

Evaluation of circulating cell-free nuclear and mitochondrial DNA levels in Syrian patients with breast tumor

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Abstract. In the last decade, the roles of circulating cell free nuclear (ccfn) and ccf mitochondrial (ccfmt) DNA as potential noninvasive biomarkers have been demonstrated in numerous different types of disease, including cancer. However, the results remain controversial. The present study aimed to investigate the roles of ccfnDNA and ccfmtDNA levels in the plasma of patients with breast cancer. A total of 84 Syrian female subjects were included in the study, who were divided into 3 groups: i) Malignant disease group (n=33); ii) benign disease group (n=26); and iii) healthy control group (n=25). CcfndNA and ccfmtDNA were determined using real-time quantitative PCR and the reactions were followed by melting curve analysis. The results indicated no significant differences in the plasma levels of ccfnDNA, ccfmtDNA or the ratio of ccfmtDNA/ccfnDNA between the study groups. Of note, a positive correlation was observed between the ccfmtDNA/ccfnDNA ratio and age in the control group ($P=0.012$; $r=0.505$). In addition, a positive correlation was identified between ccfnDNA levels and the estrogen receptor status ($P=0.045$; $r=0.416$), while a negative correlation between ccfmtDNA/ccfnDNA ratio and the progesterone receptor status was obtained ($P=0.045$; $r=-0.448$). Aging and the role of hormones in the cells may be responsible for these results. In the future, the present study should be followed up with mutation detection analyses and large-scale studies.

Introduction

Breast cancer is one of the most common malignancies, accounting for 29% of all cancer cases in females worldwide (1). In the Middle East, the prevalence of breast cancer remains lower than that in North America and Europe, where it accounts for 14-42% of all female cancer cases (2). In Syria, there are

no official statistics on the prevalence of breast cancer or other malignancies (3). According to the International Agency for Research on Cancer in 2012, the prevalence ratio of breast cancer in females in Syria is 36.4% and the mortality rate is 24.8% (4). In recent years, numerous methods have been used for screening, diagnosing and monitoring breast cancer, including physical examination, mammography and biopsy (5). However, each of these techniques has numerous drawbacks, making it necessary to identify a novel diagnostic biomarker or method (6). In addition, despite the significant progress in the development of cancer therapies, cancer-associated mortality remains high (7). Previous research has increasingly focused on the role of the quantitative and qualitative changes of circulating cell-free nuclear DNA (ccfnDNA) and circulating cell-free mitochondrial DNA (ccfmtDNA) in numerous different types of disease, but particularly in cancer (8,9). Cancer cells release their nucleic acids (both n/mDNA and RNAs) into the systemic circulation through cellular necrosis and apoptosis (10,11); this DNA is mixed with DNA released from normal cells (12). In previous studies, changes in the ccfnDNA and ccfmtDNA levels were identified not only in tissue samples but also in body fluids such as plasma, which makes them attractive noninvasive potential biomarkers (13,14). Several studies have reported elevated levels of ccfnDNA or ccfmtDNA in different types of cancer, while others reported decreased levels, thus the results remain conflicting (15-17). Due to these results, the present study aimed to determine their role in breast cancer by comparing the absolute quantities of ccfnDNA and ccfmtDNA, as well as the ccfmtDNA/ccfnDNA ratio, among malignant and benign tumor groups and healthy controls.

Materials and methods

Patients. In total, 84 Syrian females were included in the present study. Informed consent forms were signed by all participants and the study was approved by the Syrian Higher Commission for Scientific Research, Ministry of Higher Education, Damascus, Syria. The study cohort (n=84) was divided into 3 groups: i) Malignant disease group (n=33); ii) benign disease group (n=26); and iii) healthy control group (n=25). Groups 1 and 2 contained patients encountered at the general surgery department of Al-Assad University Hospital-Damascus (Damascus, Syria) for breast mastectomy; the diagnosis was confirmed by biopsy for all cases. The healthy control group used in this study had neither a history of cancer nor suffered from any other severe or autoimmune

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disease. All blood samples were taken prior to any invasive procedures or therapeutic treatments between February 2013 and June 2014. The clinical data of each patient [age, smoking status, menopausal status, cancer stage, grade, tumor size, lymph node involvement, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (Her2-neu) status] were obtained from the pathological reports (Table I). The blood samples (2 ml) were centrifuged twice, first at 1,600 x g for 10 min at 4°C and then at 16,000 x g for 10 min at 4°C (each time with the Centrifuge 5417R; Eppendorf). The supernatant was stored at -80°C until the next step. The DNA was extracted using the GeneJET Genomic DNA purification kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, except for a change of the elution buffer volume from 200 to 100 µl. DNA extracts were stored at -80°C until further use. CcfndDNA and ccfmtDNA levels were measured via real-time quantitative PCR using a LightCycler instrument (Roche Diagnostics GmbH). One primer pair specific for the nDNA (β2M) and another primer pair for the mtDNA (D-loop) were used for quantification (TIB Molbiol). The primers pairs were selected after reviewing a large number of scientific articles (15) and their specificity was analyzed using BLAST (<https://blast.ncbi.nlm.nih.gov/>; date of accession April 25th, 2019) and Primer BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>; date of accession April 25th, 2019). The primer sequences are listed in Table II. Standard curves for nDNA and mtDNA were generated using homemade synthetic target amplicons. The standards were synthesized through DNA isolation, conventional PCR, PCR product purification, DNA sequencing, measuring the concentration and finally confirming that the sample and the standard were amplified with identical efficiencies. Each sample was assayed in duplicate using FastStart DNA Master SYBR Green 1 (Roche Diagnostics GmbH) using a different primer pair each time. qPCR was performed in a total reaction volume of 20 µl, containing 3 mM MgCl₂, 0.5 µM forward primer and 0.5 µM reverse primer. The following thermocycling conditions were used: Initial denaturation at 95°C for 10 min, followed by 55 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec and elongation at 72°C for 7 sec (18). All of the reactions were followed by melting curve analysis. The copy numbers per ml of plasma for ccfndDNA and ccfmtDNA in samples were quantified using the following equation:

$$Q_{\text{nuclear}} = \frac{C}{3.3} \times \frac{V_{\text{elution}}}{V_{\text{plasma}}}$$

Q_{nuclear} is the ccfndDNA copy number per milliliter, C is the ccfndDNA concentration (pg/µl) determined by qPCR targeting the β2M gene sequence and 3.3 pg is the human haploid genome mass. V_{elution} is the volume of ccfDNA extract (µl) and V_{plasma} is the volume of plasma used for the extraction (ml).

$$Q_{\text{mitochondrial}} = \frac{C \times Na}{2 \times MW \times Ln} \times \frac{V_{\text{elution}}}{V_{\text{plasma}}}$$

$Q_{\text{mitochondrial}}$ is the ccfmtDNA copy number per milliliter and C is the ccfmtDNA concentration (pg/µl) determined by a qPCR targeting the mitochondrial D-loop. Na is Avogadro's number (6.02×10^{23} molecules per mole), Ln is the nucleotide length and MW is the molecular weight of one nucleotide (g/mol). V_{elution} is the elution volume of ccfDNA extract (µl) and V_{plasma} is the volume of plasma used for the extraction (ml).

Statistical analysis. All statistical analyses were performed using SPSS statistics 13.0 software (SPSS, Inc.). Statistical

differences between ccfndDNA or ccfmtDNA concentrations and the ccfmtDNA/ccfndDNA ratio were compared using ANOVA followed by Bonferroni's post-hoc test. The Kolmogorov-Smirnov test confirmed that the data were normally distributed. The correlation of the mean values with other parameters was assessed by calculating Pearson's and Spearman's correlation coefficients. Student's t-test was used to determine the association of ccfndDNA, ccfmtDNA or the ccfndDNA/ccfmtDNA ratio with menopause, smoking and lymph node status, while Pearson's correlation was used to study the correlation between ccfndDNA, ccfmtDNA and ccfndDNA/ccfmtDNA levels with age and tumor size. Furthermore, Spearman's correlation analysis was used to study the correlation between ccfndDNA, ccfmtDNA and ccfndDNA/ccfmtDNA levels with the tumor stage or grade, or with the ER, PR and Her2/neu receptor status. $P < 0.05$ was considered to indicate statistical significance.

Results

Plasma levels of ccfndDNA, ccfmtDNA and ccfndDNA/ccfmtDNA between the study groups. The mean ccfndDNA concentration in the breast cancer, benign tumor and the control group was 6,448, 6,197 and 6,071 copies/ml respectively, while the mean value of the ccfmtDNA concentration was 20,770,515, 6,572,829 and 5,188,000 copies/ml, respectively. The mean value of the ccfmtDNA/ccfndDNA ratio was 154,017, 2,155 and 24,192 in the breast cancer, benign tumor and control group, respectively. When comparing the plasma levels of ccfndDNA and ccfmtDNA and the ccfmtDNA/ccfndDNA ratio between the three study groups using ANOVA, no significant differences were identified between the groups ($P = 0.979, 0.542$ and 0.447 , respectively; $P > 0.05$) data not shown.

Correlation between ccfndDNA, ccfmtDNA or ccfmtDNA/ccfndDNA levels and demographic features (age, menopause and smoking status). The mean age for females with breast cancer, benign tumor and control subjects was 46 (range, 28-90), 37 (range, 21-75) and 43 (range, 24-76) years, respectively. When calculating Pearson's correlation coefficients for ccfndDNA or ccfmtDNA levels or the ccfmtDNA/ccfndDNA ratio with age, menopause or smoking status in the three study groups, a positive correlation was only identified between the ccfmtDNA/ccfndDNA ratio and age in the control group ($P = 0.012, r = 0.505$; Fig. 1). However, in the three study groups, no statistical significance was obtained for the correlation between the levels of ccfndDNA or ccfmtDNA or the ccfmtDNA/ccfndDNA ratio and demographic features (data not shown; $P > 0.05$).

Correlation between ccfndDNA, ccfmtDNA or ccfmtDNA/ccfndDNA levels in breast cancer and the clinicopathological parameters (stage, grade, lymph node involvement, tumor size, ER, PR and Her2/neu receptor status). In the breast cancer group, no statistically significant correlations between the levels of ccfndDNA or ccfmtDNA or the ccfndDNA/ccfmtDNA ratio and the stage, grade, lymph node status or tumor size were obtained ($P > 0.05$). When calculating the Spearman's correlation coefficient for the correlation between ccfndDNA or ccfmtDNA or the ccfmtDNA/ccfndDNA ratio with the ER, PR

Table I. Parameters of the study cohort.

Group	N	Age (years)	Smoking status (%)		Menopausal status (%)	
			Smoking	Non-smoker	Premenopause	Postmenopause
Malignant disease	33	46±12.8	30	70	70	30
Benign disease	26	37±13.6	19	81	89	11
Healthy control	25	43±13.6	28	72	86	32

Age is expressed as the mean ± standard deviation.

Table II. Primer sequences used for PCR.

Gene ID	Primer name	Sequence (5'-3')	Amplicon length
β2M	hβ2MF2	GCTGGGTAGCTCTAAACAATGTATTCA	94
	hβ2MR2	CCATGTACTAACAAATGTCTAAAATGGT	
D-loop	hmitoF5	CTTCTGGCCACAGCACTTAAAC	64
	hmitoR5	GCTGGTGTAGGGTCTTTGTTTT	

F, forward; R, reverse.

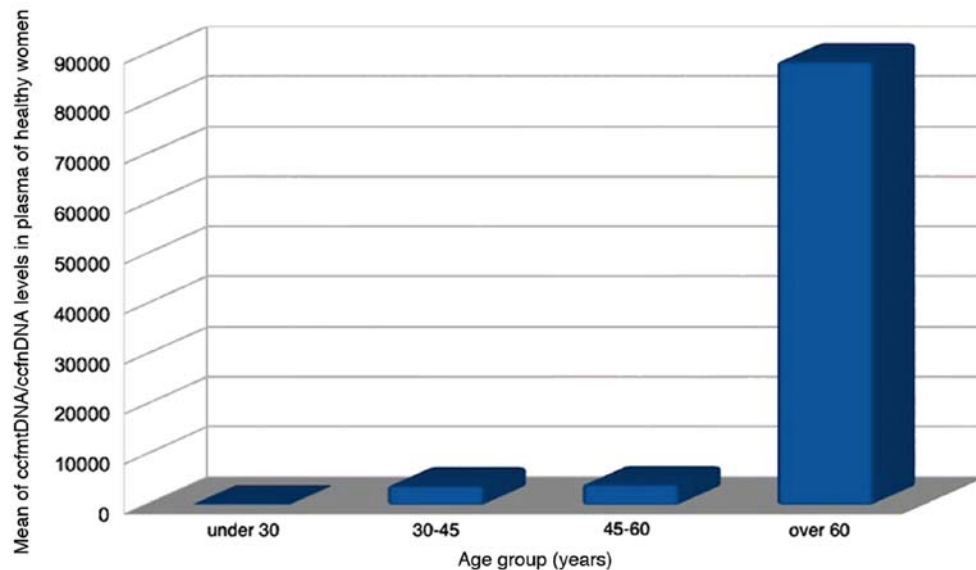


Figure 1. Association between mean values of ccfmtDNA/ccfnDNA levels in the plasma of healthy females with age. After dividing the females into four groups based on age (x-axis), a significant increase was found in the levels of ccfmtDNA/ccfnDNA (y-axis) with increasing age especially in the over 60 group. ccfmtDNA, circulating cell-free mitochondrial DNA; ccfnDNA, circulating cell-free nuclear DNA.

or Her2/neu receptors status, a positive correlation was identified between ccfnDNA levels and ER status ($P=0.045$, $r=0.416$; Fig. 2A), while a negative correlation was obtained between the ccfmtDNA/ccfnDNA ratio and PR status ($P=0.045$; $r=-0.478$; Fig. 2B and Table III).

Discussion

For breast cancer in general, but particularly in the Middle East, there remains a lack of research in the field of ccfnDNA, and it is required to further investigate its role. In the present

study, PCR with SYBR-Green I was used for the quantification and numerous previous studies were reviewed to ensure that the most specific primers for both nDNA and mtDNA were used, and their specificity was tested prior to using them. Each PCR was also followed up by melting curve analysis to ensure the accuracy of the results. Neither the concentrations of ccfnDNA and ccfmtDNA, nor the ccfmtDNA/ccfnDNA ratio were significantly different between the three study groups, which differs from the results of other previous studies (15-17). This difference may be explained through ethnic differences, limited sample sizes and differences

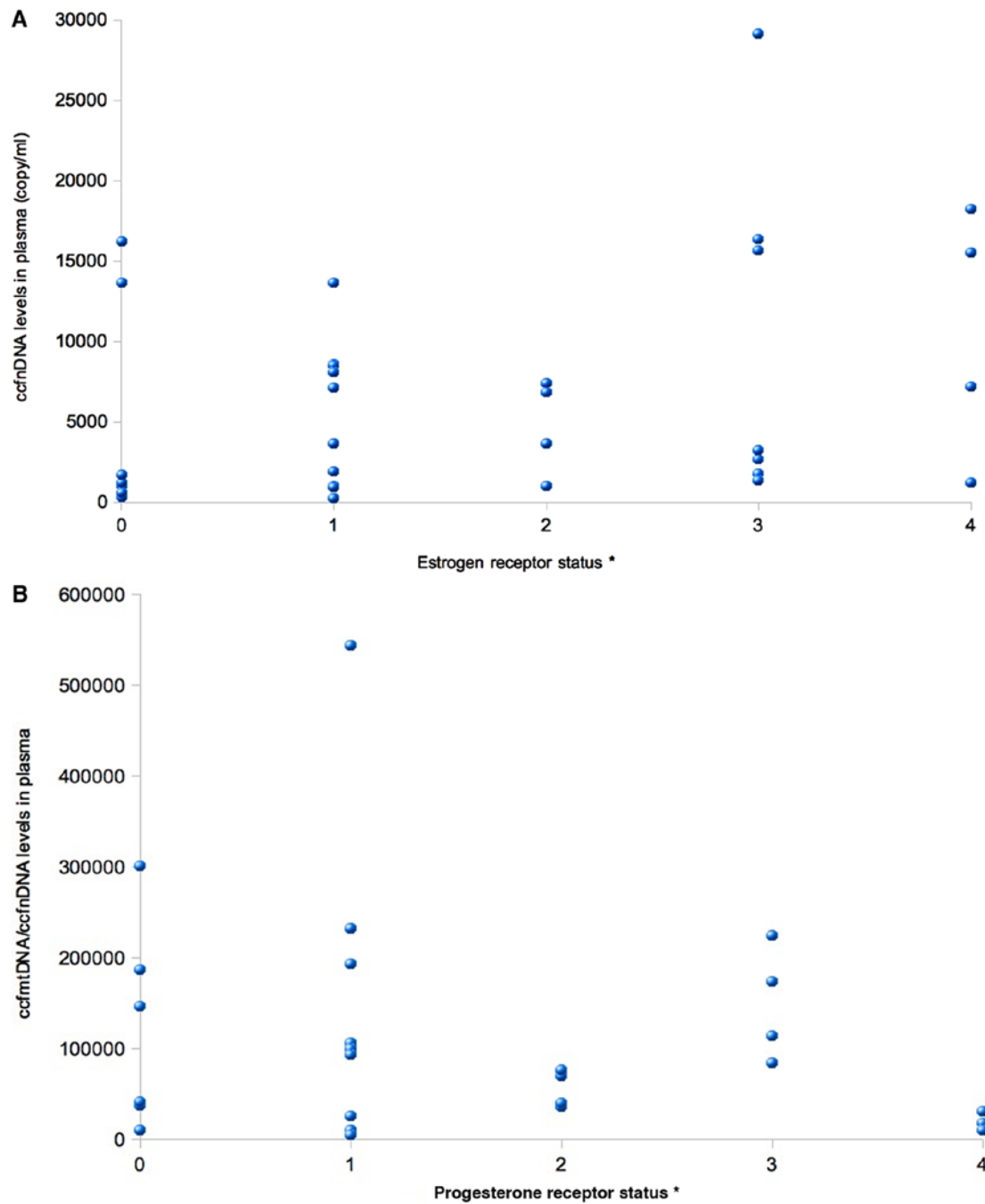


Figure 2. Scatterplots for correlation of plasma levels of ccfndDNA with ER status and ccfmtDNA/ccfndDNA levels with progesterone receptor status in patients with breast cancer. (A) After dividing patients with breast cancer into five groups based on the expression status of ER in tumor cells (x-axis), a positive correlation was found between ccfndDNA levels in plasma (y-axis) with ER status ($P=0.045$; $r=0.416$). (B) After dividing patients with breast cancer into five groups based on the expression status of PR in tumor cells (x-axis), a negative correlation was found between ccfmtDNA/ccfndDNA levels (y-axis) with PR status ($P=0.045$; $r=-0.448$). Spearman's correlation analysis was performed on the datasets. 0 indicates no expression, while the increasing numbers indicate the increasing receptor expression intensity in tumor cells. ER, estrogen receptor; PR, progesterone receptor; ccfmtDNA, circulating cell-free mitochondrial DNA; ccfndDNA, circulating cell-free nuclear DNA.

in the methods used for quantification. Furthermore, the results of the present study indicated no correlations between ccfndDNA, ccfmtDNA or ccfmtDNA/ccfndDNA levels and demographic features or clinicopathological parameters, which is consistent with the results of other previous studies, except that the present study reported a positive correlation between the ccfmtDNA/ccfndDNA ratio and age in the healthy control group. This result may be due to increased levels of ccfmtDNA, decreased levels of ccfndDNA or the impaired

interaction between mtDNA and nDNA. In addition, it may be explained through the increased levels of reactive oxygen species with aging, which is directly responsible for damaging cells and releasing their nDNA and mtDNA into the blood stream (19) or increasing the mutation rate for both nDNA and mtDNA, but with much higher rates for mtDNA (20,21). On the other hand, due to the complex relationship between mtDNA and nDNA, mutations in mtDNA may affect the way mtDNA and nDNA communicate with each other (22). The

Table III. Correlations between ccfnDNA or ccfnmtDNA levels, or the rcfnDNA/ccfnmtDNA ratio and clinicopathological parameters in the malignant disease group.

Parameter	Patients, n (%)	ccfnmtDNA (copies/ml)	P-value, r-value	ccfnDNA (copies/ml)	P-value, r-value	ccfnmtDNA/ccfnDNA	P-value, r-value
Stage ^a			0.886, 0.032		0.552, -0.139		0.641, 0.112
I	8 (24.2)	6,772,500 (1,360,000-19,500,000)		5,790.3 (2.51-16,200)		5,704,798 (9,690-39,840,637)	
II	13 (39.4)	2,647,076.9 (652,000-6,240,000)		8,767.3 (17-29,400)		8,427 (4,170-23,141)	
III	12 (36.4)	49,736,250 (945,000-549,000,000)		4,374.25 (38.6-13,600)		343,351 (3,610-300,000)	
Grade ^a			0.254, 0.246		0.282, 0.198		0.626, -0.139
I	5 (15.2)	2,914,000 (1,360,000-5,590,000)		5,950 (2.51-16,200)		8,501 (3,489-2,237.2)	
II	19 (57.6)	33,083,000 (652,000-549,000,000)		5,265.7 (17-15,600)		2,689,931 (4,170-39,840,637)	
III	9 (27.3)	4,697,777.7 (1,790,000-9,040,000)		9,221.1 (38.6-29,400)		6,496 (3,610-1,134.8)	
Tumor size (cm) ^b			0.659, 0.148		0.839, -0.051		0.296, -0.176
<2	6 (18.2)	10,800,000 (1,570,000-19,500,000)		1,800 (2.51-16,200)		52,521 (1,478-39,840,637)	
≥2- <5	16 (48.5)	2,550,000 (652,000-9,040,000)		4,510 (17-29,400)		4,105 (4,170-300,000)	
≥5	11 (33.3)	2,640,000 (652,000-549,000,000)		2,690 (38.6-13,600)		956 (3,610-300,000)	
Lymph node involvement			0.512 t(31)=-0.748		0.785 t(31)=0.490		0.365 t(26)=1.332
No	12 (36.4)	5,354,333.3 (652,000-19,500,000)		6,527.7 (2.51-16,200)		39,952 (4,170-39,840,637)	
Yes	21 (63.6)	29,579,761.9 (945,000-549,000,000)		6,402.6 (17-29,400)		17,623 (3610-300,000)	
Estrogen receptor status ^a			0.702, 0.136		0.045, 0.416		0.239, -0.311
Negative	8 (24.2)	2,390,000 (652,000-10,000,000)		7,410 (2.51-15,600)		39,276 (4,170-398,406,375)	
Positive	11 (33.3)	3,070,000 (945,000-549,000,000)		1,930 (38.6-13,100)		18,661 (1,478-30,000,000)	
+	5 (15.2)	3,010,000 (1,410,000-19,500,000)		3,590 (27.3-29,400)		543,175 (1,023-16,400,000)	
++	9 (21.3)	3,030,000 (1,490,000-8,360,000)		4,900 (1,770-15,600)		69,197 (9,550-223,729)	
+++	10 (30.3)	2,550,000 (1,500,000-549,000,000)		1,210 (2.51-15,500)	0.119, 0.334	39,276 (4,170-398,406,375)	0.045, -0.78
Progesterone receptor status ^a			0.297, -0.179		0.289, -0.228		0.985, 0.005
Negative	10 (30.3)	3,720,000 (652,000-19,500,000)		6,030 (38.6-29,400)		91,335 (955-543,175)	
Positive	7 (21.1)	1,870,000 (1,490,000-3,190,000)		5,180 (27.3-16,200)		36,100 (1,478-816,061)	
+	6 (18.2)	4,740,000 (1,360,000-7,440,000)		3,800 (1,770-8,040)		103,010 (30,909-223,729)	
++	24 (72.7)	3,050,000 (945,000-549,000,000)	0.594, -0.122	4,000 (2.51-16,200)		84,141 (4,170-398,406,375)	
+++	2 (6)	3,450,000 (1,790,000-5,100,000)		10,400 (7,100-13,600)		31,356 (25,211-37,500)	
Her2-neu receptor status ^a							
Negative	2 (6)	2,540,000 (652,000-4,420,000)		8,760 (1,910-15,600)		117,797 (4,179-231,414)	
Positive	5 (15.3)	2,090,000 (1,500,000-1,100,000)		1,210 (17-29,400)		83,969 (1,032-146,281)	

^aSpearman's correlation coefficient was used. ^bPearson's correlation coefficient was used. Plasma levels are expressed the mean (range). ccfnmtDNA, circulating cell-free mitochondrial DNA; ccfnDNA, circulating cell-free nuclear DNA.

present study also revealed a positive correlation between ccfndNA levels and ER status, which may be linked to the role of ER signaling in downregulating the DNA damage and repair pathway in mammary tissues (23). In healthy tissues, ER activity was discovered to be governed by highly regulated genetics that keep ER activity under control (24). Furthermore, dysregulated ER signals were indicated to lead to increased proliferation, the accumulation of DNA damage and eventually tumorigenesis (23,25). This increase in the rate of proliferation was reported to lead to increased levels of ccfndNA in the plasma of patients with breast cancer (17). On the other hand, a negative correlation was identified between the ccfmtDNA/ccfndNA ratio and the PR status. Similar to ER-positive cells, PR-positive cells demonstrate loss of or alterations in the DNA damage and repair pathway or checkpoint controlling system, and an increased proliferation rate, eventually leading to cancer formation (26). Of note, all previous research in this field has reported positive trends in these particular parameters, which is promising (15-17); however, the conflicting results should be followed up with large-scale studies and a mutation analysis to clarify the relationship between ccfmtDNA, ccfndNA and breast cancer.

In conclusion, a positive correlation was identified between ccfmtDNA/ccfndNA levels and age in healthy individuals. In addition, a positive correlation was observed between ccfndNA and the ER status, while a negative correlation was observed between ccfmtDNA/ccfndNA levels and the PR status. Despite the small sample size, these results suggested that the plasma levels of ccfmtDNA and ccfndNA may serve as a non-invasive biomarkers; however, large-scale studies are required to validate the results and clarify the conflicting results.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MS designed the study and performed the molecular biology studies. MS and ARN analyzed the data; MS drafted the manuscript and ARN revised it. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Informed consent forms were signed by all participants and the study was approved by the Syrian Higher Commission for

Scientific Research, Ministry of Higher Education, Damascus, Syria.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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