Detection of cancer specific mutations in early-stage non-small cell lung cancer using cell-free DNA by targeted sequencing

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Abstract. Non-small cell lung cancer (NSCLC) is a major public health problem worldwide and leads to a high mortality. NSCLC is always diagnosed in late stages because of its unapparent symptoms. However, cell-free DNA (cfDNA) may serve as a new potential biomarker to detect early stage of non-small cell lung cancer. Here we recruited 10 non-small cell lung cancer patients to obtain fresh tumor tissue, peripheral blood lymphocytes (PBLs), and plasma. CfDNAs from 13 elderly people and 7 middle-age smokers were also extracted as controls. Illumina HiSeq X10 was used to perform next-generation sequencing to evaluate differences in mutations among different samples. The result indicated that tumor DNA and its matched plasma cfDNA samples showed high concordance in their mutation patterns. Mutation rate of cfDNA was generally lower than that of tumor tissue and higher than that of PBLs. The plasma cfDNA concentration of NSCLC patients (69.2±46.9 ng/ml) is significantly higher than that of elderly people (32.5±5.2 ng/ml, t=2.96, p=0.007) and middle-aged smokers (17.9±9.1 ng/ml, t=2.83, p=0.013). Five mutations (PTEN_c.1375A>G, TP53_c.94G>A, STK11_c.816C>T, PIK3CA_c.1633 G>A, PIK3CA_c.2038G>C) were only identified in NSCLC patients but not in healthy people. Our conclusion was that cfDNA has a similar mutation pattern with its matched tumor tissue DNA. A high concentration of cfDNA and tumor specific mutations in cfDNA may serve as potential non-invasive biomarkers to detect early-stage non-small cell lung cancer.

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Introduction

It is reported that there were 1.8 million new cases of lung cancer in 2012 (12.9% of the total), 58% of which occurred in less developed regions (1). Lung cancer is estimated to be responsible for nearly one in five cancer related deaths worldwide (1.59 million deaths, 19.4% of the total), which makes it a major public health problem (2).

In most Western countries, lung cancer incidence and death rates are decreasing in men and plateauing in women (3,4); however, both incidence and mortality rates of lung cancer are still increasing in China and there were ~652,800 new cases and 597,200 deaths in China in 2015, accounting for 35.78% and 37.56% of the whole world (5). Approximately 85% of patients diagnosed with lung cancer are non-small cell lung cancer (NSCLC) and two in three of these cases are diagnosed in metastatic or advanced stages (6). NSCLC patients with early stage could have better opportunity of admirable survival (7). Therefore minimal damage, effective and convenient detection for early stage of non-small cell lung cancer is particularly necessary.

Currently there are several methods to detect NSCLC, such as tissue biopsy, which is the golden standard of diagnosis. However, it is often difficult to obtain biopsy samples, and it is very challenging or time-consuming to acquire samples especially in early-stage cases from different medical centers. Low-dose computed tomography (LDCT) and X-ray examination are two other main approaches. LDCT is an ever emerged National Lung Screening Trial (NLST) in US; however, LDCT screening has a relatively low specificity (73.4%), which results a high false-positive findings rate (96.4%) (8). LDCT screening is simple and was shown to confer a 20% reduction in lung cancer mortality and a higher proportion of early NSCLC diagnosis in a high-risk population (9), but its utility and validity are still under debate (10-12). Recently the U.S. Preventive Services Task

Force recommendation statement (USPSTF) recommended annual LDCT-screening for lung cancer in high-risk individuals and stressed the need for more research into the use of biomarkers to complement LDCT screening.

CfDNA corresponds to cell-free DNA fragments circulating in the bloodstream which can be extracted from plasma or serum. CfDNA is mostly composed of constitutive genomic DNA (13). One of the most immediate applications of circulating cfDNA has been termed 'liquid biopsy' in research studies as well as in clinical practices (14,15). Detection of genetic and specific mutations will be the most promising tool for large-scale population-based lung cancer screening when considering its safety, availability and accuracy (11). Sun *et al* found that compared with other non-invasive approaches to monitor EGFR-TKI treatment in NSCLC patients, cfDNA displayed many advantages: moderate sensitivity, high specificity, feasible on small-amount samples, rapid and low cost, and high reproducibility (16). Identified mutations in cfDNA have also been found to be potential prognostic biomarkers of NSCLC (17,18). Here we expanded the increasing interest in this approach to explore potential application in early stage screening and diagnosis of NSCLC patients.

Materials and methods

Patient information and ethics statement. Tumor and blood samples from 10 NSCLC patients were analyzed in this study. All patients, including 8 males and 2 females with an average age of 57.8±8.88, were diagnosed with stage IA, IB, IIA, and IIB NSCLC, of these 5 were adenocarcinoma, 4 were squamous cell carcinoma (SCC), and 1 was sarcomatoid carcinoma. Three of these patients have direct relatives diagnosed with cancer. Four of these patients have long term smoking history (>20 years). 20 healthy controls, including 13 elderly people and 7 middle-age smokers with an average age of 66.8±15.46, were also recruited from noncancerous out-patients of Hebei Medical University Fourth Hospital. The study was approved by Ethics Committees of Hebei Medical University Fourth Hospital. All participants, including NSCLC patients and healthy controls, provided written informed consent for this study.

Sample DNA handling. Fresh tumor tissue, peripheral blood lymphocytes (PBLs), and plasma were collected for analysis for each patient. Tubes (10 ml) containing blood samples with EDTA added were centrifuged at 1000 g for 10 min. The cell pellets containing peripheral blood lymphocytes were stored at -20˚C. The supernatants were centrifuged again at 10,000 g for 10 min, and plasma was collected and stored at -80˚C. Tiangen tissue DNA kit (Tiangen, Beijing, China) and Tiangen whole blood DNA kit (Tiangen) were used to extracted DNA from fresh tumor tissue and peripheral blood lymphocytes, respectively. QIAamp Circulating Nucleic Acid kit (Qiagen, Germany) was used to extract cfDNA form plasma. All kits were used according to the manufacturer's instructions.

Library preparation and sequencing. For each sample, DNA was quantified with the Qubit dsDNA HS Assay kit (Life Technologies, USA) according to the manufacturer's instructions. Targeted amplification and Illumina adapter-ligated library preparation was performed using Amplicon Sequencing-Illumina Compatible kit following the manufacturer's instructions (Genecrab, Beijng, China). All samples were subjected to Illumina HiSeq X-Ten for paired-end sequencing (150 bp each end). The AmpliSeq Cancer Panel covers 92 continuous region with 10,235 bp in 22 cancer-associated genes (KRAS, EGFR, BRAF, PIK3CA, AKT1, ERBB2, PTEN, NRAS, STK11, MAP2K1, ALK, DDR2, CTNNB1, MET, TP53, SMAD4, FBX7, FGFR3, NOTCH1, ERBB4, FGFR1 and FGFR2), which developed by Life Technologies Co.

Variant calling. Initial data from HiSeq X-Ten were evaluated by using fastQC (v0.11.3). Raw reads were mapped to reference genome hg19 by using BWA (0.7.12-r1039). Program Samtools and VarScan (v2.4.1) was used for variant calling: i) the average total coverage depth was defined as >1,000 and each variant coverage as >10; for called variant, at least one sample with variant frequency >1%, variant frequency of each sample >0.5%, and P-value <0.01; ii) visual examination of the mutations was performed using Samtools software (http://samtools.sourceforge.net) and possible errors specific to one DNA strand were filtered out. Software ANNOVAR (v2015-06-17) and snpEff (v4.2) was used for variant annotation.

Statistical analysis. For variant frequency <0.5%, 0 was replaced. R (hclust, v3.2.4) was used for variant frequency clustering analysis to show the types of samples from cancer patients that are more similar. Student's t-test was applied for comparison of cfDNA concentration and $p<0.05$ was considered statistically significant.

Results

Sequence QC with the Illumina X10. For the 10 NSCLC patients, tumor DNA, matched blood DNA, and plasma cfDNA were all subjected to sequencing. Of all 30 sequenced samples, sequence lengths were all set to 150 bp. The quality scores were ~40, indicated that the accuracy is very good and error rate was ~0.01% (Fig. 1A). The GC content was ~50% besides the first 1-15 bp, which was removed before further analysis (Fig. 1B). The sequencing depth in all samples ranged between 10,000x and 750,000x, and most amplications were >20,000x (Fig. 1C).

Concordance of tumor DNA and matched plasma cfDNA sample. All detected mutations are listed in Table I. Considering possible systematic error, we defined mutations with >0.5% percentages as positive mutations. Comparing each plasma cfDNA with its matched tumor DNA, concordant mutations were identified in all 10 patients.

For all concordant mutations identified in the 10 patients, mutation percentages were higher in plasma cfDNA (average 12.04%) than in tumor DNA (average 10.80%) (Table I). Mutations of 26 alleles located in 7 genes (PTEN, STK11, FGFR1, TP53, NOTCH1, ALK and MET) were identified in both tumor DNA and plasma cfDNA. Mutations in EGFR (24.72% and 18.84%), KRAS (5.74%), and FGFR2 (2.02%) were only identified in tumor DNA while no mutated genes

Table I. Mutations detected in tumor DNA and plasma cfDNA of NSCLC patients.

Patient ID	Position	Gene	Mutation	Mutation type	Blood mutation rate (reads)	cfDNA mutation rate (reads)	Tumor mutation rate (reads)
NSCLC1	chr10:89624218	PTEN	p.L171V	SUB	9.01 (11538)	0.07 (108648)	0.02 (21955)
NSCLC1	chr8:38285913	FGFR1	p.D133D	DEL	5.60 (1340)	2.22 (13695)	2.59 (9729)
NSCLC1	chr9:139399408	NOTCH1	p.L1579L	DEL	1.81 (22782)	0.70 (153196)	1.03 (34967)
NSCLC2	chr10:89720705	PTEN	p.T459A	SUB	0.13 (3197)	1.04 (220526)	0.14 (2876)
NSCLC2	chr19:1221293	STK11	p.Y272Y	SUB	0.05 (11956)	2.54 (1434088)	0.38 (11433)
NSCLC2	chr2:29443617	ALK	p.A1200A	SUB	0 (107957)	0 (1566735)	49.29 (69422)
NSCLC2	chr7:116339672	MET	p.S178S	SUB	0.09 (4232)	0.58 (504813)	0.56 (3416)
NSCLC2	chr7:55259515	EGFR	p.L858R	SUB	0.01 (43374)	0.01 (421187)	24.72 (34533)
NSCLC2	chr8:38285913	FGFR1	p.D133D	DEL	2.60 (12201)	3.46 (170139)	3.10 (7457)
NSCLC2	chr9:139399408	NOTCH1	p.L1579L	DEL	0.94 (33155)	1.11 (806291)	1.05 (22104)
NSCLC3	chr17:7577538	TP53	p.R209L	SUB	0.01 (65412)	0.01 (668018)	3.61 (62283)
NSCLC3	chr17:7579476	TP53	p.P32S	SUB	20.06 (325417)	16.12 (112725)	3.75 (266676)
NSCLC3	chr8:38285913	FGFR1	p.D133D	DEL	2.92 (20513)	2.22 (14258)	2.96 (20809)
NSCLC3	chr9:139399408	NOTCH1	p.L1579L	DEL	0.94 (46337)	0.67 (162970)	1.05 (46751)
NSCLC4	chr10:89624218	PTEN	p.L171V	SUB	12.84 (14043)	51.14 (3154)	53.31 (12299)
NSCLC4	chr19:1223125	STK11	p.F354L	SUB	3.02 (48133)	56.19 (46898)	34.46 (40610)
NSCLC4	chr3:178938796	PIK3CA	p.V680L	SUB	0.44 (4980)	2.13 (47)	0.24 (13656)
NSCLC4	chr8:38285913	FGFR1	p.D133D	DEL	4.97 (3017)	3.14 (8777)	2.72 (7789)
NSCLC4	chr9:139399408	NOTCH1	p.L1579L	DEL	1.68 (33691)	0.67 (150)	1.05 (17506)
NSCLC5	chr17:7577565	TP53	p.N200S	SUB	0.05 (27965)	0.10 (386509)	15.33 (46046)
NSCLC5	chr2:29443617	ALK	p.A1200A	SUB	47.93 (63513)	51.31 (335544)	42.54 (104652)
NSCLC5	chr3:178936091	PIK3CA	p.E545K	SUB	0 (0)	0.73 (33218)	21.64 (5084)
NSCLC5	chr8:38285913	FGFR1	p.D133D	DEL	3.04 (7144)	2.34 (36438)	3.35 (15840)
NSCLC6	chr10:123279651	FGFR2	p.G172R	SUB	0.03 (22113)	0.02 (146276)	2.02 (30992)
NSCLC6	chr12:25398285	KRAS	p.G12C	SUB	0.01 (34200)	0.01 (144676)	5.74 (37623)
NSCLC6	chr17:7578406	TP53	p.R136H	SUB	0.07 (27351)	0.05 (164157)	2.64 (37926)
NSCLC6	chr8:38285913	FGFR1	p.D133D	DEL	2.90 (10253)	2.39 (30748)	3.01 (16766)
NSCLC7	chr8:38285913	FGFR1	p.D133D	DEL	2.44 (6968)	2.63 (53550)	3.03 (7382)
NSCLC7	chr9:139399408	NOTCH1	p.L1579L	DEL	0.82 (33227)	0.82 (149833)	1.01 (28019)
NSCLC8	chr17:7578272	TP53	p.H154Y	SUB	0.04 (26414)	0.24 (330592)	28.81 (17584)
NSCLC8	chr7:116339672	MET	p.S178S	SUB	49.17 (7269)	50.86 (181222)	43.71 (4985)
NSCLC8	chr7:116340262	MET	p.N375S	SUB	50.06 (21006)	50.47 (213064)	44.18 (13086)
NSCLC8	chr8:38285913	FGFR1	p.D133D	DEL	2.74 (21644)	3.26 (188537)	2.76 (13826)
NSCLC8	chr9:139399408	NOTCH1	p.L1579L	DEL	0.96 (53785)	1.10 (543910)	0.96 (42236)
NSCLC9	chr10:89624218	PTEN	p.L171V	SUB	9.05 (4155)	0.01 (297185)	0.06 (16938)
NSCLC9	chr3:178938796	PIK3CA	p.V680L	SUB	1.14 (9864)	0.23 (304805)	0.21 (21326)
NSCLC9	chr8:38285913	FGFR1	p.D133D	DEL	5.11 (2054)	3.32 (229921)	2.79 (12710)
NSCLC9	chr9:139399408	NOTCH1	p.L1579L	DEL	1.65 (23599)	1.14 (439058)	0.84 (31467)
NSCLC10	chr17:7578534	TP53	p.K93N	SUB	0.05 (13254)	0.04 (279526)	14.22 (8318)
NSCLC10	chr7:55259515	EGFR	p.L858R	SUB	0.02 (46073)	0.17 (368684)	18.84 (36517)
NSCLC10	chr8:38285913	FGFR1	p.D133D	DEL	3.02 (14564)	3.37 (146536)	2.59 (9555)
NSCLC10	chr9:139399408	NOTCH1	p.L1579L	DEL	1.10 (34408)	1.09 (532945)	0.77 (25075)

found in plasma cfDNA but not in tumor DNA, indicated that plasma cfDNA could partially reflect the genetic condition of NSCLC tumors.

Differences in mutation pattern among tumor tissue, cfDNA and blood of NSCLC. To reveal the association between mutation pattern of tumor tissue and cfDNA, unclustered heatmap

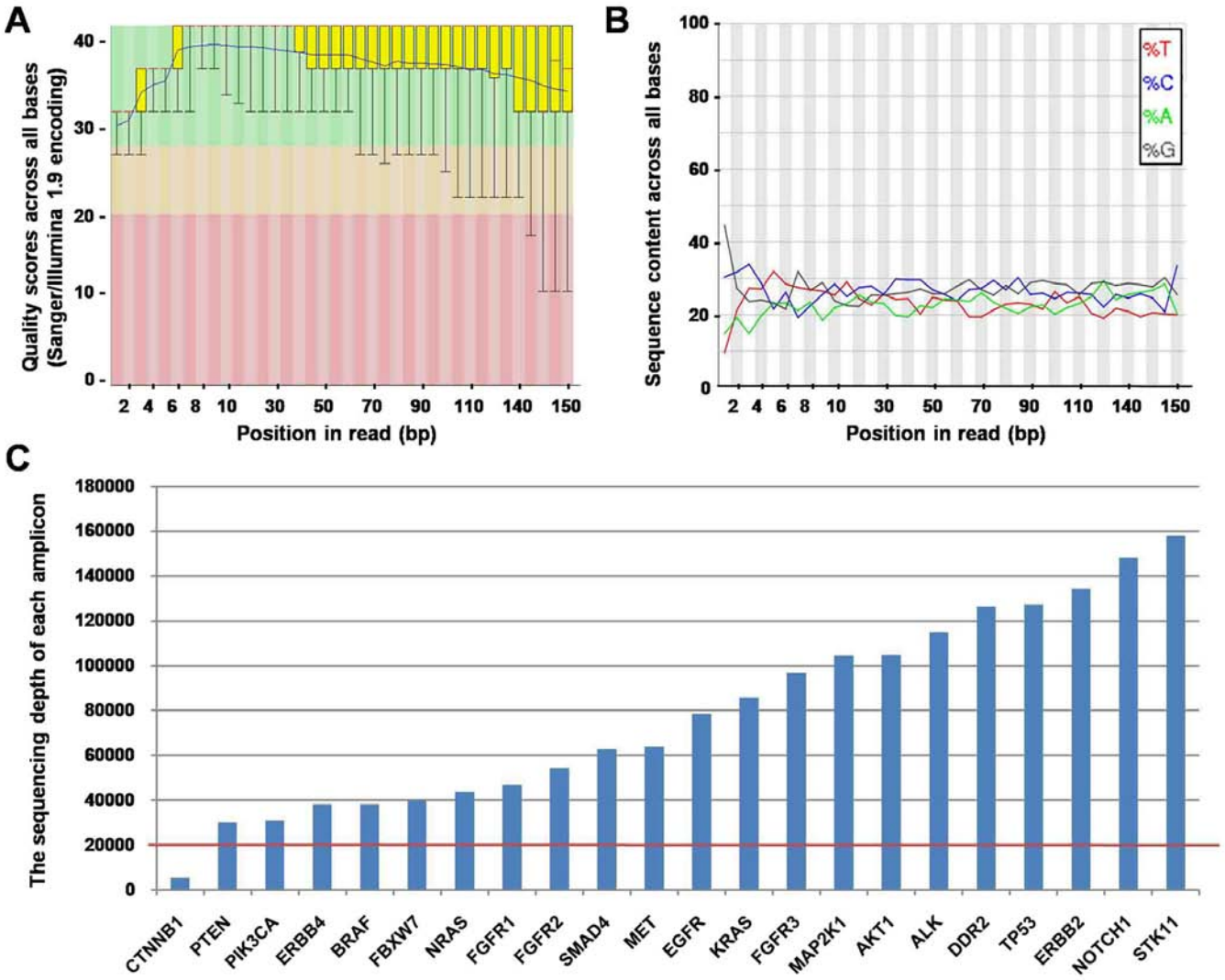

Figure 1. Sequence coverage with the Illumina X10. (A) Quality scores across all bases (every base has a quality score which indicates the accuracy of sequencing). (B) GC content across all bases. (C) The sequencing depth of each amplicon, most amplicons were >20,000x (showed by a red line).

diagrams were displayed for mutation patterns of tumor tissue, cfDNA and blood (Fig. 2A). It is clearly showed that cfDNA have a moderate mutation pattern, which is weaker than that of tumor tissue and stronger than that of blood. Mutation patterns of different tissues from the same patients were clustered by hierarchical clustering algorithm (Fig. 2B). For each representative patient, plasma cfDNA was closely clustered with tumor DNA not blood DNA, indicated that plasma cfDNA was associated with tumor genetically.

Mutations in plasma cfDNA sample of healthy people. It is widely known that mutations in NSCLC patients could be caused by senescence and smoking. To further investigate the source and specificity of mutations in NSCLC plasma cfDNA, we recruited 13 elderly people and 7 middle-age smokers and subjected their plasma cfDNA samples to sequencing.

The plasma cfDNA concentration of NSCLC patients (69.2±46.9 ng/ml) is significantly higher than that of elderly people (32.5±5.2 ng/ml, t=2.96, p=0.007) and middle-aged smokers (17.9±9.1 ng/ml, t=2.83, p=0.013). Violin plot of plasma cfDNA concentration is shown in Fig. 3A.

Nearly half of mutations (PTEN_c.511C>G, STK11_c.1062C>G, ALK_c.3600C>G, MET_c.534C>T, MET_c.1124A>G, NOTCH1_c.4735G>G) were identified in both NSCLC patients and healthy controls (elderly people and smokers). However, five mutations (PTEN_c.1375A>G, TP53_c.94G>A, STK11_c.816C>T, PIK3CA_c.1633G>A, PIK3CA_c.2038G>C) only identified in NSCLC patients, indicated that cfDNA mutation pattern was largely different between NSCLC patients and non-cancerous people.

Discussion

The 5-year survival rate following surgical resection for NSCLC at early stage is significantly higher than that at late stage (2,19). Unfortunately, NSCLC is always diagnosed at advanced stages because the symptoms are not apparent initially and the detection is difficult at stage I or II (20,21). Thus, exploring an effective approach to detect early-stage patients can observably increase survival. Even though biopsy has been the golden diagnostic method, in many cases of NSCLC, it is always difficult to obtain tissue samples in early-

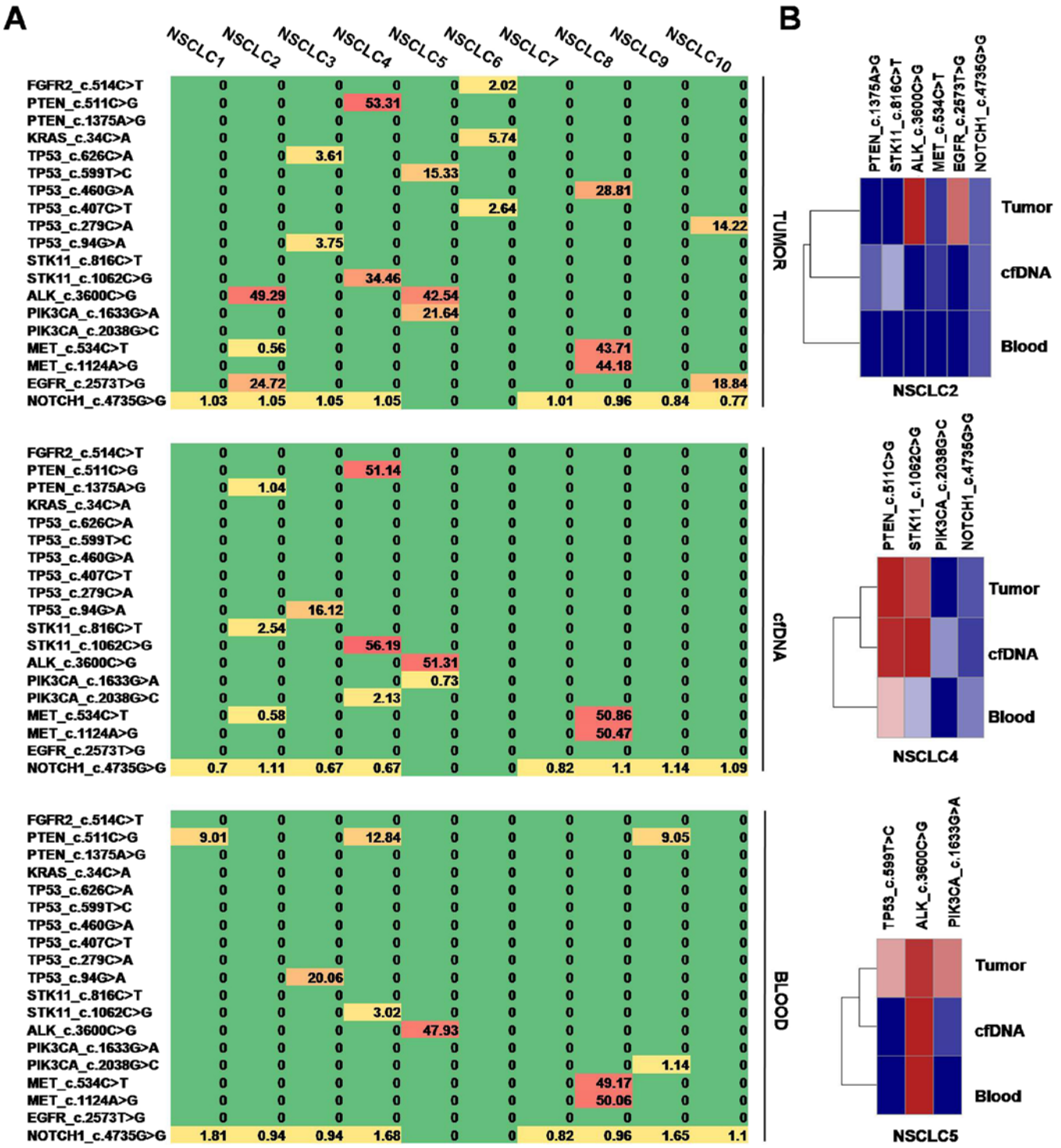

Figure 2. Differences in mutation pattern among tumor tissue, cfDNA and blood of NSCLC patients. (A) Frequencies of all mutated alleles in all samples, high frequency mutations are red and low frequency mutations are green (upper panel, tumor tissue; middle panel, cfDNA; lower panel, blood). (B) Hierarchical clustering by mutation pattern in tumor tissue, cfDNA and blood of single representative NSCLC patient (upper panel, NSCLC patient 2; middle panel, NSCLC patient 4; lower panel, NSCLC patient 5).

stage or even in advanced or metastatic stage (18). Additionally, the procedure of biopsy may increase the risk of cancer 'seeding' to other sites (22). In recent years, 'liquid biopsy' receives more and more attention but most studies involved in mutations of NSCLC are about late stages. Therefore the detection of liquid biopsy biomarkers in early-stage NSCLC patients is necessary and can provide a non-invasive way to gain genotypic information and will have broad application prospects in the large-scale in the future (18).

Several hypotheses have been proposed to explain the mechanism of cellular DNA release into the circulation. Wu *et al* proposed that tumor DNA was released into the circulation and was enriched in the plasma and serum and the increased cfDNA in plasma of cancer patients may be due

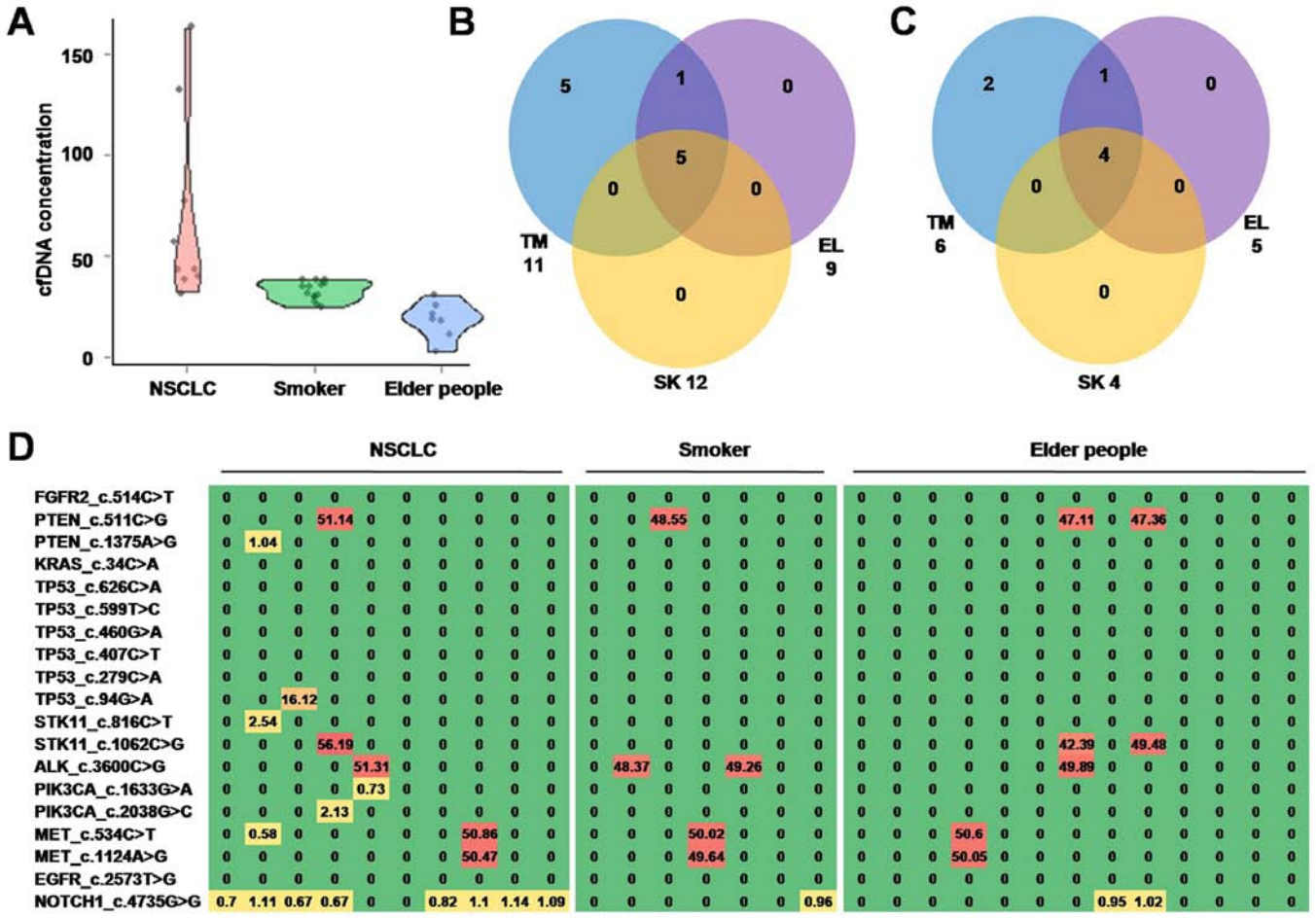

Figure 3. Differences in cfDNA mutation pattern among NSCLC patients, smokers and elderly people. (A) Plasma cfDNA concentration of NSCLC patients, smokers and elderly people. (B) Venn diagram showed overlaps in mutated alleles among NSCLC patients, smokers and elderly people. (C) Venn diagram showed overlaps in mutated genes among NSCLC patients, smokers and elderly people. (D) Frequencies of all mutated alleles in all cfDNA samples, high frequency mutations are red and low frequency mutations are green (left panel, NSCLC patients; middle panel, smokers; right panel, elderly people).

to the derivation from tumor DNA (23). Lee and colleagues hypothesized that the cfDNA was composed of small fragments of nucleic acid that are not associated with cells or cell fragments and should be from a leakage after tumor necrosis or apoptosis (24).

Jamal-Hanjani *et al* found there were ubiquitous and heterogeneous single-nucleotide variants (SNVs) between tumor DNA and cfDNA in lung cancer, incidence of which are 68% and 32%, respectively (25). Couraud *et al* reported that not all tumor mutations could be detected in cfDNA (50 mutations were identified in tumor DNA, while only 26 were detected in cfDNA) (26). Further detection of mutations in tumor DNA and cfDNA was required to define its potential use in clinical practice. In this study, we recruited 10 NSCLC patients of early stage (includes IA, IB, IIA and IIB); collected matched DNA samples and conducted further research. We found that tumor DNA and its matched plasma cfDNA samples showed high concordance in their mutation patterns.

Notably, some specific mutations were identified in tumor cfDNA, locating in genes of EGFR, KRAS and FGFR2, which is not common in lung cancer tumor samples. These results are also coherent with precious studies (17,27). However, the incidence of mutations in plasma cfDNA was roughly higher than that in tumor DNA in some previous studies (18,28). In this study, plasma cfDNA had a moderate mutation pattern, which was weaker than that of tumor tissue and stronger than that of PBLs. Generally, plasma cfDNA was closely clustered with tumor DNA not blood DNA, indicating that plasma cfDNA was associated with tumor genetically.

Many studies proposed that cfDNA concentration can reflect the tumor burden in the cases of cancer (26). In cancer patients, a relatively high concentration of cfDNA (range 0-1,000 ng/ml, average 180 ng/ml) were reported (29) compared to that of health people (range 0-100 ng/ml, average 30 ng/ml) (30), which may be predominantly originated from apoptotic cells, inflammation, tissue trauma and so on (31). Some studies even revealed that cfDNA concentration was different between early and late stages (26).

Association between mutations of cfDNA with those of tumor tissue DNA was also observed in prostate cancer and breast cancer (32,33). cfDNA is not only associated with carcinogenesis. Its concentration may also be elevated in inflammatory, infectious and other health-related conditions

such as senescence and smoking status (34). Thus further studies need to establish a standard to distinguish potential lung cancer patients with healthy subjects.

Our results suggested that the concentrations of cfDNA in NSCLC patients showed significant differences when compared with that of elderly people (t=2.96, p=0.007) and that of middle-aged smokers (t=2.83, p=0.013) although the median concentration of cfDNA can vary with senescence and smoking among healthy subjects (35). Therefore our data may suggest that the quantification of cfDNA from plasma may be a useful non-invasive technique for diagnosis and dynamic monitoring of lung cancer at early stage.

In some circumstances cfDNA alterations are detectable ahead of cancer diagnosis, raising the possibility of exploiting them as biomarkers for monitoring cancer occurrence (36). Although detection of mutations in cfDNA is difficult due to the low amount of mutant alleles in a background of wild-type DNA (37), there are still five mutations (PTEN_c.1375A>G, TP53_c.94G>A, STK11_c.816C>T, PIK3CA_c.1633G>A, PIK3CA_c.2038G>C) identified in NSCLC patients specifically, suggesting that cfDNA mutation pattern could distinguish early NSCLC patients with non-cancerous individuals. Stankovic *et al* reported that coexistence of aberrant p53 and PTEN was the most frequent marker and significantly associated with poor survival of NSCLC patients (38). Stjernström *et al* also found when analyzing all PI3K pathway related genes together, NSCLC patients would have at least one alteration (39). STK11 mutation was first found in Peutz-Jeghers syndrome patients, which is significantly associated with KRAS and EGFR (40). Mutation in STK11 was also found associated with prognosis (41).

In conclusion, we find that cfDNA has a similar mutation pattern with its matched tumor tissue DNA. Specific mutations or elevated concentration of plasma cfDNA could be useful non-invasive biomarkers through liquid biopsy in prevention and diagnosis of NSCLC. However, future investigation in utility and perspectives of cfDNA still need to be performed in large prospective cohorts.

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