ORIGINAL ARTICLE

Prevalence and clinical significance of BRAF V600E in Chinese patients with lung adenocarcinoma

Zhenxiang Li, Leilei Jiang, Hua Bai, Zhijie Wang, Jun Zhao, Jianchun Duan, Xiaodan Yang, Tongtong An & Jie Wang

The Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Thoracic Medical Oncology, Beijing Cancer Hospital & Institute, Peking University School of Oncology, Beijing, China

Correspondence
Tongtong An, Department of Thoracic Medical Oncology, Beijing Cancer Hospital & Institute, Peking University School of Oncology, Beijing 100036, China.
Tel: +86 10 88196456
Fax: +86 10 88196562
Email: antt508@sina.com

Jie Wang, Department of Thoracic Medical Oncology, Beijing Cancer Hospital & Institute, Peking University School of Oncology, Beijing 100036, China.
Tel: +86 10 88196456
Fax: +86 10 88196562
Email: zlhuxi@163.com

Abstract

Background: The purpose of this study was to investigate the prevalence, distribution, and prognostic role of v-raf murine sarcoma viral oncogene homolog B (BRAF) V600E mutations in Chinese patients with lung adenocarcinoma (ADC), and to explore the possibility of BRAF V600E mutation detection in plasma DNA.

Methods: Data from 190 patients with lung ADCs treated at the Peking University Cancer Hospital from July 2011 to March 2012 were collected. The amplification refractory mutation system was used for BRAF V600E testing and denaturing high-performance liquid chromatography for epidermal growth factor receptor (EGFR) mutation detection. In BRAF V600E-mutant cases, paired plasma DNA was tested for mutation status of BRAF V600E and EGFR. The distribution and prognostic role of BRAF V600E mutations were analyzed using SPSS 13.0.

Results: Among 190 patients with advanced lung ADC, eight (4.2%) cases carried BRAF V600E mutations. V600E mutations presented more frequently in women than in men (6 of 96, 6.3% vs. $P = 0.1$). BRAF and EGFR mutations were concomitantly presented in three patients. Five of the eight patients with BRAF V600E mutations had matched plasma DNA samples and V600E mutations were found in three plasma samples.

Conclusion: The prevalence of BRAF V600E mutations in Chinese patients with lung ADC is 4.2%. Circulating plasma DNA may be used for BRAF V600E mutation analysis in lung adenocarcinoma.

Introduction

Lung adenocarcinomas represent the major histological subtype, accounting for about 40% of lung cancer cases.[1] Target therapy based on molecular classification has achieved great success in lung adenocarcinoma.[2] It is very important to discover the appropriate driver gene as a therapeutic target, which may predict the outcome of target therapy. For instance, mutant epidermal growth factor receptor (EGFR), the most common driver gene in lung adenocarcinoma, is the most efficient predictor for EGFR tyrosine kinase inhibitor (TKI) treatment.[3,4] Driver genes with relative low frequency, such as KRAS mutation, EML4/ALK fusion, and v-raf murine sarcoma viral oncogene homolog B (BRAF) mutation, also play important roles in predicting prognosis and target therapy.[5,6] Non–small cell lung cancer (NSCLC) with anaplas-

tic lymphoma kinase (ALK) rearrangements are a molecular subgroup that could benefit from Crizotinib treatment.[5]

The BRAF code for a non-receptor serine/threonine kinase is an important member of the RAF/RAF/MEK/mitogen activated protein kinase (MAPK) signal pathway. BRAF mutations result in sustained kinase activity, causing signal pathway alteration, and are associated with the development of malignant tumors. A vast majority of these mutations correspond to the hotspot transversion mutation T1799A at exon 15, which causes the amino acidic substitution of V600E.[7] BRAF V600E has been documented in various malignant tumors, predominantly in malignant melanoma, thyroid papillary cancer, and colorectal tumors.[8–10] Previous studies have shown that the prevalence of BRAF V600E was 2–4% in lung cancer.[6,11,12] However, research regarding the frequency and prognostic role of BRAF V600E in Asian

patients with lung cancer is rare.[12,13] Several drugs targeting the BRAF kinase, such as Dabrafenib and Vemurafenib, have been developed and have shown potential clinical application in malignant melanoma.[14]

Although molecular alterations, such as mutation, rearrangement, and amplification, are usually detected in tumor tissues, it is clinically difficult to obtain such tissues, particularly for patients with recurrent and refractory NSCLC. Even in prospectively conducted clinical trials, less than 40–50% of the patients had available tumor tissues with the mutation.[15] It is important to establish convenient and noninvasive sampling methods as alternatives for these molecular detections. Our previous studies have shown that DNA abnormalities, such as EGFR and KRAS mutations, can be reliably detected in plasma samples of patients with stages IIIB to IV NSCLC and can be used as biomarkers to predict tumor response to EGFR-TKIs and progression-free survival (PFS), suggesting that plasma free DNA may be an alternative sample source for such genetic evaluation.[15,16]

In this retrospective study, we investigated the prevalence of BRAF V600E and its association with clinicopathological factors and prognosis. We determined that plasma DNA was a surrogate tissue for *BRAF* V600E detection.

Patients and methods

Patients population

Data from 190 patients with lung adenocarcinomas were collected during July 2011 to March 2012 for this study. All patients had pathologically confirmed lung adenocarcinoma and provided enough tumor tissue for *EGFR* and *BRAF* mutation detection. Patients with BRAF V600E mutation were also subject to mutation detection in plasma DNA. Clinicopathological factors including gender, age, smoking history, and staging were collected from hospital records. Staging was determined by the 2009 International Association for the Study of Lung Cancer Tumor Node Metastasis Staging System. PFS was assessed from the first day of treatment until radiologic progression or death. Overall survival (OS) was determined from the date of diagnosis of lung adenocarcinoma until the date of death as a result of the disease or final follow up. The study was reviewed and approved by the Institutional Ethic Committee. All patients signed an informed consent for participation in the study and the use of their biological tissues.

DNA extraction

For DNA extraction from tumor tissues, a total of six to eight pieces of 5-μm-thick slices were cut from paraffin-embedded tissues. The tumors were macrodissected and tumor contents were recorded for each sample using immediately adjacent sections. All of the samples had >80% tumor contents. After xylene dewaxing, we added lysate and protease K, then placed samples overnight in a 60°C water bath. DNA was extracted by phenol/chloroform/isopentanol the next day. Subsequent processes have been reported previously.[14]

Epidermal growth factor receptor (EGFR) mutation analysis

EGFR mutation detection by denaturing high-performance liquid chromatography (DHPLC) was performed to detect EGFR mutation by the Transgenomic Wave Nucleic Acid 119 Fragment Analysis System with a DNASep column (Transgenomic, Omaha, Nebraska, USA) according to our method reported previously.[14]

BRAF V600E detection by amplification refractory mutation system (ARMS)

BRAF V600E mutation was detected using a human *BRAF* gene V600E mutation fluorescence polymerase chain reaction diagnostic kit (AmoyDx, Xiamen, China). The procedure was performed under the manufacturer's instructions. Briefly, polymerase chain reaction (PCR) was performed in a 35 μL final volume reaction mixture containing 10–15 ng DNA. PCR amplification was carried out by denaturation at 95°C for five minutes, followed by 15 cycles of 95°C for 25 seconds, 64°C for 20 seconds, and 72°C for 20 seconds, and 31 cycles of 93°C for 25 seconds, 60°C for 35 seconds, and 72°C for 20 seconds. *BRAF* V600E mutation was determined by the CT value of HEX and FAM signaling collected. *BRAF* V600E mutation was considered positive when the CT value of FAM was over 28.

Statistical analyses

SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. The relationship between *BRAF* V600E mutations and relevant factors such as gender, age, stage, and smoking was examined by the chi-square test, with $P < 0.05$ as a bilateral significant difference. Survival analysis was calculated by the Kaplan-Meier method and checked using the log-rank test.

Results

Patients characteristics and BRAF V600E mutation analysis

Of the 190 patients, there were 96 women (50.5%) and 94 men (49.5%), with a median age of 62 years (range: 31–80 years). The majority of the patients involved were diagnosed stage IIIB and stage IV (87.9%, 167/190). The prevalence of
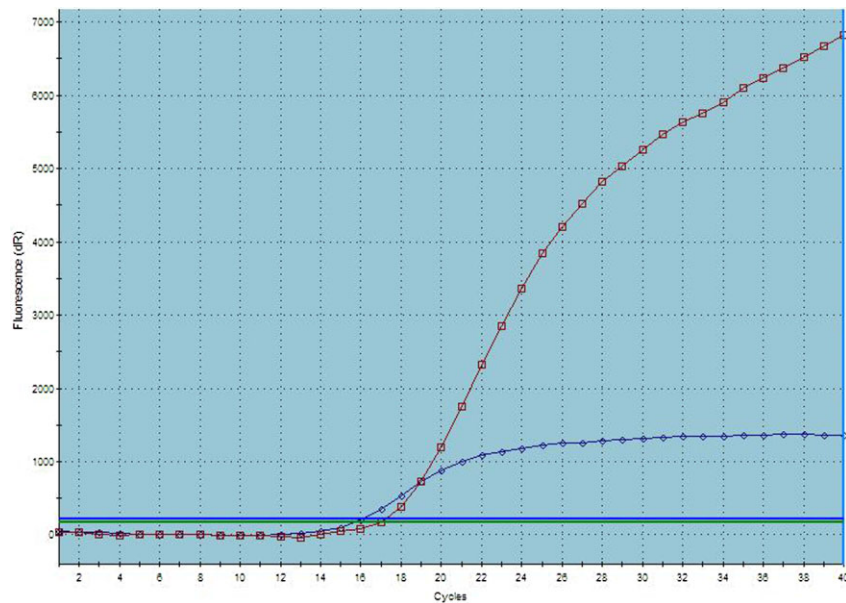
Figure 1 v-raf murine sarcoma viral oncogene homolog B (BRAF) V600E positive by amplification refractory mutation system (ARMS). The blue curve represents the HEX signal, the red curve represents the FAM signal.

BRAF V600E in the 190 lung adenocarcinomas was 4.2% (8/190) by means of amplification refractory mutation system (ARMS)-PCR (Fig 1). There were six women among the eight patients with BRAF V600E mutations (75%, 6/8). Six patients with BRAF V600E had a higher stage (stage IIIb and IV) (Table 1).

Table 1 The relationship between clinical characteristics of the patients and BRAF mutation

Characteristics	*BRAF* V600E mutation/*n* (%)			
	Total/ *n* = 190	Positive/ *n* = 8	Negative/ *n* = 182	*P* value
Age				
Mean	64.6	69.1	60.0	
Range	31–80	57–80	31–80	
Gender				0.1
Male	94 (49.5)	2	92	
Female	96 (50.5)	6	90	
Smoking status				0.8
Smoking	79 (41.6)	3	76	
Non-smoking	111 (58.4)	5	106	
TNM stage				0.6
IIa-IIIa	23 (12.1)	2	21	
IIIb-IV	167 (87.9)	6	161	
EGFR gene				0.7
Mutation	82 (43.2)	3	79	
Wild type	108 (56.8)	5	103	

BRAF, v-raf murine sarcoma viral oncogene homolog B; EGFR, epidermal growth factor receptor; TNM, tumor node metastasis.

Patients with concurrent EGFR and *BRAF* V600E mutations

DHPLC detected that 43.2% (82/190) of lung adenocarcinomas had EGFR exon 19 or 21 mutations. Among eight patients with BRAF V600E mutation, three patients also carried concurrent EGFR sensitive mutations (1 for exon 21 mutation, and 2 for exon 19 mutation). All three patients were female non-smokers and accepted Gefitinib therapy. The PFS after gefitinib treatment was 2.63, 10.23, and 17.9 months respectively (Table 2).

BRAF V600E mutation in plasma DNA and matched tumor tissue DNA

Five of the eight BRAF V600E mutant cases provided both tumor DNA and matched plasma DNA for the research. Three patients were also detected with BRAF V600E mutations in matched plasma DNA. Only one case had concurrent BRAF V600E and EGFR sensitive mutations in plasma DNA (Table 3).

Correlation of *BRAF* V600E with clinical outcome

We also analyzed the potential implication of BRAF V600E mutation status in predicting clinical outcomes in the patients with lung adenocarcinoma. There were 147 patients with complete follow up data, which was suitable for survival analysis. OS was calculated from the date of diagnosis to the

Table 2 Clinical features of patients with concurrent BRAF V600E and EGFR mutations

No.	Gender	Age	Smoking	Histology	TNM stage	Gene status	PFS
1	F	57	Non	Adenocarcinoma	IV	*EGFR* 19exon *BRAF* V600E	2.63
2	F	61	Non	Adenocarcinoma	IV	*EGFR* 19exon *BRAF* V600E	10.23
3	F	65	Non	Adenocarcinoma	IV	*EGFR* 21exon *BRAF* V600E	17.9

BRAF, v-raf murine sarcoma viral oncogene homolog B; EGFR, epidermal growth factor receptor; PFS, progression-free survival; TNM, tumor node metastasis.

date of death. The median OS was 24.0 months (95% confidence interval [CI]: 20.6–27.5) in the patients with BRAF V600E mutations *versus* 28.0 months (95% CI: 22.7–33.3) in the cases without the mutation. There was no statistically significant difference in OS between the two groups (Fig 2).

Discussion

The discovery of driver mutations, such as EGFR and ALK has led to remarkable improvement in personalized therapy for lung adenocarcinoma, indicating that it is key to explore the prevalence and clinical characters of driver genes for instructing target therapy in lung adenocarcinoma.[2–5] In the present study, the incidence of another important driver mutation, BRAF V600E, in Chinese lung adenocarcinoma was 4.2%, close to the prevalence reported previously from Western population cohorts. However, it was believed that this mutation was less frequent in Asian than in Caucasian patients.[6,12,13,17] Two possible reasons may explain this difference. Firstly, studies have indicated that V600E mutations were frequently associated with a more aggressive tumor histotype, characterized by micro-papillary features.[6] In our study, most of the patients (87.9%, 167/190) were initially diagnosed with stage IIIB and IV, whereas Sasaki *et al.* reported relatively lower frequency (0.8%) of BRAF V600E in Japanese NSCLC cohorts in which most of patients were diagnosed as stage I-II (68.2%, 88/129).[13] Secondly, several methods were developed for BRAF V600E detection, including dideoxy sequencing, colorimetric Mutector assay

(TrimGen, Sparks, Maryland, USA), allele-specific real-time PCR, pyrosequencing, high resolution melting (HRM) analysis, and co-amplification at lower denaturation temperature (COLD)-PCR, with varying in their sensitivity, assay complexity, and cost.[18] In our study, a highly sensitive method, ARMS-PCR, was used to detect the mutation and sensitivity, which was 1% for BRAF V600E mutation detection.

Previous studies have shown that V600E mutations were significantly more prevalent in women (about 70%) and lung adenocarcinomas, and were independent of smoking history. Our study found that BRAF V600E was also more frequent in women (75%, 6/8), which was consistent with the results reported. The relatively high frequency of BRAF mutations in women may be a result of hormones or environmental factors[19] and these may help to select the patients to undergo mutation screening for treatment with specific BRAF inhibitors in the future.

The prognostic value of BRAF V600E in NSCLCs remains controversial. Marchetti *et al.*[6] showed that patients with V600E mutations in the tumor had significantly shorter DFS and OS than those without mutations. Multivariate Cox regression analysis indicated an independent association of V600E mutations with poor DFS and OS. Another study found no significant difference in the OS of lung cancer patients with BRAF mutations *versus* those without mutations.[20] Our study showed that patients with BRAF V600E had a shorter OS compared to those without this type of mutation, which had no statistical difference. Because of relatively low number of patients with BRAF V600E in this study,

Table 3 The clinical features of patients with BRAF V600E mutation

No.	Gender	Age	Smoking	TNM stage	Blood BRAF	Blood EGFR	Tissue EGFR
1	F	80	1	II	1	1	0
2	M	80	1	IV	1	0	0
3	M	58	1	IV	1	0	0
4	F	65	0	IV	0	0	1
5	F	61	0	IV	0	0	1
6	F	79	0	IV	–	–	0
7	F	73	0	IIIa	–	–	0
8	F	57	0	IV	–	–	1

BRAF, v-raf murine sarcoma viral oncogene homolog B; EGFR, epidermal growth factor receptor; TNM, tumor node metastasis.
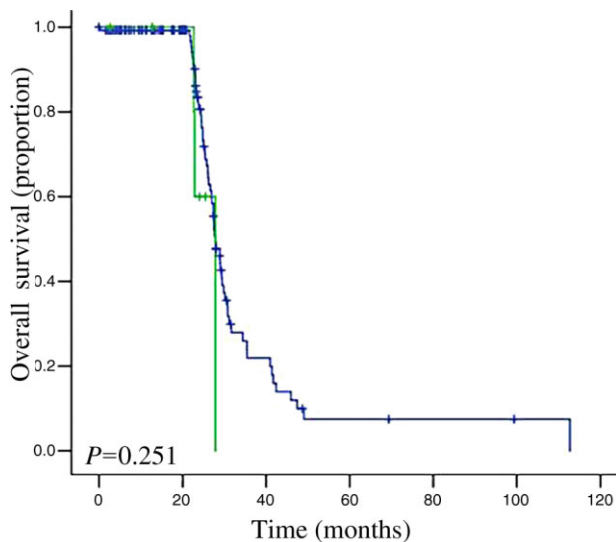
Figure 2 Survival analysis. Kaplan-Meier curves showed no statistically significant difference in overall survival of patients according to BRAF V600E mutation status. ——, BRAF V600E (+) (*n* = 8); ——, BRAF V600E (–) (*n* = 139).

the data was preliminary and requires a larger cohort and longer follow-up for confirmation.

It was thought that EGFR mutation and BRAF V600E existed mutually exclusively, representing two different subtypes of NSCLCs.[21] Our study showed that three out of eight patients concurrently harbored BRAF V600E and EGFR sensitive mutations. Another study also reported two patients with concurrent EGFR and BRAF mutations.[22] These results suggest that the two mutations may act synergistically during oncogenesis. Interestingly, in this study, the three patients shared some clinicopathological factors, including female sex, adenocarcinomas, and non-smoking, which were also common in patients with EGFR mutations. All three patients accepted gefitinib treatment: one patient exhibited primary resistance and experienced rapid disease progression; the remaining two patients had improved survival outcomes with regard to PFS (10.23 and 17.9 months, respectively) after gefitinib treatment.

It is well known that BRAF belongs to important members of the RAS/RAF/MEK/MAPK signal pathway, regulated by EGFR. BRAF mutation can result in the sustained activation of the signal pathway, conferring resistance in EGFR mutant lung cancer cells in *in vitro* studies.[23] However, in our study, two patients with concurrent EGFR and BRAF mutations still benefited from gefitinib treatment and had long PFS. This may be explained by tumor heterogeneity existing in lung cancer.[24,25] Ectopic expression of mutant BRAF in drug sensitive EGFR-mutant cells ensures that all EGFR mutant lung cancer cells have BRAF mutation (100%), in which the RAS/RAF/MEK/MAPK signal pathway cannot be inhibited by EGFR-TKIs, leading to drug resistance. In primary tumors, all kinds of het-

erogeneous cell colonies may reside in the same cancer tissues. Not all EGFR mutant tumor cells harbor BRAF mutation, whereas a fraction carry concurrent EGFR and BRAF mutations. When cancer cells with EGFR mutations only were treated with EGFR-TKIs, the cells underwent substantial apoptosis.[26] Resistant cell colonies with BRAF mutation need a long period of time before they become dominant in tumor tissues. Therefore, patients with concurrently sensitive EGFR and BRAF mutations can still respond well to EGFR-TKI treatment. Dynamic and quantitative detection of molecular alteration during the process of therapy is more meaningful and important for instructing individualized therapy.

We also demonstrated the possibility of using plasma DNA as an alternative for BRAF V600E mutation. Three out of the five BRAF V600E mutated patients who provided both tumor DNA and matched plasma DNA were detected with this gene aberrance in plasma DNA. However, it is notable that the BRAF V600E mutation in the tumor DNA samples was only found in a fraction of patients (2/5). This phenomenon has also been observed in our previous studies on EGFR and KRAS mutation detection.[15,16] One possibility for this inconsistency in mutation status is the heterogeneity of genetic abnormalities in the tumors. In such instances, tumor cells harboring BRAF V600E did not enter into blood circulation and could not be detected in plasma DNA. The lower tumor cell content in some of the tumors might also contribute to the lack of detectable mutations.

There are several limitations to our study. It is a single-institution pilot study. The number of patients with BRAF V600E mutations was limited. Therefore, the effect of this mutation on prognosis should be observed with an increased number of patients and long-term follow-up analysis.

Conclusion

In summary, the present study illustrated the prevalence and clinicopathological features of Chinese lung adenocarcinoma with BRAF V600E mutation. Some of the patients with BRAF V600E harbor concurrently sensitive EGFR mutations and respond well to initial EGFR-TKI treatment, which may define a novel subtype of lung adenocarcinoma. Our study also provides evidence to suggest that circulating plasma DNA may be used as a surrogate tissue for BRAF V600E analysis in lung adenocarcinoma.

Acknowledgments

Disclosure

No authors report any conflict of interest.

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