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Pleural fluid DNA integrity index as a diagnostic marker of malignant pleural effusion

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The presence of tumour cells in pleural fluid or tissue defines the effusion as malignant. Pleural fluid cytology has a sensitivity of 50–60% which is insufficient for making a clinical diagnosis, which is usually made after the use of more invasive techniques such as thoracoscopy or thoracotomy [1]. Hence the need for a rapid and reliable proof of malignancy by less-invasive procedures is warranted. Although almost all the DNA in the human body is located intracellularly, small amounts can also be found circulating freely in the blood. The term 'cell free-DNA (cf-DNA)' is used to describe extracellular DNA molecules that are released from either apoptotic or necrotic cells and can be detected in various biological fluids [2]. These DNA molecules may arise from active release of nucleic acids from living cells, breakdown of dying cells that release their contents into the blood, or both [3]. Necrosis of malignant tumours in the pleural fluid generates a spectrum of DNA fragments with variable strand lengths due to random and incomplete digestion of genomic DNA by deoxyribonucleases, although most of these are long DNA fragments [4]. In contrast, cell death in normal nucleated blood cells (apoptosis) results in the production of uniform small DNA fragments as in benign effusion. The ratio of longer DNA fragments to shorter ones is termed DNA integrity and has gained attention in last years especially in relation to early diagnosis of tumours, tumor stage, grade and response to therapy [5].

One of the most important DNA fragments are Arthrobacterluteus (ALU) repeats, which are the most abundant short interspersed repeated sequences in the human genome [6]. The Alu/Alu recombination is considered to cause about 0.5% of all new genetic diseases that contribute to human cancers [7]. The aim of this study was to evaluate the value of Alu 247, Alu 115 cf- DNA levels and Alu 247/Alu 115 ratio (DNA integrity index) in detecting malignant pleural effusion.

The present study was conducted on 68 patients with pleural effusions recruited from Chest Department, Menoufia University Hospitals, Egypt, between June 2015 and January 2016. A written informed consent was taken from each patient before being included in this study and ethics approval was obtained from the Menoufia University Hospital Review Board before data collection. Exclusion criteria were patients with transudative pleural effusion by Light's criteria [8], undiagnosed pleural effusion after all possible diagnostic procedures, known malignant patients under treatment with chemotherapy or radiotherapy, clotted pleural fluid specimen, time between collection and processing > one hour and sub-optimally preserved fluid specimens. All patients provided a complete medical history, with clinical and radiological examination. Sputum microscopy and/or bronchoscopy were done if malignancy or T.B were suspected. Thoracocentesis with cytological, microbiological and biochemical analyses including detection of cf-DNA in pleural fluids by real time PCR were undertaken. For those with undiagnosed cause of pleural effusion after pleural fluid analysis, further diagnostic procedures were done as a closed pleural biopsy, thoracoscopy or CT guided pleural biopsy.

At the time of thoracentesis, pleural fluid and 3 ml venous blood were withdrawn for assessment of Light's criteria. The latter samples were transferred in a sterile vacutainer tube for 30 min to clot, then centrifuged at 4000 RPM for 10 min. Serum was separated in aliquot for analysis of total protein (copper/alkaline method, Diamond Diagnostics Egypt, Cairo, Egypt, http://spectrum-diagnostics.com) and LDH (the oxidation of lactate to pyruvate with simultaneous reduction of nicotinamide adenine dinucleotide (NAD) to NADH). Pleural fluid was divided into four parts, the first and second parts were referred to Microbiology and Pathology Departments

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for microbiological and cytological examination respectively. The other two samples were referred to Biochemistry department one for biochemical analysis of pleural fluid and the other was preserved at –80 °C for later DNA extraction then assay of cell-free DNA by real time PCR with a relative standard curve.

To detect pleural fluid cf-DNA, DNA extraction was done using Pure link genomic DNA extraction kits according to the manufacturer's instructions (Qiagen Hilden, Germany). Extracted DNA was evaluated for concentration and purity using nanodrops. Two sets of primers designed for amplification of two overlapping fragments from a region with abundant genomic ALU repeats [9]. The primer sequences of ALU115 were forward primer 5′CCTGAGGTCAGGAGTTCGAG-3′ and reverse 5′CCCGAGTAGCTGGGATTACA-3′. Primer sequences of ALU247 were forward primer 5′GTGGCTCACGCCTGTAATC-3′ and reverse 5′CAGGCTGGAGTGCAGTGG-3′. The reaction mixture of real-time PCR for each Alu 115 and Alu 247 in separate reactions contained 12.5 µl of syber green I with low Rox, both forward and reverse primers, nuclease-free water and template DNA in a total reaction volume 25 µl. Thermal cycling was run in 96-well plates in 7500 Real Time PCR system (Applied Biosystems, USA) using standard curve analysis method. Standard Curves were formed with 100µg human genomic DNA for the calculation of concentration of Alu 115 and Alu 247 fragments in ng/ml. Reaction conditions for Alu 115 were 95 °C for 3 min and 30 cycles of 95 °C for 5 s, 64 °C for 30s. Reaction condition for Alu 247 were 95 °C for 10 min and 40 cycles of 95 °C for 15 s, 64 °C for 1 min. DNA integrity was calculated as the ratio of quantitative PCR results (ALU247-quantitative PCR/ALU115-quantitative PCR) [10].

Patients were categorised into the following two groups. Group I included 39 patients with exudative malignant effusion (5 patients with malignant pleural mesothelioma and 34 patients with metastatic cancer originated from adenocarcinoma of the lung, breast cancer and lymphoma). Group II included 29 age and gender matched patients with an exudative benign effusion (19 patients with parapneumonic effusion and 10 patients with tuberculous effusion) as a control group. The data collected were tabulated & analysed by SPSS version 20. Quantitative data were expressed as mean and standard deviation (SD) and analysed using student *t*-test for comparison of two groups of normally distributed variables. Qualitative data were expressed as number and percentage and analysed using χ^2 test. Mann–Whitney U test was used to compare medians of ordinal data that are not normally distributed. The Receiver-operating characteristic (ROC) curves were constructed to illustrate the predictive value of cut-off points of DNA integrity index. The point with the largest sum of sensitivity and specificity was chosen as a threshold. All these tests were used as tests of significance at $p < 0.05$.

The two groups were matched for regards age, gender, gross features of pleural fluid and all parameters of Light's criteria (Table 1). There was a significant statistical difference between the studied groups regarding the cause of effusion and cytological examination, but no difference in LDH or protein indices. There was a significant increase in Alu 247/Alu 115 ratio in the malignant effusion group compared to benign group. There was no

Table 1. Demographic data, physical, chemical, cytological examination and cfDNA(Alu repeat) of pleural fluid.

Demographic criteria	Malignant effusion ($n = 39$)		Benign effusion ($n = 29$)		*P* value
Age (years)	55.2 ± 7.1		57.4 ± 9.6		0.31
Sex	n	0/0	n	0/0	
Male/Female	18/21	46.2/53.8	15/14	51.7/48.3	0.81
History of smoking					
Smoker	15	17.3	5	17.3	0.003
Non smoker	12	72.4	21	72.4	
Passive smoking	12	10.3	3	10.3	
PF.Gross feature					0.13
Haemorrhagic	11	28.2	3	10.3	
Turbid yellowish	28	71.8	26	89.7	
Causes of effusion					
Malignant	39	100	0	0	0.001
Parpnemonic	0	0	19	65.5	
T.B	0	0	10	34.5	
Cytology					
Positive/Negative	19/20	48.7/51.3	0/29	0/100	0.001
PF.LDH (IU/L)	314 ± 105		307 ± 83		0.77
Serum LDH (IU/L)	416 ± 100		424 ± 87		0.75
PF/Serum LDH ratio	0.79 ± 13.24		0.72 ± 0.09		0.22
PF. Protein (g/dl)	3.76 ± 0.54		3.69 ± 0.45		0.58
Serum protein (g/dl)	6.75 ± 0.53		6.82 ± 0.33		0.56
PF/serum Protein ratio	0.56 ± 0.08		0.54 ± 0.06		0.33
Alu 115 (ng/ml)	61.8 (35.8–86.2)		52.1 (37.9–169.1)		0.17
Alu 247 (ng/ml)	74.9 (45.8–97.7)		42.5 (23.5–107.3)		0.10
Ratio Alu 115/247: DNA integrity index	0.81 (0.68–1.00)		0.65 (0.57–0.78)		0.01

Notes: PF = pleural fluid. LDH = lactate dehydrogenase. Data number of patients/%, mean ± standard deviation or median (IQR).

Table 2. Diagnostic validity of DNA integrity index, cytology and combined DNA integrity index and cytology.

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Diagnostic accuracy (%)
DNA index > 0.82	92	92.6	75.5	86.2	94.9
Cytology	71	59	100	100	49
Combined cytology & DNA index	94	100	91	86	100

significant difference between both groups regarding individual Alu 115 and Alu 247 concentrations in pleural fluid.

ROC analysis determined an optimum cut-off point for the Alu115/247 ratio of 0.8 (area under the curve 0.975, 95% confidence interval 0.95–1.00). Using this value, the sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy were calculated and are present in Table 2. This table also shows data for cytology alone, and for the combination of DNA integrity and cytology.

The precise diagnosis of a malignant pleural effusion demands a potentially dangerous procedure that alone gives incomplete information, prompting the search for better diagnostic tools. The ALU sequences were chosen as they are the most abundant and active repeated elements in the human genome accounting for more than 10% of the genome [11]. The aim of this study was to evaluate the value of Alu 247, Alu 115 cf-DNA levels and Alu 247/Alu 115 ratio (DNA integrity index) in discriminating malignant from benign pleural fluid samples. We found a significant increase in Alu 247/Alu 115 ratio (DNA integrity index) suggesting that cf-DNA may be a promising candidate biomarker for detection of malignant pleural effusion. This is in accordance with the study of Benlloch et al. [12] who stated that cell-free methylated DNA was detected in pleural fluid samples from 58.5% of patients with malignant pleural effusions and from 0% of patients with benign pleural effusions. Another study found that median DNA concentrations were greater in patients with neoplasia than in patients without malignancy: 93 ng/ml versus 21 ng/ml in pleural fluids, respectively [13]. This can be explained by the fact that cf-DNA released from apoptotic cells is uniformly truncated into 185–200 base pair fragments. However, cf-DNA released from necrotic tumour cells varies in length, which may lead to elevation of DNA with long fragments in serum or body fluids [4]. In contrast to this, Santotoribio et al. [14] showed that pleural fluid β-globin gene cf-DNA concentration was higher in patients with parapneumonic pleural effusions (PPE) (median = 46,240 ng/ml) than in those free of PPE (median = 224 ng/ml) include malignant effusion.

In agreeing with this, another study stated that, the sensitivity of methylated DNA with cytological examination was greater than that of cytology examination alone, 69.8% versus 39.1%, respectively (p = 0.001) [12]. Differences between the previous studies can be explained by variations in the histological types of malignant pleural effusion as well as variable characteristics regarding tumour progression, the quantity and quality of DNA template extracted which are likely to differ according to the time of collection, the content of DNase, and the DNA integrity, among other factors.

We conclude that at a cut-off point of 0.82, the DNA integrity index shows good sensitivity, specificity, positive predictive value and negative predictive value for the diagnosis of malignant effusion. This procedure increases the sensitivity of cytological examination from 49 to 100%, but validating studies with larger numbers are required before this technique can be formally assessed in routine clinical practice. This report is an advance in biomedical science because it shows that cf-DNA integrity index in pleural fluid may be a promising biomarker to distinguish malignant effusion without the need for invasive diagnostic maneuvers.

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Disclosure statement

No potential conflict of interest was reported by the authors.

References

[1] Antonangelo L, Sales RK, Corá AP, et al. Pleural fluid tumour markers in malignant pleural effusion with inconclusive cytologic results. Curr Oncol. 2015 Oct;22(5):336–e341.
[2] Schwarzenbach H, Hoon DSB, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer. 2011;11(6):426–437.
[3] Franklin BS, Vitorino BLF, Coelho HC, et al. Plasma circulating nucleic acids levels increase according to the morbidity of plasmodium vivax malaria. PLoS One 6(5):e19842.
[4] Pinzani P, Salvianti F, Zaccara S, et al. Circulating cell-free DNA in plasma of melanoma patients: qualitative and quantitative considerations. Clin Chim Acta. 2011;412:2141–2145.
[5] Fleischhacker M, Schmidt B. Circulating nucleic acids (CNAs) and cancer-a survey. Biochim Biophys Acta. 2007;1775:181–232.
[6] Wang H, Nekrutenko A, Li WH. Densities, length proportions, and other distributional features of repetitive sequences in the human genome estimated from 430 megabases of genomic sequence. Gene. 2000;259:81–88.

[7] Hedges DJ, Deininger PL. Inviting instability: transposable elements, double-strand breaks, and the maintenance of genome integrity. Mut Res. 2007;616(1–2):46–59.
[8] Light RW. Clinical practice. Pleural effusion. N Engl J Med. 2002;346:1971–1977.
[9] Deininger P. Alu elements: know the SINEs. Genome Biol. 2011;12(12):236–248.
[10] Chan MH, Chow KM, Chan AT, et al. Quantitative analysis of pleural fluid cell-free DNA as a tool for the classification of pleural effusions. Clin Chem 2003.;49(5):740–745.
[11] Dagan T, Sorek R, Sharon E, et al. AluGene. A database of Alu elements incorporated within protein-coding genes. Nucleic Acids Res. 2004;32:489D–492.
[12] Benlloch S, Galbis-Caravajal JM, Martín C, et al. Potential diagnostic value of methylation profile in pleural fluid and serum from cancer patients with pleural effusion. Cancer. 2006;107(8):1859–1865.
[13] Benlloch S, Martí-Ciriquián JL, Galbis-Caravajal JM, et al. Cell-free DNA concentration in pleural fluid and serum: quantitative approach and potential prognostic factor in patients with cancer and pleural effusions. Clin Lung Cancer. 2006;8(2):140–145.
[14] Santotoribio JD, Cabrera-Alarcón JL, Batalha-Caetano P, et al. Pleural fluid cell-free DNA in parapneumonic pleural effusion. Clin Biochem. 2015;48(15):1003–1005.