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Expression Patterns and Corepressor Function of Retinoic Acid-induced 2 in Prostate Cancer

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BACKGROUND: Revealing molecular mechanisms linked to androgen receptor activity can help to improve diagnosis and treatment of prostate cancer. Retinoic acid-induced 2 (RAI2) protein is thought to act as a transcriptional coregulator involved in hormonal responses and epithelial differentiation. We evaluated the clinical relevance and biological function of the RAI2 protein in prostate cancer.

METHODS: We assessed *RAI2* gene expression in the Cancer Genome Atlas prostate adenocarcinoma PanCancer cohort and protein expression in primary tumors (n = 199) by immunohistochemistry. We studied *RAI2* gene expression as part of a multimarker panel in an enriched circulating tumor cell population isolated from blood samples (n = 38) of patients with metastatic prostate cancer. In prostate cancer cell lines, we analyzed the consequences of androgen receptor inhibition on RAI2 protein expression and the consequences of RAI2 depletion on the expression of the androgen receptor and selected target genes.

RESULTS: Abundance of the RAI2 protein in adenocarcinomas correlated with the androgen receptor; keratins 8, 18, and 19; and E-cadherin as well as with an early biochemical recurrence. In circulating tumor cells, detection of *RAI2* mRNA significantly correlated with gene expression of *FOLH1*, *KLK3*, *RAI2*, *AR*, and *AR-V7*. In VCaP and LNCaP cell lines, sustained inhibition of hormone receptor activity induced the RAI2 protein, whereas RAI2 depletion augmented the expression of *MME*, *STEAP4*, and *WIPI1*.

CONCLUSIONS: The RAI2 protein functions as a transcriptional coregulator of the androgen response in prostate cancer cells. Detection of *RAI2* gene expression in blood samples from patients with metastatic prostate cancer indicated the presence of circulating tumor cells.

Introduction

Prostate cancer represents the most common cancer type in men, with approximately 400 000 new cases in Europe each year (1). In advanced stages, androgen deprivation therapy is a central treatment component of this hormone-sensitive disease. However, despite convincing initial responses, the vast majority of patients develop resistance to androgen deprivation therapy within 18 to 24 months and move into the castration resistant stage (2). Meaningful improvement has been achieved in the past 15 years with significant prolongation of overall survival by docetaxel, cabazitaxel, abiraterone, enzalutamide, and radium 223 in randomized Phase 3 trials. Nevertheless, life expectancy of these patients is limited with decreased response rates with each additional treatment line (3).

Better definition of prognosis to determine treatment decisions is pivotal but still represents an unmet clinical need. In this regard, liquid biopsy testing based on analysis of circulating tumor cells (CTCs) could help to better inform medical decision-making so that patient outcomes are improved (4).

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The retinoic acid-induced 2 (*RAI2*) gene was first identified as a putative metastasis suppressive gene in patients with hormone receptor positive breast cancer. Low *RAI2* expression was significantly associated with early occurring bone micrometastasis and poor patient outcome (5). Molecular characterization of the RAI2 protein in the estrogen receptor-positive luminal breast cancer cell lines suggests that the RAI2 protein acts as a transcriptional coregulator involved in differentiation of hormone-dependent breast cancer cells and might play an active part in the transcriptional network of hormonal response (5). In animal studies, integration of the mouse mammary tumor virus into the *RAI2* gene locus has been found to be associated with the emergence of recurrent and hormone-independent breast tumors (6, 7), further indicating a possible role for RAI2 inactivation in the progression from hormone-dependent to hormone-independent tumors.

Even though breast and prostate cancer develop from organs of different anatomy and physiological functions, both organs need the sex steroid hormones estrogen or androgen not only for their development but also for the rise and progression of their hormone-dependent cancers (2). The development and maintenance of the prostate is dependent on androgen receptor (AR) activity. AR remains important in the development and progression of prostate cancer and the majority of androgen-independent or hormone refractory prostate cancers express AR (8). We therefore hypothesized that RAI2 might also play a role in hormone-dependent growth of prostate cancer and response to AR-targeting therapies.

In this study, we evaluated *RAI2* gene and protein expression in primary prostate tumors. In addition, we analyzed gene expression of *RAI2* and AR pathway–related genes in CTCs from patients with metastatic prostate cancer. Furthermore, we examined the consequences of RAI2 depletion on hormone response in AR positive prostate cancer cell lines.

Methods

A detailed description of methods used for immunohistochemistry, semiquantitative gene expression analysis in CTCs, quantitative RT-PCR analysis, cell culture, and Western blot experiments are provided in the online Supplemental Methods file. Validation of used RAI2-antiserum for use in immunohistochemistry is provided in Supplemental Fig. 1. Analytical specificity and sensitivity of PCR assays used for CTC analysis is provided in Supplemental Table 2.

Results

EXPRESSION OF RAI2 IN HUMAN PRIMARY PROSTATE TUMORS

To investigate the prognostic relevance of *RAI2* gene expression in prostate cancer progression, we first evaluated *RAI2* expression in the primary prostate adenocarcinoma cohort of the Cancer Genome Atlas (TCGA) PanCancer Atlas (9, 10). In this patient cohort (n = 488), the frequency of genetic alteration in *RAI2* was found in 1.6% of samples, and only one patient had a deletion of the RAI2 locus (Fig. 1, A). We further compared all available clinical attributes in the extreme quartile groups of *RAI2* mRNA expression. In tumors with low *RAI2* gene expression, we found significant increase in the Winter hypoxia score ($P = 2.009e{-5}$) (Fig. 1, B), the Ragnum hypoxia score ($P = 2.692e{-5}$) (Fig.1, C), and the Buffa hypoxia score ($P = 2.999e{-5}$) (Fig. 1, D). In addition, we found significant increase in fractions of altered genome ($P = 2.757e{-5}$) (Fig. 1, E), the mutation count ($P = 4.412e{-5}$) (Fig. 1, F), tumor mutational burden ($P = 9.356e{-4}$) (Fig. 1, G), and microsatellite instability score ($P = 7.758e{-3}$) (Fig. 1, H) in tumors with low *RAI2* gene expression. Taken together, we found evidence that low *RAI2* gene expression was a characteristic of both hypoxic and genomic unstable prostate tumors.

We further used preprocessed *RAI2* gene expression values of the TCGA PanCancer Atlas for survival analysis of patients with prostate adenocarcinoma. Again, samples were separated into high- and low-expression groups by using the extreme quartiles of *RAI2* gene expression and differences between these groups were determined by Kaplan–Meier estimates of survival and the log-rank test. We found no significant differences in overall, disease-specific, progression-free, or disease-free survival (Supplemental Fig. 2).

Next, we evaluated RAI2 protein expression in malignant prostate cancer tissues by immunohistochemical analysis of a tissue microarray consisting of samples from 199 patients, using a purified polyclonal RAI2-specific antiserum. For 162 patients, the RAI2 concentration could be evaluated successfully in cancer tissue. For 37 patients, there was a lack of cancer tissue or samples had to be excluded due to technical issues. There was an exclusively cytoplasmic RAI2 staining in epithelial cells without expression in associated stromal cells and lymphocytes. Most samples exhibited a slight RAI2 expression that might reflect a basal protein concentration (Fig. 2, B) while 25.9% lacked RAI2 protein (Fig. 2, A) and 39 out of 162 samples (24%) showed moderate (n = 24) (Fig. 2, C) to strong (n = 15) (Fig. 2, D) RAI2 expression. Statistical analysis revealed that high RAI2 protein abundance correlated with an early biochemical recurrence ($P = 2.4e{-3}$) (Fig. 2, E). Beyond this finding, there was no significant correlation with other established prognostic factors including testosterone, nodal status, and overall survival.

Additionally, we correlated RAI2 protein expression to previously established staining results of the same patient cohort (11–13). We found that RAI2 protein abundance was significantly correlated with

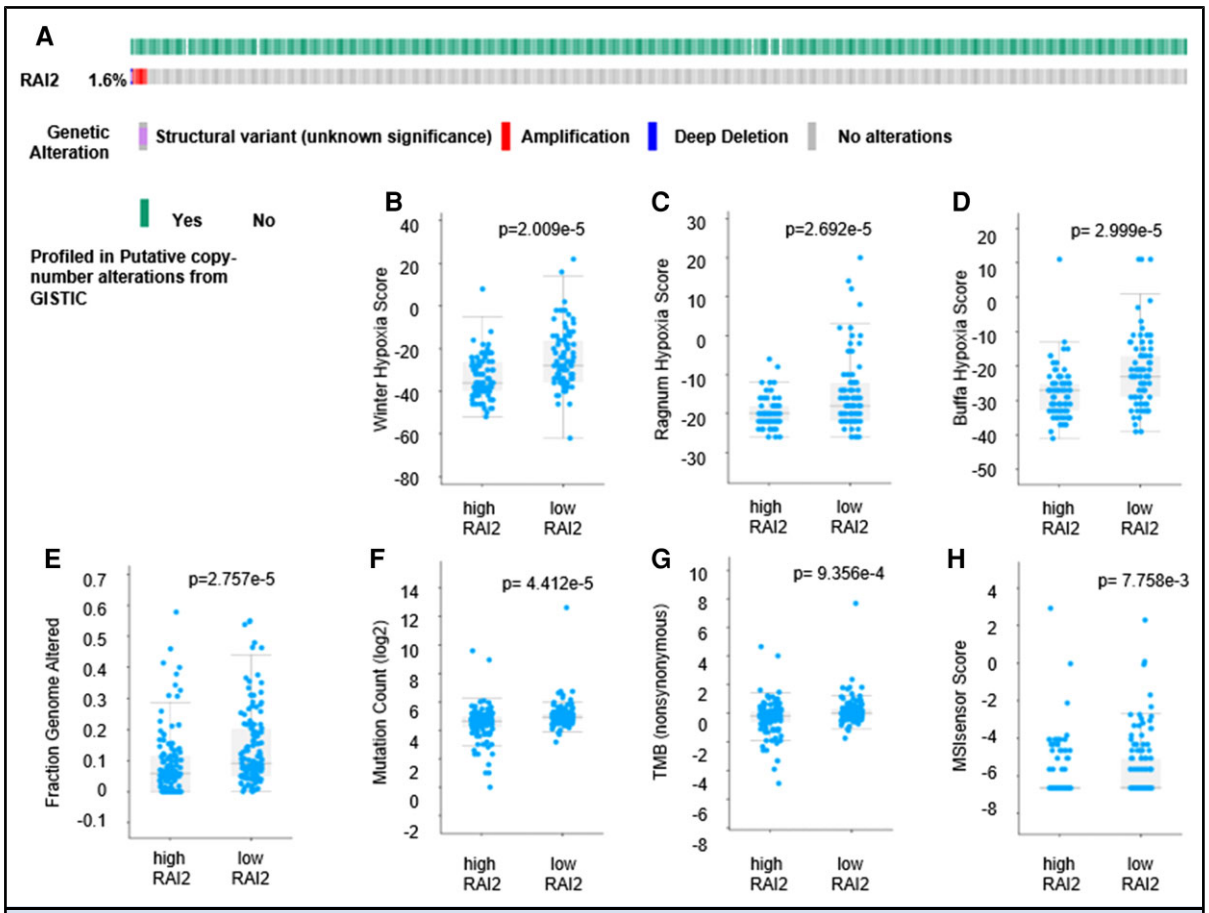

Fig. 1. Analysis of RAI2 gene expression in Prostate Adenocarcinoma TCGA PanCancer data set. (A), Oncoprint analysis summarizing genetic alterations in all samples of the RAI2 Prostate Adenocarcinoma TCGA PanCancer data set; (B–H), Comparative analysis of the indicated clinical and sample-related parameters in the extreme quartiles of RAI2 mRNA expression. *P* values were calculated with the Wilcoxon signed-rank test.

expression of keratin 8, 18, and 19 as well as with protein expression of E-cadherin and AR. We found no significant correlations between detection of the RAI2 protein and detection of EpCAM, ALDH1, EGFR, or apoptotic marker (Table 1). In summary, we found that in prostate adenocarcinomas presence of the RAI2 protein was correlated with AR protein as well as with luminal keratins and E-cadherin and high RAI2 abundance indicated early biochemical recurrence.

GENE EXPRESSION ANALYSIS IN CTCS FROM PATIENTS WITH METASTATIC PROSTATE CANCER

To find out more about *RAI2* gene expression in prostate cancer progression, we tested the feasibility of *RAI2* mRNA expression detection in blood samples in a pilot study comprising 38 samples from 32 patients with metastatic prostate carcinoma. Additionally, 10 blood samples from 10 male healthy donors were included in the analysis. Clinical characteristics of the patients involved in this study are summarized in Supplemental Table 1. CTCs were enriched using the immunomagnetic cell selection system of the AdnaTest ProstateCancerSelect system, and subsequently mRNA was isolated. After reverse transcription and preamplification of cDNA, selected transcripts were analyzed by semiquantitative RT-PCR. Next to *RAI2* gene expression analysis, each sample was profiled for expression of 11 prostate cancer–associated genes as well as 3 reference genes. The expression-based hierarchical clustering of patient and healthy donor samples grouped 6 patients together with all healthy donor samples, indicating a CTC-negative status in those samples (Fig. 3, A). CTC positivity for the patient's cohort (81.6%) was mainly characterized by positive expression of *PSMA*, *PSA*, *RAI2*, *AR*, and *AR-V7* and low *CD45* expression. In 31 of the 38 analyzed blood samples, we were

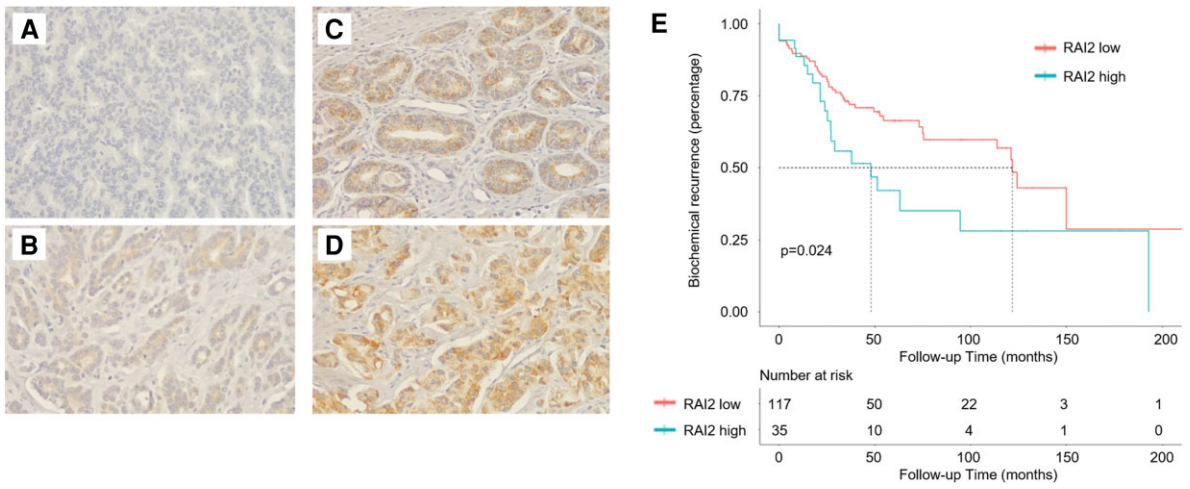

Fig. 2. Immunohistochemical analysis of RAI2 expression in primary prostate cancers. Representative images illustrative of the different staining intensities are presented: (A), no; (B), low; (C), intermediate; and (D), high RAI2 protein abundance; (E), Kaplan–Meier estimations of biochemical recurrence.

able to detect this gene expression signature, indicating that CTCs can be detected in 81.5% of the samples.

Similar to the clustering analyses, principal component analysis also distinguished the same 2 groups of samples. The principal component analysis showed a separation of the healthy donors and the CTC-negative samples from the CTC-positive samples (Fig. 3, B). Moreover, the *CD45* and *PSA*, *PSMA*, *RAI2*, and *AR* genes, as well as *AR-V7* and *KRT19,* showed highest influence on clustering and thereby a higher influence on the separation of the analyzed samples based on the transcription profile (Fig. 3, C). In comparison, the gene cluster of *MRP1* and *PIK3CA* had a lower influence on the separation of the 2 tested groups, showing a similar distribution between the samples (Fig. 3, C). Interestingly, we observed a significantly higher *RAI2* gene expression in the previously defined CTC-positive blood samples compared to healthy donors and CTC-negative prostate cancer patients (Fig. 3, D). *RAI2* expression was clustered in both multivariate analyses with expression of the AR receptors and their target genes *PSA* and *PSMA*, supporting the hypothesis of a functional link between the RAI2 protein and the hormone response.

Next, we used the Pearson correlation method to determine a possible relationship between *RAI2* gene expression and the other tested genes. A significant positive correlation was verified for *RAI2* and the hormone response–related genes such as *AR*, *AR-V7*, *PSA*, and *PSMA,* with corresponding P values < 0.05 (Fig. 4, A). We detected a weak negative correlation of *CD45* and the *RAI2* mRNA in patients with metastatic prostate cancer (Fig. 4, A). Additionally, correlation of relative *RAI2* mRNA expression was analyzed with the clinical parameters prostate-specific antigen (PSA), lactate dehydrogenase, alkaline phosphatase, or hemoglobin concentration in the patients' blood. We found a positive correlation of *RAI2* gene expression with the serum PSA concentrations ($P = 0.016$) and a negative one with the hemoglobin amount ($P = 0.009$) (Fig. 4, B). No correlation was found between *RAI2* gene expression with lactate dehydrogenase and alkaline phosphatase.

In summary, we found that *RAI2* gene expression is principally maintained in CTCs from metastatic prostate cancer patients. Furthermore, *RAI2* gene expression is detected in blood samples in which also transcripts of genes of the AR pathway such as *AR*, *AR-V7*, *PSA*, and *PSMA* are present.

ANALYSIS OF POTENTIAL COREPRESSOR FUNCTION OF RAI2 IN AR-POSITIVE PROSTATE CANCER CELL LINES

To validate a potential functional association between RAI2 and the AR response, we first analyzed *RAI2* gene expression and protein abundance in 4 prostate cancer cell lines. The highest *RAI2* gene expression was found in VCaP cells followed by LNCaP and PC-3 cells, whereas *RAI2* mRNA was not detected in DU-145 prostate cancer cells (Fig. 5, A). Additionally, in the Western blot analysis, we found the highest RAI2 protein abundance in VCaP cells followed by LNCaP with very low RAI2 protein abundance. PC-3 and DU-145 cells do not express the RAI2 protein (Fig. 5, B). In line with the previous analysis of primary tumors, we also detected a concurrent expression of E-cadherin and AR proteins in VCaP and LNCaP cells, whereas those proteins were much less abundant in

Table 1 Correlation of RAI2 protein expression to molecular data.

Characteristic of assessed protein	Protein distribution	RAI2 protein expression								*P* value
		Total cohort		Negative		Low		High		
		n	%	n	%	n	%	n	%	
Proliferation Marker	Ki-67									
	Low	279	94.3	63	96.9	170	95.5	46	86.8	
	High	17	5.7	2	3.1	8	4.5	7	13.2	
	Total	296	100							0.033
Apoptosis Marker	Apoptag									
	Low	245	83.1	60	88.2	145	81.9	40	80	
	High	50	16.9	8	11.8	32	18.1	10	20	
	Total	295	100							0.409
Luminal cell Marker	K8, 18									
	Low	103	35.2	43	66.2	52	29.5	8	15.4	
	High	190	64.8	22	33.8	124	70.5	44	84,6	
	Total	293	100							<0.001
Luminal cell Marker	K19									
	Low	153	56.3	45	83.3	82	48.8	26	52.0	
	High	119	43.8	9	16.7	86	51.2	24	48.0	
	Total	272	100							<0.001
Epithelial cell Marker	EpCAM									
	Low	83	59.3	13	65.0	51	55.4	19	67.9	
	High	57	40.7	7	35.0	92	44.6	9	32.1	
	Total	140	100							0.430
Epithelial cell Marker	E-cadherin									
	Low	109	40.8	30	58.8	65	38.7	14	29.2	
	High	158	59.2	21	41.2	103	61.3	48	70.8	
	Total	267	100							0.007
Stem cell Marker	ALDH1									
	Low	165	72.4	26	65.0	107	72.8	32	78.0	
	High	63	27.6	14	35.0	147	27.2	41	22.0	
	Total	228	100							0.415
Endocrine Marker	AR									
	Low	87	35.1	25	48.1	57	36.8	5	12.2	
	High	161	64.9	27	51.9	98	63.2	36	87.8	
	Total	248	100							0.001
Regulator of Stemness, EMT and growth	EGFR									
	Low	191	82.3	38	84.4	121	82.9	32	78.0	
	High	41	17.7	7	15.6	25	17.1	9	22.0	
	Total	232	100							0.710

PC-3 and DU-145 cells (Fig. 5, B). Due to increased protein abundance of RAI2 in both AR-positive prostate cancer cell lines, we questioned whether there was a functional relationship between RAI2 and AR expression in prostate cancer cell lines. To address this question, RNA interference technology was used to deplete the protein expression of either RAI2 or AR in LNCaP and VCaP prostate cancer cell lines.

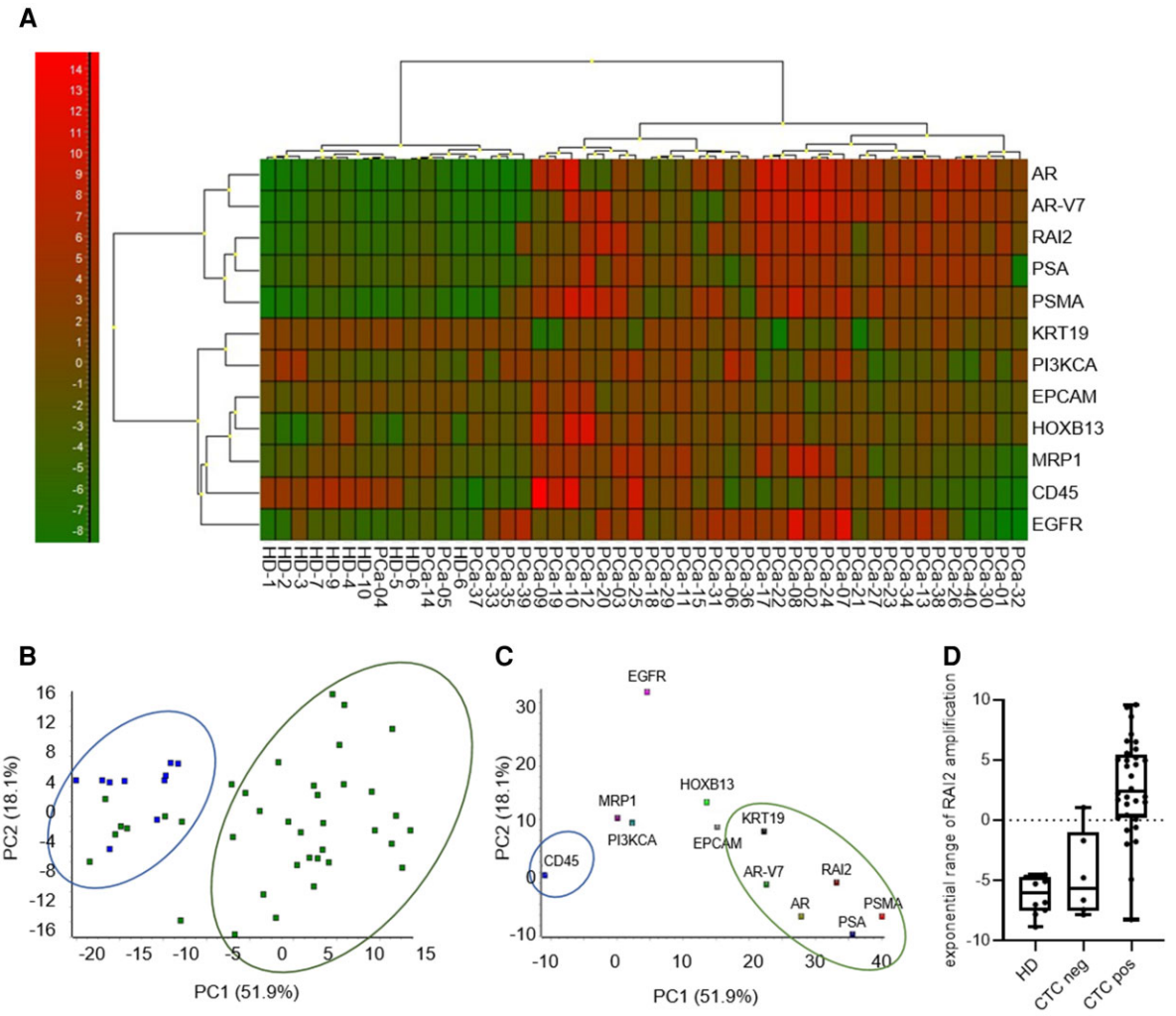

Fig. 3. Classification of analyzed genes and CTC samples from healthy donors (HD) and metastatic prostate cancer patients. (A), AdnaTest-enriched samples and genes were clustered and visualized with dendograms and a heat map showing expression levels. Red, high gene expression; green, low gene expression; (B), Samples from HD (blue squares) and patients with prostate cancer (green squares) were grouped based on their gene expression profile into 2 different clusters, separating the CTC-negative (blue encircled samples) and the CTC-positive (green encircled samples) samples by principle component analysis (PCA); (C), PCA of genes shows the most differentially expressed genes between the groups. Blue circle, genes predominantly expressed in CT-negative cluster; green circle, genes mostly expressed in CTC-positive cluster; (D), Comparison of exponential range of RAI2 amplification in indicated groups of HD (n = 10), CTC-negative (n = 6), and CTC-positive (n = 31) cancer patients.

Western blot analysis was performed to determine the mutual effect of depletion on each other's protein expression. After successful RAI2 knockdown in LNCaP cells, we observed reduced AR abundance that did not reach the set significant level of a 2-sided *t*-test [fold-change (FC)] (FC = 0.54, $P = 0.085$) (Fig. 5, C), and in VCaP cells, we found a significant reduced AR protein abundance (FC = 0.66, $P = 0.02$) (Fig. 5, C) in comparison to the respective nontarget control. Surprisingly, AR knockdown caused an increased RAI2 protein concentration in both cell lines with a significant difference in VCaP cells (LNCaP: FC = 3.34, $P = 0.055$; VCaP: FC = 2.14, $P = 0.006$) (Fig. 5, C).

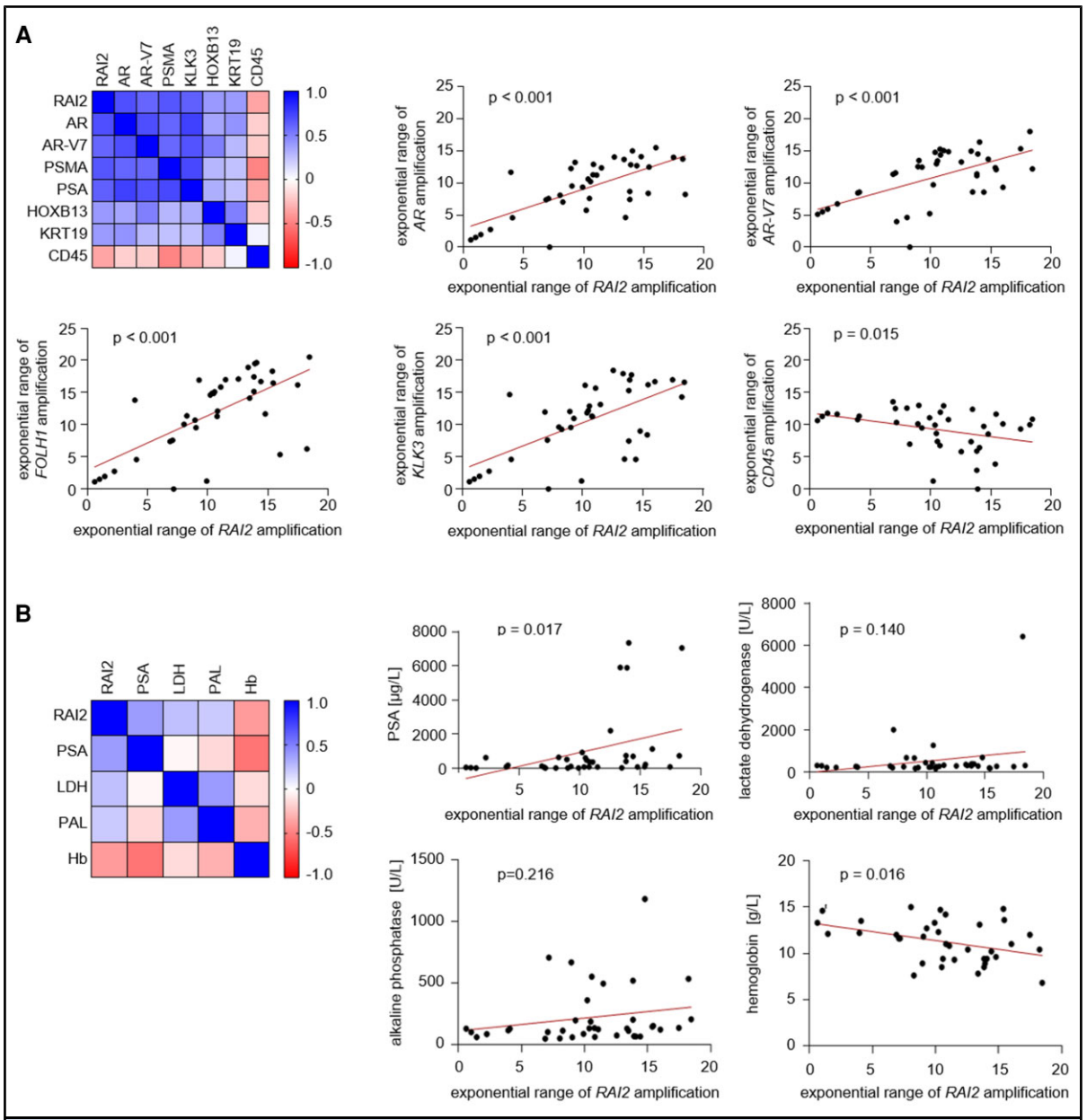

Fig. 4. Correlation of exponential range of RAI2 amplification in CTCs with the expression of other analyzed genes and clinical parameters of metastatic prostate cancer patients. (A), Pearson correlation coefficients visualized in a correlation matrix and scatterplots showing relation between exponential ranges of RAI2 and either AR, AR-V7, PSMA, PSA, or CD45 in CTCs of metastatic prostate cancer patients. Blue color indicates positive correlation and red color indicates negative correlations; (B), Correlation matrix showing the correlation between exponential range of RAI2 amplification with serum levels of clinical parameters including PSA, lactate dehydrogenase, alkaline phosphatase, and hemoglobin, which were assessed at time of blood draw. Pearson correlation coefficient is visualized within the squares. Blue color indicates positive correlation, and red color indicates negative correlations.

To further analyze the effects of AR inhibition on RAI2 and AR protein expression, parental LNCaP and VCaP cells were either grown under hormone-deprived conditions or were treated with the antiandrogens bicalutamide or enzalutamide. None of these treatment conditions induced any significant changes for RAI2 or AR

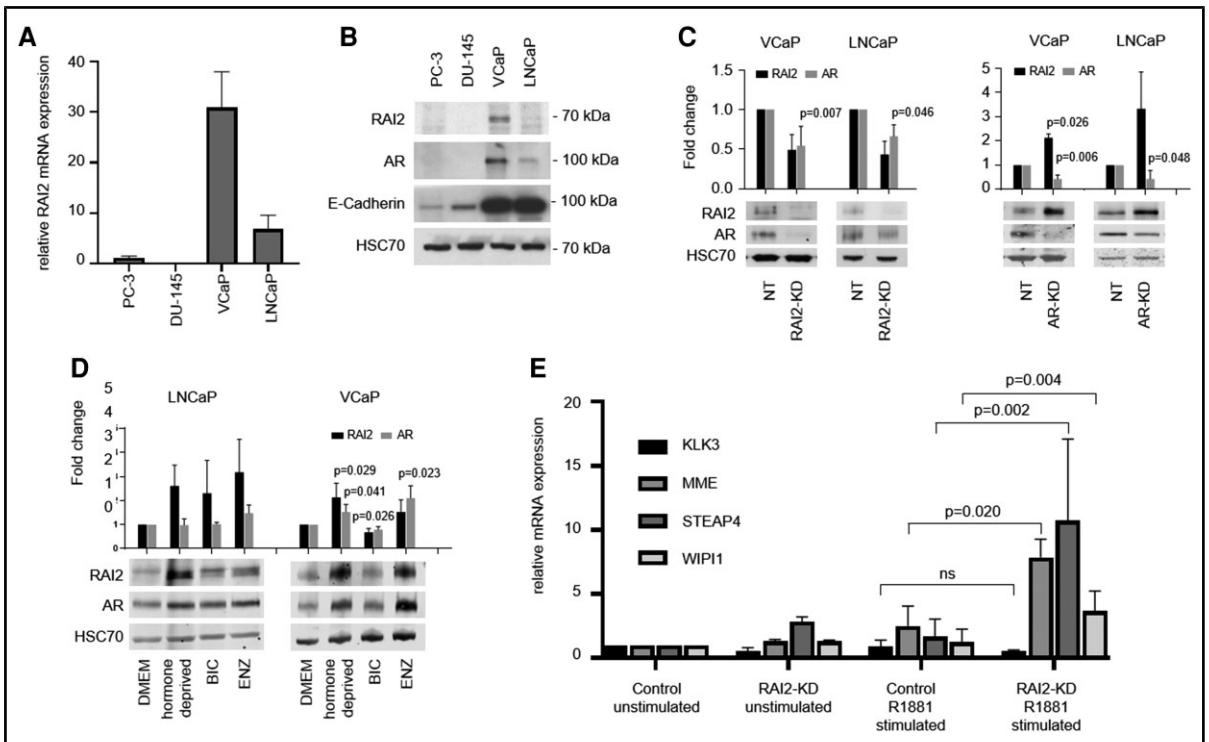

Fig. 5. RAI2 and the AR are interdependently regulated proteins. (A), RAI2 and AR mRNA expression was analyzed by RT-pPCR in PC-3, DU-145, VCaP, and LNCaP cells. Relative fold change expression is normalized to RPLPO and MCF-7 cell line expression of three independent experiments; error bars represent SD of the mean; (B), Abundance of RAI2, AR, and E-Cadherin proteins in the same panel of human prostate carcinoma cell lines was determined by Western blot analysis. Equal loading was demonstrated using an antibody recognizing HSC70; (C), Western blot analysis of RAI2 and AR protein expression in LNCaP (n = 3) and VCaP (n = 4) prostate cancer cell lines, following short hairpin RNA-mediated knockdown (KD) of RAI2 protein. Non-target (NT) short hairpin RNA served as negative control; (D), RAI2 and AR protein expression was assessed in same cell lines after hormone deprivation and treatment with either 3 μM bicalutamide (BIC) or 3 μM enzalutamide (Enz) for 10 days. Relative protein expression was determined by normalizing to HSC70 loading control and either the NT- or RPMI/DMEM protein expression using the Image Studio Lite software (Licor). Columns show the mean fold change and SD of at least three independent experiments; (E), Gene expression of AR-targeted genes was analyzed in VCaP cells after RAI2-knockdown and stimulation with the synthetic androgen R1881. The data of three biological replicates were analyzed with QIAGEN web-based software applying a 2-fold cutoff to identify significantly ($P = 0.05$) altered gene expression between stimulated and unstimulated cells.

protein abundance in LNCaP cells (Fig. 5, D). For VCaP cells, however, a significant induction of RAI2 (FC = 2.14, $P = 0.03$) and of AR (FC = 1.53, $P = 0.042$) protein abundance was observed after hormone deprivation. In VCaP cells, enzalutamide treatment resulted in significant increased AR protein expression (FC = 2.10, $P = 0.023$) but did not change RAI2 abundance. Bicalutamide treatment, however, resulted in a significant decrease of RAI2 protein (FC = 0.68, $P = 0.026$) expression in VCaP cells (Fig. 5, D).

Next, we analyzed a possible coregulator function of the RAI2 protein in the hormone response and stimulated RAI2-depleted VCaP cells with synthetic androgen R1881 and performed targeted gene expression analysis of human AR signaling targets. To this purpose, we utilized a multiplex quantitative PCR array analyzing the expression of 84 different genes that are associated with AR signaling. After hormone stimulation, we found increased expression of *MME*, *STEAP4*, and *WIPI1* in RAI2-depleted VCaP cells, whereas no significant difference was found for *KLK3 (PSA)* gene expression (Fig. 5, E, Supplemental Table 2). In summary, these results showed that RAI2 knockdown caused a downregulation of hormone receptor expression and,

conversely, inhibition of each hormone receptor–induced RAI2 expression in both tested cell lines. The altered expression of some androgen responsive genes in VCaP cell line suggested that RAI2 might act as a corepressor of hormone responses in prostate cancer cells in a gene-specific manner.

Discussion

In the present study, we found that reduced *RAI2* gene expression in primary prostate tumors was associated with increased hypoxia and markers of genomic instability but not survival. In contrast, high RAI2 protein abundance correlated with presence of AR, E-cadherin, and epithelial keratins, and with an early biochemical recurrence. In blood samples from patients with advanced prostate cancer, the *RAI2* transcript had been detected together with *AR*, *FOLH1* (prostate-specific membrane antigen), and *KLK3* (PSA) as part of a gene expression signature that indicated the presence of CTCs and AR pathway activation. In cultured prostate cancer cell lines, we confirmed a correlation between RAI2, AR, and E-cadherin protein expression, and we discovered a function as transcriptional coregulator of the AR pathway and could show that sustained AR inhibition caused increase of RAI2 protein concentrations.

One of the major findings of this study is the strong association of RAI2 expression and function with the AR pathway. First, we found a strong correlation between RAI2 and AR proteins in primary prostate adenocarcinomas, which was validated in prostate cancer cell lines. Also, in blood samples from patients with advanced prostate cancer, the *RAI2* transcript could be detected together with *AR*, *FOLH1*, and *KLK3* as part of a gene expression signature that indicated the presence of CTCs. Additionally, we established a putative corepressor function for RAI2 in the androgen response. In RAI2-depleted VCaP cells, we found increased expression of *MME*, *STEAP4*, and *WIPI1* after stimulation with the synthetic androgen R1881. Expression of *MME* and *STEAP4* is known to be directly regulated by AR (14–16), indicating that RAI2 might act as a transcriptional corepressor of the AR pathway in a gene-specific manner. A function as transcriptional coregulator is likely, mainly due to the validated interaction with C-terminal–binding proteins (5), which act as coregulators of AR-mediated transcription (17, 18).

We also found significant correlations of RAI2 protein abundance with keratins 8, 18, and 19 as well as with E-cadherin expression both in primary prostate cancer tissue as well as in cell lines. Because expression of these keratins and E-cadherin in prostate cancer specifies the luminal phenotype (19), RAI2 abundance appears to be characteristic of well-differentiated primary prostate carcinomas. In a previous study, we found that high *RAI2* expression was a feature of luminal, estrogen receptor–positive breast tumors (5). Furthermore, the *RAI2* gene has initially been described in the context of cellular differentiation of P19 embryonal carcinoma cells induced by treatment with all-trans retinoic acid (20). Because these studies have shown functional associations with different types of steroid hormone receptors, we conclude that the RAI2 protein might own a universal function as a transcriptional coregulator for different steroid receptors. However, in contrast to previous analyses in breast cancer (5, 21), we found no significant correlation between low *RAI2* gene expression and shortened overall survival. We also did not observe an association of high RAI2 protein concentrations and low Gleason score or the other clinical parameters in our tissue microarray study. Thus, in primary tumor samples, we found no evidence for a metastasis suppressive function of RAI2 in prostate cancer and conclude that our previous findings in breast cancer cannot simply be transferred to prostate cancer.

Among blood-based biomarkers, CTCs have been most intensively analyzed in prostate cancer, especially in men with metastatic castration-resistant prostate cancer, in whom CTCs are more often detected and at higher numbers (4, 22). Recent studies reporting on the enumeration and characterization of CTCs point out those CTCs can be used to develop biomarkers for a specific clinical context to improve diagnosis and better inform clinical management decisions (23). We aimed to analyze *RAI2* gene expression as part of a multimarker panel in CTC and isolated CTCs from 38 blood samples from patients with metastatic prostate cancer. A limitation of utilizing preamplified cDNA for gene expression analysis is that a quantification leading to exact fold differences might not be accurate. Thus, the results of the gene expression analysis in CTCs are regarded as a semiquantitative comparison showing relative differences between different samples. However, unsupervised hierarchical clustering of the samples based on gene expression grouped 7 prostate cancer samples together with 10 tested healthy donor samples into one cluster, indicating a negative CTC status in these samples, and the remaining 31 (81.5%) samples were considered to be positive for CTC. Compared to previous studies, the CTC positivity rate in our study is very high, even for the metastatic setting (4, 24). We think that the high CTC detection rate in our cohort is mainly the result of patient recruitment in the palliative setting as our patient cohort contains in particular metastatic cases that progress under therapy. Furthermore, our pilot study consists of only 38 samples from 32 patients, and thus our findings need to be verified with larger patient numbers. Intriguingly, prostate cancer blood samples could be discriminated from healthy control samples by gene expression of RAI2 together with the

prostate-specific transcripts *AR*, *FOLH1*, and *KLK3*, and the epithelial marker *KRT19*. A limitation of using the hierarchical clustering analysis for defining CTC-positive patients is the lack of a clear cutoff that is, for instance, provided for the commercial AdnaTest (25). However, correlation of RAI2 gene expression in CTCs with available clinical parameters showed a positive correlation with the serum PSA levels and a negative correlation with the hemoglobin amount, suggesting that RAI2 gene expression in CTCs is correlated with progressive disease (26, 27).

Molecular analysis of LNCaP and VCaP prostate cancer cell lines revealed that depletion of AR expression induced the expression of the RAI2 protein. In line with the knockdown experiment, increase of the RAI2 protein was also observed following AR inhibition in VCaP cells. Thus, sustained AR inactivation might be a possible explanation for elevated RAI2 expression in AR-dependent tumors and CTCs. Interestingly, in primary tumors increased RAI2 protein abundance correlated with early biochemical recurrence and as well *RAI2* gene expression in CTCs correlated with increased serum PSA concentrations of the corresponding patients. These findings are of particular interest because elevated *RAI2* gene and protein expression might indicate disease progression in the presence of sustained AR inhibition. However, the use of *RAI2* gene expression as part of a multimarker panel investigating therapy resistance within a liquid biopsy approach needs to be validated in longitudinal studies on larger and better-defined patient cohorts with sufficient follow-up data.

In the TCGA prostate adenocarcinoma data set, genomic rearrangements of the *RAI2* gene are rare; we assume that the observed differences in gene and protein expression are the consequence of differential transcriptional or epigenetic regulation of *RAI2* gene expression. We also have revealed that AR protein expression is downregulated after short hairpin RNA–mediated knockdown of *RAI2* expression. Thus, active AR seems to repress *RAI2* expression, while the RAI2 protein stabilizes protein expression of the hormone receptor under normal growth condition in a so far unknown manner. To our best knowledge, no AR binding sites have been identified in the *RAI2* promoter region, which implies that *RAI2* is not directly regulated by either transcription factor (15). However, little is known about the regulation of *RAI2* expression yet. Besides the previously shown influence of *RAI2* promoter methylation on RAI2 expression (28), it is assumed that *RAI2* is regulated by AP-2 transcription factors through its specific binding domains that have been identified within the *RAI2* promoter (28). Nevertheless, the molecular basis of this putative feedback regulation needs to be addressed in future studies.

This study shows that *RAI2* gene and protein expression is associated with AR activity and epithelial differentiation in primary tumor and CTCs. We found evidence that the RAI2 protein functions as a transcriptional coregulator of the androgen response and that sustained inhibition of AR activity induces augmented RAI2 protein expression. Thus, we consider that detection of *RAI2* gene or protein expression in CTCs and primary prostate tumors is a potential biomarker to refine prognosis of prostate cancer patients that progress under androgen deprivation therapy.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: CTC, circulating tumor cell; AR, androgen receptor; TCGA; The Cancer Genome Atlas; FC, fold change; PSA, prostate-specific antigen; RAI2, retinoic-acid induced 2.

Human Genes: AR, androgen receptor; AR-V7, androgen receptor splice variant V7; FOLH1, folate hydrolase 1 coding for the prostate-specific membrane antigen (PSMA); KLK3, kallikrein related peptidase coding for the prostate-specific antigen (PSA); HOXB13, homeobox B13; RAI2, retinoic acid induced 2; EGFR, epidermal growth factor receptor; EPCAM, epithelial cell adhesion molecule; KRT19, keratin 19; ABCC1; ATP binding cassette subfamily C member 1 coding for the multidrug resistance-associated protein 1 (MRP1); PI3KCA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PTPRC, protein tyrosine phosphatase receptor type C coding for the CD45 protein; ACTB, actin beta; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT1, hypoxanthine phosphoribosyltransferase 1.

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