Preliminary Experience on the Use of the Adnatest® System for Detection of Circulating Tumor Cells in Prostate Cancer Patients

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Abstract. *Background: The Adnatest® system combines immunomagnetic enrichment of epithelial cells with polymerase chain reaction for prostate cancer (PC)-specific transcripts for the detection circulating tumor cells (CTCs). We evaluated the Adnatest® in patients with castration-resistant PC receiving docetaxel chemotherapy. Patients and Methods: CTCs were assessed in 16 patients with castration-resistant PC before cycles one and three of chemotherapy. Furthermore, markers of stem cells and epithelial-mesenchymal transition were assessed. Treatment response was assessed by imaging and prostate-specific antigen measurements. Results: Before chemotherapy, 11 patients were Adnatest®-positive whereas five patients were Adnatest®-positive before cycle three. A positive Adnatest® correlated with radiological progression (p=0.02). Rates of disease progression in epidermal growth factor receptor (EGFR)-positive and -negative patients were 100% and 7.7% (p=0.03). Conclusion: In this preliminary study, the Adnatest® detected CTCs in a considerable proportion of patients with castration-resistant PC. First data on certain markers (EGFR and aldehyd dehydrogenase 1) encourage future studies investigating transcripts predicting treatment response.*

Castration-resistant prostate cancer (CRPC) is associated with poor prognosis and decreased quality of life. The first-line treatment for CRPC is docetaxel-based chemotherapy (1). Tools for evaluating treatment response and estimation of prognosis are limited (2). A large proportion of patients do not have bi-dimensionally measurable disease. Therefore, common response evaluation criteria are not applicable (3). Osteoblastic bone metastases are also difficult to assess regarding treatment response. Although it is often used as a surrogate, the correlation beween prostate-specific antigen (PSA) response and prognostic parameters is controversially discussed (2, 4). Finally, the ideal timing for the initiation of chemotherapy remains a matter of debate. The measurement of Circulating tumor cells (CTCs) has evolved as a tool for prognostication and monitoring of patients with metastatic tumors. Several techniques have been developed for the enrichment and identification of CTCs, including methods using immunomagnetic enrichment, flow cytometry, immunocytochemistry and assays based on enrichment and detection of nucleid acids such as reverse transcriptase polymerase chain reaction (RT-PCR) (5).

As the phenotype of cells in the peripheral blood, derived from the primary tumor or metastases, has not yet been identified, the sensitivity and specificity of several methods for detecting CTCs remains unclear. In PC, the clinical significance of CTCs has been discussed controversially (6). CTC trials which have been conducted in PC have used the CellSearch® system as the platform for the enrichment and analysis of CTCs. This system combines immunomagnetic enrichment of epithelial cell adhesion molecule (EpCam)-positive cells with semi-automated immunohistochemical staining and microscopy for cytokeratins and CD45 (7). The detection of CTCs using CellSearch® has been shown to be associated with impaired prognosis (8). Some technical studies have focused on the development of PCR-based detection of CTCs. However, only limited data are available regarding the prognostic relevance of this technique in PC (9). As RT-PCR is susceptible to false-positive results due to contamination and ectopic and illegitimate transcription, the combination of

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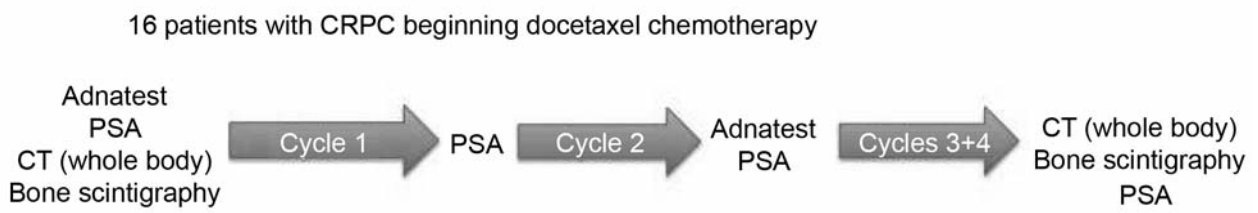

Figure 1. *Study design showing time points of prostate-specific antigen (PSA) measurement in the serum, measurement of circulating tumor cells by the Adnatest®, imaging with whole body computed tomography (CT) and whole-body bone scintigraphy. CRPC: castration resistant prostate cancer.*

immunomagnetic cellular enrichment with RT-PCR might improve the specificity of the method (10). The novel PCR-based Adnatest® assay (AdnaGen®, Langenhagen, Germany) employs RT-PCR to identify putative transcripts of genes such as *PSA*, prostate specific membrane antigen (*PSMA*), epidermal growth factor receptor (*EGFR*) in EpCAM or human epidermal growth factor receptor 2 (*HER2*)-positive cells, isolated by immunomagnetic separation. This system has been shown to be at least as sensitive as CellSearch® in breast cancer, offering the possibility for further molecular characterization of CTCs (10, 11). Furthermore, the Adnatest® allows for assessment of markers of epithelial mesenchymal transition and stem cells. Epithelial mesenchymal transition is a crucial step in the dissemination of tumor cells and promotes formation of metastasis (12). Cancer stem cells are suggested to be an essential source of metastatic spread of solid tumors (13).

Herein, we aim to investigate the use of this assay in patients with CRPC receiving docetaxel chemotherapy.

Patients and Methods

Sixteen patients with histologically proven adenocarcinoma of the prostate and evidence of metastatic disease on chest, abdominal, or pelvic computed tomography (CT) and/or bone scan were included. All patients had CRPC according to the EAU criteria (1) and fulfilled indication criteria for systemic chemotherapy due to progression, while on or after androgen deprivation therapy. Patients with prior chemotherapy were excluded. All patients had a Karnofsky score >70%. Docetaxel/prednisone was initiated on a 3-week cycle until disease progression or unacceptable toxicity (14). All patients underwent at least 3 cycles of chemotherapy. The study was approved by the local ethics committee.

PSA measurements were performed before chemotherapy and after cycles 1, 2 and 4 of chemotherapy. All patients underwent bone scans and whole-body CT within two weeks before initiation of chemotherapy and two weeks after application of cycle 4 of chemotherapy. Radiological progression of disease was interpreted due to the recommendations of the Prostate Cancer Clinical Trials Working Group (15). In patients with both soft tissue lesions and bone metastases, changes in soft tissue lesions consistent with treatment response, according to response evaluation criteria (RECIST) (3) combined with stable bone lesions, were interpreted as response. The presence of CTCs by the Adnatest® was determined on day one of cycles 1 and 3 before application of chemotherapy. Furthermore, transcripts characteristic of EMT and stem cell properties were assessed in parallel to the Adnatest®. The study design is illustrated in Figure 1.

CTC detection and criteria for positive tests. The Adnatest® PC CTC platform consists of the ProstateCancerSelect® and ProstateCancerDetect® system. The ProstateCancerSelect® system allows for an enrichment of tumor cells by an antibody-mix (anti-EpCAM, anti-Her2) linked to magnetic particles and mRNA isolated from the selected cells. The ProstateCancerDetect® System transcribes the isolated mRNA into cDNA and a multiplex PCR is performed for the analysis of tumor-associated gene expression (*PSA, PSMA, EGFR*). Blood samples (7.5 ml) were collected in AdnaCollect tubes (AdnaCollect; AdnaGen), allowing for storage for 24 h at –4°C. Within 24 h after collection, immunomagnetic separation was performed by Adnatest® ProstateCancerSelect® isolating epithelial cells by antibody-linked Dynal beads directed against EpCAM and HER2. The captured cells were lysed and mRNA was isolated using Dynal Oligo-dT beads included in the ProstateCancerDetect® kit. Subsequently, reverse transcription was performed (Sensiscript; Qiagen, Hilden, Germany) and the cDNA was then used as a template in a multiplex PCR for selected markers including *PSA, PSMA* and *EGFR* (HotStarTaq, Qiagen, Germany). Visualization and quantification of DNA was perfomed using an Agilent Bioanalyzer 2100 (Agilent, Böblingen, Germany). The Adnatest® result was considered to be positive if the fragment concentration after PCR of at least one of the markers was greater than 0,1 ng/μl.

Detection of CTC with EMT or stem cell properties. The determination of aldehyde dehydrogenase 1 (ALDH1)-positive CTCs requires the enrichment of CTC from 5 ml blood using the Adnatest® EMT1/StemCell kit prior to the singleplex PCR assay to analyze *ALDH1*, and the multiplex PCR assay to analyze EMT markers, with actin as an internal control. The immunomagnetic enrichment of CTCs was performed by incubation with anti-EpCAM and anti-mucin 1 (MUC1)-labeled magnetic beads, followed by a special washing buffer treatment to reduce leukocyte cross-contamination. Cell lysis and reverse transcription were performed according to the manufacturer's instructions. Subsequently the overexpression of EMT-related markers (Phosphatidylinositol 3-kinase alpha (*PI3Kα*), Twist related protein 1 (*TWIST1*) and *AKT2* were analyzed by PCR. The Detection of the tumor stem cell-related overexpression of *ALDH1* was determined in a singleplex PCR reaction. The resulting fragment concentrations

Table I. *Patients' characteristics and results from prostate-specific antigen (PSA) measurements, imaging and CTC determinations.*

	Baseline characteristics (before cycle 1)						Treatment response					
Patient No	Age years	Type of metastasis (1=bone, 2=lymph node, 3=visceral)	PSA baseline (ng/ml)	CTC baseline (positive transcripts)	EMT marker (PI3K, AKT2, TWIST1)	ALDH1	PSA after 4 cycles	Bone metastases (after 4 cycles)	Lymph node and visceral metastases (after 4th cycle)	CTC after 2 cycles (positive transcripts)	EMT markers (PI3Kα), AKT2, TWIST1) after 2 cycles	ALDH1 after 2 cycles
1	61	2	40	pos (PSA)	0	0	30		S	pos (PSA, PSMA)	0	1
2	69	1,2	1234	pos (EGFR, PSA, PSMA)	0	0	969	P	P	pos (PSA, PSMA)	0	1
3	71	1,2,3	449	pos (PSA)	0	0	256	S	S	pos (EGFR)	0	0
4	69	1	136	pos (PSA, PSMA)	0	0	12	P		neg	0	0
5	74	1,2	15	neg	0	0	14	S	R	neg	1 (PI3Kα)	1
6	68	1,2,3	31	pos (PSA, PSMA)	0	0	12	S	S	pos (PSA)	0	0
7	70	1	18	neg	0	0	8	S		neg	0	0
8	63	1	397	pos (PSA)	0	0	184	S		neg	0	0
9	78	2	653	pos (PSA)	0	0	594	S	S	neg	0	0
10	67	1	74	pos (PSA, PSMA)	0	0	82	S		neg	0	0
11	78	2	15	neg	0	0	3		R	neg	1 (PI3Kα)	0
12	70	1,2	27	neg	0	0	10	S	R	neg	0	0
13	79	2	137	pos (EGFR)	0	0	213		P	neg	0	0
14	71	1,2	468	pos (PSA, PSMA, EGFR)	0	1	521	P	P	pos (PSA, EGFR)	0	1
15	61	1,3	737	pos (PSA, PSMA)	0	0	1		R	neg	0	0
16	76	1	41	neg	0	0	0,28	S		neg	0	0

S: Stable; P: progressive; R: regressive; PSMA: prostate-specific membrane antigen; EGFR: epidermal growth factor receptor; ALDH1: aldehyde dehydrogenase 1.

of the EMT markers and *ALDH1* by PCR were quantified using the Agilent Bioanalyzer 2100. The results were considered positive if the cut-off values as per the manufacturer's instructions were exceeded (namely 0.2 ng/μl for *AKT2*, 0.15 ng/μl for *TWIST1*, 0.25 ng/μl for *PI3Kα* and 0.15 ng/μl for *ALDH1*).

Statistical analysis. Results of CTC detection were compared and correlated to dichotomized clinical data by contingency analysis. For correlation of PSA values and the presence of CTCs, Student's *t*-tests were used. Results of treatment response (imaging after four cycles of chemotherapy) and CTC presence were compared by contingency analyses and Cochran-Armitage tests for trend.

Results

The median patient age was 70 (61-79) years. The median interval between primary diagnosis and initiation of chemotherapy was 62 (6-174) months. The median PSA value before chemotherapy was 105 (15-1234) ng/ml. In bone scan before chemotherapy, 12 patients had evidence of metastases. In 10, CT was suspicious for lymph node involvement. Other soft tissue lesions were seen in two (one patient with peritoneal and one patient with hepatic metastases). Eight patients had lesions suitable for RECIST-assessment before chemotherapy.

Treatment response. The median PSA value before cycle 3 of chemotherapy was 21 (0.3-962) ng/ml. The median PSA ratio (*i.e.* PSA after cycle 4/PSA before cycle 1) after cycle 4 of chemotherapy was 51.6% (0.2-155%). Radiological evaluation after cycle 4 by bone scan and whole-body CT showed response in four patients, stable disease in eight patients and signs of progressive disease in four patients. Disease progression according to Prostate cancer working group 2 (PCWG2) criteria (15) (including PSA and radiological investigation) was seen in 5/16 patients.

CTC measurements. CTCs were detectable by Adnatest® in 11 out of 16 (68.8%) men before chemotherapy. Before cycle 3, 5 out of 16 patients had detectable CTCs. In 14 out of 16 positive Adnatest, transcripts of *PSA* were detected, *PSMA* was detected in 8 of 16 positive tests and *EGFR* was detected in 5 of 16 positive tests (Table I).

EMT and stem cell markers. Before chemotherapy, only one patient was positive for transcripts of the stem cell marker *ALDH1*. After two cycles of chemotherapy, four patients were positive for *ALDH1* transcripts. The EMT marker *PI3Kα* was not detected in CTCs of any patient before chemotherapy but was positive in two patients after two

Table II. *Correlations of Adnatest® (CTC+/CTC–) at baseline, markers and persistence of CTCs with therapy response. *in patients with CTCs at baseline; CTC: circulating tumor cells; EGFR: epidermal growth factor receptor; ALDH1: aldehyde dehydrogenase 1; PI3Kα: phosphatidylinositol 3-kinase alpha; PCWG2: prostate cancer working group 2; PSA: prostate-specific antigen.*

	Radiological response after 4 cycles (response/stable/progression)	*p*-Value	Ratio PSA after 4 cycles/baseline PSA	*p*-Value	Rate of progression after 4 cycles of chemotherapy (PCWG2)	*p*-Value
CTC+ (baseline)	9.1%/54.6%/36.4%	0.02	0.73	0.15	45.5%	0.06
CTC– (baseline)	60.0%/40.0%/0%		0.36		0%	
EGFR+ (baseline)	0%/0%/100%	0.006	1.11	0.02	100%	0.032
EGFR– (baseline)	30.8%/61.5%/7.7%		0.42		15.4%	
ALDH1 + after cycle 2	25%/25%/50%	0.41	0.84	0.03	50%	0.3
ALDH1 – after cycle 2	25%/58.3%/16.7%		0.41		25%	
PI3Kα + after cycle 2	100%/0%/0%	0.03	0.53	0.80	0%	0.30
PI3Kα – after cycle 2	14.3%/57.1%/28.6%		0.51		35.7%	
Persistent CTCs*	0%/60%/40%	0.53	0.73	0.90	40%	0.74
Non-persistent CTCs*	16.7%/50%/33.3%		0.68		50%	

cycles of chemotherapy (Table I). Both *PI3Kα*-positive cases and one *ALDH1*-positive case were negative for the classic Adnatest® markers *PSA*, *PSMA* and *EGFR*. *TWIST1* and *AKT2* transcripts were not detected in CTCs of any patient.

Correlation with clinical parameters and treatment response. Median PSA concentrations before chemotherapy in patients with and without positive Adnatest® before chemotherapy were 397 and 18 ng/ml (p=0.007), respectively. No differences were observed in the rate of bone (p=0.75), lymph node (p=0.88) or visceral metastases (p=0.20), between patients with and without positive Adnatest® before chemotherapy. Radiological response after four cycles of chemotherapy, PSA response and progression rate (according PCWG2 criteria) are shown in Table II. In patients with and without *EGFR* mRNA expression before chemotherapy, the median PSA values were 468 and 41 ng/ml (p=0.33). Rates of bone metastases (p=0.46), lymph node metastases (p=0.38) or other visceral metastases (p=0.56) did not differ significantly between both groups.

Correlations of presence of *EGFR*, *ALDH1* and *PI3Kalpha* transcripts with radiological response of chemotherapy, PSA response and progression rate (according PCWG2 criteria) are shown in Table II.

Discussion

Although a variety of techniques has been developed for the detection of CTCs, only one approach (CellSearch®) has acquired FDA approval (5). Nucleic acid-based methods are highly concordant with the CellSearch® assay (16) and have demonstrated good sensitivity for the detection of CTCs compared to immunological approaches (11). Several approaches have been discussed, aiming to improve the specificity of PCR-based detection, including nested PCR (17), multiplex PCR for multiple transcripts (18) and expression thresholds in quantitative PCR (19). Recently, an assay combining immunomagnetic enrichment of EpCAM- and HER-2-positive cells with multiplex PCR for tumor-associated antigens became available for breast, colorectal and prostate cancer (Adnatest®). Whereas in breast cancer first clinical studies showed similar sensitivity of the Adnatest® compared to CellSearch® (10, 11), no data are available about the feasibility and the clinical value of this test for patients with metastatic PC. In this initial study we investigated the use of Adnatest® in patients with metastatic PC undergoing chemotherapy. In addition to the standard markers of the Adnatest®, markers of EMT and stem cell properties were assessed in CTCs of the patients. Compared to studies investigating the presence of CTCs by Adnatest® in breast cancer, we had a higher rate of patients positive for CTCs (10, 11). To our knowledge, no studies using cell spiking experiments have been carried out to compare the sensitivity and specificity of the Adnatest® and the CellSearch® assay. Hence, data from other studies in PC using the CellSearch® platform cannot be compared with our results (20).

Interestingly, we had a clearly higher rate of patients with PSA transcripts than a study investigating *PSA* transcripts in mononuclear cells of 122 patients with CRPC by RT-PCR (21). However, Shariat *et al*. recorded 90% of patients with hormone-naïve metastatic PC to be positive for *PSA* by RT-PCR in peripheral blood (22). This inconsistency confirms

the concerns of many researchers that different PCR protocols, primers and detection thresholds lead to high data variability (23). The standardized protocol and fixed thresholds that the Adnatest® uses may improve reproducibility and reduce test variations.

Previously, CTC frequencies have only modestly correlated with parameters of disease burden (PSA, extent of metastases *etc*.) (8). Whereas no correlation was observed between the type of metastasis and CTC results, pre-treatment PSA was significantly higher in patients with CTCs at baseline in our study. However, this correlation was not observed after four cycles of chemotherapy.

Our results indicate that not only is the pre-treatment level of CTCs predictive for outcome, but so is the course of CTCs during therapy. This is in accordance with previous CellSearch® studies showing that a CTC decline during therapy is a positive prognostic factor (20, 24). In accordance with previous studies, our results indicate that use of CTCs may be more reliable than that of PSA regarding treatment response and outcome (24). In our cohort, *EGFR* transcripts which are routinely assessed by Adnatest® determine poor outcome. EGFR has been shown to be overexpressed in advanced PC, and EGFR inhibition has shown promising antitumor effects *in vitro* and *in vivo* (25). Analysis for *EGFR* transcripts in peripheral blood might help to identify patients with potentially increased benefit from EGFR-targeted therapy. In Adnatest®, EpCAM-positive cells are enriched for further analysis, similar to CellSearch®. In addition to EpCAM, HER2 is used as epithelial marker for CTC enrichment. Importantly, HER2 is overexpressed in PC cells (26). Since there are CTCs that do not express EpCAM, the performance of immunomagnetic enrichment will be enhanced by further surface markers (27). In contrast to the CellSearch® platform, no negative selection of CD45-positive cells is performed by the Adnatest®, which might reduce specificity (28). However, *PSMA* and *PSA*, two out of three markers for RT-PCR analysis are highly specific for prostate-derived cells.

The Adnatest® system has been recently demonstrated to be able to assess the expression of markers of stem cell properties and EMT, which is an important step in metastasis and is associated with down-regulation of several epithelial markers (29, 30). In our study population, only one patient tested positively for *ALDH1* transcripts and no patient for EMT markers before chemotherapy. By contrast, after two cycles of chemotherapy, four patients had detectable *ALDH1* transcripts and two patients *PI3Kα* transcripts. Although limited by the low number of patients included, these results raise the question as to whether systemic therapy can induce changes in the expression profile of CTCs and induce markers of stem cell properties and EMT, which has also been discussed by other others (31). The fact that some of the *ALDH1*-positive patients did not have *PSA, PSMA* or *EGFR* transcripts promotes concerns, that current CTC detection methods underestimate this important subpopulation of CTCs (32). The rate of ALDH-positive cells and EMT marker-positive cells was far lower in our study compared to a study using the same assay in 39 patients with metastatic breast cancer (29). Aktas *et al*. found clear correlation between the presence of EMT markers and *ALDH* and the response to systemic therapy (29). We observed a significantly worse PSA-response in patients with *ALDH1* transcripts and a trend towards increased rates of radiological progression. The rate of patients with transcripts of *PI3Kα* as a marker of EMT was far lower in our study compared to other studies investigating expression of this pathway in breast cancer (33). In contrast to studies in breast cancer, *PI3Kα*-positive patients with PC tended to have a better response than PI3Kα-negative patients. However, as only two patients were *PI3Kα*-positive (both negative for *PSA*, *PSMA* and *EGFR*), this observation has to be considered with caution.

The main limitation of Adnatest® and all other PCR-based methods is that CTCs cannot be exactly quantified. By determining changes of transcript levels, quantitative information can be obtained in addition to the dichotomized results (*i.e*. CTCs present *vs*. non present). However, the quantification of transcripts lacks differentiation between a high number of CTCs with low expression levels and a low number of cells with high expression levels. Furthermore, RT-PCR-based techniques hamper differentiation between viable and non-viable cells, which might also provide important information.

Our study has some limitations including a limited number of patients included. We did not compare the Adnatest® with the CellSearch® system. We only assessed the short-term response to chemotherapy and correlated CTCs with the radiological response and PSA response, which is divergently discussed in studies of PC. Further follow-up of the patients will provide important prognostic information about the predictive role of CTCs. External validation of our results is required to confirm the clinical value of Adnatest® in patients with CRPC.

Conclusion

In conclusion, this is the first study to evaluate the feasibility and clinical use of CTC detection by the Adnatest® combining immunomagnetic enrichment of epithelial cells and RT-PCR for PC-associated transcripts in patients with CRPC. The presence of CTCs and specific transcripts (*EGFR* and *ALDH1*) correlated with treatment response in this preliminary study. The nucleic acid-based method offers the possibility for further molecular characterization of CTCs, including markers of stem cell properties and EMT. The differential characteristics of CTCs and resulting clinical values need to be targeted in further studies.

Conflicts of Interest

None.

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