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Original Research

Plasma cfDNA as a potential treatment monitoring and prognostic index in patients withnon-small cell lung cancer

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Abstract: The purpose of this study was to investigate the potential of cell-free DNA (cfDNA) as a prognostic factor for non-small cell lung cancer (NSCLC). Patients with NSCLC (n = 154) treated with gefitinib were recruited over a 5-year period for this study, and served as the observation group (79 males and 75 females, mean age = 53.74 ± 10.86 years). The control group (normal healthy individuals) consisted of 30 males and 22 females, aged 44 – 64 years (mean age = 54.12 ± 9.83 years). The follow-up lasted 5 years, or until the patient relapsed and died. The plasma level of cfDNA was determined in patients 1 day before treatment, 3 days after treatment, and on the 28th day of treatment.Based on the cfDNA expression level, NSCLC patients were subdivided into high-expression and low-expression groups, and differences in survival were compared. Univariate and multivariate analyses were performed on factors affecting patients survival using COX. Total effectiveness was significantly higher in observation group (49.35%) than in control group (13.95 %) (p<0.05). The extent of disease control wasalso significantly higher in the observation group (93.51 %) than in control group (p<0.05). Plasma cfDNA level of NSCLCpatients was significantly higher than that of control group before treatment, but was significantly and time-dependently reduced after gefitinib treatment (p<0.05). Cell-free DNA (cfDNA) level increased with severity of disease (p<0.05). Patients in cfDNA low-expression group had significantly higher chances of survival than those in the high-expression group (p<0.05). The results of Cox multivariate analysis showed that pathological severity and cfDNA concentration were independent factors affecting prognosis of NSCLC (p<0.05). Plasma cfDNA is a potential prognostic index in patients with NSCLC.

Key words: Cell-free DNA; Gefitinib; Lung cancer; Prognosis; Targeted therapy.

Introduction

Lung cancer is a common malignant tumor characterized by high incidence and mortality (1, 2). It is caused by smoking, second-hand smoke, exposure to certain toxins and family history. Two major types of lung cancer are non-small cell lung cancer (NSCLC) (85 % of all cases) and small cell lung cancer (SCLC). The most common types of NSCLC are squamous cell carcinoma, large cell carcinoma, and adenocarcinoma, but there are several other types that occur less frequently. A few of the less common types are pleomorphic sarcoma, carcinoid tumor, salivary gland carcinoma, and unclassified carcinoma (3). Treatments vary, but may include surgery, chemotherapy, radiation therapy, targeted drug therapy and immunotherapy. In China, targeted therapy is the most widely used treatment for NSCLC; itis convenient for patients, allows accurate positioning, and the incidence of adverse reactions is rare (4). Gefitinib, the most commonly used drug for targeted therapy, is a tyrosine kinase inhibitor of epidermal growth factor receptor (EGFR). It inhibits tumor cell proliferation and metastasis, while promoting cell apoptosis (5). Although gefitinib is effective against NSCLC, its clinical use is however limited by the development of drug resistance within a few months or after several years (6, 7). Therefore, it has become necessary to develop novel biomarker that can accurately monitor the effectiveness of gefitinib in targeted therapy of NSCLC.

Circulating free DNA (also known as cell-free DNA, cfDNA) is derived mainly from apoptosis and necrosis of cells. Its expression level reflects various pathological and physiological processes (5, 6). The free DNA released by the tumor has the same characteristics as the DNA of the tumor cell itself, reflecting the characteristic changes of the tumor. Therefore, blood cfDNA provides a comprehensive and accurate genetic map to compensate for the heterogeneity defects associated with pathological biopsies, and reflects the disease state from a macro perspective (8).Studies have shown that the level of cfDNA is significantly higher in tumor patients than in normal healthy individuals, mostly due to tumor cell necrosis and secretion (7, 9). The level of cfDNA in patients with malignant tumor has been reported to fall significantly after treatment (10). Results of different studies suggest that cfDNA may be a potential molecular marker for assessment of the effectiveness or prognosis of patients with malignant tumors. The present study investigated the potential of cfDNA as a prognostic factor for NSCLC.

Materials and Methods

Materials

Gefitinib was obtained from AstraZeneca Limited (UK) and cisplatin was purchased from Jiangsu Haosen Pharmaceutical Co. Ltd. (China). Human genomic DNA was product of Roche Molecular Systems Inc. (USA), while QIAamp DNA Blood Mini kit was obtained from Qiagen (USA).

General information of patients

Patients with NSCLC (n = 154) treated with gefitinib were recruited over a 5-year period (February 2015 to March 2020) for this study, and served as the observation group (79 males and 75 females, mean age = 53.74 ± 10.86 years).The control group (normal healthy individuals) consisted of 30 males and 22 females, aged 44 – 64 years (mean age = 54.12 ± 9.83 years). The follow-up lasted 5 years, or until the patient relapsed and died. The follow-up methods included return to hospital review, door-to-door follow-up and telephone calls. The included patients signed written informed consent with their family members, and there were no significant differences in their clinicopathological characteristics such as sex and age. The study protocol was approved by the Clinical Research Ethics Committee of Yixing People's hospital, China.

Table 1. Patients clinicopathological data.

Parameter	Assignment
Gender	
Male	1
Female	2
Age (years)	
<60	1
≥60	2
Smoking history	
With	1
Without	2
Tumor size	
<5 d/cm	1
≥5 d/cm	2
Lymph node metastasis	
With	1
Without	2
Number of previous anti-tumor treatments	Actual number (times)
Tissue types	
Adenocarcinoma	1
Squamous carcinoma	2
Others	3
TNM stage	
TX stage	1
T1 stage	2
T2 stage	3
T3 stage	4
Pathological severity	
High differentiation	1
Medium differentiation	2
Low differentiation	3
cfDNA concentration	
Low expression	1
High expression	2
Karnofsky score	Actual score (points)

Table 2. Primer sequences used for qRT-PCR.

Primer	Sequence
Primer 1 (97bp)	Forward:5'-TGGCACATATACACCATGGAA-3' Reverse: 5'-TGAGAATGATGGTTTC-3'
β-action (186bp)	Forward: 5'-TGGCACCCAGCACAATGAA-3' Reverse: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'

***Inclusion and exclusion* criteria**

The following categories of students were included in the study: (1) patients diagnosed with NSCLC (11); (2) patients with complete clinical data; (3) patients who cooperated with treatment and follow-up; and (4) patients who signed written informed consent with their family members. Patients with other malignancies, or heart, liver, kidney and other dysfunctions, or severe cognitive impairment, were excluded from the study.

Treatment regimen

Patients in the observation group received conventional symptomatic treatment: gefitinib and cisplatin were administered for 28 days as a course of treatment, with a total of 3 courses. Cisplatin (25 mg/m^2) was administered intravenously for the first three days of each course of treatment, in addition to one tablet (250mg) of gefitinib taken orally daily.

Collection and pretreatment of patients samples

Fasting peripheral venous blood was collected early in the morning in EDTA anticoagulant bottles 24 h before treatment, 3 days after treatment, and on the 28th day of treatment. The blood sample was centrifuged at 16, 000rpm for 10 min to obtain plasma. Aliquot of the plasma (200 μL) was immediately used for DNA extraction or refrigerated at -80°C.

Determination of plasma cfDNA level

The plasma was thawed on ice and spun at 10,000 g for 3 min before DNA purification. The extracted DNA was eluted with 50 μL elution buffer using QIAamp DNA Blood Mini Kit. The purified DNA was then quantified or refrigerated at -20°C. A serially diluted standardized solution of human genomic DNA was used as a standard curve reference, and the concentration of cfDNA in each sample was extrapolated from the standard curve (12).

Real-time quantitative polymerase chain reaction (qRT-PCR)

The qRT-PCR reaction mixture (20 μL) consisted of 1μL DNA template, 0.5 μL each of forward and reverse primer (LINE1 97 or LINE1 259), 10 μL UltraSYBR Mixture, and 8 μL double-distilled water. Cycling conditions were 1 min at 95°C, and 35 cycles of 95°C for 8 sec, and 60°C for 15 sec. Each plate consisted of serum DNA sample, a negative control (distilled water) and 7 serially diluted standard DNA solutions.

Evaluation of clinical effectiveness

Clinical effectiveness was evaluated according to the Improved Solid Tumor Efficacy Evaluation Standard (mRECIST), and classified thus: *complete remission (CR), partial remission, disease stable (SD), and disease progression (PD)* (13). The conditions applicable to each classification were: *CR*: lesion disappeared, with effect lasting for 4 weeks, and no formation of new lesions; *PR*: total decrease in lesion diameter ≥ 30 %, and effect maintained for 4 weeks; *SD*: lesion reduced, failure of total diameter to achieve *PR* or increase in diameter not approaching that of *PD*; *PD*: total increase in lesion size ≥ 20 % or formation of new lesions. The total effectiveness and disease control were calculated thus:

$$Effectiveness\ of\ treatment = \frac{(CR + PR) \times 100\ \%}{n}$$

$$Disease\ control\ (DCR) = CR + PR + SD$$

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using SPSS (21.0). Groups were compared using Student's *t*-test and chi-squared test. The COX stepwise regression model was used for multivariate analysis. Statistical significance was assumed at $p<0.05$.

Results

Clinical effectiveness between the two groups

Total effectiveness was significantly higher in observation group (49.35%) than in control group (13.95%) ($p<0.05$). The extent of disease control was also significantly higher in the observation group (93.51%) than in control group ($p<0.05$; Table 3).

cfDNA level in each group

Plasma cfDNA level of NSCLC patients before treatment was significantly higher than that of the control group, but decreased significantly and time-dependently in CR and PR subgroups after treatment ($p<0.05$). Cell-free DNA (cfDNA) level increased with severity of disease ($p<0.05$). These results are shown in table 4.

Survival of patients in the two cfDNA expression groups

As shown in Table 5, patients in cfDNA low-expression

Table 3. Comparison of clinical effectiveness between the two groups (n, %).

Group	CR	PR	SD	PD	RR	DCR
Observation (n = 154)	14 (9.09 %)	62 (40.26 %)	68 (44.16 %)	10 (6.49 %)	76 (49.35 %)	144 (93.51 %)
Control (n = 52)	0 (0.00 %)	6 (13.95 %)	22 (51.16 %)	15 (34.88 %)	6 (13.95 %)	28 (65.12 %)

Table 4. Comparison of cfDNA levels between the two groups.

Time	Control(n=52)	CR (n=14)	PR (n=62)	SD (n=68)	PD (n=10)
Before treatment		15.08±5.79 [a]	16.12±5.35[a]	15.97±6.08 [a]	16.29±5.87[a]
Third day of treatment	8.15±2.07	13.34±2.45[*]	13.76±2.13[*]	13.24±2.98[*]	17.54±6.69
The 28th day of treatment		11.24±1.63[#]	11.38±1.84[#]	13.05±3.06	21.89±7.46[#]

[a]$p<0.05$ compared with the control group; [#]$p<0.05$ compared with cfDNA levels on the third day of treatment; *$p<0.05$ compared with cfDNA levels on the 28th day of treatment.

Table 5. Comparison of patient survival in the two cfDNA expression groups.

Time (years)	cfDNA high expression (n=79)	cfDNA low expression (n=75)	χ^2	*p*
<1	25 (31.65%)	37 (49.33%)	2.165	0.0304
1-3	17 (21.52%)	28 (37.33%)	2.157	0.0310
3-5	11 (13.92%)	21 (28.00%)	2.152	0.0314
>5	9 (11.39%)	18 (24.00%)	2.057	0.0397

group had significantly higher chances of survival than those in the high expression group($p<0.05$).

Cox univariate analysis for prognosis of NSCLC

The results of Cox univariate analysis showed that the prognostic factors of NSCLC were pathological severity and cfDNA concentration ($p<0.05$). Prognosis was independent of patient's gender, age, smoking history, tumor size, lymph node metastasis, previous anti-tumor treatment, tissue type, TNM stage and Karnofsky score ($p>0.05$). These results are shown in Table 6.

Cox multivariate analysis for prognosis of NSCLC

The results of Cox multivariate analysis showed that pathological severity and cfDNA concentration were independent factors that affect prognosis of NSCLC ($p<0.05$; Table 7).

Discussion

At present, the treatment options for NSCLC are surgery, chemotherapy and targeted therapy. Since the disease has insidious onset, patients often miss early diagnosis and opportunity to get proper treatment (surgical resection): They are left with no option, but to settle for non-radical treatment such as radiotherapy or chemotherapy (14). Early treatment delays disease progression, prolongs survival and improves the quality of life of patients.

Targeted therapy is a cancer treatment that uses drugs to target specific genes and proteins that are involved in the growth and survival of cancer cells. It affects the tissue microenvironment that helps a cancer grow and survive, or targets cells related to cancer growth, such as blood vessel cells. Targeted therapy produces a significant clinical effect.

Gefitinib is a drug used for certain breast, lung and other cancers. As an EGFR inhibitor, it interrupts signaling through the epidermal growth factor receptor in target cells. Therefore, it is only effective in cancers with mutated and overactive EGFR.

Gefitinib is a first-line drug for targeted therapy believed to be a major therapeutic strategy for reducing conduction ability of cancer cells. It inhibits tumor cell proliferation and metastasis, while promoting cell apoptosis (15). Gefitinib has been shown to be effective in the treatment of NSCLC (16). However, its clinical use is limited by drug resistance, which leads to poor prognosis (17, 18).

Under normal physiological conditions, the level of

Table 6. Cox univariate analysis for prognosis of NSCLC.

Indicators	χ^2	*p*
Gender	1.255	0.263
Age	2.018	0.155
Smoking history	1.580	0.209
Tumor size	0.110	0.740
Lymph node metastasis	3.253	0.071
Number of previous anti-tumor treatments	2.809	0.094
Tissue types	3.194	0.075
TNM stage	1.782	0.182
Pathological severity	6.580	0.010
cfDNA concentration	6.214	0.013
Karnofsky score	1.713	0.191

Table 7. Cox multivariate analysis for prognosis of NSCLC.

Indicators	B	S.E.	Wal	Exp(B)	95% CI	*p*
Pathological severity	0.539	0.210	6.596	1.714	1.136-2.585	0.010
cfDNA concentration	0.313	0.117	7.171	1.368	1.088-1.720	0.007

cfDNA in the human body is relatively low, but it is high in patients with malignant tumors, as a result of increased cell necrosis and apoptosis (19).More and more studies have found that cfDNA levels in patients with lung cancer, breast cancer and colon cancer are significantly higher than those in normal people, and further studies have shown that cfDNA can also be used to evaluate the treatment response of tumors and to determine the prognosis of patients with tumors. In a previous study, cfDNA level of NSCLC patients was reported to be significantly higher than that of normal healthy control before treatment, but was significantly reduced after treatment (20). In that study, cfDNA level correlated with disease progression/poor prognosis. Patients with malignant tumors with elevated peripheral blood cfDNA levels usually have poor prognosis (21). The results of previous studies suggest that plasma cfDNA level in NSCLC patients could serve as a potential indicator for evaluation of gefitinib effectiveness and prognosis. The present study investigated the potential of cfDNA as a treatment monitoring and prognostic factor for NSCLC. The results showed that plasma cfDNA level of NSCLC patients was significantly higher than that of control group before treatment, but was significantly and time-dependently reduced after gefitinib treatment. The level increased with severity of disease. These results indicate that plasma cfDNA level could reflect the state of NSCLC patients and clinical effectiveness of gefitinib, and are in agreement with reports of previous studies (21-23).

Various factors influence the prognosis of patients with NSCLC. In this study, Cox univariate and multivariate analyses were used to analyze relevant factors affecting prognosis of patients with NSCLC (24). Cell-free DNA (cfDNA) concentration and pathological severity were found to be independent risk factors for prognosis of NSCLC patients, suggesting that plasma cfDNA level may reflect clinical effectiveness of gefitinib in NSCLC treatment. Similarly, patients in cfDNA low-expression group had significantly higher chances of survival than those in the high-expression group. The results obtained in this study show that plasma cfDNA is a potential prognostic index in patients with NSCLC.

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Conflicts of interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Ying Zhang; Nan Chen,Gaofeng Li,Heng Li,Xudong Xiang, Gang Guo, Qianli Ma, Ying Zhang collected and analysed the data; Nan Chen wrote the text and all authors have read and approved the text prior to publication.

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