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Plasma cell-free DNA level and its integrity as biomarkers to distinguish non-small cell lung cancer from tuberculosis

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1. Instruction

Lung cancer remains the most common cancer worldwide. Approximately 80% of all lung cancer cases are NSCLC patients. The majority of NSCLC patients are diagnosed at late stages and have local or systemic advanced disease (stage III or IV) with 5-year survival rates of < 5% [1,2]. Therefore, there is a great demand for new diagnostic options based on specific biomarkers. The discovery of extracellular DNA circulating in blood, so called cell-free DNA (cfDNA), may greatly impact molecular diagnostics of lung cancer patients due to simple, non-invasive access to genetic material detectable in plasma and serum by sensitive molecular biology techniques [3]. cfDNA isolated from the plasma of healthy individuals and analyzed by gel electrophoresis or sequential analysis forms a ladder-like distribution, i.e. regular fragments of ~180–200 bp or a multiple of that value that represent mono-, di-, and trinucleosomes produced via enzymatic cleavage during apoptosis [4]. Interestingly, the size of the cfDNA released from dead lung cancer cells varies between small fragments of 70 to 200 base pairs and large fragments of about 21 kb and is longer than that of non-neoplastic DNA [5]. In addition, several researches have demonstrated that lung cancer patients had significantly higher cfDNA concentrations than healthy individuals [6–9]. Furthermore, in lung cancer patients, the levels of cfDNA in plasma are significantly higher than those in patients with chronic respiratory inflammation (chronic obstructive pulmonary disease, sarcoidosis, or asthma) and could be as a biomarker to distinguish lung cancer from chronic respiratory inflammation [3].

Tuberculosis, a common respiratory infectious disease, has succeeded in infecting one third of the human race though inhibition or evasion of immunity and was easily misdiagnosed as lung cancer in clinic [10]. Lots of cell apoptosis and organization necrosis, which could also produce large amount of cfDNA enter peripheral blood to some extent, were emerged in the center of tuberculosis nodules [11]. Consequently, it remains unclear that the levels of plasma cfDNA in tuberculosis patients and if plasma cfDNA level could as biomarkers to distinguish NSCLC from tuberculosis. Of note, a semi-quantitative approach to evaluate the integrity of cfDNA is measurement of the ratio of shorter (apoptotic) to longer (non-apoptotic) cfDNA fragments in plasma, which has been proposed as a potential cancer marker [12]. Although cfDNA integrity is a promising diagnostic marker in several cancer types, its clinical usefulness in distinguish NSCLC from tuberculosis has not been validated thoroughly.

The aim of this study was to evaluate the potential clinical value of the plasma cfDNA concentrations and strand integrity as an auxiliary tool for NSCLC differential diagnosis from tuberculosis in patients with solitary pulmonary nodules detected by CT.

2. Methods and materials

2.1. Subjects

All subjects were recruited from Ningbo No. 2 Hospital, the First and the Second Affiliated Hospital of Wannan Medical College. The protocol in this study was approved by the Medical Ethics Committee of Ningbo No. 2 Hospital and all subjects were provided written informed consent. This research was divided into 3 groups: NSCLC (n = 106), tuberculosis (n = 105) and healthy controls (n = 107). NSCLC and tuberculosis were diagnosed by pathology although CT-guided biopsy using World Health Organization criteria [13,14]. All subjects were without any antitumor or anti-tuberculosis treatment before. Subjects with shock, pregnancy, systemic erythema lupus, hepatitis, renal insufficiency, other malignancies and severe organic disease were excluded from the study. The inclusion criteria of the healthy controls group were good general health, insignificant past medical history.

2.2. Blood collection and DNA isolation

Before lung biopsy and treatment, peripheral venous blood of 5 ml was collected into EDTA-2K containing tubes and standed still 20 min in room temperature. To ensure cell-free plasma collection, all blood samples were centrifuged in 3000 rpm for 10 min and then 12,000 rpm

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Table 1
The comparison of clinical characteristics among non-small cell lung cancer, tuberculosis and healthy control.

	Non-small cell lung cancer (n = 106)	Tuberculosis (n = 105)	Healthy control (n = 107)	P value
Gender (male/female)	80/26	81/24	83/24	0.929
Age (years)	57.00 (56.00, 69.25)	63.00 (49.50, 73.00)	60.00 (58.00, 68.00)	0.573
CA125 (ku/l)	13.36 (5.00, 51.35)	6.72 (4.50, 18.47)	8.66 (3.46, 14.15)	0.000
NSE (ng/ml)	9.44 (5.25, 13.01)	4.31 (2.28, 7.58)	2.78 (1.07, 4.90)	0.000
CEA (ng/ml)	2.70 (1.65, 5.65)	1.88 (1.19, 3.67)	1.72 (0.78, 2.74)	0.000

CA125: carbohydrate antigen 125; NSE: neuron-specific enolase; CEA: carcino-embryonic antigen.

for 10 min. The cell-free plasmas were stored at − 80 °C until extraction. Total DNA was extracted from 400 μl cell-free plasma by using QIAamp DNA Blood Mini Kit (Germany, Qiagen) according to the Qiagen manual. The extracted DNA purity was measured by the NanoDrop ND-1000 nucleic acid quantifier ($A_{260/280\ nm}$) and only the absorbance at a ratio of 1.6–1.8 samples were usable. The extracted DNA was stored at − 80 °C.

2.3. Measurement of plasma cfDNA level and integrity

The quantization of plasma DNA fragments was performed by quantitative real-time PCR. Amplifying and quantifying shorter (115 bp) and longer (247 bp) fragments from abundant genomic ALU repeats. The primers (ALU115) amplified the shorter (115 bp) fragments and the primers (ALU247) amplified the longer (247 bp) fragments. In addition, the ratio of ALU115 to ALU247 reflected the integrity of plasma cfDNA. Sequences of ALU115 primers were forward CCTGAGGTCAGGAGTTCGAG and reverse CCCGAGTAGCTGGG ATTACA; ALU247 primers were forward GTGGCTCACGCCTGTTAATC and reverse CAGGCTGGAGTGCAGTGG. The primers were synthesized by Sangon Biotech Company (Shanghai, China). Real-time PCR reaction was performed as follows: 10 μl SYBR premix Ex Taq (Japan, Takara), 1 μl DNA sample, 1 μmol/l forward (1 μl) and reverse primers (1 μl), dd H_2O 7 μl to a final reaction volume of 20 μl. Thermal cycling was performed in LightCycle 480-II (Roche, Switzerland). The cycling program consisted of 30 s of initial denaturation at 95 °C, followed by 40 cycles at 95 °C for 5 s, 72 °C for 10 s. Following amplification, melting curve analysis was performed to confirm PCR product specificity and was carried out at 95 °C for 5 s, 60 °C for 60 s and 95 °C (0.11 °C/s and 5 points per °C). cfDNA concentrations of calibrators were calculated by an external standard curve (100 ng/ml, 10 ng/ml, 1 ng/ml, 0.1 ng/ml and 0.01 ng/ml of genomic DNA) by NanoDrop ND-1000 nucleic acid quantifier. Serial dilutions of an external standard and water blanks were included into every run. Both calibrators and samples were analyzed in triplicate.

2.4. Measurement of plasma tumor markers levels

Chemiluminescent immunoassay was used to detect plasma CA125, NSE, CEA concentrations by Roche cobas e601 (Roche, Switzerland). Reference range of CA125, NSE, and CEA were ≤ 35ku/l, ≤ 12.5 U/ml, and ≤ 5 ng/ml, respectively.

2.5. Statistical analysis

Categorical variables were abnormal distribution by Kolmogorov Smirnov test and shown as Median (Quartile) [M (P25, P75)]. Differences of plasma cfDNA concentrations and its integrity among patients with NSCLC, tuberculosis and healthy controls were analyzed by using the Kruskal-Wallis test. Multiple comparisons between two groups were derived from the Mann Whitney U test. ROC analysis was carried out to determine the AUC, sensitivity and specificity of plasma cfDNA levels and its integrity. Statistical analysis was performed by using SPSS statistical package version 16.0 (Chicago, IL, USA). Statistically significance was established at a P value < 0.05.

3. Results

3.1. General conditions

There were 106 patients with NSCLC, 105 patients with tuberculosis and 107 healthy controls in this research. Characteristics of patients grouped are reported in Table 1. There were no statistical differences in gender and age among these three groups (P = 0.929 and P = 0.573, respectively). The level of plasma CA125, NSE, CEA in patients with NSCLC were significantly higher than that in patients with tuberculosis and that in healthy controls (P = 0.000, P = 0.000, P = 0.000, respectively) (Table 1).

3.2. The comparison of cfDNA and its integrity between NSCLC, tuberculosis and healthy controls

The level of cfDNA (ALU115) in patients with NSCLC [95.67 (51.28, 238.85) ng/μl] was significantly higher than that in patients with tuberculosis [59.60 (34.25, 102.53) ng/μl P = 0.001] and that in healthy controls [44.66 (24.56, 66.54) ng/μl, P = 0.001] (Fig. 1A). The integrity of cfDNA in patients with NSCLC [5.91 (4.14, 7.45)] was also significantly higher than that in patients with tuberculosis [3.85 (2.91, 5.06), P = 0.000] and that in healthy controls [2.78 (2.18, 4.82), P = 0.000] (Fig. 1C), although there were no differences in the level of cfDNA (ALU247) among these three groups [16.74 (7.94, 53.62) ng/μl, 16.20 (8.13, 29.13) ng/μl, 14.54 (8.55, 25.41) ng/μl, P = 0.464] (Fig. 1B).

3.3. Association of cfDNA and its integrity with clinical characteristics

In patients with tuberculosis, no statistical association were found between cfDNA (ALU115) and CA125 (P = 0.426), NSE (P = 0.114) and CEA (P = 0.773), in contrast, statistical associations were found in NSCLC patients (P = 0.000, P = 0.028, P = 0.002, respectively). Similarly, there were no statistical association between integrity of cfDNA and CA125 (P = 0.144), NSE (P = 0.457) and CEA (P = 0.891) in tuberculosis patients, while, in patients with NSCLC, integrity of cfDNA were associated with CA125 (P = 0.014), NSE (P = 0.000) and CEA (P = 0.000) (Table 2). Univariate correlation analysis also found that, in NSCLC patients, cfDNA–ALU115 were related with CA125 (r = 0.221, P = 0.004), NSE (r = 0.334, P = 0.000), CEA (r = 0.373, P = 0.000), as well as integrity of cfDNA were also related with CA125 (r = 0.237, P = 0.015), NSE (r = 0.378, P = 0.000), CEA (r = 0.286, P = 0.000). However, those relationships were not appeared in tuberculosis patients and in healthy controls (Table 3–5).

3.4. cfDNA and its integrity could be used as indicators for identification of NSCLC from healthy controls

ROC curve analysis showed that cfDNA (ALU115) and its integrity could be used as indicators for identification of NSCLC from healthy people (AUC = 0.747, 95% Cl 0.680–0.814, P = 0.000, cut-off