Sequencing of circulating tumor DNA for dynamic monitoring of gene mutations in advanced non-small cell lung cancer

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Abstract. Lung cancer is the most commonly occurring type of cancer worldwide and also has the highest mortality rate. Although targeted therapy of non-small cell lung carcinoma (NSCLC) has become common, the majority of patients receiving first-line epithelial growth factor receptor (EGFR)-TKI treatment develop drug resistance. The EGFR T790M (NM_005228.4(EGFR):c.2369C>T (p.Thr790Met)) mutation accounts for half of all reported resistance cases; however, the molecular mechanism resulting in the drug resistance remains to be characterized. Circulating tumor DNA (ctDNA) isolated from plasma has great potential for identification of gene mutations in NSCLC. Collection of ctDNA is relatively non-invasive and can avoid the inherent disadvantages of tissue biopsy. In the present study, next-generation sequencing technology was used to detect the variation of ctDNA in the peripheral blood of patients administered with EGFR-TKI. The patients were monitored serially to establish a dynamic resistance gene detection system, with the rationale being to alter the treatment strategy as soon as the emergence of drug resistance gene mutations. A mutation spectrum of the group of patients was constructed. A driver gene mutation was identified in the ctDNA of each patient, and certain patients had clinically targetable gene mutations like EGFR, ROS proto-oncogene receptor tyrosine kinase and B-Raf proto-oncogene serine/threonine kinase. The dynamic monitoring of EGFR status indicated that the EGFR mutation rate was consistent with the tumor burden of patients. Overall, ctDNA detection is a useful method for the molecular genotyping of patients with cancer. The dynamic resistance gene detection system described in the present study is a sensitive and useful tool for the monitoring of gene status, which has potential to be used for direction of treatment strategy by detecting the emergence of drug resistance gene mutations.

Introduction

Lung cancer is the most commonly occurring type of cancer and the leading cause of cancer-associated mortality in adults worldwide (1). Malignant lung tumors can be subdivided into small-cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). NSCLC can be further subdivided into adenocarcinoma, squamous cell carcinoma (SCC) and large cell carcinoma using conventional histopathology. Progress in the effectiveness of conventional treatment approaches to NSCLC, including chemotherapy, radiotherapy and surgery, has plateaued. The development of genetic testing technology and genotyping has provided great insight into the molecular pathology of NSCLC. Known driver mutations are identifiable in ~60% lung adenocarcinoma and ~80% of SCC (2,3). Such mutations can trigger activating cascades in signaling pathways, including the RAS-RAF-MEK-extracellular signal-regulated kinase/mitogen activated protein kinase, phosphoinositide 3-kinase (PI3K)-AKT serine/threonine kinase-mechanistic target of rapamycin, or janus kinase-signal transducer and activator of transcription 1 pathways (4-6), leading to growth, proliferation, survival, and metastasis of cancer cells. Successful targeted therapies involve the identification and inhibition of these upregulated pathways by small molecule inhibitors or receptor monoclonal antibodies (mAb). Therapeutic targeting of the interaction between epidermal growth factor receptor (EGFR) and members of its downstream pathway has been most extensively studied. The two most common EGFR-activating mutations are an exon 19 deletion and exon 21 L858R mutation, which result in the constitutive activation of the receptor (7). It has been established that EGFR activating mutations are more common in patients

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with specific clinicopathological characteristics: Females, non-smokers, Asiatic origin and adenocarcinoma histological subtype. Mutant EGFR can be inhibited by small molecule tyrosine kinase inhibitors (TKIs), including gefitinib, erlotinib and afatinib or mAbs, including cetuximab and panitumumab. Multiple studies have demonstrated that these drugs can significantly increase therapy response rate and improve the progression free survival (PFS), and overall survival (OS) times in patients with NSCLC (8-13). The Food and Drug Administration (FDA) have approved gefitinib, erlotinib and afatinib as the first-line drugs for patients exhibiting EGFR, and the toxicity profiles of these drugs are superior to those of standard chemotherapy drugs. However, all patients with EGFR-mutation-derived NSCLC ultimately develop resistance to TKI treatment. Almost half of the reported cases of EGFR-TKI-resistance in NSCLC are due to the EGFR T790M mutation (14-16). Other mechanisms of EGFR-TKI-resistance include amplification of MET signaling (17,18), amplification of Erb-B2 receptor tyrosine kinase 2 signaling (19), mutation of PI3K catalytic subunit a (20), mutation of B-Raf proto-oncogene serine/threonine kinase (BRAF) (21) and NSCLC transformation to small-cell lung cancer (which accounts for 3-15% of all NSCLC cases and is a rare resistance mechanism which is poorly understood (22), and epithelial-mesenchymal transition (23). TKI, AZD9291, has received FDA approval and National Comprehensive Cancer Network guideline recommendations for EGFR T790M-positive patients with non-small cell lung cancer (24). However, neoplastic tissue samples are currently required to screen patients with the EGFR T790M mutation and to identify other molecular features of patients with drug resistance. Furthermore, re-biopsies following cancer progression are problematic due to factors, including the high risk of surgery, the vulnerable health of the patients and tumor heterogeneity.

Dead cells shed DNA into the bloodstream and when this DNA is selectively isolated from the plasma, it is termed cell-free DNA (cfDNA). In patients with cancerous tumors, a fraction of the cfDNA is derived from tumor cells and is referred as circulating tumor DNA (ctDNA). The presence of circulating nucleic acids in peripheral circulation was discovered >60 years ago by Mandel and Metais (25). Leon *et al* (26) later described the importance of circulating tumor DNA in cancer development. The collection of ctDNA is more feasible than the collection of whole tissue via biopsy, and it is sufficient for a general screening test to characterize the genetic profile of patients with cancer. Therefore, ctDNA has the potential to promote personalized cancer therapy. In addition, particularly through real-time tumor monitoring (27), unlike localized tumor tissues, ctDNA reflects all molecular alterations of primary tumors and metastases, potentially, inclusive of all heterogeneous mutation profiles (28). ctDNA can therefore be used for the early diagnosis of cancer (29,30), real-time assessment of risk of tumor progression (31), dynamic monitoring of patient's responses to treatment (32) and drug resistance testing (33).

In the present study, a targeted sequencing approach based on the Illumina platform was employed to detect and compare NSCLC driver gene alterations in plasma samples from 32 Chinese patients with advanced NSCLC. The purpose of this project was to elucidate the association between TKI resistance and gene mutation status, and to build a dynamic mutation detection system. A particular aim was to detect the EGFR T790M mutation, which predicts EGFR-TKI drug resistance through dynamic monitoring of ctDNA. Using continuous detection methods such as that described in the present study may be useful in the clinic to anticipate required changes in treatment strategy by detecting the emergence of drug resistant gene mutations. This has the potential to improve the prognosis, quality of life and survival time of patients with NSCLC, as well as to be cost-effective in terms of healthcare.

Materials and methods

Patients and ethical statements. The present study was approved by Shenzhen People's Hospital Ethics Committee (Shenzhen, China) and the methods were performed in accordance with their guidelines. Written informed consent was obtained from all patients for the use of blood and lung tumor tissue under the approval of the Ethics Committee. All patient samples and medical data used in this study have been irreversibly anonymized.

Patients with advanced or recurrent NSCLC derived from EGFR mutations with a treatment plan to begin TKI treatment were enrolled consecutively. The exclusion criteria were as follows: i) Pregnant or lactating women; ii) patients who have received prior treatment with an MEK inhibitor; and iii) patients with a history of clinically significant interstitial lung disease or pneumonitis.

A cohort of 32 patients with advanced NSCLC treated with TKI drugs between November 2015 and December 2016 in Shenzhen People's Hospital were enrolled into the present study. The clinical characteristics of the patients are presented in Table I. Participants in the cohort, including 18 females and 14 males, had been diagnosed with stage IIIa to IV NSCLC. Patient blood samples were taken during TKI treatment. EGFR TKI was treated until disease progression, and blood samples were taken every 3 months. Treatment effectiveness was assessed every 2 months according to the RECIST 1.1 criteria (34). Testing for EGFR mutations in blood plasma and imaging studies (including chest computational tomography, brain magnetic resonance imaging, bone scans and abdominal ultrasound) were performed by two independent research teams who were blinded from each other's results until survival outcomes were analyzed. Survival status was confirmed by telephone follow-up every 3 months. The blood samples of patient H01 were taken on the 24th December 2015 and the 7th of April 2016. The blood samples of patient H02 were taken on the 31st of December 2015 and the 11th of April 2016.

Plasma isolation and DNA extraction. Patient blood samples were collected in tubes containing EDTA and centrifuged at 1,600 x g for 10 min at 4°C within 2 h of collection. The peripheral blood lymphocyte (PBL) debris was stored at -20°C until further use. The supernatants were further centrifuged at 10,000 x g for 10 min at 4°C, and plasma was harvested and stored at -80°C until further use. DNA from PBLs was extracted using the RelaxGene Blood DNA system

Table I. Clinical characteristics of 32 patients with non-small cell lung cancer.

Characteristic	No. of patients (%)
Age (years)	
Mean (standard deviation)	59 (11.60)
Median (range)	62 (36-85)
Sex	
Male	18 (56.20)
Female	14 (43.80)
Pathological diagnosis	
Non-small cell lung cancer	32 (100.0)
Adenocarcinoma	14 (43.80)
Unknown	18 (56.20)
Tumor stage	
IIIA	5 (15.60)
IIIB	3 (9.40)
IV	24 (75.0)
Smoking history	
Smoker	10 (31.20)
Non-smoker	22 (68.80)
Treatment history	
No previous treatment	2 (6.25)
Previous treatment	30 (93.75)
Chemotherapy history	
Chemotherapy undertaken	25 (78.1)
No chemotherapy undertaken	7 (21.9)

(Tiangen Biotech Co., Ltd., Beijing, China) and ctDNA was extracted from at least 2 ml plasma using the QIAamp Circulating Nucleic Acid kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturers' instructions. DNA was quantified with the Qubit 2.0 Fluorometer and the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to manufacturer's instructions.

Next-generation sequencing (NGS) library construction. DNA collected from PBLs was sheared using enzyme dsDNA Fragmentase (New England BioLabs, Inc., Ipswich, MA, USA) according to the manufacturer's instructions. Size selection for 150-250 bp fragments was performed with AMPure XP beads (Beckman Coulter, Inc., Brea, CA, USA), which has a high recovery efficiency (the amount of DNA harvested from the size selection procedure is relatively large). DNA fragments and ctDNA were used to construct a library using the KAPA Library Preparation kit (Kapa Biosystems, Inc., Wilmington, MA, USA) according to the manufacturer's instructions. Agencourt AMPure XP beads were used for the purification of the DNA/ctDNA. Generally, following fragmentation, the End Repair and 3'-end dA-tailing ensued. T-tailed adapters were used and a 3'dA overhang was added enzymatically onto the fragmented DNA sample, using the KAPA Library Preparation kit (Kapa

Biosystems, Inc.). Ligation was performed for 15 min at 20°C. Single-step size selection was performed by adding 50 μl (1X) PEG/NaCl SPRI solution buffer (Beckman Coulter, Inc.) to enrich for ligated DNA fragments. The ligated fragments were then amplified using 1X KAPA HiFi Hot Start Ready mix (Kapa Biosystems, Inc.) and Pre-LM-PCR Oligos (Kapa Biosystems, Inc.) in 50 μl reactions and 7-8 PCR cycles, depending on the input DNA mass. The thermocycling conditions were as follows: Initial Denaturation, 98°C for 30 sec; 7-8 cycles at 98°C for 10 sec; 60°C for 10 sec; 68°C for 30 sec, and the final extension at 68°C for 60 sec. Library purity and concentration was assessed by Qubit 2.0 Fluorometer using the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Fragment length was determined on a 4200 Bioanalyzer using the DNA 1000 kit (both from Agilent Technologies, Inc., Santa Clara, CA, USA).

Library design for hybrid selection. Hybrid selection was performed with custom biotinylated DNA probes for 104 genes associated with lung cancer (Roche Diagnostics, Basel, Switzerland). The library of amplified samples was hybridized with the SeqCap EZ Library (Roche Diagnostics) according to the manufacturer's instructions for 16-20 h at 47°C. Following hybrid selection, the captured DNA fragments were amplified with 12-14 cycles of PCR using 1X KAPA HiFi Hot Start Ready mix and Post-LM-PCR Oligos in two separate 50 μl reactions. The thermocycling conditions were as follows: Initial denaturation, 98°C for 30 sec; 7-8 cycles at 98°C for 10 sec; 60°C for 10 sec; 68°C for 30 sec, and the final extension at 68°C for 60 sec. The reactions were then pooled and purified using Agencourt AMPure XP beads.

Denaturing and diluting libraries for sequencing. Multiplexed libraries were denatured by 0.2M NaOH and diluted by Tris-HCl according to the manufacturer's instructions (Illumina, Inc., San Diego, CA, USA). The libraries were sequenced using 150-bp paired-end runs using a NextSeq 500 system (Illumina, Inc.). All genes captured by NGS are listed in Table II.

Variant calling and analysis. Sequencing data were demultiplexed and aligned to the hg19 genome (GRch37) using Burrows-Wheeler Aligner (35) version 0.7.15-r1140. Pileup files for properly paired reads with mapping quality >=60 were generated using Samtools (http://www.htslib.org/) (36). Somatic variants were created using VarScan2 (http://varscan.source-forge.net/) (37) (-min-coverage=100; -min-var-freq=0.05; -somatic-P-value=0.01; -strand-filter 1; otherwise, default parameters were used). Allele Frequency (AFs) were calculated for all Q30 bases. Using a custom Python script, previously identified tumor DNA mutation were intersected with a SAMtools mpileup file generated for each plasma DNA sample, and the number and frequency of supporting reads were calculated for each mutation. For screening plasma DNA without knowledge of tumor DNA, a mutation was identified if >=4 mutant reads were revealed in plasma with >=1 read on each strand, and no mutant reads were observed in germline DNA or in a prior plasma sample with >=10-fold coverage.

Table II. The gene list for sequencing in our NGS method.

Gene name	GenBank accession no.
ABCB1	NM_000927
AKT1	NM_005163
ALK	NM_004304
APC	NM_000038
ATIC	NM_004044
ATM	NM_000051
ATRX	NM_000489
BAG4	NM_004874
BCL2L11	NM_006538
BRAF	NM_004333
BRCA1	NM_007294
BRCA2	NM_000059
C10orf10	NM_007021
C8orf34	NM_052958
CBR3	NM_001236
CCND1	NM_053056
CD74	NM_004355
CDA	NM_001785
CDK4	NM_000075
CDK6	NM_001259
CDKN2A	NM_000077
CREBBP	NM_004380
CTNNB1	NM_001904
CYP19A1	NM_000103
CYP1B1	NM_000104
CYP2C19	NM_000769
CYP2D6	NM_000106
CYP3A4	NM_017460
DDR2	NM_006182
DPYD	NM_000110
EGFR	NM_005228
EML4	NM_019063
ERBB2	NM_004448
ERBB3	NM_001982
ERBB4	NM_005235
ERCC1	NM_001983
EZR	NM_003379
FGF19	NM_005117
FGF3	NM_005247
FGF4	NM_002007
FGFR1	NM_015850
FGFR2	NM_000141
FGFR3	NM_000142
FLT3	NM_004119
GSTP1	NM_000852
HRAS	NM_005343
JAK1	NM_002227
JAK2	NM_004972
KDR	NM_002253
KEAP1	NM_012289
KIT	NM_000222
KMT2C	NM_170606

Table II. Continued.

| Gene name	GenBank accession no.
KMT2D	NM_003482
KRAS	NM_004985
LRIG3	NM_153377
MALAT1	NR_002819
MAP2K1	NM_002755
MAP2K7	NM_145185
MET	NM_000245
MLH1	NM_000249
MSH2	NM_000251
MTHFR	NM_005957
MTOR	NM_004958
MTRR	NM_002454
NF1	NM_000267
NFE2L2	NM_006164
NOTCH1	NM_017617
NRAS	NM_002524
NTRK1	NM_002529
NTRK3	NM_002530
PDE4DIP	NM_014644
PDGFRA	NM_006206
PIK3CA	NM_006218
PTEN	NM_000314
RAF1	NM_002880
RB1	NM_000321
RET	NM_020630
ROS1	NM_002944
RRM1	NM_001033
SDC4	NM_002999
SETBP1	NM_015559
SLC34A2	NM_006424
SLIT1	NM_003061
SMARCA4	NM_003072
SMO	NM_005631
SOD2	NM_000636
STAT3	NM_003150
STK11	NM_000455
STMN1	NM_005563
TACC3	NM_006342
TFG	NM_006070
TOP2A	NM_001067
TP53	NM_000546
TPM3	NM_152263
TRRAP	NM_003496
TSC1	NM_000368
TSC2	NM_000548
TUBB3	NM_006086
TYMP	NM_001953
TYMS	NM_001071
UGT1A1	NM_000463
UMPS	NM_000373
XPC	NM_004628
XRCC1	NM_006297
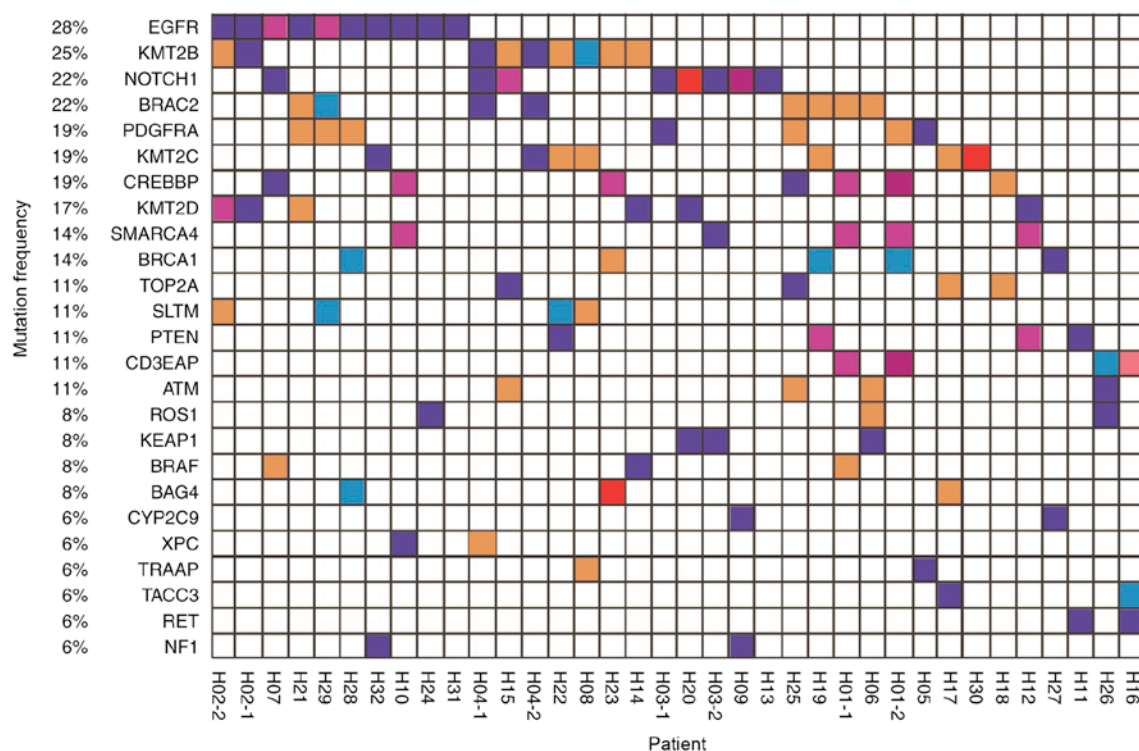
Figure 1. Mutation spectrum of 36 plasma samples from 32 patients with non-small cell lung cancer. The genes with a high mutation frequency among all patients are listed on the left and individual patients are represented by the columns. First and second blood draws are indicated by -1 and -2, respectively. Mutation types of nonsynonymous single nucleotide variant, stop-gain, frameshift insertion, non-frameshift deletion, and non-frameshift insertion are represented by purple, blue, dark red, red, orange and pink respectively.

Results

Patient characteristics. A total of 14/32 patients (43.8%) presented with adenocarcinoma, the remaining 18 patients (56.2%) were of unknown histopathology. The majority of patients were non-smokers (22/32, 68.8%), and the majority of patients were treated with EGFR-TKI (30/32, 93.75%).

The peripheral blood samples of these patients were collected during the outpatient evaluation follow-up. Secondary peripheral blood samples were collected from 4 patients, resulting in a total of 36 plasma samples from 32 patients for EGFR mutation testing.

Mutation spectrums of the 32 patients with non-small cell lung cancer. To elucidate the association between the effectiveness of EGFR-TKI treatment and gene status, NGS technology was used to detect variations in ctDNA in the peripheral blood of a cohort of 32 patients. Mutation spectrums were drawn for 36 samples from these patients (Fig. 1).

All patients exhibited 'driver gene' mutations in the ctDNA, and a number of patients exhibited actionable mutations in genes including EGFR, ROS proto-oncogene receptor tyrosine kinase and BRAF. Thus, it was demonstrated that the ctDNA tool is useful for the molecular genotyping of patients with cancer.

Dynamic detection of EGFR gene mutations in blood plasma. Patients were monitored for 24 months via consultation, with blood collected every 3 months, in order to create a dynamic resistance gene detection system. The somatic

mutation profile of all 32 patients is presented in Fig. 1. Their blood samples were collected during TKI treatment and 4 underwent a second blood draw. The results demonstrate that EGFR mutation rate was associated with disease progression.

Patient H01 presented with adenocarcinoma and an EGFR exon 19 deletion was also detected by ARMS-PCR prior to the study. After 7 months of gefitinib treatment, the ctDNA of this patient was measured, followed by a second measurement 4 months later. The ctDNA mutation landscape for each measurement is compared in Fig. 2. Upon the second measurement, the EGFR exon 19 deletion was not identified, but the mutation rate had declined, which was consistent with the partial response (PR) clinical status of the patient following gefitinib treatment. The mutation variation between first and second measurements for patients H03, and H04 was similar to that of H01.

Patient H02 presented with adenocarcinoma; however, no EGFR mutation was detected by ARMS-PCR. This patient was treated with erlotinib blindly, which was initially effective. After 7 months of erlotinib treatment, the ctDNA of this patient was measured, followed by a second measurement 3 months later. The mutation landscapes for each measurement are compared in Fig. 3. The EGFR L858R (NM_005228.4(EGFR):c.2573T>G (p.Leu858Arg)) mutation was detected upon both measurements, and may explain the PR clinical status following initial treatment with erlotinib. Furthermore, the EGFR T790M [NM_005228.4(EGFR):c.2369C>T (p.Thr790Met)] mutation was detected in the second measurement. EGFR
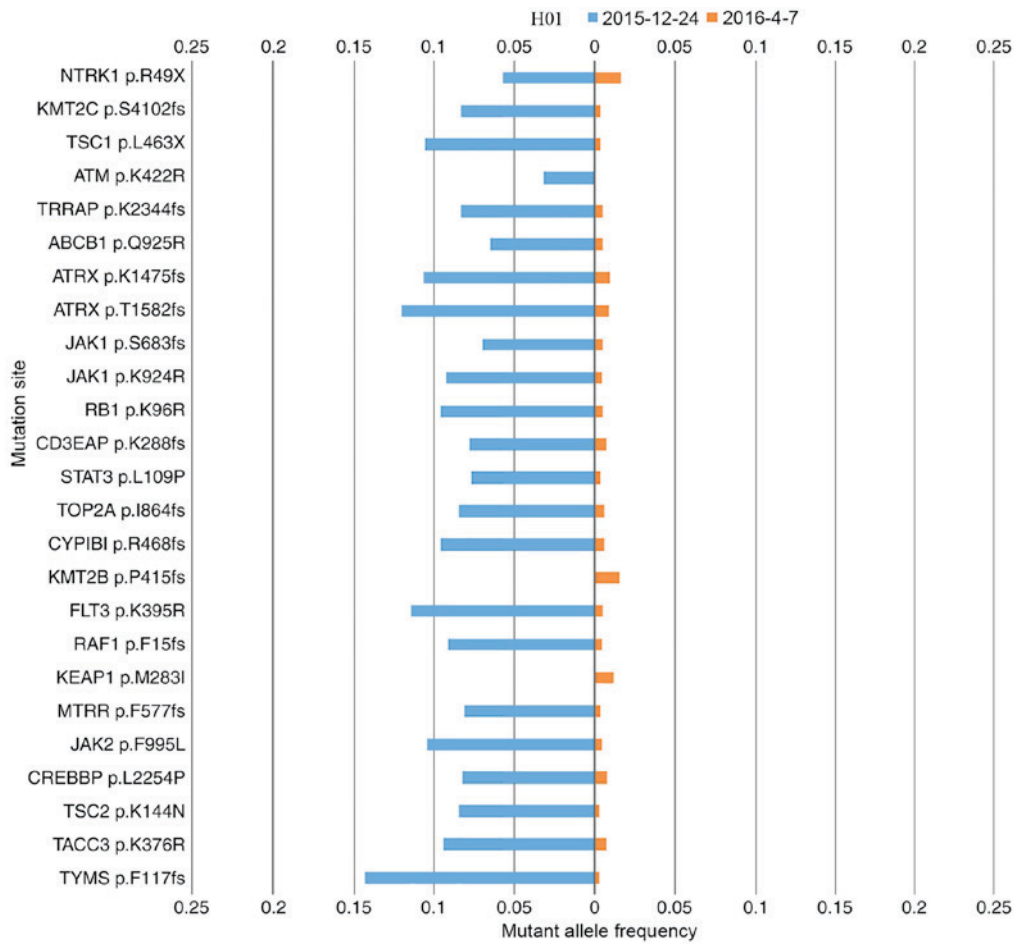
Figure 2. The cell-free tumor DNA mutation landscape of patient H01 in two different measurements. The gene mutation rate of the two cell-free DNA samples from patient 1 extracted on 2015-12-24 and 2016-04-07 are compared in blue and orange, respectively.
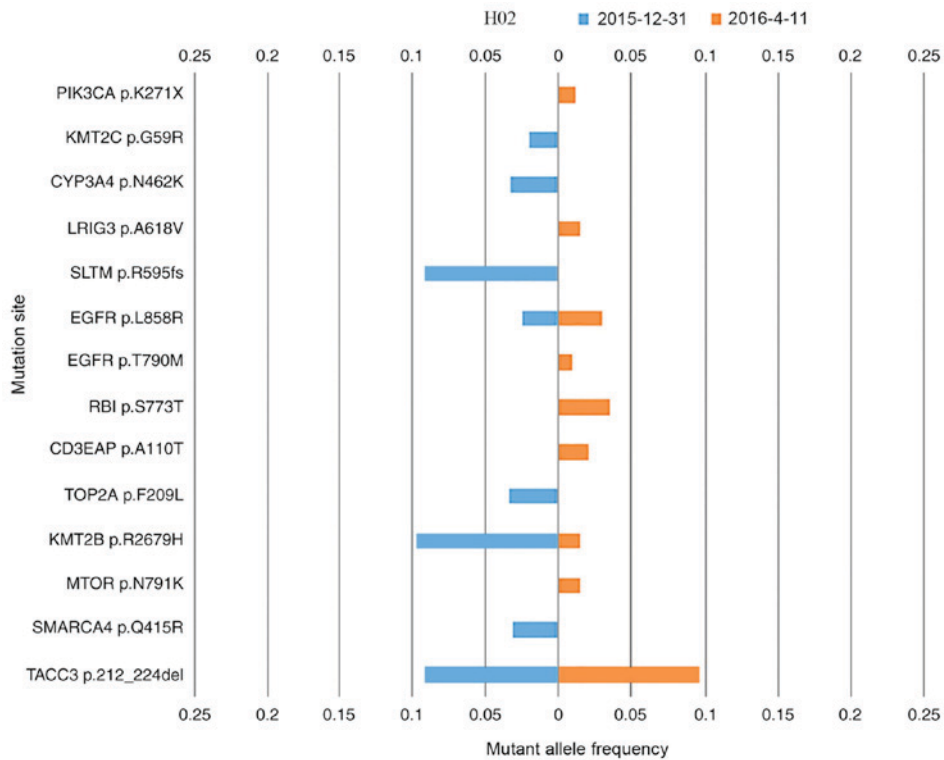

Figure 3. The cell-free tumor DNA mutation landscape of patient H02 in two different measurements. The gene mutation rate of the two cell-free DNA samples from patient H02 extracted at 2015-12-31 and 2016-04-11 are compared in blue and orange, respectively.
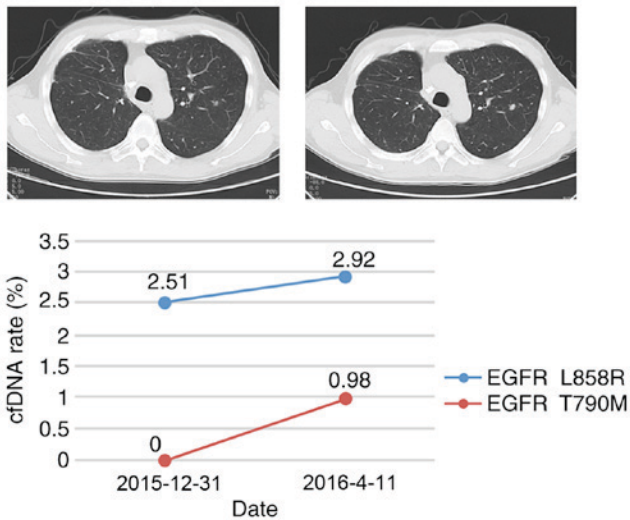
Figure 4. The driver mutations, EGFR L858R and EGFR T790M, vary in patient H02 between two measurements taken on 2015-12-31 and 2016-04-11. The computed tomography scan images were taken on these dates. The scans demonstrate disease progression. The graph illustrates the circulating tumor DNA mutation rate of EGFR L858R and EGFR T790M, indicated by the blue and red lines, respectively. EGFR, endothelial growth factor receptor; cfDNA, cell-free DNA.

T790M is detected as a 'second-site mutation' in >50% EGFR-mutation-derived types of lung cancer that have developed resistance to erlotinib or gefitinib (38). Patient H02 demonstrated a case of EGFR p.T790M-induced drug resistance. Other novel driver gene mutations were detected in the second measurement, which may explain the progressive disease clinical status assigned at this stage. ARMS-PCR for two loci is sufficient to detect the presence or absence of specific mutations; however, NGS is able to identify high numbers of simultaneous mutations (including common actionable mutations, EGFR p.L858R and EGFR p.T790M (Fig. 3) which ARMS-PCR cannot achieve (39).

Discussion

In the present study, a targeted sequencing approach based on the Illumina platform was used to test variations in NSCLC driver genes, including point mutations, insertions, deletions and gene rearrangements simultaneously in plasma samples from 32 Chinese patients with advanced NSCLC. To elucidate the association between drug effect and mutation status, mutation profiles were constructed for 32 patients with NSCLC. Furthermore, the EGFR status was dynamically monitored in order to construct a dynamic resistance gene detection system.

As the results demonstrated, for patients with NSCLC, a large number of gene mutations may occur. Besides these common actionable mutations, including NM_005228.4(EGFR):c.2573T>G (p.Leu858Arg), EGFR Exon 19 deletions and NM_005228.4(EGFR):c.2369C>T (p.Thr790Met), others may also be used as biomarkers for prognosis. Single or small numbers of target mutations can be detected using conventional single locus testing methods such as ARMS-PCR; however, such techniques do not indicate other, potentially unexpected, mutations (40). On the other hand, NGS is able to detect numerous gene mutations

simultaneously (40,41). The present study investigated >25 genes, constructed overall mutation landscapes for individual patients and performed blood collection rather than tissue biopsy. Another advantage of the present study is the use of continuous samples from the same individuals instead of isolated samples.

In recent years, ctDNA has been demonstrated to be an effective material for tumor DNA monitoring. In the present study, it was demonstrated that ctDNA may be used to measure tumor burden (number of cancer cells, the size of a tumor or the amount of cancer in the body). The concordance between ctDNA and tissue immunohistochemistry testing of mutation variation is ~50%; considering that the occurrence of novel mutations and the disappearance of existing mutations is constant due to evolving tumors, this is a relatively high similarity rate (42). This may be due to release of genomic DNA from necrotic white blood cells into blood, and diluted ctDNA in the plasma (43). The amount of ctDNA in patients is also likely to be associated with metastasis, vascularity, cellular turnover and response to therapy (44,45). In addition, ctDNA may be influenced by clearance, degradation and other physiological filtering events involving blood, and lymphatic circulation (43).

While the sample size of 36 is not large, all samples represented advanced NSCLC and were associated with complete clinical information. Therefore, the samples used in the present study were of high quality and consistency. The clinical consequence of EGFR-mutation-derived NSCLC demonstrated inter-patient variations following the initially rapid response to EGFR-TKIs. For example, certain patients experience a rapid disease flare just subsequent to the cessation of EGFR-TKI treatment, while others do not (46). Repeated treatment with an EGFR-TKI is effective in certain patients, while ineffective in others (47). These results indicate that the contribution of driver mutations vary between patients and change during the clinical courses. Therefore, the monitoring of EGFR gene mutation status is critical. Currently, re-biopsy is recommended to achieve such monitoring by tissue sample sequencing. However, tumor biopsy is usually invasive and difficult to repeat in solid tumors. Since genetic changes appear to accumulate over time in patients with NSCLC and different portions of a tumor carry different genetic profiles, single biopsy of a tumor is spatially and temporally limited, and may fail to monitor dynamics of cancer progression (48,49). The advantages of using ctDNA for dynamic genetic monitoring studies are demonstrated in this study, which suggests that this method should be applied more widely in clinical practice.

Acknowledgements

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