

Combination of Multiple Markers Predicts Prostate Cancer Outcome

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Abstract

Today we are facing a large problem of overtreatment in men with prostate cancer (PCa) due to the current lack of reliable prognostic biomarkers. Aberrations including ETS family gene rearrangements, phosphatase and tensin homolog (PTEN) deletions, enhancer of zeste homolog 2 (EZH2) overexpression and changes in the androgen receptor (AR) are commonly described in PCa and are believed to have an important role in progression of the disease. The aim of this study is to analyze if the protein expression of ERG, AR, PTEN and EZH2, and the deletion status of the *PTEN*, either alone or in combination can predict clinical outcome.

Our cohort consists of 214 men that have undergone radical prostatectomy at the University Hospital of Örebro, Sweden between 1989-2005. Immunohistochemistry was used to detect AR, ERG, PTEN and EZH2 antigen and *PTEN* deletion was assessed using chromogenic *in situ* hybridization.

The overall frequency showed AR-, ERG- and EZH2 expression in 99.5%, 52.9%, and 92.3% respectively. *PTEN* deletion was seen in 37.4% of the cases, where homozygous and heterozygous deletion was present in 18.1% and 19.2%, respectively. Our results show that there was a significant association between the combined ERG- and EZH2 expression and *PTEN* deletion with PCa specific death ($p=0.035$). This significant association was also seen in the group of cases that harbored both ERG expression and *PTEN* deletion ($p=0.036$). Cases expressing ERG, exhibiting *PTEN* loss (either hetero- or homozygous loss) and a Gleason score ≥ 8 showed a significantly higher rate of developing castration-resistant PCa (CRPC) and dying of PCa.

The current lack of a reliable prognostic tool available for PCa is a large problem, the results from this study and others shows the great potential in using multiple biomarkers to predict PCa outcome.

Keywords: Prostate cancer; Combination therapy; Protein expression; Multiple biomarker

Introduction

The consistent problem of overtreatment in men suffering from prostate cancer (PCa) is persistently a major challenge due to the absence of reliable prognostic biomarkers that can distinguish between indolent and aggressive forms of the disease. Since the introduction of prostate specific antigen (PSA) testing, the lifetime risk of being diagnosed with PCa has increased almost two-fold between the years 1985-2007 and the number of newly diagnosed cases has increased four-fold between the years 1996-2005 [1]. Studies have shown that a large number of PCa, identified through PSA testing, seldom progress into a clinically relevant stage. Hence, there is an urgent need for the identification of reliable prognostic markers that can be used at time of diagnosis to improve patient care.

Over the years, several genetic aberrations have been identified in PCa, which have raised the hope of finding biomarkers of prognostic value. Some of the most commonly described genetic aberrations include ETS rearrangements, loss of the tumor suppressor phosphatase and tensin homolog (PTEN), overexpression of enhancer

of zeste homolog 2 (EZH2) and changes of androgen receptor (AR) functions. AR is well-known to play a key role in both normal and malignant development of the prostate [2-4]. Tumors having the *ERG* rearrangement with concurrent *PTEN* loss have been proposed in several studies to be a sign of a more aggressive subtype of PCa [5-8] while EZH2 overexpression is commonly seen in metastatic PCa as well as being associated with aggressive disease [9-11].

Lately, studies have shown that potential cross-talk and interactions involving two or more of the above mentioned aberrations are of importance in PCa progression [12]. In a recent study by Mullholland et al. it was shown that *PTEN* loss enhances the expression of EZH2, which in turn had a negative effect on the AR transcription factor activity, enabling the tumor to proceed to a castration resistant state [13]. It has been further shown that ERG can activate EZH2, resulting in cancer progression [12] as well as regulating AR transcription in tumors with *PTEN* loss [14].

The aim of this study was to analyze the protein expression of ERG, AR and EZH2, including the deletion status of the *PTEN* gene. We aimed to investigate if any of these genes, both alone or in combination, correlate with and can predict clinical outcome.

Materials

Clinical samples

The cohort consists of 214 men who have undergone radical prostatectomy (pT1a-T3, Nx, Mx) at the University hospital of Örebro in Sweden between the years 1989-2005. Clinical and pathological data was obtained through patient records in October 2012. Study end-point were PCa specific death and castration resistant PCa (CRPC). Median follow-up time for CRPC and PCa specific death was 122 months and 110 months for PSA relapse. Secondary therapies included radiation and hormonal treatment, where 25/214 received radiation, 42/214 hormonal treatment and 8/214 a combination of both treatments. Whole mount tissue specimens were reviewed by the study pathologist (SP) and all tumor foci were identified prior to selecting three randomly representative 0.6 mm cores each from the circled tumor and benign foci for TMA construction. The study was approved by the Regional Ethics Board in Uppsala/Örebro (Dnr 2009/016).

Methods

Immunohistochemistry (IHC)

Immunohistochemical detection of AR, ERG and EZH2 antigen was conducted with the Ventana automated staining system (Ventana Medical Systems, Tucson, AZ, USA). Paraffin-embedded TMA blocks were sectioned into 4 µm slices, deparaffinized and antigens demasked in EDTA buffer, pH 8.4 (AR, ERG and EZH2) or Natrium citrate buffer, pH 6.0 (PTEN). Staining of AR was performed using the monoclonal rabbit Ig antibody against AR (Clone SP107, Ventana), ERG staining using the monoclonal rabbit Ig antibody against ERG (clone EPR3864, Ventana), PTEN monoclonal antibody (clone SP218, Spring Bioscience) was used for PTEN staining. A biotinylated Ig cocktail (UltraMap anti-rabbit HRP; Ventana) was used as secondary staining.

EZH2 staining was performed using rabbit monoclonal primary antibody directed against human EZH2 (SP129, Ventana). Secondary staining was performed using ultra View Universal DAB Detection Kit (Ventana). Finally, slides were counterstained with Hematoxylin II, followed by Bluing Reagent (Ventana). The intensity of the protein expressions was scored (by M.A.S) as negative (0), weak (1+), moderate (2+) or strong (3+) staining.

Chromogenic in situ Hybridization (CISH)

PTEN deletion was assessed using CISH conducted with the Ventana Discovery XT automated staining system (Ventana Medical Systems, Tucson, AZ, USA). Paraffin-embedded TMA blocks were sectioned into 4 µm slices, deparaffinized and pre-treated using enzymatic treatment (Protease 3, Ventana). Hybridization with PTEN probe and chromosome 10 reference probe (PTEN DNP Probe and Chromosome 10 DIG Probe, Ventana) was performed prior to detection (ultraView SISH DNP-and ultraView Red ISH DIG Detection Kit, Ventana). Slides were then counterstained with Hematoxylin II, followed by Bluing Reagent (Ventana). PTEN deletion was evaluated (by M.A.S) and reported as no deletion, heterozygous deletion or homozygous deletion.

Statistics

The cases were divided into three subsets, first containing all cases in the cohort, second containing only unifocal cases and third containing only multifocal cases. A focus was considered positive if one of the cores were stained positive for the marker. For each subset, Spearman's correlation test and Chi-2 tests were used to evaluate correlations/associations between the markers and tumor characteristics. Cox regression was used in order to identify predictors of clinical outcomes. Multivariate models were adjusted for age at diagnosis, calendar year of diagnosis (1989-1992, 1993-1996, 1997-2000 and 2001-2005), Gleason score at diagnosis, T-stage, R-status and PSA at diagnosis. In all statistical tests, a p-value <0.05 was considered significant. All statistical tests were performed in SPSS version 21.0.

Results

Prostatectomy samples from 214 men with PCa were assessed for AR-, ERG- and EZH2 protein expression and PTEN genomic deletion. The patient characteristics are shown in Table 1. AR protein expression was seen in 99.5% (211/212) of the evaluable tumor. The one case without AR expression was not among the cases that had PSA-relapse at the last time of follow-up, and was also negative for EZH2 and ERG protein expression. Furthermore, PTEN deletion could not be assessed for this case due to insufficient hybridization. ERG expression and EZH2 expression was present in 52.9% (110/208) and 92.3% (193/209) of the cases, respectively. Loss of PTEN protein expression was identified in 37.6% (80/213) and PTEN deletion was identified in 37.4% (68/182) of the assessable tumor cases. Heterozygous PTEN deletion was seen in 19.2% (35/182) while 18.1% (33/182) of the cases showed homozygous PTEN deletion. There was a significant association between ERG expression and PTEN loss (p=0.006). Concurrent ERG expression and loss of PTEN expression/PTEN deletion (either hetero- or homozygous deletion) was seen in 24.5% (51/208) and 25.8% (46/178) of the tumor cases, respectively.

We further investigated the assessed markers on possible associations with clinical outcome. Based on the numbers of identified tumor areas in each case, we divided the cohorts into two groups, unifocal and multifocal. Two-thirds of the cohort had a single identified tumor area (unifocal) while the remaining cases showed two or more (maximum of four) tumor areas (multifocal), (Table 1). A multifocal case was considered positive for protein expression/PTEN deletion if at least one tumor focus harbored the protein expression/PTEN deletion.

Amongst the unifocal cases, concurrent ERG expression and heterozygous PTEN deletion was significantly associated with PCa specific death (p=0.036). This significant association was also present in cases that harbored concurrent EZH2 expression, ERG expression and heterozygous PTEN loss (p=0.035). This association was not seen when comparing concurrent ERG expression and loss of PTEN expression.

Furthermore, cases expressing ERG, exhibiting PTEN loss (either hetero- or homozygous loss) and a Gleason score ≥8 showed a significantly higher rate of developing CRPC (p=0.016 (HR: 19.43, 95% CI 1.73-218.17)) compared to cases not having this phenotype. This finding remained significant after adjusting for clinical variables (Table 2a). Cases with the above described signature also showed a significantly higher rate of dying of their PCa (p=0.020 (HR: 17.99,

95% CI 1.57-205.66)), which also remained significant after adjustment of clinical variables (Table 2b).

Characteristics		Overall (n=214)	Unifocal cases (n=143)	Multifocal cases (n=71)	p-value
Age at Diagnosis		62.8 (45-74)	62.6 (45-74)	63.4 (52-74)	0.066
PSA at Diagnosis	<4 ng/ml	12 (6.1)	7 (5.4)	5 (7.2)	0.283
	4-10 ng/ml	111 (56.1)	75 (58.1)	36 (52.2)	
	>10 ng/ml	75 (37.9)	47 (36.4)	28 (40.6)	
	n/a	16	14	2	
Gleason Score	2-6	50 (23.4)	33 (23.1)	17 (23.9)	0.221
	3+4	56 (26.2)	34 (23.8)	22 (31.0)	
	4+3	83 (38.8)	59 (41.3)	24 (33.8)	
	8-10	25 (11.7)	17 (11.9)	8 (11.3)	
pT-stage	T1a	1 (0.5)	1 (0.7)	0 (0)	0.92
	T1b	6 (2.8)	4 (2.7)	2 (2.8)	
	T1c	111 (51.9)	73 (51.0)	38 (53.5)	
	T2	85 (39.7)	59 (41.3)	26 (36.6)	
	T3	11 (5.1)	6 (4.2)	5 (7.0)	
R-status	R0	102 (47.7)	71 (49.7)	31 (43.7)	0.697
	R1	49 (22.9)	32 (22.4)	17 (23.9)	
	RX	63 (29.4)	40 (28)	23 (32.4)	
CRPC	Yes	16 (8.0)	13 (10.0)	3 (4.3)	0.095
	No	183 (92.0)	117 (90.0)	66 (95.7)	
	n/a	15	13	2	
PCA specific Death	Yes	15 (7.5)	11 (8.4)	4 (5.8)	1
	No	185 (92.5)	120 (91.6)	65 (94.2)	
	n/a	14	12	2	
PCA relapse	Yes	55 (29.4)	37 (30.3)	18 (27.7)	0.241
	No	132 (70.6)	85 (69.7)	47 (72.3)	
	n/a	27	21	6	
Secondary treatment	Radiation	25 (11.7)	18 (12.6)	7 (9.9)	0.059
	Hormonal	42 (19.6)	31 (21.7)	2 (2.8)	0.283
	Radiation +Hormonal	8 (3.8)	6 (4.2)	2 (2.8)	1

Table 1: Selected characteristics of the study cohort

The combination of ERG expression-, EZH2 expression- and heterozygous deletion of PTEN were significantly associated with PCA specific death after adjusting for clinical variables, ($p=0,016$ (HR: 40.70, 95% CI 2.00-827.98)) (Table 2b). A significant association with

PCa specific death was also seen in patients harboring ERG expression- and heterozygous *PTEN* loss, ($p=0,016$ (HR: 44.05, 95% CI 2.01-964.37)) (Table 2b).

	HR (95% CI) (unadjusted)	P-value (unadjusted)	HR (95% CI) (adjusted)	P-value (adjusted)
2a CRPC				
ERG expression/PTEN deletion*/GS≥8	19.43 (1.73-218.17)	0.016	40.445 (2.94-557.32)	0.006
2b PCa specific death				
ERG expression/PTEN deletion*/ GS≥8	17.99 (1.57-205.66)	0.020	64.21 (4.22-976.52)	0.003
ERG expression/EZH2 expression/PTEN deletion**	ns		40.70 (2.00-827.98)	0.016
ERG expression/PTEN deletion**	ns		44.05 (2.01-964.37)	0.016
* Hetero- or homozygous deletion ** Heterozygous deletion Adjusted for PSA at diagnosis, T-stage, Gleason score, age at diagnosis, calendar year of diagnosis GS=Gleason score, ns=not significant				

Table 2: Combination of markers and clinical outcome

These findings did not reach statistical significance when investigating the *PTEN* expression instead of the *PTEN* deletion status.

Among the multifocal cases, *EZH2* expression alone was significantly associated with PCa specific death ($p=0.007$). This significant association, however, was not seen among the unifocal cases ($p=0.414$). When combining unifocal and multifocal cases we observed a trend towards an association of *EZH2* expression and PCa specific death ($p=0.080$) and CRPC ($p=0.081$). A trend towards association between PSA-relapse in cases that showed ERG expression and normal *PTEN* status (i.e. no deletion) ($p=0.065$) was also seen.

No association was found between intensity of any of the markers (alone or in combination) with clinical parameters (data not shown).

Discussion

PCa is a heterogeneous disease, making it difficult to distinguish the indolent from the aggressive form of the disease. This challenge often results in the overtreatment of patients. In this study we have assessed four markers known to be involved in PCa progression in order to investigate if a combination of these markers could be helpful in predicting PCa outcome. In agreement with previous studies, we observed ERG expression in 52.9% and *PTEN* loss in 37.4% of the cases [7,8,15-19]. There was also a significant association between ERG protein expression and *PTEN* loss ($p=0.006$), similar to the findings by Leinonen et al. [8]. *EZH2* expression was observed in the majority (92.3%) of cases. In this cohort, all cases, except one, were positive for AR protein expression. *EZH2* was the only single marker that was able to predict PCa progression (i.e. PSA-relapse or CRPC). We found, among the multifocal cases, that *EZH2* expression was significantly associated with PCa specific death ($p=0.007$). However this was only restricted to the multifocal cases and was not seen among the unifocal cases ($p=0.414$), suggesting that a patient with multiple cancer foci expressing *EZH2* has worse prognosis compared to unifocal PCa expressing *EZH2*. Studies have associated *EZH2* expression with aggressive disease and metastasis [10,11] and in a study by Varambally et al. they found that the highest *EZH2* expression was seen in metastatic PCa compared with benign prostate tissue and localized

PCa [9]. We found the combination of *EZH2* expression, ERG expression and *PTEN* heterozygous loss to be significantly associated with PCa specific death among the unifocal cases ($p=0.035$). Furthermore, unifocal cases harboring simultaneous ERG expression and *PTEN* heterozygous loss was significantly associated with PCa specific death ($p=0.036$), suggesting that the driving alterations leading to worse outcome, among the unifocal cases, involves ERG and *PTEN*. Our results further show that patients with Gleason score ≥ 8 , harboring ERG expression and *PTEN* heterozygous loss have a significantly higher rate of developing CRPC and of dying of the disease, compared to patients not having the above mentioned aberrations. This significant finding remained after adjusting for clinical variables and further supports the role of ERG and *PTEN* in PCa progression, but did not reach statistical significance when evaluating the loss of *PTEN* protein expression in the same manner as above mentioned combinations with *PTEN* deletion. The sequential order of *ERG* rearrangements and *PTEN* aberrations is currently unsolved but [6,19-21] concurrent *ERG* rearrangement and *PTEN* loss have been shown to promote PCa [5,6] and have been associated with PCa outcome. Leinonen et al. found that patients harboring aberrant ERG with simultaneous *PTEN* loss, were associated with shorter progression-free survival [8] and Yoshimoto et al., found the combination to be predictive of earlier biochemical recurrence [7], supporting the results found in this study. Somewhat contradicting, Reid and colleagues found that worst cause specific survival was seen in patients with *PTEN* loss lacking *ERG/ETV1* rearrangements while lacking both *PTEN* loss and *ERG/ETV1* rearrangements was a sign of better prognosis [22].

In order to be able to predict PCa outcome, several studies, including this study, supports the idea of using multiple markers to identify biological features of aggressive PCa especially with evidence of cross-talk and interactions involving common described aberrations. One problem with conducting biomarker studies for aggressive PCa is that it requires large cohorts with long follow-up time in order to include "hard" end-points such as CRPC and PCa specific death. The cohort size of this study is reasonably large, enabling us to study the combination of common aberrations in PCa and we could identify signatures that might be signs of a more aggressive type of disease. A caveat of this cohort is that the number of

hard outcome events is on the low side. In order to pin-point the potential implications of our significant findings, a cohort consisting of an increased number of outcome events would be required. Upon validation on a larger cohort with increased outcome events, the use of multiple biomarkers could prove to be a useful additional tool when evaluating biopsy material.

Conclusion

After investigating 214 radical prostatectomy samples, all tumor foci included, we found the combination of ERG expression, *PTEN* deletions and *EZH2* expression to be a sign of aggressive disease. Furthermore, PCa cases with Gleason score ≥ 8 harboring ERG expression and *PTEN* loss was also a sign of a more aggressive subtype. The current lack of a reliable prognostic tool available for PCa is a large problem, the results from this study and others shows the great potential in using multiple biomarkers to predict PCa outcome.

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