RESEARCH ARTICLE

High Resolution Melting Analysis for Epidermal Growth Factor Receptor Mutations in Formalin-fixed Paraffin-embedded Tissue and Plasma Free DNA from Non-small Cell Lung Cancer Patients

Chang-Wen Jing[1&], Zhuo Wang[1&], Hai-Xia Cao[2], Rong Ma[1], Jian-Zhong Wu[1*]

Abstract

Background: The aim of the research was to explore a cost effective, fast, easy to perform, and sensitive method for epidermal growth factor receptor (EGFR) mutation testing. Methods: High resolution melting analysis (HRM) was introduced to evaluate the efficacy of the analysis for dectecting EGFR mutations in exons 18 to 21 using formalin-fixed paraffin-embedded (FFPE) tissues and plasma free DNA from 120 patients. Results: The total EGFR mutation rate was 37.5% (45/120) detected by direct sequencing. There were 48 mutations in 120 FFPE tissues assessed by HRM. For plasma free DNA, the EGFR mutation rate was 25.8% (31/120). The sensitivity of HRM assays in FFPE samples was 100% by HRM. There was a low false-positive mutation rate but a high false-negative rate in plasma free DNA detected by HRM. Conclusions: Our results show that HRM analysis has the advantage of small tumor sample need. HRM applied with plasma free DNA showed a high false-negative rate but a low false-positive rate. Further research into appropriate methods and analysis needs to be performed before HRM for plasma free DNA could be accepted as an option in diagnostic or screening settings.

Keywords: EGFR mutation - high resolution melting analysis - plasma free DNA - non-small cell lung cancer

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Introduction

Lung cancer ranks the first cause of cancer deaths in the world. Non-small cell lung cancer (NSCLC), as the most common histologic type of lung cancer, has a very poor prognosis and 5-year survival rate (Govindan et al., 2006).

The epidermal growth factor receptor (EGFR), a member of the ErbB receptor tyrosine kinase family, is related with many human tumors (Nicholson et al., 2001). EGFR is a key regulatory molecule in a diverse range of cellular signalling pathways, promoting cell proliferation and inhibiting apoptosis (Scaltriti and Baselga, 2006).

Recently, epidermal growth factor receptor- tyrosine kinase inhibitor (EGFR-TKI) such as Iressa is the most widely used targeted therapy in lung cancer patients and it has significantly improved the overall survival of Chinese lung cancer patients (Thatcher et al., 2005). Unfortunately, only one part of lung cancer patients associated with female gender, never-smokers, Asian ethnicity and adenocarcinoma histology are sensible to EGFR-TKI (Shigematsu et al., 2005). Some studies have reported that EGFR mutation status leading to structural modifications of the ATP-binding site in the tyrosine kinase domain of EGFR may implicate the effect of EGFR-TKI and future survival for patients (Maemondo et al., 2010; Mitsudomi et al., 2010; Zhou et al., 2011). The two mutations, in exon19 deletions and exon21 (L858R), account for up to 90% of all EGFR mutations. According to American national comprehensive cancer network guidelines (NCCN) for non-small cell lung cancer (version 2.2012), EGFR-TKI is considered as first-line treatment of NSCLC patients with activating mutations located in EGFR exon 19 and 21.

Direct sequencing is used as standard for detecting EGFR mutations. However, sequencing is limited by high cost, time consuming and low sensitivity, especially it has high demandingness on sufficient amount of tumour tissue and high quality which is usually difficult to obtain from cancer patients with advanced NSCLC. Therefore, a more cost effective, faster, easier to perform, and more sensitive method for EGFR mutations testing is required. High resolution melting (HRM) analysis is a newly developed method for DNA polymorphism detection. HRM analysis using a DNA binding dye, is to record the progressive change in fluorescence from a DNA duplex when it is denatured by increasing the temperature and collect a high resolution melting curve. The presence of a sequence variant can be identified according to melting curve aberrations.The recent application of HRM has shown great promise for assessment of gene mutations, genotyping and methylation (Nomoto et al., 2006; Krypuy

[1]Clinical Cancer Research Center, [2]Department of Epidemiology, Jiangsu Cancer Hospical, Nanjing, China [&]Equal contributors
**For correspondence: wujzh1528@126.com*

Table 1. Patients Characteristics (n=120)

Variables	Number	Percent(%)
Sex		
Male	69	57.5
Female	51	42.5
Age		
<62 years	57	47.5
≥62 years	63	52.5
Histological type		
Adenocarcinoma	70	58.3
Non- Adenocarcinoma	50	41.7
Histopathological grading		
High-median	58	48.3
Low	62	51.7
TNM staging		
I-II	38	31.7
III-IV	82	68.3

et al., 2007; Wojdacz and Dobrovic, 2007).

Compared with tumor tissues, plasma is easy to get and contains abundant biological information. Circulating free DNA is present in serum or plasma as usually short fragments (<1000 bp) and its concentration is normally low and varies considerably between individuals (range from 1–100 ng/ml in human). In the plasma of patients with metastatic cancers, circulating free DNA levels are significantly elevated (Kimura et al., 2006a). Several studies have shown the sensitivity of EGFR mutations between tissue and serum (Pathak et al., 2006; Hu et al., 2012). Therefore, circulating free DNA has the potential to be biomarkers for certain cancers and disease states and surrogate tumour tissues for detecting genetic alterations. In this study, we introduced HRM assays to evaluate the efficacy of the analysis for dectecting EGFR mutations in exons 18 to 21 using FFPE tissues and plasma free DNA from 120 non-small lung cancer patients.

Materials and Methods

Patients characteristics

One hundred and twenty patients who were diagnosed as NSCLC and hospitalized in Jiangsu cancer hospital from January 2010 to December 2012, were enrolled in the research. Clinical information of all patients, including gender, age, tumor histology, clinical stage and histopathological grading were recorded in Table 1. Among them, 69 were male and 51 were female, with a median age of 62 years (range, 36–85 years). The histological diagnosis of all samples (70 were adenocarcinomas, the others were non-adenocarcinomas) was confirmed by the pathologists. Tumor stage was determined according to the TNM classification of malignant tumors, a cancer staging system that describes the extent of cancer. 38 patients were classified as at Stage I and Stage II, while 82 were at Stage III and Stage IV. Informed consent was signed by the patients to participate in this study and permission was obtained for the use of their tissues and plasma samples.

DNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue

The tumour-rich samples were acquired when the patients were on operation. DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue sections using DNA FFPE tissue kit (Qiagen) according to the described protocol and then stored at -20˚C before use. The concentration was detected by a spectrophotometer (one drop).

Table 2. Primers for Direct Sequencing

Exon	Primer name	Sequence
18	EGFR 18S F	AGCATGGTGAGGGCTGAGGTGAC
	EGFR 18S R	ATATACAGCTTGCAAGGACTCTGG
19	EGFR 19S F	CCAGATCACTGGGCAGCATGTGGCACC
	EGFR 19S R	AGCAGGGTCTAGAGCAGAGCAGCTGCC
20	EGFR 20S F	GATCGCATTCATGCGTCTTCACC
	EGFR 20S R	TTGCTATCCCAGGAGCGCAGACC
21	EGFR 21S F	TCAGAGCCTGGCATGAACATGACCCTG
	EGFR 21S R	GGTCCCTGGTGTCAGGAAAATGCTGG

Table 3. Primers for HRM Analysis

Exon	Primer name	Sequence
18	EGFR 18H F	CATGGTGAGGGCTGAGGTGA
	EGFR 18H R	CCAGAGGACTGTGCCAGGGAC
19	EGFR 19H F	GTGCATCGCTGGTAACATCCA
	EGFR 19H R	AAAGGTGGGCCTGAGGTTCA
20-1	EGFR 20H F	AAGCCACACTGACGTGCCTCT
	EGFR 20H R	GCGTGATGAGGTGCACGGT
20-2	EGFR 20H F	CCTCCACCGTGCACCTCATC
	EGFR 20H R	CCCGTATCTCCCTTCCCTGA
21	EGFR 21H F	CCTCACAGCAGGGTCTTCTCTG
	EGFR 21H R	TGGCTGACCTAAAGCCACCTC

Free DNA extraction from plasma

Plasma samples were collected in EDTA tubes and stored at -80°C after centrifugation. Free DNA was extracted from plasma by DNA Blood mini kit (Qiagen) following the manufacturer's instructions. Absolute real-time PCR was used to measure the levels of plasma DNA.

Primers for direct sequencing

Primers sequences were synthetized according to the published reference (Do et al., 2008) and listed in Table 2 and 3.

DNA direct sequencing

For DNA sequencing, PCR was performed. The reaction mixture contained 1× Hotstar PCR mixture (Takara), 2.5 mM MgCl2, 100 nM of each primer and 100 ng DNA template. The PCR reaction was performed in a PCR Amplifier (Biometra).The products were purified by AxyprepTM PCR cleanup kit (Axygen) followed by a sequencing reaction with Big Dye Terminator v3.1 (Applied Biosystems). Then the samples were denatured by highly deionized-formamide (Applied Biosystems) before analysed in 3500 DNA sequencer (Applied Biosystems). The data was shown using sequencing analysis V5.4 software.

HRM analysis

Samples were assayed by Roche LightCycler 480 system using LC480 HRM master mix (Roche). The first step of the HRM protocol is the amplification of the target genes in the presence of a specialized double-stranded DNA binding dye. This specialized dye is highly fluorescent when bound to dsDNA and poorly

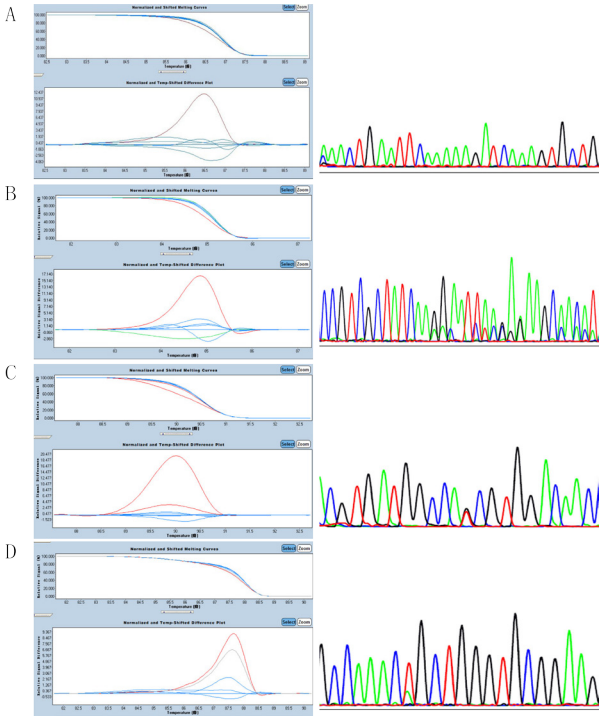

Figure 1. . Difference Plots and Sequence Traces for EGFR Exons 18 and 19. Two types of HRM figures were exhibited. One is normalized and temp-shifted melting curves, the other is normalized and temp-shifted difference plot in which samples are grouped in dependence of curve shape relating to their sequence. Panel A-D: The difference plots of EGFR exon 18 to 21 showed melting profiles for one sample (wild-type in blue, mutation in red). A, Exon 18 mutations, genotype is E-18 2126A>C. B, Exon 19 mutations, genotype is 2235-2244 deletion. C, Exon 20 mutations, genotype is 2303G>T. D, Exon 21 mutations, genotype is 2573T>G

fluorescent in the unbound state. Each reaction system contained DNA template, LightCycler HRM Master Reaction Mix (Roche), 3 mM $MgCl_2$, and 200 nM primers. After completion of the PCR step, the amplified target is gradually denatured by increasing the temperature in small increments, in order to produce a characteristic melting profile, this is termed melting analysis. The amplified target denatured gradually, releasing the dye, which results in a drop in fluorescence. All samples were plotted according to their melting profiles. The data of the normalised graph and the difference graph were evaluated by Gene Scanning software. The normalised graph shows the degree of reduction in fluorescence over a temperature range (typically 70°C to 95°C). Normalised and temperature-adjusted melting curves of samples and wild type whose profiles were converted to a horizontal line were compared. DNA variants forming mismatched heteroduplices whose fluorescence would decline more sharply than that of wild type. The results were clearly displayed in the difference graph.

Statistical analyses

SPSS statistical software (version 17) was used for statistical analysis. The relationship between EGFR mutations and clinical features was analyzed using Chi-square test (χ^2 test). A two-tailed *p*-value of <0.05 was statistically significant.

Table 4. EGFR Mutations Detected by Sequencing and HRM

Exon	Sequencing positive	HRM for FFPE positive	HRM for plasma DNA positive
Total	37.5% (45/120)	40.0% (48/120)	25.8% (31/120)
18	2.50%(3/120)	3.33%(4/120)	1.67%(2/120)
19	18.3%(22/120)	20.0%(24/120)	12.5%(15/120)
20	1.67%(2/120)	1.67%(2/120)	0.83%(1/120)
21	20.0%(24/120)	20.0%(24/120)	10.8%(13/120)

Table 5. The Sensitivity and Specificity of HRM Assays

Exon	Sensitivity (%)		Specificity(%)	
	HRM for FFPE	HRM for plasma DNA	HRM for FFPE	HRM for plasma DNA
Total	100.0(45/45)	66.4 (29/45)	96.0 (72/75)	97.3(73/75)
18	100.0(3/3)	66.7 (2/3)	99.1(116/117)	100.0(117/117)
19	100.0(22/22)	59.1 (13/22)	98.0(96/98)	98.0(96/98)
20	100.0(2/2)	50.0 (1/2)	100.0(118/118)	100.0(118/118)
21	100.0(24/24)	54.2(13/24)	100.0(96/96)	100.0(96/96)

Results

Relationship between EGFR mutations and clinical features

EGFR mutations were detected more frequently in females than males (25/51 versus 20/69, χ^2 =5.02, *P* <0.05), and in adenocarcinoma compared to squamous carcinoma (32/70 versus 13/50, χ^2 =4.84, *P* <0.05). EGFR mutation status did not show any significant association with age, histopathological grading and TNM staging (*P* >0.05).

The requirement of HRM analysis for DNA template amount

Usually, direct sequecing needs large amount and good quality of tissue DNA. HRM assay is outstanding for requirement of a small amount of DNA template. Mutations in sample with only 1 ng of template could be distinguished from wild-type .

Direct sequencing and HRM analysis for EGFR mutations

The total EGFR mutation rate was 37.5% (45/120) dectected by direct sequencing. There were 3 samples in exon 18, 22 in exon 19, 2 in exon 20 and 24 in exon 21 (Table 4) . We found that four samples had mutations in more than one exon. There was three samples with double mutations in exons 18 and 19, in exons 18 and 21, and in exons 19 and 21. One sample harboured quadruple mutations.

Compared to the results of direct sequencing, the positive rate was higher with HRM analysis. There were 48 mutations in 120 FFPE tissues, with 4 in exon 18, 24 in exon 19, 2 in exon 20 and 24 in exon 21. For plasma free DNA, the EGFR mutation rate was 25.8% (31/120). There were 2, 15, 1 and 13 mutations that were scored as HRM positive in exons 18 to 21 respectively (Table 4). Figure 1 showed the HRM assays and sequence traces for EGFR mutations.

The sensitivity and specificity of HRM assays

In our research, we difined sensitivity as the capacity of HRM analysis to detect positive samples. Our results

demonstrated that HRM assays for EGFR mutations in FFPE samples are more sensitive than direct sequencing. All positvie samples in FFPE tissues proved by direct sequencing were verified by HRM analysis. In other words, the sensitivity of HRM assays in FFPE samples was 100%. However, there were differences between the results of 20 patients by direct sequecing and HRM assays in plasma DNA samples. Four mutation-positvie samples in two or more exons found by direct sequencing were verified in less exons by HRM. One mutation-positive in exon 18 samples were identified as negative by HRM. Nine patients who had mutations in exon 19 were diagnosed as mutation-negative while two negative samples became positive by HRM. Similarly, there were 1 and 11 false-negative samples in exon 20 and 21, respectively. Overall the total sensitivity of HRM assays in plasma DNA samples was 77.8% (29/45). As for specificity, it is 96.0% (72/75) and 97.3% (73/75) in FFPE samples and plasma samples, respectively.

Discussion

EGFR mutations are considered to be one of the best predictive markers for the efficacy of EGFR-TKI treatment. Those patients who has mutations in EGFR exon 19 and 21 may benefit from EGFR-TKI, while mutations in exon 20 confer drug resistance to EGFR-TKI (Maheswaran et al., 2008). The heightened accuracy and convenience of dectecting EGFR mutation would help to reduce waste of medical resources, enhance drug efficacy and decrease toxicity.

In accord with former studies, EGFR mutations were detected more frequently in females than males and in adenocarcinoma compared to squamous carcinoma (Shigematsu et al., 2005).

Wildly applicated methods to detect EGFR mutations included direct sequencing, the peptide nucleic acid (PNA)-mediated polymerase chain reaction (PCR) clamping method, the scorpion-amplified refractory mutation system (SARMS), denaturing high-performance liquid chromatography (dHPLC), Taqman PCR, high resolution melting (HRM) and so on (Mack et al.,2009; Jian et al., 2010; Kim et al., 2013). With a view to low cost and speediness, we compared HRM with gold standard in the research. The most important application of HRM is gene scanning - the search for the presence of unknown variations in PCR amplicons prior to or as an alternative to sequencing. Mutations in PCR products are detectable by high resolution melting because they change the shape of DNA melting curves.

HRM is a suitable to test FFPE samples as well as samples with a very low quantity of DNA and has sensitivities approaching 100%. However, there were three false positive samples. HRM analysis has a relatively low false positive rate in FFPE samples. Our results were consist with the previous studies and demonstrated the application of HRM for the detection of somatic mutations in clinical samples and for screening of samples prior to direct sequencing (Do et al., 2008; Takano et al., 2008).

Plasma samples which frequently contain circulating free DNA derived from tumor tissues for EGFR mutation analysis has been actively studied recently since most patients did not have or had inadequate pathological samples for direct EGFR sequencing analysis. Over the past several years, many reports suggested that using such methodology to predict response to gefitinib and have shown promising results (Kimura et al., 2006b; Maheswaran et al., 2008; Kuang et al., 2009; Jiang et al., 2011). However, there are differences in the previous reports. Some experts revealed that EGFR mutations were detected by HRM analysis in 22 serum samples from 24 tissue EGFR mutation-positive patients. The concordance rate between serum and tissue in EGFR mutation screening was 91.67% (Hu et al., 2012). In another report, EGFR mutation positive rate was lower when assessed using pretreatment cfDNA (23.7%) versus tumor tissue-derived DNA (61.5%). Circulating free DNA results identified no false positives but a high rate of false negatives (Goto et al., 2012). The detection rate of EGFR mutations from plasma was not so high by PNA-mediated PCR clamping (Kim et al., 2013). In our research, the total sensitivity of HRM assays in plasma DNA samples was 77.8% (29/45). The results varies due to sample factor covering amout and source country and different methods.

The amout of circulating DNA was measured by quantitative real-time PCR as described previously (Sozzi et al., 2003). The content of DNA in plasma depends on: condition of the donor, sampling and handling of the blood, plasma preparation, DNA isolation method, DNA quantification method and so on. In our research, the concentration range was from 10 to 2310 ng/ml. The concentration was very low in some plasma samples and primer dimer impaired our judgement. Therefore, the positive rate of EGFR mutations in plasma free DNA was not so high as that detected by direct sequencing. However, depite the relative low sensitivity, the specificity of HRM analysis used in free DNA was higher than it in FFPE tissues.

The results suggested that more exploration in enrichment and detection methods of free DNA are required. Further research into appropriate methods and analysis needs to be performed before it could be accepted as an option in the diagnostic or screening setting. Moreover, in the further studies, we should monitore EGFR mutation status detected by HRM assay and the clinical response to EGFR-TKIs to assess the clinical outcome of the test.

In conclusion, our results highlight that HRM analysis is qualified with small tumor content need. HRM applicated in plasma free DNA has shown a high false-negative rate but a low false-postive rate. More data about EGFR mutations in free DNA are required to evaluate sensitivity, stability and clinical applicability.

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