



How localized is pathologically localized prostate cancer? The use of secondary circulating prostate cells as a marker of minimal residual disease and their association with patient outcome

Patolojik olarak lokalize prostat kanseri ne derece lokalize? Minimal rezidüel hastalık belirteci olarak dolaşımdaki ikincil prostat hücrelerinin kullanılması ve hasta sonuçlarıyla ilişkileri

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ABSTRACT

Objective: To determine the prognostic value of secondary circulating prostate cells (CPCs) in men with pT2 prostate cancer treated with radical prostatectomy.

Material and methods: Prospective observational study was performed in men with pathologically confined prostate cancer who had been treated with radical prostatectomy. CPCs were obtained by differential gel centrifugation from 8 mL venous blood and identified by standard immunocytochemistry using anti-Prostate Specific Antigen (PSA) monoclonal antibody. A positive test was defined as ≥ 1 PSA staining cell/blood sample. Biochemical failure was defined as a serum PSA > 0.2 ng/mL. Age, PSA at diagnosis, pT2a versus pT2b/c, Gleason score and the presence/absence of CPCs were compared with patient outcomes using Kaplan-Meier curves and Cox's hazard model.

Results: Hundred and ninety-one men participated in the study, 107 (44.0%) had pT2b/c disease, 25 (13.1%) had a Gleason score ≥ 7 , and 39 (20.4%) were positive for CPCs. Biochemical failure occurred in 39 (20.4%) patients which was associated with a Gleason score ≥ 7 and CPCs (+). Survival rates at 3, 5 and 10 years for men with CPC (-) and CPC (+) were 100%, 100% and 89.6%, and 74.4%, 64.1% and 18.5% respectively (HR: 18.70). The median time to failure was 5.1 years in CPC (+) men versus 8.1 years in CPC (-) patients.

Conclusion: Secondary CPC is a marker for minimal residual disease and it is associated with a worse prognosis. The lead time to failure over serum PSA is approximately 5 years. However they do not define whether the failure is local or systemic.

Keywords: Biochemical failure; circulating prostate cells; minimal residual disease; pathologically organ confined; prostate cancer; radical prostatectomy.

ÖZ

Amaç: Radikal prostatektomiyle tedavi edilmiş pT2 prostat kanserli hastalarda dolaşımdaki ikincil prostat hücrelerinin (İPH'ler) prognostik değerini belirlemek.

Gereç ve yöntemler: Bu prospektif gözlemsel çalışmada patolojik olarak prostata sınırlı prostat kanseri nedeniyle radikal prostatektomi yapılan erkekler değerlendirildi. Hastaların 8 ml venöz kan örneğinden diferansiyel jel santrifüjleme tekniğiyle İPH'ler elde edildi ve anti-prostat spesifik antijen (PSA) monoklonal antikorları kullanılarak standart immünohistokimyasal yöntemle tanımlandı. Kan örneğinde ≥ 1 PSA ile boyanan hücrenin varlığı pozitif test olarak tanımlandı. Serum PSA düzeyinin > 0.2 ng/mL olması biyokimyasal başarısızlık olarak tanımlanmıştır. Hasta yaşı, tanı anında PSA, pT2b/c'ye karşın pT2a, Gleason skoru, İPH'lerin varlığı veya yokluğu Kaplan-Meier eğrileri ve Cox risk modeli kullanılarak hasta sonuçlarıyla karşılaştırılmıştır.

Bulgular: Çalışmaya katılan 191 erkeğin 107'sinde (%44,0) pT2b/c hastalık mevcut olup, 25'inde (%13,1) Gleason skoru ≥ 7 ve 39'unda (%20,4) İPH pozitif. Saptanan biyokimyasal başarısızlık Gleason skoru (≥ 7) ve İPH'lerinin varlığıyla ilişkiliydi. İPH (-) erkeklerde 3, 5 ve 10 yıllık sağkalım oranları sırasıyla %100, %100 ve %89,6 iken İPH (+) olanlarda sırasıyla %74,4, %64,1 ve %18,5 idi (HR: 18,70). İPH (+) ve İPH(-) erkeklerde başarısızlığa kadar geçen ortalama süre sırasıyla 5,1 ve 8,1 yıl idi.

Sonuç: Dolaşımda İPH'lerinin varlığı minimal rezidüel hastalık belirteci olup kötü prognozla ilişkilidir. Serum PSA tespitinden başarısızlığa kadar geçen süre ortalama 5 yıldır. Ancak İPH'ler tedavi başarısızlığının lokal veya sistemik olup olmadığını tanımlanamamaktadır.

Anahtar Kelimeler: Biyokimyasal başarısızlık; dolaşımdaki prostat hücreleri; minimal rezidüel hastalık; patolojik olarak organa sınırlı; prostat kanseri; radikal prostatektomi.

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Introduction

The use of prostate specific antigen (PSA) as a screening test has resulted in detection of the prostate cancer (PCa) at an earlier stage, with the majority of men being diagnosed with non-palpable, clinically localized disease.^[1,2] Although the percentage of patients with pathologically organ-confined tumors has substantially increased^[3], 4-32% of these men will eventually relapse following radical prostatectomy (RP).^[4-6] Multivariate analyses have reportedly revealed that the pathological tumor grade (Gleason score) and preoperative serum PSA are highly predictive of outcome following RP for pathologically localized PCa.^[7] More recently, it has been reported that even men with high grade Gleason scores of 8-10 have long-term outcomes similar to those men with more favorable disease characteristics when the disease is pathologically organ confined.^[8]

The presence of secondary circulating prostate or tumor cells (CPCs) detected in peripheral blood after RP, has been associated with a worse prognosis with a seven-fold increase in biochemical failure.^[9] The presence of CPCs after RP implies the persistence of PCa or minimal residual disease (MRD), which is not detected by imaging studies. We present a study performed in men with pathologically organ confined PCa who had been treated by RP and in whom CPCs were detected 1 month after surgery and CPCs association with patient outcome were analyzed.

Material and methods

Between January 2002 and December 2014 all patients who underwent open retro-pubic RP for PCa were enrolled in the study. After obtaining their informed written consent, for each patient the following data were recorded: date of surgical treatment, age, PSA at diagnosis measured using the Siemens Advia CentaurXR[®] assay, pathological Stage pT2a or pT2b/c and Gleason Scores of RP specimens estimated by a dedicated genitourinary pathologist.

Exclusion criteria:

- 1) Patients with extra-capsular extension (ECE); defined as cancer cells in contact with the prostatic capsule.
- 2) Patients with a positive surgical margin; defined as cancer cells in contact with the inked surface of the surgical specimen.
- 3) Previous treatment or consideration for treatment with androgen blockade
- 4) Consideration for adjuvant radiotherapy
- 5) Infiltration of the seminal vesicles and/or regional lymph nodes with cancer.

The pathological stage was defined as organ confined if all the cancer was confined within the prostate. Patients were classified as pT2a or pT2b/c. Biochemical failure was defined as a serum PSA >0.2 ng/mL on at least two occasions separated by a least a three week interval. Patients were followed up with serial total PSA levels, three monthly for the first year and six monthly thereafter.

Detection of secondary circulating prostate cells: One month after surgery an 8 mL venous blood sample was taken and collected in a tube containing EDTA (Beckinson-Vacutainer[®]). Samples were maintained at 4°C and processed within 48 hours. Presence of CPC was independently evaluated by an independent biochemist being blinded to the clinical details.

Collection of CPCs: Mononuclear cells were obtained by differential centrifugation using Histopaque 1,077 (Sigma-Aldrich), washed, and re-suspended in a 100 μ L aliquot of autologous plasma. Twenty-five microliter aliquots were used to make slides (silanized, DAKO, USA). These aliquots were dried in air for 24 hours and fixed in a solution of 70% ethanol, 5% formaldehyde, and 25% phosphate buffered saline (PBS) with a pH 7.4 for five minutes and finally rinsed three times in PBS.

Immunocytochemistry: Secondary CPCs were detected using a monoclonal antibody directed against PSA, clone 28A4 (Novocastro Laboratory, UK), and identified using an alkaline phosphatase-anti alkaline phosphatase based system (LSAB2, DAKO, USA), with new fuchsin as the chromogen. Positive samples underwent a second process with anti-CD45 clone 2B11 + PD7/26 (DAKO, USA) and were identified with a peroxidase based system (LSAB2, DAKO, USA) with DAB (3,3 diaminobenzidine tetra hydrochloride) as the chromogen. A secondary CPC was defined according to the criteria of ISHAGE (International Society of Hemotherapy and Genetic Engineering).^[10] A secondary CPC was defined as a cell that expressed PSA but not CD45; a leucocyte that did not express PSA but expressed CD45. A test was considered positive for secondary CPCs when at least 1 cell per 8 mL of blood was detected, and the number of CPCs detected per 8 mL blood sample was registered.

Statistical analysis

The analysis was performed using the program Stata (Stata/SE 14.0 for Windows, Stata Corp Lcc, 20159 Tx, USA) which statistically analyzed nature and distribution of the quantitative and ordinate variables with measurements of central tendency (mean and median) and of dispersion using the inter-quartile range (IQR) and standard deviation (SD).^[11] The Shapiro-Wilk Test was used to define the null hypothesis with respect to the normal distribution. The nominal dichotomous variables were described as proportions with their respective confidence intervals.^[12]

Age, PSA at diagnosis, Gleason Score, Pathological Stage (pT2a or pT2b/c) and presence of secondary CPC were compared, to detect the presence of relapse.

From the predictors: A multivariable Cox regression analysis considering age, PSA at diagnosis, pathological stage (pT2a or pT2b/c), Gleason Score greater than 6 and presence of secondary CPCs was performed in order to evaluate the relapse predicted during the ten-year follow-up. The Cox regression analysis was conducted by means of a stepwise backward selection approach.

The constructed final model was established with predictors whose coefficients showed statistical significance (p value <0.01) and the fulfillment of assumptions concerning proportional risk, adequate calibration and discrimination).

In addition, this model was tested for compliance to proportional hazards by means of log-log plots, Therneau and Grambsch test and testing for a cohort time interaction.^[13] The appropriate specification of the final model was studied through the Linktest.^[12] The calibration aspect of the model refers to agreements between the predicted outcome and observed outcome.^[14] In this model, this is assessed by graphical methods, which include observed versus predicted values for probabilities and predictions and cumulative hazard of Cox-Snell residuals.^[12] Also, performed a comparison of survival using the Cox and Kaplan-Meier models.^[12]

The discrimination of a prognostic model reflects its ability to distinguish between patient outcomes. We calculated Harrell's C discrimination index^[15,16] and the Gönen and Heller's concordance coefficient^[17] which are scored on a scale of 0 to 1. Also, for the final model the log likelihood (LL), Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC), predictor coefficient and its predictive value^[11,12] were determined.

Results

Three hundred and twenty-one men underwent RP for PCa of which 191 men complied with the study criteria of having pathologically organ confined PCa. The mean age of these 191 men was 64.86 ± 8.14 years. The PSA at diagnosis had a median value of 5.20 ng/mL and and IQR of 1.62 ng/mL.

Of the 191 patients, 107 (43.98%; 95% CI: 36.94-51.029) were classified as having T2b/c disease. Among 191 men, biochemical

failure was observed in 43 men (22.51%; 95% CI: 16.59 - 28.44), 39 (20.42%; 95%CI: 14.70 - 26.14) men were positive for secondary CPCs, and 25 men (13.09%; 95% CI: 8.31 - 17.87) had a Gleason score of >6 .

Table 1 shows a significant statistical association between biochemical failure and PSA at diagnosis, Gleason Score greater than 6, pathological stage T2b/c and secondary CPC. The frequency of detection of secondary CPCs was significantly higher in patients with a Gleason score <6 , pathological stage pT2b/c and biochemical failure but not correlated with age or serum PSA at diagnosis (Table 2).

After 3, 5 and 10 years of follow up, the Kaplan-Meier biochemical failure-free survival rates for the whole group were 94.72% (95% CI: 90.41 - 97.13), 92.37% (95% CI: 87.45-95.42) and 71.57% (95% CI: 62.53-78.80), respectively. For CPC (-) patients the 3, 5 and 10-year survival rates were 100%, 100% and 89.6% respectively and for CPC (+) patients corresponding survival rates were 74.4, 64.1 and 18.5%, respectively (Table 3). The Cox regression model showed a hazard ratio for secondary CPCs as 18.70 (95% CI: 8.82-39.63) (for all $p < 0.0001$). For this model, the values for LL were -182.46, AIC 295.15 and BIC of 298.40.

For the built final model, the log-log plots for $-\ln(-\ln)$ survival versus $\ln(\text{time})$ using Kaplan-Meier estimates, show parallel log curves. The Therneau and Grambsch test and testing for a cohort time relation on the model was not significant.

The cumulative hazard of Cox-Snell residuals for the model studied, showed adequate degree of goodness of fit. During 10 years, comparison between predicted (according to the model of Cox) versus observed survival rates (model Kaplan-Meier) showed agreement (Figure 1). The Harrell's C concordance coefficient was 0.83 (excellent concordance).

Table 1. Clinicopathological findings in 191 subjects with organ- confined prostate cancer

Characteristics	BF (-) n=148	BF (+) n=43	p	RR/OR
Age (years) mean \pm SD	64.4 \pm 8.4	66.6 \pm 6.8	0.121 ^a	
PSA at diagnosis (ng/mL) Median; IQR	5.17; 1.42	5.50; 2.32	0.014 ^b	
PSA \leq 10 ng/mL	141	39		
PSA $>$ 10 ng/mL	7	4		
Gleason score >6 , n (%)	14 (9.5%)	11 (25.6%)	$<0.006^c$	RR=2.70 (95% CI 1.33-5.52) OR=3.29 (95% CI 1.37-7.92)
pT2b/c, n (%)	52 (35.1%)	32 (74.4%)	$<0.0001^c$	RR=2.12 (95% CI 1.60-2.80) OR=5.37 (95% CI 2.50-11.53)
CPC (+)	7 (4.7%)	32 (74.4%)	$<0.0001^c$	RR=11.34 (95% CI 6.30-20.41) OR=58.60 (95% CI 21.08-162.9)

SD: standard deviation; IQR: interquartile range. ^aThe p-value of the Shapiro-Wilk test was <0.15 for these variables; ^bStudent's t-test assuming equal variance (Variance ratio test p-value >0.05); ^cMann-Whitney U Test; ^dPearson's Chi RR: relative risk; OR: odds risk; PSA: prostate specific antigen; CPC: circulating prostate cells; BF: biochemical failure

The mean time to failure was 5.1±2.8 years for CPC (+) patients versus 8.1±1.3 years for CPC (-) patients (p<0.002) with a median lead-time of 4.7 years (IQR 2-7 years) before PSA defined failure occurred.

Discussion

Radical prostatectomy is an effective therapy for PCa, especially when the cancer is organ confined at final pathological analysis. Failure of RP in these patients is not easily understood, and has been reported to occur in 5-23% of the patients.^[4-6] In patients who achieve PSA nadir of 0.01 ng/mL post-surgery, as in our series, it is even harder to explain failure of RP. Although an erroneous pathological classification may, in terms of either the cancer penetrating the PCa (pT3) or an anatomically incorrect dissection plane (unrevealed positive margin), which left behind microscopic amounts of PCa and then subsequently progressed, explain some cases but not the majority. The presence of sub-clinical micrometastasis not detected by conventional imaging is a more logical explanation of these cases. These microscopic

foci left behind after radical surgery is termed minimal residual disease. In this study we analyzed the use of secondary CPC detection as a measure of minimal residual disease and their association with treatment outcome in patients with pathologically confined PC treated by radical prostatectomy.

In the study group, a higher PSA at diagnosis, a Gleason score of ≥7 and pT2b/c were associated with a higher frequency of biochemical failure; age *per se* was not a significant prognostic factor as has been previously reported.^[18] Forty-three (43/191; 22.5%) patients in the study group underwent biochemical failure within 10 years which is consistent with the reported literature.

The presence of secondary CPCs has been reported to be associated with decreased biochemical free survival^[9], and adverse prognostic factors, higher Gleason score, higher stage cancer and higher PSA values at diagnosis.^[9] In men with pathologically confined PCa, biochemical failure was associated with higher Gleason score, T2b/c disease, higher PSA at diagnosis and the presence of secondary CPCs. Multivariate analysis showed that

Table 2. Association between clinicopathological findings and frequency of secondary CPC detection

Characteristics	CPC (-) n=152	CPC (+) n=39	p	RR/OR
Age (years) mean±SD	64.7±8.4	65.7±6.9	0.477 ^a	
PSA at diagnosis (ng/mL). Median; (IQR)	5.18; 0.76	5.49; 0.72	0.51 ^b	
PSA ≤10 ng/mL	143	37		
PSA >10 ng/mL	9	2		
Gleason > 6 n (%)	14/152 (9%)	11/39 (28%)	0.0036 ^c	RR 3.06 (95% CI: 1.56-6.21); OR 3.87 (95% CI: 1.59-9.41)
pT2b/c n (%)	60/152 (39%)	25/39 (64%)	0.007 ^c	RR 1.62 (95% CI: 1.20-2.21); OR 7.40 (95% CI: 1.32-5.69).
Biochemical failure n (%)	11/152 (7%)	32/39 (82%)	<0.0001 ^c	RR 17.82 (95% CI: 8.52-37.28); OR 94.69 (95% CI: 31.04-288.89).

SD: standard deviation; IQR: interquartile range. ^aThe p-values of the Shapiro-Wilk test were <0.15 for these variables; ^bStudent's t-test assuming equal variance (Variance ratio test p-value >0.05); ^cMann-Whitney U Test; ^dPearson's Chi RR: relative risk; OR: odds ratio; PSA: prostate specific antigen; CPC: Circulating prostate cells

Table 3. Three, five and ten year biochemical failure-free survival rates according to the presence of secondary CPCs; comparisons between observed survival (Kaplan-Meier) and predicted survival rates (Cox model) in 191 patients with pathologically organ confined prostate cancer

Presence of secondary CPCs	Years survival	Observed survival rates (95% CI)	Predicted survival rates (95% CI)
Negative	3	100*	98.7 (97.0-99.5)
	5	100*	98.1 (95.9-99.1)
	10	89.6 (80.7-94.5)	90.7 (82.9-95.0)
Positive	3	74.4 (80.7-94.5)	78.7 (63.9-88.0)
	5	64.1 (47.0-76.9)	70.0 (54.3-81.2)
	10	18.5 /7.6-33.0)	16.0 (6.7-28.9)

+ biochemical failure was not observed. CPC: circulating prostate cells

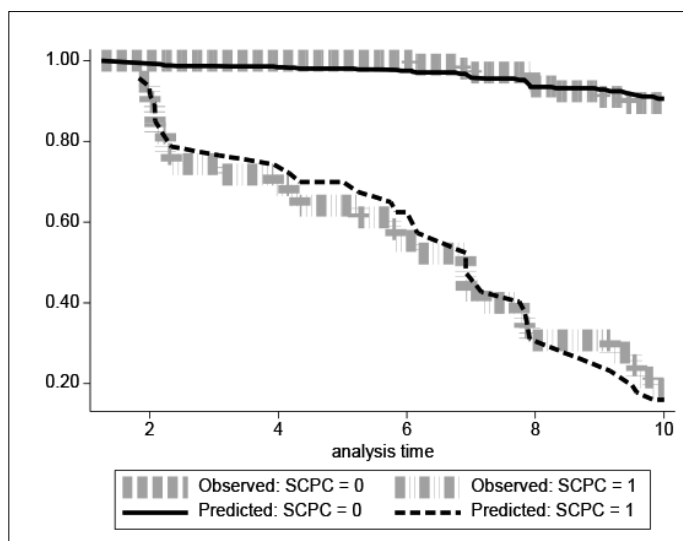


Figure 1. Ten year biochemical failure-free survival rates for prostate cancer according to presence of secondary circulating prostate cell (SCPC) comparing the observed survival (Kaplan-Meier) with predicted survival (Cox Model) in 191 subjects with organ-confined prostate cancer

only the presence of secondary CPCs and Gleason score >7 were significantly associated with biochemical failure. An association between higher Gleason scores and risk of biochemical failure is consistent with previously reported data, and that patients with Gleason scores of 8-10 are uncommon in these patients, in our study representing only 2.1% (4/191) of all patients.^[19]

The importance of secondary CPC detection is that it identifies a group of patients with a high risk of disease progression before serum PSA increases, providing a median lead time of 4.8 years which is an advantage over the PSA relapse criteria. It was independent when compared to conventional risk factors (HR 18.70 $p < 0.0001$) with a specificity of 95.2% (95% CI 90.1-97.9), sensitivity of 82.1% (95% CI 65.9-91.9), a positive predictive value of 82% (95% CI 65.9-91.9) and negative predictive value of 95.3% (95% CI 90.1-97.9).

The optimal use of secondary CPCs has not been established; neither in terms of the method used to detect them nor in terms of defining a positive/negative test or cut-off values. We used a positive/negative definition of the CPC test and not a cut-off value using a specific number of cells/samples detected, in order to simplify clinical decisions. The only FDA approved CTC detection system ie. the CellSearch® system, which uses a cutoff of 5 cells/sample in metastatic disease, 1-5 cells/sample for non-metastatic disease a cutoff of between 1-5 cells/sample has been suggested.^[20] The EpiSPOT® is an EpCAM independent assay that enriches CTCs by negative depletion of leukocytes and detects viable cells based on their active secretion of PSA^[20], with this assay 40% of T1c cancers were positive for CTCs.^[20]

The clinical utility of secondary CPC based early detection of recurrence could be associated with the success of postoperative radiotherapy. Radiotherapy has the potential to eliminate microscopic residual disease and cure local recurrence. At present high-risk pathological features (extracapsular extension, seminal vesicle infiltration and positive surgical margins) are used to select patients for possible radiotherapy. High-risk patients treated with adjuvant radiotherapy had an improved biochemical relapse-free survival^[21,22] and overall survival.^[23] These studies showed a benefit of radiotherapy over observation, but did not address the fundamental question of adjuvant versus early salvage radiotherapy. Salvage radiotherapy is more effective when initiated at lower PSA values. A meta-analysis showed that the success of salvage radiotherapy decreased by 2.5% with every increment of 0.1 ng/mL PSA.^[24]

However, the presence of secondary CPCs does not distinguish between local and systemic disease. Thus although they identify patients at high risk of treatment failure their presence does not specify which treatment option, local or systemic could be beneficial to the patient. With regards to the election of systemic therapy, CPCs can be classified by their phenotypic characteristics, expression of the androgen receptor splice variant -7 (Arv7)^[25] or HER-2 expression^[26] in predicting the efficacy of hormonal therapy. This may aid in the decisions of treatment selection, and as such personalized medicine. However a more important question, which is beyond the scope of this study, is whether patients with pathologically confined PCa and positive for secondary CPCs would benefit from early adjuvant therapy.

In conclusion, among men with pT2 organ confined disease, a significant number will undergo biochemical failure within 10 years of radical prostatectomy. The detection of secondary CPCs identifies a subgroup of these men with a significantly higher risk of treatment failure, with a median lead time of 4 years before an increase in serum PSA is detected. However, their presence as a biomarker of minimal residual disease does not help in the selection of treatment in that their detection does not distinguish between local and systemic micrometastatic disease.

Ethics Committee Approval: Authors declared that the research was conducted according to the principles of the World Medical Association Declaration of Helsinki “Ethical Principles for Medical Research Involving Human Subjects”, (amended in October 2013).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

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