

Circulating Cell-free DNA in Serum as a Marker for the Early Detection of Tumor Recurrence in Breast Cancer Patients

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Abstract. *Background/Aim:* Circulating cell-free DNA (cfDNA) isolated from serum by noninvasive procedures can serve as a potential biomarker for the early detection of many cancers. The aim of this study was to implement a simple, yet effective quantitative method for measuring the cfDNA in serum and to investigate the relationship between cfDNA and the occurrence of recurrence in breast cancer (BrCa) patients. *Patients and Methods:* A total of 240 cases were selected, which comprised different subtypes of BrCa patients and control individuals. We selected 20 serum samples from patients which showed recurrence after 4-7 years of disease-free survival. SYBR green was used as a reporter molecule to estimate the amount of cfDNA in these serum samples. *Results:* A global Wilcoxon analysis was performed to compare the cfDNA abundance between non-recurrent and recurrent patients. The amount of cfDNA was

higher in recurrent patients (recurrent vs. non-recurrent ratio=1.3; $p=0.03$; $AUC=0.76$) compared to non-recurrent patients. The data between normal/healthy controls and non-recurrent patients indicated no significant differences ($n=20$ in each group, healthy to non-recurrent ratio=1.03; $p=0.20$; $AUC=0.61$). *Conclusion:* We implemented a straightforward one-step technique to measure the amount of cfDNA in serum, which can translate into a clinical diagnostic tool in the near future. The high levels of cfDNA in the serum of recurrent BrCa patients compared to non-recurrent BrCa patients indicates a possible uncovered role for circulating genetic information, which either contributes to the cancer recurrence phenomenon or at the very least, serves as an identifier for the potential of recurrence.

The “liquid biopsy” is a non-invasive diagnostic test, which provides information about disease conditions (1). With respect to several cancers, the prognostic importance of circulating cell-free DNA (cfDNA) from peripheral blood samples is an emerging field of research (2-9). In patients with cancer, the concentration of cfDNA increases, and structural, sequence, and epigenetic changes to DNA can be observed during the disease process and therapy (10-15). Therefore, cfDNA allows non-invasive assessment of cancer in real time. Furthermore, cfDNA released by the tumor contains the same variants as those in the tumor cells (7, 10, 13, 16). The origin and biological relevance of cfDNA can be found in both healthy individuals and cancer patients (2). However, previous reports have indicated a correlation between cancer and increased cfDNA, especially in cases of metastatic cancer (10, 11, 17). The main cause of this apparent increase in cfDNA likely comes from both apoptotic and necrotic cell death, a common phenomenon in tumors (18, 19). Additionally, one theory is that necrotic cell death is the more prominent source

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of cfDNA, as this allows for longer, less degraded DNA release compared to the apoptosis pathway (18). Moreover, another source of cfDNA is cellular secretion, which has been shown to be increased in BrCa samples (20). There also appears to be a correlation between the G1 Phase of the cell cycle and the secretion of cfDNA in BrCa cells (20). Interestingly, cfDNA extracted from the supernatant of the BrCa cell line MDA-MB-231 was shown to increase the proliferation of two other BrCa cell lines, T47-D and MCF-7, through the TLR9-NF-κB pathway (20).

Therefore, it is hypothesized that cfDNA may have a role in metastasis and an importance as a non-invasive diagnostic assay for cancer. Considering that cfDNA quantification has yet to become a clinical marker for cancer or recurrence, additional evidence is required to demonstrate efficacy. In this current study, we established cfDNA quantification as a viable tool for predicting BrCa recurrence, using a conventional and simple method to estimate cfDNA levels in a patient.

Patients and Methods

Patients. This clinical study was approved by the Institutional Review Board (IRBNet Board, Institute- USUHS and Location- Walter Reed National Military Medical Center (WRNMMC), Bethesda, MD). The title of the project was ‘race specific molecular determinants of recurrence in BrCa subtypes’ (PI- Dr. Meera Srivastava and Project ID# 403173-1 and effective date: May 29, 2014). Upon IRB approval, serum samples were obtained from the Murtha Cancer Center, Walter Reed National Military Medical Center, Bethesda, MD, USA. This is a retrospective study and details of patient recruitment; study design and exclusion methods have been described in our earlier publications (21-24). The BrCa serum samples were obtained five or more years prior to the experiment and were categorized as either recurrent or non-recurrent BrCa samples (20 recurrent serum samples out of a total 240 patient samples) (Table I).

Isolation and characterization of CfDNA in serum. CfDNA was isolated from a control serum sample using a QIAamp DNA Mini Kit from QIAGEN (catalog No. #51304, Qiagen, Germantown, MD, USA). The protocol for “DNA Purification from Blood” was followed with 200 µl of serum as recommended by the manufacturer. The cfDNA was eluted from the column with a total volume of 50µl of deionized water. To demonstrate the presence of DNA in the serum, real-time PCR (RT-PCR) was performed with the isolated DNA and two controls (PBS control and water control). The components of the PCR mixture were as follows: 20 µl master mix (iTaq Universal Probes Supermix, catalog No. 172-5130, Bio-Rad, Hercules, CA, USA), 5 µl isolated DNA or control (1× PBS), 2 µl primer solution containing FAM (18s Ribosome-TaqMan Assay ID: Hs99999901_s1, endogenous control, Thermo Fisher, Waltham, MA, USA), and 13 µl molecular grade water. The parameters of the RT-PCR were as follows: 95°C for 10 min followed by (95°C for 15 s followed by 60°C for 1 min) ×40 cycles.

CfDNA standard curve and quantification. The cfDNA quantification experiment was conducted by mixing a sample with SYBR Green I (final concentration of 1×) (Cat # S33102, Invitrogen, Waltham, MA,

Table I. *The distribution of total 240 samples within the 24 patient groups.*

Patient/Tumor characteristics	AA samples	CA samples
Control, pre-menopausal	10	10
Control, post-menopausal	10	10
TN, pre-	15	10
TN, post-	14	10
HER2+, pre-	6	10
HER2+, post-	1	10
LA, pre-	10	10
LA, post-	8	10
LB1, pre-	15	10
LB1, post-	22	10
LB2, pre-	5	10
LB2, post-	4	10

AA: African American; CA: Caucasian American; TN: triple-negative subtype (ER-PR-/HER-); Her2+: human epidermal growth factor receptor 2 subtype (ER-PR-/HER2+); LA: Luminal A subtype (ER+, Ki67-); LB1: Luminal B1 subtype (ER+, HER2+); LB2: Luminal B2 subtype (ER+, HER2+).

USA) and by measuring the fluorescent intensity at an excitation wavelength of 495 nm and an emission wavelength of 520 nm. The buffer consisted of TBS (Tris buffered saline) with 0.1% Tween 20 (0.1% polyethylene glycol sorbitan monolaurate, Catalog # P-9416, Sigma Aldrich, St. Louis, MO, USA). All measurements were performed in duplicate.

A standard curve for DNA concentration was then constructed, while, importantly, compensating for how the protein in the serum may impact the measurements. To compensate for protein in the serum samples, BSA was added at a constant final concentration for each dilution of DNA in the standard curve (0.175 mg/ml BSA final concentration, consistent with the average serum albumin of 35-55 g/l, taking into account the total 1:200 sample dilution). The standard curve consisted of the following DNA concentrations (total added): 1.6, 3.1, 6.3, 12.5, 25, 50, 100, and 200 ng.

The BrCa and control samples were measured under the sample conditions as mentioned above and with a final dilution of 1:200 (serum: SYBR Green I/TBS solution).

Statistical analysis. All statistical analyses were performed using the program GraphPad Prism (GraphPad Software, San Diego, CA, USA). For each figure, the type of statistical analysis performed is indicated, with the corresponding statistical value (such as *p*-value). To compare groups, ROC curves were generated (with a corresponding AUC score) and *t*-tests were performed (to determine a *p*-value) as in earlier studies (21-24).

Results

Characterization of the circulating DNA in serum. To characterize the circulating DNA in serum, DNA was isolated from blood samples using a Qiagen kit. The isolated DNA was characterized by RT-PCR to measure 18S Ribosomal subunit (186bp) (Figure 1A and B). Both water and PBS served as negative controls.

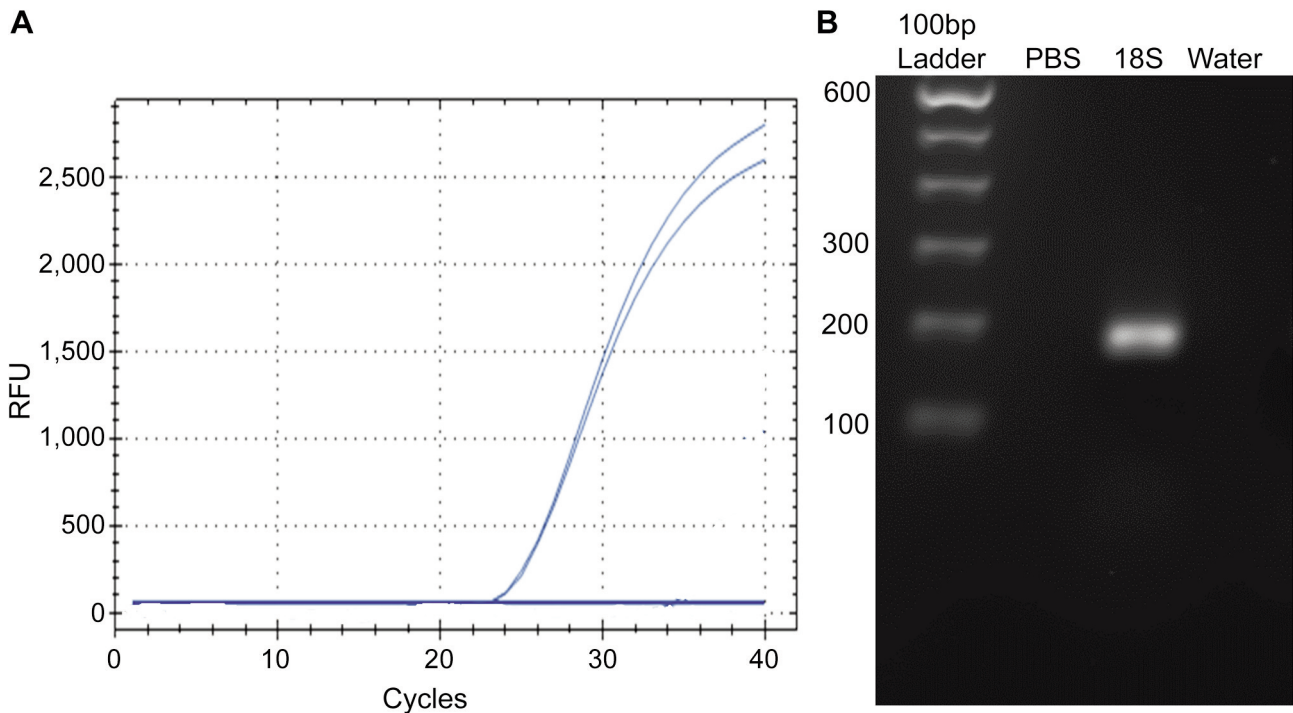


Figure 1. Isolation and characterization of the circulating cell free DNA (cfDNA) from serum. A. qPCR Ct values (18S, duplicates) for 18S DNA along with negative controls and B. Amplified 18S on agarose gel. DNA was isolated from 200 μ l of serum samples.

Standardization of SYBR Green I detection method. To determine the efficiency of SYBR Green I as an indicator of cfDNA in serum, a series of controls were conducted. First, we sought to determine the effect of protein on SYBR Green I fluorescence. Surprisingly, it appears that SYBR Green I fluorescence is quenched by protein, as the fluorescence of SYBR Green I mixed with BSA was roughly 40% lower than that of SYBR Green I alone (Figure 2A). Additionally, when BSA was added to the mixture of serum sample and SYBR Green I, the fluorescence was on average 5% lower than that of the same mixture in the absence of added BSA. While this demonstrates that SYBR Green I fluorescence is slightly negatively impacted by the presence of protein, a simple correction was made to account for this phenomenon. To account for protein's quenching ability, when constructing the standard curve, BSA was added at a relatively similar concentration to the concentration of BSA in serum. Second, we attempted to determine the linearity of DNA under this detection method. This is demonstrated in the standard curve of DNA mixed with BSA (Figure 2B), which has an R² value of 0.9928 and ranges from below the lowest and above the highest cfDNA fluorescent values of the serum samples.

Comparison of recurrent BrCa, non-recurrent BrCa, and controls. The relative fluorescence intensity of the healthy control, patient with BrCa, and patient with non-recurrent or

recurrent cancer serum samples are shown in Figure 3A. ROC (Receiver Operator Curve) analysis was performed to measure the *p*-value and area under the curve (AUC) for healthy control *vs.* cancer samples and for non-recurrent *vs.* recurrent serum samples (Figure 3B and C). The ROC analysis demonstrated an AUC of 0.6175, and a *p*-value=0.2036 (Figure 3B) for the healthy control *vs.* cancer samples. The ROC analysis for non-recurrent *vs.* recurrent demonstrated an AUC of 0.7693, and a *p*-value=0.0346 (Figure 3C). The difference between the recurrent BrCa group and the two other groups, non-recurrent BrCa and controls, was statistically significant (*p*<0.0001); whereas the difference between non-recurrent BrCa and control samples was not statistically significant (*p*=0.20) (Figure 3B). The mean concentration of cfDNA in the serum of each group is as follows: recurrent BrCa patients: 23 \pm 4 ng/ml, non-recurrent BrCa patients: 18 \pm 3 ng/ml, and control individuals: 18 \pm 2 ng/ml (Figure 4A). Furthermore, when we analyzed the patients based upon racial classifications (African Americans *vs.* White), it was determined that African American patients tended to have lower levels of cfDNA than White patients (*p*<0.005) (Figure 4B).

Discussion

There are several risk factors, which eventually lead to poor prognosis for several cancers including BrCa (25, 26).

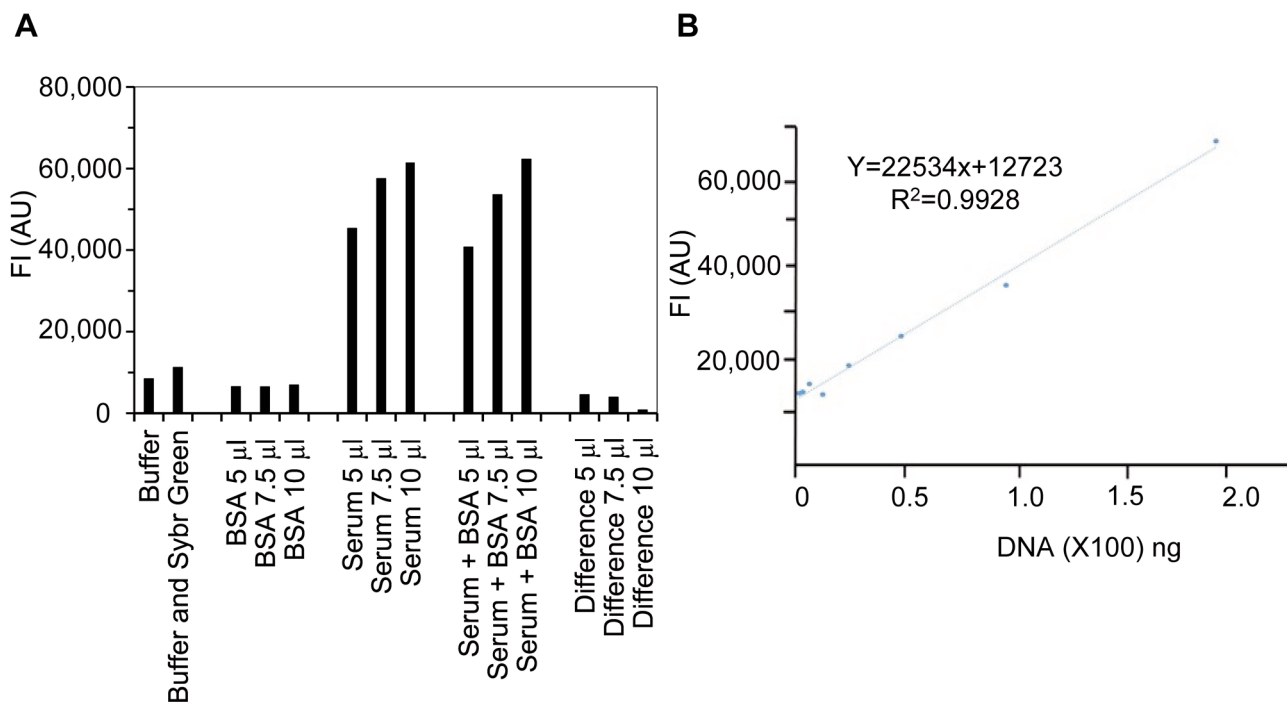


Figure 2. Binding of SYBR-Green I to circulating cfDNA with various controls for compensation and standard curve generation. A) Fluorescent intensities of SYBR Green I at various controls including in the presence of BSA and serum cfDNA. All samples contain SYBR Green I, except the first sample defined as Buffer. B) Standard curve for quantitative measurement of cfDNA.

Considering that recurrent BrCa patients are at a higher risk of death, with one study suggesting an overall 5-year survival rate of 63% after local recurrence, there exists a need for identification of methods, which can proactively detect BrCa recurrence (1, 23, 26-29). Current methods of detecting BrCa recurrence involve radiographic and clinical evaluations, with mammograms being the most common identification method. However, mammograms are not always accurate, leading to a false reassurance or a false-positive result and tend to be less sensitive regarding younger women (age 40-49 years) (26). This causes a lower reduction in mortality for younger women who rely on mammograms compared with older women (26).

Considering the non-invasive nature of liquid biopsies, otherwise known as the analysis of blood, we primarily focused on this method of detection. Building on previous work, which showed a relationship between high cfDNA levels and BrCa relapse in patients with triple-negative BrCa, we sought to provide further validation for the use of cfDNA as a marker of BrCa recurrence (30). Herein, we demonstrate the ability of cfDNA as a possible indicator of BrCa recurrence. We used a simple and straightforward approach in which serum was incubated with SYBR Green I, a DNA binding and fluorescent molecule, to quantify the amount of cfDNA in serum. This enables the comparison of cfDNA

levels between samples. Compared with non-recurrent BrCa patients, there was a 23% increase ($p < 0.0001$) in cfDNA concentration in recurrent BrCa patients. Additionally, there was a 28% increase in cfDNA ($p < 0.001$) concentration in recurrent BrCa patients compared to control. However, there was no apparent statistically significant difference between non-recurrent BrCa patients and controls.

There are several reasons as to why cfDNA levels may be an accurate indicator of BrCa recurrence. For instance, cfDNA originates from necrotic and apoptotic cell death, as well as passive secretion. Considering that high-grade breast cancers have a significantly higher apoptotic index, likely due to a higher growth rate, it is understandable that there may be more cfDNA in the blood because of increased apoptosis. This increased rate of apoptosis, as well as necrosis, is also correlated with lower survival rates. As such, higher cfDNA levels may be an indication of a more aggressive and higher tumor growth rate. Additionally, cfDNA extracted from the medium of cultured MDA-MB-231 cells, a BrCa cell line, was able to induce proliferation in two other BrCa cell lines (20). This suggests that higher cfDNA levels may also cause increased tumor growth, which in turn may lead to the survival of tumor cells following BrCa treatment.

Interestingly, we found higher levels of serum cfDNA in non-Hispanic white BrCa patients (Figure 4B). Previous

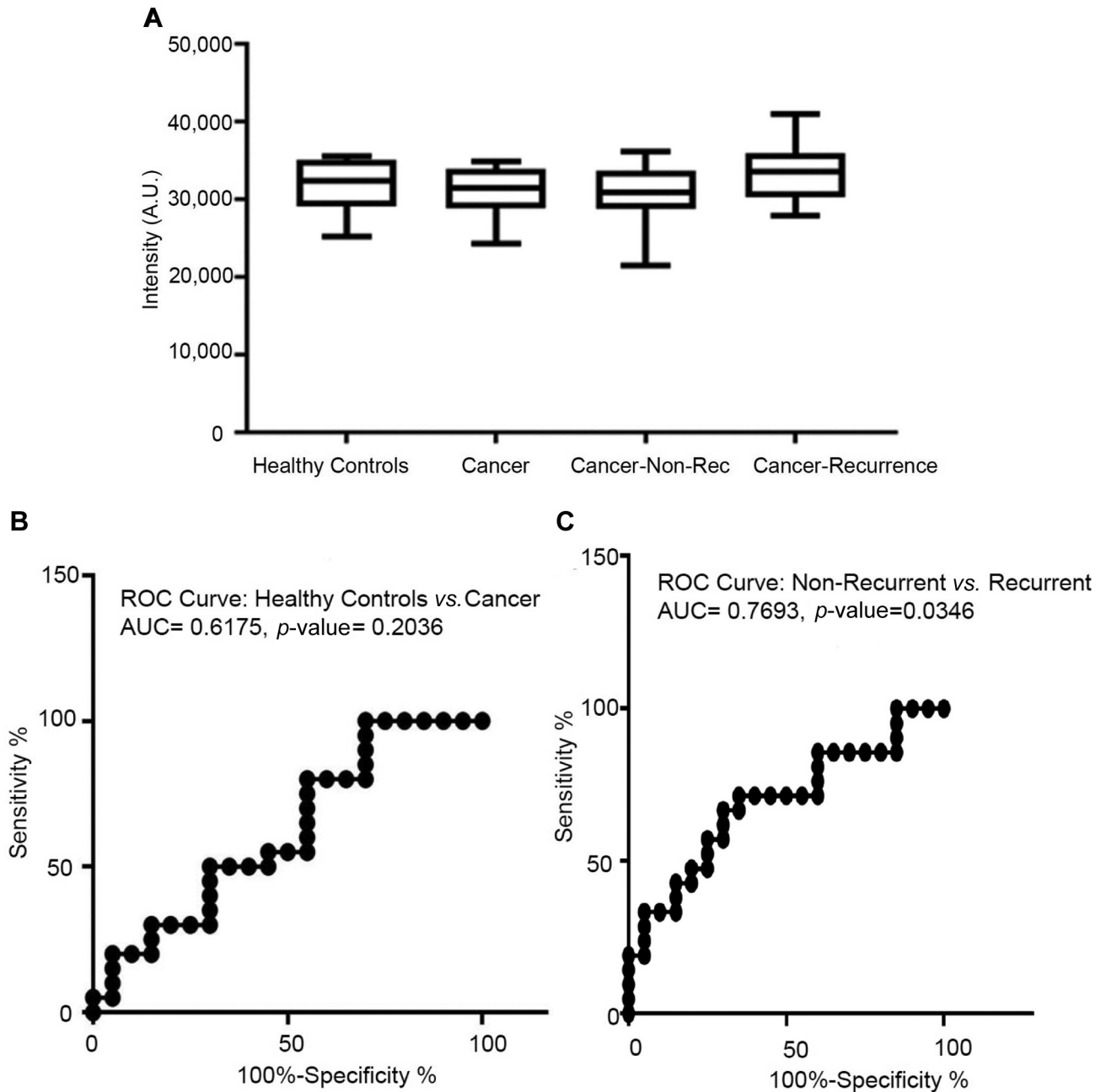


Figure 3. Visual and statistical analysis of circulating cfDNA in BrCa patients compared to control. A) Box and Whisker plot for healthy controls, cancer patients, cancer-non-recurrent patients, and cancer-recurrence patients. B and C) ROC curves comparing healthy vs. cancer patients and non-recurrent vs. recurrent patients.

studies indicated that compared to non-Hispanic whites, African-American women tend to be diagnosed with BrCa at an earlier age, with aggressive characteristics, and eventually higher mortality rates. However, the extent to which differences in clinical traits account for the black/white disparity in BrCa mortality is unclear (31, 32). Therefore, moving forward, if cfDNA quantification was to transition

into clinical use, differences in baseline cfDNA values between racial groups may be an important variable to study and understand for both the accuracy of the results and the elimination of the disparity in cancer mortality rates.

There are some limitations to this study. One limitation is the small sample size of recurrent BrCa samples ($n=0$); however, it is difficult to procure a large sample base of recurrent BrCa

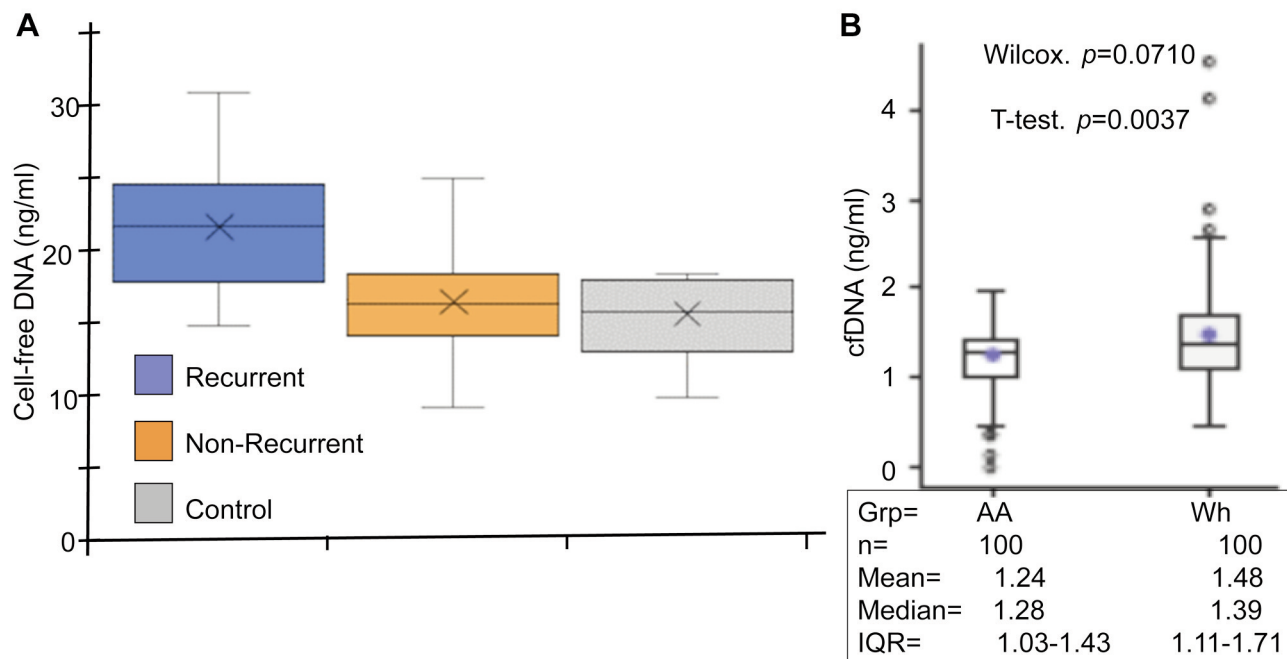


Figure 4. Quantification of the cfDNA in female serum (healthy controls and BrCa patients). A. Quantification of circulating cfDNA using the standard curve generated from Figure 2. B. Several cfDNA concentration comparison studies were performed in serum samples from patients with different sub-types of BrCa. cfDNA levels are higher in non-Hispanic white patients compared to African American women. Differential levels of cfDNA in serum is indicating BrCa racial disparities.

patient samples as many patients may attend other facilities instead of continuing at the study-site location. This makes it difficult to track which patients ended up having a recurrence and which did not. Another limitation is the absence of multiple time-points for each patient, such as one serum sample being obtained each year. This would allow us to track the cfDNA and possibly match it with the progression of the recurrence.

In conclusion, the results of this study suggest that the quantification cfDNA in serum may be a viable detection method for identifying BrCa recurrence and racial disparities.

Conflicts of Interest

All Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization: Alakesh Bera and Meera Srivastava; Data collection and analysis: Alakesh Bera, Eric Russ, John Karaian, Adam Landa, Surya Radhakrishnan, Madhan Subramanian, Matthew Hueman, Harvey Pollard, Hai Hu, Craig Shriver, Meera Srivastava; Critical reading and writing: Alakesh Bera, Eric Russ, John Karaian, Adam Landa, Surya Radhakrishnan, Meera Srivastava.

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