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Real-time PCR assay with high resolution melting for EGFR and BIM mutation of lung cancer

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Abstract. – OBJECTIVE: To establish and develop a reliable and simple Real-time PCR assay with high resolution melting (Real-time PCR-HRM) method for detection epidermal growth factor (EGFR) and BIM mutation of lung cancer and looking for effective targeted drugs to control lung cancer.

PATIENTS AND METHODS: A total of 6858 participants (2538 cases with lung cancer and 4275 healthy controls who took part in the study by doing the physical examination in Shanghai Xuhui community) were recruited in the study. Clinical characteristics and 5 ml peripheral blood were collected from each participant, and the DNA has been extracted, which were determined the EGFR and BIM mutation by Real-time PCR-HRM. Data were recorded and Statistical analyses.

RESULTS: All samples completed the study. BIM deletion polymorphism was no related with age, sex, and smoking or EGFR mutation.

CONCLUSIONS: There were no relations among BIM deletion polymorphism, EGFR mutation or lung cancer risk. HRM is a novel procedure and provides rapid, sensitive, specific and simultaneous detection for gene mutation of cancer patients for predicting the efficacy of targeted therapy.

Key Words
Real-time PCR assay with high resolution melting (Real-time PCR-HRM), BIM deletion polymorphism, Epidermal growth factor mutation (EGFR), Lung cancer.

Introduction

Lung cancer is the main cause of malignant tumors death in China. According to the last statistics, the morbidity of lung cancer has grown to 26.9%. Improvement the progression-free survival (PFS) and finding effective target therapy are all urgent tasks in the worldwide[1,2]. BIM (also known as BCL2L11) is a pro-apoptotic member of the Bcl-2 protein family and important for apoptosis of targeted therapy[3-5]. BIM deletion polymorphism between exons 3 and exons 4, named 2903 bp, is related to the epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) resistance of lung cancer[6]. Previous reports assumed that BIM is a key modulator of apoptosis triggered by EGFR-TKIs. EGFR (also known as ERBB1 and HER1) is a transmembrane tyrosine kinase receptor (RTK)[7]. It has been found in a variety of human malignancies[8]. EGFR-TKIs pointed out benefits in lung cancer as many studies have mentioned[9]. While there are several patients who have a high incidence of EGFR mutations which reside in exons 19 and 21 and directly influence the efficacy of EGFR-TKIs[10]. There are also more patients thet became drug resistant[11]. While some reports presumed a discrepancy between EGFR mutation and drug resistance in lung cancer[12]. As the down-regulation of EGFR, BIM is the key for the targeted therapy of EGFR-TKIs and influence the efficacy[13].

In this study, we randomly selected a wide range of participants and used Realtime PCR assay with high resolution melting (Real-time PCR-HRM) to detect BIM and EGFR mutation, looking for a better, quicker, and more accurate way for patients to detect the mutation, and expected for solving the drug resistance of lung cancer and extend the life of patients.

Patients and Methods

Participants and Sample Collection

This study took place in Shanghai Chest Hospital and completed between August 2012 and August 2014. Ethical approval for the study was obtained from Human Research Ethics Committee of

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Table I. Clinical characteristics of participants.

Participants' characteristics	Combined, n (%)	Cancer, n (%)	Control, n (%)
Total NO. participants = 6858		2583 (37.7)	4275 (62.3)
Median age in years	65	59	69
Gender			
Male	3746 (56.1)	1598 (66.7)	2148 (50.2)
Female	2926 (43.9)	799 (33.3)	2127 (49.8)
Smoke status			
Never	3939 (66.8)	1096 (50.2)	2843 (76.6)
Previous	364 (6.2)	83 (3.8)	281 (7.6)
Current	1593 (27.0)	1006 (46.0)	587 (15.8)
Drink			
No	4611 (91.3)	1931 (91.4)	2680 (91.2)
Yes	442 (8.7)	182 (8.6)	260 (8.8)
Tumor			
Adenocarcinoma	1800 (26.2)	1800 (69.7)	-
Squamous cell carcinoma	527 (7.7)	527 (20.4)	-
Others	256 (3.7)	256 (9.9)	-
Cancer-free	4275 (62.3)	-	4275 (100)
Stage			
Ia	32 (2.4)	32 (2.4)	-
Ib	80 (5.9)	80 (5.9)	-
IIa	56 (4.1)	56 (4.1)	-
IIb	45 (3.3)	45 (3.3)	-
IIIa	208 (15.3)	208 (15.3)	-
IIIb	175 (12.9)	175 (12.9)	-
IV	765 (56.2)	765 (56.2)	-

Shanghai Chest Hospital. All the participants were informed about the study and gave a written consent. 2583 patients with lung cancer and 4275 participants took part in the study by doing the physical examination in Shanghai Xuhui community. Patients of the experimental group suffered from lung cancer diagnosed in Shanghai Chest Hospital.

Smoking status was divided into three categories: never, previous and current. Former smokers were defined as stop smoking at least 1 year before the study. The general condition of the two groups was listed in Table I.

Sample Collection and DNA Extraction

DNA was extracted by the salting out method. In the protocol, the blood was drawn from the peripheral blood. Each 2 ml blood samples were added with 2 ml red blood cell lysis buffer (10 mM Tris-HCl, 20 mM NaCl, 1 mM EDTA) 10min on ice for incubation, centrifuged at 8000 rpm 5 min at 4°C, discarded the supernatant liquid and centrifuged at 8000 rpm 5 min at 4°C again, added 410 μl lysis buffer STE (10 mM Tris-HCl, 20 mM NaCl, 1 mM EDTA, pH 8.10), 90 μl 10% SDS, 5 ul Proteinase K (20 ug/μl), gently inverting the tube to mix thoroughly. The lysate was ready for DNA isolation. The cell lysates were treated with proteinase K (150 μg/ml) at 65°C for 15h to digest. Next, each sample added 300 μl saturated NaCl and slightly shaking for 3 min, added 340 μl chloroform slightly shaking for a few seconds, followed by centrifugation at 1200 rpm 20 min at 20°C, remained the supernatant liquid to a new centrifuge tube and added 650 μl isopropyl alco-

Table II. BIM and EGFR sequencing primer sequences.

Primer name	Sequence
BIM upstream	5'-ATACCATCCAGCTCTGTCTTCATAG-3'
BIM downstream 1	5'-CCCAACCTCTGACAAGTGACC-3'
BIM downstream 2	5'-TTGGTGGGAATGTAAAATGGC -3'
EGFR (exon19) upstream	5'-GTGCATCGCTGGTAACATCCA-3'
EGFR (exon19) downstream 1	5'-GGAGATGAGCAGGGTCTAGAGCA-3'
EGFR (exon19) downstream 2	5'-AAAGGTGGGCCTGAGGTTCA-3'
EGFR (exon21) upstream	5'-TGGCATGAACATGACCCTGAA-3'
EGFR (exon21) downstream 1	5'-CAGCCTGGTCCCTGGTGTC-3'
EGFR (exon21) downstream 2	5'-TGGCTGACCTAAAGCCACCTC-3'

hol at 12000 rpm 16 min at 4°C. Next, leaved the isopropyl alcohol and added 500 μl 75% ethanol at 12000 rpm 5 min at 4°C. Repeating twice and then moderately dried. The dried DNA pellet was dissolved into 70 μl Mili Q water and stored at 4°C. Taking 1 μl DNA to quantify by NanoDrop D8000 to make sure the DNA concentration among 5 to 10 ng/μl, the value of D260/D280 between 1.8 and 2.0 would be regarded as qualified production.

DNA Sequencing and Real-Time PCR

BIM gene sequence was obtained from GeneBank (Gene ID: 10018) and EGFR gene sequence was obtained from GeneBank (Gene ID: 1956) by LightCycler 480 High Resolution Melting Master. All the primer sequences were listed in Table II. A 10 μl PCR reaction was set up, containing 5-10 ng DNA, 5 μl 1 × Master Mix, $MgCl^2$ (3.0 mmol/L), 1 μl each primer (0.2 μmol/ L). The amplification and HRM analyses was performed by LightCycler 480 II Real-time PCR (Roche, Basel, Switzerland). The real-time PCR process used touchdown mode as follow: predenaturation at 95°C for 10 min, then denaturation at 95°C for 15s, annealing from 65 to 55°C with a degression of 0.5°C per cycle for 15s, and extension 20 cycles at 72°C for 20s, and then denaturation at 95°C for 15s, annealing at 65°C for 15s, lastly extension 25 cycles at 72°C for 20s. The melting of amplification products immediately follow the next procedure: heating to 95°C for 15s, then cooled to 40°C for 1 min, lastly heating to 65°C for 1s again. Fluorescence collection was performed 20 times/°C from 65°C heating to 95°C, and cooled to 40°C lastly. HRM was analyzed by the LightCycler Software version 1.5 (Roche, Basel, Switzerland).

Statistical Analysis

Data were presented as mean. *t*-test was used to determine the significant difference between the experiment group and control group. As more than two groups, data were analyzed by one-way analysis of variance (ANOVA) and used post hoc test when p-value < 0.05. Pearson test was used to establish whether there was a linear relationship. A p-value of < 0.05 was considered as significant difference.

Results

There were 6858 participants joined in the study. The age gender, smoke status and drink of participants' characteristics were recorded and evaluated by ANOVA as Table I had listed. The characteristics of participants were compared, there were no significant differences between the controls and cancers.

In this study, we used touchdown PCR on primers amplification, and all samples of DNA produced were analyzed by HRM. It showed that there were four outcomes of BIM: non-detection of 290 3bp BIM (NA), non-deletion of 2903 bp BIM (homozygous non-deletion type DNA, II), 2903 bp deletion BIM (homozygous deletion type DNA, DD) and heterozygote (ID). The BIM deletion polymorphism in the two groups had listed in Table III. Compared the BIM deletion polymorphism between the controls and cancers, it showed that there were no statistical differences between the controls and cancers. The frequency of BIM deletion polymorphism shown a lack of statistically significant association with lung cancer.

To determine the association between BIM deletion polymorphism and each characteristic of participants, we analyzed the BIM deletion polymorphism with characteristics of controls and cancers. There were no differences in frequency of BIM deletion polymorphism were shown with any characteristic of participants either in the control or the cancers (Table IV).

For further validation the relationship between BIM deletion polymorphism and lung cancer, we analyzed the data of BIM deletion polymorphism with different background of cancer subtype and stage. While there were no significant differences in any type of lung cancer patients (Table V).

For authenticated the dependability between BIM deletion polytheism and EGFR somatic mutation, we analyzed the data of BIM deletion polymorphism with different background of EGFR somatic mutations. It showed that there was no association between BIM deletion polymorphism and EGFR somatic mutations (Table VI).

Table III. Association between BIM deletion polymorphism.

2903-bp deletion	Combined, n (%)	Cancer, n (%)	Control, n (%)
II	5796 (84.5)	2168 (83.9)	3628 (84.9)
ID	949 (13.8)	343 (13.3)	606 (14.2)
DD	34 (0.5)	18 (0.7)	16 (0.4)
NA	79 (1.2)	54 (2.1)	25 (0.6)

Table IV. Association between 2903-bp deletion and participants' characteristics.

	Combined				Control				Cancer			
	II, n (%)	ID, n (%)	DD, n (%)	ID+ DD, n (%)	II, n (%)	ID, n (%)	DD, n (%)	ID+ DD, n (%)	II, n (%)	ID, n (%)	DD, n (%)	ID+ DD, n (%)
Gender												
Male	3142 (85.1)	534 (14.5)	18 (0.5)	552 (14.9)	1818 (85.1)	311 (14.6)	7 (0.3)	318 (14.9)	1324 (85.0)	223 (14.3)	11 (0.7)	234 (15.0)
Female	2491 (85.9)	394 (13.6)	14 (0.5)	408 (14.1)	1810 (85.6)	295 (14.0)	9 (0.4)	304 (14.4)	681 (86.8)	99 (12.6)	5 (0.6)	104 (13.2)
Smoke status												
Never	3353 (86.2)	517 (13.3)	22 (0.6)	539 (13.8)	2427 (85.9)	385 (13.6)	12 (0.4)	397 (14.1)	926 (86.7)	132 (12.4)	10 (0.9)	142 (13.3)
Previous	301 (84.1)	56 (15.6)	1 (0.3)	57 (15.9)	231 (83.4)	45 (16.2)	1 (0.4)	46 (16.6)	70 (86.4)	11 (13.6)	0 (0)	11 (13.6)
Current	1323 (84.4)	238 (15.2)	6 (0.4)	244 (15.6)	490 (83.8)	94 (16.1)	1 (0.2)	95 (16.2)	833 (84.8)	144 (14.7)	5 (0.5)	149 (15.2)
Positive	1624 (84.4)	294 (15.3)	7 (0.4)	301 (15.6)	721 (83.6)	139 (16.1)	2 (0.2)	141 (16.4)	903 (84.9)	155 (14.6)	5 (0.5)	160 (15.1)

Discussion

The study random collected a large number of samples to determine the BIM deletion polymorphism and EGFR somatic mutations by Real-time PCR-HRM. It showed that BIM deletion polymorphism was a lack of association with age, gender, drink, smoke or lung cancer subtype[14]. There were no associations between BIM deletion polymorphism and EGFR somatic mutations either[15].

Reports have revealed that cancer patients with BIM deletion mutation have an average of about six and a half month of PFS[16]. While those patients without BIM deletion mutation have nearly 12 months of PFS. The upregulation of BIM is required for apoptosis induced by EGFR which is a promising target in the therapy of lung cancer[17]. The lacking of BIM isoforms leads to low expression of BIM and sufficient to EGFR-TKIs resistance in lung cancer. EGFR-TKIs downregulate MAPK signaling which leads to upregulation of BIM expression and induces cancer cell apoptosis as several studies have shown[18]. The 2903bp of BIM deletion would lead to loss of BIM subtypes BH3 structure expression and restrain EGFR-TKIs efficacy, which is commonly found in the East Asian population[19]. While in this study we did not found a relation between BIM deletion polymorphism and EGFR somatic mutations[20]. The outcomes were contrary to someone of the

Table V. Association between BIM deletion polymorphism and lung cancer subtype.

	II, n (%)	ID, n (%)	DD, n (%)	ID+DD, n (%)
Adenocarcinoma	1513 (85.8)	236 (13.4)	14 (0.8)	250 (14.2)
Squamous cell	432 (84.2)	80 (15.6)	1 (0.2)	81 (15.8)
Others	223 (88.1)	27 (10.7)	3 (0.1)	30 (10.8)

Table VI. Association between BIM deletion polymorphism and EGFR somatic mutations

	Control	EGFR(-)	EGFR(+)	EGFR-Exon19(-)	EGFR-Exon19(+)	EGFR-Exon21(-)	EGFR-Exon21(+)
II	3628 (85.4)	103 (82.4)	92 (83.6)	151 (82.1)	44 (86.3)	147 (83.1)	48 (82.8)
ID	606 (14.3)	22 (17.6)	17 (15.5)	33 (17.9)	6 (11.8)	29 (16.4)	10 (17.2)
DD	16 (0.4)	0 (0)	1(0.9)	0 (0)	1 (2.0)	1 (0.6)	0 (0)
ID+DD	622 (14.6)	22 (17.6)	18 (16.4)	33 (17.9)	7 (13.7)	30 (16.9)	10 (17.2)

literatures[10,21,22]. It seems that BIM was regulated by other oncogenes such as MYC and cyclin D1 and probably conducted through the MEK/ERK pathway[23,24]. Loss expression of BIM interfered the therapy of EGFR-TKIs progressing[23,25-27] and affected the PFS of lung cancer patients[23,28]. BIM could be one target gene to wrench the resistance of EGFR-TKIs.

In this study, we did not found the frequency of BIM deletion polymorphism is linked with lung cancer risk, either with lung cancer subtypes or stage. It indicates that the mutation of BIM is not necessary for predicting the incidence of lung cancer in Chinese[29]. While BIM deletion polymorphism is essential to targeted medication therapy and necessary for the lung cancer patients to detect, especially for the patients who are resistance to EGFR-TKIs therapy[30,31] and expect to improve the quality life of patients.

In the study, we used Real-time PCR-HRM a novel procedure to determine gene mutation. It showed that Real-time PCR HRM for the BIM deletion polymorphism and EGFR mutation was a sensitive, accurate, rapid and high-throughput method. In the previous, the primer of a traditional detection method for 2903 bp deletion of BIM is designed on the both side of the 2903 bp deletion region; after amplification, the length of production were detected by electrophoresis[14,32]. The longer one would be the normal production, while the shorter one to be the deletion production (Figure 1). For the deletion mutation of BIM gene, we designed a novel method to detect the deletion polymorphism: two primers of upstream and one primer of downstream were designed and amplification. It could amplify out two different kinds of production as there were two pairs of primers in the same reaction[33]. For better outcomes we used touchdown PCR mode to produce the amplification. After amplified the productions were detected by HRM (Figure 2). HRM could detect the change of sequence by detecting the fluorescence intensity combined with the double-stranded DNA. As the DNA melting with temperature increases, the fluorescence intensity is free into the system and weakening detected by HRM. Compared with normal PCR, HRM could implement PCR reaction in one procedure with no extension or electrophoresis identification. HRM method could detect specific sites of mutation and screen the amplified sequence changes for detecting any sequence changes in the primer. HRM is suitable to test a very low quantity of DNA. Cancer patients could test the gene mutation by a few peripheral blood. In our study, nearly almost samples were successfully amplified and analyzed. The sensitivity and availability could provide a genetic testing option for cancer patients.

Lastly, in this study, we did not analyze the association between BIM deletion polymorphism and the stages of lung cancer as there were a few data to analyze. Most lung cancer patients were diagnosed as the advanced stage when it appeared clinical symptoms[34]. Finding an available method to diagnosis lung cancer is always a puzzle for the world. Thus, right now effective therapy has become the most need of lung cancer. It has been showed in this study that the there was any correlation between BIM deletion polytheism and EGFR. We proposed that there were another oncogene or a signal way to priming the BIM and induced resistance of EGFR-TKIs[3,16,34]. The molecular biology of lung cancer has been showed to be essential in the progression of disease[1,35]. In the future, we plan to study the molecular biology of BIM under different stages of lung cancer, looking for an accurate target therapy for lung cancer and extend the PFS of lung cancer. Although the predict of lung cancer is no possible at present, we need further studies for molecular biology of lung cancer and find out the predicate to estimate the incidence of cancer in the community.

Figure 1. The traditional detection of PCR amplification for deletion polymorphism.

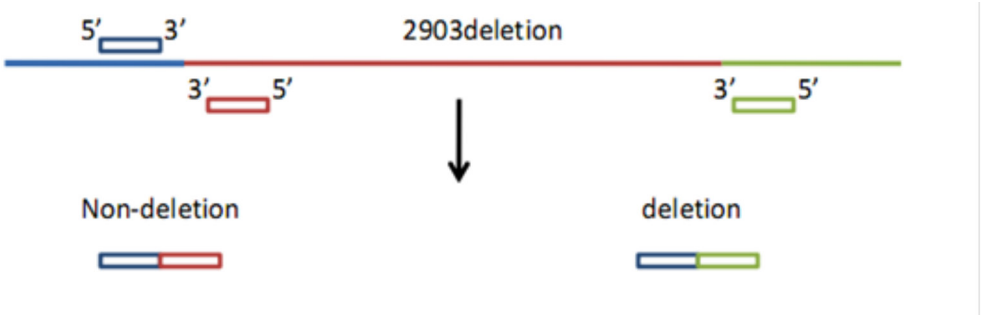

Figure 2. The novel detection of PCR amplification for deletion polymorphism.

Conclusions

Our results suggested that there was no relation between BIM deletion polymorphism and EGFR somatic mutations, and no significant link with age, gender, smoke, or drink in lung cancer. Real-time PCR HRM could be the novel method determined deletion polymorphism of gene for diagnosis, research and studies.

Conflict of Interests:

1. Shanghai Municipal Commission of Health and Family Planning (No. 20144Y0236); 2. Chinese National Science Foundation (Grant No. 81302004); 3. Natural Science Foundation of Shanghai, China (Grant No. 12ZR1428800); 4. Key Basic Research Program of Shanghai Committee of Science and Technology, China (Grant No. 11JC1412200); 5. China National Key Basic Research Program (Grant No. 81172104); 6. Science and Technology Leading Talent Foundation of Shanghai.

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