Serum circulating cell free DNA as potential diagnostic and prognostic biomarker in non small cell lung cancer

Shimaa El-Shafey Soliman⁎, Alshimaa Mahmoud Alhanafy, Mona Salah El-din Habiba, Mohamed Hagagc, Reda Abdel Latif Ibrahemed

a Department of Medical Biochemistry & Molecular biology, Faculty of Medicine, Menoufia University, Shebin El-Kom, Menoufia Postal Code: 32511, Egypt
b Department of Clinical Oncology and Nuclear Medicine, Faculty of Medicine, Menoufia University, Egypt
c Department of Cardiothoracic Surgery, Faculty of Medicine, Menoufia University, Egypt
d Department of Public Health and Community Medicine, Faculty of Medicine, Menoufia University, Egypt

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ABSTRACT

Background: Non-small cell lung cancer (NSCLC) is leading cause of cancer related death and the survival rate for patients with NSCLC remain poor so early diagnosis of NSCLC represents the best opportunity for cure. Cell-free DNA (cf-DNA) is extracellular nucleic acids found in cell-free plasma/serum of humans, given the recent approval of a liquid biopsy in lung cancer, the use of circulating tumor DNA as a novel non-invasive diagnostic and prognostic biomarker is promising.

Objectives: Studying whether the concentrations of circulating Cell Free DNA in serum can be used as a diagnostic and prognostic biomarker for NSCLC patients.

Method: This study was carried out on 140 subjects included 60 patients with non small cell lung cancer, 40 patients with Chronic Obstructive Pulmonary Disease (COPD) and 40 healthy controls. Quantitative analysis of serum circulating cf-DNA was done by ALU-based quantitative real time PCR. Serum level of CEA was measured by ELISA.

Results: NSCLC patients demonstrated significantly higher values of each of ALU 215, ALU 247, and DNA integrity than both COPD patients and controls. On ROC curve analysis, the total accuracy of ALU 247, ALU 115, DNA integrity (92.1%, 83.6%, 56.4%) at cutoff points (325, 565 & 0.48) respectively. On combining both DNA integrity and CEA, improved sensitivity to 93.3% was noted. For NSCLC patients, ALU 115 & ALU 247 increased significantly with more advanced stage and highest level was noticed in metastatic patients. Regarding survival there was better overall survival among patients with low DNA integrity.

Conclusion: Serum cf-DNA concentrations and integrity index may be valuable tool in early diagnosis of NSCLC and prediction of prognosis of those patients.

1. Introduction

Lung cancer is driving reason of cancer-related deaths in the world and almost one million new cases in the world and about 8000 new cases in Egypt are expected annually by 2025. The greater part of patients is determined late with local or systemic advanced disease (stage III or IV) [1,2]. Non-small-cell lung cancer represents 80% of cases, including major subtypes such as lung adenocarcinoma, squamous cell carcinoma (SCC) and large cell carcinoma (LCC) [3]. Although novel treatment has been developed, 5-year survival rate was still under 15% because of its late diagnosis and poor outcome [4].

Lung, cervix, colon and breast cancers showed change in the patients’ outcome with screening programs and early detection. Yearly low dose computed tomography (CT) is the prescribed technique for screening for high risk populations for lung cancers; over 50 years old and heavy smokers but connected with radiation exposure and false positive and negative results, also COPD patients have higher hazard for developing lung cancer particularly smoker patients [5]. Employment of a sensitive assay that can help in early diagnosis of cancer by non-invasive, safe method is ideal for early detection. Carcinoembryonic antigen (CEA) is known as tumor marker for many cancers including lung cancer, however, not all lung cancer cases can be diagnosed by CEA alone owing to unstable detection and incremental concentrations in benign diseases [6].
Circulating cell-free DNA in plasma, which may be derived from cell necrosis, apoptosis, and/or digestion by macrophages, to determine the utilization of cfDNA as a new tool for diagnosing malignancy, checking treatment or even estimating prognosis this method is referred to as ‘fluid biopsy’ [7]. Indeed, even small tumors containing as few as 50 million cells discharge adequate DNA to be distinguished in the blood, whereas tumors of this size fall well below the detection limit of standard radiological methods [8].

Circulating DNA is for the most part discharged from degrading cells after cleavage by endonucleases that cut the chromatin into the essential nucleosomes, which monitors them from proteolytic digestion in blood [9]. In a healthy individual, cfDNA enters circulation via apoptosis of lymphocytes and other nucleated cells. Apoptosis has an interesting DNA ‘ladder’ pattern that show banding at 200 bp resulted from endonuclease-mediated double-strand cleavage between nucleosomes [10]. While in cancer, cfDNA results from tumor necrosis, but other mechanisms include lysis of circulating malignant cells or of micro-metastases, or because of active release. Necrosis yields DNA fragments with various lengths, because of random and incomplete digestion of genomic DNA by DNases [11].

Clinical applications of cfDNA in early-stage NSCLC include early tumor discovery, checking of tumor burden and monitoring of minimal residual disease. cfDNA can be acquired from minimally invasive procedures and reflects the genetic alterations found in tumor tissue, cfDNA analysis is considered a potential tool for NSCLC diagnosis and monitoring. Thus, the aim of this study was to study whether the concentrations and integrity index of cfDNA in serum can be used as a diagnostic and prognostic biomarker for NSCLC patients [12].

2. Subjects and methods

This study was carried out by cooperation between Medical Biochemistry & Molecular Biology, Clinical Oncology & Nuclear Medicine, Cardiothoracic Surgery and Public Health and Community Medicine Departments, Faculty of Medicine, Menoufia University in the period from October 2015 to September 2017. It included 140 individuals were categorized into; Group I: 60 patients with histopathologic diagnosis of NSCLC, Group II: 40 patients with COPD and Group III: 40 age and gender matched healthy controls.

2.1. An informed written consent was obtained from all participant approved by the Ethical Committee of Medical Research, Faculty of Medicine, Menoufia University

Patients with bad performance status 3 and 4, or with impaired liver or kidney functions, Patients with another malignancy and patients with autoimmune disease, tissue injury, viral diseases or trauma excluded from this study.

All studied participants were subjected to history taking, physical examination, For NSCLC patients; baseline computed chest, abdomen and pelvis with contrast, brain magnetic resonance imaging and bone scan were done for staging. Staging was done according to the American Joint Committee on Cancer. All patients received standard treatment; platinum based chemotherapy regimen in first line treatment for adjuvant, neoadjuvant and metastatic setting, concurrent chemoradiotherapy for advanced stages; surgery for early operable and resectable cases, palliative radiotherapy and/or bisphosphonates for metastatic patients. Data collection of histopathological subtype (adenocarcinoma, squamous cell carcinoma and large cell type, other subtypes) and grade (I, II, III) were done. Survival was calculated from date of diagnosis to date of death or date of last contact or date of data collection.

Laboratory investigations including: detection of serum CEA level by ELISA and Real-time ALU-qPCR to assess the concentration and integrity index of serum cfDNA.

2.2. Assay method

5 ml of venous blood were withdrawn by venipuncture, transferred into plain tube, left to clot, centrifuged at 4000 r.p.m for about 10 min. The serum obtained was stored at −80 °C until analysis of serum CEA and DNA extraction for quantitative detection of cfDNA. Serum CEA was determined by enzyme linked immunosorbent assay method (ELISA) using Human CEA PIokine ELISA kit following the manufacturer’s instructions.

2.2.1. Quantitative measurement of serum cfDNA by real time PCR technique

It was done in two main steps including:

2.2.2. DNA extraction step

Genomic DNA was extracted from serum by GeneJET Viral DNA and RNA Purification Kit, Thermo Scientific, (USA) [13].

2.2.3. Real time PCR step

To assess the concentration and integrity index of serum cfDNA, both short fragment (115 bp) and long fragment (247 bp) from a consensus sequence with genomic ALU repeats were amplified and quantified. The ALU-qPCR result obtained with ALU115 primers represents the total amount of serum DNA. DNA integrity index was calculated by the ratio of ALU-qPCR result (ALU247 and ALU115) using the 2 × SensiFAST™ SYBR® LO ROX kit (Bio Reagents Ltd.) on Applied Biosystems 7500 Real-Time PCR systems.

The ALU115 primers were: (5′-CCTGAGTCCAGGTTGAG-3′) (forward) and (5′-CCCCGATGTACGTTGATTACA-3′) (reverse). ALU247 primers were: (5′-GGCTAGCCGCAGGTATATC-3′) (forward) and (5′-CAGGGTGGAGTCGTCGG-3′) (reverse). The reaction mix was prepared by mixing 10 µl Master Mix, 1 µl of each primer (sigma) and 3 µl of DNase-free water in a total reaction volume of 20 µl. For each unknown reaction, 5 µl (0.1 µg/µl) of DNA extract was added and for the negative control reaction, 5 µl of DNase-free water was added. The cycling conditions of ALU (247 bp) were set as follows: Initial denaturation for 10 min at 95 °C, 1 cycles of denaturation at 95 °C, followed by 35 cycles of melting for 10 s at 95 °C and annealing/collection for 1 min at 64 °C using the Applied Biosystems 7500 software version 2.0.1. The cycling parameters of ALU (115 bp) were: initial denaturation step for 3 min at 95 °C, 1 cycles of denaturation at 95 °C, followed by 30 cycles of melting for 5 s at 95 °C and annealing/collection for 30 s at 62 °C using the Applied Biosystems 7500 software version 2.0.1.

The amount of serum DNA fragments in each sample was determined using a standard curve with serial dilutions (from 0.222 to 25,000 ng/ml) of human genomic DNA. A negative control in each reaction plate was run. Standard curves were created for both ALU115 and ALU247 primer sets by PCR amplifying 10-fold serially diluted human genomic DNA samples [14].

2.3. Statistical analysis

The data were collected, tabulated, and analyzed by SPSS (statistical package for social science) version 17.0 on IBM compatible computer (SPSS Inc., Chicago, IL, USA). The data are presented as number and percentage for qualitative variables and mean, standard deviation and range for quantitative one. χ2 test was the test of categorical data comparison. Student t-test was used to compare to sets of normally distributed quantitative data while Mann Whitney U test was used for not normally distributed data. Kruskal Wallis test is the test of multiple group comparison in not normally distributed data while spearman correlation was used to assess correlation between parameters in the studied groups. Kaplan – Meier curve analysis was used to evaluate survival functions among the studied cases. In all analyses, a two-sided P value of < 0.05 was considered statistically significant.
3. Results

This case control study was conducted on 60 lung cancer patients, 40 COPD patients and 40 healthy controls; they showed mean age as (56.8 ± 9.8, 52.9 ± 11.1 & 53.7 ± 9.0) for the three studied groups respectively with non-significant difference between them. Sex distribution was uniform among the groups with insignificant difference while smoking status recorded a significantly higher percentage of smokers and their smoking index among lung cancer patients than both COPD and control groups. Lung cancer patients demonstrated significantly higher values of each of ALU 215, ALU 247, DNA integrity and CEA than both COPD patients and controls, with non-significant difference between the last two groups (Table 1).

The ROC curve analysis demonstrated that the total accuracy of ALU 247 was 92.1% at a cutoff point 325, 0.98 AUC, 96.7% sensitivity, 88.7% specificity, 86.6% PPV & 97.3% NPV, while ALU 115 recorded (0.93, 90%, 78.7%, 76.1, 91.3 & 83.6) for AUC, sensitivity, specificity, PPV, NPV and accuracy respectively at a cutoff point 565. It was noticed that DNA integrity has AUC 0.65, sensitivity 75%, specificity 42.5%, 49.5% PPV, 69.4% NPP and total accuracy 56.4% at a cutoff point 0.48. Lastly CEA recorded 0.75 AUC, 70% sensitivity, 67.5% specificity, 61.8 PPV, 75% NPV and 68.6% accuracy at a cutoff point 6.5. On combination of both DNA integrity and CEA in parallel, they showed improved level of sensitivity 93.3% (Fig. 1 & Table 2).

Among lung cancer patients, ALU 115 & ALU 247 increased significantly in advanced tumor and more elevation was noted in metastatic one also ALU 247 was significantly higher in NSCIC than SCIC type (Table 3).

Regarding survival, after median follow up duration of 20 months, Overall survival of NSCLC cancer patients in relation to ALU 247, ALU 115 & DNA integrity documented a significant relationship between low DNA integrity and better survival of those patients (Table 4 & Fig. 2).

4. Discussion

Lung cancer, basically comprised of non-small cell lung cancer (NSCLC), is the most often as possible diagnosed carcinoma and the leading cause of cancer-related death around the world. Regardless of it has a little change, the prognosis of lung cancer is still poor, with under 18% of patients surviving over 5 years, partly due to the fact that greater part of cases are diagnosed at a late stage [1]. The survival of these patients depends remarkably on diagnosis. Therefore, identification of new biomarkers for the early diagnosis of NSCLC is critical for the patients to get ideal therapeutic regimen as early as possible [15]. Treatment of metastatic NSCLC greatly depends on histological subtyping and molecular testing for selection of patient for first line...
treatment, according to National Comprehensive Cancer Network (NCCN) Guidelines [16]. Just little biopsies are accessible for both histologic diagnosis/subtyping and genetic testing in the large part of advanced stage patients, and the tissue often becomes lacking for genomic analysis after initial histology diagnosis ± stains for subtyping. To decide on the following typing and genetic testing in the large part of advanced stage patients, a ratio of males to females was 71.7% and 28.3% in the NSCLC patients and with YI et al. [18] and Heitzer et al. [19]. In this study, the proportion of the three studied groups regarding age and gender. This is in agreement with gender and YI et al. [18] reported that there was no association between the level of cfDNA and clinical parameters such as age, gender, histology, smoking or pulmonary inflammatory conditions.

The present findings revealed that there was significant difference between NSCLC patients and both of COPD and control subjects regarding serum levels of CEA. However, there was no statistically significant difference in the level of CEA between COPD and control subjects. This is matched with Zaher et al. [23] Stated that CFDNA represented a highly sensitive and specific marker to discriminate cancer patients from control and benign individuals when compared to conventional tumor markers used in various types of cancer diagnosis. AUC of CEA in this study was 0.75 Szpechcinski et al. [21] stated that diagnostic power of the quantitative cfDNA assay exceeds many other serological markers used in lung cancer, for example CEA (AUC value: 0.591). Our results showed that combined detection of CEA and integrity index (ALU247/115) showed improved the sensitivity level to 93.3%.

In the present study, there were significant higher levels of ALU 115, ALU 247 and DNA integrity (ALU247/115) in NSCLC patients when compared with COPD and control subjects, whereas there was non-significant difference in these levels between COPD and control subjects. This perhaps could be due to exclusion of the patients with diseases that may increase ccf-DNA concentrations as autoimmune or viral diseases. This is matched with Szpechcinski et al. [21] found that NSCLC patients had significantly higher mean plasma DNA concentrations than in individuals with chronic respiratory inflammation and healthy controls; this strongly suggest that elevated plasma cfDNA

### Table 2
Diagnostic validity of ALU115, ALU247 and DNA integrity (ALU247/115) in diagnosis of lung cancer cases.

|                | ALU 247 | ALU 115 | DNA integrity | CEA | Combined CEA & DNA integrity |
|----------------|---------|---------|---------------|-----|-----------------------------|
| **AUC**        | 0.98    | 0.93    | 0.65          | 0.75| –                           |
| **P value**    | < 0.001 | < 0.001 | < 0.002       | < 0.001 | –                           |
| **95% CI**     | 0.97 – 1.0 | 0.89 – 0.98 | 0.56 – 0.74 | 0.67 – 0.83 | –                           |

AUC = area under the curve, CI = confidence interval, PPV = positive predictive value, NPV = negative predictive value.

### Table 3
ALU 115 & ALU 247 in relation to tumor character among lung cancer group.

| Clinical stage | Total No (%) | ALU 115 X ± SD | P value | ALU 247 X ± SD | P value | DNA integrity X ± SD | P value |
|----------------|--------------|----------------|---------|----------------|---------|----------------------|---------|
| Early stage (I,II) | 6 (10.0) | 573.0 ± 91.7 | 36.9 | 433.3 ± 185.7 | 25.5 | 0.27 ± 0.17 | 2.99 |
| Advanced stage (III) | 22 (30.7) | 1080.5 ± 408.6 | < 0.001 | 614.7 ± 254.5 | (< 0.001) | 0.64 ± 0.25 | 0.22 |
| Metastatic stage (IV) | 32 (53.3) | 1836.1 ± 279.3 | 103.3 | 205.2 | 0.59 ± 0.15 | 0.12 |
| Histopathological type | | | | | | | |
| Adenocarcinoma | 40 (66.7) | 1487.3 ± 602.1 | 1.80 | 839.3 ± 329.1 | 1.28 | 0.62 ± 0.17 | (0.91) |
| Other (SCC, LCC) | 20 (33.3) | 1323.7 ± 449.8 | (0.07) | 733.0 ± 266.2 | (0.20) | 0.64 ± 0.25 | |
| Grade | | | 2.10 | | | | |
| 1 | 4 (6.7) | 1824.5 ± 121.8 | (0.36) | 1107.5 ± 216.5 | 6.03 | 0.67 ± 0.13 | 3.18 |
| II | 40 (66.7) | 1395.7 ± 603.4 | | 802.0 ± 344.8 | (0.049) | 0.65 ± 0.20 | 0.20 |
| III | 16 (26.7) | 1427.3 ± 477.9 | | 732.5 ± 181.3 | | 0.56 ± 0.19 | |
| Metastases | | | | | | | |
| Non metastatic | 22 (36.7) | 949.3 ± 439.7 | 29.7 | 597.5 ± 266.7 | < 0.001 | 0.68 ± 0.23 | 2.22 |
| Synchronous metastases | 28 (46.7) | 1825.3 ± 284.7 | < 0.001 | 1007.3 ± 202.9 | | 0.60 ± 0.16 | 0.33 |
| Metachronous metastases | 10 (16.7) | 1397.4 ± 539.6 | | 688.0 ± 313.4 | | 0.59 ± 0.20 | |
| Surgery | | | 1.32 | | | 0.34 |
| Not operated | 57 (95.0) | 1458.0 ± 541.4 | (0.19) | 808.4 ± 313.4 | 0.73 | 0.62 ± 0.19 | 1.20 |
| Operated | 3 (5.0) | 953.3 ± 774.4 | | 716.7 ± 320.4 | | 0.77 ± 0.23 | 0.23 |
| Chemotherapy Received | 2 (3.3) | 1020.0 ± 0.0 | (0.14) | 810.0 ± 0.0 | (1.0) | 0.62 ± 0.20 | 0.10 |
| Not received | 58 (96.7) | 1439.3 ± 563.6 | | 795.4 ± 313.4 | | 0.84 ± 0.07 | 0.11 |

X = mean, SD = standard deviation.
levels in NSCLC patients result primarily from tumor development raising the potential clinical implications for lung cancer screening and early analysis. da Silva Filho et al. [24] who showed a significant increase in ALU 115 and ALU 247 levels in cancer patients when compared with control. Moreover, Zaher et al. [23] found that the mean level of CFDNA in cancer patients was around 10-fold that of controls and around 5-fold that of benign group. This might be because of the release of a considerable amount of genomic DNA into the systemic

Table 4
overall survival of lung cancer patients in relation to ALU115, 1LU 247 & DNA integrity.

|                | Mean (months) Value | 95%CI Value | Median (months) Value | 95%CI Value | Log Rank | P value |
|----------------|---------------------|-------------|-----------------------|-------------|----------|---------|
| ALU 247        |                     |             |                       |             |          |         |
| High (≥ 810)   | 18.9                | 16.4–21.3   | 20                    | 15.9–24.0   | 1.61     | 0.20    |
| Low            | 21.6                | 18.5–24.7   | 21                    | 16.2–24.8   |          |         |
| ALU 115        |                     |             |                       |             |          |         |
| High (≥ 1660)  | 18.8                | 16.3–21.3   | 18                    | 14.2–21.8   | 1.83     | 0.18    |
| Low            | 21.7                | 16.6–24.8   | 20                    | 16.7–23.9   |          |         |
| DNA integrity  |                     |             |                       |             |          |         |
| Low            | 14.36               | 10–18.5     | –                     | –           | 2.33     | 0.04    |
| High (≥ 0.55)  | 20.51               | 15.5–24.1   | 22                    | 12.1–24.6   |          |         |

Fig. 2. Overall survival of NSCLC patients in relation to ALU 247 & ALU 115 showed better survival for low level versus high level patients but the difference didn’t reach statistically significant level $p = 0.20$ and $p = 0.18$ respectively, however a statistically significant relationship between low DNA integrity and overall survival between those patients ($P = 0.04$) with longer survival for patients with low levels.
circulation from tumor cells either by necrosis or active release. And there was non-significant statistical difference in the short and long fragment levels and DNA integrity between benign and control groups.

In contrast to our results, Hao et al. [9] reported that a significant difference in ALU115, ALU247 levels and DNA integrity was found between COPD and control subjects.

Considering the diagnostic validity of each of the ALU247, ALU115, DNA integrity index (ALU247/115) and CEA serum levels, we observed that the ALU247 cutoff point is 325 ng/ml with highest diagnostic accuracy 92.1%, with 96.7% sensitivity, 88.7% specificity, 86.6% positive predictive value (PPV) & 97.3% negative predictive value (NPV). This is similar to the study done by Szpechcinski et al. [21] found that the diagnostic power of the quantitative cfDNA assay to discriminate NSCLC from non-malignant inflammatory diseases and healthy individuals, ROC curve analysis was performed; with highest accuracy was obtained at a cutoff point of 42.80 ng/ml, which compared to to sensitivity and specificity of 90% and 80.5%, respectively, PPV of 85% and NPV of 87%. This is coordinated with Zhang et al. [23] who found that the diagnostic curve of cfDNA assay for lung cancer was (1.0–0.949) The cutoff point from the ROC curve of cancer versus control groups was 100 ng/I, with 75% specificity and 100% sensitivity, another cutoff value was 600 ng/l, which gave a corresponding 100% specificity and 53.4% sensitivity. Hence, patient with cfDNA P600 ng/l could be straightforwardly diagnosed as a cancer patient. But, patients that had cfDNA concentrations, between 100 and 600 ng/l, could not be diagnosed as cancer patients, because of the overlap with benign subjects, cfDNA integrity index may resolve this discrepancy.

Discrepancies across various studies may be related to many factors as selection of subjects or pre-analytical and analytical procedures, such as type of sample, method of cfDNA isolation and quantification, as well as in the characteristics and numbers of patient populations [21].

In this study, ALU115 & ALU247 increased significantly in advanced tumor and more elevated in metastatic cases. This is matched with Gautschi et al. [25] who reported that the plasma cfDNA concentration at advanced tumor stages was higher compared with that at early tumor stages. This is matched with Sozzi et al. [22] who stated that there was a significant relation between long fragment levels in serum and DNA integrity (ALU247/115) levels with size of invasive cancer and the presence of lymphovascular invasion. That may be explained by the fact that DNA released from malignant tumors into the blood stream was enhanced by vascular invasion, so direct lymphatic or blood flow through the tumors enabled dissemination of viable tumor cells and enhanced diffusion of DNA released from necrotic or living tumor cells into the blood stream.

In contrast there was no association between cfDNA concentration and tumor stage Yi et al. [18]. Szpechcinski et al. [21] found that Plasma DNA levels were not significantly related to the NSCLC stage (I–IIIA) or histology (ADC or SCC) but several groups have evaluated plasma DNA levels in resectable NSCLC patients. This is similar to the study done Zaher et al. [23] did not find any correlations between clinicopathological parameters (e.g. tumor size, stage, grade, metastasis) and cfDNA concentration or integrity index.

In the current study, there was a significant relationship between low DNA integrity and better survival of those patients. Yi et al. [18] reported that a high level of cfDNA was correlated with poor overall survival (OS) in the group of NSCLC patients with stage III or IV disease, while there was no correlation between the level of cfDNA and OS in the group of patients with stage I-II disease. Drift et al. [26] found a high circulating plasma DNA concentration at the time of diagnosis in NSCLC patients was a prognostic factor for poorer survival and the median DNA concentration of the patients who died was significantly higher compared to the patients that survived.
5. Conclusion

This study concluded that serum cfDNA offers an interesting prospect for non-invasive blood test screening in NSCLC with the ability to discriminate normal individuals from patients with COPD and lung cancer, also a high level of cfDNA is associated with short survival for NSCLC patients.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2018.06.002.

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