

## Cell-free Tumor DNA in Blood Plasma As a Marker for Circulating Tumor Cells in Prostate Cancer

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**Abstract Purpose:** Circulating cell-free DNA in the blood of cancer patients harbors tumor-specific aberrations. Here, we investigated whether this DNA might also reflect the presence of circulating tumor cells (CTC).

**Experimental Design:** To identify the source of cell-free DNA in blood, plasma derived from 81 patients with prostate cancer was examined for CTCs and cell-free DNA. An epithelial immunospot assay was applied for detection of CTCs, and a PCR-based fluorescence microsatellite analysis with a panel of 14 polymorphic markers was used for detection of allelic imbalances (AI).

**Results:** The plasma DNA levels significantly correlated with the diagnosis subgroups of localized (stage M0,  $n = 69$ ) and metastasized prostate cancer (stage M1,  $n = 12$ ;  $P = 0.03$ ) and with the tumor stage of these patients ( $P < 0.005$ ). AI was found on cell-free DNA in plasma from 45.0% and 58.5% of M0 and M1 patients, respectively. Detection of CTCs showed that 71.0% or 92.0% of the M0 and M1 patients harbored 1 to 40 CTCs in their blood, respectively. The occurrence of CTCs correlated with tumor stage ( $P < 0.03$ ) and increasing Gleason scores ( $P = 0.04$ ). Notably, significant associations of the number of CTCs with the AI frequencies at the markers D8S137 ( $P = 0.03$ ), D9S171 ( $P = 0.04$ ), and D17S855 ( $P = 0.02$ ) encoding the cytoskeletal protein dematin, the inhibitor of the cyclin-dependent kinase CDKN2/p16 and BRCA1, respectively, were observed.

**Conclusions:** These findings show, for the first time, a relationship between the occurrence of CTCs and circulating tumor-associated DNA in blood, which, therefore, might become a valuable new source for monitoring metastatic progression in cancer patients.

Early occult dissemination of prostate cancer (PCa) cells from the primary tumor through the bloodstream into secondary organs is a critical step in tumor progression. Currently, repeated measurements of PSA blood serum levels are done after the primary treatment of PCa (e.g., surgery or radiotherapy). However, an increase in PSA levels (also called biochemical relapse) cannot distinguish between a local or metastatic relapse. Moreover, even if overt metastases are subsequently confirmed by current imaging technologies (e.g., bone scans) the patient has become already incurable. Thus, a biomarker indicating early spread of tumor cells as the potential seed for future metastases is highly desirable.

Over the past 10 years, sensitive molecular and immunocytochemical assays have been developed that enable the early

detection of single PCa cells in the background of one million normal cells (1). By applying these assays, it has become evident that bone marrow seems to be an important reservoir for PCa cells as well as tumor cells released by other epithelial tumors (2, 3). Recently, we have revealed that the presence of DTCs in bone marrow of PCa patients was correlated to the detection of cell-free tumor-specific DNA in the same bone marrow sample (4), suggesting that this DNA might be a suitable biomarker for detection of DTCs. However, bone marrow aspiration is an invasive procedure, and therefore, many groups have focused on the detection of circulating tumor cells (CTC) in the peripheral blood of PCa patients (5–13). The clinical utility of CTC counts in PCa is under active investigation with some promising clinical data from single institutions (5, 7, 8, 10). The possibility of detecting tumor cell dissemination years before the onset of metastasis may allow an early curable therapeutic intervention and improve the clinical management of PCa patients. However, the development of a cell-free method that works on blood plasma or serum and targets tumor-specific genomic aberrations would be desirable.

The molecular mechanism of DNA release into the bloodstream is not completely clarified. The presence of high levels of circulating DNA in blood of tumor patients has been suggested to be caused by apoptosis and necrosis of tumor cells, or release of intact cells into the bloodstream and their subsequent lysis (14–16). Correlations between the occurrence of cell-free DNA in blood of tumor patients and malignancy of their disease

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## Translational Relevance

Approximately 25% of patients with clinically localized prostate cancer will eventually experience biochemical evidence of tumor recurrence after surgical resection of the primary tumor. A possible explanation for this clinical observation may be an early onset of tumor cell dissemination to the blood in these men. In view of the lack of tumor-specific molecular markers applicable to the detection of early micrometastatic spread, we selected a prostate-relevant panel of microsatellite markers and searched for tumor-derived cell-free DNA in blood of patients with localized primary tumors and metastatic disease and, additionally, for circulating tumor cells. There is mounting evidence that the presence of circulating tumor cells in blood is associated with an unfavorable outcome in tumor patients. Besides the development of a new detection system for monitoring systemic tumor cell spread, the molecular analysis of cell-free DNA may lead to a new noninvasive approach to detect micrometastatic spread in cancer patients.

were reported (17, 18). Based on the low DNA levels in blood of healthy controls and the high cellular turnover in tumors, the circulating DNA fragments might originate from a primary tumor, which discharges its DNA into the blood circulation (17). However, concordance and discordance between the genetic aberrations of the primary tumor and the alterations of the circulating DNA in blood were detected and described by several laboratories (15, 19–22). The lacking concordance may partly be assigned to micrometastatic cells in blood, which can also shed their DNA into the blood circulation. These observations suggest that cell-free tumor DNA in blood of cancer patients may be also a biomarker for tumor cell dissemination. Therefore, thus far, no report testing this important assumption exists. Recently, Maheswaran et al. (23) claimed that genotyping of free plasma DNA in lung cancer patients for epidermal growth factor receptor activating mutations seemed to be less sensitive than direct analysis of CTCs, but only 12 patients were analyzed.

In the present study, we have searched for blood plasma-based and tumor-specific molecular markers relevant for the detection of early micrometastatic spread in PCa patients by combining the detection of CTCs with the PCR-based microsatellite analysis of cell-free DNA in blood. Allelic imbalances (AI), such as loss of heterozygosity (LOH) and microsatellite instability, occur commonly in primary tumors and with increased frequency in metastases (15, 24). The use of microsatellite analysis has shown that AI maps to tumor suppressor genes playing a key role in PCa progression (24). Here, we investigated the occurrence of AI on cell-free DNA and its relationship to the presence of CTCs detected by an immunometric technique (designated EPISPOT) with proven specificity and sensitivity (11, 12).

## Materials and Methods

**Patients and clinical parameters.** Blood samples were collected from a collective of 69 patients with localized PCa and 12 patients with

metastatic disease between February 2004 and July 2005. A second previous or simultaneous cancer of the PCa patients was ruled out before enrolling the patients in the study. For all patients, blood samples have been taken before any treatment or after treatment during the relapse. All patients were referred to the Department of Urology at the Beau Soleil Clinic of Montpellier, France. Informed consent was obtained from all patients and the study was approved by the local Montpellier research ethics committee.

Of the PCa patients 19.0%, 35.0%, 40.5%, and 5.5% had clinical stages pT1, pT2, pT3, and pT4, respectively. The median prostate-specific antigen (PSA) value was 10.5 ng/mL, ranging up to 1,121.0 ng/mL, and the median %fPSA value was 17%, ranging up to 80%. In the prostatectomy specimens, Gleason scores ranged from 2+3 to 5+5, and Gleason scores of  $\leq 3+5$ , from 4+3 to 4+5 and of  $\geq 5+4$  were recorded in 51.0%, 37.5%, and 10.5% of the men, respectively.

**Preparation of blood plasma and peripheral blood mononuclear cells.** For the cell-free DNA analysis, blood collected into EDTA tubes was centrifuged at 2,500 g, 4°C, for 10 min. The upper phase corresponding to the plasma was immediately stored at -80°C until analysis. In parallel, peripheral blood mononuclear cells (PBMC) were isolated from 2 mL blood by Ficoll-Hypaque density gradient centrifugation (Pharmacia). The cell pellet was washed in 50 mL PBS supplemented with 2% FCS and 2 million of PBMC were stored at -80°C. For the EPISPOT assay, blood collected into EDTA tubes was depleted of hematopoietic cells using the RosetteSep procedure (RosetteSep Circulating Epithelial Tumor Cell Extensive Enrichment Cocktail; StemCell) according to the manufacturer's instructions.

**DNA extraction and fluorescence-labeled PCR.** For the LOH analyses, we used our former method without extended fractionation step because the fractionation technique, which separates plasma DNA in short and long DNA fragments (25), was not yet established when blood samples were collected between 2004 and 2005.

Genomic DNA was extracted from PBMC and plasma of peripheral blood using the QIAamp Blood DNA Mini kit (Qiagen) and a vacuum chamber (Qiagen QIAvac24) according to the manufacturer's instructions. Quantification and quality of the extracted DNA were determined spectrophotometrically using the BioPhotometer (Eppendorf) or the NanoDrop Spectrometer ND-1000 (NanoDrop). To determine the lowest portion of tumor-specific DNA that can be flawlessly detected, dilution experiments were done. For this study, we mixed and amplified known quantities and proportions of normal (PBMC) DNA and plasma DNA, as described before (25).

Ten nanograms of the DNA samples derived from PBMC and plasma were amplified in a 10  $\mu$ L-reaction volume containing PCR Gold buffer, 2.5 mmol/L MgCl<sub>2</sub> (Applied Biosystems or Bio-Rad), 20 nmol/L deoxynucleotide triphosphates (Roche), 0.4 pmol/L of primer sets (Sigma), and 0.2 units of AmpliTaq Gold DNA-Polymerase (Applied Biosystems). PBMC DNA of each patient served as reference DNA. The markers used are listed and described in Table 1. Each sense primer was fluorescence-labeled (HEX, FAM, or TAMRA) at the 5' end. The reaction was started with activation of the DNA polymerase for 5 min at 95°C, followed by 40 cycles of PCR amplification. For optimizing the PCR conditions, 0.1 mmol/L tetramethylammonium chloride was added to each reaction. To confirm the microsatellite alterations, each PCR was repeated at least twice.

**Evaluation of PCR products.** The fluorescence-labeled PCR products were separated by capillary gel electrophoresis and detected on an automated Genetic Analyzer 310 (Applied Biosystems). Fragment length and fluorescence intensity were evaluated by the GeneScan software. The 500-ROX size marker (Applied Biosystems) served as an internal standard. The AI incidence was determined by calculating the ratio of the intensities of the two alleles from a plasma sample corrected by that from the corresponding PBMC sample, which served as reference DNA. LOH was interpreted if the final quotient was  $<0.6$  or  $>1.67$ . Microsatellite instability was defined by the occurrence of a number of additional peaks. Homozygous and nonanalyzable peaks were designated as noninformative cases.

**Table 1.** Characterization of the primer sets used for the analysis of AI

Marker	Chrom. Region	Description
D6S474	6q21-22	BFD4 (Body fluid distribution QTL 4)
D6S1631	6q16	Transforming growth factor- $\beta$ -activating kinase
D7S522	7q31.1	Caveolin 1
D8S87	8p12	Neuregulin 1
D8S137	8p21.1	Dematin
D8S286	8q21.3	HNF-4 $\gamma$ (Hepatocyte nuclear factor-4 $\gamma$ )
D8S360	8p21	n.a
D9S171	9p21-22	CDKN2/p16
D9S1748	9p21	CDKN2/p16
D10S1765	10q23.3	PTEN (Phosphatase and tensin homologue)
D10S541	10q23	PTEN
D11S898	11q22	n.a.
D11S1313	11q12-13	CORS2 (Cerebello-oculo-renal syndrome 2)
D17S1855	17q21	BRCA1 (Breast cancer type 1)

Abbreviations: Chrom. region, chromosome region; n.a., not analyzed.

**Cell Lines and culture conditions.** The PCa cell lines LNCaP (American Type Culture Collection number: CRL-1740) and PC3 (American Type Culture Collection number: CRL-1435) were used as positive and negative controls in the PSA-EPISPOT assay, respectively (26, 27). LNCaP and PC3 cells were maintained in DMEM (Biochrom KG) supplemented with 1% Glutamax (Life Technologies), 10% FCS (Life Technologies), 500 U/mL penicillin, and 500  $\mu$ g/mL streptomycin (Life Technologies) in a humidified incubator containing 5% CO<sub>2</sub> at 37°C.

**PSA-EPISPOT assay.** Immobilon-P membrane 96-well plates (MAIPN450; Millipore Corporation) were coated overnight at 4°C with the anti-PSA mouse monoclonal antibody PS2 (10  $\mu$ g/mL). The plates were then blocked with PBS supplemented with 5% FCS (Sigma Co.) for 1 h at room temperature and washed thrice with PBS. Next, 10<sup>4</sup> to 2  $\times$  10<sup>5</sup> of enriched CD45<sup>+</sup> cells from PCa patients were seeded in different wells of the plates. After 48 h of cell culture at 37°C in a 5% CO<sub>2</sub> incubator, unbound cells were removed by washing thrice with PBS containing 0.2% Tween 20 and 6 times with PBS alone, then the secondary Alexa<sup>555</sup>-labeled anti-PSA monoclonal antibody PS6 was added (1  $\mu$ g/mL), and the plates were incubated overnight at 4°C. The red fluorescent immunospots represented the fingerprints of viable cells secreting PSA. Subsequently, immunospots were counted by video camera imaging and computer assisted analysis (KS ELISPOT-Axio Imager.M1; Carl Zeiss Vision). Quality control was done under standardized ELISPOT assay conditions by including positive and negative controls in each experiment (2  $\times$  10<sup>2</sup> LNCaP and PC3 cells in duplicate, respectively).

The labeling of the anti-PSA monoclonal antibody PS6 used in this study was realized using the Alexa Fluor<sup>555</sup> Monoclonal Antibody Labelling kit (Molecular Probes; Invitrogen) according to the manufacturer's recommendations.

**Statistical analysis.** The statistical analysis was done using the SPSS software package, version 13.0 (SPSS, Inc). Chi Square test, Fischer's exact test and univariate binary logistical regression were used to identify possible associations of the occurrence of CTCs with the AI patterns and the following established risk factors of the PCa patients: age, clinical stage, tPSA values, a combination of tPSA and %fPSA values (tPSA\*%fPSA), Gleason scores, tumor stage, total prostate volume, and surgical margin. High-risk tPSA\*%fPSA values were tPSA of 4 to 10 ng/mL plus fPSA < 15% and tPSA > 10 ng/mL plus fPSA <

10%. Low-risk tPSA\*%fPSA values were tPSA of 4 to 10 ng/mL plus fPSA > 15% and tPSA>10 ng/mL plus fPSA > 21%. A P value of <0.05 was considered as statistically significant.

## Results

**Quantification of cell-free plasma DNA.** Spectrophotometrical quantification of the DNA revealed a wide range of DNA yields of 11 to 1,746 ng/mL plasma of patients with localized PCa (stage M0, n = 69) and 108 to 5,556 ng/mL plasma of patients with overt metastases (stage M1, n = 12). As shown in the box plot of Fig. 1A, M1 patients had significantly higher DNA levels in their blood than M0 patients (P = 0.03). The mean (957 ng/mL) and median (562 ng/mL) values of M1 patients were approximately twice to thrice higher than the mean (451 ng/mL) and median (186 ng/mL) values of M0 patients.

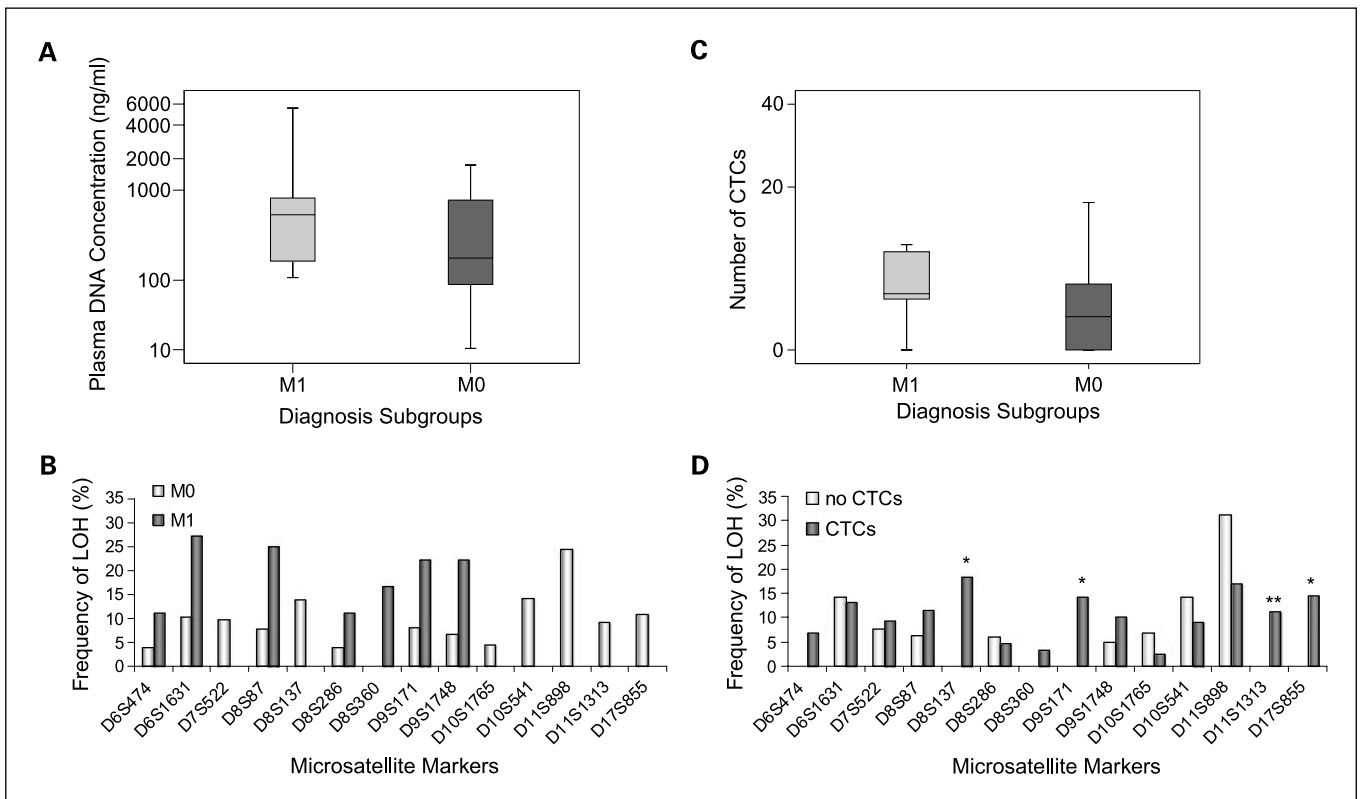
Moreover, we measured the DNA concentrations of a control cohort of 10 healthy men. The range of the measured DNA yields was between 4 and 44 ng/mL with the same mean and median value of 21 ng/mL plasma (data not shown), indicating that only minor DNA amounts circulate in the blood of healthy individuals.

The statistical evaluations of the DNA concentrations in blood of the tumor patients showed that rising DNA yields significantly paralleled with advanced tumor stages of these patients (P < 0.005; Table 2) but did not correlate with the presence of CTCs in blood.

**AI frequency and its relationship to established risk factors.** To examine the relationship of the presence of CTCs in blood to the occurrence of LOH on cell-free plasma DNA, DNA was extracted from blood plasma and PBMC of 81 PCa patients and amplified by PCR using a panel of 14 different polymorphic microsatellite markers (Table 1). Table 3 specifies the distribution of AI detected on cell-free plasma DNA for both patient subgroups M0 and M1, tumor stages, Gleason scores, and tPSA\*%fPSA values. AI (LOH and microsatellite instability) was found on cell-free DNA in plasma from 45.0% and 58.5% of M0 (n = 69) and M1 (n = 12) patients, respectively (Table 3). Figure 1B depicts the distribution of LOH at the different microsatellite markers in M0 and M1 patients. The bar chart distinctly shows that more markers were affected by LOH in the M0 than M1 subgroup. However, among the informative cases, only the marker D11S898 (24.5%), which was M0-specific, showed a LOH rate of over 20% in the M0 patients, whereas 4 markers (D6S1631, 27.5%; D8S87, 25.0%; D9S171, 22.0%; D9S1748, 22.0%) exceeded this frequency in the M1 patients. The marker D8S360 was only affected by LOH in M1 patients, whereas several markers (D7S522, D8S137, D10S1765, D10S541, D11S898, D11S1313, and D17S855) displayed LOH only in M0 patients (Fig. 1B). The overall frequency of microsatellite instability was 1%, and as a rare event, microsatellite instability occurred at the microsatellite markers D6S1631, D8S87, D9S171, D11S898, and D11S1313 (data not shown).

In addition, we analyzed plasma derived from the 10 healthy individuals for AI and did not find AI at any microsatellite marker used (data not shown). These findings indicate that our marker panel was tumor-specific.

Statistical evaluations showed a significant association between advanced tumor stages and the AI frequency at the marker D9S171 (P < 0.05; Table 2).



**Fig. 1.** *A*, the box plot shows the comparison of plasma DNA concentrations in patients with localized M0 ( $n = 69$ ) and M1 ( $n = 12$ ). *B*, comparison of the LOH frequencies detected at 14 different microsatellite markers in blood plasma samples from M0 and M1 patients. The frequency of LOH at each marker was calculated by division of the number of LOH by the number of informative cases. *C*, the box plot shows the numbers of CTCs in peripheral blood of patients with localized M0 ( $n = 69$ ) and M1 ( $n = 12$ ). *D*, comparison of the LOH frequencies detected at 14 different microsatellite markers in the blood plasma samples according to the absence and presence of CTCs in blood. \*, statistical significance; \*\*, borderline significance.

**Associations of CTCs with the AI frequency and established risk factors.** We applied a novel technique for the detection of single CTCs derived from prostate tumors. The enzyme-linked epithelial immunospot assay (EPISPOT) detects tumor cells at an individual cell level (11). Using our patient cohort, we found CTCs in blood of 71% and 92% of the M0 and M1 patients, respectively. In the box plot of Fig. 1C, the distribution of CTCs in both subgroups is depicted. Whereas M0 patients had up to 17 CTCs, M1 patients had up to 40 CTCs in their blood. The mean and median values of CTCs were 3.5 and 2.0 for M0 patients and 10.0 and 4.0 for M1 patients, respectively.

Our control group of 10 healthy individuals had no CTCs in their blood (data not shown), indicating the specificity of our

PSA-EPISPOT detection method, and confirming our previous analysis of a different control group (12).

Statistical evaluations of the number of CTCs in blood were done with the established risk factors for PCa patients and in particular with the occurrence of AI on cell-free plasma DNA of the patients. Table 3 shows the distribution of CTCs in the diagnosis subgroups M0 and M1, tumor stages, Gleason scores, and tPSA\*%fPSA values. The statistical evaluations showed significant correlations between the detection of CTCs and increasing Gleason scores ( $P = 0.04$ ) as well as advanced tumor stages ( $P < 0.03$ ) of the patients (Table 2). When we compared the genetic alterations on cell-free plasma DNA with the presence and absence of CTCs, we detected significant associations of the AI frequency at the microsatellite markers

**Table 2.** Associations between presence of CTCs, plasma DNA yields, LOH frequencies at the microsatellite markers and established risk factors

Parameters	Presence of overt metastasis	Detection of CTCs	Tumor Stage (pT)	Gleason Score
CTCs	No	∕.	$P < 0.03$	$P = 0.04$
Plasma DNA Yields	$P = 0.03$	No	$P < 0.005$	No
LOH Frequency at D8S137	No	$P = 0.03$	No	No
LOH Frequency at D9S171	No	$P = 0.04$	$P < 0.05$	No
LOH Frequency at D17S855	No	$P = 0.02$	No	No

NOTE: No, no significant correlation; ∕., identical variable.



**Table 3.** Patient characteristics and distribution of AI and CTCs in blood

Clinical parameters	Patients total	No. of patients with	
		AI*	CTCs*
Diagnosis subgroup			
M0	69 (85.0%)	31 (45.0%)	49 (71.0%)
M1	12 (15.0%)	7 (58.5%)	11 (92.0%)
Total	81 (100%)	38 (47.0%)	60 (74.0%)
Tumor stage			
pT1-pT2	40 (54.0%)	16 (40.0%)	27 (67.5%)
pT3-pT4	34 (46.0%)	18 (53.0%)	28 (82.5%)
Total	74 (100%)	34 (46.0%)	55 (74.5%)
Gleason score			
≤3+4	41 (54.0%)	18 (44.0%)	28 (68.5%)
≥4+3	35 (46.0%)	18 (51.5%)	29 (83.0%)
Total	76 (100%)	36 (47.5%)	57 (75.0%)
tPSA*%fPSA values †			
Low risk ‡	22 (32.0%)	11 (50.0%)	17 (77.5%)
High risk §	47 (68.0%)	22 (47.0%)	34 (72.5%)
Total	69 (100%)	33 (48.0%)	51 (74.0%)

\*Detection of one or more AI and CTCs was considered as positive.  
 †tPSA\*%fPSA, a combination of tPSA and %fPSA values.  
 ‡Low-risk tPSA\*%fPSA values: tPSA = 4-10 ng/mL plus fPSA>15% and tPSA>10 ng/mL plus fPSA>21%.  
 §High-risk tPSA\*%fPSA values: tPSA = 4-10 ng/mL plus fPSA<15% and tPSA>10 ng/mL plus fPSA<10%.

D8S137 ( $P = 0.03$ ), D9S171 ( $P = 0.04$ ), and D17S855 ( $P = 0.02$ ) with the occurrence of CTCs in the total patient cohort (Table 2). In Fig. 1D, the bar chart shows the frequency of LOH in the absence and presence of CTCs and the detected significant associations between both variables (marked by \*). AI at the markers D6S474, D8S137 ( $P = 0.03$ ), D8S360, D9S171 ( $P = 0.04$ ), D11S1313, and D17S855 ( $P = 0.02$ ) was only detected in the presence of CTCs. The relationship between AI at the marker D11S1313 and occurrence of CTCs was of borderline significance (Fig. 1D, \*\*).

**Discussion**

In the current study, we assessed the relationship of the occurrence of AI on cell-free DNA and DNA concentration with the presence of CTCs in peripheral blood of 81 PCa patients. Our data showed a positive correlation between DNA levels and tumor stages. AI on cell-free DNA and CTCs were frequently found and correlated with established risk factors. However, the key finding was the significant correlation between the presence of CTCs and the detection of LOH at specific genomic regions.

Tumor-associated DNA in blood is supposed to originate from apoptotic and necrotic cells of the primary tumor, which discharge their DNA early during tumorigenesis. It is conceivable that cell-free tumor-specific DNA could also be related to the rate of turnover of CTCs (10). Using the EPISPOT assay, we found CTCs in blood of nearly each M1 patient, and frequently even in M0 patients without overt metastases. However, the mean number of detected CTCs was significantly lower in blood of M0 patients than in blood of M1 patients. The specificity and sensitivity of the applied EPISPOT assay has been shown in

previous investigations (11, 12). By using reverse transcription-PCR and prostate-specific membrane antigen as mRNA marker for monitoring CTCs in clinically localized PCa patients during radical prostatectomy, a previous report also showed significant correlations of CTCs with advanced tumor stages and increasing Gleason scores (28). Furthermore, the isolation of CTCs by immunomagnetic capture and flow cytometry fluorescence-activated cell sorting showed that the CTC counts were strongly associated with survival of patients with progressive castration-resistant or hormone-refractory PCa (5, 10). The use of a diagnostic antibody targeting the insulin-like growth factor-IR epitope showed that insulin-like growth factor-IR-positive CTCs were most common in advanced hormone-refractory PCa (29). These findings suggest that CTCs may serve as a marker for tumor progression.

The most important finding of the present investigation was the significant relationship between the presence of CTCs and cell-free tumor-specific DNA in blood of the PCa patients analyzed. Different patterns of LOH may affect cancer progression toward metastases, and these genetic aberrations are detectable on cell-free DNA in blood. The occurrence of CTCs significantly correlated with the increase in AI at the microsatellite markers D8S137, D9S171, and D17S855, which are located in the chromosomal regions of the cytoskeletal protein dematin (30, 31), the inhibitor of the cyclin dependent kinase CDKN2/p16 (31) and BRCA1 (32), respectively. The biological relevance of LOH in these regions might contribute to a better understanding of the early steps of the metastatic cascade in PCa. Dematin is localized in the junctional complex bundling actin filaments in a phosphorylation-dependent manner. Its biological function is to regulate the cell shape (30), and changes in cell plasticity are thought to be important for the dissemination of tumor cells (33). CDKN2/p16 is a cell cycle regulatory protein and regulates the G<sub>1</sub>-S phase transition. It is inactivated by mutations, deletions, or transcriptional silencing during pathogenesis of a variety of human malignancies and seems to be involved in the tumorigenesis of PCa (34). BRCA1 has been implicated in a number of cellular processes including DNA repair and recombination, cell cycle checkpoint control, chromatin remodeling, ubiquitination, and apoptosis (35). Deletions in BRCA1 have been recently implicated in tumor metastasis (36, 37). Schmidt et al. (38) did a comparative genetic profiling of isolated PSA-positive CTCs and multifocal prostate tumors and showed that detection of LOH at BRCA1 locus in CTCs and primary tumors was associated with an early biochemical recurrence.

The present study indicates, for the first time, that the detection of cell-free circulating DNA is linked to the presence of CTCs in the peripheral blood of PCa patients. This observation is consistent with our previous findings that showed a link between specific genetic alterations of cell-free DNA in bone marrow samples and the presence of disseminated tumor cells (4). However, the set of the two AI patterns associated with disseminated cancer cells in blood or bone marrow were not overlapping, which may reflect that tumor cell circulation and homing into bone marrow are two distinct biological steps in the metastatic cascade (1, 2). Moreover, LOH at locus D9S171 was significantly correlated to the presence of CTCs and tended to be also more frequent in M1 patients (Fig. 1B and D), suggesting that this genomic aberration might be a marker for both blood-borne dissemination of tumor cells

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and formation of overt metastases. In contrast, LOH at loci D11S1313 and D17S855 were only detected in M0 but not in M1 patients and was only associated with the presence of CTCs, suggesting that the genes in these regions might be involved in tumor cell dissemination but not in metastatic outgrowth. However, these assumptions need to be tested in future functional studies.

The observed correlations between free plasma DNA and CTCs may be explained by the assumption that cell-free tumor DNA in the peripheral blood (or bone marrow) may not only be derived from the primary tumor but also from circulating (or disseminated) tumor cells. To further support this assumption, comparative genetic profiling of the primary tumors, CTCs, and cell-free plasma DNA is required, which, however, might be hampered by the considerable genetic heterogeneity of PCa cells. An alternative explanation is that primary tumors with a high metastatic potential have particular genetic aberrations, which can be detected on cell-free DNA in the blood of the respective patients carrying these tumors. Recently, Holcomb et al. (39) have characterized genomic alterations of DTCs in bone marrow of patients with localized (M0) and metastatic (M1) PCa using an array-based comparative genomic hybridization approach. Genetic losses in DTCs included two areas (8p23 and 10q25.1) that were close to the regions investigated in our study. In general, M0 patients had fewer and less striking genomic alterations than the paired primary tumors and the DTCs of M1 patients (39, 40). The authors explained this interesting finding by the assumption that tumor cells may disseminate early before the primary tumor has acquired multiple genomic changes (39) and that the DTCs can acquire key molecular changes that may allow them to form an overt metastasis (1).

Besides the DNA losses assessed in the present study, the inactivation of tumor suppressor genes can also occur by

promoter hypermethylation. An early epigenetic event during prostate carcinogenesis is the alteration in the methylation profile of the genome. Hypermethylation of the promoter region of the pi-class glutathione S-transferase gene is the most common somatic genome abnormality in PCa. Because cell-free methylated DNA can be detected in the blood of PCa patients, especially DNA hypermethylation of pi-class glutathione S-transferase gene, may be an additional blood-based biomarker for PCa (41, 42). The epigenetic alterations may also affect the *PTEN* locus and explain why AI of *PTEN* was rather a rare event in our cohort. Apart from DNA deletions, the activity of *PTEN* may be repressed by promoter methylation silencing at high frequency in PCa (43). In this context, it is interesting that promoter hypermethylation in circulating blood cells seems to identify PCa progression (44).

In conclusion, the present investigation indicates that genetic analyses of cell-free DNA in blood plasma might become a valuable new source for monitoring metastatic progression in cancer patients and may therefore contribute to the molecular staging of PCa. Future studies on larger numbers of cancer patients with sufficient follow up time are now required to further validate the clinical utility of free plasma DNA.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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