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Cysteine-rich secretory protein 3 overexpression is linked to a subset of *PTEN*-deleted *ERG* fusion-positive prostate cancers with early biochemical recurrence

Katharina Grupp[1*], Sebastian Kohl[2*], Hüseyin Sirma[2], Ronald Simon[2], Stefan Steurer[2], Andreas Becker[3], Meike Adam[3], Jakob Izbicki[1], Guido Sauter[2], Sarah Minner[2], Thorsten Schlomm[3,4] and Maria Christina Tsourlakis[2]

[1]*General, Visceral and Thoracic Surgery Department and Clinic, University Medical Center Hamburg-Eppendorf, Hamburg, Germany;* [2]*Institute of Pathology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany;* [3]*Martini-Clinic, Prostate Cancer Center, University Medical Center Hamburg-Eppendorf, Hamburg, Germany and* [4]*Department of Urology, Section for translational Prostate Cancer Research, University Medical Center Hamburg-Eppendorf, Hamburg, Germany*

The aim of this study was to determine whether cysteine-rich secretory protein 3 (CRISP3) expression is linked to clinically or molecularly relevant subgroups of prostate cancer. A tissue microarray representing samples from >10 000 prostate cancers from radical prostatectomy specimens with clinical follow-up data were analyzed for CRISP3 expression by immunohistochemistry. CRISP3 expression was also compared with key genomic alterations of prostate cancer. CRISP3 staining was found as weak in 15%, moderate in 8.5%, and strong in 7.2% of prostate cancers, whereas no expression was detected in normal prostate. Strong CRISP3 expression was linked to advanced tumor stage, high Gleason score, and positive surgical margin status ($P<0.0001$ each). There was a marked accumulation of high CRISP3 expression in *PTEN*-deleted ERG-positive tumors ($P<0.0001$). A total of, 21.7% of ERG-positive and *PTEN*-deleted cancers had strong CRISP3 expression, but only 10.4% of ERG-positive cancers without *PTEN* deletion ($P<0.0001$). The rate of high CRISP3 expression was 2.5% in *ERG*-negative cancers ($P=0.0001$; *vs ERG*-positive cancers). Accordingly, CRISP3 overexpression was associated with early prostate-specific antigen recurrence in all tumors ($P=0.0013$) as well as in ERG-negative ($P=0.004$) and ERG-positive cancers ($P=0.0318$). CRISP3 expression did not retain prognostic significance in models also involving *PTEN* deletions. Strong CRISP3 expression is associated with unfavorable tumor phenotype and early recurrence in prostate cancers. The tight link of strong CRISP3 expression to the *ERG* fusion-positive prostate cancers with *PTEN* deletions provides further evidence for the existence of molecularly distinct subgroups of prostate cancers.
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Prostate cancer is, with about one million new cases and 250 000 deaths per year worldwide, a major cause of morbidity and mortality linked to human cancer.[1] As prostate cancer often has a slow disease progression and typically affects elderly men, it is evident, that not every patient requires potentially curative but aggressive treatment. Therapeutic options vary from active surveillance to surgical or radiation therapy. The only established pretreatment prognostic parameters currently include Gleason grade, tumor extent on biopsies, preoperative prostate-specific antigen (PSA), and clinical parameters. These data are statistically powerfull but still not sufficent for optimal treatment decisions in individual patients. There is a considerable hope, that the analysis of molecular features will better enable an individual prediction of tumor aggressiveness in the future.

Correspondence: Dr MC Tsourlakis, Institute of Pathology, University Medical Center Hamburg-Eppendorf, Martinistr. 25, 20246 Hamburg, Germany.
E-mail: m.tsourlakis@uke.de
[*]These authors contributed equally to this work.
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Cysteine-rich secretory proteins, including cysteine-rich secretory protein 3 (CRISP3) are poorly characterized extracellular proteins, which are preferentially expressed in cells of the reproductive tract and immune system. CRISP3 appears to be linked to innate immunity and inflammation (reviewed in Gibbs *et al*[2]). Several studies recently suggested a role for CRISP3 in prostate cancer. CRISP3 is expressed at low levels in normal human prostate and strongly upregulated in a fraction of prostate cancers.[3–6] The clinical relevance of CRISP3 overexpression is controversially discussed. Some studies found CRISP3 overexpression to be associated with increased risk of tumor recurrence,[7] unfavorable tumor phenotype,[8,9] castration-resistant prostate cancer, and metastasis,[9,10] whereas others could not find any clinically relevant associations.[11,12]

We and others recently identified a tight link of CRISP3 expression to positive *ERG* fusion status in RNA expression screening studies.[8,13] Associations of possible prognostic biomarkers with molecularly distinct tumor subtypes raise the question, whether their prognostic relevance may be limited to certain tumor subgroups. Such phenomenons could also explain the varying outcome of earlier studies involving relatively small patient sets.

To evaluate the potential clinical utility of CRISP3 measurement in prostate cancer we took advantage of a prostate cancer tissue microarray containing samples of >10 000 patients with clinical follow-up information and an attached molecular database. Our data indicate a strong predilection of high-level CRISP3 expression in *PTEN*-deleted *ERG* fusion-positive prostate cancers. Accordingly, high CRISP3 expression is significantly linked to clinical outcome and tumor aggressiveness in prostate cancer.

Table 1 Composition of the prognosis tissue microarray containing 11 152 prostate cancer specimens

	No. of patients	
	Study cohort on tissue microarray (n = *11152*)	*Biochemical relapse among categories* (n = *1824*)
Follow-up (mo)		
Mean	53.4	—
Median	36.8	—
Age (years)		
<50	318	49
50–60	2768	460
60–70	6548	1081
>70	1439	232
Pretreatment PSA (ng/ml)		
<4	1407	142
4–10	6735	827
10–20	2159	521
>20	720	309
pT category (AJCC 2002)		
pT2	7370	570
pT3a	2409	587
pT3b	1262	618
pT4	63	49
Gleason grade		
≤3 + 3	2859	193
3 + 4	1565	573
4 + 3	6183	849
≥4 + 4	482	208
pN category		
pN0	6117	1126
pN+	561	291
Surgical margin		
Negative	8984	1146
Positive	1970	642

Abbreviation: AJCC, American Joint Committee on Cancer.
NOTE: Numbers do not always add up to 11 152 in the different categories because of cases with missing data.

Materials and methods

Patients

A set of tissue microarrays was made from 11 152 prostatectomy specimens from patients undergoing surgery between 1992 and 2011 at the Department of Urology, and the Martini Clinics at the University Medical Center Hamburg-Eppendorf according to our institutional standard.[14,15] The tissue microarray manufacturing process was described earlier in detail.[16] In short, one 0.6 mm core was taken from a representative tissue block from each patient. The tissues were distributed among 24 tissue microarray blocks, each containing 144–522 cores. Clinical follow-up data were available for 9695 of 11 152 arrayed tumors. Median follow-up was 36.8 months ranging from 1 to 228 months. PSA values were measured following surgery and recurrence was defined as a postoperative PSA of 0.2 ng/ml and increasing at first of appearance. The detailed composition of the tissue microarray and the attached histopathological and clinical data are outlined in Table 1. Presence or absence of cancer tissue was validated by immunohistochemical AMACR and 34BE12 analysis.[17] The molecular database attached to this tissue microarray contained results on ERG expression in 8538, *ERG* break apart fluorescence *in situ* hybridization analysis in 1440 (extended from[18]), *5q21* deletions (*CHD1*) in 3023 (Burkhardt L, unpublished data), *6q15* deletions (*MAP3K7*) in 2458 (Kluth *et al*; in press),[19] *PTEN* deletions in 4088,[20] and *3p13* deletions (*FOXP1*) in 1828 tumors (Krohn A, unpublished data).

Immunohistochemistry

Freshly cut tissue microarray sections were analyzed in 1 day and in one experiment. A polyclonal

CRISP3 antibody (rabbit, Abcam; at 1/450 dilution; cat#ab105951, Cambridge, UK) was used for detection of CRISP3 expression. Slides were deparaffinized and exposed to heat-induced antigen retrieval for 5 min in an autoclave at 121 °C in pH 7.8 buffer. Bound primary antibody was visualized using the DAKO EnVision Kit (Dako, Glostrup, Denmark). CRISP3 staining was evaluated according to the following scoring system as previously described:[21–23] The staining intensity (0, 1+, 2+, and 3+) and the fraction of positive tumor cells were recorded for each tissue spot. A final score was built from these two parameters as follows: Negative: absence of CRISP3 staining; weak: intensity of 1+ in ≤70% of tumor cells or staining intensity of 2+ in ≤30% of tumor cells; moderate: intensity of 1+ in >70% of tumor cells, or staining intensity of 2+ in >30% but ≤70% of tumor cells or staining intensity of 3+ ≤30% of tumor cells; strong: intensity of 2+ in >70% of tumor cells or staining intensity of 3+ in >30% of tumor cells.

Statistics

For statistical analysis, the JMP 9.0 software (SAS Institute, NC, USA) was used. Contingency tables were calculated to study the association between CRISP3 expression and clinicopathological variables, and the χ^2- (Likelihood) test was used to find significant relationships. Kaplan–Meier curves were generated for PSA recurrence-free survival. The log-Rank test was applied to test the significance of differences between stratified survival functions. COX proportional hazards regression analysis was performed to test the statistical independence and significance between pathological, molecular, and clinical variables.

Results

Technical Aspects

CRISP3 analysis was successful in 9240/11 152 arrayed cancers (82.9%). Immunohistochemistry was non-informative in 1912 (17.1%) tumors because lack of unequivocal tumor cells in the tissue spots or missing tissue spots on the tissue microarray section.

CRISP3 Expression in Prostate Cancers

CRISP3 protein was virtually not detectable in the epithelium of normal prostatic glands and stromal cells by immunohistochemistry. CRISP3 immunohistochemistry in 9240 informative tumors (82.9%) revealed 1387 (15%) tumors with weak, 790 (8.5%) with moderate, and 662 (7.2%) with strong staining, whereas 6401 (69.3%) tumors did not show any staining (CRISP3 negative). Representative images of CRISP3 immunohistochemistry are shown in Figure 1. CRISP3 overexpression was significantly linked to advanced tumor stage, high Gleason grade, and positive surgical margins ($P<0.0001$; each) (Table 2).

TMPRSS2–ERG Fusion Status and ERG Protein Expression

The relationship of CRISP3 expression and 'fusion-type' prostate cancer was analyzed by two independent methods. There were subsets of 1216 cancers with available *ERG* rearrangement data obtained by fluorescence *in situ* hybridization and 8032 cancers with ERG immunohistochemistry data for which CRISP3 data were also available. Strong CRISP3 staining was more frequent in ERG immunohistochemical positive (461/3547; 13%) as compared with ERG immunohistochemical negative cancers (114/4485; 2.5%, $P<0.0001$) (Figure 2a). Utilizing genomic *ERG* rearrangement data obtained by using an *ERG* break apart probe yielded similar results.[18] Strong CRISP3 staining was markedly more frequent in tumors with *ERG* rearrangement (67/593; 11.3%) as compared with tumors without *ERG* rearrangement (11/623; 1.7%) ($P<0.0001$; Figure 2b). A subset analysis in 3547 *ERG* fusion-positive cancers (Table 3) and 4485 *ERG* fusion-negative (Table 4) revealed that all associations of CRISP3 overexpression with unfavorable tumor phenotype held true for both ERG-negative and -positive cancers. The associations of CRISP3 in *ERG* fusion-positive and -negative subgroups are summarized in Tables 3 and 4.

Associations of CRISP3 with Key Genomic Deletions in *ERG* Fusion-Positive and -Negative Prostate Cancers

As several genomic deletions define distinct subgroups of *ERG*-negative and *ERG*-positive cancers, we next evaluated whether CRISP3 expression might be linked to one of these genomic aberrations. Data for these deletions were available to us from previous studies (PTEN,[20] Kluth *et al*[19]; Burkhardt L, unpublished data; Krohn A, unpublished data), applying fluorescence *in situ* hybridization to parallel sections from our TMA. Deletion calling was at a threshold of 50% deleted tumor cells per spot in all these studies (PTEN,[20] Kluth *et al*;[19] Burkhardt L, unpublished data; Krohn A, unpublished data). The overall deletion frequency was 20.2% for *PTEN*, 16.5% for *3p13*, 18.5% for *6q15*, and 8.7% for *5q21*. The relationship of CRISP3 expression with these key deletions is shown in Figures 3–5 for all tumors and the subsets *ERG* fusion-negative and -positive prostate cancers.

As all these deletions are known to be strongly linked to *ERG* fusion status, the overall association found for all these deletions with CRISP3 was expected. The subset analysis of ERG-positive

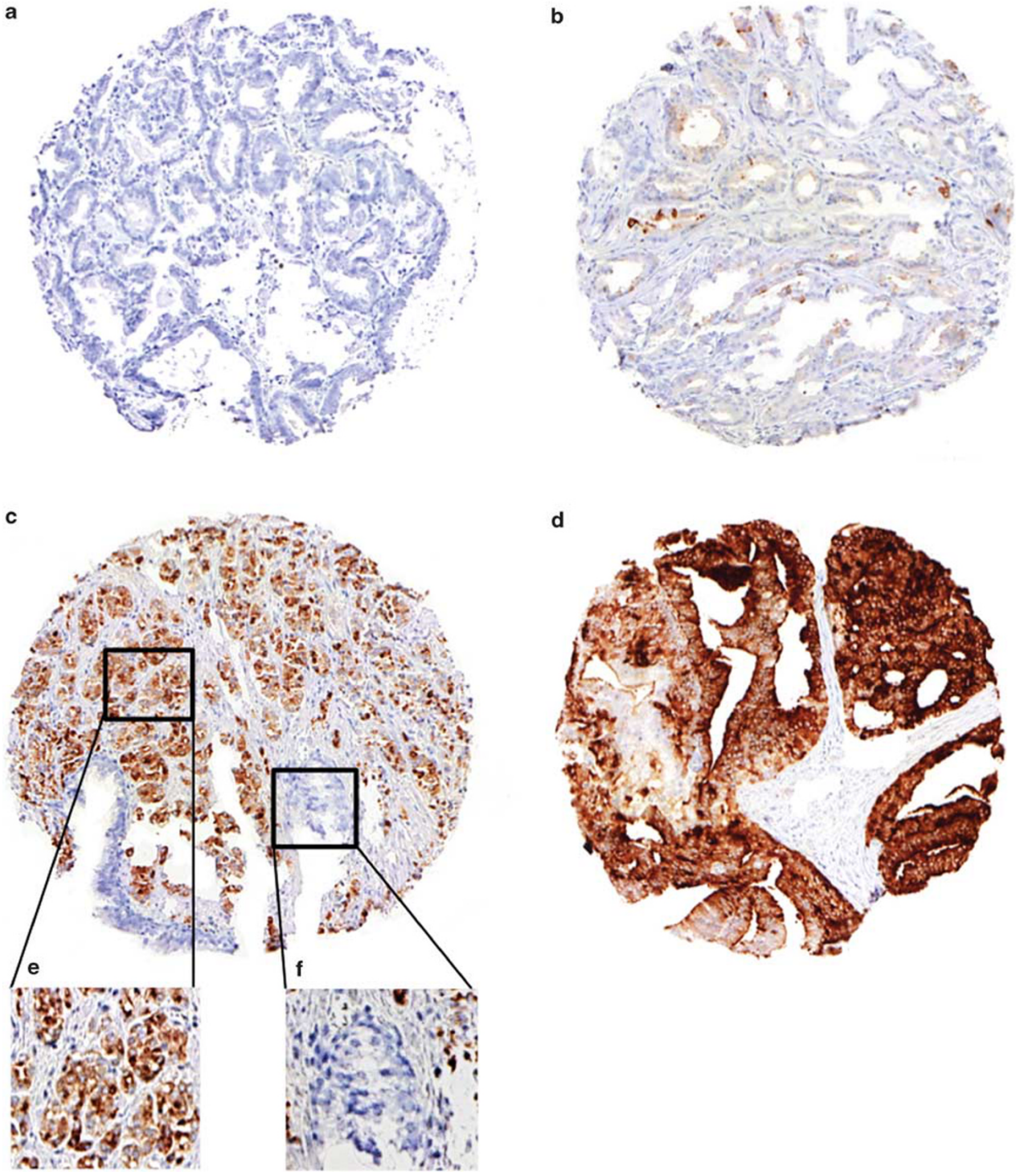

Figure 1 Representative pictures of CRISP3 immunostaining in prostate cancer. (**a**) Negative, (**b**) weak, (**c**) moderate, (**d**) strong staining, (**e**) magnification of moderate staining in cancer cells, and (**f**) magnification of negative staining of stromal tissue.

and ERG-negative cancers revealed, however, that only PTEN deletions were tightly linked to CRISP3 expression within these subgroups. Within ERG-positive cancers, strong CRISP3 immunostaining was found in 119 of 549 (21.7%) *PTEN* deleted as compared with 119 of 1149 (10.4%) cancers without *PTEN* deletion (Figure 4a). Within ERG-negative cancers, strong CRISP3 immunostaining was found in 9 of 246 (3.7%) *PTEN*-deleted cancers as compared with 33 of 1481 (2.2%) cancers without *PTEN* deletion ($P=0.0019$; Figure 5a).

Table 2 Associations between CRISP3 immunostaining results and prostate cancer phenotype in all cancers

Parameter	n *Evaluable*	*CRISP3 immunohistochemistry result*				P *value*
		Negative (%)	*Weak (%)*	*Moderate (%)*	*Strong (%)*	
All cancers	9240	69.3	15.0	8.5	7.2	
Tumor stage						
pT2	5972	72.61	14.38	7.38	5.63	<0.0001
pT3a	2082	62.73	16.28	11.14	9.85	
pT3b	1091	63.52	16.04	10.17	10.27	
pT4	54	59.26	18.52	9.26	12.96	
Gleason grade						
≤3+3	2195	73.58	14.81	7.02	4.6	<0.0001
3+4	5222	67.81	15.55	9.42	7.22	
4+3	1358	66.72	14.21	8.54	10.53	
≥4+4	414	72.22	12.08	6.52	9.18	
Lymph node metastasis						
N0	5159	68.75	14.81	8.96	7.48	0.016
N+	485	62.68	17.32	9.07	10.93	
Surgical margin						
Negative	7362	70.13	15.12	8.22	6.53	<0.0001
Positive	1712	64.89	14.84	10.22	10.05	

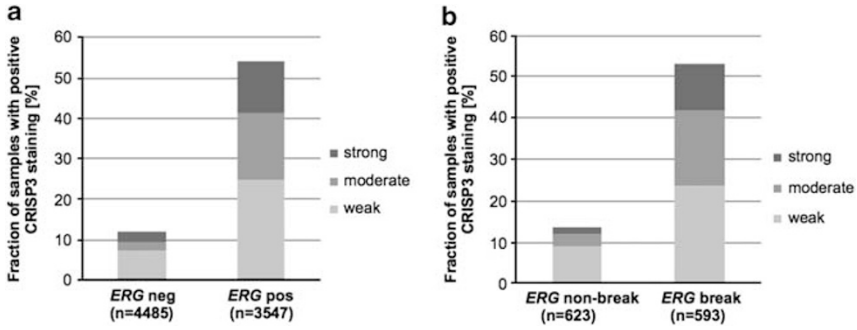

Figure 2 Association between positive CRISP3 immunostaining and *ERG* fusion. *ERG* fusion was probed by both (**a**) immunohitochemistry and (**b**) fluorescence *in situ* hybridization analysis ($P<0.0001$; each).

Prognostic Relevance of CRISP3 Expression

Follow-up data were available from 8019 cancers. Strong CRISP3 staining was significantly linked to early biochemical recurrence in all cancers ($P=0.0013$, Figure 6a). This association also hold true in 3835 *ERG* fusion-negative ($P=0.004$, Figure 6b) and in 3080 *ERG* fusion-positive cancers ($P=0.0318$, Figure 6c). A combined analysis of *PTEN* deletion and CRISP3 expression status revealed that the prognostic impact of CRISP3 expression was mainly driven by its association with *PTEN* deletion. CRISP3 expression lost its prognostic relevance in subsets of 2428 *PTEN*-normal ($P=0.1796$, Figure 6d) and 751 *PTEN*-deleted cancers ($P=0.5291$, Figure 6e). Multivariate analysis including only *PTEN* and CRISP3 status revealed a strong prognostic impact of *PTEN* deletions on outcome ($P<0.0001$) whereas CRISP3 expression provided no additional prognostic impact ($P=0.6014$). Multivariate analysis including also pT stage, Gleason grade, and preoperative PSA values revealed independent prognostic relevance for tumor stage ($P<0.0001$), Gleason grade ($P<0.0001$), PSA ($P<0.0001$), and *PTEN*-deletion ($P=0.001$), but not for CRISP3 expression ($P=0.618$).

Discussion

The results of our study indicate that strong CRISP3 expression is highly linked to *ERG* fusion-type prostate cancer and—within this subgroup—to presence of *PTEN* inactivation. Accordingly, high CRISP3 expression is also tied to adverse tumor phenotype and early PSA recurrence.

Earlier studies using microarrays had found massive CRISP3 overexpression in a subset of prostate cancers. Ernst *et al*,[3] described CRISP3 to be overexpressed >20-fold in microdissected

Table 3 Associations between CRISP3 immunostaining results and ERG-positive prostate cancer phenotype

Parameter	n *Evaluable*	*CRISP3 immunohistochemistry result*				P *value*
		Negative (%)	*Weak (%)*	*Moderate (%)*	*Strong (%)*	
All cancers	3547	45.9	24.8	16.7	13.0	
Tumor stage						
pT2	2120	48.96	24.76	15.52	10.75	<0.0001
pT3a	933	40.94	24.76	18.54	15.76	
pT3b	452	41.59	24.12	16.59	17.7	
pT4	23	26.09	43.48	13.04	17.39	
Gleason grade						
≤3+3	776	52.19	24.87	14.18	8.76	<0.0001
3+4	2112	43.8	25.14	18.04	13.02	
4+3	512	43.36	24.41	14.06	18.16	
≥4+4	124	48.39	19.35	13.71	18.55	
Lymph node metastasis						
N0	2047	44.11	24.72	17.49	13.68	0.1631
N+	205	43.9	25.37	12.68	18.05	
Surgical margin						
Negative	2776	47.12	24.71	16.07	12.1	0.001
Positive	703	40.11	25.18	17.92	16.79	

Table 4 Associations between CRISP3 immunostaining results and ERG-negative prostate cancer phenotype

Parameter	n *Evaluable*	*CRISP3 immunohistochemistry result*				P *value*
		Negative (%)	*Weak (%)*	*Moderate (%)*	*Strong (%)*	
All cancers	4485	87.9	7.1	2.4	2.5	
Tumor stage						
pT2	2999	89.63	6.97	1.67	1.73	<0.0001
pT3a	916	85.26	6.99	3.93	3.82	
pT3b	523	82.6	8.41	4.4	4.59	
pT4	28	85.71	0	3.57	10.71	
Gleason grade						
≤3+3	962	90.64	6.96	1.56	0.83	<0.0001
3+4	2539	88.54	7.17	2.13	2.17	
4+3	707	83.45	6.51	4.67	5.37	
≥4+4	254	83.07	8.66	3.15	5.12	
Lymph node metastasis						
N0	2646	88.21	6.73	2.49	2.57	0.0017
N+	227	79.3	9.69	4.85	6.17	
Surgical margin						
Negative	3572	88.3	7.42	2.21	2.07	<0.0001
Positive	821	85.75	5.97	3.65	4.63	

prostate cancer as compared with adjacent normal prostate epithelium. Others found a 20–2000-fold upregulation of CRISP3 mRNA in cancerous prostate.[5,6] As low-level CRISP3 expression does also occur in normal prostate epithelium,[4] our immunohistochemistry protocol was designed to identify high CRISP3 expressers and not to detect tissues containing any level of CRISP3 protein. Utilizing such an experimental setup, we identified 16% of prostate cancers with moderate-to-strong CRISP3 expression, whereas the remaining 84% of tumors had weak or absent CRISP3 immunostaining. This fraction of cancers exhibiting high-level CRISP3 expression fits well with data by Hoogland *et al* describing 13% of their prostate cancers showing a striking CRISP3 positivity at low magnification in their tissue microarray study.[11] Previous studies have reported higher rates of CRISP3 overexpression ranging between 19 and 96% of prostate cancers.[3,7–9,12] We assume that these authors partly

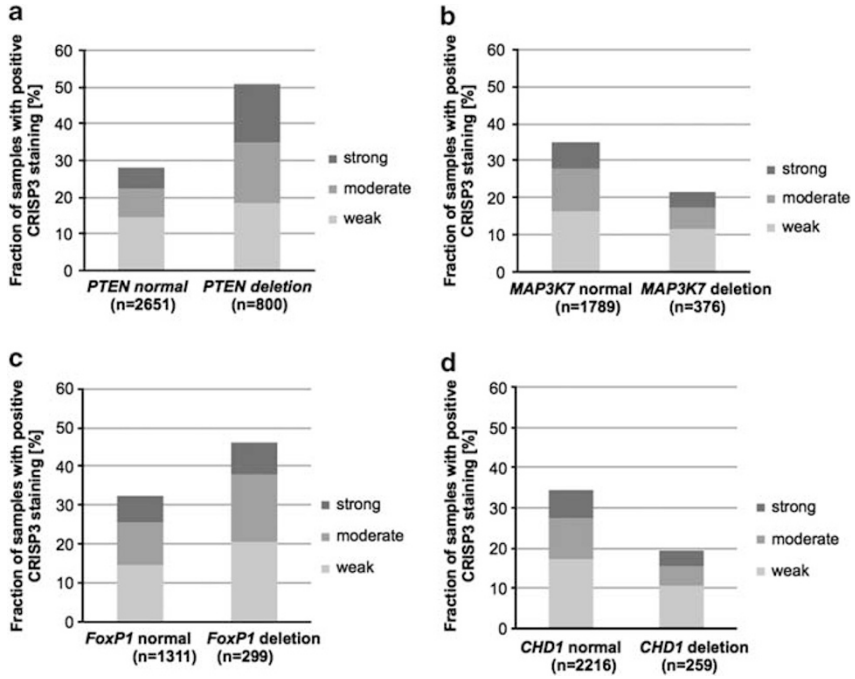

Figure 3 Association between positive CRISP3 immunostaining and (**a**) *PTEN*, (**b**) *MAP3K7*, (**c**) *FOXP1*, and (**d**) *CHD1* deletion probed by fluorescence *in situ* hybridization analysis in all cancers ($P<0.0001$; each).

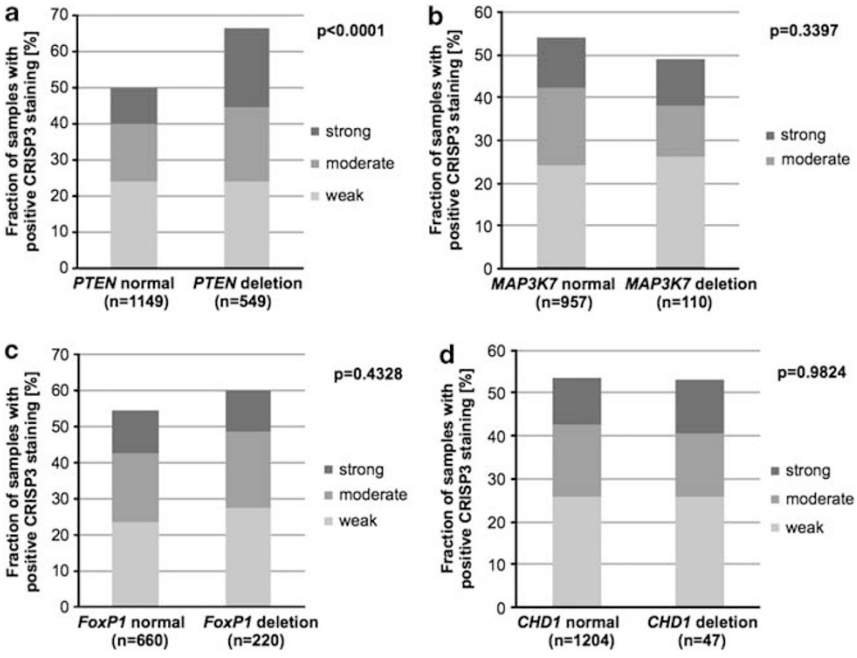

Figure 4 Association between positive CRISP3 immunostaining and (**a**) *PTEN*, (**b**) *MAP3K7*, (**c**) *FOXP1*, and (**d**) *CHD1* deletion probed by fluorescence *in situ* hybridization analysis in *ERG* fusion-positive cancers.

used markedly more sensitive immunohistochemistry approaches than selected for our project. The relatively low rate of CRISP3 positivity seen in our study cannot be caused by representative issues of our tissue microarray approach. Earlier immunohistochemical studies using tissue microarrays reported 74–98% CRISP3 positivity,[7,9,12] including one study analyzing a subset of the tissue microarrays employed in this project.[12] It is possible, that automated TMA reading with unusually sensitive thresholds has contributed to the high expression rate.[12]

High CRISP3 expression was strongly linked to *ERG* fusion-positive cancers. Finding this association

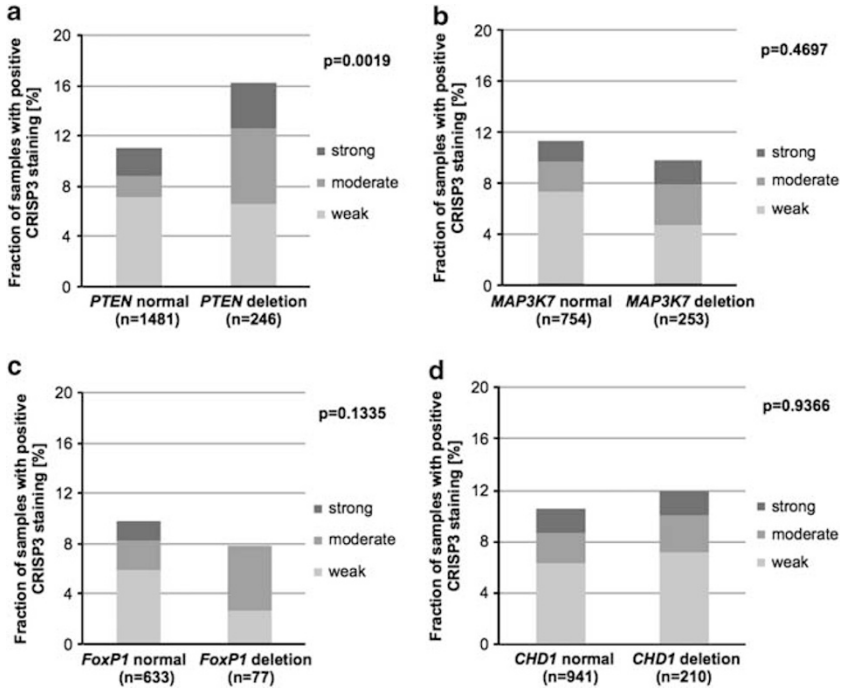

Figure 5 Association between positive CRISP3 immunostaining and (**a**) *PTEN*, (**b**) *MAP3K7*, (**c**) *FOXP1*, and (**d**) *CHD1* deletion probed by fluorescence *in situ* hybridization analysis in *ERG* fusion-negative cancers.

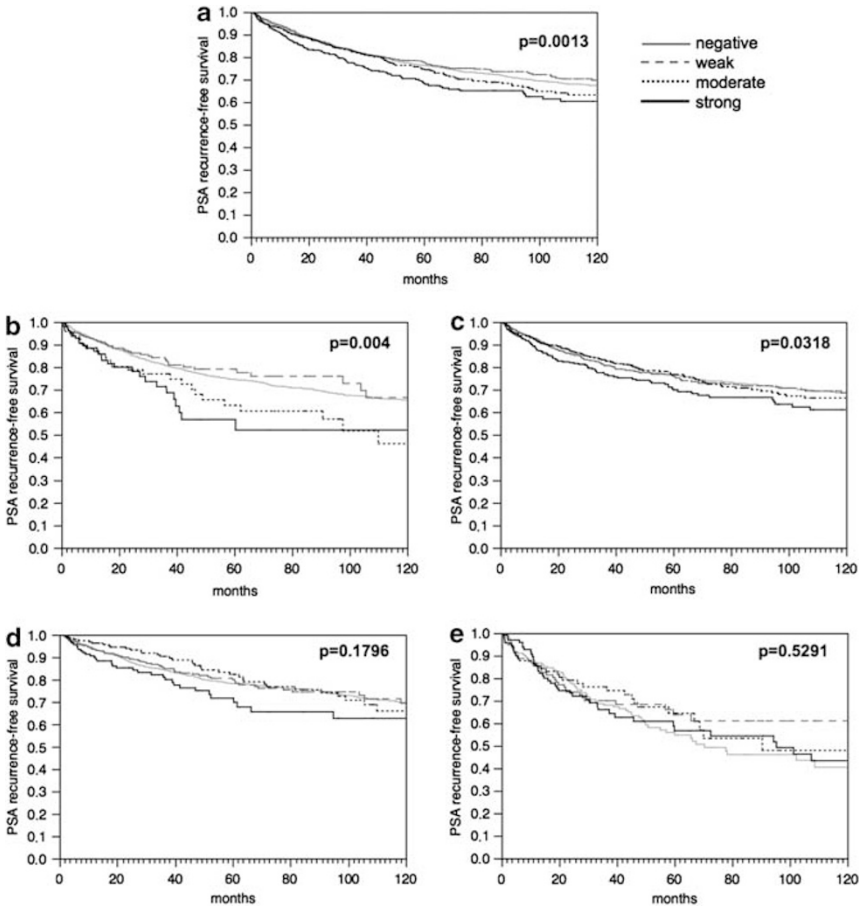

Figure 6 Association between CRISP3 immunostaining intensity and biochemical recurrence in (**a**) all cancers ($n=8019$), (**b**) *ERG* fusion-negative cancers ($n=3835$), (**c**) *ERG* fusion-positive cancers ($n=3080$), (**d**) cancers without *PTEN* deletion ($n=2428$), and (**e**) cancers with *PTEN* deletion ($n=751$).

by two independent approaches for *ERG* fusion detection (immunohistochemistry/fluorescence *in situ* hybridization) largely excludes a false-positive association due to inefficient immunostaining in a subset of damaged non-reactive tissues. Earlier data derived from mRNA expression screening studies by Brase *et al*[13] and Ribeiro *et al*[8] had already indicated an association between *ERG* fusion and CRISP3 expression in prostate cancer. Activation of ERG expression, mostly caused by *TMPRSS2:ERG* gene fusion, results in aberrant activation of different signaling cascades including upregulation of several metabolic enzymes, as well as extracellular/transmembrane proteins involved in cell adhesion, matrix remodeling, and signal transduction.[13,24–26] Our study identifies CRISP3 as another extracellular protein, which is strongly upregulated in *ERG* fusion-positive cancers. The functional role of CRISP3 in prostate cancer development and/or progression is unclear. It may be speculated, that CRISP3 exerts a role in cancer through beta-microseminoprotein (MSMB), a protein with proapoptotic activity and tumor inhibitory effects in prostate cancer cell lines.[27–29] CRISP3 forms complexes with MSMB. The MSMB is strongly downregulated in prostate cancer and thus prostate cancer progression may be critically affected by the amount of unbound CRISP3.[30]

Earlier genomic studies had identified chromosomal deletions that were tightly linked to either *ERG*-positive or *ERG*-negative prostate cancer. In particular, deletions at *3p13* and of *PTEN* were found to be associated with *ERG*-positive and *5q21* and *6q15* to *ERG*-negative cancers.[31–33] Our comparison of CRISP3 expression with these genomic deletions revealed, that high-level CRISP3 expression is particularly linked to ERG-positive/*PTEN*-deleted prostate cancer. These data may indicate, that either activation of a pathway that also induces CRISP3 overexpression may facilitate *PTEN* inactivation or else, that *PTEN* inactivation—at least in *ERG* positive cancers—may facilitate development of certain molecular features eventually leading to CRISP3 overexpression.

As 22% of all strong CRISP3 expressing *ERG*-positive prostate cancers had *PTEN* inactivation—a major negative prognosticator in prostate cancer—in our study, it was not surprising, that high-level CRISP3 expression was strongly associated with unfavorable tumor phenotype and early PSA recurrence in this study. Earlier studies had also suggested a negative prognostic role of high CRISP3 levels in serum and prostate cancer tissue.[7,9] Using immunohistochemistry on biopsies Bjartell *et al*[9] described CRISP3 as overexpressed in high-grade (Gleason scores 4/5) prostate cancer. It was also found in one study, that patients with CRISP3 overexpression had a slightly higher risk of recurrence after radical prostatectomy.[7] Using a consecutive series of 200 prostatectomy samples, Ribeiro *et al*[8] found upregulation of CRISP3 mRNA to be associated with stage pT3. The clinical utility of CRISP3 expression analysis remains questionable, as our multivariable analysis including also stage, grade, preoperative PSA values, and *PTEN* deletions did not suggest an independent prognostic role of CRISP3 overexpression. Other authors also failed to find an independent prognostic role of CRISP3 expression in prostate cancer.[8,10–12]

In summary, high CRISP3 expression identifies a small subset of prostate cancer harboring *ERG* fusion and *PTEN* deletion, which is clinically characterized by unfavorable tumor phenotype and early PSA recurrence.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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