Gleason subgroup and radio-recurrent PCA group showed a medium effect size (Cohen's *d*: 0.55), indicating a significant difference between these groups (Table 3). The other proteins were more similarly expressed in the low- and high-risk groups but different in the radio-recurrent PCA group. The corresponding immunostainings are provided in Figure 1a.

Next, the staining characteristics between radio-recurrent PCA and the highest Gleason pattern of each PRP specimen were compared (Table 4). Kruskal-Wallis test showed significantly different staining intensities for ALDOA ($p = 0.001$), aldo-keto reductase family 1 member C3 (AKR1C3) ($p = 0.047$), CAV-1 ($p = 0.008$), and CXCR4 ($p = 0.033$). However, the post hoc test to calculate pairwise ratios for each significant differentially expressed protein only showed a small effect size for Gleason patterns 4 and 5 compared to the cohort of radio-recurrent PCA for AKR1C3 (Cohen's *d*: 0.05 and 0.10), indicating similarity, whereas for the Gleason pattern 3 subgroup and radio-recurrent PCA group, only a medi-

Table 3. Pairwise ratio analysis using the Dunn-Bonferroni test was performed on immunostainings which showed significant results for the Kruskal-Wallis test (Table 2)

Expression	Kruskal-Wallis	Pairwise ratio (*p* value) (effect size by Cohen's *d*)		
		low vs. high	low vs. recurrence	high vs. recurrence
ALDOA	**0.001**	0.304 (0.22)	<0.001 (0.85)	0.011 (0.63)
AR	**0.016**	0.015 (0.51)	0.017 (0.55)	0.851 **(0.05)**
CAV-1	**0.003**	0.606 (0.11)	0.004 (0.72)	0.002 (0.70)
CXCR4	**0.012**	0.326 (0.21)	0.003 (0.69)	0.05 (0.48)
DAB2IP	**0.029**	0.724 (0.08)	0.011 (0.60)	0.035 (0.52)

Cohen's *d* indicates the intergroup effect size (≤0.10 = relatively small, 0.20 = typical, ≥0.30 = relatively large). Only expression levels of AR were similar between radio-recurrent and Gleason high-risk patients. AR, androgen receptor. Significant *p* values are highlighted in bold.

um effect size (Cohen's *d*: 0.69) was observed, which indicates a subgroup difference (Table 5). Immunostainings of AKR1C3 are shown in Figure 1a.

Validation of AR and AKR1C3 Using ELISA

Considering AR, we found similar expression levels for SRP and Gleason high-risk PRP but significant differences between SRP and Gleason low-risk PRP ($p = 0.002$) and the tumor-free control group ($p = 0.031$) (Fig. 1b). Findings for AKR1C3 were similar. Expression levels for SRP and Gleason high-risk PRP were comparable. However, significant differences were revealed for SRP and Gleason low-risk PRP ($p = 0.021$) and the tumor-free control group ($p = 0.002$) (Fig. 1c).

Discussion

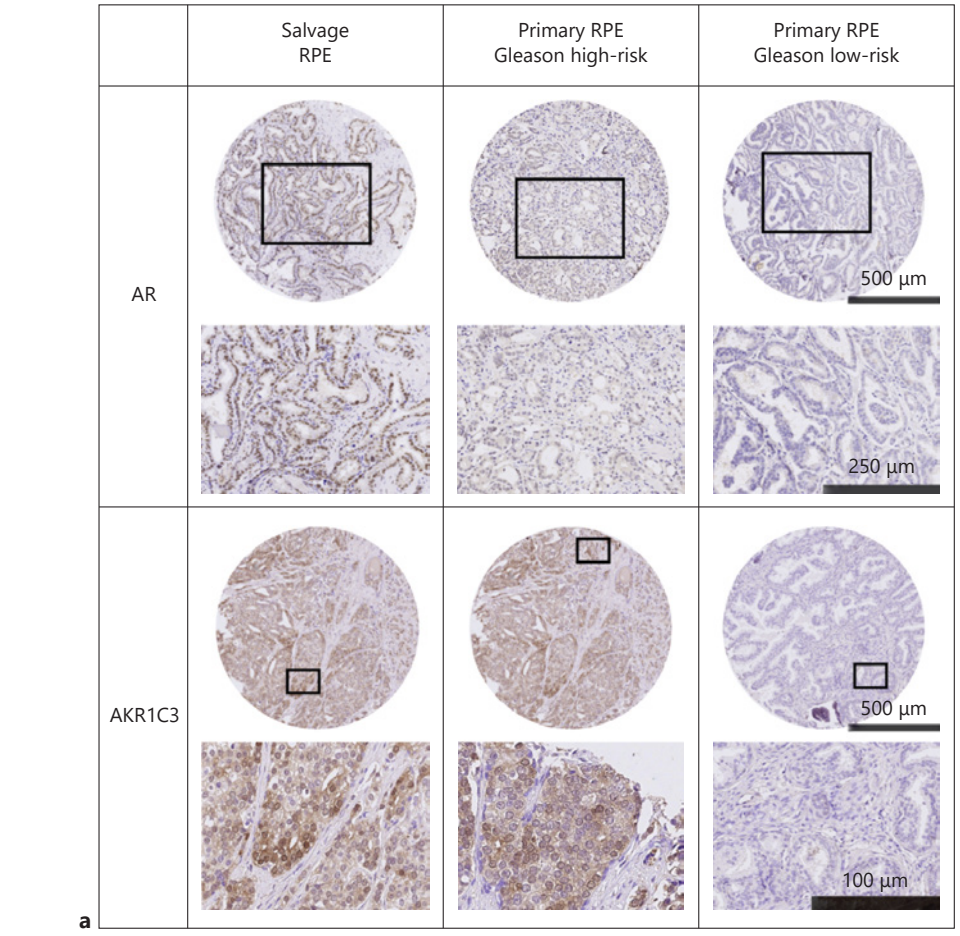
In order to reduce the risk of local recurrence, identification of a preexisting, intrinsic radioresistance in PCA would be of utmost importance for the selection of the optimal treatment strategy for localized PCA in terms of a personalized therapy. In this study, we investigated expression levels of 15 different proteins in order to identify a biomarker to predict radiation-resistant PCA. These proteins were previously described in vitro. We compared PCA tissue of patients who had undergone SRP due to local recurrence after radiotherapy to a control group of PCA patients who had undergone PRP. We found that radio-recurrent PCA at the time of SRP more closely mirrors primary aggressive PCA.

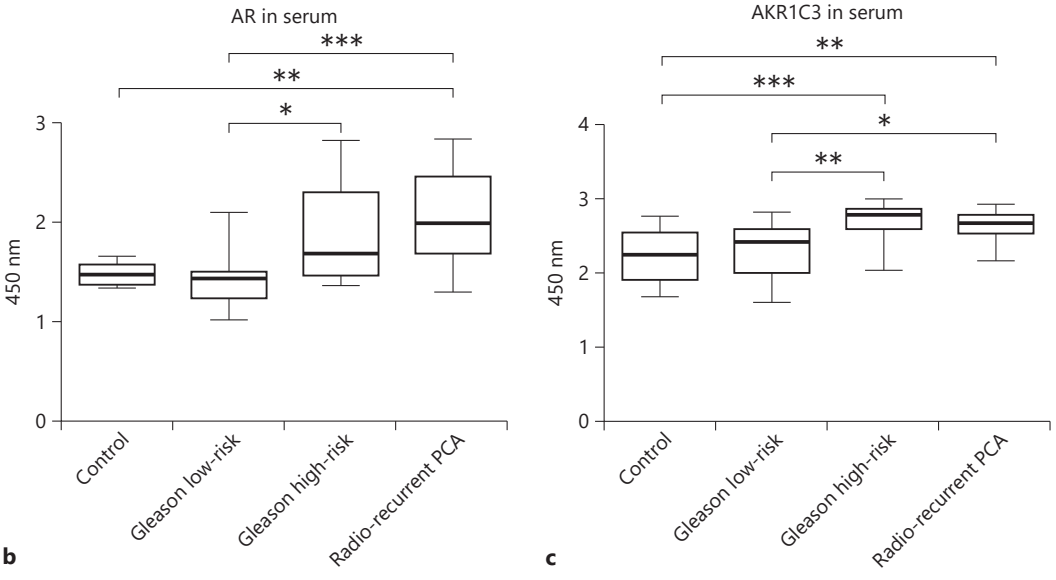
Protein expression analysis demonstrated that AR overexpression in most radio-recurrent PCA closely resembled the majority of the PCA of the high-risk Gleason group but only a smaller subgroup of patients in the low-risk Gleason group. AR is known to be significantly upregulated in more aggressive PCA with Gleason scores ≥3 + 4 compared to a low-risk Gleason score [32]. Previously it has been shown that upregulated AR led to an enhanced transcription of genes that are involved in DNA repair like AR cofactor PARP 1, homologous recombination (RAD54B and RAD51C), mismatch repair (MSH2 and MSH6), or the Fanconi pathway (FANCI, FANCC, and USP1). Consequently, second-generation antiandrogen therapy results in downregulation of DNA repair genes [33]. This finding is consistent with the time-honored clinical knowledge that in high-risk prostate cancer patients, the 10-year recurrence risk is significantly reduced if radiotherapy is combined with long-term antiandrogen therapy when compared with radiotherapy alone [34–36]. Paximadis et al. [13] identified AR as a downstream target of *PTEN* and *PDGF D* and demonstrated the relevance of AR leading to radioresistance in LNCaP cells, which could be blocked by enzalutamide. Also, these preclinical findings underline the fact that androgen deprivation therapy increases the efficacy of radiotherapy in intermediate, high-risk, and locally advanced PCA [34–36]. In line, it has to be assumed that patients with AR overexpression already prior to radiotherapy are at risk for radioresistance as proposed by our study.

Comparing radio-recurrent PCA and primary PCA separated by the highest Gleason patterns showed a significant correlation for AKR1C3, but the group differences were less distinct than those of the Gleason risk groups. Only a tendency for different expression levels was noted between the RPE low-risk group and radioresistant PCA group. AKR1C3 overexpression has been re-

Fig. 1. a Immunostainings of AR and AKR1C3 for SRP and PRP cancer tissue separated by Gleason high risk and low risk. Overviews are in 5-fold magnification, details as shown. ELISA of AR (**b**) and AKR1C3 (**c**) on serum prior to PRP (separated by Gleason high risk and low risk), SRP, and a control group without malignant disease. Data are presented as mean ± standard deviation (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; 1-way ANOVA, Bonferroni post hoc comparisons). AR, androgen receptor; AKR1C3, aldo-keto reductase family 1 member C3; SRP, salvage radical prostatectomy; PRP, primary radical prostatectomy; PCA, prostate cancer.

(For figure see next page.)





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Color version available online

Table 4. Comparison of different staining intensities between the groups of primary PCA, which were separated by the individual highest Gleason patterns (3–5), and the radio-recurrent PCA using the Kruskal-Wallis test

Expression	Group	Highest Gleason pattern	TMA *n* (evaluable patients)	Staining intensity				*p* value
				0 % (*n*)	1 % (*n*)	2 % (*n*)	3 % (*n*)	
AKR1C3	Primary PCA	Pattern 3	18	88.9 (16)	–	–	11.1 (2)	**0.047**
		Pattern 4	61	52.5 (32)	–	–	47.5 (29)	
		Pattern 5	2	50 (1)	–	–	50 (1)	
	Radio-recurrent PCA		31	54.8 (17)	–	–	45.2 (14)	
ALDOA	Primary PCA	Pattern 3	21	57.1 (12)	–	42.9 (9)	–	**0.001**
		Pattern 4	65	28.5 (25)	–	47.7 (31)	13.8 (9)	
		Pattern 5	3	66.7 (2)	–	33.3 (1)	–	
	Radio-recurrent PCA		29	20.7 (6)	–	34.5 (10)	44.8 (13)	
AR	Primary PCA	Pattern 3	17	35.3 (6)	–	–	64.7 (11)	0.199
		Pattern 4	74	21.6 (16)	–	–	78.4 (58)	
		Pattern 5	2	0	–	–	100 (2)	
	Radio-recurrent PCA		29	10.3 (3)	–	–	89.7 (26)	
BCL2	Primary PCA	Pattern 3	18	100 (18)	–	–	0	0.210
		Pattern 4	72	88.9 (64)	–	–	11.1 (8)	
		Pattern 5	3	66.7 (2)	–	–	33.3 (1)	
	Radio-recurrent PCA		30	93.3 (28)	–	–	6.7 (2)	
BIRC5	Primary PCA	Pattern 3	18	11.1 (2)	–	–	88.9 (16)	0.718
		Pattern 4	72	8.3 (6)	–	–	91.7 (66)	
		Pattern 5	3	–	–	–	100 (3)	
	Radio-recurrent PCA		29	3.4 (1)	–	–	96.6 (28)	
CAV-1	Primary PCA	Pattern 3	19	36.8 (7)	–	63.2 (12)	–	**0.008**
		Pattern 4	67	43.3 (29)	–	56.7 (38)	–	
		Pattern 5	3	33.3 (1)	–	66.7 (2)	–	
	Radio-recurrent PCA		31	12.9 (4)	–	77.4 (24)	9.7 (3)	
CLU	Primary PCA	Pattern 3	17	41.2 (7)	–	35.3 (6)	23.5 (4)	0.235
		Pattern 4	73	53.4 (39)	–	30.1 (22)	16.4 (12)	
		Pattern 5	3	33.3 (1)	–	33.3 (1)	33.3 (1)	
	Radio-recurrent PCA		30	26.7 (8)	–	56.7 (17)	16.7 (5)	
CXCR4	Primary PCA	Pattern 3	19	42.1 (8)	–	–	57.9 (11)	**0.033**
		Pattern 4	67	50.7 (34)	–	–	49.3 (33)	
		Pattern 5	3	66.7 (2)	–	–	33.3 (1)	
	Radio-recurrent PCA		30	20.0 (6)	–	–	80.0 (24)	

Table 4 (continued)

Expression	Group	Highest Gleason pattern	TMA *n* (evaluable patients)	Staining intensity				*p* value
				0 % (*n*)	1 % (*n*)	2 % (*n*)	3 % (*n*)	
CyclinD1	Primary PCA	Pattern 3	20	10.0 (2)	–	–	90.0 (18)	0.891
		Pattern 4	69	10.1 (7)	–	–	89.9 (62)	
		Pattern 5	3	100 (3)	–	–	0	
	Radio-recurrent PCA		30	6.7 (2)	–	–	93.3 (28)	
DAB2IP	Primary PCA	Pattern 3	19	5.3 (1)	10.5 (2)	36.8 (7)	47.4 (9)	0.068
		Pattern 4	66	3.0 (2)	7.6 (5)	39.4 (26)	50.0 (33)	
		Pattern 5	3	–	33.3 (1)	–	66.7 (2)	
	Radio-recurrent PCA		29	–	–	24.1 (7)	75.9 (22)	
HBP1	Primary PCA	Pattern 3	18	61.1 (11)	–	–	28.9 (7)	0.22
		Pattern 4	72	68.1 (49)	–	–	31.9 (23)	
		Pattern 5	3	33.3 (1)	–	–	66.7 (2)	
	Radio-recurrent PCA		29	48.3 (14)	–	–	51.7 (15)	
HIF1a	Primary PCA	Pattern 3	27	51.9 (14)	–	–	48.1 (13)	0.473
		Pattern 4	58	48.3 (28)	–	–	51.7 (30)	
		Pattern 5	1	0	–	–	100 (1)	
	Radio-recurrent PCA		28	35.7 (10)	–	–	64.3 (18)	
LDH5	Primary PCA	Pattern 3	26	15.4 (4)	–	–	84.6 (22)	0.504
		Pattern 4	53	5.7 (3)	–	–	94.3 (50)	
		Pattern 5	1	0	–	–	100 (1)	
	Radio-recurrent PCA		31	12.9 (4)	–	–	87.1 (27)	
PIK3R1	Primary PCA	Pattern 3	20	80.0 (16)	–	–	20.0 (4)	0.690
		Pattern 4	60	85.0 (51)	–	–	9.0 (9)	
		Pattern 5	0	0	–	–	0	
	Radio-recurrent PCA		31	78.1 (25)	–	–	21.9 (7)	
PTEN	Primary PCA	Pattern 3	20	30.0 (6)	10.0 (2)	25.0 (5)	35.0 (7)	0.456
		Pattern 4	65	35.4 (23)	9.2 (6)	12.3 (8)	43.1 (28)	
		Pattern 5	3	–	–	33.3 (1)	66.7 (2)	
	Radio-recurrent PCA		29	31.0 (9)	3.4 (1)	6.9 (2)	58.6 (17)	

PCA, prostate cancer; AR, androgen receptor; AKR1C3, aldo-keto reductase family 1 member C3; TMA, tissue microarray. Significant *p* values are highlighted in bold.

ported in some radioresistant cancers such as esophageal cancer or non-small-cell lung cancer [37, 38]. Consistently with these findings, AKR1C3 knockdown significantly enhanced radiosensitivity in cancer cells [37, 38]. In PCA, Sun et al. [10] observed that an overexpression of AKR1C3 is associated with radiation resistance by activation of the MAPK pathway, while inhibition of AKR1C3 restored the radiosensitivity.

Table 5. Pairwise ratio analysis using the Dunn-Bonferroni test was performed on those immunostainings which showed significant results for the Kruskal-Wallis test

Expression	Kruskal-Wallis	Pairwise ratio (p value) (effect size by Cohen's d)					
		3 vs. 4	3 vs. 5	3 vs. recurrence	4 vs. 5	4 vs. recurrence	5 vs. recurrence
AKR1C3	**0.047**	0.006 (0.74)	0.291 (0.79)	0.20 (0.69)	0.945 (0.58)	0.827 **(0.05)**	0.893 **(0.10)**
ALDOA	**0.001**	0.078 (0.44)	0.815 (0.14)	<0.001 (1.07)	0.32 (0.59)	0.005 (0.63)	0.045 (1.21)
CAV-1	**0.008**	0.620 (0.13)	0.910 (0.07)	0.036 (0.61)	0.736 (0.20)	0.001 (0.74)	0.372 (0.54)
CXCR4	**0.033**	0.502 (0.17)	0.425 (0.50)	0.128 (0.45)	0.586 (0.32)	0.005 (0.62)	0.120 (0.94)

Cohen's d indicates the intergroup effect size (≤0.10 = relatively small, 0.20 = typical, and ≥0.30 = relatively large). "3," "4," and "5" indicate the highest Gleason patterns (Table 4). Only the expression levels of AKR1C3 were similar between radio-recurrent and Gleason pattern (4 and 5) patients. AKR1C3, aldo-keto reductase family 1 member C3. Significant p values are highlighted in bold.

Remarkably, most of the studied proteins did not show significant group differences. Reasons might include that our analysis was conducted at the protein level, whereas most in vitro analyses were based on mRNA. Additionally, highly controlled and uniform condition in vitro models are often not directly transferable to heterogenous human tissue.

Validation of the most promising proteins AR and AKR1C3 using ELISA on serum samples supported our results. Again, AR and AKR1C3 were similarly enriched in PRP high-risk patients and SRP compared to PRP low-risk patients and tumor-free controls.

Taking into account patient characteristics, the majority of patients from the SRP group were in the low-risk Gleason group considering their initial prostate biopsy. However, it is likely possible that these patients have been understaged. Most patients of this group were initially diagnosed based on conventional sextant biopsies, which might have missed high-risk clones within a PCA, in contrast to current and more accurate multiparametric MRI fusion biopsies [39]. Additionally, discordance between needle biopsies and histological findings after RPE is up to 40% and, therefore, only comparable to a limited extent [40]. It must also be noted that radiation of the patients was carried out in external institutes, and possibly today's standards in radiation, such as IGRT or IMRT, were not used at that time. Therefore, it is at least conceivable that today's radiation methods for the treatment of PCA are more efficient and effective.

In vivo research on radioresistant PCA has its natural limitations. Studies based on tumor specimens obtained for initial diagnosis bare the risk that the radioresistant PCA cell clones are not represented in the biopsies. Currently, studying PCA after radiation therapy seems to be the only feasible way to gain deeper insight into radioresistant PCA in human tissue, at least regarding the screening for different markers. Another limitation might be an inappropriate application of radiation therapy, especially by omitting parts of the cancer located at regions of functional importance like the urethra [41]. In those patients, it is impossible to discriminate whether their PCA reoccurred due to a resistant clone or inappropriate treatment.

This is the first study investigating expression levels of different proteins in order to identify a predictive biomarker for radiation-resistant PCA in human tissue. By comparing radiation-resistant PCA in SRP specimens with primary RPE specimens, we identified AR and AKR1C3 as the most promising markers for PCA at high risk of recurrence after radiotherapy.

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Statement of Ethics

All experimental protocols in the study were approved by the local ethical committee of the University Hospital of Cologne (17-426) and performed according to the Declaration of Helsinki.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest to disclose.

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Author Contributions

Tim Nestler: designed the research study, analyzed the data, interpreted the data, and wrote the paper. Maike Wittersheim: evaluated tissue staining and contributed to critical revision of the paper. Stephan Schäfer: evaluated tissue staining and contributed to critical revision of the paper. Martin Hellmich: analyzed the data and contributed to critical revision of the paper. David Pfister: designed the research study and contributed to critical revision of the paper. Margarete Odenthal: contributed to establish new antibodies, critical revision of the paper. Melanie von Brandenstein: performed ELISA and contributed to critical revision of the paper. Reinhard Büttner: contributed to establishing new antibodies and critical revision of the paper. Axel Heidenreich: designed the research study, interpreted the data, and contributed to critical revision of the paper.

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