Oncology and Translational Medicine
DOI 10.1007/s10330-019-0335-5

June 2019, Vol. 5, No. 3, P103–P108

ORIGINAL ARTICLE

Quantitative monitoring and mutations of ctDNA before and after non-small cell lung cancer radical surgery*

Xiangqi Chen[1], Tingyan Lin[1], Zhisen Gao[1], Jiangjiang Xu[3], Rui Feng[2], Sheng Yang[2], Shuchen Chen[3], Mingqiang Kang[3] (✉)

[1] Department of Respiratory, Fujian Medical University Union Hospital, Fuzhou 350001, China
[2] Department of Oncology, the Union Hospital of Fujian Medical University, Fuzhou 350001, China
[3] Department of Thoracic surgery, Fujian Medical University Union Hospital, Fuzhou 350001, China

Abstract

Objective The aim of this study was to study the quantitative expression of circulating tumour DNA (ctDNA) in patients with non-small cell lung cancer (NSCLC) before and after radical operation and to explore the correlation between gene mutations in non-small cell lung cancer tissues and those in ctDNA.

Methods We randomly assigned 5 NSCLC patients from the Department of Thoracic Surgery of Fujian Medical University Union Hospital. All the patients had undergone radical surgery. Venous blood samples were collected from the 5 NSCLC patients at two time points (before the operation and 21–37 days after the operation) for monitoring ctDNA levels. This was done by isolating plasma from venous blood using high velocity centrifugation, extracting DNA from the plasma using the QIAamp Circulating Nucleic Acid kit, and then quantifying the ctDNA levels. The results were analyzed using the Wilcoxon Rank Sum Test. Moreover, the ctDNA levels were compared with those of carcinoembryonic antigen (CEA), which was detected simultaneously with the ctDNA. Then, DNA samples from the tumor tissues and peripheral blood cells and ctDNA were sequenced using the Hiseq2000 sequencing platform (Illumina) and the mutant genes were screened out. Mutations that occurred within the tumor tissues were used as positive control, whereas those found in the pre-operative blood cells were used as a negative control. Based on the mutational analysis of ctDNA genes, a total of 508 cancer-related genes were screened.

Results The median values of the pre- and post-operative ctDNA levels in the 5 patients with NSCLC were 0.612 (0.518–0.876) and 0.430 (0.372–0.612) ng/μL, respectively. There was a significant difference between the two groups ($P < 0.05$). The pre-operative CEA level was slightly higher than the post-operative level ($P > 0.05$). In one of the cases, LC tissues showed multiple mutations, consistent with pre-operative ctDNA. Moreover, isogenic mutations of the same type were not detected in post-operative ctDNA or peripheral blood cells.

Conclusion Mutations found in the lung cancer (LC) ctDNA gene were consistent with the mutation type of LC tissue. Hence, the quantitative and qualitative analysis of ctDNA is a promising novel molecular biomarker for the evaluation of tumor burden changes in NSCLC.

Received: 17 March 2019
Revised: 5 April 2019
Accepted: 20 May 2019

Key words: ctDNA; non-small cell lung cancer (NSCLC); mutant genes; molecular markers

✉ Correspondence: Mingqiang Kang. Email: drkang2011@163.com
* Supported by grants from the Key Specialty Discipline Construction Program of Fujian and Nation, P.R.C and Fujian Provincial Department of Health's Key Middle-aged and Young Talents Program (No. 2013-ZQN-JC-14).

Lung cancer (LC) is a type of malignant cancer with a very high mortality rate worldwide. In China, its morbidity and mortality rates are the highest and non-small cell lung cancer (NSCLC) is the most common form of this disease. ctDNA is a type of circulating cell-free DNA (ccfDNA) fragment found in the plasma or serum and it is released due to tumor cell apoptosis, necrocytosis, or proactive release [1–2]. ccfDNA is extracellular in nature and is found at extremely low levels in normal human tissues. In 1977, Leon et al. discovered that the DNA levels in the plasma of tumor patients was greatly increased and based on this revelation, several studies further investigated the applications of ccfDNA for the early diagnosis of tumors, real-time monitoring of therapeutic effects, and prediction of relapse [3]. ccfDNA levels were also found to be abnormally increased in patients with ovarian, gastric, and breast cancers [4–6]. Newman et al. reported that the ctDNA level can reflect the changes in the tumor burden in early or terminal NSCLC after various types of treatments [7]. Therefore, ctDNA is a promising novel molecular biomarker for the early diagnosis and evaluation of malignant tumors.

Materials and methods

Clinical data and inclusion criteria for relevant mutant genes

All patients included in the study had no previous history of other tumors, distant organ metastasis, chronic liver, kidney, endocrine and immune system diseases before the operation, and had not received any radiotherapy, chemotherapy or immunotherapy (Table 1).

According to the catalog of somatic mutations in cancer (COSMIC) database, the genes that qualified the inclusion criteria (a total of 508 genes) referred to the high frequency mutant genes in various tumors and signaling pathways related to cancer occurrence and development.

Methods

Separation of tumor, plasma and blood cell samples

Venous blood (5 mL) was drawn into EDTA-coated anticoagulant tubes, centrifuged for 10 min at 1600 g (4 °C), and the supernatant containing the plasma was separated. The blood cells were collected and stored at –80 °C. The plasma was further centrifuged for 10 min at 16000 g (4 °C) to remove the residual cells and stored at –80 °C until further use.

Fresh tissues were removed immediately after tumor resection, approximately 0.5 cm^3 of tumor tissue mass was cut and then, these samples were stored at -80 °C.

Extraction of sample DNA and quantitative determination of ctDNA

DNA extraction from all the tissues and peripheral blood cell samples was performed using QIAGEN QIAamp DNA and blood mini kits (QIAGEN, USA), according to the manufacturer's instructions.

The extraction of free DNA from the plasma was performed using the QIAamp circulating nucleic acid kit, according to the manufacturer's instructions, and the extracted DNA was quantified using the Qubit (Invitrogen, the Quant-iTTM dsDNA HS Assay Kit) quantometer.

Detection of gene mutations

A customized chip 140119_HG19_CAN_panel_EZ_HX3 (1.7M) was obtained from Roche and was used to capture sequence hybridization. Hiseq2000 sequencing platform (Illumina) was used for sequencing. GATK (2.3.9) and other software were used to identify somatic SNV, INDEL and CNV.

Quantitative determination of CEA

Venous blood (3 mL) was drawn, and CEA was detected using automated immunoassay system (E601, Roche) using the original reagents according to the manufacturer's instructions.

Results

ctDNA And CEA levels in patients with NSCLC

As shown in Table 2, the median values of pre- and post-operative ctDNA levels in the five patients with NSCLC were 0.612 (0.518–0.876) and 0.430 (0.372–0.612) ng/μL, respectively. The pre-operative ctDNA level was significantly higher than the post-operative level ($P < 0.05$; Table 2).

The median values of pre- and post-operative CEA levels in the five patients were 3.90 (2.25–6.20) and 1.90 (1.55–4.95) ng/mL, respectively. The pre-operative CEA

Table 1 Clinical data of five patients with non-small cell lung cancer (*n*)

No.	Gender	Age (years)	Pathology	TNM Stage*	Clinical stage
1	Female	59	Invasive adenocarcinoma	T1N2M0	IIa
2	Male	62	Median differentiated squamous carcinoma	T2N0M0	Ib
3	Male	56	Median differentiated squamous carcinoma	T2N1M0	IIb
4	Male	61	High differentiated squamous carcinoma	T1N1M0	IIa
5	Male	61	Invasive adenocarcinoma	T2N0M0	Ib

* TNM staging was developed by American Joint Committee on Cancer (AJCC) and Union for International Cancer Control (UICC) in 2002

Table 2 Concentrations of ctDNA and CEA

Group	ctDNA (ng/μL) (Quartile range)	CEA (ng/mL) (Quartile range)
Before operation	0.612 (0.518–0.876)	3.90 (2.25–6.20)
After operation	0.430 (0.372–0.612)	1.90 (1.55–4.95)
Z	–2.023	–0.944
P	0.043*	0.345**

* Comparison of pre- and post-operative ctDNA levels after 28 days in five patients with non-small cell lung cancer ($P < 0.05$); ** Comparison of the first and second CEA levels in patients with non-small cell lung cancer ($P > 0.05$)

level was slightly higher than the post-operative level ($P > 0.05$).

Although there was a difference in the detection time of ctDNA and CEA before operation, all patients had non-excised lung tumor tissue before operation. The median detection time of both indices in blood samples after the operation was 26 days, assuming that the sampling occurred at the same time.

Pre-operative ctDNA and CEA levels were higher than the post-operative levels, but only the ctDNA levels showed significant difference ($P < 0.05$; Table 2). The comparison of pre- and post-operative ctDNA and CEA levels in five patients is shown in Fig. 1.

Mutations found in NSCLC tissue, peripheral blood cells, and ctDNA

The samples from five patients showed several mutant genes in tumor tissues as well as pre- and postoperative ctDNA (Table 3). A total of 85 mutant genes were detected, with 42 genes detected in the lung tumor tissue, 26 in pre-operative ctDNA, 15 in post-operative ctDNA, and two in pre-operative peripheral blood cells. No mutant genes were found in the post-operative peripheral blood. The mutant genes found were either

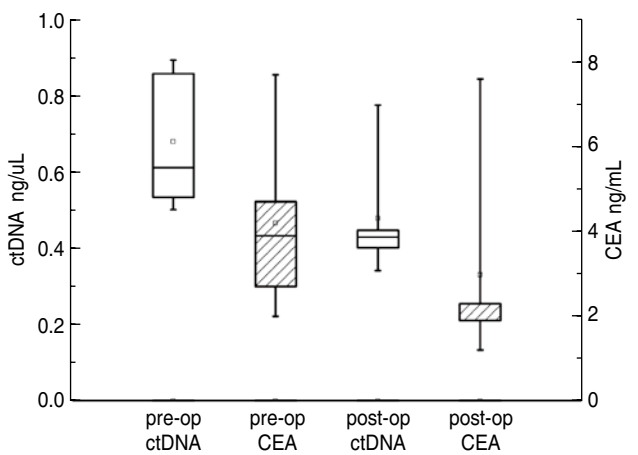

Fig. 1 Comparison of pre- and post-operative ctDNA and CEA levels in five patients

Table 3 Number of mutant genes detected in samples

No.	Pre-operative ctDNA	Post-operative ctDNA	Tumor tissue	Peripheral blood cells
1	4	1	3	0
2	9	2	12	0
3	6	0	5	0
4	4	6	17	1
5	3	6	5	1

oncogenes or tumor suppressor genes involved in tumor cell signal transduction, regulation of gene transcription, cell proliferation, differentiation, apoptosis, migration, invasion, and metastasis (Table 3).

The mutations in the pre-operative ctDNA were compared with those found in the tumor tissues, post-operative ctDNA and pre-operative blood cells, and the results were as follows: LC tissue showed mutations consistent with pre-operative ctDNA in one case (case no. 2), and the gene and the site location of the mutations were found to be similar. There were eight pairs of identical mutant genes between tumor tissues and pre-operative ctDNA (TP53, PTEN, KMT2D, TET2, CDK12, PRPF40B, RUNX1T1, and NTRK3) in this patient. Moreover, after excision of LC tissue, the same type of isogenic mutation was not detected in ctDNA of this patient. A consistent gene mutation (ASXL1) was also detected between pre- and post-operative ctDNA in one case (No. 5). However, this mutation was not detected in the tumor tissue or peripheral blood cells, suggesting that it might not be derived from the excised tumor tissue.

The average numbers of mutant genes found in the four sample types were in the following order: tumor tissue > pre-operative ctDNA > post-operative ctDNA > peripheral blood cells. There were significant differences in the number of mutant genes between the tumor tissue and peripheral blood cells, and between pre-operative ctDNA and peripheral blood cells ($P < 0.05$). In case of the other pairwise comparisons, especially the pre- and post-operative ctDNA showed no statistical difference ($P > 0.05$).

No significant positive or negative correlation was found in the number of mutant genes in ctDNA and their individual levels. In only three cases, a uniform decrease was seen in the pre- and post-operative ctDNA levels, and two cases of these cases actually showed decreased level but increased number.

We detected three genes (KMT2D, EGFR and TP53) in this study that were amongst the top 20 mutanted genes for LC according to the COSMIC database. The TP53 mutation was detected in three cases, for one of which it was found in the pre-operative ctDNA. Four KMT2D mutations were detected in two cases. Besides the similar mutation types of KMT2D found in ctDNA and tumor, we also found two different types of mutations in KMT2D

in one of patient's tumor tissue. Two mutations in EGFR were detected, and the mutation of L858R, which is a drug target site, was also detected in the tumor tissues of two patients (Table 4).

Discussion

LC is a malignant tumor with a high morbidity rate worldwide. While the preferred treatment for early-stage LC is surgical resection, only about 50% patients achieve a high long-term survival rate with a good quality of life. In particular, patients with NSCLC easily relapse and develop metastasis within a short duration after operation [8]. Since there are no sensitive tumor biomarkers available for imaging examination, the search for an efficacious, simple, and highly accurate clinical biomarker is a research hotspot in tumor biology.

Compared to the traditional protein tumor biomarkers, when ctDNA with specific mutations and other cancer-related genetic changes is used as a marker, the probability of false positivity is significantly reduced [9] and better specificity is also observed. Clinical tumor markers are easily influenced by other lesion factors. However, the half-life of ctDNA is shorter than those of protein markers, therefore, it represents the current status of the tumor [10] and is more appropriate for real-time monitoring of tumors. Detection of CEA levels in the serum is also very important for the evaluation of prognosis of LC. For example, Yang *et al* found that the CEA level continuously declined in patients with non-relapsed NSCLC after operation, and the post-operative CEA level greatly decreased within one month [11]. Tan *et al* reported that the post-operative CEA level was significantly different from the pre-operative level within seven days of operation [12]. Similarly, in our study, we noticed that the CEA level decreased as compared to the pre-operative level and could be detected up to 26 days after the operation. However, there was no significant difference between the two levels and this can perhaps be due to the small sample size. The ccfDNA level in the tumor samples is higher than in normal samples, as demonstrated by the studies on ctDNA and tumors [4–5]. Furthermore, ctDNA level shows obvious correlation with tumor cell burden [13]. Imberger et al. also found that the post-operative ctDNA levels was greatly reduced and even reached normal levels in patients with ovarian cancer after successful operation. However, increased ctDNA levels after operation might be caused by ineffective treatment or systemic disease [14]. Among the five cases investigated in our study, the post-operative ctDNA level was significantly reduced compared to pre-operative ctDNA level, consistent with the previous reports. Jung *et al* showed that the continuously increasing cfDNA level could reflect the possibility of a relapse earlier than that predicted using the CEA marker or imaging examination in patients who have undergone esophageal resection [15]. In our study, the quantitative comparison of pre- and post-operative ctDNA and CEA levels in NSCLC patients showed a decreasing trend, which was significantly different for ctDNA, suggesting that ctDNA was more sensitive for detection of surgical efficacy. Hence, the quantitative analysis of ctDNA has potential application

Table 4 Genes mutated ≥ two times out of the 508 genes and their corresponding distribution (*n*)

Gene	Total number of times	Number of cases/person	Tumor tissue/time	Pre-operative ctDNA/time	Post-operative ctDNA/time
TP53	4	3	3	1	0
KMT2D	4	2	2	1	1
CHD4	3	2	1	0	2
NTRK3	3	2	2	1	0
GNAQ	2	2	0	2	0
ASXL1	2	1	0	1	1
ABL2	2	2	1	1	0
EGFR	2	2	2	0	0
NOTCH2	2	2	1	1	0
PTEN	2	1	1	1	0
TET2	2	1	1	1	0
NOTCH1	2	2	1	1	0
PRPF40B	2	1	1	1	0
CDK12	2	1	1	1	0
RUNX1T1	2	1	1	1	0
EPHB2	2	2	1	0	1
PTCH1	2	2	2	0	0
KMT2C	2	1	0	2	0
AXL	2	2	0	2	0

in the early diagnosis of LC; however, the evaluation of its clinical efficacy and the improvement of its relevance in translational medicine are required. However, in our study, the sample size was relatively small and future studies using larger sample sizes should be performed.

A total of 508 genes were analyzed in our study, including the highly comprehensive detection of genes relevant to LC. The genes detected were either oncogenes or tumor suppressor genes involved in tumor cell signaling transduction, regulation of gene transcription, cell proliferation, differentiation, apoptosis, migration, invasion, and metastasis. In this study, we found that the mutation frequency of TP53 was high. Previous studies have reported that point mutations of TP53 are detected in the ctDNA of patients with colorectal, lung, liver, breast, and head and neck cancers [16–17]. Recently, Chen *et al* reported that the co-exist of tumor mutations and ctDNA mutation was up to 26% of breast cancer cases [18]. Similarly, in our study, KMT2D gene was detected several times, which is one of the genes that shows high mutation rate, according to the COSMIC database. However, there are few reports about its effective mechanism in tumors, or co-existence of its mutation with tumor in ctDNA detection. Sahoo *et al* demonstrated that the mutation rate of EGFR was 30%–50% in Asian NSCLC patients [19]. Peng *et al* reported that the mutation rate of EGFR was 17.7% in plasma ctDNA in 96 pairs of LC [20] .Furthermore, Lee *et al* showed that the EGFR mutation type found in the plasma was the same as that found in the tumor with a coincidence rate of 59.6%, compared to the LC tissue [21]. Taken together, these three studies suggested that approximately half of the mutation-positive EGFR genes found in NSCLC patients could be detected using peripheral ctDNA: an EGFR gene mutation was detected in LC tissues in two cases, but no mutation was detected in ctDNA. Hence, the detection of the mutations in the ctDNA gene can be used for tumor efficiency monitoring, prognosis evaluation, prediction of disease progression and survival time, drug selection, and drug resistance. Due to the low sensitivity and specificity of single-gene diagnosis and evaluation, the presence of a mutant gene cannot be used as the sole marker for tumor diagnosis. However, a combination of quantitative and qualitative analyses of ctDNA with other tumor markers has shown higher accuracy for the diagnosis and prognosis of tumors, compared to a single indicator.

Notably, ctDNA is highly relevant in tumors, and the mutation of the LC ctDNA gene is found to be consistent with the mutation type of LC tissue, further proving the tumor derivation of ctDNA. When compared to traditional tumor markers, ctDNA is sensitive to tumor burden changes, which indicates that the quantitative and qualitative analysis of ctDNA is a promising novel molecular biomarker for the evaluation of tumor burden changes in NSCLC. Our study had some limitations, such as a small sample size and short follow-up period. Having said that, this was just a preliminary study. Further high quality, multicenter, clinical studies with large sample sizes focusing on the correlation of ctDNA and LC diagnosis are needed to provide a novel strategy for the prophylaxis and treatment of LC.

Conflicts of interest

The authors declare no conflicts of interest.

References

1. Lianos GD, Mangano A, Kouraklis G Roukos DH. Dynamic sequencing of circulating tumor DNA: novel noninvasive cancer biomarker. Biomark Med, 2014, 8: 629–632.
2. Jiang WF, Zhang HL. Enrichment and detection of circulating tumor cells in peripheral blood. Oncol Transl Med, 2011, 10: 240–244.
3. Leon SA, Shapiro B, Sklaroff DM, *et al*. Free DNA in the serum of cancer patients and the effect of the rapy. Cancer Res, 1977, 37: 646–650.
4. Cheng X, Zhang L, Chen Y, Qing C. Circulating cell-free DNA and circulating tumor cells, the “liquid biopsies” in ovarian cancer. J Ovarian Res, 2017,10: 75.
5. Todenhöfer T, Struss WJ, Seiler R, *et al*. Liquid Biopsy-Analysis of Circulating Tumor DNA (ctDNA) in Bladder Cancer. Bladder Cancer, 2018, 4: 19–29.
6. Gao J, Wang H, Zang W, *et al*. Circulating tumor DNA functions as an alternative for tissue to overcome tumor heterogeneity in advanced gastric cancer. Cancer Sci, 2017, 108: 1881–1887.
7. Newman AM, Bratman SV, To J *et al*. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med, 2014, 20: 548–554.
8. Liu ZZ, Xie XD. Whole process control and precision therapy in lung cancer. Oncol Transl Med, 2017, 3: 91–92.
9. Ellen Heitzer, Peter Ulz, Jochen B. Circulating Tumor DNA as a Liquid Biopsy for Cancer. Clin Chem, 2015, 61: 112–123.
10. Zhao H, Chen KZ, Hui BG, *et al*. Role of circulating tumor DNA in the management of early-stage lung cancer. Thorac Cancer, 2018, 9: 509–515.
11. Yang D, Xia W, Ma R. Significance of observing serum tumor markers before and after operation of NSCLC. Chin J Surg Oncol (Chinese), 2014, 6: 25–27.
12. Tan W, Yang S, Bao Y, *et al*. Changes of serum NSE, CYFRA21-1 and CEA in patients with lung cancer before after operation and related clinical significance analysis. Int J Lab Med, 2012, 33:16–17.
13. Sausen M, Parpart S, Diaz LA. Circulating tumor DNA moves further into the spotlight. Genome Med, 2014, 6: 35.
14. Wimberger P, Roth C, Puntel K, *et al*. Impact of platinum-based chemotherapy on circulating nucleic acid levels, protease activities in blood and disseminated tumor cells in bone marrow of ovarian cancer patients. Int J Cancer, 2011,128: 2572–2580.
15. Jung K, Fleischhacker M, Rabien A. Cell-free DNA in the blood as a solid tumor biomarker-acriticalappraisal of the literature. Clin Chim Acta, 2010, 411: 1611–1624.
16. Park YR, Kim Y-M, Lee SW, *et al*. Optimization to detect TP53 mutations in circulating cell-free tumor DNA from patients with serous epithelial ovarian cancer. Obstet Gynecol Sci, 2018, 61: 328–336.
17. Wade M, Li YC, Wan GM. MDM2, MDMX and p53 in oncogenesis

and cancer therapy. Nat Rev Cancer, 2013, 13: 83–96.
18. Chen Z, Fang J, Buzin CH, *et al*. Analysis of cancer mutation signatures in blood by a novel ultra-sensitive assay: monitoring of therapy or recurrence in non-metastatic breast cancer. PloS One, 2009, 4: e7220.
19. Sahoo R, H arini VV, Babu VC, *et al*. Screening for EGFR mutations in lung cancer a report from India. Lung Cancer, 2011,73: 316–319.
20. Peng Q, Zhao J, Qin H, *et al*. Detection of EGFR mutation in peripheral blood and its clinical significance in lung cancer patients. Chin J Surg Oncol (Chinese), 2011, 38: 263–267.
21. Lee Y, Park S, Kim WS, *et al*. Correlation between progression-free survival, tumor burden, and circulating tumor DNA in the initial diagnosis of advanced-stage EGFR-mutated non-small cell lung cancer. Thorac Cancer, 2018: doi:10.1111/1759-7714.12793.

DOI 10.1007/s10330-019-0335-5
Cite this article as: Chen XQ, Lin TY, Gao ZS, *et al*. Quantitative monitoring and mutations of ctDNA before and after non-small cell lung cancer radical surgery. Oncol Transl Med, 2019, 5: 103–108.