Prognosis of Castration-resistant Prostate Cancer Patients – Use of the AdnaTest® System for Detection of Circulating Tumor Cells

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Abstract. *Background: Detection of circulating tumor cells (CTC) in patients with castration-resistant prostate cancer (CRPC) may improve the estimate of chemotherapy response. We evaluated the AdnaTest® system in patients receiving docetaxel. Patients and Methods: CTC analysis was carried out in 37 patients by immunomagnetic separation. Correlation between serum prostate-specific antigen (sPSA) change and CTC presence and the influence of each parameter on the overall survival (OS) were evaluated. Results: We detected CTCs in 32 and 16 patients before and after three docetaxel cycles, respectively. The sPSA level correlated with CTC positivity during docetaxel therapy (p=0.0031). The longest OS was in patients negative for CTCs in both samples (p=0.0228). Change in sPSA levels was associated with treatment response (p=0.033). Conclusion: We detected CTCs in a considerable number of patients with CRPC. The absolute change of sPSA level correlated with OS. CTC presence during docetaxel therapy was associated with shorter OS.*

Currently, 10-20% of patients with prostate cancer enter the castration-resistant stage within approximately 5 years of follow-up during androgen deprivation therapy (1). Castration-resistant prostate cancer (CRPC) is burdened with poor prognosis and impaired quality of life. Historically, the estimated mean survival of patients with CRPC was 9-36 months, according to the extent of metastatic disease and presence of symptoms (2). These estimates have been modified by new hormonal and cytotoxic regimes (*e.g.* abiraterone, enzalutamide or cabazitaxel). Prognostic tools in CRPC are mainly based on retrospective evaluation of completed chemotherapy trials, resulting in either definition of risk factors (3) or construction of nomograms (4). Currently, CRPC represents quite a heterogeneous disease, with many patients not having radiographically measurable disease. Osteoblastic bone metastases are the commonest site of metastatic spreading of prostate cancer, yet are difficult to assess regarding treatment response. Moreover, the decline in serum levels of prostate-specific antigen (sPSA) or relief from painful symptoms as surrogate markers remain a matter of debate, even though some results indicate that they are associated with prolonged survival (5).

Circulating tumor cells (CTCs) are rare cancer cells that derive from the primary tumor or metastases and enter peripheral blood (6). Genetic analyses have shown, that *e.g.* in colorectal cancer, CTCs are similar to cells of the main tumor and might be used immediately as a source of tumor cells for different tests (7). Another practice is to perform biopsy of the tumor lesion before and during therapy and to tailor the sequencing regimen based on the molecular profile analysis. Bone metastasis biopsies might be challenging and not adequately reliable, therefore this approach is not routinely used in patients with CRPC. Detection of CTCs, often called 'liquid biopsy', can be used for the assessment not only of tumor burden, but also for the prognosis before initiation of and during systemic therapy (8).

Currently, the most commonly used system for CTC detection is CellSearch™ (Veridex LLC). The assay enriches CTCs by using antibodies to epithelial cell adhesion molecule (EpCAM) conjugated to a magnetic bead. The cells are further classified by immunohistochemical staining of cytokeratins and exclusion of leukocytes (CD45 staining). The final result is given as the number of CTCs in 7.5 ml of whole blood (9). The absolute number of CTCs in CRPC has been shown to be

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Key Words: AdnaTest, castration-resistant prostate cancer, circulating tumor cells, chemotherapy, prognosis.

clinically relevant. Samples with five or more CTCs are marked as 'unfavorable', due to this having a direct impact on patient survival (10). Other methods of CTC detection are based on the assessment of expression of tumor-associated genes by a reverse transcription and polymerase chain reaction (RT-PCR), which enables further molecular analysis of CTCs (11). However, the RT-PCR-based studies of different prostate cancer stages have shown conflicting results regarding the proportion of positive samples and clinical value of CTC detection (12, 13). These inconsistencies may be caused mainly by different laboratory protocols and analytical methods, *i.e.* lack of standardization, and different reporting of RT-PCR results.

A novel RT-PCR method for CTC detection, the AdnaTest (Adnagen AG, Langenhagen, Germany) was recently tested in patients with breast, prostate and colorectal cancer (14-16). The AdnaTest® platform is a two-step method that consists of the ProstateCancerSelect® and ProstateCancerDetect® systems. This commercially available kit enables immunomagnetic enrichment of CTCs from whole blood (ProstateCancerSelect®) followed by reverse transcription and multiplex PCR analysis of tumor-associated mRNAs (ProstateCancerDetect®. In the present work, we assessed CTC detection by AdnaTest method in patients with CRPC before and during docetaxel therapy. We evaluate predictive parameters of cytotoxic therapy efficacy and overall survival (OS) and describe an association between the sPSA level and CTC detection.

Patients and Methods

Patient selection and study procedures. A total of 37 patients with histologically proven prostatic adenocarcinoma and evidence of metastatic disease on either bone scintigraphy or computed tomographic (CT) scan were included in this study. All patients fulfilled criteria of CRPC according to the European Association of Urology Guidelines (2) and all of them were indicated for cytotoxic therapy due to either symptomatic or radiographic progression. Patients either incapable of or with former/ongoing cytotoxic therapy were excluded. All patients had performance status (PS) of 2 or less. Docetaxel at 75 mg/m^2 was administered in a 3-week cycle regime with 5 mg prednison orally twice daily. For three patients, a 1-week regime was indicated either due to PS of 2 or impaired creatinine clearance. sPSA values were assessed before initiation of docetaxel and in every other cycle. Bone scintigraphy was performed in all patients before initiation of docetaxel. In cases of negative bone scan and rising sPSA, abdominal CT scan was added to confirm visceral/lymph node metastasis. Bone scan during docetaxel therapy was thereafter performed at 3-month intervals; CT scans were performed at the discretion of the attending oncologist. Biochemical response was evaluated by a decline in PSA level. Best response was assessed by the oncologist according to the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 (17). Retrospective data, *i.e.* sPSA value or Gleason score (GS) at the time of diagnosis were retrieved from patients' charts. The study was approved by the Institutional Ethics Committee (34/10 IGA) and all participants gave their written informed consent.

Detection of CTCs and positive test criteria. Whole peripheral blood (7.5 ml) was drawn into collecting tubes with stabilizing agent, enabling blood processing within 24 h when stored in a refrigerator (4-8˚C). In the first step, by using AdnaTest® ProstateCancerSelect®, the tumor cells were enriched by the binding of monoclonal antibodies [anti-epithelial cell adhesion molecule (anti-EpCAM), anti-human epidermal growth factor receptor 2 (anti-HER2)] conjugated with Dynabeads™ magnetic particles (Dynal Biotech ASA, Oslo, Norway) to tumor-specific antigens on the surface of tumor cells. These cells were then separated from peripheral blood in the magnetic field and lysed subsequently. In the second step, the lysed fraction was used for mRNA isolation by Dynabeads oligo(dT)25-particles included in the ProstateCancerDetect® kit and cDNA was synthetized by a reverse transcription (Sensiscript RT kit, Qiagen, Hilden, Germany). Subsequent multiplex-PCR (HotStarTaq Master Mix kit; Qiagen) was performed by using a PrimerMix from the ProstateCancerDetect® kit (AdnaGen). The presence of a control gene (actin) and three tumor-related genes [prostate-specific membrane antigen (PSMA), PSA and epidermal growth factor receptor (EGFR)] was monitored. A PCR-product analysis was performed by Agilent DNA 1000 kit on a 2100 Bioanalyzer (AdnaGen). CTC presence was positive if at least one of the tumor-related PCR fragments was present at a concentration higher than 0.15 ng/μl.

Statistical analysis. Standard descriptive statistics were used to describe frequency distributions for categorical data, and medians and interquartile ranges for other variables. The primary objective was to assess the OS, which was compared by age, initial sPSA value, GS and stage, extent of metastatic disease, number of total cycles of docetaxel administered and sPSA and tumor-related genes transcripts values measured before and during docetaxel therapy. Survival data were censored at the last date the patient was known to be alive or at the analysis cut-off date, whichever was first. Kaplan-Meier plots were generated for OS based on CTCs measurement at baseline and follow-up blood collections for survival analyses. Survival curves were compared using log-rank test. Univariate and multivariate hazard ratios for OS were determined by Cox proportional hazards regression and Wilcoxon test. The secondary objective was to assess association of the sPSA levels and detection of CTCs. Due to the non-Gaussian distribution of sPSA and CTC transcript values, Spearman's correlation coefficient was used. Statistical significance was determined at an alpha=5%. Statistical analysis was performed using SAS 9.4 software (Cary, NC, USA).

Results

Patients' characteristics are summarized in Table I. The median age was 71 (range=54-82) years. A total of 16 patients had a GS greater than 7. Nine and five patients underwent radical prostatectomy or radiotherapy, respectively, as primary treatment. The median time from diagnosis to castration and from castration to initiation of docetaxel was 4.3 (range=0.5-209.1) and 25.0 (range=3.0-213.5) months, respectively. All but four patients had positive bone scans before chemotherapy. Seven patients had oligometastatic disease (maximum three lesions on bone scan). In twelve patients, the CT scan was positive for lymph node metastasis. These patients were suitable for RECIST assessment before and during chemotherapy. No patient had evidence of other soft-tissue lesions.

Table I. *Study group characteristics*.

	N	%
All patients	37	100.0
Median age (range), years	71 (54-82)	
Gleason score		
≤6	7	18.9
7	12	32.4
≥8	16	43.3
Unknown	2	5.4
Primary treatment		
Radical prostatectomy	9	24.3
Radical radiotherapy	5	13.5
Castration only	23	62.2
Bone metastasis before docetaxel	33	89.2
≤3 Lesions	8	21.6
Multiple lesions	25	67.6
Lymph node metastasis before docetaxel	12	32.4
Mean PSA (range), ng/ml		
At diagnosis	105.8 (3.2-782)	
Before Dtx	96.9 (2.2-770.0)	
During Dtx	53.9 (0.8-1243.0)	

Dtx: Docetaxel chemotherapy; sPSA: serum prostate-specific antigen.

Table II. *Detection of circulating tumor cell (CTC) transcripts and correlation with serum prostate-specific antigen (sPSA) levels before and during docetaxel therapy.*

Transcript present	Mean concentration of transcript (range), ng/μl	CTC-positive samples, n (%)	Correlation with sPSA*
Before Dtx (n=37)			
PSA	9.2 (0.0-41.8)	31 (83.8)	0.439†
PSMA	0.8 (0.0-11.3)	21 (56.8)	0.348†
EGRF	0.1 (0.0-0.5)	3 (8.1)	0.305
During Dtx (n=30)			
PSA	3.6 (0.0-39.3)	15 (50.0)	0.563†
PSMA	0.4 (0.0-7.4)	5 (13.5)	0.430†
EGRF	0.0 (0.0-0.5)	3 (10.0)	0.374†

Dtx: Docetaxel chemotherapy; PSMA: prostate-specific membrane antigen; EGRF: epidermal growth factor receptor; *assessed by Spearman correlation coefficient, †statistically significant correlation.

Table III. *Univariate analysis of associations with survival by Cox regression hazard model*.

Variable	HR	95% CI	*p*-Value
Age	0.72	(0.28-1.84)	0.4892†
sPSA at diagnosis	0.49	(0.19-1.29)	0.1368†
sPSA before Dtx	1.64	(0.64-4.22)	0.3010†
sPSA during Dtx	2.00	(0.73-5.51)	0.1712†
CTC-positive before Dtx	1.75	(0.40-7.71)	0.4556†
CTC-positive during Dtx	2.17	(0.79-5.98)	0.1246†
Multiple bone metastasis	2.21	(0.77-6.35)	0.0421*

sPSA: Serum prostate-specific antigen; Dtx: docetaxel chemotherapy; CTC: circulating tumor cells; HR: hazard ratio; CI: confidence interval; †log-rank test; *Wilcoxon test (†p=0.1306).

Treatment response and OS. Follow-up data were available in 30 out of 37 (81.1%) patients. These patients' data were used for the OS analysis. Four patients were lost to follow-up, one patient died of cardiac failure after one cycle of docetaxel, one patient refused to undergo chemotherapy after first CTC assessment and one patient had not finished three cycles of chemotherapy at the time of data analysis. A decline of more than 30% in sPSA was detected in 16 out of 30 (53.3%) evaluable patients. The median number of administered docetaxel cycles was eight (range=3-25). Best response was assessed as follows: partial remission in 12 (40.0%), stable disease in 13 (43.3%) and progressive disease in five (16.7%) out of 30 evaluated patients.

CTC detection. Circulating tumor cells were detected in 32 out of 37 (86.5%) and in 16 out of 30 (53.3%) men before and during chemotherapy, respectively. Eleven patients became CTC-negative and 15 remained CTC-positive during the therapy. All three patients who were CTC-negative at the beginning and underwent the second CTC analysis remained negative. Before chemotherapy, transcripts of *PSA, PSMA* and *EGFR* were detected in 83.8%, 56.8% and 8.1%, respectively. After three cycles of docetaxel, transcripts of *PSA, PSMA* and *EGFR* were detected in 50.0%, 13.5% and 10.0%, respectively (Table II). During docetaxel, *EGFR* was detected in three patients who did not express it before the treatment.

Correlation of OS with clinical parameters and CTC detection. During the median follow-up of 14.6 months, a total of 20 patients died, 13 were alive and four were lost to follow-up. Thirty patients received at least three cycles of docetaxel and were thus evaluated for survival. The median survival of these patients was 15.3 (range=0.9-35.2) months. Fifteen patients died of disease progression. Regarding clinical parameters, a borderline statistically significant difference in OS was observed in patients with no or only limited *vs*. multiple bone metastasis (p=0.0421; Table III). Patients with multiple bone metastases were at 2.2-times higher risk of dying from all causes (hazard ratio=2.21; 95% confidence interval=0.77-6.35; p=0.0421). The median sPSA level at the time of the first and second CTC assessment was 100 and 50 ng/ml, respectively. Patients with a sPSA level below the median value before chemotherapy lived longer than those with sPSA level above the median value (median=16.8 *vs*. 12.9 months), however, the difference was not significant (p=0.301). A similar trend was observed in patients with sPSA level below the median value

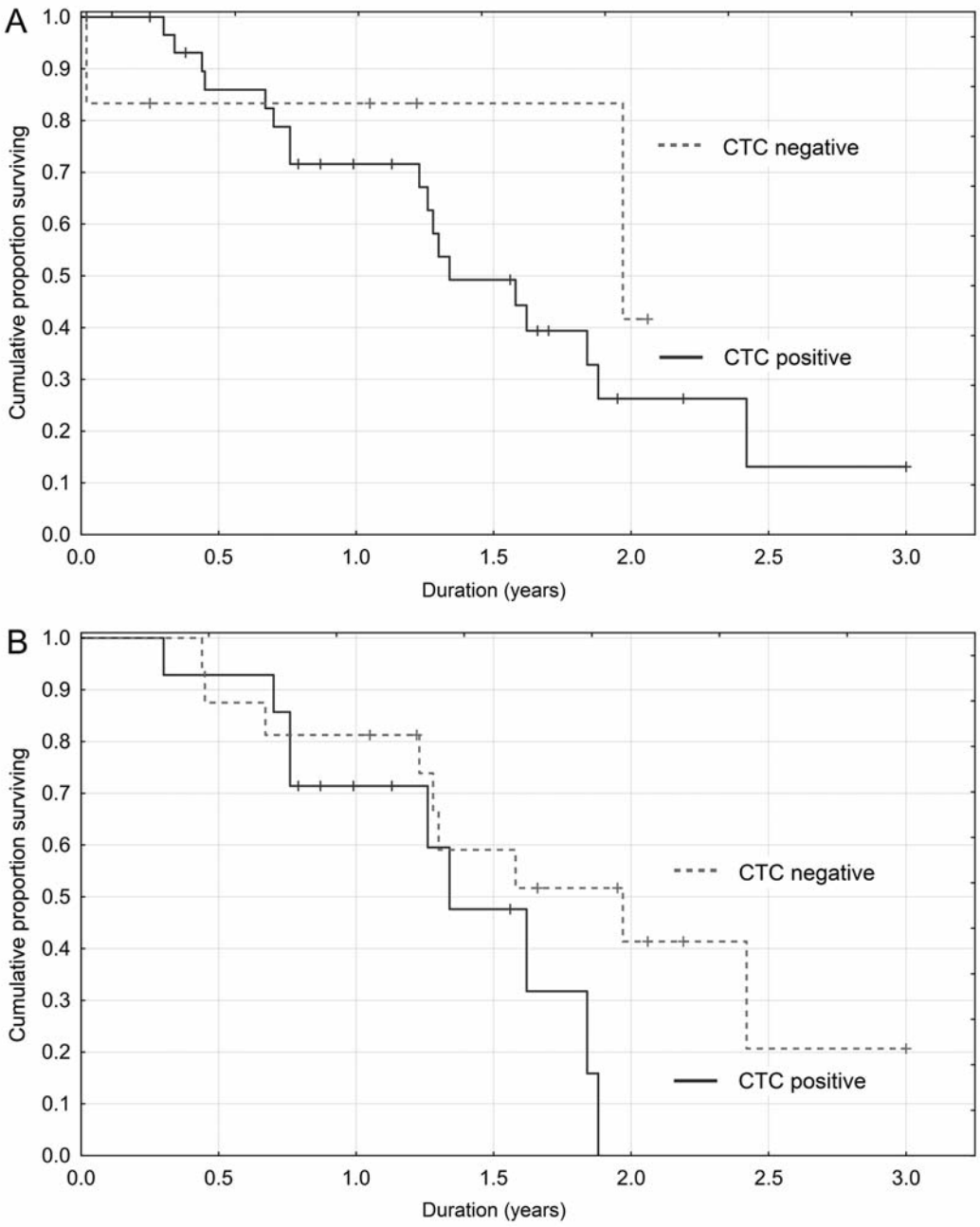

Figure 1. *Kaplan–Meier estimates of overall survival probability in patients with castration-resistant prostate cancer by circulating tumor cell (CTC) status before initiation of docetaxel therapy (A) and during docetaxel therapy (B).*

during chemotherapy (median=19.4 *vs*. 13.7 months; p=0.1712). We observed no difference in OS in patients evaluated as CTC-positive before docetaxel in comparison with CTC-negative patients (median OS=14.8 *vs*. 13.6; p=0.4556; Figure 1A). Patients with CTC-negative samples during docetaxel lived longer than patients who remained CTC-positive after four cycles of docetaxel, however, the difference in OS was not statistically significant (median OS=17.3 *vs*. 12.7 months; p=0.1246; Figure 1B). The longest OS was observed in four patients who had CTC-negative samples both before and during docetaxel therapy (n=4). Overall survival in these four patients and those who were CTC-positive both before and during docetaxel (n=14) was significantly different (median=19.2 *vs*. 12.7 months; p=0.0228; Figure 2).

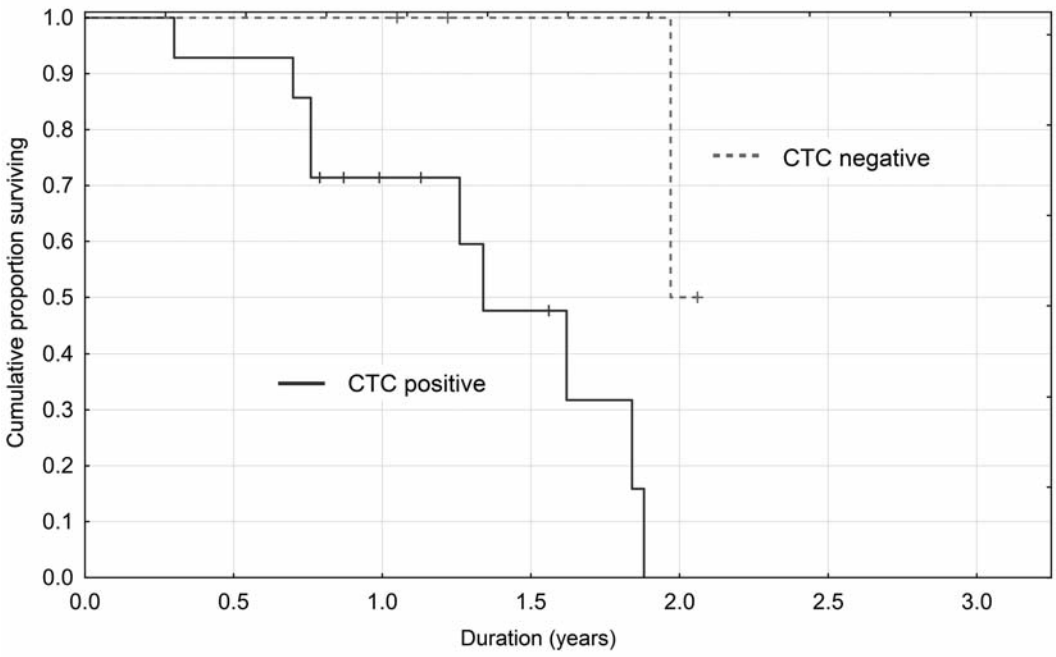

Figure 2. *Kaplan–Meier estimates of overall survival probability in patients with castration-resistant prostate cancer with negative circulating tumor cell (CTC) status both before and during chemotherapy versus patients with both samples CTC-positive.*

Correlation of therapy efficiency/disease progressions and CTC detection. The sPSA level before docetaxel therapy was associated with the level of CTC fragments for PSA (p=0.0084) and PSMA (p=0.0404). The association with sPSA level during chemotherapy was seen for all antigens. We found no correlation between sPSA level and CTC positivity before docetaxel (p=0.1101), however, during docetaxel therapy, the sPSA levels in CTC-positive patients were significantly higher (p=0.0031). On the contrary, a change in sPSA level before and during docetaxel therapy was not significantly different in patients who stayed CTC-positive *versus* those who became or stayed CTC-negative (Figure 3). No difference in OS was observed for patients with more than 30% decline in sPSA level during docetaxel therapy *versus* those with worse biochemical response (log-rank, p=0.1534). The same was found when a 50% sPSA decline was considered a favorable response (log-rank, p=0.9076). No difference in CTC detection before or during docetaxel therapy was found between patients with partial remission or stable disease *versus* those with progressive disease (Chi-square, p=0.5979). However, an absolute change in sPSA level was significantly associated with the best response status (Chi-square, p=0.033) (Table IV).

Discussion

In our study, we assessed prognostic parameters in a rather heterogeneous group of patients with CRPC. Only multiple bone metastasis was significantly associated with worse OS. Patients with CTC-negative samples both before and during docetaxel therapy had the longest survival, that was significantly different from that of patients who remained CTC-positive during chemotherapy. Change in sPSA levels correlated well with best treatment response.

Numerous methods of CTC detection have been tested outside the clinical praxis (18). Only limited data are available about their performance in patients with CRPC. The first and only clinically validated blood test for enumerating CTCs, CellSearch®, was approved by the United States Food and Drug Administration for monitoring of metastatic prostate cancer in 2008 based on the results of one multicenter, prospective clinical trial (10). The studies comparing the AdnaTest®, used in our study, and CellSearch® for the CTC detection were carried out in patients with breast cancer. Two of them showed a concordance of over 80% (11, 20), and two of them showed a concordance of 64% (19, 21). Only one of these studies was performed to compare the prognostic impact of CTC positivity assessed with the CellSearch® and AdnaTest® platform. In this multivariate analysis of the same cohort of patients with metastatic breast cancer, the presence of CTCs detected by CellSearch® was an independent predictor of OS, contrary to the AdnaTest®, where the correlation of CTC positivity with OS was not proven (21). Few studies proved the clinical efficacy of AdnaTest® in patients with prostate cancer. Todenhöfer *et al*. found that the CTC positivity before initiation of chemotherapy was associated with radiological response and rate of radiological

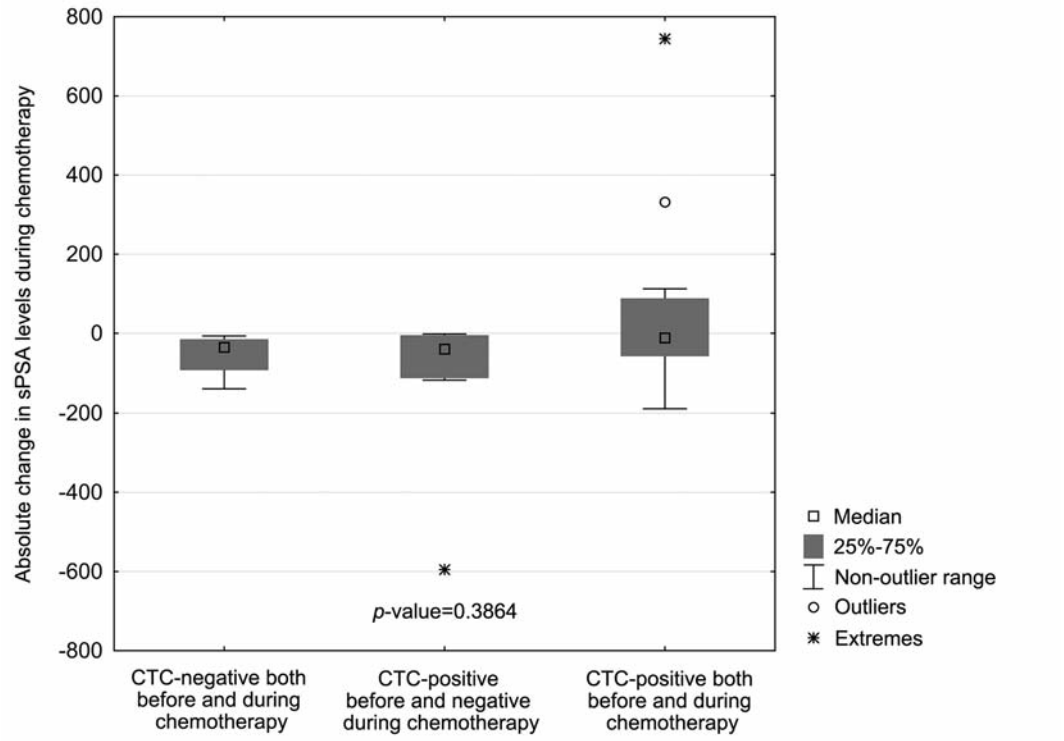

Figure 3. *Change in serum prostate-specific antigen (sPSA) levels before and during docetaxel therapy.*

Table IV. *Association of circulating tumor cell (CTC) detection and changes in serum prostate-specific antigen (sPSA) levels with the best response during docetaxel therapy (Dtx).*

	Number of patients			
	PR	SD	PD	*p*-Value*
CTC-negative before and during Dtx (n=4)	2	2	0	0.8976
CTC-positive before and -negative during Dtx (n=12)	5	5	2	
CTC-positive before and during Dtx (n=14)	5	6	3	
Decline in sPSA level ≤30% (n=9)	6	2	1	0.1233
Decline in sPSA level >30% (n=19)	5	10	4	
		Mean change (%)		
sPSA level during *vs*. before Dtx	44	76	144	0.0333†

PR: Partial remission; SD: stable disease; PD: progressive disease; *Chi-square test; †statistically significant.

progression after four cycles of docetaxel. However, persistence of CTCs during chemotherapy had no impact on the difference for either objective (15).

In our study, the patients with CTC-negative samples both before and during docetaxel therapy had the longest survival, which was significantly different from that of patients with both samples positive. In addition, those patients who became CTC-negative during chemotherapy had better OS than CTC-positive patients, although the difference was not statistically significant. Another study assessed a potential usefulness of CTC detection in follow-up of patients with low- and high-risk prostate cancer and individuals without any sign of prostate cancer. All healthy individuals and patients without metastasis were CTC-negative. In the high-risk group, all responders to various types of therapy were CTC-negative, whereas CTCs were detected in all three non-responders (22). We did not find a significant correlation between CTC detection and best response to therapy as is commonly assessed by an oncologist. However, no patient with a CTC-negative sample both before and during chemotherapy showed progression. In our opinion,

the main benefit of CTC detection during difficult and often expensive therapy of metastatic CRPC is the possibility of identifying those patients who have a poor prognosis (in the case of CTC persistence), as previously shown (10), and for whom it is advisable to choose best supportive care or search for another treatment (*e.g.* novel antiandrogens) instead of continuing with chemotherapy.

To the best of our knowledge, our study is the first to assess the prognostic value of the AdnaTest® platform regarding the OS in patients with metastatic CRPC. We studied a group of 37 patients indicated for standard docetaxel therapy due to progression of metastatic CRPC. Follow-up data were available in 30 patients and all of them received at least three cycles of docetaxel (median of eight cycles). We observed a higher CTC detection rate in comparison to previous studies assessing the AdnaTest® platform in patients with breast cancer, as well as in comparison with a rate of CellSearch®-positive samples (10, 11, 20). This can be explained by the cut-off of five and more CTCs established for CellSearch® (10), which is higher than the detection limit of AdnaTest® (11). Due to this fact, the result achieved by these methods cannot be directly compared. It also has to be emphasized, that contrary to the CellSearch® platform, the AdnatTest® system does not report the absolute number of CTCs, but determines only their presence or absence in a patient's blood. This method provides information about the expression of monitored tumor-associated genes in a patient's CTCs and enables further molecular characterization of these cells (15).

Comparing with the only study using AdnaTest® in a similar group of patients with CRPC (15), we found more CTC-positive cases (86.5% *vs*. 68.8%). This could be explained by the lower proportion of patients with only lymphadenopathy in our study (10.8% *vs*. 25.0%) and by the relatively small number of patients in the study of Todenhöfer *et al*. Another recently published study claimed only 46% CTC-positive patients with CRPC identified by the Cellsearch® method and 53% patients positive for the tumor-associated mRNA in whole blood. However, some of the patients from this study were not chemotherapy-naive which can, together with the different method used, explain the discrepancy (23). Different RT-PCR protocols and definitions of CTC positivity thus make current studies incomparable. The manufacturer's protocol and clearly defined threshold in the AdnaTest® platform could lead to better inter-study comparison.

Currently, serum markers, such as PSA, have only a limited use in CRPC. The relationship between the detection of CTCs and the levels of these markers is not well studied and its prognostic significance is unclear. In our study, the sPSA levels correlated well with almost all transcripts both before and during docetaxel, however, the impact of such correlation remains unclear. We found that the sPSA level and overall CTC status before docetaxel therapy did not correlate, however, those patients who remained CTC-positive also had significantly higher sPSA levels during docetaxel therapy. On the contrary, we did not find that changes in sPSA levels during chemotherapy were significantly different between patients who remained CTC-negative, became CTC-negative or remained CTC-positive. In the aforementioned study, a change from 'unfavourable' to 'favorable' CTC number considerably improved the prognosis of patients regarding OS. Moreover, the change in CTC number predicted OS better than the reduction in sPSA level (10). Similarly in our study, the patients who were CTC-negative during therapy had better OS. Based on our results, we cannot conclude that the change in sPSA level is inferior to the change in CTC status.

The role of EGFR in promotion of prostate cancer bone metastasis has been extensively studied and this receptor is currently one of the potential molecular targets both for radionuclide-based imaging and novel systemic therapy (24). In the study of Todenhöfer *et al*., all three patients with baseline positive EGFR transcript showed progression during docetaxel therapy (15). Contrary to these results, EGFR prognostic significance in CTCs in our study is difficult to assess. Three patients were EGFR-positive before docetaxel therapy. Two of them died of progressive disease at 4.1 and 8.4 months, respectively. One patient died of pulmonary embolism at 16 months, with no sign of cancer progression. Three patients became EGFR-positive during chemotherapy. Two of them were alive at the time of analysis (both with partial remission), one died of progression at 22.6 months.

The limitation of our study is mostly due to a relatively low number of patients. The statistically significant difference in OS was thus proven only between CTC-positive and CTC-negative patients during the therapy. The difference in sPSA levels between these groups was not proven.

Conclusion

We can conclude that CTC detection in CRPC by a new RT-PCR method, AdnaTest®, is feasible and could be used in the clinical praxis. The detection of CTCs by this method can provide information about prognosis, therapy efficacy and OS of patients with CRPC. Considering the low number of patients in our study regarding OS, multicenter studies with larger cohorts of patients with CRPC in the context of modern systemic therapy are warranted.

Acknowledgements

This study was supported by the project IGA NT 12205-5/2011.

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Received January 2, 2016
Revised February 29, 2016
Accepted March 3, 2016