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Efficient ten-gene analysis of NSCLC tissue samples by next-generation sequencing

Xiuhuan Ji[a,1], Nanying Che[b,1], Rixu lin[a], Jianou Chen[c], Xiuling Wu[a,*]

[a] Pathology Department, The First Affiliated Hospital, Wenzhou Medical University, 325003, Wenzhou, China
[b] Pathology Department, Beijing Chest Hospital, Capital Medical University
[c] Pathology Department, Wenzhou Hospital of Traditional Chinese Medicine, Zhejiang University of Traditional Chinese Medicine

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ABSTRACT

In the era of personalized medicine, lung cancer is a typical disease which can be treated strategically based on the patient's histological and molecular diagnosis. Immunohistochemistry (IHC), fluorescence in-situ hybridization (FISH), Sanger sequencing and real-time PCR are techniques commonly used in clinical laboratories. Many patients are required to use several of the above technologies to get a complete diagnosis, which is expensive and timeconsuming. Next generation of sequencing (NGS) has the advantage to simultaneously analyze multigene mutations. The average cost for each patient is affordable if each run contains a certain number of samples. In this study, we tested a 10-gene, 32-mutation detection NGS method, which was used to test 195 samples from non-small cell lung cancer (NSCLC). Sanger sequencing and Amplification-refractory Mutation System (AMRS) PCR were employed to verify Epidermal Growth Factor Receptor (EGFR) and Anaplastic Lymphoma Kinase (ALK) results. This NGS method was partially proved to have a higher sensitivity to detect mutations with low abundance than Sanger sequencing and even ARMS PCR. Using genomic DNA to detect gene fusions may have some disadvantages to miss low abundance or large fragment fusions. As compared to using a few different technologies to analyze multigene mutations, small NGS analysis panel is a clinically applicable, efficient and affordable choice for NSCLC patients.

1. Introduction

The incidence and mortality rates of lung cancer, the leading cause of cancer death, have significantly increased in recent years. Globally, it is estimated that 2.09 million new cases of lung cancer were diagnosed in 2018 [1]. In China, the age-standardized incidence rate of lung cancer was 36.71 per 100,000, while the age-standardized mortality rate was 28.49 per 100,000 in 2014 [2]. Ten years ago, most patients of non-small cell lung cancer (NSCLC) used to receive surgical and/or chemotherapy treatment, which had a very low 5-year survival rate and poor quality of life [3]. Recent advances in molecularly targeted therapy and immunotherapy offer a glimmer of hope for potentially realizing the dream of personalized therapy for lung cancer patients [4]. Since the first generation of tyrosine kinase inhibitors (TKIs) have been approved by the National Medical Products Administration (NMPA) of China, a few of targeted therapy drugs and immune checkpoint inhibitors have provided patients with better response rate, lower side effect and longer overall survival, which include Gefitinib, Erlotinib, Afatinib, Icotinib, Crizotinib, Ceritinib, Osimertinib, Ipilimumab, Nivolumab etc [5].

Most small molecular inhibitors and antibodies which are mentioned above normally have significant therapeutic effects on patients with certain targets, such as Epidermal Growth Factor Receptor (EGFR) TKI sensitivity mutations, EGFR T790M,Anaplastic Lymphoma Kinase (ALK) fusions, ROS Proto-Oncogene 1 (ROS1) fusions, Tyrosine-protein kinase Met(C-Met) mutations, Programmed Death (PD1)/ Programmed Death-ligand 1 (PDL1) expression, high Tumor Mutation Burden (TMB) [5,6]. Therefore, analyzing druggable targets in pathological samples became necessary for tailoring personalized treatment. Although Polymerase Chain Reaction (PCR), Sanger sequencing, Florescence In Site Hybridization(FISH) and Immunohistochemistry (IHC) are the common technologies which are used in pathological laboratories [7], but multi-gene analysis by using those common methods is time-consuming and cost inefficient. An advanced method of NGS offers an unbiased analysis of multi-mutations, complex structural changes including translocation and gene copy number changes and TMB, thus

* Corresponding author.
E-mail address: wwxxll718@163.com (X. Wu).
[1] Joint first authors.

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providing more complete and accurate information about clinical samples, which may guide more appropriate clinical decision-making [8].

In this study, we verified a ten-gene NGS panel and analyzed 195 NSCLC pathological tissue samples. Sanger sequencing was used to verify EGFR, ALK mutation status, meanwhile, Amplification-refractory Mutation System (AMRS) PCR was also employed for some inconsistent samples. Our data suggest that this small panel of multi-gene analysis by NGS could be an efficient and clinically applicable method to provide adequate genetic information for patients and oncologists.

2. Materials and methods

2.1. Patients and samples

There are 214 formalin-fixed, paraffin-embedded tumor tissue specimens from NSCLC patients in the pathology departments of the First Affiliated Hospital, Medical School, Wenzhou University and Beijing Chest Hospital, Capital Medical University. The specimens were all surgically harvested from newly diagnosed patients without any TKI treatment and 199 of them were included in this study. The protocol was approved by Ethics Committee of Bio-medicine Research in the hospitals. Informed consents were obtained from all the patients of this study. Diagnosis of NSCLC was established histologically and immunohistologically according to World Health Organization (WHO) criteria [9].The eighth version of TNM staging was used [10].

2.2. DNA and RNA extraction

Ten 4 μm tissue sections from paraffin-embedded tumor blocks were macro-dissected according to their consecutive H&E stained slides to enrich over 70% abundance of tumor cells in each sample. DNA and RNA were isolated simultaneously using a Formalin-fixed paraffin-embedded DNA & RNA extraction kit (AmoyDx, Xiamen, China). Concentrations of DNA and RNA were determined by SmartSpec Plus Spectrophotometer (Bio-Rad Life Science, CA, USA).

2.3. NGS analysis

The library of 18 targeted regions of ten genes was prepared by the multi-gene mutation analysis kit from AmoyDx (Xiamen China) according to the kit manual. Briefly, genomic DNA was processed through fragmentation, end-repairing, ligation of adapters, library amplification and hybridization capturing. The targeted library was analyzed by the Illumina Mid Output V2 Reagent kit and BerryGenomics NextSeq CN500. The subsequent Variant Call Files (VCF) were subjected to cross sample background filtering with potential artifacts removed below 3 standard deviations of the mean background noise for each position. Filter criteria for variant calling for absolute mutated allele read counts were set to the valid depth $\geq 500\times$, percentage of mutated copies $\geq 0.4\%$. All the mutations which were included in this kit were listed in Table 1.

Table 1
Mutation panel of the NGS test.

Targeted Areas	Mutations	Base Changes	Cosmic ID
EGFR Exon18	G719A	2156G > C	6239
EGFR Exon18	G719S	2156G > A	6252
EGFR Exon18	G719C	2156G > T	6253
EGFR Exon19	E746_A750del	2235-2249del15	6223
EGFR Exon19	E746_A750del	2236-2250del15	6225
EGFR Exon19	L747_P753delinsS	2240-2257del18	12370
EGFR Exon19	E746_L752delinsV	2237-2255delinsT	12384
EGFR Exon19	L747_P753delinsP	2239-2248delinsC	12382
EGFR Exon19	L747_T751del	2240-2254del15	12369
EGFR Exon20	T790M	2369C > T	6240
EGFR Exon20	S781I	2303G > T	6241
EGFR Exon20	D770_N771insG	2310_2311insGGT	12378
EGFR Exon21	L858R	2573T > G	6224
EGFR Exon21	L861Q	2582T > A	6213
KRAS Exon2	G12D	35G > A	521
KRAS Exon2	G12A	35G > C	522
KRAS Exon2	G12V	35G > T	520
KRAS Exon2	G12S	34G > A	517
KRAS Exon2	G12C	34G > T	516
KRAS Exon3	Q61H	183A > C	554
NRAS Exon2	G12D	35G > A	564
NRAS Exon3	Q61R	182A > G	584
NRAS Exon3	Q61K	181C > A	580
PIK3CA Exon21	H1047R	3140A > G	775
BRAF Exon15	V600E	1799T > A	476
HER2 Exon20	A775_G776insYVMA	2324_2325ins ATACGTGATGGC	20959
MET Exon14	–	3082 + 1G > T	24687
ALK Exon20	Fusion (M1)	EML4 Exon13-ALK Exon20	463
ALK Exon20	Fusion (M2)	EML4 Exon6-ALK Exon20	474
ALK Exon20	Fusion (M3)	EML4 Exon20-ALK Exon20	465
ROS1 Exon34	Fusion	CD74 Exon6-ROS1 Exon34	1201
ROS1 Exon35	Fusion	GOPC Exon8-ROS1 Exon35	1251
RET Exon12	Fusion	KIF5B Exon15-RET Exon12	1233

2.4. Sanger sequencing of EGFR and ALK

EGFR mutation Sanger sequencing was carried on in the same laboratory using the published protocol [11]. Four separate PCR amplifications targeting exons 18–21 were prepared. Targeted PCR products were separated and purified. 20 ng of separated PCR products were sequenced by repeating 25 cycles of 96°C 10s, 50°C 5s and 60°C, 4 min in a 20μl mixed solution, including 8 μl of Big Dye Terminator v3.1 (Life Technologies, CA, USA)and 2 pmol of forward or reverse sequencing primers respectively, by using an Applied Biosystems (ABI) PRISM 3130 DNA Analyzer (Applied Biosystem, Tokyo, Japan).

ALK mutation Sanger sequencing used two rounds of PCR after cDNA was prepared. The reverse transcription reaction was completed by using a mixture of 23.45μl Diethyl Pyrocarbonate (DEPC) H2O, 12.5μl reverse transcription buffer, 1 μl AvianMyeloblastosis Virus (AMV)reverse transcriptase (Tagene Biotechnology, Xiamen, China), 1μl dNTP (25 mM), 0.05μl reverse transcription primer (ALK-E20-R: AGCTCCATCTGCATGG CTTG, 100μM), 12μl RNA (1 μg/ml) under 42°C 1 h and 95°C 5 min. The first round of PCR was carried out by using a mixture of 17.79 μl H2O, 8 μl 5× buffer, 8 μl Mg^{2+}(25 mM), 0.4μl dNTP(25 mM), forward primers 0.064 μl each (Echinoderm Microtubule Associated Protein Like 4(EML4) E13: AAAACTACTGTAGAGCCCACACCT, EML4 E6: CCAAGCAAAAATGT CAACTCGCG, EML4 E20:TAATGTCTAACTCGGGAGACT, 100μM each), reverse primer 0.064 μl (ALK-E20-R: AGCTCCATCTGCATGGCTTG, 100μM), Bovine Serum Albumin (BSA) 0.096μl (5mg/ml), HS-Taq polymerase 0.25μl (5U/μl, TAKARA, Dalian, China), formamide 0.4 μl, cDNA 5μl under 95°C 5 min pre-denaturing, 15 cycles of 95 °C 25 s, 64 °C 20 s, 72°C 20 s, 31 cycles of 93°C 25 s, 60°C 35 s, 72°C 20 s, and 72 °C 10 min. The second round of PCR was carried out by using a mixture of 17.726μl H2O, 8μl 5× buffer, 8μl Mg^{2+} (25 mM), 0.4μl dNTP (25 mM), forward primers 0.064μl each (EML4 E13-2:CGCCAGGGTTTTCCCAGTCACGACG CCCACACCTGGGAAAGGAC CTAAAG, EML4 E6-2: CGCCAGGGTTTTCC CAGTCACGACAGACAAGCATAAAGATGTCATCATC AACCAA,EML4 E20-2: CGCCAGGGTTTTCCCAGTCACGACTCTAACTCGGGAGACTATGAAATA TTGTACT, 100μM each), reverse primer 0.064μl (ALK-E20-R-2: AGCGG ATAACAATTTCA CACAGGACTTGCAGCTCCTGGTGCT, 100μM), BSA 0.096μl (5 mg/ml), HS-Taq polymerase 0.25μl (5U/μl, TAKARA, Dalian, China), formamide 0.4μl, first round PCR product 5μl under 95°C 5min pre-denaturing, 50cycles of 95°C 20s, 60°C 20s, 72°C 20s and 72°C 5min. PCR product was sequenced using the same protocol as EGFR mutation sequencing. ALK sequencing primers were ALK-Seq-F: GCCAGGGTTTTC CCAGTCACG, ALK-Seq-R: GCGGATA ACAATTTCACAC.

All the electropherograms were analyzed by visual inspection by a highly experienced scientist (> 1000 sequences/year). The nucleotide sequences were aligned by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.

cgi). The known drug response related mutations of EGFR and ALK (Table 1) were reported.

2.5. EGFR ARMS PCR analysis

EGFR mutation ARMS analysis was conducted by using the NMPA approved EGFR mutation detection kit (AmoyDx, Xiamen, China) following the manufacturer's instruction on theApplied Biosystems™ 7500 Real-Time PCR System (Applied Biosystem, Tokyo, Japan) in our certified laboratory. Different cutoff values were used to analyze 29 EGFR mutations according to the kit manual [11].

2.6. Statistical analysis

All statistical data were analyzed using SPSS 20.0 statistical software (SPSS Inc. Chicago, IL). The χ^2 test was used to evaluate the difference between the different methods.

3. Results

3.1. Clinicopathologic data

A total of 214 candidate NSCLC patients were available for this study. However, 15 of them were excluded due to inadequate tumor cells in their donated tissue samples. Therefore, only199NSCLC patients were included in this study. Their pathological information was shown in Table 2, which included sex, age, histological types, and clinical stages.

3.2. genes NGS analysis

Four of the 199 samples were excluded due to the low amount of DNA samples before the NGS analysis. One sample was excluded during the NGS experiment because of its low DNA quality. A total of 194 samples were successfully analyzed by the 10 genes NGS method. The results were shown in Fig. 1. No mutations were found in 42 samples. EGFR mutation were found in 105 samples, which consisted of 100 single mutation and 5 double mutations among the four exons. The majority of EGFR mutations were Exon21 point mutation (53%) and Exon19 deletions (34%). ALK fusions (10%),Kirsten rat sarcoma 2 viral oncogene homolog (KRAS) mutations (6.5%), Human Epidermal Growth Factor Receptor2 (Her2) mutations (2.5%), Rearranged During Transfection (RET) fusions (1.5%), Neuroblastoma RAS Viral Oncogene homolog (NRAS mutation) 0.5% and Phosphoinositide-3-kinase, Catalytic, Alpha Polypeptide (PIK3CA) mutation (0.5%) were also found. Four samples were found to have two genes mutations, which were EGFR-T790M, ALK M1, KRAS G12A, EGFR G719A, KRAS G12C, ALK M2, KRAS G12V, PIK3CA H1047R.

Table 2
Clinicopathological Data.

Characteristics	Numbers (Percentage)
Sex	
Male	96 (48.2%)
Female	103 (51.8%)
Age	
≤50 years	36 (18.1%)
51-60	57 (28.6%)
61-70	78 (39.2%)
> 70	28 (14.1%)
Histological Subtypes	
Adenocarcinoma	173 (86.9%)
Squamous Cell Carcinoma	22 (11.1%)
Large Cell Carcinoma	3 (1.5%)
Adenosquamous Carcinoma	1 (0.5%)
Clinical Stages	
I	130 (65.3%)
IA	111
IB	19
II	18 (9%)
IIA	2
IIB	16
III	31 (15.6%)
IIIA	20
IIIB	9
IIIC	2
IV	14 (7%)
IVA	12
IVB	2
Unknown	6 (3%)
Total	199

Clinicopathological Data.

3.3. Sanger sequencing and ARMS PCR analysis of EGFR mutation

Three DNA samples were found to be inadequate after NGS analysis. A total of 191 samples were analyzed by EGFR Sanger sequencing successfully. The data of comparison study was summarized in Table 3. No EGFR mutated sample were found by Sanger sequencing among the 84 NGS EGFR wildtype samples. Five samples with low abundance of EGFR mutated reads (< 5%) were found to be negative by Sanger method. One sample with EGFR T790M and L858R double mutations was found to be only L858R mutated. However, four out of the above six samples were proved to be EGFR mutation positive by the ARMS PCR method, as shown in Table 4. Two samples with 0.67% EGFR-E746_A750del and 0.49% EGFR T790M, respectively were concluded as wildtype by both Sanger and ARMS PCR methods. The χ^2test showed that this NGS method for EGFR mutation analysis has no significant difference as compared to Sanger sequencing.

3.4. Sanger sequencing and ARMS PCR analysis of ALK fusions

All the RNA samples were analyzed by ALK Sanger sequencing. Table 5 showed the comparison study with NGS. All the NGS ALK fusion positive samples were proved by Sanger sequencing. Only one NGS ALK fusion negative sample was found to be positive by Sanger sequencing, and it was confirmed as positive by the follow-up ARMS PCR test. The χ^2test showed that this NGS method for ALK mutation analysis has no significant difference as compared to Sanger sequencing.

4. Discussion

In addition to surgery and chemotherapy, NSCLC patients have many other targeted therapy choices, which includes three generations of EGFR TKIs, ALK, ROS1, c-MET inhibitors, immunotherapies [12].In most cases, companion diagnosis of genetic status is needed simultaneously with the histopathological diagnosis for lung cancer patients. To a certain extent, molecular diagnosis is more important than pathological diagnoses for those patients who prefer to use targeted therapy than chemotherapy due to the less side-effect and better responses they can get [13,14]. Normally, pathologists request ALK, PD1/PDL1 IHC first and then ALK FISH if necessary. EGFR mutation test is highly recommended for adenocarcinoma patients. If ALK and EGFR are both wild types, ROS1, c-MET, v-raf murine sarcoma viral oncogene homolog B1 (BRAF), Her2, RET,V-akt Murine Thymoma Viral Oncogene Homolog 1(AKT1), Neurotrophic Tyrosine Kinase (NTRK)may need to be tested by IHC, Sanger sequencing, fluorescent quantitative PCR or FISH. More genes could be screened in the follow-up steps [15]. The whole procedure of molecular pathological diagnosis of NSCLC patients takes a few weeks and costs a high amount of expense from our health care system. Therefore, a highly efficient and relatively low-cost detection method which can simultaneously test most NSCLC therapeutic targets is urgently needed.

Recently, NGS has become a cutting-edge platform to analyze multigene mutations, fusions, amplifications and even mutation

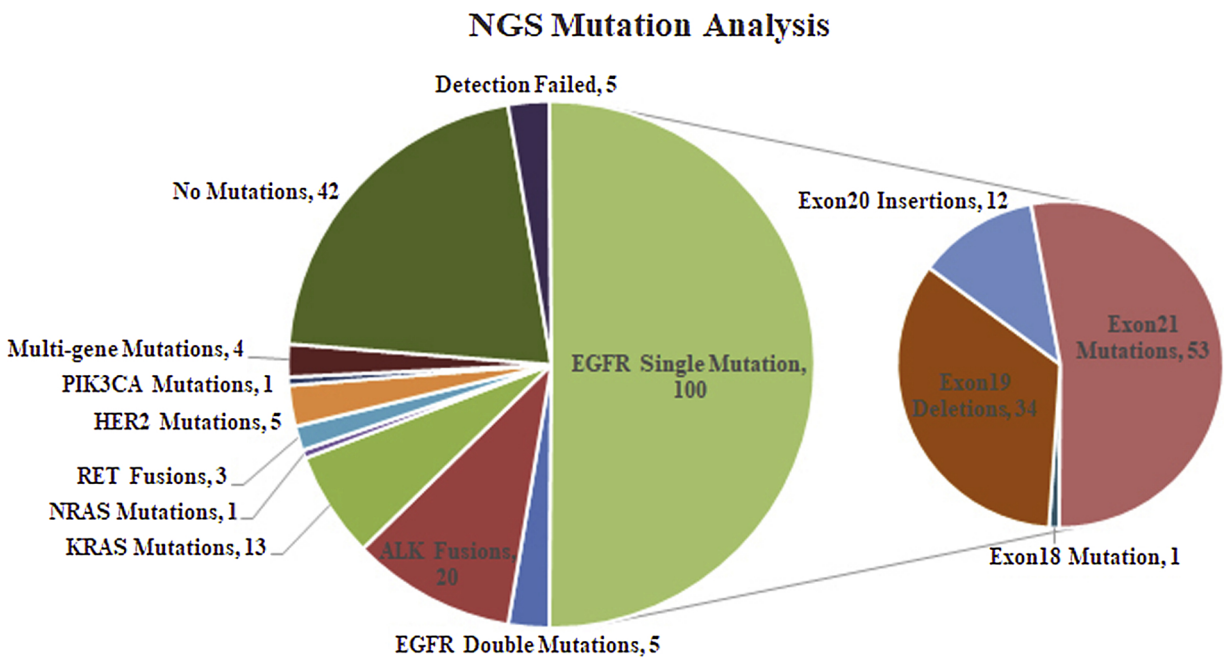

Fig. 1. The pie chart on the left shows the pattern of all the mutations in this study. The pie chart on the right shows the pattern of EGFR single mutation.

Table 3
Comparison study of NGS and Sanger sequencing.

	NGS	Sanger sequencing
EGFR Wild type	84	89
EGFR Mutated	107	102

$\chi^2 = 0.2641$, $P = 0.6073$.

Table 4
ARMS PCR Verification.

Sample No.	NGS	Mutated reads	ARMS PCR
S5	EGFR-T790M	1.36%	Positive
S12	EGFR-L858R	4.34%	Positive
S23	EGFR-E746_A750del	2.45%	Positive
S59	EGFR-L858R	2.99%	Positive
S64	EGFR-E746_A750del	0.67%	Negative
S95	EGFR-T790M	0.49%	Negative

ARMS PCR Verification.

Table 5
Comparison study of NGS and Sanger sequencing.

	NGS	Sanger sequencing
ALK WT	169	168
ALK Mutated	22	23

$\chi^2 = 0.0252$, $P = 0.8739$.

burdens in tumor samples. This technology is changing our way to make diagnosis for tumor patients. Medical laboratories now can provide adequate genetic information of a patient sample as much as needed by clinicians [16]. However, due to the high incidence of cancer patients and insufficient health care budget in China, most patients have difficulties affording the existing diagnostic tests, which made a relative affordable multigene analysis method necessary.

In this study, we tested this NGS mutation analysis method, which covered 32 popular mutations of 10 NSCLC therapy related genes. Due to the high volume of lung cancer patients in China, this small panel of mutation test can provide enough genetic information for the patients while minimizing the average expense for each sample. We excluded TMB analysis mainly because it would increase the average expense significantly as the data for each sample are increased for TMB analysis. Additionally, the relationship between TMB and immunotherapy response is still controversial [17]. Sanger sequencing is commonly used as a reference method to detect gene mutations, especially for abundant mutations. EGFR and ALK Sanger sequencing were performed in our study, which produced consistent data with that of the NGS method, except for 6 samples with inconsistent EGFR results and 1 sample with conflicting ALK results. It is obvious that Sanger sequencing was unable to identify the mutated signal of these 6 samples with low EGFR mutation reads. Four of them were proved to be positive by the highly sensitive ARMS PCR method. The two with negative results from both Sanger sequencing and ARMS PCR has only 0.67% EGFR-E746_A750del and 0.46% mutatedT790M reads respectively according to the NGS data. It indicated that the NGS method with adequate sequencing depth may have higher sensitivity than ARMS PCR. In addition, for EGFR-E746_A750del analysis, this specific sample had a higher Cycle threshold (Ct) value than the cutoff value to call it is E746_A750del positive. The amplification efficiency in this case may be not high enough due to that the multi-plex PCR has a complex reaction system which needs higher quality of DNA samples. For T790M analysis, the ARMS PCR probe designing is relatively hard due to the nearby Single Nucleotide Polymorphism (SNP) Q787Q, which could cause difficulties to test low-frequency mutations [11]. Furthermore, our study also found that one case had ALK fusion negative result was proved positive by both Sanger sequencing and ARMS PCR. A possible reason is that we used genomic DNA to do the NGS analysis, which could miss large fragment translocations and fusions. NGS method may still have false calls or missed rearrangements that are present at low frequencies and could not distinguish real fusions from potential fusion artifacts, low-level contamination, or background noise associated with library preparation. The same phenomenon was found in another study [18]. We are trying to gather drug response data for all the patients, especially for those patients with inconsistent results from different detection methods. Drug response is convincing evidence of patients' genetic status, especially for those rare mutations: RET and Her2 mutations founded in this study. Unfortunately, most of our patients have class I lung cancer, which does not require extra therapies after surgery. Targeted therapy or chemotherapy may be considered until signs of

progress are found.

In conclusion, this ten-gene panel of NGS testing platform is a well-designed and clinical applicable solution for NSCLC patients companion diagnosis. It was at least partially proved to have high efficiency and adequate sensitivity with affordable cost, providing enough therapeutic target information for NSCLC patients.

Conflict of interests

The authors have no conflicts of interest to declare.

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