Int J Clin Exp Pathol 2013;6(9):1880-1889
www.ijcep.com /ISSN:1936-2625/IJCEP1307047

Original Article

Screening for *EGFR* and *KRAS* mutations in non-small cell lung carcinomas using DNA extraction by hydrothermal pressure coupled with PCR-based direct sequencing

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Received July 26, 2013; Accepted August 16, 2013; Epub August 15, 2013; Published September 1, 2013

Abstract: *EGFR* and *KRAS* mutations correlate with response to tyrosine kinase inhibitors in patients with non-small cell lung carcinoma (NSCLC). We reported a hydrothermal pressure method of simultaneous deparaffinization and lysis of formalin-fixed paraffin embedded (FFPE) tissue followed by conventional chaotropic salt column purification to obtain high quality DNA for mutation analysis using PCR-base direct sequencing. This study assessed the feasibility of using this method to screen for exons 18-21 of *EGFR* and exon 2 of *KRAS* gene mutations in surgical resection and core needle biopsy specimens from 251 NSCLC patients. *EGFR* mutations were identified in 140 (55.8%) NSCLC patients (118 in adenocarcinoma, 11 in squamous cell carcinoma, 7 in adenocarcinoma and 4 in NSCLC-not otherwise specified), including four novel substitutions (L718M, A743V, L815P, V819E). EGFR mutations were frequently present in female patients (72 of 113, 63.7%) and NSCLC with adenocarcinoma component (125/204, 61.3%) with statistical significance. Twenty-one patients had multiple mutations at different exons of *EGFR*, in which seventeen patients had deletions in exon 19. *KRAS* mutations were found in 18 (7.2%) patients (15 in adenocarcinoma, 2 in squamous cell carcinoma and one in NSCLC-not otherwise specified), including an uncommon substitution G13C. Deparaffinization and lysis by hydrothermal pressure, coupled with purification and PCR-based sequencing, provides a robust screening approach for *EGFR* and *KRAS* mutation analysis of FFPE tissues from either surgical resection or core needle biopsy in clinical personalized management of lung cancer.

Keywords: *EGFR*, *KRAS*, FFPE, hydrothermal pressure, lung cancer, mutation analysis

Introduction

As a transmembrane receptor, epidermal growth factor receptor (*EGFR*) is a key regulator of epithelial cell proliferation. Excessive EGFR signaling upsets the balance between cell growth and apoptosis contributing to tumor genesis in a wide variety of solid tumors including non-small cell lung cancer (NSCLC) [1]. Somatic mutations in the tyrosine kinase domain (exons 18-21) of *EGFR* gene can bring about constitutive activation of *EGFR* tyrosine kinase activity. Most of these mutations, such as deletion mutations in exon 19 that affect the conserved LREA motif and a single amino acid substitution at codon 858 (Leucine to Argine; L858R) of exon 21, are associated with sensitivity to the small molecule tyrosine kinase inhibitors (TKIs), erlotinib and gefitinib. These drug-sensitive mutations are found in up to 60% of Asian patients with lung adenocarcinoma [2]. However, minor mutations, such as T790M and S768I, are associated with resistance to TKI therapy and have been reported in about 50% of patients with disease progression [3, 4].

Approximately 15-20% of unselected NSCLC harbor mutations in the exon 2 of Kirsten rat sarcoma viral oncogene homolog (*KRAS*) [5-7]. Although most of current literatures suggest that *EGFR* and *KRAS* mutations are mutually exclusive [8], as a downstream signal molecule of *EGFR* pathway, *KRAS* mutation may be a pre-

Table 1. Sequences of primers and PCR amplicons

Gene	Exon	Primer Sequences (from 5' to 3')	Amplicons
EGFR	18	Forward: TTTCCAGCATGGTGAGGG	263 bp
		Reverse: ACAGCTTGCAAGGACTCT	
EGFR	19	Forward: AGCATGTGGCACCATCTC	224 bp
		Reverse: AGACATGAGAAAAGGTGG	
EGFR	20	Forward: CATGTGCCCCTCCTTCTG	325 bp
		Reverse: CTATCCCAGGAGCGCAGA	
EGFR	21	Forward: AATTCGGATGCAGAGCTT	293 bp
		Reverse: TACAGCTAGTGGGAAGGC	
KRAS	2	Forward: AACCTTATGTGTGACATGTTCTAAT	232 bp
		Reverse: CTGTATCAAAGAATGGTCCTGC	

dictor for primary resistance to TKIs therapy in NSCLC [9]. As a prognostic marker, *KRAS* mutations in resected NSCLC were associated with shorter overall survival than those with *EGFR* mutations. As a result, clinically adequate work-up of lung cancer cannot be limited to histotype classification, but should include a series of molecular biology analyses (*EGFR* and *KRAS*) to define distinct subgroups with different responses to *EGFR*-targeted therapies [10].

Direct sequencing of PCR products corresponding to target sequences is a popular and acceptable approach to assess genetic mutation status [11, 12]. However, using traditional DNA extraction methods, biopsy specimens usually provide suboptimal tumor material and frequently insufficient for routine molecular diagnosis in clinical pathology labs. Although the commercial nucleic acid extraction kits based on chaotropic salt purification mechanism are commonly used, analysis of DNA extracted from formalin-fixed paraffin embedded (FFPE) samples remains difficult, costly, biohazardous and time consuming. Unlike standard protease K digestion, Zhong et al has reported a reliable and efficient method of simultaneous deparaffinization and lysis of FFPE tissue using hydrothermal pressure to obtain high quality DNA for PCR and subsequent direct sequencing. As we previously described, the quality and integrity of extracted DNA have been certified by successful amplification of variable-sized amplicons in tissue samples archived from 0.2 to 22 years [13]. This is particular important to maximize the DNA recovery for mutation analyzing in FFPE tissues, especially small-sized bronchoscopic or transthoracic core needle biopsies. Here we screened for the mutations of *EGFR* exons 18-21 and *KRAS* exon 2 in 251 FFPE samples derived from surgical resections and core needle biopsies of NSCLC patients in routine clinical practice using a single assay on the principles of hydrothermal pressure extraction and direct sequencing.

Materials and methods

Patients and histological evaluation

Between January 2010 and October 2012, paraffin-embedded tissues from 251 patients with histologically confirmed NSCLC were obtained. Of these, 136 specimens were from surgical resection and 115 specimens were from core needle biopsies, in which 70 were from CT-guided transthoracic biopsy, 27 from bronchoscopic biopsy and 18 from metastatic lymph node biopsy. There were 113 women and 138 men. The median age was 65 (range, 21-88 years) and 93 patients (37.1%) were older than 70 years. This study was approved by the Peking University Institutional Review Board with the approval No. IRB00001052-10004.

The biopsy procedure was performed using an 18-gauge or 20-gauge Chiba aspiration needle. One to three separate needle insertions are typically needed to obtain biopsy samples approximately 0.5-0.75 inches long (approximately 1.2-2.0 cm) and 0.04-0.06 inches (approximately 0.1-0.15 cm) in diameter. After the surgery or biopsy, all the samples are immediately sent to the pathology laboratory for diagnosis. In our standard clinical protocols, immunohistochemistry staining was performed for establishing a precise diagnosis for each NSCLC patient. The original histopathologic diagnoses were reviewed and confirmed by two pathologists according to the 2004 WHO classification of lung tumors and the 2011 International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) lung adenocarcinoma classification. None of these patients had received tyrosine kinase inhibitor treatment or chemotherapy before mutation analysis.

DNA extraction and quantitation

For each sample, a total of 8 sections of 5 μm thickness and one corresponding hematoxylin

Table 2. *EGFR* mutations detected in non-small cell lung carcinoma patients

Histology	*EGFR*		Number of cases
	Mutation (Amino Acid)	Exon	
Adenocarcinoma	E709K + E746_A750 *del*	18, 19	2
Adenocarcinoma	E709_T710 *delins* D	18	1
Adenocarcinoma	L718M + E746_A750 *del*	18, 19	1
Adenocarcinoma	G719C	18	1
Adenocarcinoma	G719A + L747S	18, 19	1
Adenocarcinoma	G719A + R776S	18, 20	1
Adenocarcinoma	G719S + S768I	18, 20	1
Adenocarcinoma	P741L	19	1
Adenocarcinoma	A743V	19	1
Adenocarcinoma	E746_A750 *del*	19	62
Adenocarcinoma	E746_A750 *del* + A767V	19, 20	1
Adenocarcinoma	E746_A750 *del* + L788F + V802I	19, 20	1
Adenocarcinoma	E746_A750 *del* + V819E	19, 20	1
Adenocarcinoma	E746_A750 *del* + L858R	19, 21	5
Adenocarcinoma	E746_A750 *del* + L858W + E868V	19, 21	1
Adenocarcinoma	E746_T751 *delins* A	19	2
Adenocarcinoma	E746_S752 *delins* V	19	4
Adenocarcinoma	L747_T751 *del*	19	1
Adenocarcinoma	L747_T751 *del* + S768I	19, 20	1
Adenocarcinoma	L747_S752 *del*	19	1
Adenocarcinoma	L747_P753 *delins* S	19	4
Adenocarcinoma	S753_I759 *del*	19	1
Adenocarcinoma	S768I + L858R	20, 21	1
Adenocarcinoma	G857E	21	1
Adenocarcinoma	L858R	21	21
Adenosquamous carcinoma	E746_A750 *del*	19	1
Adenosquamous carcinoma	E746_A750 *del* + Y801C	19, 20	1
Adenosquamous carcinoma	E746_A750 *del* + L858R	19, 21	1
Adenosquamous carcinoma	L858R	21	3
Adenosquamous carcinoma	L861Q	21	1
NSCLC, not otherwise specified	L718M	18	1
NSCLC, not otherwise specified	E746_A750 *del*	19	3
Squamous cell carcinoma	G719D + E746_A750 *del*	18, 19	1
Squamous cell carcinoma	E746_A750 *del*	19	9
Squamous cell carcinoma	E746_A750 *del* + L815P	19, 20	1

and eosin (H.E.) stained section were obtained. One anatomic pathologist reviewed H.E. stained histological sections to exclude necrosis or hemorrhage and determine the quantity of tumor cells for molecular testing. In order to enrich tumor cells, the tumor foci from the marked areas were selectively scraped from the corresponding unstained FFPE sections and collected into 1.5 ml centrifuge tubes for DNA isolation. After manual dissection, samples contained more than 60% tumor cells as estimated from the H.E. stained slide.

As previously described by Zhong et al, genomic DNA was extracted from tumor tissue lysed by hydrothermal pressure [13]. Briefly, tissue samples were submerged into 250 μL lysis solution (0.1 M NaOH with 5% Chelex-100). Before capping, an orifice was created through the centrifuge tube cap by passing through a

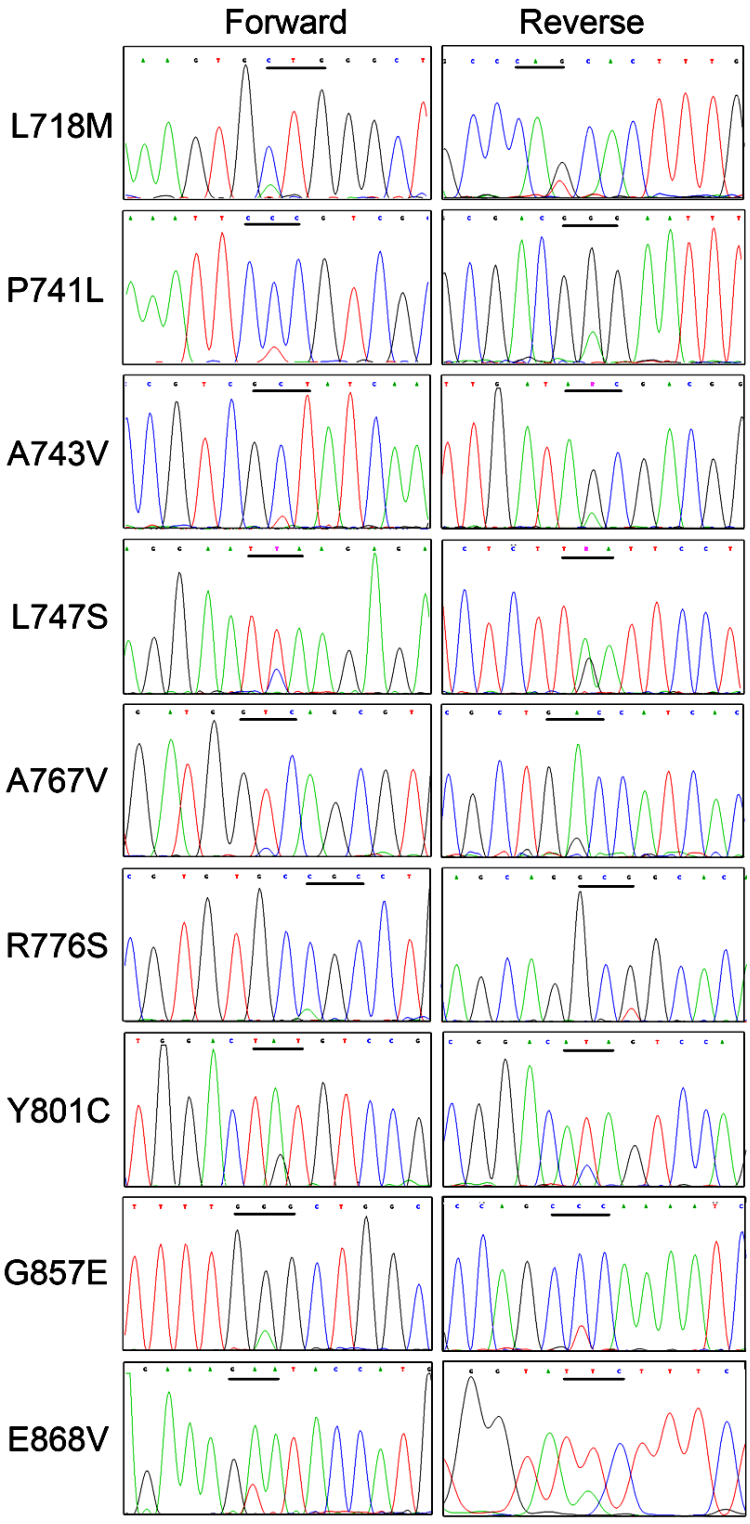

Figure 1. Sequence chromatograms displaying partial types of *EGFR* uncommon mutations found in this study. The amino acid changes were corresponded to the codon alternations as following: L718M (CTG→ATG) in exon 18; P741L (CCC→CTC), A743V (GCT→GTT) and L747S (TTA→TCA) in exon 19; A767V (GCC→GTC), R776S (CGC→AGC) and Y801C (TAT→TGT) in exon 20; G857E (GGG→GAG) and E868V (GAA→GTA) in exon 21.

23-gauge hypodermic needle (0.64 mm in diameter). Hydrothermal pressure treatment was performed for 30 minutes using a conventional pressure cooker at 80 Kilopascal (kPa) working pressure setting. After treatment, centrifuge tubes were centrifuged at 14000 rpm for 5 minutes at 4°C and 200 μL upper liquid phase was transferred into a new tube. Genomic DNA was extracted by adding 200 μL AL lysis buffer, a component of the Qiagen Blood and Tissue Kit (Qiagen Inc, Valencia, CA) and vortexed, followed by adding 200 μL 100% ethanol before transferring into the Qiagen purification column. The column was then washed and DNA was purified into instruction into 30 μL AE buffer according to the manufacturer's instruction. DNA was quantified by spectrophotometric absorbance at 260 nm using the NanoDrop® apparatus (Thermo Scientific Inc., Wilmington, DE, USA).

Analysis of EGFR and KRAS mutations

The *EGFR* exons 18-21 and *KRAS* exon 2 were amplified by polymerase chain reaction (PCR) using Promega GoTaq® Hot Start Colorless Master Mixes (Promega Corporation, Madison, WI, USA). The specific primers and sizes of the expected amplicons are presented in **Table 1**. Genomic DNA of 50-100 ng was amplified in a 50 μL reaction containing 25 μL of Hot Start Colorless Master Mix and 5 μL of 10 μM primer mix. The PCR reaction consisted of 2 minutes at 95°C, followed by 40 cycles of 94°C for 30 seconds; 56°C for 40 seconds and 72°C for 1 minute, finished by 72°C for 7 minutes. Five microliter of the PCR prod-

Table 3. *KRAS* mutations detected in non-small cell lung carcinoma

Histology	*KRAS*		Number of cases
	Mutation (Amino Acid)	Exon	
Adenocarcinoma	G12A	2	2
Adenocarcinoma	G12C	2	4
Adenocarcinoma	G12D	2	5
Adenocarcinoma	G13C	2	1
Adenocarcinoma	G13D	2	3
Squamous cell carcinoma	G12C	2	1
Squamous cell carcinoma	G12D	2	1
NSCLC, not otherwise specified	G12C	2	1

uct was analyzed by 1.2% agarose gel with 100 to 600 bp DNA marker. Gels were visualized on a BioRad Gel Doc 2000™ system and Quantity One software (BioRad, Hercules, CA, USA). The resulting PCR amplicons were purified and sequenced in both directions using the BigDye Terminator kit and an ABI Prism 3500 DNA Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Mutant cases were validated by second independent PCR and sequencing. In every experiment, ddH_2O substituted for DNA template was used as a negative control to rule out the possibility of contamination. The sequencing results were observed by ABI Sequence Scanner software and compared with the reference sequence of *EGFR* and *KRAS* gene from NCBI database to mark the position of nucleotide change.

Statistical evaluation

The associations between the presence of *EGFR* or *KRAS* mutations and the clinicalpathological characteristics were assessed by chi-square test using SPSS11.0 software package. A threshold of $P < 0.05$ was defined as statistically significant.

Results

Morphological diagnosis and immunoprofile

Histological type of each patient was determined by a combination of morphology and immunohistochemical profile according to the NCCN Guidelines® for Non-Small Cell Lung Cancer (Version 3.2012) and the guidelines for the diagnosis of adenocarcinoma published by IASLC/ATS/ERS. In the case of adenocarcinoma, diagnosis was confirmed by expression of TTF-1, Napsin A or CK7 and negativity for CK5/6 and p63. Histological characteristic and expression of p63 favored squamous cell carcinoma diagnosis. CD56, Chromogramin A and synaptophysin were used to identify neuroendocrine tumors. Undifferentiated NSCLC by morphology that also lacked expression of differentiation markers TTF-1, Napsin A or p63 resulted in the diagnosis of NSCLC-NOS (not otherwise specified). Based on these criteria, 193 of the 251 patients (76.9%) were diagnosed with adenocarcinoma, 37 (14.7%) with squamous cell carcinoma, 11 (4.4%) with adenosquamous cell carcinoma, 10 (4.0%) with NSCLC-NOS. In small biopsy specimens, adenosquamous cell carcinoma was not diagnosed because of inadequate sampling.

Mutations analysis

All 251 FFPE samples were successfully tested by hydrothermal pressure extraction and PCR amplification of amplicons ranging from 224 to 325 bp. The PCR products were subject to direct Sanger sequencing and all produced informative data for mutation analysis.

Mutations in the *EGFR* gene were detected in 140 of 251 NSCLC patients (55.8%). Details of the mutations detected in *EGFR* gene are listed in **Table 2**. Deletions were almost detected in exon 19, while a short in-frame deletion (c.2127_2129 *del* AAC) in exon 18 was found in one patient with adenocarcinoma. Deletions in exon 19 and L858R substitution in exon 21 were the hotspots mutations with the higher frequency 75.0% (105 of 140) and 22.1% (31 of 140). As shown in **Figure 1**, infrequent point mutations were also found in exon 18-20 and other codons of exon 21, in which most mutations had been collected by Catalogue of Somatic Mutations in Cancer (COSMIC). Other four less common single amino acid substitutions, one in exon 18 (L718M) and three in exon 20 (A743V, L815P, V819E), were not previously reported or found in the SNP database. It is unlikely that the novel mutations detected in this study are due to PCR artifacts, as all mutations were confirmed in separate amplifications and no mutations were detected in negative control.

Table 4. Distribution of patients with molecular results according to clinical and pathologic factors

Variable	No.	*EGFR*		KRAS	
		Mutation (%)	*P*	Mutation (%)	*P*
Gender					
Male	138	68 (49.3)	0.022#	13 (9.4)	0.127
Female	113	72 (63.7)		5 (4.4)	
Age					
≤ 70	154	85 (55.2)	0.815	13 (8.4)	0.326
> 70	97	55 (56.7)		5 (5.2)	
Histology					
Adenocarcinoma + Adenosquamous	204	125 (61.3)	0.000#	15 (7.4)	0.935
Squamous cell carcinoma + NSCLC-NOS*	47	15 (31.2)		3 (6.4)	
Sampling procedures					
Core needle biopsy	115	72 (62.6)	0.059	9 (7.8)	0.712
Surgical resection	136	69 (50.7)		9 (6.6)	
Derivation of tumor					
Primary carcinoma	233	132 (56.7)	0.315	18 (7.7)	0.453
Metastatic carcinoma^	18	8 (44.4)		0 (0)	

Note: *Refers to not-otherwise specified; #Refers to $P < 0.05$; ^Refers to tumor samples from lymph node biopsy.

As shown in **Table 2**, twenty-one patients (21 of 140, 15.0%) had multiple mutations in different exons of *EGFR* gene, in which 17 patients were adenocarcinomas, two were adenosquamous carcinomas and two were squamous cell carcinomas. Seventeen patients (17/21, 81.0%) had both deletion in exon 19 and point mutation in other exon. In these patients, six harbored L858R substitution in exon 21 as the simultaneous mutation. S768I was found in three adenocarcinoma patients with simultaneous sensitive mutation G719A, L747_T751 deletion and L858R respectively. But T790M mutation in exon 20 was not detected in our patient cohort. Repeating the extraction, amplification and sequencing protocol from the same FFPE tissue block confirmed all multiple mutations.

KRAS gene mutations were found in 18 of 251 (7.2%) NSCLC patients. Single amino acid substitutions involving codon 12 and 13 of KRAS were identified in fifteen and three patients respectively (**Table 3**). The most common mutations were G12D (6/18, 33.3%) and G12C (6/18, 33.3%). An uncommon mutation G13C was found in an adenocarcinoma patient.

Association of EGFR and KRAS mutations with clinical features

Table 4 summarized the patient characteristics for all patients as well as the frequency of *EGFR* or *KRAS* gene mutations in each category. As reported previously, *EGFR* mutations were more frequent in female patients (72 of 113, 63.7%) than in male patients (68 of 138, 49.3%) ($p = 0.022$). The frequencies of *EGFR* mutation were higher in adenocarcinomas (118/193, 61.1%) and adenosquamous carcinomas (7/11, 63.6%), compared with those in NSCLC-NOS (4/10, 40.0%) and squamous cell carcinomas (11/37, 29.7%). If considering the adenocarcinoma and adenosquamous carcinoma as one group, its mutation frequency were significant higher than that of the other non-adenocarcinoma group (squamous cell carcinoma and NSCLC-NOS) ($p = 0.0002$).

There was no significant difference between the mutation frequency of core needle biopsy and surgical resection samples ($p = 0.059$). Core biopsies may provide reliable samples for the mutation analysis of NSCLC. But most of the squamous cell carcinoma samples with *EGFR* mutation (9 of 11, 81.8%) and all the four NSCLC-NOS samples with *EGFR* mutation were from core needle biopsy. This indicated that the possibility of an adenocarcinoma component could not be excluded because of incomplete sampling. In the core biopsy samples, 18 were metastatic tumors from jugular lymph nodes and *EGFR* mutations was found in 8 samples (8/18, 44.4%). However, there was no statistical difference between the mutation frequencies of primary and metastatic tumors.

KRAS mutation was detected in fifteen adenocarcinoma patients (15/193, 7.8%), two squamous cell carcinoma patients (2/37, 5.4%) and one NSCLC-NOS (1/10, 10.0%). We also examined the relationship between the presence of *KRAS* mutation and the preceding clinicopathologic parameters, but no significant difference was found (**Table 4**).

Discussion

The potential of new molecular techniques to search for biomarkers, such as *EGFR* and *KRAS*, in predicting response to therapy could improve the treatment and survival of lung carcinoma [14, 15]. Instead of invasive staging procedures, CT-guided needle biopsies of peripheral primary lung tumors or metastatic tumors in lymph nodes have been proven useful to diagnose lung carcinomas or stage patients. Increasing data supports that core biopsies may provide reliable samples for the biological characterization of NSCLC [16]. Therefore, mutation analysis of both resection and biopsy NSCLC samples in the initial pretreatment evaluation are the standard clinical procedures in our hospital.

Using standard extraction procedures and sequencing, Chen et al and Zhuang et al reported that *EGFR* gene mutations were found in fresh-frozen CT-guided biopsies from 12 of 17 (70.6%) adenocarcinoma patients and 23 of 43 (53.5%) NSCLC patients respectively [17, 18]. As the reported sensitivity of direct sequencing is 20-25%, therefore, cancer cell enrichment by the manual dissection of the morphologically confirmed cell population based on H & E staining on FFPE slide should be performed to assure the sensitivity of mutation detection. FFPE slide is likely to be more suitable than fresh-frozen sample for NSCLC tissues in somatic mutation analysis.

There are currently a number of methods that have been developed to detect *EGFR* mutations in FFPE small samples. Some real time PCR assays with Amplification Refractory Mutation System (ARMS) or locked nucleic acid probe screen for specific mutation with sensitivities of 1%-10%. But using traditional protease K digestion and ethanol-precipitating extraction, Ellison et al reported that 215 of 433 NSCLC FFPE samples yielded detectable amount of genomic DNA for EGFR gene analysis by ARMS. On the other hand, nine mutations, neither L858R nor delE746-A750, could only be detected by sequencing but not ARMS [19]. Loop-hybrid mobility shift assay was adopted by Nakajima et al to successfully analyze EGFR mutation in 93.5% of primary lung cancer samples obtained by EBUS-TBNA [20]. Other strategies that rely on techniques such as high resolution melting and denaturing high performance liquid chromatography detect most mutations without specifying the precise amino acid substitution.

There is currently no general agreement on which of these represents the best method for mutation analysis in NSCLC. However, strategies based on DNA amplification and direct sequencing are the most comprehensive as they can screen not only for known but also novel mutations. It is recommended that at least 40% tumor cells need to be present with more than 20% mutant DNA for efficient mutation screening relying on standard PCR and sequencing protocols [21]. In our routine clinical practice, manual dissection of tumor cells from tissue slides marked by anatomic pathologist was performed prior to DNA extraction.

Although the FFPE samples are adequate for PCR-based mutation analysis, DNA extraction remains difficult and costly as a result of cross-link introduced by the fixation and embedding. Although commercially available, current protocols with xylene deparaffinization and protease K digestion are generally manual, biohazardous and time consuming. The hydrothermal pressure approach with chaotropic column purification may significantly resolve such problems and produce comparable quantity and better quality of DNA [13]. Here we report the feasibility of this rapid extraction procedure for screening *EGFR* and *KRAS* mutations in FFPE tissue samples by Sanger sequencing. Complete evaluation of exons 18-21 of *EGFR* and exon 2 of *KRAS* were performed in all of clinical biopsy and resection samples.

Our extraction procedure involves hydrothermal pressure treatment in 0.1 M NaOH with 5% Chelex-100. Instead of the xylene deparaffinization and protease K digestion, the hydrothermal pressure process with alkaline lysis buffer disrupt cell membranes and retrieve the cross-link of DNA in one step and reduce potential tissue loss. Chelex-100, a chelating resin, is

popular used to successfully extract DNA from many forensic samples. Schuurbier et al used the 2 μg/μL proteinase K digestion with 5% Chelex-100 and successfully analyzed 77% of all FFPE cellblock samples by standard PCR and sequencing of exons 18-21 of *EGFR* [22]. Chelex-100 accommodates PCR-quality products by ensuring the complete removal of PCR inhibitors (contaminating metal ions that catalyze the digestion of DNA). After centrifuging, the resin and cellular debris were separated from the supernatant containing the released DNA. With the chaotropic column purification, high quantity and better quality of DNA would be sufficient for amplification and sequencing.

Using this extraction method and PCR-based sequencing, we found *EGFR* gene mutations in 140 of 251 (55.8%) NSCLC samples. This mutation frequency was slightly higher than those in the results of two previous large studies that examined hundreds of Chinese NSCLC patients using scorpions amplified refractory mutation system or the SurPlex®-xTAG70plex platform, 49.8% and 41.0% respectively [23, 24]. Four of the mutations (L718M, A743V, L815P and V819E) detected in our patient cohort were novel and more other less common mutations (such as L747S, R776S, P741L, A767V, G857E, Y801C and so on) would not have been detected by ARMS assay. Distinguishing novel EGFR mutations that are clinically relevant from those that are functionally silent or artifacts is clearly important, particularly as diverse responses to EGFR tyrosine kinase inhibitor (TKI) therapy of patients with NSCLC harboring uncommon EGFR mutations were recently reported. According to Wu et al, the less common mutations at codon G719 and L861, which were found in five patients of our study, would be sensitive to *EGFR* TKI therapy [25]. But the adenocarcinoma patient simultaneously harbored another mutation at the codon 747 (L747S) or codon 768 (S768I) has been linked to acquired resistance to TKI therapy. We also identified 19 patients with doublet mutations and two patients with triplet mutations in different exons. Doublet mutations accounted for 6%of EGFR mutations, with approximately half of these occurring at five codons: E709, G719, S768, T790 and L861 [26]. The exon 19 deletion, which has been linked to favorable response to gefitinib, was the most common mutation in the patients with multiple mutations (17/21, 81.0%) in present study. It is much regretted that we have no information regarding the responsiveness of our multiple mutations to TKI therapy.

In this study we also found *KRAS* mutations in fifteen lung adenocarcinoma patients and three other NSCLC patients. The frequency of *KRAS* mutations in Chinese NSCLC samples analyzed in this study (18/251, 7.2%) is in keeping with previous studies that reported *KRAS* mutation frequency of up to 8%, predominantly in adenocarcinomas (7.1% and 9.9% respectively) [23, 24]. The frequency of *EGFR* mutations in Chinese NSCLC patients is similar to that in East Asian patients [27, 28], but higher than that in Caucasian populations, and the frequency of KRAS mutation is quite opposite [5]. Importantly, *KRAS* mutations are associated with lack of response to *EGFR* inhibitor therapy in NSCLC [8]. This provides a rationale to predict relatively high response rates to *EGFR*-TKIs in Chinese patients, based on the relatively high *EGFR* mutation rate and low *KRAS* mutation rate. Taken together, our results demonstrate that by combining *EGFR* and *KRAS* mutation analysis in NSCLC patients, decisions on appropriateness of *EGFR* TKI therapy can be made in half of our patient cohort.

In conclusion, deparaffinization and lysis by hydrothermal pressure offers an unprecedented simplicity and speed of DNA release from FFPE tissue. After purification, high quality DNA can be sufficient for PCR-based direct sequencing. This method offers opportunities for rapid nucleic acid extraction for *EGFR* and *KRAS* mutation analysis of FFPE NSCLC specimens from either surgical resection or core needle biopsy in clinical diagnostic practice. Continual detection of novel *EGFR* mutations may also be useful in screening for molecular abnormality related to primary or acquired *EGFR* resistance.

Disclosure of conflict of interest

None.

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