Expression of tumour progression-associated genes in circulating tumour cells of patients at different stages of prostate cancer

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Objective

To evaluate the presence of circulating tumour cells (CTCs) at different stages of prostate cancer using the AdnaTest® ProstateCancerDetect kit (Qiagen). Moreover, we aimed to assess the expression of transcripts that are specific for cancer stem cells (AdnaTest StemCell) and epithelial–mesenchymal transition (EMT) in CTCs (AdnaTest EMT), as well as additional genes that are known to promote prostate cancer progression.

Patients and Methods

In this prospective study, we included 81 patients who underwent treatment for prostate cancer between 07/2014 and 02/2015, including: Group A, 18 patients (22.2%) with low-risk clinically localised prostate cancer; Group B, 25 patients (30.9%) with high-risk clinically localised prostate cancer; Group C, 11 patients (13.6%) with metastatic castration-sensitive prostate cancer (mCSPC); and Group D, 27 patients (33.3%) with metastatic castration-resistant prostate cancer (mCRPC). AdnaTest ProstateCancer and AdnaTest StemCell/EMT were performed in all cases. In addition, expression of the androgen receptor (*AR*), *c-met*, *c-kit* and thymidylate synthase (*TYMS*) in CTCs was assessed using specific polymerase chain reaction assays.

Results

A positive AdnaTest ProstateCancer was present in three (16.7%), two (8.0%), six (54.5%) and 19 (70.5%) patients in groups A, B, C and D, respectively ($P < 0.01$, chi-squared test). The AdnaTest EMT and AdnaTest StemCell were positive in zero (0.0%), zero (0.0%), one (9.1%), and two (7.4%); and in five (27.8%), four (16.0%), three (27.3%), and 11 (40.7%) patients in groups A, B, C and D, respectively, with no significant differences noted between groups. CTCs expressing *TYMS* (44.4% and 50.0% vs 13.9%) or *AR* (18.2% and 25.9% vs 0.0%) were seen more commonly in patients in groups C and D vs patients with non-metastatic disease (all $P < 0.05$). Expression of *c-kit* and *c-met* were rare events, with only two patients positive for either marker.

Conclusions

AdnaTest ProstateCancerDetect exhibits positive results mainly in patients with metastatic disease. Expression of *AR* and *TYMS* are frequent events in CTCs of patients with advanced disease, whereas *c-met* and *c-kit* gene expression is seen in only a small proportion of patients. The implications of these results for the use of CTC analysis as a decision factor for personalised treatment strategies in advanced prostate cancer remain to be determined.

Keywords

AdnaTest®, circulating tumour cells, epithelial mesenchymal transition, prostate cancer, stem cell, #PCSM, #ProstateCancer

Introduction

Whereas most cases of prostate cancer are diagnosed in an early phase, patients with metastatic disease and an initial response to androgen deprivation typically exhibit progression to a castration-resistant prostate cancer (CRPC) stage within 18–24 months [1]. Various studies have shown that the blood of patients with metastatic prostate cancer may contain circulating tumour cells (CTCs) derived from the primary tumour and different metastatic sites [2–5]. The presence of CTCs in patients with prostate cancer is mainly dependant on the platform used for CTC detection. To date, various

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techniques have been established for CTC enrichment or detection, including techniques that are based on immunomagnetic enrichment and microscopy, such as the CellSearch assay (Janssen Diagnostics, Raritan, NJ, USA), the only platform that has achieved USA Food and Drug Administration approval [6]. Moreover, PCR-based techniques with or without previous enrichment steps have been assessed in various studies [7]. The AdnaTest® ProstateCancer (Qiagen, Hilden, Germany) combines immunomagnetic enrichment of epithelial cells with PCR for tumour-associated transcripts [2]. The initial CTC-detection kit is based on the analysis of the expression of *PSA*, prostate-specific membrane antigen (*PSMA*), and epidermal growth factor receptor (*EGFR*). To date, only limited evidence exists regarding the diagnostic and prognostic potential of this panel [4,6]. One major advantage of the platform is the potential inclusion of genes of interest that are not included in the AdnaTest ProstateCancerDetect panel. In this context, the platform has received significant attention following the publication of a study by Antonarakis et al. [8] demonstrating that the presence of splice variant 7 of the androgen receptor (*AR-V7*) detected by the AdnaTest platform (as an add-on to the ProstateCancerDetect kit) is associated with resistance to second-generation anti-hormonal drugs. In the study, only patients with CTCs (detected by the AdnaTest ProstateCancer) were included. Several studies subsequently followed using the AdnaTest for detection of *AR-V7*. Although the first studies indicate that the detection rate of the platform is superior compared with other tests [6], only limited data on the dependence of the detection rate of this platform on clinical stage have been reported to date. We therefore aimed to assess the presence of CTCs at different stages of prostate cancer using the ProstateCancerDetect kit. Moreover, we assessed the presence of CTCs with stem cell- or epithelial–mesenchymal transition (EMT)-like features using the commercially available AdnaTest EMT-2/StemCell panel. To assess the potential of the platform to include further transcripts of interest, we additionally assessed the expression of mRNAs that are relevant for prostate cancer progression or treatment of advanced prostate cancer (*AR*, thymidylate synthase [*TYMS*], *c-kit,* and *c-met*) [6,9–11].

Patients and Methods

Patients

In this prospective study, we included a consecutive series of patients who were treated for the following prostate cancer conditions at our institution between 07/2014 and 02/2015 and were willing to participate in the study: Group A, low-risk cM0 prostate cancer; Group B, high-risk cM0 prostate cancer; Group C, metastatic castration-sensitive prostate cancer (mCSPC); and Group D, metastatic CRPC (mCRPC).

Low-risk cM0 prostate cancer has been defined as patients with a Gleason score ≤6, PSA level <10 ng/mL, and clinical stage ≤T2a, whilst high-risk cM0 prostate cancer has been defined as the presence of a Gleason score >7 or PSA level >20 ng/mL or clinical stage ≥T2c with no signs of distant metastases on imaging [12]. In groups C and D, both patients with and without the primary tumour in place were included.

Blood samples for CTC analysis were collected before treatment (before prostatectomy in case of localised disease and before initiation of a new systemic treatment in patients with metastatic disease). All patients with metastatic disease had disease progression prior to study inclusion. The study was approved by local Ethics Committee No. 124/2011BO2.

CTC Enrichment and Analysis

The determination of AdnaTest results and detection of CTCs with EMT or stem cell properties have been previously reported [4]. The immunomagnetic enrichment of CTCs was performed by incubation with anti- epithelial cell adhesion molecule (EpCAM) and anti-mucin 1 (MUC1)-labelled magnetic beads, followed by a special washing buffer treatment to reduce leucocyte cross-contamination. Cell lysis and reverse transcription were performed according to the manufacturer's instructions. The presence of CTCs was assessed using the AdnaTest ProstateCancerDetect kit, which detects the over-expression of *PSA*, *PSMA* and *EGFR* mRNAs. Actin expression was used as the housekeeping gene in the multiplex PCR. Moreover, CTCs with EMT-like or stem cell phenotypes were analysed using the AdnaTest EMT-2/StemCellSelect kit. Expression of *AR*, *c-met, c-kit* and *TYMS* was assessed using specific PCR assays.

The determination of aldehyde dehydrogenase 1 (*ALDH1*)-positive CTCs requires the enrichment of CTC from 5 mL blood using the AdnaTest EMT-2/StemCellSelect kit (Qiagen) and the multiplex PCR assay to analyse EMT markers using actin as an internal control. Subsequently, the expression of EMT-related markers (phosphatidylinositol 3-kinase alpha [*PI3Kα*], twist-related protein 1 [*TWIST1*] and AKT serine/threonine kinase 2 [*Akt-2*]) were analysed by PCR. The resulting fragment concentrations of the EMT markers and *ALDH1* by PCR were quantified using the Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA). The results were considered positive if the threshold values indicated by the manufacturer's instructions were exceeded, namely 0.15 ng/μL for *ALDH1* and *TYMS* and 0.25 ng/μL for *PI3Kα*, *Akt-2* and *TWIST*. For *AR*, *c-kit* and *c-met*, a threshold of 0.15 ng/μL was applied.

Statistical Analysis

Continuous variables are presented as median and interquartile range (IQR), and differences between groups A, B, C and D were assessed using the Student's independent *t*-test or the Mann–Whitney *U*-test based on their normal or non-normal distributions, respectively (normality of variable

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distribution was assessed using the Kolmogorov–Smirnov test). Categorical variables were assessed using the chi-squared test. Multiple comparisons were performed by ANOVA and *post hoc* analyses using the Bonferroni test. All variables were also dichotomised according the manufacturer's threshold.

All statistical analyses were completed using the Statistical Package for the Social Sciences (SPSS®) software, version 19 (SPSS Inc., IBM Corp, Somers, NY, USA). For all statistical comparisons, significance was considered as $P < 0.05$.

Results

In all, 81 patients were included with a median (range) age of 66.0 (48.0–86.0) years. Of these, 18 patients (22.2%) had low-risk cM0 prostate cancer (Group A), 25 (30.9%) had high-risk cM0 prostate cancer (Group B), 11 patients (13.6%) had mCSPC (Group C), and 27 patients (33.3%) had mCRPC (Group D) (Table 1).

A total of 30 (37.0%) and nine (11.1%) patients had positive AdnaTest ProstateCancer results, whereas the AdnaTest StemCell and EMT results were positive in 23 (28.4%) and three (3.8%) patients, respectively. *AR, TYMS, c-kit* and *c-met* transcripts were detected in 15 (18.5%), 24 (31.6%), two (2.6%) and two (2.6%) patients, respectively. *TYMS, c-kit* or *c-met* analysis was not performed in five patients.

The AdnaTest ProstateCancer was positive in three (16.7%), two (8.0%), six (54.5%), and 19 (70.5%) patients in groups A, B, C and D, respectively ($P < 0.01$; Fig. 1). The differences between Group C vs groups A and B, and between Group D vs groups A and B, were statistically significant (both $P < 0.01$).

Table 2 shows the clinical variables associated with positivity of the AdnaTest ProstateCancer. Patients' positive at the time of testing had increased rates of positive lymph node status (59.1% vs 30.5%) and clinical metastasis (83.3% vs 25.5%) (both $P < 0.01$) (Table 2).

We did not identify statistically significant differences in the positivity rate of AdnaTest EMT and AdnaTest StemCell in patients in Group C (9.1% and 27.3%) and Group D (7.4% and 40.7%) vs Group A (27.8% and 0.0%) and Group B (16.0% and 0.0%) ($P = 0.31$ and $P = 0.27$, respectively).

Table 1 Baseline characteristics of the total cohort and groups A–D.

Characteristic	Total cohort	Group A	Group B	Group C	Group D	*P* value between all groups
Number of patients	81	18	25	11	27	
Median (IQR)						
Age, years	65.0 (59.3–71.0)	62.0 (54.5–66.0)	65.0 (60.2–66.0)	65.0 (61.0–73.5)	70.0 (61.0–76.0)	0.72
PSA level, ng/mL	10.4 (6.0–40.7)	6.5 (4.2–8.1)	10.0 (5.6–21.1)	21.0 (1.3–113.5)	77.3 (31.9–168.0)	<0.01
LDH level, mg/dL	194.5 (162.7–225.7)	176.0 (156.5–195.0)	178.0 (156.5–201.2)	206.0 (143.0–241.5)	226.0 (203.2–292.9)	<0.01
AP level, mg/dL	75.5 (60.2–92.7)	72.0 (60.2–79.2)	67.5 (55.2–80.0)	87.0 (75.0–110.5)	94.0 (75.0–175.0)	<0.01
N (%)						
Biopsy Gleason score						
6	19 (23.5)	17 (94.4)	1 (4.0)	0 (0.0)	1 (3.7)	<0.01
7	8 (9.9)	1 (5.6)	1 (4.0)	3 (27.3)	3 (11.1)	
8	23 (28.4)	0 (0.0)	16 (64.0)	2 (18.2)	5 (18.5)	
9–10	15 (18.5)	0 (0.0)	6 (24.0)	2 (18.2)	7 (25.9)	
Unknown	16 (19.8)	0 (0.0)	1 (4.0)	4 (36.4)	11 (40.7)	
Pathological Gleason score						
6	14 (17.2)	13 (72.2)	1 (4.0)	0 (0.0)	2 (7.4)	<0.01
7	9 (11.1)	1 (5.6)	1 (4.0)	4 (36.4)	4 (14.8)	
8	26 (32.1)	0 (0.0)	15 (60.0)	4 (36.4)	4 (14.8)	
9–10	18 (22.3)	0 (0.0)	8 (32.0)	3 (27.3)	8 (29.6)	
Unknown	10 (12.3)	4 (22.2)	0 (0.0)	0 (0.0)	6 (22.2)	
Pathological stage						
pT2	31 (38.3)	14 (77.8)	10 (40.0)	1 (9.1)	6 (22.2)	<0.01
pT3a	16 (19.8)	2 (11.1)	6 (24.0)	2 (18.2)	6 (22.2)	
pT3b–pT4	24 (29.6)	0 (0.0)	9 (36.0)	8 (72.7)	7 (25.9)	
Unknown	10 (12.3)	2 (11.1)	0 (0.0)	0 (0.0)	8 (29.6)	
Positive lymph node status	27 (33.3)	0 (0.0)	9 (36.0)	7 (66.4)	11 (40.74)	<0.01
Clinical metastasis	38 (46.9)	0 (0.0)	0 (0.0)	11 (100.0)	27 (100.0)	<0.01
Secondary treatment						
Abiraterone	8 (9.9)	0 (0.0)	0 (0.0)	0 (0.0)	8 (29.6)	<0.01
Chemotherapy	6 (7.4)	0 (0.0)	0 (0.0)	1 (9.1)	5 (18.5)	
Enzalutamide	2 (2.5)	0 (0.0)	0 (0.0)	0 (0.0)	2 (7.4)	
ADT	18 (22.2)	0 (0.0)	4 (16.0)	4 (36.4)	10 (37.0)	

LDH, lactate-dehydrogenase; AP, alkaline phosphatase; ADT, androgen-deprivation therapy. Group A, low-risk clinically localised prostate cancer; Group B, high-risk clinically localised prostate cancer; Group C, cM1 CSPC; Group D, cM1 CRPC.

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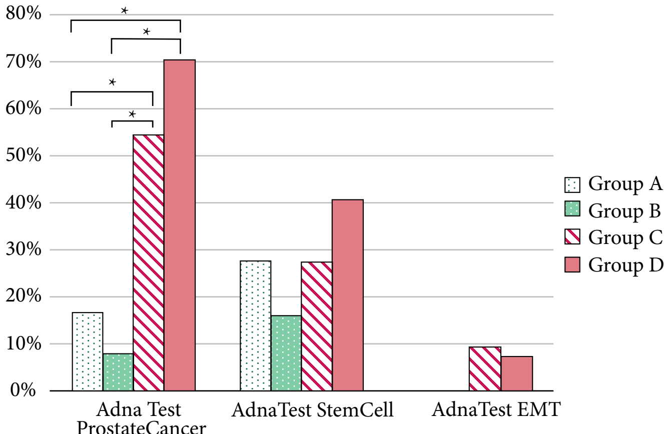

Fig. 1 Distribution of positivity using AdnaTest ProstateCancer, AdnaTest StemCell and AdnaTest EMT between groups. A–D. $*P < 0.01$ chi-squared test. Group A, low-risk clinically localised prostate cancer; Group B, high-risk clinically localised prostate cancer; Group C, cM1 CSPC; and Group D, cM1 CRPC.

Table 2 Comparison of clinical variables according to the AdnaTest ProstateCancer result.

Variable	Negative AdnaTest	Positive AdnaTest	*P*
Number of patients	51	30	
Median (IQR)			
Age, years	65.0 (59.2–70.0)	66.0 (61.0–75.0)	0.25
PSA level, ng/mL	9.4 (6.0–27.0)	37.0 (7.0–147.0)	<0.01*
LDH level, mg/dL	179.5 (156.0–206.0)	224.0 (202.0–269.0)	<0.01*
AP level, mg/dL	71.5 (56.2–82.2)	93.0 (73.0–147.0)	<0.01*
N (%)			
Biopsy Gleason score			
6	15 (29.4)	4 (13.3)	0.04†
7	6 (11.8)	2 (6.7)	
8	16 (31.4)	7 (23.3)	
9–10	9 (17.6)	6 (20.0)	
Unknown	5 (9.8)	11 (36.7)	
Pathological Gleason score			
6	10 (23.8)	4 (16.0)	0.60†
7	6 (14.3)	3 (12.0)	
8	17 (40.5)	9 (36.0)	
9–10	9 (21.4)	9 (36.9)	
Pathological stage			
pT2	24 (47.1)	7 (23.3)	0.04†
pT3a	11 (21.6)	5 (16.7)	
pT3b–pT4	13 (25.5)	11 (36.7)	
Unknown	3 (5.9)	7 (23.3)	
Positive lymph node status	14 (30.5)	13 (59.1)	<0.01†
Clinical metastasis	13 (25.5)	25 (83.3)	<0.01†

*LDH, lactate dehydrogenase; AP, alkaline phosphatase. *Mann–Whitney U-test; †chi-squared test.*

Assessing the single transcripts of AdnaTest ProstateCancer, we found statistically significant increased positivity rates for *PSA, PSMA* and *EGFR* in Group C (45.5%, 25.9% and 37.0%, respectively) and Group D (63.0%, 27.3% and 27.3%, respectively) vs the cM0 groups A+B (7.1%, 2.4% and 7.0%, respectively) (all $P < 0.05$).

The analysis of additional transcripts revealed an increased rate of *AR*-positive patients in groups C (18.2%) and D (25.9%) vs groups A+B (0.0%, both $P < 0.05$) (Fig. 2). *TYMS* gene expression was significantly more frequent in groups C (44.4%) and D (50.0%) vs groups A+B (13.9%, both $P < 0.05$).

Expression of *c-kit* or *c-met* was present in two patients, separately. For *c-met*, both patients with positive expression were metastatic, whereas *c-kit* expression was also detected in one patient with low-risk localised disease.

Table 3 shows the presence of single transcripts according to AdnaTest ProstateCancer. A significant association between AdnaTest ProstateCancer results and the detection of *AR* and *TYMS* expression was observed. EMT-associated transcripts and *c-met* were exclusively detected in patients with positive AdnaTest results.

Discussion

Several platforms exist allowing for the detection and characterisation of CTCs. The CellSearch platform is the most broadly available platform with data from multiple studies, whereas only limited data are available from the AdnaTest platform [2]. This platform allows the detection of CTC-associated mRNAs after immunomagnetic enrichment of epithelial cells [2,13], including *PSA* and *PSMA* for detection of prostate cancer-associated CTCs. However, the panel included in the test can be supplemented by additional markers with potential relevance for prostate cancer biology and treatment of patients, such as *AR-V7* [2,13]. Studies on *AR-V7* as a marker of resistance for second-generation anti-hormonal drugs have drawn attention to the AdnaTest [14]. The present study was initiated due to the lack of data on the presence of CTCs detected by the AdnaTest at different stages of prostate cancer. We aimed to compare the rate of positive patients using the classic AdnaTest ProstateCancer panel in different clinical stages of prostate cancer. In addition, we assessed CTCs using panels designed for detection of CTCs with EMT-like and stem cell characteristics (AdnaTest EMT/StemCell). As the major advantage of the AdnaTest is the potential addition of other markers that may be relevant for prognosis and treatment [6]. We assessed whether mRNAs that have been shown to correlate with prostate cancer aggressiveness (such as *AR* and *TYMS*) or represent potential targets for therapy (*c-met* or *c-kit*) exhibit differential expression patterns according to the clinical stage.

As expected, we found a clearly increased rate of positive AdnaTest results in patients with metastatic disease compared with patients with localised disease. The highest rates of positive AdnaTest results were seen in patients with CRPC, demonstrating that the presence of CTCs detected by the

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Fig. 2 Distribution of positive transcripts between groups A+B (cM0), Group C (cM1 CSPC) and Group D (cM1 CRPC). *$P < 0.05$ chi-squared test between all groups.

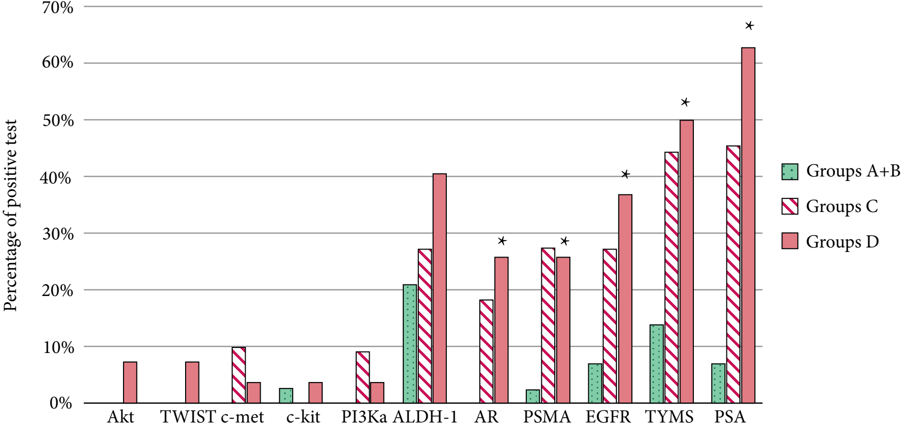

Table 3 Comparison of single transcripts according to AdnaTest ProstateCancer test.

Single transcripts, *n* (%)	Negative AdnaTest (*n* = 51)	Positive AdnaTest (*n* = 30)	*P*
c-met	0 (0.0)	2 (6.7)	0.07
c-kit	1 (2.0)	1 (3.3)	0.73
AR	1 (2.0)	8 (26.7)	<0.01
PI3Kα	0 (0.0)	2 (6.7)	0.06
Akt-2	0 (0.0)	2 (6.7)	0.06
TWIST	0 (0.0)	1 (3.3)	0.19
ALDH1	11 (21.6)	12 (40.0)	0.08
TYMS	8 (15.7)	18 (60.0)	<0.01

AdnaTest may be a surrogate parameter for disease aggressiveness.

The proportion of patients with a positive AdnaTest in the group of patients with mCRPC is consistent with previously published literature. Antonarakis et al. [15] detected CTCs using the AdnaTest ProstateCancerDetect panel in 73.8% of patients with mCRPC starting abiraterone or enzalutamide. Steinestel et al. [16] detected CTCs in 79% of patients with mCRPC using the AdnaTest. To date, results from only one study are available comparing positivity rates of the AdnaTest with CellSearch results in 40 patients with CRPC [17]. The rate of patients with a positive AdnaTest was increased (65%) compared with the CellSearch platform (55%). However, this higher positivity rate should not be interpreted as a higher sensitivity given that the true rate of CTCs is not known. Previous studies have shown that a positive AdnaTest is associated with inferior outcome. Pous et al. [18] recently analysed the oncological outcomes of the prospective Spanish Oncology Genitourinary Group (SOGUG) clinical trial (Phase II Multicenter Study to Analyse the Predictive Value of Fusion Gene *TMPRSS2-ETS* in Response to Enzalutamide in Patients With Metastatic CRPC not Previously Treated With Chemotherapy [PREMIERE] study) in 98 asymptomatic or oligo-symptomatic chemotherapy-naïve patients with mCRPC receiving enzalutamide. All patients were assessed using the AdnaTest before treatment. The authors reported that the detection of CTCs at baseline was associated with worse PSA progression-free survival (hazard ratio [HR] 3.67; $P < 0.001$), radiological progression-free survival (HR 7.61; $P < 0.001$), and overall survival (HR 9.51; $P = 0.040$). Moreover, CTC-positive patients were less likely to exhibit a ≥90% reduction in PSA (odds ratio 2.88; $P = 0.02$). Our group has previously observed inferior survival in patients with mCRPC treated with docetaxel who had a positive AdnaTest before treatment [3].

The present study is the first assessing CTCs, using the AdnaTest, in patients with clinically localised disease. We observed a positive AdnaTest in 11.6% of patients. Previous studies have shown that the proportion of positive-CTC results in localised prostate cancer is strongly dependant on the platform used. In general, platforms using microfluidic techniques for enrichment or PCR for detection have exhibited increased rates compared with the CellSearch platform [5]. Most of these studies did not identify an association with outcome after prostatectomy [5,19]. Whether this also applies to the AdnaTest remains to be elucidated in further studies.

In addition to the prostate cancer-related transcripts *PSA*, *PSMA* and *EGFR* that are used in the context of the original but open-ended AdnaTest ProstateCancerDetect, we also used a kit allowing detection of CTCs with EMT/stem cell characteristics. It is assumed that a significant proportion of CTCs lose features of epithelial cells and undergo EMT to promote disease progression. The detection of these cells represents a significant challenge for CTC research [20]. In the present study, EMT-like gene expression was exclusively present in patients with metastatic disease, whereas *ALDH1*, as a putative stem-cell marker, was even increased in patients

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with localised disease. Whether this finding indicates a specific issue of the test cannot be clearly concluded from the present study as no 'gold standard' enabling the detection of these cells is available. However, these data clearly show that more information on the use of the AdnaTest to assess CTCs with EMT and stem cell characteristics is urgently needed. Whereas several studies have assessed the role of CTCs with stem cell and EMT characteristics in gynaecological cancers and colorectal cancer, only limited evidence is available from studies including patients with prostate cancer [20].

Armstrong et al. [21] analysed the expression of the stem-cell marker CD133 in combination with EMT markers by immunocytochemistry in CTCs from 41 patients with CRPC. Most of the CTCs co-expressed the stem-cell marker CD133 with epithelial markers (e.g., EpCAM, cytokeratins, and E-cadherin) and mesenchymal markers (vimentin, N-cadherin and O-cadherin). Another group used a quantitative PCR method to detect EMT (TWIST1 and vimentin) and stem cell gene expression (ATP-binding cassette transporter G2 [ABCG2], CD133, prostate stem cell antigen [PSCA]) in peripheral blood from 70 patients with metastatic prostate cancer, in addition to CTC enumeration to validate whether this method could complement plain CTC enumeration via the CellSearch system. They found that expression of stem cell-related genes indicates poor prognosis, whereas EMT-related expression does not [22].

The role of the *AR* in CTCs, as a major target for prostate cancer therapy, has been assessed in various studies [2]. In the present study, the expression of full-length *AR* was assessed. We observed that in patients with positive AdnaTest results, *AR* expression rates increased with clinical stage. Although no patient with a positive AdnaTest in the localised disease setting had *AR* transcripts, 33.3% and 57.9% of patients with mCSPC and mCRPC, respectively, had *AR* expression in CTCs. This finding is in accordance with multiple studies suggesting that *AR* expression in mCRPC is upregulated compared with treatment-naïve prostate cancer [23]. The main limitation of the AdnaTest compared with other platforms employing immunohistochemistry-based *AR* detection in CTCs is that subcellular localisation of the *AR* cannot be analysed. Previous studies have indicated strong intercellular heterogeneity of *AR* expression in CTCs and the potential relevance of intracellular localisation [24].

We also assessed the expression of another potential target for prostate cancer treatment, the tyrosine kinase *c-met*. *C-met* is a driver for prostate cancer progression and castration resistance. Moreover, *c-met* is an important promoter of bone metastases [25]. Although the phase III trial of cabozantinib, a specific *c-met* inhibitor, did not show an overall survival benefit in patients with mCRPC, phase II and phase III data indicate that a subset of patients have significant responses to *c-met* inhibition. To date, no data are available on *c-met* analysis in liquid biopsies. However, data from our present study indicate that only a low proportion of patients' exhibit *c-met* expression in CTCs.

TYMS plays an essential role in the biosynthesis of the DNA-component thymidylate (dTTP) and is required for DNA replication and repair [26]. Recently, the expression of *TYMS* has been shown to be significantly associated with unfavourable tumour phenotypes, rapid tumour cell proliferation, and early PSA recurrence [9]. This finding is in accordance with our present study demonstrating a strong correlation between *TYMS* expression in peripheral blood and clinical stage. Whether *TYMS* expression in peripheral blood may serve as a prognostic marker in patients with prostate cancer (in accordance with tissue expression) remains to be elucidated.

In addition to *TYMS* expression, other alterations, such as *c-met* and *c-kit*, also contribute to the development of the castration-resistant phenotype [27,28]. Clinical studies indicate that elevated *c-met* expression is frequently seen in metastatic and CRPC tissues [29].

Moreover, receptor tyrosine kinase pathways have been implicated in the development or progression of prostate cancer, including the proto-oncogenes *c-kit* and *c-met*. Tumorigenesis induced by prostate cancer stem cells is accompanied by the increased expression of *c-kit* [30]. Interestingly, Di Lorenzo et al. [10] observed a trend for an increased risk of relapse amongst patients with high-risk prostate cancer with *c-kit*-positive samples at radical prostatectomy. Moreover, *PI3Kα* can also be indirectly activated by *c-kit* through its binding to the tyrosine phosphorylated adaptor protein GAB2 (growth factor receptor bound protein 2-associated protein 2). *PI3Kα* activation in response to *c-kit* is followed by the phosphorylation of downstream signalling molecules in the *PI3Kα* cascade [28].

Similarly, androgen deprivation is connected with a more aggressive phenotype and leads to increased *c-met* expression [31]. In fact, *c-met* signalling obviously has an important role in maintaining survival and proliferation in *AR*-independent prostate cancer cells.

Based on these results, we hypothesise that in advanced prostate cancer, CTCs mainly express single transcripts associated with progression and metastatic disease.

The present study has important limitations. The most significant limitation is the small sample size. One major limitation of the AdnaTest platform and other CTC-enrichment techniques is that they use epithelial markers instead of disease-specific markers for CTC enrichment, limiting further molecular characterisation and analyses of cells that have undergone the EMT with complete loss of epithelial characteristics. We were not able to correlate CTC

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results with outcomes and treatment response, as no follow-up data of the patients were available.

In conclusion, the AdnaTest ProstateCancerDetect revealed positive results mainly in patients with metastatic disease. *AR* and *TYMS* expression are frequent events in CTCs of patients with advanced disease, whereas *c-met* and *c-kit* gene expressions are only observed in a low proportion of patients. The prognostic and predictive implications of specific gene expression profiles detected by the AdnaTest remain to be determined. The platform is open and can be easily used for the analysis of genes of interest in the context of metastatic prostate cancer.

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None.

Conflict of Interest

Siegfried Hauch and Jens van de Flierdt are employees of Qiagen GmbH, Hilden. Qiagen GmbH provided non-financial support for the study.

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Abbreviations: (m)CRPC, (metastatic) castration-resistant prostate cancer; (m)CSPC, (metastatic) castration-sensitive prostate cancer; Akt-2, AKT serine/threonine kinase 2; ALDH1, aldehyde dehydrogenase 1; AR, androgen receptor; CTC, circulating tumour cell; EGFR, epidermal growth factor receptor; EMT, epithelial–mesenchymal transition; EpCAM, epithelial cell adhesion molecule; HR, hazard ratio; IQR, interquartile range; *PI3Kα*, phosphatidylinositol 3-kinase alpha; PSMA, prostate-specific membrane antigen; TWIST1, twist-related protein 1; TYMS, thymidylate synthase.

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