Cell-Free DNA: A Novel Biomarker for Patients with Prostate Cancer?

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Abstract: The presence of small amounts of circulating DNA in plasma was demonstrated 60 years ago. Since then, cell-free DNA has been tested for quantity, fragmentation pattern and tumor-specific sequences in patients with various malignancies. The introduction of improved detection methods showed that all these alterations are regularly detectable in many cancer patients and the investigation of cell-free DNA may provide useful diagnostic and prognostic information. Herein, we review the recent findings on cell-free DNA alterations in patients with prostate cancer and discuss its diagnostic and prognostic potential.

Keywords: Cell-free DNA, prostate cancer, biomarker, serum, methylation.

INTRODUCTION

The presence of circulating cell-free DNA in human plasma was reported in 1948 by Mendel and Metais [1]. The development of improved detection methods led to the discovery of increased circulating DNA levels in cancer patients compared to healthy individuals [2]. It was later confirmed that cancer patients' DNA levels are not only increased compared to healthy individuals, but also to patients with various non-malignant diseases [3]. Finally, it was shown that the circulating DNA carried tumor-specific alterations [4]. The amount of circulating DNA is within the range of nanograms, and thus the detection of circulating DNA was laborious until the development of the polymerase chain reaction (PCR). After development of PCR and quantitative PCR techniques, knowledge of cell-free circulating DNA was rapidly growing: It was shown for many tumor entities that cell-free circulating DNA levels are increased and allow distinguishing patients with non-malignant disease from healthy individuals (e.g. lung [5], colon [6], cervical [7], ovarian [8], breast [9], testis [10], bladder [11] and prostate cancer [12]). Cell-free DNA may therefore serve as a non-invasive universal cancer biomarker.

Despite the 60 years of research, the origin of these DNA fragments in cancer patients remains largely unknown. While the tumor contributes to the circulating DNA, the vast majority originates from healthy cells [13, 14]. Jahr, *et al*. further showed that neither endothelial nor tumor-infiltrating T-cells are causative for the increase of cell-free DNA [13]. The induction of apoptosis (anti-CD95 antibody) or liver necrosis (acetaminophen) in a mice model resulted in a distinct increase of cell-free DNA with either high-molecular DNA (necrosis) or short, mono- and di-nucleosomal DNA fragments (apoptosis) [13]. Cancer patients' DNA shows an apoptotic as well as a necrotic pattern [13, 15], and the pattern seems to be different in various cancer entities: DNA integrity was increased in patients with colon [6], testicular [10], head and neck [16], breast [17] cancer patients indicating predominantly necrotic breakdown. On the other hand, mainly apoptotic DNA fragments were detected in patients with prostate [18] and bladder [11] cancer. Interestingly, tumor-specific sequences were more frequently observed in low-molecular weight than in high-molecular cell-free DNA of prostate cancer patients [19]. The clearance of cell-free DNA from the bloodstream occurs rapidly: fetal DNA disappeared from the blood of mothers after delivery with a half-life time of 16.3 minutes [20]. It is known that cell-free DNA is sensitive to plasma nucleases (e.g. DNase 1), but renal [21] and hepatic [22] clearance are also involved in the elimination of cell-free DNA.

So far, it is unknown if the release of cell-free DNA has any biological effects. Cultured cells have been shown to release double stranded DNA into the media [23], and cell-free DNA might be incorporated into cells [24]. These findings led to the introduction of the concept of "genometastasis" which postulates a horizontal transfer of tumor DNA with transforming potential into stem cells in distant organs [25]. However, this hypothesis remains to be proven.

Circulating DNA can be isolated from both plasma and serum, but serum contains an approximately 6-times higher DNA concentration. It was debated for a long time whether these higher levels are due to a contamination by leukocytes. Recently, Umetani, *et al*. showed that less than 10% of the 6-fold higher serum DNA levels were due to contamination by other sources (i.e. release from leucocytes during the separation of serum) [26]. The reason for higher serum levels remains unknown; but a loss of DNA in plasma during purification procedures was excluded [26]. Higher DNA levels

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favor the use of serum over plasma, but it is not as simple as that: delayed processing (i.e. >6 hours) of blood samples resulted in significantly increased serum but not in plasma DNA concentration [27]. In addition, the size of cell-free DNA increased if the blood was stored longer than 6 hours prior centrifugation, thereby indicating contamination by leukocyte DNA during storage [28]. Thus, the analysis of cell-free DNA requires highly standardized processing procedures.

Cell-free circulating DNA harbors the potential of a useful cancer biomarker. DNA levels, fragmentation patterns and tumor-specific alterations offer interesting possibilities for diagnostic and prognostic purposes. We will discuss the recent findings on cell-free DNA in prostate cancer (PCA) patients with regard to the diagnostic and prognostic information.

DNA LEVELS

So far, cell-free DNA levels have been assessed in patients with prostate cancer in ten studies [12, 18, 29-36]. Pooled data from several studies including more than 650 PCA patients and 350 control subjects suggest that cell-free DNA levels provide helpful diagnostic (Table **1**) and prognostic (Table **2**) information. However, the evaluation of the performance of cell-free DNA as diagnostic/prognostic marker is difficult because different DNA isolation procedures and detection methods have been applied.

Jung, *et al*. published the first study on cell-free DNA levels in patients with prostate cancer [12]. DNA levels were similar in patients with benign prostate hyperplasia (BPH) and clinically localized PCA, but patients with metastatic PCA had significantly increased cell-free DNA levels. Maybe the use of the less sensitive fluorometric assay is the reason for missing differences between BPH and localized PCA.

Recent studies employed a more sensitive real-time PCR to compare the concentration of cell-free DNA in PCA patients and controls [18, 29-31, 33]. The largest study so far (localized PCA n=168, BPH n=42, healthy controls n=11) was published by Ellinger, *et al*. [18]. The detection of cell-free DNA provided a sensitivity of 88% and a specificity of 64% (area under curve 0.824). A somewhat better discrimination was observed by Altimari, *et al*. [33] with a sensitivity of 80% and a specificity of 82% (localized PCA n=64, BPH

Table 1. DNA Levels: Diagnostic Value

Study		n PCA)	n (Control)	Sensitivity	Specificity	AUC	Method
Jung 2004	[12]	91†	93	n.s.	n.s.	n.s.	fluorometric assay
Allen 2004	[29]	27‡	10	85%	73%	n.r.	PCR
Papadopoulou 2004	[30]	12	18	58%	94%	0.840	dipstick
Papadopoulou 2004	[30]	12	13	58%	92%	0.708	PCR
Boddy 2005	[31]	78	99	n.s.	n.s.	n.s.	PCR
Chun 2006	[32]	142	19	n.r.	n.r.	n.r.	spectrophotometry
Ellinger 2008	[18]	168	42	88%	64%	0.824	PCR
Altimari 2008	[33]	64	45	80%	82%	0.881	PCR
Cherepanova 2008	[35]	5	59	n.r.	n.r.	n.r.	fluorometric assay
Schwarzenbach 2009	[36]	69	10	n.r.	n.r.	n.r.	spectrophotometry

Abbreviations: AUC, area under curve; n.s. not significant; n.r., not reported
Note: † mixed cohort of patients with metastatic and localized disease; ‡ including 12 patients with high grade PIN.

Table 2. DNA Levels: Prognostic Value

Study		n (PCA)	Observation	Method
Jung 2004	[12]	91†	predictor of cancer specific survival, increased in metastatic PCA	fluorometric assay
Bastian 2007	[34]	192	predictor of PSA recurrence, correlated with Gleason Score, surgical margin status and extraprostatic extension	PCR
Ellinger 2008	[18]	168	predictor of PSA recurrence	PCR
Altimari 2008	[33]	64	correlated with pT-stage	PCR

Abbreviations: AUC, area under curve; n.s. not significant; n.r., not reported
Note: † mixed cohort of patients with metastatic and localized disease.

n=45; area under curve 0.881). Both studies reported approximately 3-fold increased DNA levels in PCA patients. The smaller series published by Allen *et al.* (n=15) and Papadopoulou, *et al*. (n=12) showed similar results [29, 30]. Interestingly, Papadopoulou, *et al*. also reported that a semi-quantitative dipstick method (Invitrogen DNA DipStick), which may be less cost-intensive and more rapidly to accomplish, provided similar diagnostic information as a real-time PCR-assay. Using spectrophotometry Chun, *et al*. also found increased DNA levels in PCA patients, and the predictive value of a multivariate model (including total PSA, free/total PSA, cell-free DNA) was significantly improved by 5.6% to 78.3% [32].

In contrast to the above findings, Boddy, *et al*. failed to detect significant differences between patients with PCA and non-malignant prostate disease [31]. This could be due to the fact that PCA was not definitively excluded at the time of blood withdrawal by histological examination, and thus elevated levels may be due to undetected PCA: Ellinger, *et al*. also reported increased levels of cell-free DNA in patients with incidental PCA (See Table **1** for details) [18].

In addition to the diagnostic information, cell-free DNA levels may also help to identify PCA patients with poor prognosis. Altimari, *et al.* showed significant correlation with pathological stage [33]. It was also recently shown that plasma DNA levels of patients with metastatic disease were higher than in patients with localized PCA [36]. Bastian, *et al.* [34] and Ellinger, *et al*. [18] demonstrated that cell-free DNA levels were significant predictors of PSA recurrence following radical prostatectomy. Jung, *et al.* showed that cell-free DNA levels were a predictor of PCA specific survival in patients with metastatic disease (See Table **2** for details) [12].

DNA FRAGMENTATION

The fragmentation pattern allows conclusions about the underlying cell-death entity of the circulating DNA. Hanley, *et al.* combined a hybridization-capture technique with a PCR to measure ten different sized plasma DNA fragments (200bp to 10kb) and to calculate the DNA integrity. The DNA integrity was increased in PCA patients (n=123) compared to controls (n=67; sensitivity 70%, specificity 68%, area under curve 0.788) [37]. Two other studies employed a quantitative real-time PCR to amplify two different sized PCR products (124bp and 271bp [18], 105bp and 356bp [38]) to assess the fragmentation pattern. Ellinger, *et al*. reported shorter DNA fragments in patients with PCA (n=168) than in patients with BPH (n=42; specificity 81%, sensitivity of 68%, area under curve 0.786). Furthermore, the presence of short DNA fragments was a predictor of PSA recurrence following radical prostatectomy [18]. In contrast, Boddy, *et al.* did not observe a different cell-free DNA fragmentation pattern in PCA (n=61) and BPH patients (n=62) or a correlation with clinical-pathological parameters [38].

The somewhat confusing results may be explained by the use of plasma [37, 38] vs. serum [18] and the different detection methods. However, the studies by Hanley, *et al.* and Ellinger, *et al*. suggest a diagnostic potential and future studies are necessary to clarify the role of DNA fragmentation in patients with PCA.

MITOCHONDRIAL DNA

Two studies investigated plasma/serum cell-free mitochondrial DNA. Interestingly, the concentration of genomic and mitochondrial DNA were not correlated to each other, maybe due to different compartmentalization and degradability of mitochondrial and genomic DNA [39]. Mehra, *et al*. reported a 3-fold increase of mitochondrial DNA in patients with metastatic PCA compared to controls [39], whereas Ellinger, *et al*. did not observe differences between patients with clinically localized PCA and BPH [40]. However, mitochondrial DNA levels were an independent predictor of PSA recurrence following radical prostatectomy for clinically localized PCA [40] and high levels of mitochondrial DNA were correlated with PCA-specific survival in patients with advanced PCA [39]. In summary, a diagnostic potential is questionable, but there is a potential role as prognostic biomarker.

TUMOR-SPECIFIC ALTERATIONS

Tumor-specific sequences in serum/plasma cell-free DNA were first reported in 1989 [4]. Recent studies also confirmed that prostate cancer patients' cell-free DNA includes tumor-specific DNA [19, 30, 33, 34, 41-51]. The amount of tumor-specific DNA is usually limited to a fraction of less than 10%, [18, 52, 53]). An increase of cell-free DNA seems to be a universal feature of neoplasia, and thus the detection of PCA-specific alterations may be helpful in order to distinguish PCA patients from patients with other malignancies. Recent studies in PCA patients focused on either the detection of epigenetic (DNA hypermethylation) or genetic DNA alterations (allelic imbalances).

DNA Hypermethylation

DNA hypermethylation is one of the most common and earliest alterations during prostate carcinogenesis. For instance, DNA hypermethylation of the *GSTP1* promoter was observed in approximately 90% of PCA tissues [54]. The detection of aberrantly methylated cytosine is feasible following bisulphite treatment, which induces conversion of unmethylated cytosine to uracil, whereas methylated cytosine remain unchanged. The sequence differences are then detectable using a PCR ("methylation specific PCR") [55]. However, approximately 90% of the DNA is lost during bisulphite treatment, and thereby limiting the sensitivity [56]. A "methylation sensitive PCR" may be more sensitive: methylation-sensitive restriction enzymes cleave unmethylated DNA, and methylated DNA sequences are thereafter detected using PCR [52].

A number of studies have assessed the presence of *GSTP1* hypermethylation in cell-free circulating DNA [18, 30, 33, 43-45, 49-53]. The sensitivity of these studies ranged from 11-100% and the specificity was >93%; the frequency of *GSTP1* hypermethylation was 30% in the pooled analysis of approximately 900 PCA. A multigene analysis increased the sensitivity if three or four gene sites were analysed in combination [44, 49, 51, 53]. It was also shown that a DNA hypermethylation profile allowed to distinguish tissue of different carcinomas [57]. Our recently published studies on patients with prostate [42], bladder [14] and testicular cancer [58] suggest that a multigene analysis may also be applicable

in cell-free DNA. See Table **3** for a detailed summary of the diagnostic information of cell-free DNA hypermethylation.

The analysis of cell-free DNA hypermethylation may also provide prognostic information (see Table **4**). Bastian, *et al.* demonstrated that *GSTP1* hypermethylation was the strongest predictor of PSA recurrence following radical prostatectomy in patients with clinically localized PCA [52]. Reibenwein, *et al* reported a correlation between *GSTP1* methylation and the Gleason Score respectively the metastatic load in patients with hormone-refractory PCA [44]. Finally, it was recently published that *RASSF1A*, *RARB2* and *GSTP1* hypermethylation correlated with the Gleason Score and the serum PSA; *RARB2* and *GSTP1* methylation also correlated with the AJCC stage [49].

Allelic Imbalances

So far, five studies have investigated the presence of allelic imbalance (i.e. loss of heterozygosity, LOH; microsatellite instability, MSI). Allelic imbalance requires the comparison of cell-free DNA with patients' leukocyte DNA, and differences are assessed using a PCR. The high background of normal DNA within the circulation limits the sensitivity of the method and is the major drawback. Furthermore, the tests require relatively high amounts of cell-free DNA, and thus the number of informative of analyses varies between 40 and 80% [48, 49]. Separation into high and low-molecular DNA during the DNA isolation procedure seems to be an improvement: Muller, *et al.* demonstrated a higher sensitivity using low- instead of high-molecular DNA for the LOH analyses [19].

Schwarzenbach, *et al.* were the first to show that the analysis of LOH in cell-free DNA is feasible [46]: However, LOH/MSI was also detected in cell-free DNA of patients with BPH [46, 47]. A panel of 13 polymorphic markers provided a sensitivity of 57% and a specificity of 70% [47]. Sunami, *et al.* used a panel of eight markers and observed the presence of at least one LOH in 47% of PCA patients, whereas LOH was not observed in healthy controls [49]. A recent study by Schwarzenbach, *et al.* demonstrated a 45% sensitivity of AI in cell-free plasma DNA of patients with localized PCA [36]. See Table **5** for a summary of the diag-

Table 3. DNA hypermethylation: Diagnostic value

Study		Gene Site	n (PCA)	n (Control)	Sensitivity	Specificity	Method
Goessl 2000	[41]	*GSTP1*	32†	22	72%	100%	MSP
Jeronimo 2002	[43]	*GSTP1*	69	31	36%	100%	MSP
Jeronimo 2002	[43]	*GSTP1*	69	31	13%	100%	qMSP
Papaopoulou 2004	[30]	*GSTP1*	31‡	9	52%	100%	MSP
Bastian 2005	[52]	*GSTP1*	213†	35	11%	100%	qMS-PCR
Reibenwein 2007	[44]	*GSTP1, AR, 14-3-3-Sigma*	76‡	49	30%	100%	MSP
Chuang 2007	[50]	*GSTP1*	36	27	31%	93%	MSP
Bryzgunova 2008	[45]	*GSTP1*	5	10	100%	100%	BS
Ellinger 2008	[53]	*GSTP1, TIG1, PTGS2, RPRM*	168	42	47%	93%	qMS-PCR
Altimari 2008	[33]	*GSTP1*	18†	22	33%	95%	MSP
Bastian 2008	[51]	*GSTP1, MDR*1*	192	35	32%	100%	qMS-PCR
Sunami 2008	[49]	*GSTP1, RARB2, RASSF1A*	83‡	40	28%	100%	MSP

Abbreviations: MSP, methylation-specific PCR; BS, bisulphite sequencing; qMS-PCR, quantitative methylation-sensitive PCR; qMSP, quantitative methylation-specific PCR
Note: † including patients (<25%) with metastatic or hormone-refractory prostate cancer; ‡ including patients (>60%) with metastatic or hormone-refractory prostate cancer; * *EDNRB, CD44, NEP, PTGS2, RASSF1A, RARB* and *ESR1* were unmethylated in patients with localized PCA

Table 4. DNA Hypermethylation: Prognostic Value

Study		n (PCA)	Observation	Method
Bastian 2005	[52]	192	*GSTP1* was a predictor of PSA recurrence	MS-PCR
Reibenwein 2007	[44]	76‡	*RASSF1A, RARB2, GSTP1* correlated with Gleason Score and PSA; *RARB2, GSTP1* correlated with AJCC stage	MSP
Sunami 2009	[49]	83‡	*GSTP1* correlated with Gleason Score and metastasis in HRPC patients	MSP

Abbreviations: MSP, methylation-specific PCR; qMS-PCR, quantitative methylation-sensitive PCR
Note: ‡ including patients (≥70%) with metastatic or hormone-refractory prostate.

Table 5. Allelic Imbalance: Diagnostic Value

Study		Microsatellite Marker	n (PCA)	n (Control)	Sensitivity	Specificity
Muller 2006	[46]	D3S3703, THRB, D6S474, D6S1631, D7S522, D8S87, D8S137, D8S286, D8S360, D9S171, D9S1748, D10S1765, D11S898, D11S1313, TP53.6	65	36	34%	78%
Schwarzenbach 2008	[47]	THRB, D6S474, D61631, D7S522, D8S87, D8S137, D8S286, D8S360, D9S171, D9S1748, D10S1765, D11S898, D11S1313	230	43	57%	70%
Sunami 2008	[49]	D6S286, D8S261, D8S262, D9S171, D10S591, D18S70	83‡	40	47%	100%
Schwarzenbach 2009	[36]	D6S474, D6S1631, D7522, D8S87, D8S137, D8S286, D8S360, D9S171, D9S1748, D10S1765, D10S541, D11S898, D11S1313, D17S1855	69	10	45%	100%

Note: ‡ including 58 (70%) patients with metastatic prostate cancer.

nostic potential of cell-free DNA. Furthermore, the AI frequency was higher in patients with metastatic disease [36], and genetic alterations also indicated a higher Gleason Score [48].

CONCLUSIONS

Circulating DNA is a promising non-invasive biomarker in the diagnosis and prognosis of PCA. The detection of tumor-specific alterations as well as DNA fragmentation patterns may further increase the diagnostic information obtained by pure analysis of DNA quantities. However, the evaluation of cell-free DNA needs meticulous standardization, and prospective studies are necessary to confirm the clinical value of circulating DNA fragments in PCA.

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