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PCR-sequencing is a complementary method to amplification refractory mutation system for EGFR gene mutation analysis in FFPE samples

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ABSTRACT

Amplification Refractory Mutation System (ARMS) is the most popular technology for EGFR gene mutation analysis in China. Cutoff Ct or ΔCt values were used to differentiate low mutation abundance cases from no mutation cases. In this study, all of 359 NSCLC samples were tested by ARMS. Seventeen samples with larger Ct or ΔCt than cutoff values were retested by PCR-sequencing. TKI treatment responses were monitored on the cases with ARMS negative and PCR-sequencing positive results. One exon 18 G719X case, 67 exon 19 deletion cases, 2 exon 20 insertion cases, 1 exon 20 T790M case, 60 exon 21 L858R cases, 5 exon 21 L861Q cases and 201 wild type cases were identified by ARMS. Another 22 cases were evaluated as wild type but had later amplification fluorescent curves. Seventeen out of these 22 cases were retested by PCR-sequencing. It turns out that 3 out of 3 cases with exon 19 deletion later amplifications, 2 out of 2 cases with L858R later amplifications and 4 out of 12 cases with T790M later amplifications were identified as mutation positive. Two cases with exon 19 deletion and L858R respectively were treated by TKI and got responses. Our study indicated that PCR-sequencing might be a complementary way to confirm ARMS results with later amplifications.

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1. Introduction

Global incidence and mortality attributed to lung cancer climb up dramatically recently (Jemal et al., 2011; Ridge et al., 2013). The registered lung cancer mortality rate increased by 464.84% in the past 3 decades in China, which imposes an enormous burden on patients, health-care professionals and the society (She et al., 2013). Tobacco usage and environment pollution are considered as the major risk factors for this malignancy (Han et al., 2013; She et al., 2013). Besides surgery for early-stage patients, chemo-therapy with dissatisfied response rate is the only treatment for advanced and relapsed patients ten years ago in China (Lam and Watkins, 2007; Molina et al., 2008). The development of orally active small molecule inhibitors, gefitinib or erlotinib, of the epidermal growth factor receptor (EGFR) has led to a new treatment option for non-small cell lung cancer (NSCLC) patients (Peters et al., 2014). Patients with EGFR-activating mutations show sensitivity to and clinically benefit from treatment with EGFR tyrosine kinase inhibitors (TKIs) (Kobayashi and Hagiwara., 2013). Furthermore, commercially available crizotinib and other drugs under clinical trials are providing or will provide treatment options for those patients with other specific targets (Ulivi et al., 2013). Gene status must be clarified before these drugs are prescribed according to National Comprehensive Cancer Network (NCCN) guideline (Horn., 2014).

EGFR gene mutation test is becoming a routine test for every NSCLC patient with available histological or cytological samples (Zhang et al., 2014). The EGFR mutation detection kit from the Amoy Diagnostics (Xiamen, China) which was proved to have high sensitivity and short turn-around-time is now the most popular detecting method in China and Southeast Asia, replacing the reference PCR-sequencing method (Liu et al., 2014; Liu et al., 2011). This kit uses Amplification Refractory Mutation System (ARMS) technology to differentiate EGFR mutated DNA from non-mutated DNA with a so-called 1% sensitivity. Ct cutoff values or ΔCt cutoff values from external control Ct minus Ct of the mutation detected wells are used to differentiate low abundance mutated cases from wild type cases. However, in our daily service, we noticed that some cases with larger Ct values or ΔCt values than cutoff values had typical amplification curves, which should be evaluated as wild type samples by the criteria on the kit manual. As pathologists, we knew that the results of EGFR mutation testing were critical to patients. Other reliable methods must be used to clarify these equivocal samples with later amplifications.

In this study, we used PCR-sequencing to retest these later amplified samples. It turns out that these later amplifications of exon 19 deletion

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and exon 21 L858R were specific, which was proved by TKI treatment to a certain extent. Several exon 20 T790M later amplifications were specific and the others were non-specific amplifications which might be related to exon 20 Q787Q. Our data indicated that for these later amplified cases by AmoyDx EGFR mutation detecting kit, PCR-sequencing was a necessary and complementary analysis method.

2. Materials and methods

2.1. Patients and samples

We studied 359 formalin-fixed, paraffin-embedded tumor tissue specimens from NSCLC patients in the pathology department, Sir Run Run Shaw Hospital, Medical School, Zhejiang University, China. The protocol was approved by the Ethics Committee of Bio-medicine Research in the hospital. All the selected patients gave informed consents for this study. Diagnosis of NSCLC was established histologically and immunohistologically according to World Health Organization (WHO) criteria (Travis WD, 2004). All the patients had not received TKI treatment before EGFR mutation analysis.

2.2. DNA isolation

Five FFPE tissue specimen sections (4 μm each) from 359 NSCLC patients FFPE tumor blocks were macro-dissected according to their H&E stained slides to enrich over 20% abundance of tumor cells in each sample by us. DNA was extracted using a QIAamp FFPE DNA extraction kit (Qiagen, Germany). The extraction was performed according to the manufacturer's instructions for genomic DNA purification from paraffin-embedded tissues with the following modifications: Xylene was added twice. The residual ethanol for Xylene removal was evaporated at 56 °C for 5 min to save time. DNA on QIAamp MiniElute column was eluted by 40 μl of ATE to increase the DNA concentration. Qualities and concentrations of the harvested DNA samples were determined by SmartSpecPlus Spectrophotometer (Bio-Rad Life Science, CA, USA).

2.3. EGFR mutation analysis by ARMS

EGFR mutation status was analyzed by the China Food and Drug Administration (CFDA) approved EGFR mutation detection kit (AmoyDx, Xiamen, China) following the manufacturer's instruction on the LightCycler® 480II(Roche, Switzerland) in our certified laboratory. G719S/A/C in exon 18, deletions in exon 19 and 3 insertions in exon 20 were not distinguished in each exon respectively. Different cutoff values were used to analyze different mutations (Table 1). Briefly, if the Ct value of one detection well is less than 26, this sample should be called positive of that mutation, while if it is bigger than 29 for most mutations (28 for T790M mutation), the sample is EGFR wild type. On the other hand, if the Ct value is between 26 and 29 (28 for T790M mutation), ΔCt value from external control Ct minus Ct of the mutation detection well should be used to differentiate these low mutation abundance cases from no mutation cases. The ΔCt cutoff values for different mutations could be found in Table 1.

2.4. EGFR mutation analysis by general-PCR and Sanger sequencing

General-PCR and Sanger sequencing were carried on in the same laboratory. The protocol we used has been published in our previous study (Xu et al., 2014). Briefly, the frequently mutated regions of EGFR: exon 18–21 were amplified by four separate PCR reactions. Four pairs of primers were: Exon18F:GCTGAGGTGACCCTTGTCTCTG TGT, Exon18R:ATACAGCTTGCAAGGACTCTGGGCT, Exon19F:CAGCATGT GGCACCATCTCACAAT, Exon19R:AGACATGAGAAAAGGTGGGCCTGAG, Exon20F:GAAGCCACACTGACGTGCCTCTC, Exon20R:GCTCCTTATCTCCC CTCCCCGTAT, Exon21F:ATCTGTCCCTCACAGCAGGGTCTTC, Exon21R:G CAGCCTGGTCCCTGGTGTC. Then, gel-cutting and purification was used to harvest the specific PCR band after electrophoresis. Purified PCR products were sequenced by Big Dye Terminator v3.1 (Life Technologies, CA, USA) and the ABI PRISM 3130 DNA Analyzer (Applied Biosystem, Tokyo, Japan). All the sequencing reactions were performed bi-directionally and all the electropherograms were analyzed by a highly experienced observer (>1000 sequences/year). The known TKI response related mutations were reported (Sharma et al., 2007). The nucleotide sequences were aligned by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).To exclude false results, mutations were only accepted if they presented in both forward and reverse sequencing traces.

2.5. Clinical surveillance

Tumor response data were collected for efficacy assessment in patients who received at least one 4-week cycle of icotinib treatment. Efficacy was assessed by tumor response and defined as complete remission (CR) or partial response (PR) according to computerized tomography (CT) scanning. Treating physicians assessed tumor responses using the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. Tumor responses were evaluated one month after the first administration of Icotinib.

3. Results

3.1. Clinicopathologic data

All the clinicopathologic data of these 359 cases were summarized in Table 2, which included sex, age, diagnosis, stage, and sample types.

3.2. EGFR mutation pattern

Of the 359 cases, 1 exon 18 G719X case, 67 exon 19 deletion cases, 2 exon 20 insertion cases, 1 exon 20 T790M case, 60 exon 21 L858R cases, 5 exon 21 L861Q cases and 201 wild type cases were identified by ARMS method, which were shown in Fig. 1.A. Another 22 cases have later amplification fluorescent curves (Fig. 1.C) with larger Ct values or larger ΔCt values than cutoff values, which should be evaluated as EGFR wild type by the criteria in Table 1. Three out of these 22 cases have exon 19 deletion later amplifications, 2 cases have exon 21 L858R later amplifications, and 17 cases have exon 20 T790M later amplifications.

Table 1
EGFR mutation evaluation criteria from the kit manual.

Tube label		A	B	C	D	E	F	G
Mutation		19-Del	L858R	T790M	20-Ins	G719X	S768I	L861Q
Positive (mutated DNA abundance > 5%)	Ct Value	Ct < 26	Ct < 26	Ct < 26	Ct < 26	Ct < 26	Ct < 26	Ct < 26
Weak positive	Ct value	26 ≤ Ct < 29	26 ≤ Ct < 29	26 ≤ Ct < 28	26 ≤ Ct < 29	26 ≤ Ct < 29	26 ≤ Ct < 29	26 ≤ Ct < 29
(mutated DNA abundance < 5%)	ΔCt Cut-off	11	11	7	9	7	8	8
Negative	Ct Value	Ct ≥ 29	Ct ≥ 29	Ct ≥ 28	Ct ≥ 29	Ct ≥ 29	Ct ≥ 29	Ct ≥ 29

Ct: cycle threshold; ΔCt: external control Ct minus detecting well Ct.

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