Received: 9 June 2020 | Revised: 31 August 2020 | Accepted: 27 September 2020

DOI: 10.1002/mc.23261

RESEARCH ARTICLE

Molecular Carcinogenesis WILEY

Diagnostic potential of circulating cell-free nuclear and mitochondrial DNA for several cancer types and nonmalignant diseases: A study on suspected cancer patients

Kristina Sundquist | Jan Sundquist | Anna Hedelius | Ashfaque A. Memon

Center for Primary Health Care Research, Lund University, Malmö, Sweden

Correspondence

Ashfaque A. Memon, Wallenberg Laboratory, Center for Primary Health Care Research, Skåne University Hospital, 6th floor, Inga Marie Nilsson's gata 53, S-205 02 Malmö, Sweden.

Email: ashfaque.memon@med.lu.se and ashfaque.a.memon@gmail.com

Funding information

Allmänna Sjukhusets i Malmö Stiftelse för Bekämpande av Cancer; ALF region Skåne; Vetenskapsrådet

Abstract

Circulating cell-free nuclear DNA (nDNA) has been implicated in individual cancer types with a diagnostic value; however, the role of cell-free mitochondrial DNA (mtDNA) in cancers is controversial. We aimed to investigate and compare the diagnostic potential of both nDNA and mtDNA for multiple cancers and to investigate their ability to distinguish multiple cancers from healthy controls and from nonmalignant diseases. We also investigated the prognostic value of both nDNA and mtDNA. The absolute copy number of circulating DNAs in suspected cancer patients (n = 286) referred to a cancer diagnostic center and healthy controls (n = 109) was quantified by droplet digital polymerase chain reaction. Among the suspected cancer patients, 66 (23%) were diagnosed with various cancers, 193 (67%) with nonmalignant diseases, and 27 (10%) with no active disease. Levels of nDNA were significantly higher in cancers (copies/μl; mean ± *SD*, 21.0 ± 14.2) as compared with nonmalignant diseases (15.2 ± 10.0) and controls (9.3 ± 4.1). In contrast, levels of mtDNA were significantly lower in cancers (copies/μl; mean ± *SD*, 68,557 ± 66,663) and nonmalignant diseases (60,174 ± 55,831) as compared with controls (98,714 ± 77,789). Receiver operating curve analysis showed that nDNA not only could distinguish multiple cancers from controls (area under curve [AUC] = 0.78; 95% confidence interval [CI] = 0.70–0.86) but also from nonmalignant diseases (AUC = 0.68; 95% CI = 0.59–0.76). However, mtDNA could only differentiate cancers from controls (AUC = 0.65; 95% CI = 0.56–0.73). Higher levels of nDNA were also associated with increased mortality in the cancer patients (hazard ratio = 2.3; 95% CI = 1.1–4.7). Circulating cell-free nDNA, but not the mtDNA, could distinguish multiple cancers from nonmalignant diseases and was associated with poor survival of cancer patients.

KEYWORDS

biomarker, cancer, circulating DNA, diagnostic, mitochondrial DNA, nuclear DNA

1 | INTRODUCTION

Circulating cell-free DNA (ccf DNA) is a group of DNA fragments present in the blood in both healthy individuals and those with pathological conditions.[1] The presence of circulating nucleic acids (CNAs) in peripheral blood was discovered in 1948[2]; however, its association with cancer was described later by Leon et al.[3] in the 1970s. The substantially increased amount of ccf DNA in blood of cancer patients is believed to originate from tumor cells by apoptosis or necrosis, active secretion from tumor cells, and/or direct release from circulating tumor cells.[1,4,5] With the emergence of recent advanced technologies, levels of ccf DNA have been proposed as useful noninvasive diagnostic and prognostic biomarkers of cancer, and its utility in assessment of treatment response and relapse risk has been investigated in clinical settings.[6] Moreover, studies have also focused on detection of tumor-specific DNA (by analyzing tumor-specific mutations in ccf DNA), termed as liquid biopsy, which is a fraction of total ccf DNA[7] that is expected to correlate with tumor burden and also with the ccf DNA. However, as total ccf DNA includes both tumor-specific DNA as well as DNA released from surrounding tissues and peripheral cells,[1,8,9] total ccf DNA may provide a more detailed picture of the complex biology of cancer pathophysiology. Levels of both ccf nuclear DNA (nDNA) and ccf mitochondrial DNA (mtDNA) have been investigated in individual cancer types and their diagnostic value have been evaluated by comparisons mainly with healthy controls and in few studies with nonmalignant diseases as well.[10–13] However, their role as a universal cancer diagnostic marker has not been established. Moreover, as a result of considerable differences in DNA purification and analytical techniques, it is difficult to compare the levels of ccf DNA across studies. To the best of authors' knowledge, the roles of both nDNA and mtDNA as diagnostic biomarkers have not previously been evaluated simultaneously in the same study population and for multiple cancer types. Our aim was to simultaneously quantify both nDNA and mtDNA and investigate their role as universal cancer diagnostic biomarkers by investigating their levels in newly diagnosed multiple cancer types and nonmalignant diseases as well as in healthy controls. We also aimed to investigate their role in the overall survival of cancer patients and their correlations with clinicopathological parameters of cancer.

2 | MATERIALS AND METHODS

2.1 | Study population

In October 2012, a project was initiated within the primary healthcare in Region Skåne in southern Sweden for fast-track diagnosis of patients with suspected cancers but with nonspecific cancer symptoms rather than specific ones; in these efforts, a diagnostic center (DC) for early detection and better prognosis for cancer patients was established, as described previously.[14] Briefly, primary healthcare centers were invited to refer patients who were 18 years or older with one or more of the following symptoms: fatigue, weight loss more than 5 kg, pain/joint pain, prolonged fever, unexplained pathological lab results, for example, erythrocyte sedimentation rate, serum alkaline phosphatase, serum calcium, hemoglobin, or suspected metastasis.

When a baseline investigation at the primary health care centres could not explain the symptoms, patients with suspected cancer were referred to the DC for a faster and more extensive examination. The study was performed according to the Declaration of Helsinki. The regional ethical committee at Lund University approved the study (approval no. 2012/449) and written informed consent was given by all the participants in the study after full explanation of the purpose and nature of all procedures.

As of December 2015, a total of 393 consecutive suspected cancer patients were referred to the DC. Of these, 103 patients were excluded due the following reasons: declined participation ($n = 23$), had severe psychological disorders or dementia or were too ill for outpatient investigation ($n = 38$), did not fulfill the referral criteria, as mentioned above ($n = 17$), did not speak Swedish ($n = 15$), and/or referral from another unit than a primary healthcare center ($n = 10$). The remaining 290 patients were objectively evaluated at the DC and 286 had blood samples available for ccf DNA quantification. Of 286 consecutive patients investigated at DC, a total of 66 patients were diagnosed with cancer (solid tumors, $n = 50$ and hematological malignancies, $n = 16$), 193 with nonmalignant diseases, and 27 were judged to be healthy; a full classification of the study population is shown in Figure S1. In addition to the 27 subjects at the DC judged as healthy, 109 additional healthy blood donors were also included as a control group. This group comprised of volunteer blood donors and were recruited from Skåne blood center. All the individuals met the Swedish Blood donor criteria for blood donation and must answer several questions provided by the blood donation authority.[15] Blood donors in Sweden are not paid any money for their blood donation. DC population has been used in our previous study where we developed and optimized the droplet digital polymerase chain reaction (ddPCR) method for accurate quantification of mtDNA obtained from peripheral blood.[14] In this study, we have used samples from the same population; however, here we have quantified cell-free circulating mtDNA and nDNA obtained from plasma samples and investigated and compared diagnostic value of both cell-free nDNA and mtDNA to distinguish multiple cancers from healthy controls and from nonmalignant diseases.

2.2 | DNA purification

Whole blood samples were collected in ethylenediaminetetraacetic acid tubes and were centrifuged at 2000*g* for 10 min within 8 h of sample collection. Plasma was carefully removed in 1-ml fresh tubes and stored at −80°C for DNA extraction. Double centrifugation of plasma has been suggested for quantification of cell-free DNA.[16] We prepared our samples with and without additional centrifugation (16,000*g* for 10 min) and quantified nDNA and mtDNA in both pellet and supernatant with ddPCR. Pellet obtained after a second centrifugation showed a significant amount of mtDNA (90%) as well as nDNA (30%; Figure S2A), most probably due to aggregation of extracellular vesicles in pellet, which may contain a significant amount of DNA, as also shown in a

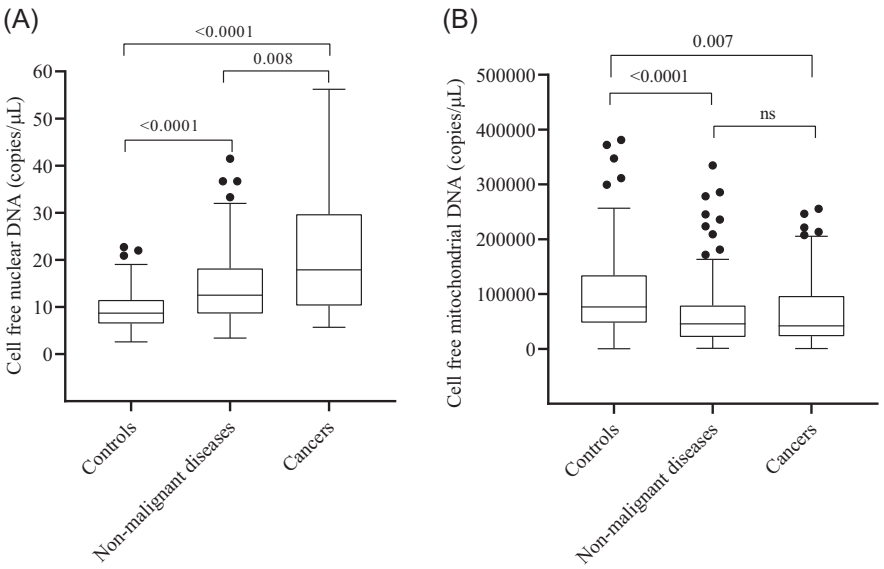

FIGURE 1 Tukey box and whiskers plot showing the levels of (A) nuclear DNA and (B) mitochondrial DNA in controls, cancers, and other diseases diagnosed at the diagnostic center. The line within the boxes indicates the median value, whereas the outer boxes represent the 25th and the 75th percentiles and whiskers show the nonoutlier range. *p* Values are calculated by the two-tailed *t*-test (Mann–Whitney *U*-test)

previous study.[16] Furthermore, another study on quantification of nDNA by ddPCR also suggests that a high-speed second centrifugation is not required.[17] Therefore, in this study, we did not perform second centrifugation of samples. We also compared purification kits previously used for DNA purification such as Qiagen Blood Mini Kit with QIAamp CNA Kit from Qiagen. Our results show that Qiagen Blood Mini Kit used in a previous study[16] was not suitable for small amplicon size (<200 base pairs [bps]) and resulted in a significantly lower yield. However, CNA Kit showed a purification yield efficiency of more than 85% even for amplicon size less than 200 bp (Figure S2B); these results are also in agreement with a previous study.[18]

Total ccf DNA was extracted from 1 ml of plasma samples obtained from 286 suspected cancer patients (Figure 1) and 109 healthy controls using CNA Kits, Qiagen, according to the manufacturer's instructions. Each plasma sample was spiked in with synthetic DNA template (TATAA Universal DNA Spike 166 bp; TATAA Biocenter), which is not present in any known living organism. DNA was eluted in 100 µl of AVE buffer (RNase-free water + 0.04% NaN3). The Spike 166-bp assay amplifies a 69-base region of the synthetic template, and to check the efficiency of DNA purification, synthetic spiked-in DNA was quantified in each sample by ddPCR and samples with more than 90% yield were included.

2.3 | Droplet digital PCR

The absolute copy number of nDNA and mtDNA was quantified simultaneously by ddPCR. In this study, ddPCR system included an automated droplet generator and reader from Bio-Rad, (QX200 ddPCR; Bio-Rad), which fractionates samples into ~20,000 droplets. For mtDNA quantification, primers and probes targeting the mitochondrially encoded NADH dehydrogenase 1 (*MT-ND1*) were used, and for nDNA quantification, primers and probes targeting the eukaryotic translation initiation factor 2C, 1 (*EIF2C1*), also known as argonaute 1, RISC catalytic component (Gene ID: 26523), were used for quantification of the absolute copy number by ddPCR. All primer and probes were obtained from Bio-Rad. Sequence and other information about primers and probes are available at www.bio-rad.com with the following ID numbers: MT-ND1 (assay ID: dHsaCPE5029120, sequence accession number: NC_012920.1, and context sequence: CTCTAGCCTAGCCGTTTACTCAATCCTCTGATCAGGGTGAGCATCAAACTCAAACTACGCCCTGATCGGCGCACTGCGAGCAGTAGCCCAAACAATCTCATATGAAGTCACCCTAGCCATCATTCTACTATCAACATTACTAATAA), EIF2C1 (assay ID: dHsaCP1000002, sequence accession number: NM_012199.2, and context sequence: TGGTTCGGCTTTCACCAGTCTGTGCGCCCTGCCATGTGGAAGATG ATGCTCAACATTGATGGTGAGTGGGGAGAGCTATGGAGCCAGGGGCACCCCAAGTCCAGTGACCACACTCCCAGCCTC).

All probes had Iowa Black® FQ quencher. Amplicon sizes were 86 and 117 bp for mtDNA and nDNA, respectively. The ddPCR method was performed according to manufacturer's instructions, with some modifications as described below. First, amplification was performed in a 20 µl multiplex reaction purified ccf DNA from plasma and 900 nM of primers and 250 nM of probes, 2X ddPCR Supermix for probes (no UTP). Samples were subjected to droplet generation by an automated droplet generator, and later, endpoint PCR was performed. Cycling steps for the ddPCR were as follows: Initially, an enzyme activation at 95°C for 10 min (1 cycle), followed by 40 cycles of denaturation and annealing (each cycle at 94°C for 30 s and 60°C for 1 min), and finally enzyme deactivation at 98°C for 10 min (1 cycle). The PCR plate was incubated overnight at 4°C. This additional step significantly improved the droplet recovery to maximum (19,000–20,000 droplets). Finally, droplets were read on a droplet reader and data were analyzed using QuantaSoft™ Software that determines the numbers of droplets that were positive and negative for each fluorophore in each sample. The fraction of positive droplets was then fitted to a Poisson distribution in QuantaSoft Software to determine the absolute copy number in units of copies/µl. DNA preparation and PCR experiments were performed in separate designated rooms. All samples were randomly quantified among plates

TABLE 1 Characteristics and levels of cell-free nDNA and mtDNA (copies/μl) in DC samples and healthy donors

Classification	Age Median (min–max)	Gender %Men/women	nDNA Mean ± *SD*	*p*-Value	Adjusted *p*-value[a]	mtDNA Mean ± *SD*	*p*-Value	Adjusted *p*-value[a]
Controls	48 (20–87)	57/43	9.3 ± 4.1	**<.0001**[b]	**.001**[b]	98,714 ± 77,789	**.007**[b]	.41[b]
Cancer (all)	71 (20–89)	59/41	21.0 ± 14.2	**.008**[c]	**.04**[c]	68,557 ± 66,662	.8[c]	.98[c]
Hematologic	71 (52–82)	56/44	27.6 ± 17.4			81,962 ± 85,947		
Solid	72 (20–89)	56/44	19.0 ± 12.7			64,267 ± 59,632		
Other diseases (all)	68 (18–90)	46/54	15.2 ± 10.0	**<.0001**[d]	**<.0001**[d]	60,174 ± 55,831	**<.0001**[d]	**<.0001**[d]
Clinicopathological parameters								
Distant metastasis								
No	70 (61–83)	50/50	15.3 ± 16.5	.3	.24	72,831 ± 44,335	.6	.6
Yes	71 (65–82)	71/29	23.2 ± 13.2			88,734 ± 75,906		
Nodal involvement								
No	70 (61–83)	54/46	15.1 ± 9.8	0.3	.27	102,406 ± 79,634	.4	.5
Yes	72 (67–79)	73/27	21.0 ± 13.3			77,177 ± 58,077		
T stage								
Ta–T1	69 (61–79)	57/43	11.4 ± 7.0	**.04**	.07	81,242 ± 70,656	.9	.7
T2–T4	72 (65–83)	65/35	24.0 ± 15			86,859 ± 70,026		

Abbreviations: DC, diagnostic center; mtDNA, mitochondrial DNA; nDNA, nuclear DNA.

[a]Adjusted for age and sex, logistic regression analysis.

[b]Control vs. cancer.

[c]Cancer vs. other diseases.

[d]Control vs. other diseases.

and each plate was included with two positive controls (high and low) and two negative controls.

To account for a possible overestimation of the circulating DNA by leukocyte (genomic DNA) contamination in the preanalytical phase, a unique B-cell immunoglobulin DNA rearrangement (PBC) was quantified, as described previously.[19] This approach improves the quality of the analysis and lowers the risk of falsely increased values. A total of 16 samples were PBC-positive, that is, contaminated with genomic DNA (>0.1% of the total ccf DNA), and were excluded as described previously[19]; nine were of controls, five of other diseases, and two of cancers.

2.4 | Statistics

Characteristics of the participants and ccf DNA levels, that is, both nDNA and mtDNA, in each group are presented in Table 1. Pearson χ^2 test was used to compare dichotomized variables and continuous variables were compared using Mann–Whitney *U*-test. Multivariate logistic regression analysis was performed to adjust for age and sex (Table 1). Linearity of the assays was tested by linear regression and R^2 was calculated for the best fit. We used receiver operating characteristic (ROC) curves to analyze the diagnostic potential of both the nDNA and mtDNA. This curve plot represents the true positive rate (also known as sensitivity) against the false positive rate (1-specificity), and the accuracy is measured by the area under the curve (AUC). An AUC value of 1 represents a perfect predictive power, whereas an AUC of 0.5 indicates no predictive power. The Kaplan–Meier curve was plotted to calculate the overall survival of cancer patients and log-rank test was used to compare the groups with high (>median) and low (≤median) ccf DNA. Overall survival was calculated from the date of inclusion until death from any cause. Multivariate Cox regression analysis was performed to further examine whether the ccf DNA was associated with survival when controlling for T-stage of the cancer, age, and sex. Statistical analyses were carried out in SPSS software version 23 (IBM). Graphs and figures were prepared in the GraphPad prism software version 8. 1.2. The data that support the findings of this study are available from the corresponding author upon reasonable request.

3 | RESULTS

3.1 | Quantification of nDNA and mtDNA in cancer and nonmalignant diseases

Participants' characteristics and levels of nDNA and mtDNA are presented in Table 1, the latter as copies/μl. Of the healthy controls, 57% were males and 43% were females with median age of 47 (range: 20–87) and 47 (20–80), respectively. Of the cancer patients, 59% were males and 41% were females, with a median age of 71 (range: 20–89) and 73 (61–89), respectively. Finally, of the patients diagnosed with nonmalignant diseases, 46% were males and 54% were

females, with a median age of 68 (range: 21–86) and 69 (18–90), respectively. No significant difference in the distribution of gender was found across the groups (χ^2 test for trend; $p > .05$). However, the controls were younger (median age [range], 48 [20–87]), compared with the cancer patients (71 [20–89]; $p < .0001$) and patients with nonmalignant diseases (68 [18–90]; $p < .0001$). Compared with the controls (mean ± *SD*; 9.3 ± 4.1 copies/μl), the levels of nDNA were significantly higher in all cancers (21.0 ± 14.2; $p < .0001$) and nonmalignant diseases (15.2 ± 10.0; $p < .0001$). Levels of nDNA were also significantly different between cancer and nonmalignant diseases ($p = .008$), as shown in Figure 1. Levels of nDNA were positively correlated with age ($\rho = 0.4$; $p = <.0001$) –and mtDNA levels were negatively correlated with age ($\rho = -0.14$; $p = .005$). Among clinicopathological parameters of cancer, nDNA levels were significantly associated with advanced stage of cancer (T2–T4) as compared with early stage (Ta–T1) but not with nodal involvement or distant metastases (Table 1). All the abovementioned differences were adjusted for age and sex by multivariable logistic regression analysis, and results showed that the differences between controls, nonmalignant, and cancer groups were independent of age and sex (Table 1). Moreover, levels of nDNA were higher in hematologic cancers (27.6 ± 17.4) as compared with solid cancers (19.0 ± 12.7); however, these differences were not significant ($p = .11$). Therefore, further analysis on cancer patients was performed in a combined group. Comparisons of nDNA levels between each group are shown in Figure 1.

In contrast to nDNA, levels of mtDNA were significantly lower in cancers (68,557 ± 66,662; $p = .007$) and nonmalignant diseases (60,174 ± 55,831; $p < .0001$) as compared with controls (98,714 ± 77,789); however, there was no significant difference in mtDNA levels between cancer and nonmalignant diseases ($p = .8$), as shown in Table 1. Logistic regression analysis indicated that the differences in mtDNA levels between the controls and the cancers were confounded by age (0.41), as shown in Table 1. Levels of mtDNA were not associated with any clinicopathological cancer parameter analyzed in this study (Table 1). Comparisons of mtDNA levels between each group are shown in Figure 1.

As the individuals in the control group were younger than in the cancer and nonmalignant groups, we also stratified and compared our data according to the age group (20–40, 41–70, and >70), and our results show that, compared with healthy controls, the cDNA levels were higher in the groups with nonmalignant diseases. In addition, the cancer group had even higher levels of nDNA than both the healthy controls and those with nonmalignant diseases in the different age groups. The levels of mtDNA were higher in the healthy controls aged 20–40 and 41–70 years and in the cancer group; however, the differences were lost when compared in the older age group (data are not shown). These data are in line with the results presented in Table 1 where we show that the differences in mtDNA levels in the controls and in the cancer group are confounded by age.

Data were also stratified according to the common cancer types diagnosed in this study, and the results show that colon cancer ($n = 8$, mean ± *SD*, 34,013 ± 37,206) had the lowest mtDNA levels, which were significantly different from the controls (98,714 ± 77,789). Levels of mtDNA in lung cancer ($n = 13$, 91,309 ± 76,103) and hematologic cancers ($n = 16$, 81,962 ± 85,947) were significantly higher than in colon cancer but were not significantly different from the controls ($p > .05$). Levels of mtDNA in the remaining cancer types (60,492 ± 37,206; $p = .001$) were also significantly lower than in the controls.

3.2 | Diagnostic potential of nDNA and mtDNA as universal cancer biomarkers

To evaluate the diagnostic potential of nDNA and mtDNA, we performed an ROC curve analysis. AUC values with 95% confidence interval (CI) were calculated. ROC curve analysis showed that nDNA not only could distinguish the cancers from controls (AUC = 0.78; 95% CI = 0.70–0.86) but also from nonmalignant diseases (AUC = 0.68; 95% CI = 0.60–0.76; Figure 2, upper panel). However, mtDNA could only distinguish cancers from healthy controls and that also with a lesser efficiency than the nDNA (AUC = 0.65; 95% CI = 0.56–0.73). Furthermore, mtDNA had no diagnostic value in distinguishing cancers from nonmalignant diseases (AUC = 0.55; 95% CI = 0.47–0.63), as shown in Figure 2, lower panel. We also investigated whether the mtDNA and nDNA ratio (MNR) could be a better diagnostic biomarker. Our results showed that the MNR, compared with nDNA alone, was not superior in distinguishing cancer from healthy controls (AUC = 0.76; 95% CI = 0.69–0.83) or cancers from nonmalignant diseases (AUC = 0.62; 95% CI = 0.54–0.70).

3.3 | Association between overall survival of cancer and levels of nDNA and mtDNA

Kaplan–Meier survival curve analysis showed that patients with higher baseline nDNA levels (median > 10.9 copies/μl) had significantly higher overall mortality in cancer patients as compared with lower nDNA levels (hazard ratio [HR] = 2.3; 95% CI = 1.1–4.7, log-rank test = 0.046; Figure 3). Multivariate Cox regression analysis adjusted for age, sex, and T-stage of the cancer also showed an association between higher levels of nDNA and higher overall mortality (HR = 2.3; 95% CI = 0.84–6.5); however, results did not reach statistical significance ($p = .1$). We also investigated baseline mtDNA as a predictor of overall mortality of cancer patients, and our results showed that it was not significantly associated with overall mortality (HR = 0.8; 95% CI = 0.4–1.7, log-rank test $p = .53$).

4 | DISCUSSION

In this study, we have quantified both ccf nDNA and mtDNA and investigated their role as universal cancer diagnostic biomarkers by comparing their levels in several cancers, nonmalignant diseases, and healthy controls. Our results showed that nDNA could distinguish

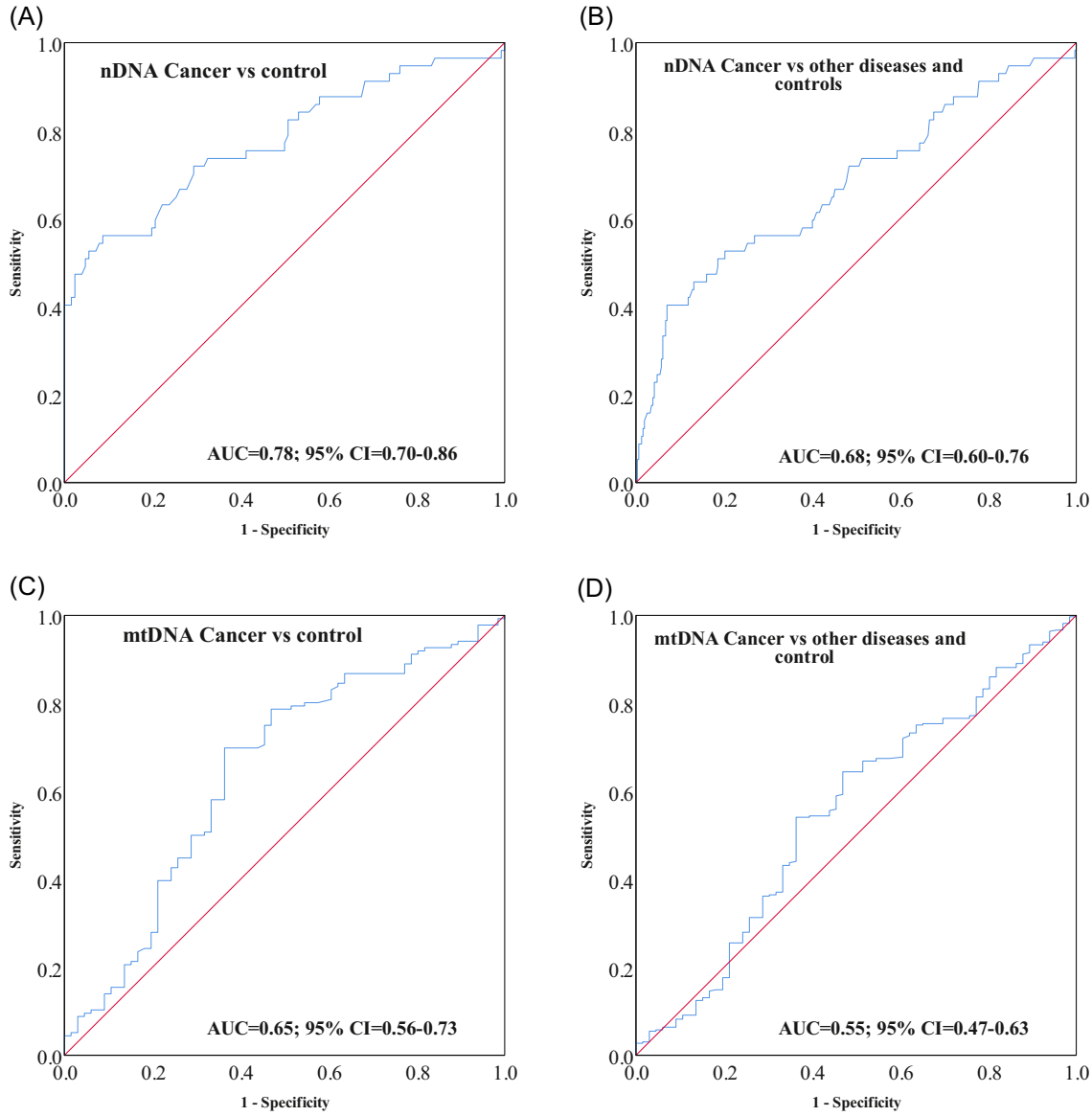

FIGURE 2 Receiver operating characteristic (ROC) curve analysis for diagnostic potential of nuclear DNA (nDNA) for distinguishing (A) cancers from controls (sensitivity = 72% and specificity = 71%) and (B) cancers from other nonmalignant diseases and controls (sensitivity = 72% and specificity = 52%). ROC curve analysis for diagnostic potential of mitochondrial DNA (mtDNA) for distinguishing (C) cancers from controls (sensitivity = 70% and specificity = 54%) and (D) cancers from other nonmalignant diseases and controls. Levels of mtDNA were unable to distinguish cancer from other diseases and controls. Blue lines indicate ROC curves and red lines indicate reference. AUC, area under the curve; CI, confidence interval [Color figure can be viewed at wileyonlinelibrary.com]

cancers from controls as well as from nonmalignant diseases, and it was also associated with poor overall survival of cancer patients. Levels of mtDNA could distinguish between cancers and controls but had no diagnostic value in distinguishing cancers from nonmalignant diseases. To the best of authors' knowledge, this is the first study in which the diagnostic and prognostic potential of both nDNA and mtDNA have been investigated in several cancers by comparisons with both nonmalignant diseases and healthy controls.

Cancer is a multifactorial heterogeneous disease and its early diagnosis is important for better management and outcome. In Sweden, DCs have been established to prioritize earlier cancer diagnoses once the first symptoms have occurred.[20] These centers spend huge resources to perform prioritized thorough investigations to diagnose cancer as early as possible. One of these centers is based in our county where the suspected cancer patients, in our study, were sent for an early diagnosis. Among the referred patients, only 23% were diagnosed with cancer, whereas 67% were diagnosed with other nonmalignant diseases. Therefore, there is a need to develop biomarkers that can help to distinguish several cancers from nonmalignant diseases to optimize the use of healthcare resources.

The role of ccf DNA, and especially nDNA, in cancer has been well studied; however, it is mostly investigated in individual cancers

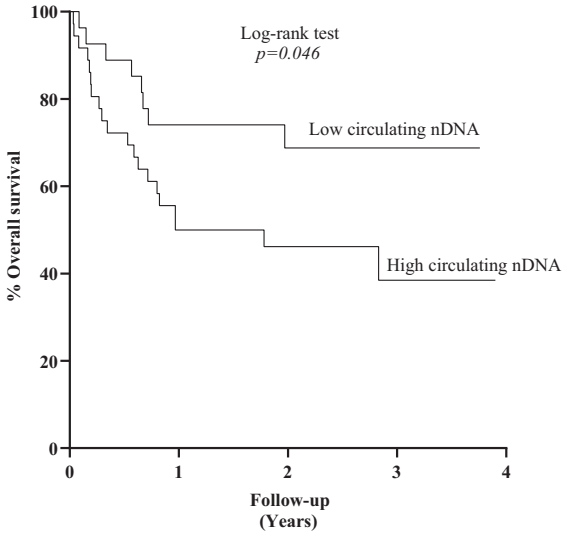

FIGURE 3 Analysis of Kaplan–Meier curve plotted between high (>median) and low nuclear DNA (nDNA; ≤median) to calculate overall survival of patients diagnosed with multiple cancer types. Higher levels of nDNA were associated with significantly shorter overall survival of cancer patients as compared with lower levels of nDNA. Levels of mitochondrial DNA were not associated with overall survival (data are not shown)

and compared only with healthy controls. The main dilemma of cancer diagnostics is to distinguish cancers from nonmalignant diseases, which may, on the first clinical assessment, appear to be malignant. In this study, we have not only compared the levels of both nDNA and mtDNA among several cancers, nonmalignant diseases, and healthy controls but also investigated their diagnostic potential in distinguishing cancer patients from nonmalignant diseases. There are very few studies published on ccf DNA in multiple cancers. For instance, Bettegowda et al.[10] have performed a well-designed study in which they have compared nDNA levels in early and late stages of various cancer types. They found significantly higher levels of nDNA in most of the late-stage cancers, which is in accordance with the higher levels of nDNA found in the invasive stage of cancers (T2–T4) in the present study. To further this, we also showed that nDNA can distinguish multiple cancer types from nonmalignant diseases as well as from healthy controls; nDNA thus has a potential to be used as a universal cancer biomarker.

We could find only one study where both the nDNA and mtDNA have been investigated but only in breast cancer. In line with our study, Kohler et al.[21] also found significantly higher levels of nDNA in breast cancer patients as compared with the healthy controls as well as in nonmalignant diseases. Moreover, for mtDNA, the authors found significantly lower levels of mtDNA in breast cancer patients when compared with healthy controls diseases, which is also consistent with our findings. Interestingly, the authors found even lower levels of mtDNA in nonmalignant diseases than in cancer. In contrast, we did not find any significant difference in mtDNA levels between cancer and nonmalignant diseases. In addition, a study on colorectal cancer also showed significantly higher levels of nDNA when compared with nonmalignant diseases and healthy controls.[11] Consistent with our results, they also showed that higher levels of nDNA were associated with shorter overall survival.[11] Among clinicopathological parameters, we observed an association between nDNA and T-stage only, whereas, in agreement with previous results, no associations were found between nDNA and nodal involvement and distant metastasis.[21]

The role of mtDNA is controversial in cancers and is attributed to technical issues[22]; however, most studies suggest a depletion of mtDNA content in cancers,[23] which corresponds with the findings in this study. However, we found a significant variation in mtDNA levels across cancer types. In addition, levels of mtDNA were confounded by age. Our results suggest that the association between mtDNA and cancers depends on cancer type and age. In agreement with our results, another study on ovarian cancers could not find any significant difference in levels of circulating mtDNA between controls and those with cancer.[24] Furthermore, inability of mtDNA in distinguishing cancer patients from nonmalignant diseases suggests that mtDNA levels may not be a useful cancer diagnostic biomarker for all cancer types. Our results demonstrate that the mtDNA depletion is a hallmark of some cancers and also of nonmalignant diseases; however, its role as a diagnostic biomarker needs further investigation.

Previous studies suggest that MNR may better distinguish healthy controls from cancer patients[16]; however, our results suggest that MNR was not superior to the individual levels of nDNA in distinguishing cancers from healthy controls or from nonmalignant diseases.

ddPCR provides an accurate and absolute quantification of ccf DNA quantification without a standard curve.[14] We performed few modifications in the sample preparation, which could affect an accurate quantification of ccf DNA in plasma. For example, we did not perform double centrifugation on plasma samples, as suggested previously for ccf DNA extraction.[16] In another study on nDNA, double centrifugation was not recommended.[17] However, none of the above studies compared the effect of double centrifugation on extraction efficiency of mtDNA. Our results show that double centrifugation results in a significant loss of mtDNA (90%) in pellet. Finally, comparison of extraction kits revealed that the CNA Kit was ideal for purification of smaller DNA (<200 bp) fragments from plasma. Qiagen Blood Mini Kit used in a previous study[16] for purification resulted in a lower DNA yield, as shown in this study and also confirmed by others.[18] Taken together, we do not recommend double quantification, especially for cell-free mtDNA quantification and DNA extraction before quantification of ccf DNA, which should be performed by kits suitable for purification of smaller DNA fragments, for example, CNA Kit.

The main strength of this study is that we investigated the diagnostic potential of nDNA and mtDNA by simultaneously quantifying their levels in multiple pathologies in a single study where patients were clinically diagnosed at the same DC without inclusion biases as patients were consecutively included in the study. There were some limitations with the study as well; for

instance, due to the case-control nature of the study, the causal relationship between the exposure and the effect could not be determined. Our results should be interpreted with caution, as the number of cancer patients was relatively small; therefore, further studies are required to investigate the clinical applicability of nDNA as general cancer diagnostic biomarkers.

In conclusion, we have investigated the role of both nDNA and mtDNA as universal cancer diagnostic biomarkers. Our results suggest that levels of nDNA have the potential to distinguish cancers from both nonmalignant diseases and healthy controls, whereas levels of mtDNA rather seem to be a biomarker of general illness.

ACKNOWLEDGMENTS

This study is supported by the Swedish Research Council and ALF funding from Region Skåne to Jan Sundquist and Kristina Sundquist. Allmänna Sjukhusets i Malmö Stiftelse för bekämpande av cancer to Ashfaque Memon. Authors would like to thank Patrick Reilly for proofreading the article.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Kristina Sundquist, Jan Sundquist, and Ashfaque A. Memon conceived and designed the study; Anna Hedelius and Ashfaque A. Memon performed the experiments and Kristina Sundquist and Ashfaque A. Memon performed the statistical analysis; Jan Sundquist, Anna Hedelius, and Ashfaque A. Memon collected the samples and clinical data. Kristina Sundquist, Jan Sundquist, and Ashfaque A. Memon performed the data analysis and interpretation; Kristina Sundquist and Ashfaque A. Memon wrote the first draft and Jan Sundquist, Anna Hedelius, and Ashfaque A. Memon revised the article and approved the final version.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the Swedish National Board of Health and Welfare; however, restrictions apply to the availability of these data, which were used under license for the current study, and so they are not publicly available.

ORCID

Ashfaque A. Memon http://orcid.org/0000-0002-1081-3553

REFERENCES

1. Jahr S, Hentze H, Englisch S, et al. DNA fragments in the blood plasma of cancer patients: Quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res*. 2001;61:1659-1665.
2. Mandel P, Metais P. [Nuclear acids in human blood plasma]. *C R Seances Soc Biol Fil*. 1948;142:241-243.
3. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res*. 1977;37:646-650.
4. Thierry AR, Mouliere F, Gongora C, et al. Origin and quantification of circulating DNA in mice with human colorectal cancer xenografts. *Nucleic Acids Res*. 2010;38:6159-6175.
5. Stroun M, Maurice P, Vasioukhin V, et al. The origin and mechanism of circulating DNA. *Ann N Y Acad Sci*. 2000;906:161-168.
6. Perkins G, Yap TA, Pope L, et al. Multi-purpose utility of circulating plasma DNA testing in patients with advanced cancers. *PLoS One*. 2012;7:e47020.
7. Diaz LA, Jr, Williams RT, Wu J, et al. The molecular evolution of acquired resistance to targeted egfr blockade in colorectal cancers. *Nature*. 2012;486:537-540.
8. Diehl F, Li M, Dressman D, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. *Proc Natl Acad Sci USA*. 2005;102:16368-16373.
9. Spindler KL, Pallisgaard N, Andersen RF, Brandslund I, Jakobsen A. Circulating free DNA as biomarker and source for mutation detection in metastatic colorectal cancer. *PLoS One*. 2015;10:e0108247.
10. Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med*. 2014;6:224ra24.
11. Spindler KL, Appelt AL, Pallisgaard N, Andersen RF, Brandslund I, Jakobsen A. Cell-free DNA in healthy individuals, noncancerous disease and strong prognostic value in colorectal cancer. *Int J Cancer*. 2014;135:2984-2991.
12. Kumar M, Srivastava S, Singh SA, et al. Cell-free mitochondrial DNA copy number variation in head and neck squamous cell carcinoma: a study of non-invasive biomarker from northeast india. *Tumour Biol*. 2017;39:101042831773664. https://doi.org/10.1177/1010428317736643
13. Afrifa J, Zhao T, Yu J. Circulating mitochondria DNA, a non-invasive cancer diagnostic biomarker candidate. *Mitochondrion*. 2019;47:238-243.
14. Memon AA, Zoller B, Hedelius A, et al. Quantification of mitochondrial DNA copy number in suspected cancer patients by a well optimized ddPCR method. *Biomol Detect Quantif*. 2017;13:32-39.
15. Blood donation in Sweden. https://geblodnu/english-engelska/
16. Meddeb R, Dache ZAA, Thezenas S, et al. Quantifying circulating cell-free DNA in humans. *Sci Rep*. 2019;9:5220.
17. Cavallone L, Aldamry M, Lafleur J, et al. A study of pre-analytical variables and optimization of extraction method for circulating tumor DNA measurements by digital droplet PCR. *Cancer Epidemiol Biomarkers Prev*. 2019;28:909-916.
18. Devonshire AS, Whale AS, Gutteridge A, et al. Towards standardisation of cell-free DNA measurement in plasma: controls for extraction efficiency, fragment size bias and quantification. *Anal Bioanal Chem*. 2014;406:6499-6512.
19. Pallisgaard N, Spindler KL, Andersen RF, Brandslund I, Jakobsen A. Controls to validate plasma samples for cell free DNA quantification. *Clin Chim Acta*. 2015;446:141-146.
20. Stenman E, Palmer K, Ryden S, et al. Diagnostic spectrum and time intervals in sweden's first diagnostic center for patients with nonspecific symptoms of cancer. *Acta Oncol*. 2019;58:296-305.
21. Kohler C, Radpour R, Barekati Z, et al. Levels of plasma circulating cell free nuclear and mitochondrial DNA as potential biomarkers for breast tumors. *Mol Cancer*. 2009;8:105.
22. Andreu AL, Martinez R, Marti R, Garcia-Arumi E. Quantification of mitochondrial DNA copy number: pre-analytical factors. *Mitochondrion*. 2009;9:242-246.
23. Yu M. Generation, function and diagnostic value of mitochondrial DNA copy number alterations in human cancers. *Life Sci*. 2011;89:65-71.

24. Keseru JS, Soltesz B, Lukacs J, et al. Detection of cell-free, exosomal and whole blood mitochondrial DNA copy number in plasma or whole blood of patients with serous epithelial ovarian cancer. *J Biotechnol*. 2019;298:76-81.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Sundquist K, Sundquist J, Hedelius A, Memon AA. Diagnostic potential of circulating cell-free nuclear and mitochondrial DNA for several cancer types and nonmalignant diseases: A study on suspected cancer patients. *Molecular Carcinogenesis*. 2020;59:1362–1370. https://doi.org/10.1002/mc.23261