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SHORT COMMUNICATION

Seminal Cell-Free DNA Assessment as a Novel Prostate Cancer Biomarker

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Abstract

Cell-free DNA (cfDNA) includes circulating DNA fragments, which can be obtained from different human biological samples. cfDNA originates either from apoptotic and/or necrotic cells or is actively secreted by cancer cells. As yet, a quantification and size distribution assessment of seminal plasma cfDNA from prostate cancer patients has never been assessed. To discover a novel, sensitive, non-invasive biomarker of prostate cancer, through the fluorometric quantification and the electrophoretic analysis of seminal cfDNA in prostate cancer patients compared to healthy individuals. The concentration of seminal plasma cfDNA in prostate cancer patients was 2243.67 ± 1758 ng/μl, compared to 57.7 ± 4.8 ng/μl in healthy individuals ($p < 0.05$). Electrophoresis sites distribution patterns were different; ladder fragmentation was associated with prostate cancer patients and apoptotic electrophoretic fragmentation with healthy individuals. Human seminal fluid can be a valuable source of cfDNA in the setting of liquid biopsy procedures for the identification of novel oncological biomarkers. Seminal plasma cfDNA in prostate cancer patients is significantly more concentrated than that of age-matched, healthy controls. Fluorometric measurement and electrophoretic assessment allow a reliable quantification and characterization of seminal plasma cfDNA, which can be used routinely in prostate cancer screening programs.

Keywords Seminal plasma cfDNA · Spermal cfDNA · Prostate cancer · Urological biomarkers · Fluorimetry · Prostate cancer screening · Liquid biopsy

Introduction

Cell-free DNA (cfDNA) includes circulating DNA fragments in human biological fluids originated from either apoptotic and/or necrotic cells, or actively secreted by cancer cells [1]. In clinical oncology, the plasma genotyping of cfDNA for diagnostic and prognostic biomarkers has rapidly grown given the non-invasive nature of the assessment [2].

cfDNA can be obtained from different biological samples, such as serum, plasma, urine and other biologic fluids, including male semen [3, 4]. The latter is a mixture of prostate and bulbourethral glands secretions produced from the male urethra, and secretions from testes, epididymis and seminal vesicles. The chemical composition of human seminal plasma include cell free nucleic acids, such as DNA, long single stranded RNA, small RNAs-miRNA and piRNA [5, 6]. cfDNA has been detected in human semen at higher concentrations with respect to other biologic liquids [3].

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Seminal cfDNA has been studied in terms of male infertility, as it has been associated with sperm parameters linked to normal spermatocyte function [6] and has been shown to be higher in the seminal plasma of azoospermic compared to normozoospermic patients [4]. However, potential associations between seminal cfDNA concentrations and urogenital malignancies, especially prostate cancer, are yet to be assessed.

In the setting of urologic malignancies, especially for prostate cancer, routine biopsy may not be feasible because of the risks of morbidity and potential low yield. The currently available diagnostic and prognostic biomarkers, such as PSA, are limited by a low specificity and sensitivity, resulting in frequent misdiagnosis [7]. Hence, there is an urgent need for clinically relevant biomarkers for prostate cancer screening, diagnosis and prognosis.

Different methods can be applied to cfDNA extraction and quantification, both in blood samples (plasma and serum) and seminal fluid; the most important techniques being UV spectrophotometry (NanoDrop), fluorochrome binding to single and/or double stranded DNA molecules (Qubit) and qPCR [2]. In our recent studies, we demonstrated that the fluorometric approach is an easy and reliable procedure to quantify cfDNA plasma concentrations in patients with advanced melanoma and prostate cancer [4, 8].

The aim of our study was to assess the quantification and size distribution patterns of seminal plasma cfDNA in prostate cancer patients and healthy individuals, by fluorometry and electrophoresis. The specific aim of this research was to identify a novel, sensitive, non-invasive biomarker for prostate cancer screening, diagnosis and management.

Materials and Methods

Ethics Statement

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

Six prostate cancer patients and three healthy individuals were recruited and enrolled into the study in the Urology Department of the University of Modena and Reggio Emilia. The research protocol was approved by the ethics committee of the University of Modena and Reggio Emilia and informed consent was obtained from each recruited subject.

The inclusion criteria specified an age range between 50 and 75, histological proof of prostate cancer, before any radical prostatectomy and/or chemotherapy or radiotherapy. For each enrolled patient, records of pathological anamnesis, histopathological characteristics of the tumors and biochemical marker levels were collected. A seminal sample was collected from each enrolled patient prior to any radical prostatectomy and/or chemotherapy or radiotherapy.

Extraction of cfDNA

Within an hour from the collection, 1 to 0.2 ml of seminal fluid was processed. Seminal plasma was obtained by two centrifugations: 400 rcf for 10 min and 16,000 rcf, for 5 min, at room temperature [3]. Aliquots of 0.2 ml of seminal plasma were stored at −80 °C.

cfDNA manual extraction was performed using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions in 2.5 h. The cfDNA was diluted in 120 μl Buffer AVE, as specified by the Qiagen protocol and cryopreserved at −20 °C.

Quantification of cfDNA

The Quantification of cfDNA through fluorometric assay was performed using the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, California, US). According to manufacturer instructions, 1 μl of each sample was examined using the Qubit ssDNA Assay Kit (Life Technologies, Carlsbad, California, US) able to detect both single and double strand DNA [4].

Measurement of the Size Distribution of cfDNA

Size distribution of cfDNA was evaluated by DNA electrophoresis (at 80 V for 70 min) using a 1% agarose gel stained with ethidium bromide [9]. For each electrophoresis lane, a total of 200 ng of purified DNA was loaded. The size range of the DNA fragments was evaluated using ready-to-use O'GeneRuler 1Kb ladder (Fermentas Life Science, St. Leon-Rot, Germany). Gels were viewed under UV light on a Gel Doc 1000 system (Bio-Rad, Munich, Germany).

Statistical Analysis

Descriptive statistics, average seminal cfDNA concentrations, Student t-test with Welch's correction was performed with Prism 6.0 (GraphPad software, La Jolla, CA, USA).

Results

Patient Population

Six prostate cancer patients and three healthy individuals were enrolled. The median age of prostate cancer patient population was 66 (range between 58 and 76), mean PSA level was

5.84 mg/dl (range between 4.02 and 8.7 mg/dl) and Gleason Score of 6.5 (range between 6 and 7) (Table 1).

Quantification of cfDNA

The concentrations of seminal plasma cfDNA in prostate cancer patients, (2243.67 ng/μl; SD: ± 1758; range 508–4800) was significantly higher than that of healthy individuals (57.7 ng/μl; SD: ± 4.8, range 52.9–62.5), $p < 0.05$ (Table 1).

Size Distribution of cfDNA

Electrophoresis patterns were different between prostate cancer patients and healthy individuals (Fig. 1). Especially distinct characteristic DNA pattern fragmentations were verified for cfDNA of all prostate cancer patient samples (smears ranged from 250 bp to 10,000 bp) compared to healthy individuals (ladder ranged from 100 bp to 2000 bp), presenting a high molecular weight DNA in prostate cancer patients.

Statistical Analysis

Student t-test with Welch's correction Concentrations of cfDNA in seminal plasma of seminal cfDNA concentrations of healthy individuals and prostate cancer patients were resulted in a statistically different significant difference between two group between healthy individuals and patients ($p < 0.05$).

Discussion

As yet, seminal cfDNA has never been investigated in association with oncological diseases. This is the first time that extraction, fluorometric quantification and electrophoretic analyses have been performed on seminal cfDNA in cancer patients. The main findings from the current study highlight that firstly, human seminal fluid can be a valuable source of cfDNA in the setting of liquid biopsy procedures and is a critical human biologic fluid for the identification of novel oncological biomarkers; secondly, seminal plasma cfDNA from prostate cancer patients is significantly more concentrated than age-matched healthy individuals; thirdly, fluorometric and electrophoretic assessments allow a reliable quantification and qualification of seminal plasma cfDNA, that could be routinely adopted for prostate cancer screening programs.

Traditionally, prostate cancer diagnosis is based both on PSA determination and histology. However, debate has recently pinpointed problems associated with PSA testing for prostate tumor screening and diagnostics. Biopsy collection is often considered a complex procedure, due to the unsuitability of the patient for surgical interventions. Further, biopsy collection in prostate cancer is associated with patient morbidity [7]. In this clinical scenario, cfDNA, through the liquid biopsy approach, could be a more effective, alternative method for cancer detection and monitoring.

The analysis of cfDNA is a promising area of investigation in the detection of disease-specific molecular alterations, through fast and non-invasive procedures. At present, the analysis of blood cfDNA is opening a new clinical and laboratory setting, with several recently identified cancer

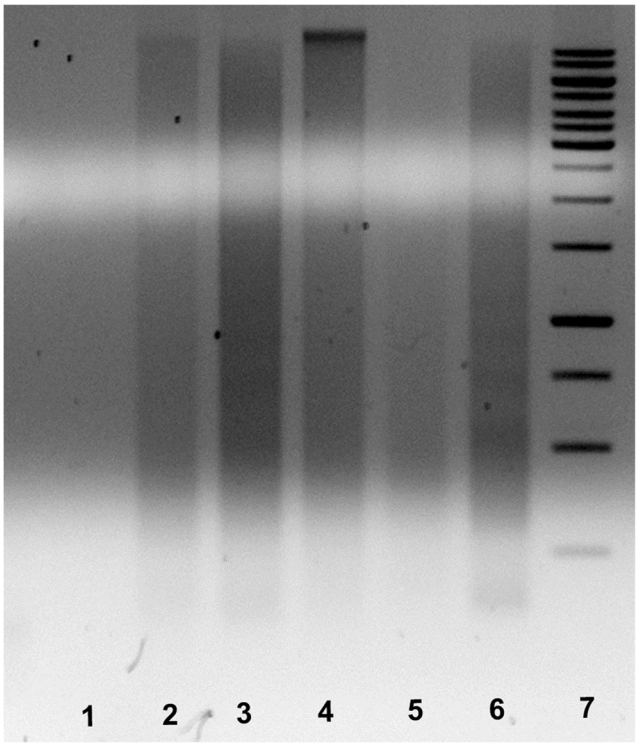

Fig. 1 Size distribution of cfDNA in semen plasma of prostate cancer patients (1–6). Distinct characteristic DNA smear fragmentation was observed in all cancer patients (lane 1–6). Lane 7 was relative to DNA ladder marker. The specific necrotic pattern distribution of cfDNA fragment ranging from 100 bp to 10,000 bp

Table 1 Clinical, pathological and biomolecular characterizations and PSA values of prostate cancer patients (Number 1–6) and healthy individuals (C1, C2, C3). Gleason scores of prostate cancer patients were reported

Patients number	Qubit ssDna (ng/μl)	PSA (ng/ml)	Gleason score
C1	62,5	< 3.0	–
C2	52,9	< 3.0	–
C3	57,7	< 3.0	–
1	3635	8,7	7 (4 + 3)
2	4800	7,05	6 (3 + 3)
3	1100	6,2	7 (3 + 4)
4	508	4,02	6 (3 + 3)
5	2732	4,37	7 (4 + 3)
6	687	4,73	7 (3 + 4)

biomarkers, cardiovascular and neurological diseases management and prenatal diagnosis, without the disadvantages of traditional tissue biopsy [2, 4]. In oncology, neoplastic blood cfDNA has been detected in several tumor types, such as cancers of the oral cavity and pharynx, digestive system, respiratory system, skin, endocrine system, breast and urogenital system, being a promising biomarker of the neoplastic process [2].

Circulating cell-free nucleic acids can be extracted from male semen, obtaining higher concentration respect to other biological fluids [3]. Seminal cfDNA was studied in male infertility, being associated with sperm parameters linked to normal sperm functions such as velocity or morphology [6] In a previous study, azoospermic patients had significantly higher seminal cfDNA values with respect to normospermic controls [6]. The total amount of seminal plasma cfDNA of prostate cancer patients was found to be significantly higher when compared to healthy individuals in the current study and compared to the values assessed in azoospermic patients in literature (2.56 ng/μl) [4]. In our study, seminal cfDNA of prostate cancer patients yielded higher values than those of age-matched healthy volunteers, 2243.67 ng/μl and 57.7 ng/μl, respectively. This allows a reliable characterization and differentiation between the cohort of patients affected by prostate cancer and the age-matched control group. The aforementioned average seminal cfDNA is notably higher than average blood cfDNA concentrations of prostate cancer patients: in our cohort of patients, the average seminal cfDNA concentration was 2.243×10^3 ng/μl, which is approximately 10^2 higher compared to mean blood cfDNA values reported in scientific literature (1.8–35 ng/μl) [6, 7].

However, several technical issues regarding sample collection, processing, and molecular techniques have still to be addressed in order to allow the acquisition of uniform and reliable results. The standardization of the extraction and quantification methods is necessary, in order to allow the application of the technique to the daily clinical and laboratory practice. Technically, after DNA extraction from body fluids, a preliminary quantification of total circulating DNA should be performed before of downstream applications. Different methods can be applied for this preliminary evaluation, among them, the most important ones are based on PCR, UV spectrophotometry (NanoDrop) or fluorochrome reactions with the single and/or double stranded DNA helix (Qubit) [2].

Fluorometric quantification techniques based on single strand fluorometric probes, which detect both single and double strand DNA [4], can be easily applied to seminal plasma, as is the case for blood plasma cfDNA quantification, through a fast, reliable and cost-effective procedure [4, 8]. This approach to quantify seminal plasma cfDNA concentration, may therefore be applied to screening programs, diagnosis of prostate cancer and to therapeutic monitoring. Future studies are needed to address these specific fields of applications of seminal cfDNA biomarker application in different settings: before and after surgical procedures, during therapy and as a prognostic biomarker.

The characterization of seminal cfDNA can be easily carried out through standard agarose gel-electrophoresis. In detail, prostate cancer patients result in a wide range of molecular weight DNA (smears ranging from 250 bp to 10,000 bp) typically released from necrotic cells (Fig. 1), while age-matched healthy controls are characterized by a size-distribution pattern of typical DNA ladder fragmentation (ranging from 100 bp to 2000 bp). Similarly, regarding blood plasma cfDNA, it has been proposed that high molecular weight DNA is released from necrotic, dying cells, while the multiples of 180-bp fragments are associated to the DNA ladder specific of apoptosis [10].

Concerning the modality of electrophoresis procedures, in a previously study on seminal cfDNA Li et al. proposed a step wise approach [3] consisting of three sequential voltage increases during the electrophoretic procedures. In our research a different approach was applied with a voltage of 80 V cm^{-1} for 70 min, in order to achieve a better resolution of cfDNA fragments.

A limitation of the study is the small number of subjects enrolled, but a higher number of prostate cancer patients and healthy individuals will be enrolled in future studies.

In the current study, seminal plasma cfDNA was identified as a novel, sensitive, non-invasive biomarker, which is able to reliably discriminate between patients affected by prostate cancer and healthy individuals, both recruited into this study and in other studies in literature. Future prospective studies to define if seminal cfDNA concentration predicts disease relapse, aggressiveness of the tumor and response to therapy are needed, but our preliminary results are encouraging. This is the first time that seminal cfDNA has been applied in oncology and described as a biomarker of prostate cancer. Given the relatively easy laboratory techniques for seminal cfDNA extraction and quantification and the non-invasive modality of fluids collection, in the very near future, seminal cfDNA may be routinely applied to prostate cancer screening and management, with a promising clinical application.

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