

ORIGINAL ARTICLE

Circulating cell-free DNA integrity as a diagnostic and prognostic marker for breast and prostate cancers

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Abstract

Background: Cancer incidence and its related mortality is rising and is currently the second leading cause of death globally. In Africa, breast and prostate cancer in females and males, respectively, are the worst globally. However, biomarkers for their early detection and prognosis are not well developed. This study sought to investigate circulating cell-free DNA (ccfDNA) integrity and its potential utility as diagnostic and/or prognostic biomarker. Circulating cell-free DNA (ccfDNA) is degraded DNA fragments released into the blood plasma. In healthy individuals, the source of ccfDNA is solely apoptosis, producing evenly sized shorter DNA fragments. In cancer patients, however, necrosis produces uneven longer cell-free DNA fragments in addition to the shorter fragments originating from apoptosis. DNA integrity, expressed as the ratio of longer fragments to total DNA, may be clinically useful for the detection of breast and prostate cancer progression.

Methods: Sixty-four (64) females, consisting of 32 breast cancer patients and 32 controls, and 61 males (31 prostate cancer patients and 30 controls) were included in the study. Each participant donated 5 ml peripheral blood from which sera were separated. Real-time qPCR was performed on the sera to quantify ALU 115 and 247 levels, and DNA integrity (ALU247/ALU115) determined.

Results & Conclusion: ALU species 115 and 247 levels in serum were elevated in breast and prostate cancer patients compared to their counterpart healthy controls. DNA integrity was higher in prostate cancer patients than in the control, but in breast cancer patients was lower compared to

Received November 27, 2018; received in revised form April 15, 2019; accepted April 21, 2019

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their controls. In prostate but not in breast cancers, DNA integrity increased with disease severity and higher staging.

Keywords Circulating cell-free DNA, DNA integrity, Breast cancer, Prostate cancer.

Introduction

The incidence of cancer and its related mortality is on the rise and has become a major public health concern. Currently, it is the second leading cause of death globally and was responsible for about 8.82 million deaths in, 2015 [1]. The international Agency for Research on Cancers of the WHO in 2012 projected that by the year 2030, there would be approximately 21.7 million new cancer cases and 13 million cancer deaths globally due to population growth and increased life expectancy [2]. Cancer incidence and mortality over the years have also seen a steady rise on the African continent with breast and prostate cancers among the leading causes of cancer deaths in females and males, respectively [1,3]. Breast cancer is the world's commonest cancer in women, and similarly, the foremost cancer in women in Ghana with high mortality rate [3,4]. WHO reported that about 2000 Ghanaian women were diagnosed with the disease in 2012 out of which 1000 (50%) died. Breast cancer has been identified as the second leading cause of cancer deaths in Ghana, with about 2900 cases being diagnosed annually and at least one of eight women with the disease dying [3]. Prostate cancer is also, a leading cause of cancer-related death in men globally and particularly in Africa [5,6]. In 2012, there were 1094,916 incident cases of prostate cancer with 307,481 deaths worldwide [7]. In sub-Saharan Africa, the prevalence of prostate cancer is gradually becoming a health burden particularly among men 70 years old and above [8]. In Ghana, even though it remains the commonest cancer in men with high mortality, there is low awareness of the disease resulting in poor attitudes towards it with concomitant grave outcomes [9].

In light of these alarming trends, more pragmatic effort must be put in place to contain and manage cancers. A major challenge in Africa with cancer management, is late detection, late report to hospitals and sometimes poorly resourced health facilities to manage the disease. Effective management of cancers results in higher survival rates in patients especially with early detection [10] necessitating vigorous research into new and effective ways of detecting the disease. These new ways of detection and/or outcome prediction should be relatively less expensive, easy to carry out, reliable and universally acceptable. Furthermore, it provides an opportunity for non-invasive sampling of tumour/tissue DNA.

Circulating cell-free DNA (ccfDNA) are degraded DNA fragments released into the blood stream [11–13]. Chronicling its discovery, Iqbal et al. [14] states that, circulating cell-free DNA (cfDNA) in human plasma was rediscovered in 1966 by Tan et al. [15] in autoimmune disorders and later by Leon et al. [16] in 1977 in cancers after its initial discovery in 1948 by Mandel and Metais [17]. The source of ccfDNA in healthy individuals is solely by apoptosis, producing evenly sized shorter DNA fragments. In cancer patients however, necrosis produces uneven longer DNA fragments in addition to the shorter fragments from apoptosis. [12,18,19]. Therefore, elevated levels of longer fragments of DNA in the blood stream has been targeted as a good marker for the presence of malignant tumour DNA [18–20]. The levels of ccfDNA in blood serum are

higher in cancer patients compared with healthy individuals [21–24]. Consequently, DNA integrity, the ratio of longer to shorter fragments has been explored for its usefulness in diagnosis and prognosis of cancers. It has been suggested to be increased in cancer patients and particularly in metastatic cases than in non-metastatic cases. It has been found to predict tumour progression, and regional lymph node metastases in primary breast cancer patients [19,24,25].

The use of DNA, within the cell or in circulation, as a biomarker in clinical medicine for early diagnosis, prognosis and monitoring of therapy has been a significant advancement in the biomedical field [12,13,26, 27]. Circulating DNA as a biomarker will be easily accessible and cost effective, overcoming infrastructural limitations that face many developing countries. In this study, ccfDNA levels and DNA integrity were assessed in blood sera of breast and prostate cancer patients and compared against that of apparently healthy individuals.

Methods

Participants and study sites

The study involved females and males who had been clinically diagnosed with breast cancer and prostate cancer, respectively, and also healthy individuals who consented to be part of the study. Breast cancer patients were recruited from the Department of Surgery of the Korle-Bu Teaching Hospital (KBTH), Accra, Ghana. It is the largest department of the hospital with a bed capacity of 612. Annually, it receives an average of 15,100 cases at Surgical Specialist clinic; 10,600 cases at the Genito-Urinary clinic; and 4600 cases at the Neurology clinic. Prostate cancer patients were recruited from the MDS-Lancet laboratory, East Legon, Accra, Ghana. MDS-Lancet is a leading medical laboratory in Ghana and receives prostate cancer patients from major hospitals in Ghana for diagnosis. Normal controls were apparently healthy individuals attending the respective health facilities who had gone through routine medical screening and had no visible form of illness or any clinical signs of any form of cancer.

Participant selection

By convenience sampling, we included all breast and prostate cancer patients attending the respective health facilities between February and May of 2017 who consented to be part of the study. It included newly diagnosed breast and prostate cancer patients as well as those on treatment who were 18 years and above. Newly diagnosed patients were participants who had been diagnosed for the first time of either breast or prostate cancer based on relevant medical and laboratory examination and were yet to be placed on any form of medical treatment. Patients on treatment included those whose condition had been diagnosed before the study begun and

were already receiving medical treatment of any form including chemotherapy and radiotherapy. Cancer patients enrolled into the study were those who had been referred to the health facilities by qualified physicians for various forms of laboratory investigations. Patients with multiple cancers were excluded from the study. A written informed consent form was obtained from all participants. Demographic and relevant clinic-pathological data of all the participants were also taken.

Blood samples collection and serum preparation

Samples (5 ml) of venous blood were taken from the median cubital vein of each study participant into labelled serum separator tubes. Fifteen (15) minutes thereafter, each sample was centrifuged at $1000 \times g$ for 15 min to obtain the serum. The serum was removed and aliquoted for storage at -20°C until use. Samples were also obtained from gender and age-matched healthy volunteers as controls.

Sample preparation for quantitative PCR (qPCR)

Frozen serum samples were thawed at room temperature on ice. The sample preparation for qPCR was done according to the method described by Iqbal et al. [14]. A preparation buffer containing 2.5% Tween-20, 50 mmol/L Tris, and 1 mmol/L EDTA was constituted. To each 20 μL of serum sample, 20 μL of the preparation buffer was added to deactivate proteins that bound to template DNA or DNA polymerase which could invalidate qPCR results. To the mixture, 20 μg of Proteinase K (Inqaba Biotec, South Africa) was subsequently added for protein digestion at 56°C for 50 min followed by heat inactivation at 95°C for 5 min. The mixture was centrifuged at $1000 \times g$ for 5 min and 0.2 μL of the supernatant was used as template for each qPCR reaction.

qPCR conditions and quantification of ALU fragments

The reaction mixture for each direct qPCR consisted of a template sequence, 0.2 $\mu\text{mol/L}$ of the forward and reverse primers (Inqaba Biotec, South Africa) for both ALU115 and ALU247, one unit of iTaq DNA polymerase, 0.02 μL of fluorescein calibration dye and $1 \times$ concentration of SYBR Green in a total reaction volume of 20 μL with 5 mmol/L Mg^{2+} . Real-time PCR amplification was performed with pre-cycling heat activation of DNA polymerase at 95°C for 10 min, followed 35 cycles of denaturation at 95°C for 30 s, annealing at 64°C for 30 s and extension at 72°C for 30 s using QuantStudio5 Real-time PCR. The absolute equivalent amount of DNA in each sample was determined by a standard curve with serial dilutions (10 ng – 0.01 pg) of gently prepared genomic DNA obtained from peripheral blood leukocytes of healthy donor volunteers. The purpose of using DNA from peripheral blood leukocytes of a healthy donor was to serve as external standard. A negative control (sample without targetable DNA) was performed in each plate.

To achieve the highest sensitivity for DNA quantification, a qPCR assay that utilizes primer sets was applied to amplify the consensus ALU sequences (ALU115 and ALU247). Two

sets of ALU primers were designed: the primer set for the 115bp amplicon (ALU115) amplified both shorter (truncated by apoptosis) and longer DNA fragments, whereas the primer set for the 247bp amplicon (ALU247) amplified only longer DNA fragments. The sequences of ALU115 primers were forward: 5'-CCTGAGGTCAGGAGTTCGAG-3' and reverse: 5'-CCCGAGTAGCTGGGATTACA-3'; ALU247 primers were forward: 5'-GTGGCTCACGCCTGTAATC-3' and reverse: 5'-CAGGCTGGAGTGCAGTGG-3'. β -actin was used as normalizer for all qPCR assays. The sequences of β -actin primers used were forward: 5'-GACCTCTATGCCAACACAGT-3' and reverse: 5'-AGTACTTGCGCTCAGGAGGA-3'.

DNA integrity determination

DNA integrity was calculated as the ratio of ALU247-qPCR to ALU115-qPCR. ALU115-qPCR values represented the total amount of free serum DNA. ALU247-qPCR values represented the total amount of DNA released from non-apoptotic cells. Since the annealing sites of ALU115 are within the ALU247 annealing sites, the qPCR ratio (DNA integrity) is 1.0 when the template DNA is not truncated and 0.0 when all template DNA is completely truncated into fragments smaller than 247 bp [14].

Data analysis

Results were analyzed statistically using Statistical Package for Social Sciences version 20 statistical software for windows. Data was expressed as mean and standard deviation. Differences in the study parameters between groups were assessed using student *t*-test. A *p*-value ≤ 0.05 was considered as significant. The mean ALU values of two replicates each of two independent tests were computed and reported as Mean $\pm$ SD to two decimal places.

Ethical approval

Approval for the conduct of the study was given by the Ethical and Protocol Review Committee of the School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana (SBAHS: MD./10550649/AA/5A/2016–2017) and the Institutional Review Board (IRB) of the Korle-Bu Teaching Hospital (STC/IRB/000100/2016).

Results

Demographic characteristics of study participants

A total of 64 females, 32 breast cancer patients and 32 controls were recruited into the breast cancer group of the study. The breast cancer patients (cases) were between the ages of 36 and 70 years and the healthy individuals (controls) were between the ages of 44 and 62 years. The mean ages of the cases and the controls, respectively, were 50.6 ± 10.2 years and 53.2 ± 6.2 years. Majority of the cases were 41–50 years (51.0%) and for the controls were 51–60 years (50.0%). For the prostate cancer component, 61 males were

Table 1 Demographic and clinical parameter of participants.

Parameter	Breast cancer		Prostate cancer	
	Cases	Controls	Cases	Controls
Age (years)				
Min	36	44	63	43
Max	70	66	83	67
Mean	50.6 ± 10.2	53.2 ± 6.2	71.4 ± 5.8	55.8 ± 6.8
Occupation				
Government/Private workers	7	11	10	18
Petty traders	10	5	2	–
Unemployed/Retirees	15	4	11	8
Fashion industry/ Artisanship	–	10	5	4
Unskilled labour	–	2	3	1
Breast cancer grade				
Grade I	1		17	
Grade II	25		11	
Grade III	4		3	
Grade IV	–		–	
Grade X	2		–	
Diagnosis				
Invasive ductal carcinoma	30		–	
Metastatic carcinoma	2		–	

Table 2 Mean ALU levels and DNA Integrity among cancer cases and controls.

	Breast cancer (ng/ml)		Prostate cancer (ng/ml)	
	Cases	Control	Cases	Control
ALU 115	0.06 ± 0.04	0.01 ± 0.008	0.04 ± 0.03	0.03 ± 0.02
ALU 247	0.08 ± 0.06	0.02 ± 0.01	0.04 ± 0.02	0.02 ± 0.01
* DNA Integrity	1.32	2.20	1.00	0.67

* DNA Integrity was calculated as ratio of mean ALU 247 to mean ALU 115 and rounded off to two decimal places.

involved: 31 prostate cancer patients and 30 controls. The mean age of prostate cancer patients was 71.4 ± 5.8 years with a maximum age of 83 years and a minimum age of 63 years whilst the mean age for healthy controls was 55.8 ± 6.8 years with a maximum age of 67 years and a minimum age of 43 years.

Clinical characteristics of study patients

As indicated in Table 1, 30 (95%) out of the 32 breast cancer patients had invasive ductal carcinoma, whereas 2 (5%) had metastatic carcinoma. Furthermore, 25 (78%) out of the 32 breast cancer patients were cancer grade II while 4 (13%) and 1 (3%) were cancer grade III and grade I, respectively. There was no grade IV breast cancer but 2 (6%) grade X. Out of the 31 prostate cancer cases, 17 (55%), 11 (35%) and 3 (10%) were stage I, stage II and stage III, respectively.

ALU115 levels in cancer patients and controls

For the breast cancer group, the mean ALU 115 levels were 0.06 ± 0.04 ng/ml and 0.01 ± 0.008 ng/ml for cases and controls, respectively (Table 2). The breast cancer patients had a significantly higher ($p=0.005$) ALU 115 level than the controls. For the prostate cancer group also, higher ALU 115

levels were observed in cases than in controls but the difference was not significant. ALU 115 levels were 0.04 ± 0.03 ng/ml and 0.03 ± 0.02 ng/ml for cases and controls, respectively. For the breast cancer stages, there was no clear pattern in the ALU 115 concentration among them. However, of the two stages encountered in this study, ALU 115 concentration was lower in stage II (0.03 ± 0.01 ng/ml) than in stage III (0.06 ± 0.07 ng/ml) with $p=0.026$. Among the prostate cancer patients, ALU 115 levels decreased with increasing stage progression. The mean ALU 115 concentration for stage I, stage II and stage III were 0.06 ± 0.03 ng/ml, 0.02 ± 0.01 ng/ml and 0.01 ± 0.008 ng/ml, respectively (Table 3). Comparing ALU 115 levels in breast cancer participants to those of the prostate cancer participants, the former was higher than that of the latter.

ALU247 levels in cancer patients and controls

In the breast cancer group, the mean ALU 247 was 0.08 ± 0.06 ng/ml for the cases and 0.02 ± 0.01 ng/ml for the controls with a statistically significant difference ($p=0.01$) (Table 2). Among the stages, ALU 247 level was significantly lower ($p=0.035$) in stage II (0.03 ± 0.02 ng/ml) than in stage III (0.04 ± 0.02 ng/ml) (Table 3). ALU247 levels were also elevated in prostate cancer patients (0.04 ± 0.02 ng/ml) when compared to 0.02 ± 0.01 ng/ml of the controls with a statistically significant difference ($p=0.037$) (Table 2). For the can-

Table 3 Mean ALU levels and DNA Integrity among cancer stages.

	Breast Cancer				Prostate Cancer		
	ALU 115 (ng/ml)	ALU 247 (ng/ml)	* DNA Integrity		ALU 115 (ng/ml)	ALU 247 (ng/ml)	* DNA Integrity
Control State	0.10 ± 0.01	0.22 ± 0.13		Control State	0.03 ± 0.002	0.02 ± 0.01	
I	–	–	–	I	0.06 ± 0.03	0.05 ± 0.03	0.83
II	0.03 ± 0.01	0.03 ± 0.02	1.33	II	0.02 ± 0.01	0.03 ± 0.02	1.50
III	0.06 ± 0.21	0.04 ± 0.02	0.67	III	0.01 ± 0.008	0.02 ± 0.01	2.00
IV	–	–	–	IV	–	–	–

* DNA Integrity was calculated as ratio of mean ALU 247 to mean ALU 115 and rounded off to two decimal places.

cer stages, ALU 247 concentration decreased with prostate cancer stage progression. Stage I, stage II and stage III, had mean ALU 247 levels of 0.05 ± 0.03 ng/ml, 0.03 ± 0.02 ng/ml and 0.02 ± 0.01 ng/ml, respectively (Table 3). A pattern of ALU 247 levels similar to ALU 115 was observed among breast cancer participants and prostate cancer participants. ALU 247 was higher in the female breast cancer participants compared to male prostate cancer participants.

DNA integrity [ALU 247/ALU 115]

The mean DNA integrity was lower in breast cancer patients (1.22) than in the controls (1.48) as indicated in Table 2 though the difference between them was not statistically significant ($p > 0.05$). However, a comparison of the breast cancer stages revealed that DNA integrity was higher in breast cancer stage III patients than in stage II patients who respectively, had 1.33 and 0.67 ($p = 0.03$). In the prostate cancer group, DNA integrity was increased from 0.67 in healthy controls to 1.00 in the cases ($p = 0.02$). Among the different prostate cancer stages, DNA integrity showed an increasing pattern with disease stage progression. The stages I, II and III, respectively had DNA integrity of 0.83, 1.50 and 2.00 (Table 3).

Discussion

In this study, the serum ccfDNA levels in breast and prostate cancer patients and healthy individuals were determined. Serum was preferred over plasma because serum has been reported to contain higher levels of ccfDNA than plasma [19,25–27] and therefore is much suitable for the purpose. The mean age of the breast cancer patients was 50.6 ± 10.20 years and that for prostate cancer patients was 71.4 ± 5.8 years. These are consistent with established trends that 81% of all female breast cancers occur in women who are 50 years and above [28]. Prostate cancer is said to be disease of the aged and as such common in men 60 years and above [29,30].

In our study, the pattern of serum ccfDNA levels and DNA integrity among cancer patients and healthy controls, and with disease severity was consistent with earlier studies [12–14,19,26,27] with regard to the prostate and endometrial cancer groups. In the prostate cancer group, DNA integrity was higher in cases than in controls and also exhibited increasing trends with disease stage progression. ALU 115 which represents total serum circulating cell-free DNA, was

higher in prostate cancer patients compared to apparently healthy controls. ALU 247, representing circulating cell-free DNA released by dying cells (both apoptotic and necrotic), was also elevated in prostate cancer patients as compared to healthy controls. This is most likely the result of increased amounts of truncated fragments of DNA released by apoptotic cells into the bloodstream of prostate cancer patients as compared to healthy men [12,14,15,19,31]. A striking observation was the high levels of ALU species in breast cancer participants compared to their prostate cancer counterparts (Tables 2&3). Though this study did not investigate the observation and an immediate reason is not in sight, we speculate the following: (1) that females may have higher levels of ALU than males based on hormonal differences, with possible pre- and post-menopausal influences and (2) the influence of age of ALU expression. Mean age of female breast cancer participants was lower than that of the male prostate cancer participants. These were not investigated in this study and are subjects for further investigations.

DNA integrity was relatively higher in prostate cancer patients with a mean of 1.00, as compared to healthy men who had a mean of 0.67. DNA integrity was lower in healthy men probably due to low necrotic activity in body tissues, thereby lowering the concentration of longer DNA fragments in the bloodstream. When DNA integrity among the prostate cancer stages was analyzed, stage III had the highest level, followed by stage II, then stage I. This observation may be explained by the increased amount of DNA released into the bloodstream arising from tissue necrotic activities as disease severity increases. Other reports [12–14,31] observed similar trends and were convinced of a significant positive association between DNA integrity and prostate and endometrial cancer development and progression.

Among the breast cancer patients, the DNA integrity was higher in controls than in the cases, an observation divergent from the pattern observed in the prostate cancer arm of this study and other published reports. In general, both ALU species 115 and 247 had higher concentrations in breast cancer patients than in healthy individuals but this did not reflect in the DNA integrity. The difference in DNA integrity between the cases and the control was however, not statistically different ($p > 0.05$). One of the limitations of this study is the relatively small sample size (32 breast cancer patients) and this may contribute to the divergent observation. Secondly, in our study, we did not investigate the presence of comorbidities in participants which could also contribute to elevation of ALU 247 species through necrosis. Additionally, the DNA integrity compared between the breast cancer stages II and III did not conform to patterns observed by

others. DNA integrity was rather higher in the stage III than in the stage II. Whilst all the contributing factors to our observed pattern may not be readily clear, some of the above-mentioned limitations could be useful in planning future studies. Factors worth investigating for further studies include (1) the influence of cancer treatment on ccfDNA levels and DNA integrity and (2) influence of malaria and HIV on ccfDNA levels and DNA integrity. These factors, though not investigated in this study, we suspect, may have some notable effects on ccfDNA levels and DNA integrity and thus may account for some of the differences noted in our results. Ghana has high endemicity for malaria and HIV and these diseases are noted for derangement effects on the immune system.

Conclusion

In this study we have shown ALU species 115 and 247 levels in serum are elevated in breast and prostate cancer patients compared to their counterpart healthy controls. DNA integrity was higher in prostate cancer patients than the control but in breast cancer patients it is lower compared to their control counterparts. In prostate but not in breast cancers, DNA integrity increased with disease severity and higher staging. These results largely conform to published reports, and further boosts hope of the diagnostic and prognostic utility of ccfDNA and DNA integrity in cancers. It holds potential and more research effort should be dedicated to the area. We however, express that our investigations are preliminary and need an extension into a larger sample size for validation.

Acknowledgments

We are thankful to staff of MDS Lancet Laboratory Ghana, and Department of Surgery and the Chemotherapy Unit of the Korle-Bu Teaching Hospital who helped tremendously with patient recruitment and sample collection. The laboratories of the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, were used for successful laboratory experimentation and we appreciate the faculty and all support staff (administrative and laboratory) who helped in wonderful ways to make this project a success.

Conflict of interest

The authors declare that they have no conflict(s) of interest regarding this manuscript.

Author contribution

Conceptualization: BAB, RMB, NAA

Design, Data Generation & Data Analysis: ESDS, ABD, ANAA, EDAO, BAB

Manuscript Development: NAA, BAB, EAT, ANAA, EDAO

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.cancerger.2019.04.062](https://doi.org/10.1016/j.cancerger.2019.04.062).

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