Seminal Cell Free DNA Concentration Levels Discriminate Between Prostate Cancer and Benign Prostatic Hyperplasia

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Abstract. *Background/Aim: Seminal plasma cfDNA (scfDNA) was recently proposed as a novel PCa biomarker. Our aim was to evaluate whether scfDNA could discriminate PCa from benign prostate hyperplasia (BPH) patients. Patients and Methods: A cohort of 43 patients (18 and 25 pathology proven PCa and BPH patients), and 13 healthy age-matched control subjects were enrolled. scfDNA quantification was performed. Data were analyzed through ANOVA testing. Results: Average scfDNA concentrations were 1,407.83 ng/μl, 128.13 ng/μl and 78.09 ng/μl for PCa patients, BPH patients and healthy subjects, respectively. Statistical analysis showed a significant difference among the groups, allowing for distinction of patients with optimal accuracy. A cut-off level of 450 ng/μl scfDNA was identified for the differentiation of PCa and BPH patients. Conclusion: scfDNA concentrations are significantly different between PCa patients and BPH patients. scfDNA is a promising biomarker with several applications in PCa diagnosis, screening programs and therapeutic monitoring.*

Prostate cancer (PCa) is the most common malignant disease and the second most frequent cause of cancer-

related mortality amongst men worldwide (1). The advent of liquid biopsy techniques and the isolation of circulating tumor DNA (ctDNA) from blood or seminal fluid, may represent a major step forward in PCa diagnosis and management compared to traditional diagnostic techniques involving prostate-specific antigen (PSA), digital rectal examination (DRE), transrectal ultrasound (TRUS) and biopsy (2-5).

The use of PSA as a serum marker has revolutionized PCa diagnosis. However, this antigen is not cancer-specific and may be elevated in other non-malignant conditions, such as benign prostatic hyperplasia (BPH) and prostatitis (6, 7). Several new biological markers such as BRCA2 (8, 9), TMPRSS2-Erg fusion and PCA3 (10) and four-kallikrein panel (11) have been shown to add sensitivity and specificity to PSA, thereby decreasing the incidence of PCa over-diagnosis. Current evidence regarding biomarkers is too limited for the development of recommendations. Further, a population-based survival benefit has not yet been demonstrated.

Within the setting of liquid biopsy, circulating free (cfDNA) and ctDNA quantification and sequencing allow the collection of important data regarding cancer diagnosis and characterization, patient prognosis and management in order to determine therapeutic strategies and the subsequent follow-up (12, 13). Higher blood plasma cfDNA levels in PCa patients with respect to healthy subjects have been reported (3-5), as well as significant differences in cfDNA levels and integrity between PCa and BPH patients (14, 15). Previous studies have identified urine cell-free DNA (UCF-DNA) integrity as a potential marker for early diagnosis of non-invasive prostate cancers, even though there are no large cohort studies supporting this hypothesis (16, 17).

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Table I. Clinical, pathological features of prostate cancer patients, BPH patients and healthy individuals.

PC Patients	Age	PSA (ng/ml)	Gleason Score	Qubit ssDna (ng/μl)	BPH Patients	Age	PSA (ng/ml)	Qubit ssDna (ng/μl)	Healthy	Qubit ssDna (ng/μl)
1	67	8.7	7 (4+3)	1,635.00	1	67	4.49	215.40	1	62.50
2	61	7.05	6 (3+3)	2,100.00	2	55	4.9	76.10	2	52.90
3	65	6.2	7 (3+4)	978.00	3	52	7.52	110.00	3	43.20
4	74	4.02	6 (3+3)	780.00	4	66	9.5	64.00	4	124.60
5	76	4.37	7 (4+3)	2,340.00	5	64	7.37	278.00	5	112.30
6	63	4.73	7 (3+4)	879.00	6	63	4.13	74.00	6	67.90
7	69	7.02	7 (4+3)	980.00	7	67	6	67.80	7	85.40
8	58	14	7 (3+4)	789.00	8	51	5.77	276.00	8	56.73
9	64	6.06	6 (3+3)	856.00	9	60	3.7	76.65	9	45.67
10	66	12.12	7 (4+3)	2,280.00	10	57	4.14	89.00	10	87.54
11	71	6.14	8 (5+3)	975.00	11	65	4.55	135.00	11	115.24
12	74	5.37	6 (3+3)	871.00	12	61	4.42	63.50	12	62.56
13	71	12.66	6 (3+3)	1,120.00	13	58	4.02	93.70	13	98.67
14	65	7.15	7 (3+4)	1,482.00	14	60	4.52	75.40		
15	59	6.89	6 (3+3)	2,123.00	15	63	3.89	218.00		
16	56	18	7 (3+4)	2,230.00	16	61	4.2	150.00		
17	68	6.5	6 (3+3)	1,167.00	17	67	4.88	69.70		
18	70	7.4	6 (3+3)	1,756.00	18	67	3	76.50		
					19	55	4.25	115.30		
					20	65	7.12	134.20		
					21	68	5.75	87.90		
					22	70	4.2	232.80		
					23	64	3.4	89.70		
					24	68	5.6	145.20		
					25	68	6.3	189.40		
Mean	67	8.02		1,407.83		62.48	5.10	128.13		78.09
SD	5.6	3.07		585.11		4.29	1.28	68.00		27.73

So far, no research has been dedicated to cfDNA assessment in seminal plasma of PCa and BPH patients. Seminal fluid, which also originates from prostatic secretions, can be a privileged source of biomarkers for PCa diagnosis. cfDNA has been detected in human semen at higher concentrations compared to other biological fluids (3, 4, 18). Seminal cfDNA is a potentially strong biomarker with several oncologic applications for PCa risk stratification, diagnosis and therapeutic monitoring. Recently, we reported that the fluorometric quantification and the electrophoretic characterization of cfDNA in seminal plasma allow the discrimination between PCa patients and age-matched healthy individuals, demonstrating significantly higher concentrations of cfDNA in the former group, with a distinct electrophoretic pattern (4). In these studies, we demonstrated that the fluorometric approach is an easy and reliable method to quantify cfDNA concentration in PCa patients. Furthermore, the electrophoresis technique is an efficient and cost-effective method, which is particularly suitable for the quantification of DNA size distribution patterns and integrity (13). The aim of this study was to evaluate fluorimetric quantification of seminal cfDNA as a biomarker for discrimination of PCa from BPH patients and healthy individuals.

Materials and Methods

Ethics statement. The study was approved by the ethics committee of the University of Modena and Reggio Emilia and informed consent was obtained from each recruited subject.

This study was conducted according to the principles of the Helsinki Declaration of 1975, amended in 1996 (http://www.wma.net/e/humanrights/policy_meetings.htm).

Sample collection. The Urology Department of the University of Modena and Reggio Emilia enrolled patients with histopathologically proven prostate cancer, and BPH. Healthy individuals were enrolled as a control group. Patient eligibility criteria included age between 50 and 75 years, seminal fluid samples collected at least one month after prostate biopsy, availability of clinical data and PSA levels. Healthy individual's eligibility criteria included the absence of prostate pathology.

Patient seminal samples were collected prior to radical prostatectomy and/or chemotherapy or radiotherapy.

Extraction of cfDNA. As the samples ranged from 0.65 to 3.0 ml, a standardized sample volume of 0.65 ml was analyzed for each sample collected. Samples processed within 2 h from collection were centrifuged at room temperature in 2 steps: 400 rcf for 10 min and 16,000 rcf, for 5 min (18). Seminal plasma samples were then stored at -80°C awaiting cfDNA extraction.

cfDNA was then extracted with the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Aliquots of 120 µl cfDNA were cryopreserved at -20°C until quantification.

Quantification of cfDNA. cfDNA concentration levels were measured using the Qubit ssDNA Assay Kit according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA).

Statistical analysis. This article presents the statistical analysis of concentrations of cfDNA in seminal plasma in each group (PCa, BPH and healthy individuals).

MedCalc Statistical Software version 14.8.1 (MedCalc Software bvba, Ostend, Belgium; <http://www.medcalc.org>; 2014) was used to perform statistical analysis. The results are presented as the mean±standard deviation (SD) for each group. The mean effect of these groups was determined using analysis of variance (ANOVA). Box-and-whisker plot was plotted to compare the results.

Prior to the ANOVA test, Levene's Test for Equality of Variances was performed. If the Levene test was positive ($p < 0.05$), the variances in the different groups were different (the groups are not homogeneous) and logarithmic transformation for the data was applied. The program performs the calculations on the logarithms of the dependent variable, but the different means are back-transformed and reported as the geometric means.

ANOVA test, F statistic and associated p -value were calculated. If p -value was less than 0.05, then the hypothesis is accepted that there is an influence of the qualitative factor on the dependent data, or that the means of at least two of the subgroups differ significantly.

If the ANOVA test was positive a post hoc test was performed (using Tukey-Kramer's method) for pairwise comparison of subgroups. A p -value < 0.05 was considered significant.

Results

Patient population. Patients enrolled included 18 PCa patients, 25 BPH patients and 13 healthy individuals. The median age of the PCa patient population was 67 years (range=58-76 years), of the BPH patient population was 62.48 years (range=51-70 years) and of the healthy individuals was 66 years (range=59-74 years). Other clinical and pathological data are outlined in Table I.

Quantification of cfDNA. Median seminal cfDNA levels in PCa patients were higher than in BPH patients and healthy individuals ($p < 0.001$). Mean cfDNA level for PCa patients was 1,407.83 ng/µl (range=780-2,340 ng/µl), for BPH patients was 128.13 ng/µl (range=63.5-278 ng/µl) and for healthy individuals was 78.09 ng/µl (range=43.2-124.6 ng/µl), Table I. Seminal cfDNA concentrations were similar in BPH patients and healthy volunteers.

Comparison among groups. In this study, a significant difference was found between the concentrations of cfDNA in seminal plasma in PCa patients *versus* BPH patients

Figure 1. The Box-and-whisker plot displays a graphical statistical summary of a variable. In the basic Box-and-whisker plot, the central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. The horizontal line extends from the minimum to the maximum value.

(1,407.8 vs. 128.1, ANOVA $p < 0.001$) and PCa patients *versus* healthy individuals (1,407.8 vs. 78.0, ANOVA $p < 0.001$). Box-and-whisker plot representing scfDNA values among groups are represented in Figure 1.

Discussion

In the present study scfDNA levels were evaluated as a potential clinical biomarker in early PCa diagnosis, using the total scfDNA amount in order to discriminate benign from malignant-prostate disease.

cfDNA can be found in biological fluids, such as plasma (2) urine (3), cerebral spinal fluid (4), pleural fluid (5), and saliva (6). ctDNA/cfDNA levels in blood samples seems to be dependent on tumour type. ctDNA was detected in over 75% of patients with advanced pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular, and head and neck cancers. However, levels below 50% were found in primary brain tumours, kidney, prostate, and thyroid cancers (7). These findings may be due to the influence of other factors, such as inflammatory or autoimmune processes that increase non-tumour cfDNA fraction, thereby diluting the ctDNA concentration.

The identification, isolation and quantification of ctDNA from non-blood biological fluids, allows prompt diagnosis and a reliable genetic characterization of the cancer molecular signature. Thus, potential identification of ctDNA in non-blood fluids is of interest especially for cancers with low levels of ctDNA in blood samples. Some tumours are often in direct contact with specific non-blood bodily fluids, such as urine, saliva, cerebrospinal fluid, pleural effusion or

seminal liquid, and release into these fluids a significant amount of ctDNA. These fluids therefore constitute a privileged source of ctDNA.

Concerning the relationship among cfDNA and ctDNA, a percentage of cfDNA in PCa patients was derived from non-cancerous cells because of the induction of apoptosis by proapoptotic cytokines released from prostate cancer cells (8). Therefore, the quantification of total cfDNA level may be a more useful marker for cancer detection (8, 9). Today, it is possible to assess the precise amount of ctDNA through the identification of specific genetic mutations, present in cancer cells' nucleic acids. However, the precise differentiation between the amount of cfDNA derived from non-cancerous cells and the amount of ctDNA requires laboratory techniques such as qPCR or next generation sequencing (NGS) which are expensive and time consuming. These techniques enable the identification of DNA mutations and/or methylation that characterize different cancers (19, 20). However, the fluorimetric technique allows a fast and convenient quantification of the total amount of cfDNA, without distinction between the non-neoplastic and neoplastic fraction. It is plausible that the measurement of the total amount of cfDNA could be a more sensitive biomarker of the neoplastic process because the total amount of cfDNA includes also the fraction derived from apoptosis and necrosis (10-12).

It has been demonstrated that a higher amount of cfDNA is released in the blood and can be isolated from serum and plasma of PCa patients, and that the level of cfDNA in PCa is significantly higher than in patients with BPH (13). Gordian *et al.* proposed a cut-off of 180 ng/ml cfDNA to differentiate PCa from BPH patients with PSA levels less than 10 ng/ml (9) whilst Feng J *et al.* proposed cfDNA plasma levels of 10 ng/ml to differentiate PCa from BPH (13).

Previous studies identified urine cell-free DNA (UCF-DNA) integrity as a potentially good marker for the early diagnosis of non-invasive prostate cancer, even though large cohort studies are lacking (14, 15). Van Nestle L *et al.* proposed a urine risk score based on the mRNA liquid biopsy assay (16). Additionally, a urinary three-gene panel (*HOXC6*, *TDRD1* and *DLX1*) has been proposed as a promising tool to identify patients with aggressive PCa, also among those with low PSA values (17).

However, so far, no research has been dedicated to cfDNA assessment in the seminal plasma of PCa and BPH patients. cfDNA has been detected in human semen at higher concentrations with respect to other biological fluids (11, 12, 18). Recently, we reported that the quantification and the electrophoretic characterization of cfDNA in seminal plasma enables the discrimination between PCa patients and age-matched healthy individuals, demonstrating significantly higher concentrations of scfDNA in the former group, with a distinct electrophoretic pattern (11, 12). In the

present study this analysis was expanded to include BPH patients, revealing a significant difference in the concentration levels of cfDNA in PCa and BPH patient cohorts (Figure 1). Specifically, a possible cut-off level of 450 ng/ μ l seminal cfDNA is suggested to discriminate between the two distinct groups.

Several methods of cfDNA quantification in serum/plasma have been reported, including qPCR, spectrophotometry, radioimmunoassay and fluorometry. Recently, we compared the standard method of qPCR amplification to the fluorometric assessment by Qubit and we showed high levels of correlation among the two methods (10), confirming that the fluorometry and electrophoresis are both fast, cost-effective and reliable procedures to quantify cfDNA and assess its quality (11, 12).

This study has inherent limitations associated with a single institution study. The study has a limited number of participating patients and healthy individuals and requires future studies that will include larger cohorts to confirm the external validity of the study results. Further, seminal fluid cfDNA can only be applied to patients capable of producing a seminal sample.

Conclusion

Several non-blood biological fluids are potential sources for quantification and characterization of cfDNA in the clinical setting. Among these, seminal fluid is a precious source of nucleic acids, characterized by higher values of cfDNA compared to blood. Seminal fluid, which also contains prostatic secretions, can be adopted as a useful source of biomarkers for differential diagnosis between PCa and BPH patients. Seminal fluids of PCa patients were characterized by significantly higher values of cfDNA whilst BPH patients had low seminal cfDNA levels, similar to those observed in healthy volunteers. A cut-off level of 450 ng/ μ l seminal cfDNA was able to discriminate PCa from BPH patients. Seminal cfDNA is a biomarker which can potentially be applied for PCa risk stratification and therapeutic monitoring.

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