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Cancer Prognosis Defined by the Combined Analysis of 8q, PTEN and ERG

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Abstract

Overtreatment is a major concern in men diagnosed with prostate cancer. The aim of this study was to evaluate the combined prognostic role of three frequent molecular alterations in prostate cancer, namely relative 8q gain, ERG overexpression, and loss of PTEN expression, in a series of 136 patients with prostate cancer treated with prostatectomy and with a long follow-up. Fluorescent *in situ* hybridization was used to detect the relative copy number of 8q and immunohistochemistry was used for quantitative assessment of ERG and PTEN expression. During a median follow-up period of 117.8 months, 66 (49%) patients had disease recurrence. Relative 8q gain, ERG overexpression, and loss of PTEN expression were observed in 18%, 56%, and 33% of the cases, respectively. No association with patient recurrence-free survival was found for relative 8q gain or ERG overexpression on their own, whereas loss of PTEN expression was associated with worse recurrence-free survival ($P = .006$). Interestingly, in the subgroup of patients with normal PTEN expression, we found that the combined relative 8q gain/ERG overexpression is associated with high risk of recurrence ($P = .008$), suggesting that alternative mechanisms exist for progression into clinically aggressive disease. Additionally, in intermediate-risk patients with normal PTEN expression in their tumors, the combination of 8q gain/ERG overexpression was associated with a poor recurrence-free survival ($P < .001$), thus indicating independent prognostic value. This study shows that the combined analysis of 8q, ERG and PTEN contributes to an improved clinical outcome stratification of prostate cancer patients treated with radical prostatectomy.

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Introduction

Prostate cancer (PCa) remains a major health burden in men, being the second most common non-skin cancer and the fifth leading cause of death from cancer worldwide [1]. These tumors display a heterogeneous spectrum of molecular abnormalities that arguably explains the variable clinical outcome [2]. Prostate-specific antigen (PSA) is an important clinical tool for early PCa detection, but has poor specificity and limited prognostic value [3–5]. Additionally, no tissue markers of aggressiveness other than Gleason score (GS) are available at diagnosis and many non-lethal cancers are treated

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aggressively [6,7]. Therefore, there is a need for more reliable diagnostic markers to complement PSA, as well as better prognostic markers to differentiate aggressive from indolent disease.

Gene fusions involving the erythroblastosis virus E26 transformation-specific (ETS) family of transcription factors are a highly specific and early molecular event in PCa [8,9] and studies have shown that about 50% of localized PCa patients harbor the *TMPRSS2-ERG* gene fusion [10–12]. The impact of *ERG* rearrangements in PCa prognosis remains controversial to date, both for authors using biochemical recurrence (BCR) as a clinical endpoint [13–15] and those using disease-specific survival [16–18]. On the other hand, ETS gene fusions seem to be insufficient to induce cancer formation on their own, and secondary chromosomal changes appear to be important in clinically aggressive PCa [19]. Chromosomal 8q gain has been associated with tumors in advanced stage [20] and a worse clinical outcome [21]. We have previously shown that PCa with relative 8q gain is associated with poor disease-specific survival, independently of Gleason score (GS) [22] and *TMPRSS2-ERG* gene fusion status [23]. Relative 8q gain was also strongly predictive of BCR in radical prostatectomy (RP) treated patients, independently of GS and TNM stage [24], thus supporting the role of relative 8q gain as a biomarker for aggressive PCa.

Genomic deletion of phosphatase and tensin homolog (*PTEN*), a tumor suppressor gene located at 10q23, is another commonly observed event associated with the prognosis of PCa [25]. *In vivo* studies have shown that complete loss of this gene recapitulates the major hallmarks of aggressive PCa, namely local tumor invasion, metastases and castration resistance [26]. Moreover, the role of PTEN in PCa progression has been supported by multiple studies showing that loss of the *PTEN* gene is a frequent event in castration-resistant metastatic prostate cancer [27–29]. Furthermore, loss of *PTEN* gene has been associated with *TMPRSS2-ERG* positive PCa tumors [30,31] and these genetic alterations combined have been suggested as a biomarker of early recurrence [32]. Nevertheless, it is unknown whether loss of *PTEN* in apparently localized tumors can help to identify which men are at increased risk of future castration-resistant PCa.

In the present work, we assessed the relative 8q copy number status and the expression profiles of ERG and PTEN in 136 RP treated PCa patients with long-term follow-up to evaluate their combined prognostic value.

Table 1. Characteristics of the 136 Prostate Cancer Patients Treated by Radical Prostatectomy

Parameters	Recurrence	
	Yes	No
Age, mean (range)	63 (47–73)	65 (50–74)
PSA (ng/mL), median		
≤ 6	9	16
≥ 6.01 and ≤10	5	10
≥ 10.01 and ≤20	22	22
> 20	27	22
Gleason score		
< 7	0	2
7 (3 + 4)	6	26
7 (4 + 3)	21	25
> 7	39	17
Pathological stage		
pT2	4	13
pT3	49	52
pT4	13	5
Time to recurrence (months), median (range)	72 (12–64)	155 (9–226)

Material and Methods

Prostatectomy Specimens and Clinical Data

The studied cohort consisted of 170 patients that underwent retropubic radical prostatectomy at the Norwegian Radium Hospital, Oslo University Hospital HF (between 1988 and 1996). A tissue microarray (TMA) block including two 0.6 μm punches from each of these patients was constructed. Of the 170 patients of the initial series, tumor material enough for the combined analysis of 8q, ERG and PTEN was available for 136 patients. Relevant clinical data at diagnosis was obtained from clinical records and are summarized in Table 1. Patient age ranged from 47 to 74 (mean and median 64) and pre-operative PSA levels from 1 to 96 ng/mL (median 17). Of the 136 prostatectomy specimens, 1.5% had GS lower than 7, 57.3% GS = 7, and 41.2% GS>7. After prostatectomy, 13% of the patients had the disease classified as pathological stage pT2, 74% as pT3, and 13% as pT4. The clinical endpoint of this study, assessed after a median of 117.8 months of follow-up (range, 8.6 to 226.3), was disease recurrence, which was defined as local recurrence, distant metastasis or prostate cancer death (death cause registry) and was assessed with biopsy, digital rectal examination or imaging modalities.

To stratify the patients in different risk groups of disease recurrence, we calculated the Cancer of the Prostate Risk Assessment post-Surgical (CAPRA)-S score [33]. Beyond preoperative PSA, this postoperative analogue to the CAPRA score incorporates additional pathological data, such as GS, surgical margin (SM) status, presence or absence of extracapsular extension (ECE), seminal vesicle invasion (SVI), and lymph node involvement (LNI). The CAPRA-S score was categorized in three groups: low (CAPRA-S 0 to 2), intermediate (CAPRA-S 3 to 5), and high (CAPRA-S≥6) risk of recurrence [33].

Fluorescence in Situ Hybridization (FISH)

Five-micrometer-thick sections of formalin fixed, paraffin-embedded (FFPE) prostatectomy specimens were processed and hybridized as previously described [22,23,34]. For the relative 8q24 gain assessment, a commercial dual-color break-apart probe flanking *MYC* at 8q24 (ZytoVision, Bremerhaven, Germany) and a centromeric probe for chromosome 18 (CEP18) labeled with SpectrumAqua (Vysis, Downers Grove, IL, USA) were used, as previously described [22,23,34]. Slides were counterstained with DAPI (Vector Laboratories, Burlingame, CA, USA) and fluorescent signals were captured in a Zeiss Axioplan fluorescence microscope (Zeiss, Oberkochen, Germany) coupled with a Cohu 4900 CCD camera using a Cytovision system version 7.4 (Leica Biosystems Richmond, Inc., USA). A ratio between *MYC* and CEP18 signals within individualized nuclei of a representative cancer cell population was computed for each sample. A sample was categorized as negative for relative 8q gain whenever *MYC*/CEP18<1.5 and as positive when *MYC*/CEP18≥1.5 [22]. Additionally, cases with *MYC*/CEP18≥2 were deemed amplified. An abnormal signal pattern was considered representative when present in a minimum of 50 morphologically intact, non-overlapping nuclei. FISH analysis was performed at the Department of Genetics of IPO Porto.

Immunohistochemistry

Staining for ERG and PTEN was performed on 3 μm tissue sections using the Dako Envison FLEX+ system (K8002; Dako, Glostrup, Denmark) and Dako Autostainer Link 48. The sections were incubated for 30 minutes with the rabbit anti-ERG monoclonal antibody (1:400, EPR 3864, Epitomics, Burlingame, CA, USA), or

for 120 minutes with the rabbit anti-PTEN monoclonal antibody (1:200, 138G6, Cell Signaling Technology, Danvers, MA, USA). The slides were dehydrated and counterstained with hematoxylin for 10 seconds before mounting. The slides were scanned by the NanoZoomer 2.0 Digital Slide Scanner (Hamamatsu Photonics KK, Japan).

The ERG expression was classified according to a four-tier grading system: negative, weak positive, moderate positive and strong positive expression, with the later three categories being lamped as positive (ERG+) for statistical analysis. The PTEN expression was evaluated manually as either positive (PTEN+) or negative (PTEN–). Both ERG and PTEN scores were performed by a pathologist (MP) at Oslo University Hospital.

Statistical Analysis

Pearson's chi-squared test was used to evaluate associations between categorical variables and Student's *t*test was used to compare continuous variables. Comparison of recurrence-free survival among subgroups of patients defined by different molecular alterations was performed using the log-rank test and plotted as Kaplan-Meier curves. Statistical significance was defined as two-sided $P < .05$. Statistical analyses were performed using SPSS, version 22.0 (Chicago, IL, USA).

Results

8q Copy Number Status

FISH analysis was successful in 124 of the 136 prostatectomy specimens analyzed. Tumor cell populations with 8q24 copy number increase were found in 65 of 124 (52%) of the specimens (Supplementary Table 1). Twenty-two (18%) specimens had a relative 8q24 copy number gain (8q+; *MYC*/CEP18≥1.5) (Figure 1*A*), with six of these samples displaying *MYC* amplification (*MYC*/CEP18 ≥ 2) (Figure 1*B*). Additionally, a putative structural rearrangement involving the *MYC* gene was found in one prostatectomy specimen (case P148T), showing a split of 3′and 5′*MYC* flanking probes (Figure 1C).

ERG and PTEN Expression

Immunohistochemistry (IHC) was performed in 136 PCa samples, of which seven and four were deemed not analyzable for ERG and PTEN protein expression, respectively. ERG expression was evaluated in the nucleus of tumor cells, with 57 (44%) of the cases showing a negative (ERG-) protein expression (Figure 2*A*). Positive expression of ERG (ERG+) was detected in the remaining 72 (56%) cases (17 weakly positive, 22 moderately positive and 33 strongly positive) (Figure 2*B*). Loss of PTEN expression, considered when it was not detected in both nucleus and cytoplasm of tumor cells, was found in 44 (33%) of the cases (Figure 2*C*), whereas normal PTEN expression was observed in 88 (67%) of the cases (Figure 2*D*).

Prognostic Value of 8q, ERG, and PTEN

After a median follow-up time of 117.8 months post-surgery, no evidence of disease was observed in 70 of the 136 patients (51%). The remaining 66 patients (49%) relapsed within a median follow-up time of 72 months, occurring significantly earlier in those with higher disease grade ($P < .001$, Supplementary Figure 1*A*) and more advanced disease ($P = .027$ Supplementary Figure 1*B*).

When assessing the prognostic value of the studied markers individually, no significant association with recurrence-free survival was found for either 8q + ($P = .489$, Supplementary Figure 2*A*) or ERG+ ($P = .514$, Supplementary Figure 2*B*), whereas patients displaying loss of PTEN expression had higher recurrence rate ($P = .006$, Supplementary Figure 2*C*) (Table 2). Additionally, in the subgroups of patients with GS = 4 + 3 and pT3/T4 tumors, loss of PTEN expression predicted a worse outcome ($P = .009$ and

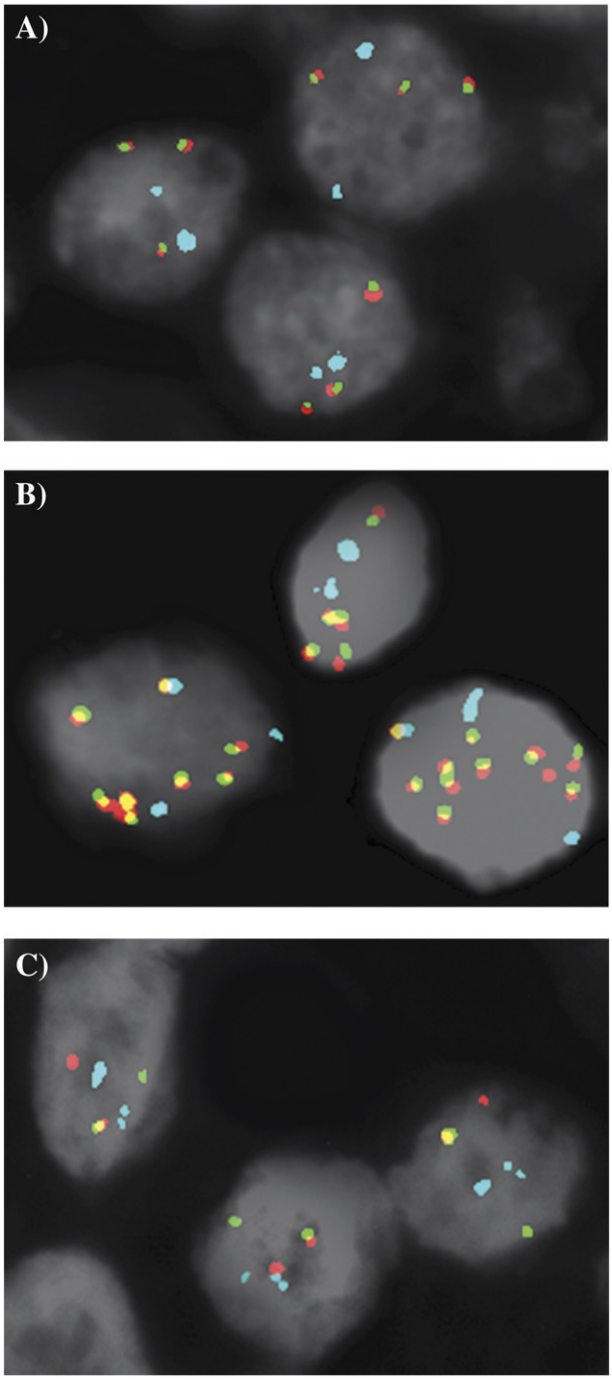

Figure 1. Representative FISH images from selected prostatectomy specimens analyzed with a commercial dual-color break-apart probe flanking *MYC* at 8q24 and with a chromosome 18 centromeric probe. A) Case P051T with nuclei displaying relative copy number increase of *MYC* (*MYC*/CEP18 = 1.5), illustrated by three co-localized signals of *MYC* (red and green for 5′and 3′, respectively) and two centromeric signals of chromosome 18 (aqua). B) Case P150T presenting *MYC* amplification (*MYC*/CEP18 ≥ 2). C) Case P148T showing nuclei with one co-localized signal of *MYC*, an additional split between red and green signals, indicating a *MYC* structural rearrangement, and two centromeric signals of chromosome 18 (aqua) (*MYC*/CEP18 = 1.5).

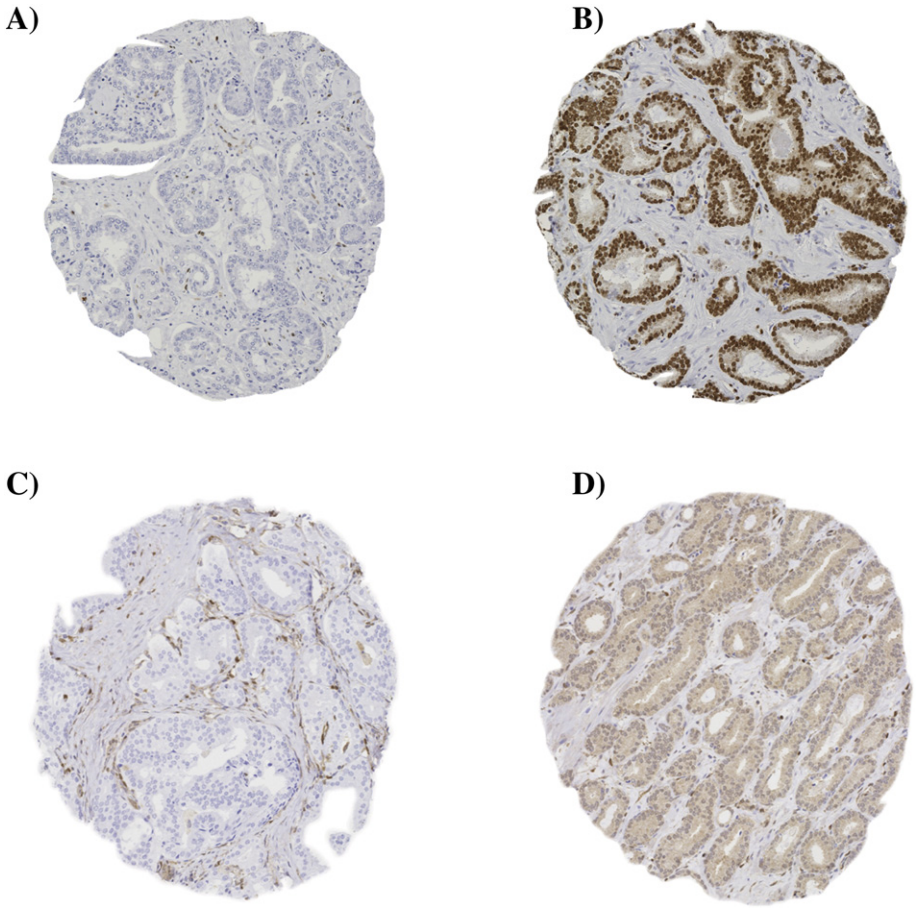

Figure 2. Illustrative IHC patterns of ERG and PTEN expression in the TMA of prostatectomy specimens. A) Negative ERG expression. B) Positive ERG expression. C) Negative PTEN expression. D) Positive PTEN expression.

P = .003, respectively, Supplementary Figure 3). When patients were stratified into subgroups according to relative 8q copy number alterations and ERG expression status, those patients with 8q + and ERG+ showed a tendency towards worse recurrence-free survival (P = .104, Supplementary Figure 4). Among the patients with normal PTEN expression, the 8q+/ERG+ patients had significantly worse prognosis (P = .008, Figure 3*A*) (Table 2), an association that was also statistically significant when comparing 8q+/ERG+/PTEN+ patients with all the other (P = .047, Supplementary Figure 5), but not in the subgroup with loss of PTEN expression (data not shown).

The patients were categorized by CAPRA-S score in groups of low (n = 5), intermediate (n = 41) and high (n = 87) risk of progression. We observed a significantly higher relapse rate in patients with higher CAPRA-S score (CAPRA-S ≥ 6, P < .001, Figure 3*B*). When we evaluated the prognostic value of isolated 8q, ERG and PTEN changes in the three risk groups defined by CAPRA-S, we found that intermediate risk patients with 8q + and high-risk patients with PTEN- showed a trend towards worse clinical outcome (P = .059 and P = .077, respectively, Supplementary Figure 6, *A* and *B*).

Interestingly, the combination of the two molecular markers 8q + and ERG+ added prognostic value in the intermediate-risk group (P = .026, Supplementary Figure 6*C*), with statistical significance being even more evident when considering only patients having a normal PTEN expression (P < .001, Figure 3*C*; P = .001, Supplementary Figure 7).

Other Clinicopathological Associations

Both relative 8q copy number gain and loss of PTEN expression were significantly associated with seminal vesicle infiltration (P = .047 and P = .033, respectively) and loss of PTEN expression was associated with GS>7 tumors (P = .017). Associations were also found between high-risk CAPRA-S score and tumors with PTEN- or 8q+/ERG+/PTEN+ (P = .028 and P = .004, respectively). No other statistically significant associations were found between clinicopathological variables and molecular features (Table 3 and Supplementary Table 2).

Discussion

In this study, we investigated the prognostic significance of the combination of three common molecular alterations in PCa, namely relative 8q copy number gain, ERG overexpression and loss of PTEN expression. Relative 8q copy number gain (*MYC*/CEP18≥1.5) was found in 18% of PCa patients, six of which harbored *MYC* amplification (*MYC*/CEP18≥2). Our results are therefore in agreement with other studies showing that relative 8q copy number gain is relatively frequent in PCa [23,24,35]. Although we have previously shown that relative 8q gain is a marker of poor prognosis in diagnostic prostate cancer biopsies [22,23,34], the present data in prostatectomy specimens from patients with long-term follow-up showed no overall differences in recurrence-free survival in 8q+patients, although intermediate risk 8q + patients showed a

Table 2. Association Between Disease Recurrence and Relative 8q Copy Number Gain, ERG Overexpression, and Loss of PTEN Expression in Prostate Cancer Patients

Markers		Number of Cases (%)	Number of Cases With Recurrence (%)	*P* Value
8q+	yes	22 (18%)	13 (59%)	0.489
	no	102 (82%)	47 (46%)	
ERG+	yes	72 (56%)	36 (50%)	0.514
	no	57 (44%)	26 (46%)	
PTEN-	yes	44 (33%)	27 (61%)	**0.006**
	no	88 (67%)	35 (40%)	
8q+/ERG+	yes	15 (13%)	10 (67%)	0.104
	other[a]	102 (87%)	47 (46%)	
PTEN-	8q+/ERG+	6 (16%)	3 (50%)	0.633
	other[a]	32 (84%)	22 (69%)	
PTEN+	8q+/ERG+	9 (12%)	7 (78%)	**0.008**
	other[a]	69 (88%)	27 (39%)	

8q+: relative 8q copy number gain; ERG+: ERG overexpression; PTEN-: loss of PTEN expression; PTEN+: PTEN normal expression.

[a] Includes all possible combinations regarding relative 8q gain and ERG expression status besides 8q+/ERG+.

trend to worse prognosis. Contrarily, Fromont and colleagues [24] found that relative copy number increase of *MYC* (*MYC*/CEN8≥1.5) was a strong predictive marker of BCR after RP, being independent of other known prognostic factors such as TNM stage and GS. In addition to differences related to the control probe used (CEN8) and the number of cases analyzed (n = 242), perhaps the most relevant explanation for the different conclusions regarding relative 8q copy number gain as an independent prognostic marker in patients treated with RP is related to the study design: in the study of Fromont et al. [24], all the patients that recurred were matched with patients free of recurrence according to age, PSA, GS and pT stage.

It has previously been demonstrated that, by using a specific antibody, overexpression of ERG can be used as surrogate marker for the presence of an ERG fusion gene [36,37]. We observed ERG+ expression in 72 cases (56%), which is in accordance with the rearrangement frequency of 44–65% reported by previous IHC studies on RP specimens [38,39]. We further observed that positive ERG expression was not associated with any of the clinicopathological variables analyzed (GS and pT stage) and had no prognostic value evaluated by recurrence. This lack of prognostic significance in surgically treated patients was observed in some reports [39–41] but not in others [15,42,43], which might be explained by differences in the endpoint used. When we stratified patients by relative 8q copy number and ERG protein status in the entire series, the patients with 8q+/ERG+ tumors did not show a significant difference in disease relapse compared to the other groups, indicating that the 8q+/ERG+ combination by itself is not an independent prognostic factor.

Earlier findings showed a strong link between deletion of *PTEN* and adverse tumor features, suggesting that PTEN down-regulation confers substantial malignant potential to PCa cells [25]. Reid and co-workers [44] combined IHC and FISH to detect alterations at the *PTEN* locus and reported a complete loss of PTEN protein expression in 58% of the tumors that had normal *PTEN* copy number by FISH. As alternative mechanisms could result in PTEN protein loss [45] and this molecular feature has been shown to be associated with PCa survival [28], we performed IHC in our series of patients who presented clinical criteria to be surgically treated by RP. We showed that 33% of the cases had loss of PTEN expression, which is within the range of 16 to 44% reported in previous studies [15,39,46]

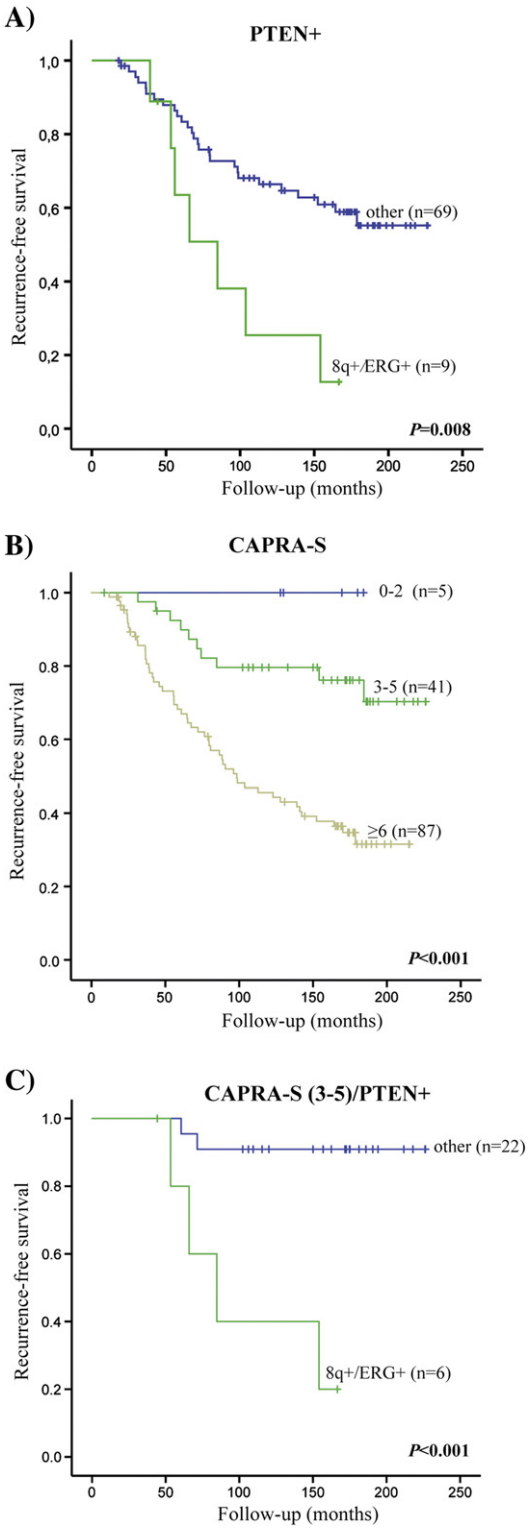

Figure 3. Kaplan–Meier curves illustrating recurrence-free survival. A) Comparison of patients with both relative 8q + and ERG+ with all other cases, among the patients with normal PTEN expression. B) Overall recurrence-free survival stratified by grouped CAPRA-S score: 0–2 indicates low risk, 3–5 intermediate-risk, and ≥6 high-risk of disease progression. C) Comparison of patients with both relative 8q + and ERG+ with all other cases, among the patients with normal PTEN expression and with an intermediate-risk of recurrence.

Table 3. Clinicopathological Associations with Relative 8q Copy Number Gain, ERG Overexpression and PTEN Loss of Expression in Prostate Cancer Patients

	8q + (n = 124)		ERG (n = 129)		PTEN (n = 132)	
Clinicopathological Parameter	No	Yes	No	Yes	No	Yes
Age (y), mean (range)	64 (48–73)	63 (47–74)	65 (48–73)	63(47–74)	63 (49–74)	64 (47–73)
	P = .486		*P* = .193		*P* = .610	
PSA (ng/mL), median						
≤6	16	5	7	17	7	17
≥6.01 and ≤10	14	1	7	6	3	11
≥10.01 and ≤20	35	7	16	27	18	26
>20	34	9	26	21	16	31
	P = .559		*P* = .117		*P* = .538	
Gleason score						
<7	0	1	1	1	0	2
7 (3 + 4)	23	6	12	17	5	26
7 (4 + 3)	35	8	21	22	13	31
>7	44	7	22	31	26	29
	P = .145		*P* = .885		*P* = **.017**	
Pathological stage						
pT2	11	4	7	10	5	12
pT3	74	17	44	51	36	62
pT4	17	1	6	11	3	14
	P = .259		*P* = .676		*P* = .285	
Surgical margins						
Negative	39	5	16	25	16	28
Positive	63	17	40	46	28	60
	P = .168		*P* = .427		*P* = .602	
Extraprostatic extension						
Negative	12	4	7	11	5	13
Positive	90	18	50	61	39	75
	P = .415		*P* = .626		*P* = .591	
Seminal vesicle infiltration						
Negative	66	19	40	47	25	66
Positive	36	3	16	24	19	22
	***P* = .047**		*P* = .529		***P* = .033**	
Lymph node invasion						
Negative	95	20	55	65	40	83
Positive	7	2	2	7	4	5
	P = .715		*P* = .169		*P* = .464	
CAPRA-S score						
0–2 (low)	4	1	3	2	2	3
3–5 (intermediate)	29	8	13	26	7	33
≥ 6 (high)	66	13	40	43	35	49
	P = .793		*P* = .233		***P* = .028**	

8q+: Relative 8q gain; ERG+: ERG overexpression; PTEN-: loss of PTEN expression; PTEN+: PTEN normal expression; CAPRA-S score: Cancer of the Prostate Risk Assessment.

Furthermore, we observed that loss of expression of PTEN was associated with markers of aggressive disease, including higher GS and seminal vesicle infiltration (Table 3), which is in line with previous reports [27,47]. PTEN down-regulation, alone or stratified for GS and pT stage, independently predicts worse recurrence-free survival. Our data is in agreement with that of others [48–50], but observations have been conflicting on whether PTEN inactivation is a prognostic marker in PCa [15,29,51], even when using the same clinical endpoint. This might in part be explained by the type of tumor samples used, as two of the studies reporting no significant association between loss of PTEN expression and BCR [29,51] were performed in biopsy samples. Furthermore, *PTEN* genomic loss has been identified as one of the most common concomitant events with *TMPRSS2-ERG* rearrangement [2,25,44], an interaction that has been validated by *in vivo* studies in mice [30,31,52]. The combination of these two alterations was described as predictor of early recurrence [32], something that we could not validate in this study.

Interestingly, although patients with normal PTEN expression in general presented better prognosis than those showing loss of PTEN expression, we here show for the first time that among the former there seems to be a subgroup of patients with tumors showing combined relative 8q gain and *ERG* overexpression (strongly associated with the *TMPRSS2-ERG* fusion gene) who are at high risk of recurrence. Moreover, for the patients that have an intermediate CAPRA-S score, the combination of these two molecular markers adds prognostic value, thus allowing differentiating a subgroup of patients that are at high risk of recurrence and another with good prognosis. The reason for the poor prognosis for the 8q+/ERG+ combination specifically in the background of normal PTEN expression is unknown, but may represent alternative mechanisms of PCa progression, one associated with loss of PTEN expression and another with overexpression of one or more target genes at 8q in a background of *ERG* rearrangement and normal PTEN expression. This study further indicates that it is unlikely that a single molecular prognostic marker is able to fully capture the clinically aggressive PCa cases, as alternative progression pathways and interactions between molecular alterations exist. Although further studies are necessary to fully characterize the molecular mechanisms of clinically aggressive PCa, the data we here present contribute significantly to molecular subtyping of the disease, with significant prognostic information that, if validated in biopsy specimens in large prospective studies with current standard treatment strategies, may

allow better treatment stratification if confirmed in independent studies.

Disclosure/Conflict of Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tranon.2016.08.005.

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