A Multiplex PCR-Based Assay for Detection of Plasma Cell Free DNA Integrity as a Marker of Bladder Cancer

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ABSTRACT
Background: Cell free DNA (cfDNA) analysis in patient plasma has been suggested for screening of different cancers. Healthy cells may shed more degraded DNA than non-apoptotic tumor cells.
Objectives: Our goal was to check whether DNA fragments extracted from patient's plasma with bladder cancer (BC) had higher integrity than DNA purified from plasma of healthy controls.
Methods: cfDNA was extracted from the plasma of a healthy control group and patients with BC. The association between clinical status and length of DNA fragments was examined. Plasma cfDNA integrity was examined using multiplex PCR with specific primers producing progressively long PCR fragments (200, 400 & 800-bp). Length and concentration of PCR amplicons obtained from BC patients and healthy individuals were compared.
Results: Using a multiplex PCR assay, the used *p53* primers efficiently amplified the increasingly long fragments. High molecular weight DNA fragments (400 & 800-bp) in the plasma cfDNA are associated with the presence of cancer. Moreover, elevation of the 200-bp fragment concentration in the plasma cfDNA is significantly (p < 0.002) associated with the presence of cancer.
Conclusions: The detected long DNA fragments in plasma cfDNA is associated with BC indicating that the multiplex PCR assay of plasma cfDNA integrity could be a useful future marker for the detection of BC.
Keywords: Bladder cancer, cell free DNA, Multiplex PCR, DNA integrity.

INTRODUCTION

Required tumor biopsies for diagnosis of cancers usually got by invasive techniques. In addition, the present screening methods sometimes fail to recognize early stages of cancers, leading to late discovery of cancers when clinical symptoms begin showing. Thus, a noninvasive screening tool is needed to detect cancer in early stages. At the same time, the screening test should be safe, inexpensive, precise and easy to use in a large population to detect the disease [1, 2]. In the last GLOBOCAN report 2018 [3], with an estimated 549,000 new cases and 200,000 deaths, bladder cancer is the 10th most prevalent form of cancer globally. Bladder cancer is 4 times less common in women than in men, with respective incidence and mortality rates of 9.6 and 3.2 per 100,000 men worldwide [3]. By the year 2050, Egyptian population is expected to grow to nearly 160% the 2013 population size taking the incidence of BC cases up to 290%. Cystoscopy, an invasive method, is currently the main tool to diagnose and follow-up bladder cancer. However, its result could be negative and /or misleading in flat malignancies such as carcinoma in situ. Thus, developing a noninvasive, sensitive and low-cost screening tool to detect BC is urgently required. Histological or imaging examination have disadvantages compared to the preferred and minimally invasive blood-based biomarkers. Moreover, numerous samples can be collected during

a specific period [4]. Currently, plasma cfDNA has drawn research interest. Various molecular features of cancer-associated DNA including epigenetic changes, aberrations of copy number, mutations of single-nucleotide and rearrangement of chromosomes have been studied extensively [5]. Likewise, cfDNA integrity has been utilized as a prognostic and diagnostic biomarker in different cancers [6].

As a biomarker, cfDNA integrity has many advantages. For instance, cfDNA can be easily obtained (serum, plasma or other body fluids). The established and fast PCR methods can be used, which is cost efficient and requires a small quantities of blood [7]. cfDNA can mirror the tumor real-time status as its half-life in blood circulation is short [8, 9]. Although cfDNA integrity is an attractive marker for patients with different cancers [10], few studies have focused on studying its clinical importance for BC patients. In our study, the cfDNA integrity of locally BC patients was figured as a marker for detection of BC. Interestingly, we observed the DNA fragments with high molecular weight specifically in plasma cfDNA of BC patients suggesting that cfDNA integrity could be a possible marker for detection of BC.

MATERIALS AND METHODS

Patients (35) with BC attending the Urology Clinic, Faculty of Medicine, Assiut University,

Assiut, Egypt, during the period from May 2016 to October 2017 were involved in this study. A total of 15 healthy controls were involved. None of the controls had a previous history of cancer or any disease that could be associated with the presence of *p53* mutagenesis. All participants submitted a written informed approval before being enrolled in the study after explaining all procedures.

Sample collection

10 ml samples of peripheral blood were obtained from participants using EDTA tubes. Plasma was separated by double centrifugation at 2000 rpm for 15 min. Supernatant plasma were collected into sterile 1.5 ml Eppendorf tubes and centrifuged again at 14000 rpm for 15 min. All centrifugation steps were carried out at 4 °C. Sample aliquots were immediately kept at -80 °C until extraction was performed.

DNA extraction

Plasma cfDNA extraction: 0.3 ml of plasma was used for cfDNA isolation. Extraction was carried out using Sigma-Aldrich commercial kit (GenElute™ Mammalian Genomic DNA Miniprep Kit Protocol, Sigma-Aldrich, USA) according to the manufacturer's instructions, with minor modifications to accommodate the sample volume.

Genomic DNA extraction: 0.2 ml of whole blood was used for gDNA isolation. Extraction was carried out using Qiagen commercial kit (QIAamp DNA Mini Kit, Qiagen, Germany) following the manufacturer's instructions**.**

PCR amplification

cfDNA integrity was examined by multiplex PCR. 200, 400, and 800-bp for *p53* gene were amplified (Fig. 1A). Different sized fragments were amplified using a forward primer; F-5'CACCTCCACCACCTCCTCAA3', and three reverse primers; Rev1-5'GTATCAGCATCTGGAAGAA3', Rev2-5'CATCATCATCTGAATCATCT3' and Rev3-5'TCACCTGACTGTGCTCCTCC3' producing 200, 400 and 800-bp respectively [11]. PCR was carried out in a 50 µl total volume; 25 ul of iNtRON's Maxime 2X PCR master mix solution (i-Taq™) kit containing 2.5 U of i-Taq™ polymerase (5U/µl), PCR reaction buffer 1X, gel loading buffer 1X, dNTPs 2.5 mM each, 1.0 µl of each primer, 5.0 µl of DNA template and the volume was completed with deionized water. Thermal cycling was as following: one denaturation step of 5 min at 94 °C, followed by 41 cycles of 94 °C for 45 s, 59 °C for 60 s, 72 °C for 60 s and a final extension at 72°C for 7 min. PCR products were visualized by UV after being separated by gel 1.5% agarose gel electrophoresis and stained by ethidium bromide. The gel was finally photographed and analyzed by Gel-Pro Analyzer software 3.1.

Semi-quantitative analysis of fragment concentration

Image J software was used for the determination of DNA concentration in each gel band by comparing band intensity to one sharp band of ladder marker (Cat. # DM001, enzynomics, South Korea). DNA concentration in each band of the ladder marker was predetermined by the manufacturer.

Statistical analysis

SPSS (Statistical Package for the Social Sciences) software package, version 22 (IBM Corporation, Armonk, NY, USA) was used to perform the statistical analysis. Mean values were compared by Mann–Whitney U-test. A *p*-value was considered statistically significant if less than 0.05.

Ethical approval

A signed written informed consent of all the subjects was obtained, explaining that the study is a research and declared the details of the procedure and the anticipated benefits and complications.

RESULTS

Patient characteristics

Thirty-five patients with BC (6 females and 29 males) were included in the current study. Patients were aged between 46 – 71 years, with an average of 56 years. Patients were grouped according to WHO TNM to 25.7% Ta, 22.9% T1 and 51.4% T2 stages with 31.4% low and 68.6% high grades based on histopathologic results (Table 1).

Table (1): Pathological patient profile

Patient characteristics		BC patient	Control
		N (%)	*N* (%)
No. of patients		35	15
Age (y), mean ± SD		56 ± 6.2	51 ± 5.5
Tumor pathology			
Stage	Ta	9 (25.7)	N/A
	T1	8 (22.9)	
	T2	18 (51.4)	N/A
Grade	High	24 (68.6)	
	Low	11 (31.4)	

## *p53* primers efficiently amplify the targeted fragments

We checked the set of the four *p53* primers separately to evaluate their efficiency to amplify three targeted fragments (Fig. 1A) using gDNA as a template. The set of the four used primers successfully amplified the target fragments at expected molecular weights (Fig. 1B). We further checked the primers in a multiplex

PCR. We also observed differential amplification of 200, 400 and 800-bp (Fig. 1C).
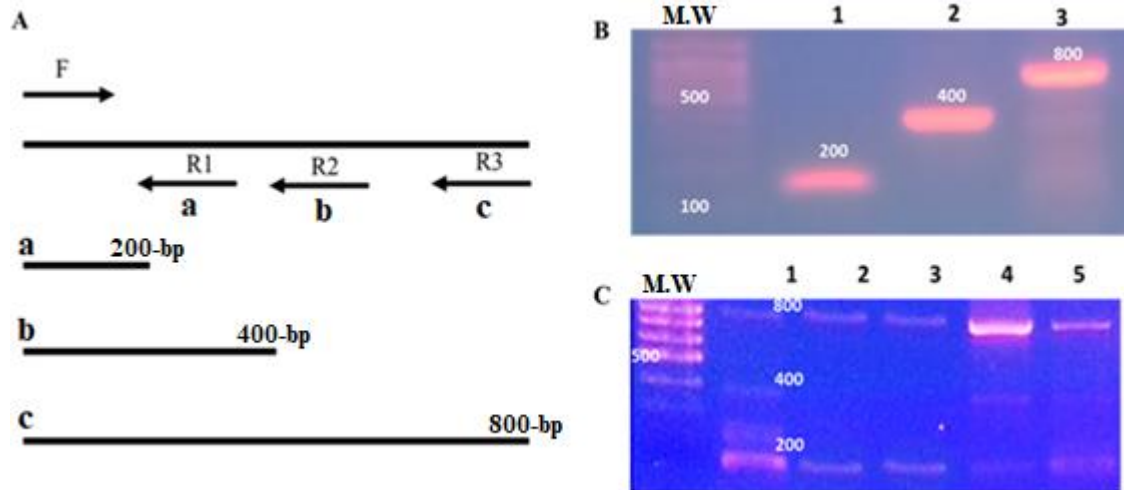
Fig. (1): DNA integrity analysis

A. PCR amplification scheme for DNA integrity analysis; One forward primer and three reverse primers (a-c) were used to amplify three different *p53* gene fragments (200, 400 and 800-bp). **B. Conventional PCR analysis**. The primers were tested separately to produce target fragments; 200, 400, 800-bp. **C. Multiplex PCR analysis**. The primers were tested in one PCR tube reaction to produce target fragments: 200, 400, 800-bp. M.W., molecular weight marker.
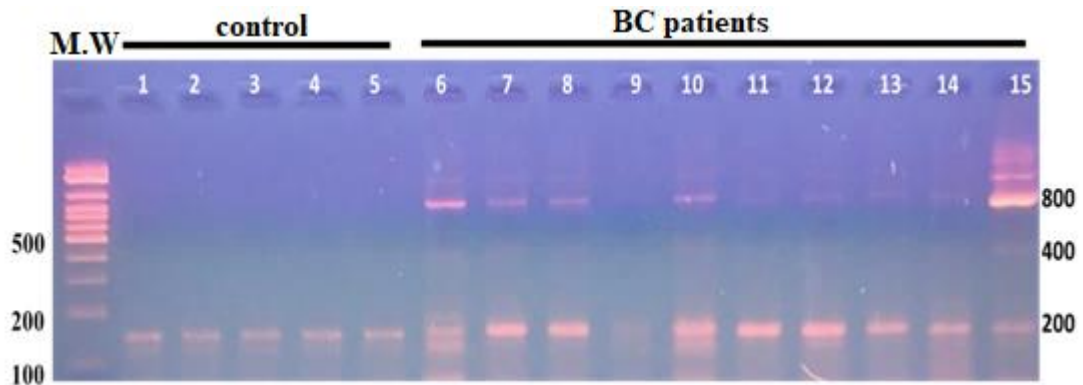
Fig. (2): Plasma cfDNA integrity assay

Multiple reverse primers and one forward primer were used to detect 800-bp, 400-bp, and 200-bp fragments of the *p53* gene in these samples. Examples of plasma cfDNA profiles are shown from normal controls (*lanes 1–5)* and from BC patients (*lanes 6–15).*

Long DNA fragments in the plasma cfDNA are associated with the presence of cancer.
We targeted amplifying 200, 400 and 800-bp fragments within the *p53* gene sequence using purified plasma cfDNA from cancer and healthy subjects (Fig. 2). The 200-bp fragments were present in all checked samples (100%). The 400-bp fragments were observed in 31 of 35 (88.6%) of patients and observed only in 5 of 15 (33.3%) of control. The 800-bp fragment was detected in all cancer cases 35 of 35 (100%) while only detected in 1 of 15 (6.7%) of control subjects (Table 2).

To go further, the frequency of 400 and 800-bp fragments in different stages or grades of BC patients was calculated. Results showed that neither the frequency of 400 nor 800-bp fragments showed a significant difference among different stages or grades in BC patients (Table 2).

Table (2): Frequency of DNA fragments in plasma cfDNA of bladder cancer patients and normal controls

Patient characteristics		DNA Fragment *N* (%)		
		200-bp	400-bp	800-bp
Control *N* = 15		15 (100)	4 (26.7)	0 (0)
Patient *N*= 35		35 (100)	31(88.6)	35 (100)
Stage Grade	Ta *N* =9	9 (100)	9 (100)	9 (100)
	T1 *N* =8	8 (100)	6 (75)	8 (100)
	T2 *N* =18	18 (100)	16 (88.9)	18 (100)
	High *N*=24	24 (100)	21 (87.5)	24 (100)
	Low *N* =11	11 (100)	10 (90.9)	11 (100)

Elevation of DNA concentration in the 200-bp fragments in the plasma cfDNA is also associated with the presence of cancer

Although differential amplification of 400 and 800-bp was observed, both of cancer and healthy samples contained the 200-bp fragment. The relative concentration of 200-bp fragment in the PCR product of healthy and BC cfDNA was compared. Results indicated that samples from patients tended to have higher DNA concentrations compared to healthy samples with a statistically significant difference between groups ($p < 0.002$) as shown in Figure (3).
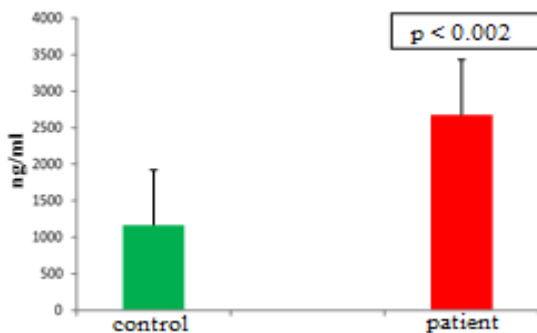
Fig. (3): Comparison of DNA concentration in of 200-bp fragment in bladder cancer patient and control group. DNA concentration in each DNA fragment was estimated using Image J software. DNA concentration in bladder cancer patients were found to be significantly higher than that in the control groups.

DISCUSSION

Bladder cancer is of the most common tumors of genitourinary [12–15] mainly due to *p53* gene mutations [16]. It is well known that mutated *p53* gene tumors are characterized by high rates of recurrence and may develop to invasive stages, which are associated with poor prognosis. BC patients would undoubtedly benefit from early diagnosis and nowadays, cystoscopy still the most sensitive approach for detecting BC, despite of its invasiveness, painfulness, requiring anesthesia or sedation. BC can also be detected using voided urinary cytology, but it has high variability and low sensitivity. For such limitations, a limited number of the existing methods have a satisfactory implementation for clinical use [17,18]. Therefore, novel molecular markers are still urgently needed to help in early diagnosis of BC. Within the BC arena, cfDNA isolated from serum, plasma, and urine have been used searching for possible changes associated with BC. Such kind of changes include the detection of epigenetic and mutational changes focused on specific gene targets and cfDNA quantification [19–25]. Fortunately, a simple and inexpensive assay for cancer detection has been confirmed, indicating high levels of cfDNA in patients with cancer compared to controls. We found that the 400-bp fragment was detected in 88.6% and 800-bp fragments were present in all BC patients as a large fragment. It was hypothesized that apoptotic cells could release different short fragments of cfDNA in healthy controls, however, in cancer patients, malignant cells undergoing autophagy and necrosis are responsible for releasing long cfDNA fragments [26]. In the case of apoptosis, DNA can normally be cleaved into 180- to 210-bp fragments [27]. One possible explanation for the existence of long DNA fragments during the examination of plasma cfDNA from cancer patients is that somehow a group of cells could escape apoptosis-induced DNA pathway of degradation. The necrotic tumor cells that have avoided apoptosis may represent a lot of the cells discarded into the blood circulation. Another possible explanation is that longer DNA fragments may be due to a kind of nuclease protection. Accordingly, DNA of necrotic cells may not be available to nuclease-mediated cleavage because of the undegradable nature of its chromatin structure. Thus, it is most likely that the detection of DNA fragments with high molecular weight clearly associated with cancer. In our study, we found that the 200-bp and 800-bp fragment were present in all BC patients as a large fragment resulting from necrotic effect due to tumorigenesis and present only in one of fifteen controls, so our finding deals with cfDNA integrity is a promising diagnostic tool for BC detection. The 200-bp fragment was detected in all subjects of the study with a remarkable increase in the intensity of the band correlated with the hypothesis suggested that necrosis increases the level of short fragments[28]. The other possibility is that the patient plasma contains a high amount of cfDNA than control plasma which would lead to a higher amount of DNA as a template in PCR reaction.

CONCLUSION

cfDNA integrity succeeded as a discriminative tool between BC and healthy subjects but it did not show any difference between BC patients in different stages or grades. Hence, we can claim that cfDNA

integrity could represent a possible diagnostic marker for BC detection. However, a larger scale study is needed to define precisely the required criteria that facilitate the application of cfDNA integrity as a future screening test for BC.

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