Detection of Circulating Tumor Cells in Locally Advanced High-risk Prostate Cancer During Neoadjuvant Chemotherapy and Radical Prostatectomy

MARK THALGOTT[1], BRIGITTE RACK[3], THOMAS HORN[1], MATTHIAS M. HECK[1], MATTHIAS EIBER[2], HUBERT KÜBLER[1], MARGITTA RETZ[1], JÜRGEN E. GSCHWEND[1], ULRICH ANDERGASSEN[3*] and ROMAN NAWROTH[1*]

Departments of [1]Urology, and [2]Radiology, Technical University of Munich, Rechts der Isar Medical Center, Munich, Germany;
[3]Department of Gynaecology and Obstetrics, Ludwig Maximilian University, Munich, Germany

Abstract. *Aim: Circulating tumour cells (CTCs) may be prognostic for biochemical recurrence-free survival (bRFS) in patients with locally advanced high-risk prostate cancer (LAPC) undergoing neoadjuvant chemohormonal therapy (NCHT) and radical prostatectomy (RP). Patients and Methods: CTCs were detected before and after NCHT, after RP and at follow-up using the CellSearch™ -System for 59 blood samples (20 ml) from patients with LAPC (n=15) and, additionally, for 15 control samples. Results: The median 5-year progression risk was 90%. CTCs (≥1/20 ml) were detected in 53.3% of patients, with a detection rate of 18.6% in sample-adjusted analysis. CTCs were detected at baseline in 20% of patients with LAPC and 6.7% of controls (p=0.6). CTC findings displayed no association with clinico-pathological characteristics. The median bRFS of CTC-negative vs. CTC-positive patients was 43.7 (95% confidence interval not reached) vs. 29.2 months (95% confidence interval=26.8-60.6 months), without statistical significance (p=0.76). Conclusion: During NCHT and RP, longitudinal CTC presence seems to some extent stochastic, although patients with persistant CTCs post-RP developed biochemical recurrence. No significant association with clinicopathological characteristics or bRFS was observed in patients with LAPC, despite a trend for reduced bRFS in patients with detectable CTCs.*

*These Authors contributed equally to this study.

Correspondence to: Dr. Mark Thalgott, Department of Urology, Rechts der Isar Medical Center, Technical University of Munich, Ismaninger Straße 22, 81675 Munich, Germany. Tel: +49 0894140-9763, e-mail: mark.thalgott@lrz.tum.de

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For localized prostate cancer, there is no approved blood-derived molecular marker amenable for stratification or treatment monitoring. Therefore, there is a crucial unmet need to identify clinically significant prostate cancer potentially resulting in progressive or even life-threatening disease (1, 2). Despite the routinely used diagnostic procedures and precautionary measures, up to 40% of patients present at diagnosis with locally advanced stage disease and are prone to biochemical recurrence despite radical prostatectomy (RP) (3, 4). In order to improve the clinical outcome, multimodal therapeutic strategies are investigated in locally advanced high-risk prostate cancer (LAPC) (5). Nevertheless, in patients with LAPC, there is a lack of stratification tools for indicating systemic perioperative therapies. Prostate-specific antigen (PSA), as the only approved serum marker of prostate cancer, has a positive predictive value of 47% and does not correlate with tumor characteristics or progression (6-9). Potential novel molecular markers are circulating tumor cells (CTCs) that have been detected in venous blood in patients with locally confined as well as metastatic malignancies (10-13). The only medically approved device for CTC detection is the CellSearch™ System (Veridex LLC, Johnson and Johnson) that has been approved in metastatic prostate cancer due to a strong correlation with prognosis, superior to PSA decrement algorithms (9, 14).

Especially in LAPC on the progression from localized to metastatic disease, CTCs may provide additional information on disease progression. However, reports on CTC presence in localized cancer vary notably, resulting in a controversy about their diagnostic implications and possible value in localized prostate cancer (7, 10-13).

Since very little is known about the behaviour of CTCs in localized prostate cancer, especially during different treatment modalities, we investigated CTC detection with the CellSearch™ System in patients with LAPC during neoadjuvant chemohormonal therapy (NCHT) followed by

RP. In order to exclude one of the frequently mentioned pitfalls of the system namely the use of a low analytical sample blood volume of 7.5 ml, in our cohort we investigated an almost three-fold higher blood volume of 20 ml in patients with LAPC and healthy volunteers. Our main objective was to assess CTC detection rates in longitudinal landmark analyses and to explore the association of CTC counts with clinicopathological features, as well as biochemical recurrence-free survival (bRFS). To our knowledge, this is the first study that aimed to detect CTCs in patients with LAPC during different steps of therapy, including NCHT and RP.

Patients and Methods

Study design. This study was a prospective sub-group analysis of longitudinal CTC assessments in peripheral blood of patients with LAPC who were treated with NCHT and RP during a phase II trial that was conducted at the Department of Urology, Technical University of Munich (5). The study was approved by the Institutional Review Board (approval number: 2219/08) and was performed in accordance with the ethical standards of the Declaration of Helsinki. All participants gave their written informed consent. Preoperative NCHT included a complete androgen deprivation with trimestral buserelin (9.45 mg) and bicalutamide 50 mg/day, as well as three cycles of docetaxel (75 mg/m^2) in a 3-weekly schedule, as reported earlier (5). All patients underwent RP including bilateral pelvic lymph node dissection. Adjuvant treatment was individualized according to pathological results. Primary eligibility criteria were non-metastatic locally advanced adenocarcinoma of the prostate with a biochemical 5-year recurrence risk of >40%, according to Kattan's preoperative nomogram (4, 5). Additionally, 15 healthy volunteers were accrued for a single CTC assessment.

In patients with LAPC, blood was collected before (CTC1) and after NCHT (CTC2), 7±3 days post-RP (CTC4), and during follow-up 8-16 weeks following RP (CTC4) (Figure 1).

The main objective was to assess CTC detection rates in longitudinal landmark analyses in patients with LAPC during NCHT followed by RP, and to explore the association with clinicopathological results, as well as bRFS.

Blood sampling and CTC isolation. CTC isolation and enumeration of epithelial-derived cells from peripheral venous blood was carried out as extensively described earlier using the FDA-approved CellSearch™ - System (Janssen Diagnostics, LLC, Raritan, NJ, USA). In brief, 20 ml blood was collected at the clinical site into CellSave™ Preservative Tubes and was processed within 96 hours. Cells were selectively captured by a ferromagnetic antibody to epithelial cancer adhesion molecule (EpCAM) followed by fluorescent staining, using an automated CellTracks™ AutoPrep-System and a CellSearch™ Epithelial Cell Reagent Kit (Janssen Diagnostics, LLC, Raritan, NJ, USA). Cells were transferred into a CellSpotter™ Analyzer (Janssen Diagnostics, LLC, Raritan, NJ, USA) for automated imaging capture. CTCs were characterized by an oval or round form and positive staining for nucleic acid and cytokeratin (9, 15, 16). Samples were evaluated in a blinded fashion at the University of Munich, Germany. Routine laboratory studies were performed using the same blood draw and included PSA determination.

Table I. *Pre-treatment clinical characteristics*.

Clinical characteristic	Value
Patients (n)	15
Age (years)	
Median (mean)	70 (68)
Range	55-76
ECOG	
0	15 (100%)
Prostate-specific antigen (ng/ml)	
Median (mean)	23 (40.7)
Range	5.7-260.0
Gleason score at diagnosis	
6	1 (6.0%)
7	6 (40.0%)
8	4 (26.7%)
9	4 (26.7%)
Time from biopsy (days)	
Median (mean)	46 (50)
Range	20-103
Clinical stage	
cT2c	1 (6.7%)
cT3a	4 (26.7%)
cT3b	10 (66.7%)
Kattan score	
Median (mean)	170 (169)
Range	135-200
Probability of 5-year bRFS (%)	
Median (mean)	10 (14.7)
Range	0-45

bRFS, Biochemical recurrence-free survival; ECOG, eastern cooperative oncology group performance status.

Clinical and pathological evaluation. Metastatic disease was disclosed by routine staging procedures with bone scintigraphy and computed tomographic (CT) scans. Local clinical tumour staging included digital rectal examination and magnetic resonance imaging (MRI) of the prostate with endorectal coil (1.5T, Magnetom Avanto, Siemens, Germany) before and after NCHT. On MRI, an independent board-certified radiologist analysed the T2-weighted sequences in three planes, an axial T1-weighted and a dynamic-contrast-enhanced sequence. Pathological specimens were classified according to criteria of the Union internationale contre le cancer (version 2002) including local stage, resection status, lymph node involvement and Gleason score (17). Following RP, patients with PSA values of less than 0.07 ng/ml were deemed therapy responders (5). Biochemical failure was defined by two consecutive PSA increases of more than 0.2 ng/ml (18).

Statistical analysis. The detection rate was defined as the number of patients with detectable CTCs (≥1 per 20 ml of blood). Continuous measures were compared using the Mann–Whitney U-test and categorical variables using Chi-square test. Association analyses were performed using the Fisher's exact test for categorical variables and the Eta coefficient (η) for the association of categorical with metric variables. An η of more than 0.3 indicated a strong association. The prognostic value of CTC counts for bRFS

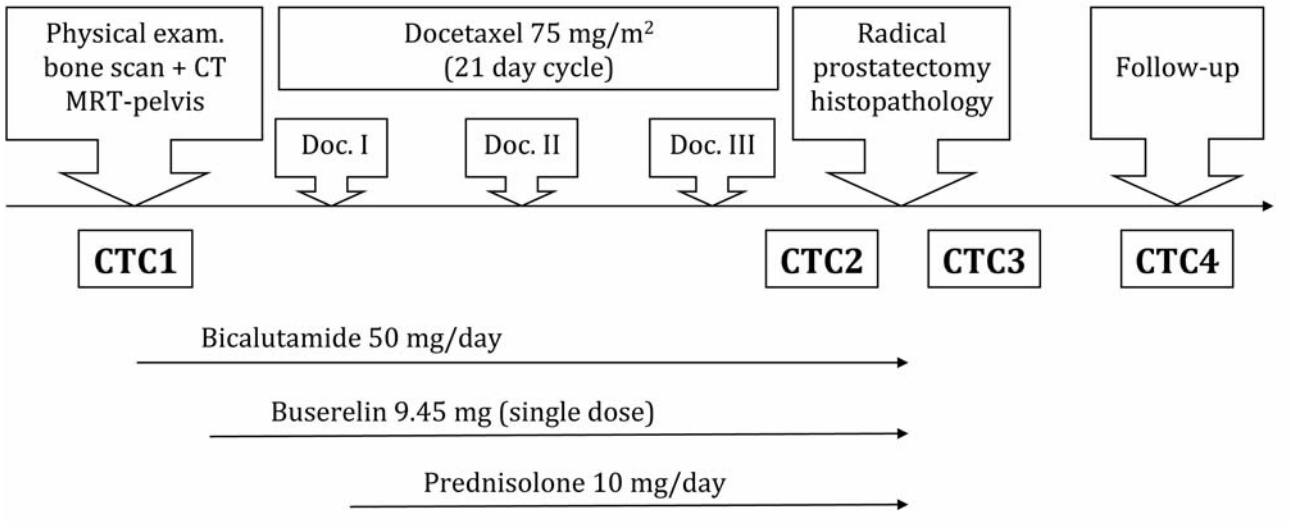

Figure 1. *Study design*.

was analysed using the Kaplan–Meier method. Differences were calculated using the log-rank test. bRFS was defined as the time from the first study treatment to biochemical (PSA) progression (19). A value of $p \leq 0.05$ was considered statistically significant. Analyses were performed and figures prepared using SPSS version 20 (SPSS Inc., Chicago, IL, USA).

Results

Patients' demographics. Landmark analyses for CTC counts in patients with LAPC were performed on a total of 59 blood samples. Concomitant staging analyses were carried-out in all patients. The clinical characteristics of the enrolled patients are summarized in Table I.

LAPC patients had a median risk of recurrence of 90% (range=55-100%). The median time from transrectal biopsy to the first blood draw was 46 days (range=20-103). All patients underwent three cycles of neoadjuvant chemotherapy except for one receiving only two cycles, due to a lumbar disk herniation with consecutive spinal surgery. All patients underwent RP and one patient additionally underwent adjuvant radiation therapy due to positive surgical margins. Postoperative pathological results revealed positive surgical margins or lymph node metastases in four cases (36.4%) and locally advanced disease greater than pT3 in 53.34% of all cases. Controls (n=15) consisted of healthy volunteers with a median age of 28 (range=23-83) years.

CTC detection during therapy and association with clinical characteristics. Overall, CTCs (≥1/20 ml) were detected in 8 out of 15 patients with LAPC (53.3%) during the different steps of therapy in at least one blood draw. In sample-adjusted analyses, the CTC detection rate was 18.64% (11 out of 59 blood draws). However, in healthy controls, one individual was positive for CTCs (6.67%). Similar to other reports, this results in a statistical difference for patients with LAPC which failed to reach significance ($p=0.6$).

Table II presents the clinical and pathological characteristics of the CTC-positive patients and also the frequency and total number of CTC-positive blood draws. In a patient-adjusted analysis, the presence of CTCs was inconsistent over time and rather stochastic (Table II). Out of the patients with detectable CTCs before NCHT, none had positive CTC counts during subsequent time points. Of the initially CTC-negative patients (n=12), three patients became CTC-positive after NCHT, with two of them harbouring CTCs post RP.

Assessing the association of CTCs with clinical characteristics, no significant correlation of CTC detection with clinical characteristics was found, including a correlation with initial Gleason score (≤7 *vs*. ≥8; $p=0.12$) and clinical tumor stage (≤cT2 *vs*. cT3a *vs*. ≥cT3b; $p=0.08$). Equally, CTCs displayed no statistical association with the PSA value ($\eta=0.21$) or time from biopsy ($\eta=0.06$). Similarly, the pathological characteristics of surgical specimens revealed no association with the presence of CTCs, including pathological stage (≤pT2 *vs*. pT3a *vs*. ≥pT3b; $p=0.12$), surgical margin status (R0 *vs*. R1; $p=0.66$), lymph node involvement (pN0 *vs*. pN1; $p=0.28$) or Gleason-score (≤7 *vs*. ≥8; $p=0.3$). Of note, all patients with detectable CTCs had an initial advanced clinical stage of cT3 or more, with 75% presenting with seminal vesicle invasion.

Table II. *Clinical and pathological characteristics of circulating tumor cell (CTC) positive patients.*

									Timepoint of CTC count*				
n	iPSA	iGl-sc	cT	PD-Risk	pT	R	pN1	pGl-sc	1	2	3	4	PD
1	26.6	8	3b	95	3b	0	0	8	3	0	0	0	No
2	39.6	7	3b	95	3b	1	0	7	0	1	4	1	Yes
3	14.1	9	3b	85	3b	0	1	9	0	0	0	1	No
4	19.2	7	3a	85	2c	0	0	7	0	1	2	0	Yes
5	13.9	7	3a	82	2c	0	0	9	1	0	0	0	No
6	31.1	9	3b	95	2a	1	0	9	0	2	0	0	Yes
7	15.9	8	3b	90	3b	0	0	7	1	0	0	n.a.	No
8	260	8	3b	100	3b	0	0	8	0	0	2	0	Yes

cT, Clinical stage; pT, pathological stage; iGl-sc, initial Gleason score at diagnosis; pGl-sc, pathological Gleason score; iPSA, initial prostate-specific antigen value; n.a., not applicable; pN1, pathological lymph node involvement (1 positive; 0 negative); R, surgical resection margin (1 positive; 0 negative); RP, radical prostatectomy; PD-Risk, risk of biochemical recurrence (%) according to Kattan's preoperative nomogram; PD, patients with biochemical recurrent prostate cancer. *1: Before neoadjuvant chemohormonal therapy (NCHT), 2: post-NCHT, 3: post-RP, 4: at follow-up.

Association of CTC counts with time to PSA progression. At the time of analysis, eight (53.3%) patients had recurrent disease, whereas seven (46.7%) had PSA levels below the detection limit. After a median follow-up of 44.3 (range 25-52) months all patients are still alive. As a function of CTC count, the median bRFS was 43.7 months (95% confidence interval not reached) in CTC-negative patients compared with 29.2 months (95% confidence interval=26.8-60.6) in patients with detectable CTCs (≥1). Despite the obvious difference in the median bRFS durations, there was no significant difference (p=0.76) (Figure 2). Interestingly, patients with persistant CTCs post-RP presented biochemical failure during follow-up.

Discussion

In this prospective landmark analysis, we examined the CTC detection rates in patients with LAPC treated with NCHT and RP. The main objective was to assess the association of CTC presence with clinicopathological results, as well as bRFS. Although CTC counts have been assessed earlier in mostly small cohorts with localized prostate cancer, to our knowledge we are the first to present a longitudinal analysis during a defined course of therapy including NCHT for prostate cancer. Moreover, we analyzed a higher blood volume (20 ml) than that initially established for patients with metastatic disease of 7.5 ml of blood. Finally, we included patients with a very high median risk of disease recurrence of 90%, therefore being on the crossover to metastatic disease and likely to harbour clinically have been unapparent micrometastases.

With respect to CTC detection rates at any time point during therapy, 53.3% of the included patients had detectable CTCs during therapy, indicating the highest presented detection rate in patients with localized prostate cancer compared to other reports. However, the sample-adjusted analyses revealed a detection rate of 18.6% similarly to earlier reports, demonstrating in localized and LAPC a CTC detection rate of 5-27% (15, 20, 21). We confirm the results of other studies that failed to demonstrate significantly elevated CTC counts in patients with localized prostate cancer when compared to controls (7, 15, 20). Folkersma *et al.* reported the highest detection rate at 27% in a group that mainly comprised intermediate risk prostate cancer, whereas 10% of controls displayed CTCs (20). Nevertheless most studies detected CTCs in 7.5 ml of blood, whereas we investigated a higher volume of 20 ml, indicating a very low detection rate in the sample-based analyses. A systematic operating error resulting in the mentioned negative results can be excluded since assay controls were performed concomitantly. Similarly Davis *et al.* presented CTC counts per 22.5 ml blood, with equal detection rates in healthy volunteers and patients with prostate cancer of about 20% (7, 21). In summary, we and others have been unable to confirm initial published data that demonstrated significant elevated CTC counts in patients with localized prostate cancer (10). Moreover an increase of the collected blood volume seems not to result in improved detection rates or information.

Focusing on the longitudinal CTC course of each individual patient, our data indicate an uncertain clinical value of CTC presence in patients with LAPC. The presence of CTCs seems to be to some extent a rather stochastic than a systematic finding. A potential explanation for the inconsistent CTC findings could be a shedding of CTCs into the circulation during the different steps of therapy, or insensitivity of the CellSearch™ System for CTC counts in this low range. Subsequently, we found no significant correlation of CTC findings with clinicopathological

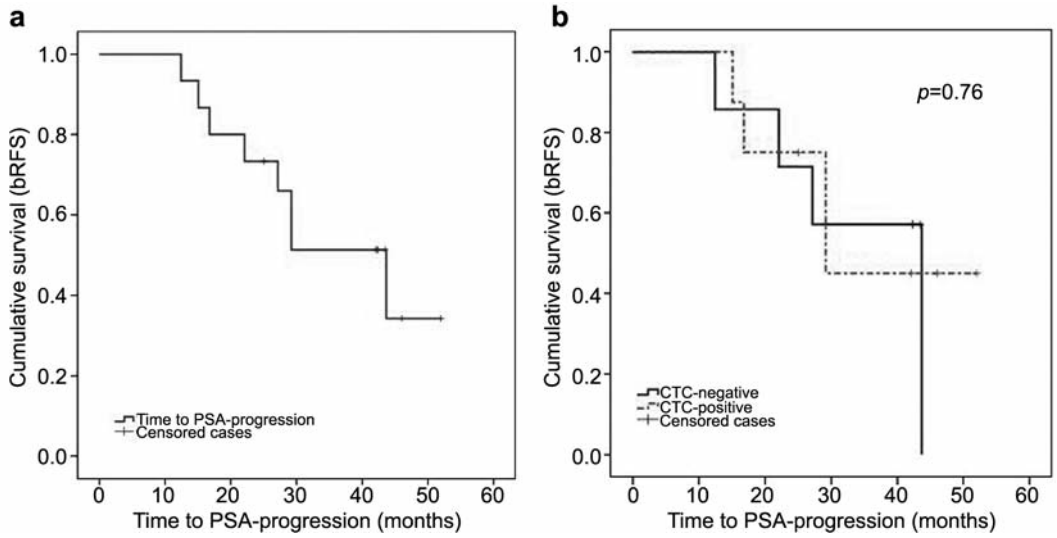

Figure 2. *Kaplan–Meier plot for biochemical recurrence-free survival (bRFS) in the complete cohort of patients (a) and in circulating tumor cell (CTC) positive (CTC ≥1) and CTC-negative patients.*

characteristics. Similarly in the largest presented cohort, in patients with mainly low- and intermediate-risk prostate cancer, no correlation of CTC counts with clinicopathological characteristics was found. Of note, out of 20 patients with detectable CTCs at diagnosis, only two still had CTCs after RP (7). Similarly, more recent studies were unable to demonstrate a correlation between CTC positivity and clinicopathological characteristics (20, 21).

With respect to the prognostic value of CTC presence, we found a median bRFS of 43.7 months in CTC-negative patients compared to 29.2 months in CTC-positive patients. Despite an obvious difference, significance was lacking for our cohort. Similarly Steuber *et al*. demonstrated a trend towards an increased risk of biochemical recurrence after RP in patients with one or more CTCs before RP. Nevertheless significance was lacking marginally, perhaps due to a short-term median follow-up of 24 months (22).

Considering the current literature, the CellSearch™-System seems not to be applicable for staging or diagnosis in localized prostate cancer. Whether the occurrence of CTCs in these patients is stochastic or rather predictive for survival and disease recurrence remains to be evaluated by larger prospective series with a longer follow-up (15, 23). The low detection rate of CTCs in localized prostate cancer might be due to several aspects. Firstly, at this stage, not every cancer specimen might effectively progress to invasiveness. Secondly, the detection system is dependent on localization of the adhesion molecule EpCAM to the cell surface. Although EpCAM has been demonstrated to be expressed ubiquitously in primary prostate cancer tissue, its expression level and its sub-cellular localization during the epithelial-to-mesenchymal transition has not been examined in detail. In addition, a certain sub-population of CTCs may actually be characterized by a lack of EpCAM. Since there is reliable evidence of elevated CTC counts using reverse transcription polymerase chain reaction (RT-PCR) or microchip technologies in localized prostate cancer, the current CellSearch™-System might be inefficient in the capture of CTCs in localized disease (7, 10, 12, 13, 24). This is supported by results that were presented for localized breast and bladder cancer which demonstrated prognostic significance of CTCs. In addition, CTCs persisted in circulation for several years, despite surgical procedures and an absence of metastatic disease, indicating an early shed of CTCs into the circulation and an early involvement in the metastatic process (23, 25-27). Considering that CTCs could provide additional prognostic information and thus potentially indicate an early need for systemic therapies, further effort is needed to improve CTC detection techniques in order to establish CTCs as a supplemental molecular marker in localized prostate cancer (23, 28). Potentially, the newly-described methods, including microchip technologies or filtration devices, such as MetaCell®, or improved and standardized RT-PCR protocols are capable of answering the remaining questions (12, 29). But to the best of our knowledge, there is no approved system currently available.

The main limitation of our study was the very small study cohort. Further validation in larger cohorts is warranted, especially with regard to prognostic value. Possibly a longer follow-up may reveal the prognostic value of CTCs for

bRFS. However, this study provides additional evidence that CTCs are present in the peripheral blood of patients with localized prostate cancer, even though at very low frequencies, and indicate as a trend that this molecular parameter might be useful for predictive use. Projecting into the future, CTCs may serve as a liquid biopsy, predictive of treatment sensitivity and offering a window towards personalized strategies (5, 23).

Conclusion

To our knowledge, this is the first study aiming to detect CTCs in LAPC during the course of neoadjuvant therapy followed by RP. Detection rates during different steps of treatment were variable in each individual, and had an uncertain clinical value. Subsequently, no significant association with clinicopathological characteristics or bRFS was found. However, more longitudinal studies with improved technologies are needed to validate CTCs as a tool for stratification and treatment guidance in localized prostate cancer.

Conflicts of Interest

The Authors have no actual or potential conflict of interest to declare in relation to this research and article. The study was supported by Sanofi-Aventis, Frankfurt, Germany, and the Siegfried Gruber-Foundation, Munich, Germany.

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