Clinical significance of BRAF^{V600E} mutation in circulating tumor DNA in Chinese patients with melanoma

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Abstract. The present study aimed to assess the B rapidly accelerated fibrosarcoma (BRAF^{V600E}) status in plasma from Chinese patients with melanoma, and evaluated its prognostic value following treatment with BRAF inhibitors. Mutation-specific 3D digital polymerase chain reaction (dPCR) was used to quantify BRAF^{V600E} in circulating tumor DNA (ctDNA) in 58 patients with melanoma, prior to treatment with BRAF inhibitors. Correlations between baseline ctDNA levels and clinicopathological characteristics and clinical benefits were then statistically analyzed. The concordance and sensitivity of BRAF^{V600E} between ctDNA and tumor tissue were 70.2% and 76%, respectively, in 58 patients with melanoma. BRAF^{V600E} mutation in ctDNA correlated with lactate dehydrogenase concentration ($P=0.04$) and Eastern Cooperative Oncology Group score ($P=0.04$). There was no correlation between BRAF^{V600E} of ctDNA with response, progression-free survival (PFS), or overall survival (OS) following targeted therapy. The objective response rate, PFS and OS stratified by BRAF^{V600E} of ctDNA were 30.0% vs. 56.7%, ($P=0.3$), 8.1 months vs. 6.7 months, ($P=0.38$) and 65.6 months vs. 42.3 months ($P=0.52$), respectively, for undetectable and mutant types. In conclusion, 3D dPCR is appropriate for ctDNA detection and BRAF^{V600E} in ctDNA is a non-invasive biomarker in patients with melanoma.

Introduction

Malignant melanoma is the most aggressive skin cancer and has poor prognosis. Its incidence and mortality have increased rapidly worldwide and in Asia. In China, the estimated new cancer cases and deaths of melanoma in 2015 were 8,000 and 3,200 respectively (1). The overall survival (OS) rate at 5 years for patients with stage IV disease is <5% (2).

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Somatic genetic aberrations have provided a framework for developing targeted therapy in advanced cancer. One of the most validated treatments in this area is B rapidly accelerated fibrosarcoma (BRAF) inhibition. BRAF mutations are present in 50% (3) of cases of Caucasian melanoma. The most common mutation is B rapidly accelerated fibrosarcoma (BRAF^{V600E}) (4-6). BRAF inhibitors, such as vemurafenib and dabrafenib, lead to tumor regression in 60-80% of patients with melanoma (7). However, in Chinese patients with melanoma, the incidence of somatic mutations within the BRAF genes is 25.5% (8). Melanoma has diverse clinicopathological characteristics, especially in BRAF mutation frequencies in different ethnic groups and pathologic subtypes.

Tumor surgical or biopsy tissue is the standard material used to determine the presence of somatic mutations before the start of targeted therapy. However, mutation status is unstable and often changes (9). It is difficult to obtain tumor tissue from rebiopsies owing to discomfort and high risk and cost for patients. A liquid biopsy from blood samples, included the circulating tumor DNA (ctDNA) obtained from cell-free DNA (cfDNA), cellular tumor cells (CTCs) and others, overcome the invasive nature and heterogeneity (10). The concordance between ctDNA for BRAF^{V600E} and BRAF^{V600E} status in tissue has been shown to be ~70%. CtDNA for BRAF^{V600E} has a sensitivity of 38-79% and a specificity of 40-100% (9,11-13). BRAF^{V600E} mutated DNA was detected in CTCs of 77% of melanoma patients with recorded mutated tumor tissues (14). In colorectal cancer, a report before showed that among 23 matched CTCs and ctDNA samples, the concordance was 73.9% for BRAF mutations (15). But the disadvantage of CTCs is that the repetitive rate and the success rate of PCR in CTCs were really low in the detection of BRAF mutations (14,16). So the higher concentrations of ctDNA, which is correlated with tumor burden, can be a great alternative to rebiopsy. CtDNA can provide the genetic landscape of all cancerous lesions (primary and metastases) as well as offering the opportunity to systematically track genomic evolution which is used for evaluating response after treatment and monitoring disease recurrence. However, there is no reports associated with BRAF^{V600E} in ctDNA and Chinese melanoma patients.

3D digital PCR (dPCR) is a third-generation dPCR technique that minimizes the use of DNA template. It can be used for true real-time quantitative detection and can

detect mutation frequencies as low as 0.005-0.01% (17). In the present study we use 3D dPCR to detect ctDNA with BRAF[V600E] in 58 Chinese patients with melanoma and also determine whether the levels of ctDNA with BRAF[V600E] at baseline before BRAF inhibitor therapy correlate with treatment response and survival.

Materials and methods

Patients and sample collection. Paired tissues and blood samples from 58 patients with melanoma who were hospitalized between 2012 and 2015 in the Renal Cancer and Melanoma Department of Beijing Cancer Hospital were used in our study. Written consent was obtained from the patients or patients' parent/carer. All tissue samples were confirmed to be positive for melanoma using hematoxylin and eosin (H&E) staining. Of all the patients, the mean age is 43 years, ranged from 17 to 74 years. We obtained the following information from the patients' clinical histories, as recorded by their oncologists: disease stage, tissue mutation, Eastern Cooperative Oncology Group (ECOG) performance status, and lactate dehydrogenase (LDH) values. Blood samples were collected before BRAF inhibitor treatment and tumor response was evaluated in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST version 1.1). The latest follow-up date was October 1, 2016. The present study was approved by the Medical Ethics Committee of the Beijing Cancer Hospital and Institute and was conducted according to the Declaration of Helsinki Principles.

Sample collection and cfDNA extraction. Blood samples from patients with melanoma were collected in ethylene diamine tetraacetic acid (EDTA) vacutainer tubes and stored at 4°C within 24 h before isolation. After centrifugation at 2,500 rpm for 10 min and a further centrifugation at 1,200 rpm for 15 min, plasma samples were collected. CfDNA was extracted from 2 ml plasma stored at -80°C using the QIAamp Circulating Nucleic Acid kit (Qiagen China Co., Ltd.) according to the manufacturer's protocols. CfDNA was eluted in 30 μl of Buffer AVE (Qiagen China Co., Ltd.).

CfDNA detection using 3D digital polymerase chain reaction (dPCR). The TaqMan® BRAF SNP Genotyping assay (cat. no. 4465804; Life; Thermo Fisher Scientific, Inc.) was used for dPCR detection. Each 15 μl dPCR reaction contained 0.375 μl BRAF assay, 7.5 μl QuantStudio™ 3D Digital PCR Master Mix 2X (Life; Thermo Fisher Scientific, Inc.), 6.125 μl nuclease-free water (Life, Thermo Fisher Scientific, Inc.), and 1 μl cfDNA. The dPCR reactions were then loaded onto a QuantStudio™ 3D Digital PCR Chip v2 (Life; Thermo Fisher Scientific, Inc.). The chips were preformed using QuantStudio™ 3D Digital PCR System (Life; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The dPCR cycling was performed as follows: Initial denaturation at 96°C for 10 min, followed by 39 cycles of annealing at 60°C for 2 min, denaturation at 98°C for 30 sec, and elongation at 60°C for 2 min. The levels of cfDNA were determined using QuantStudio™ 3D AnalysisSuite™ Software (Life; Thermo Fisher Scientific, Inc.) by analyzing the numbers and proportions of positive droplets.

Table I. BRAF[V600E] in ctDNA and tumor tissue.

BRAF[V600E] mutation	Tumor tissue			
	WT	MT	NA	Total
ctDNA				
UT	2	12	0	14
MT	5	38	1	44
Total	7	50	1	58

Concordance and sensitivity of BRAF mutation status between ctDNA and tumor tissue were 40/57=70.2% and 38/50=76%, respectively. WT, wild-type; UT, undetectable type; MT, mutant type; NA, not available.; ctDNA, circulating tumor DNA; BRAFV600E, B rapidly accelerated fibrosarcoma.

Table II. Correlation between BRAF[V600E] in ctDNA and characteristics of patients.

Clinical charateristics	BRAF[V600E] mutation of ctDNA		
	UT (n=15,%)	MT (n=43,%)	P-value
Sex			
Male	5 (26.3)	14 (73.7)	1.00
Female	10 (25.6)	29 (74.4)	
Age, years			
>60	1 (14.3)	6 (85.7)	0.46
≤60	14 (27.5)	37 (72.5)	
Subtype			
Acral + Mucosal +Uveal	3 (20)	12 (80)	0.68
CSD + non-CSD	10 (30.3)	23 (69.7)	
Unknown	2 (25)	8 (75)	
Stage			
I-III	8 (29.6)	19 (70.4)	0.54
IV	7 (22.6)	24 (77.4)	
Thickness, mm			
<4	3 (37.5)	5 (62.5)	0.76
≥4	3 (27.3)	8 (72.7)	
Ulcer			
With	5 (29.4)	12 (70.6)	1.00
Without	6 (26.1)	17 (73.9)	
LDH			
<240 IU/L	12 (36.4)	21 (63.6)	0.04
≥240 IU/L	1 (6.7)	14 (93.3)	
ECOG			
0	12 (42.9)	16 (57.1)	0.04
1	3 (12)	23 (88)	
2	0 (0)	4 (100)	

UT, undetectable type; MT, mutant type; CSD, chronic sun-induced damage; LDH, lactate dehydrogenase; ECOG, Eastern Cooperative Oncology Group; BRAF[V600E], B rapidly accelerated fibrosarcoma; ctDNA, circulating tumor DNA.
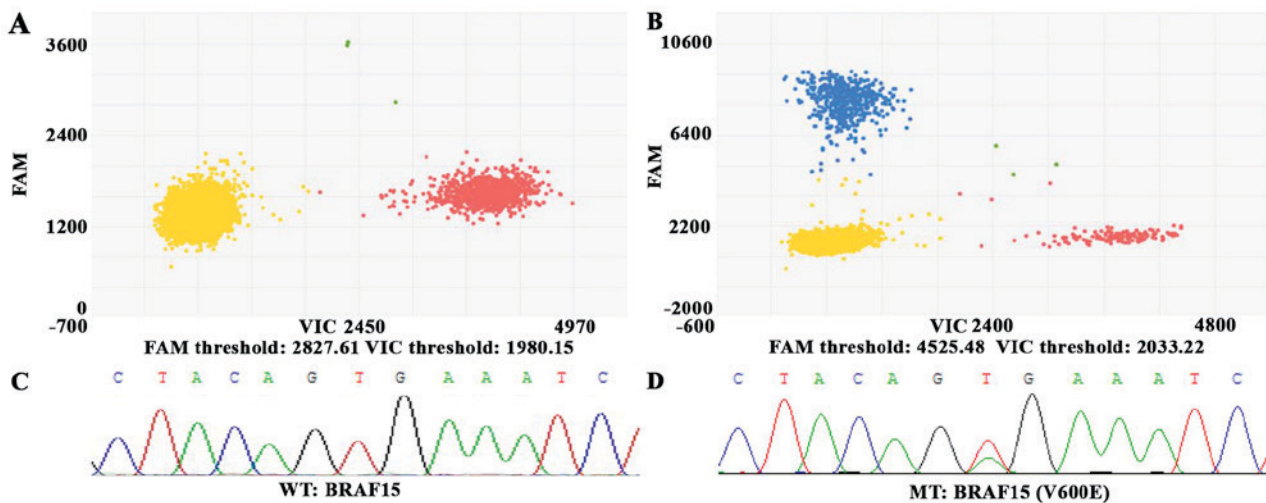
Figure 1. A negative sample from a melanoma patient of ctDNA in 3D dPCR (A) and the other positive for this mutation (B). A negative sample in tumor tissue by Sanger Sequencing (C) and the other positive for this mutation (D). In plot of 3D dPCR, yellow cluster corresponds to negative droplets, red cluster corresponds to droplets positive for wild-type, blue cluster corresponds to droplets positive for mutant BRAF[V600E], and green cluster corresponds to droplets both positive for wild-type and mutant BRAF[V600E]. In plot of Sanger Sequencing, the nucleotide refers to 1799T >A and the amino acid refers to V600E. dPCR, digital PCR; BRAF[V600E], B rapidly accelerated fibrosarcoma.

Statistical analyses. Statistical analysis was performed using SPSS 20.0 for Windows (IBM Corp, Armonk, NY, USA). Qualitative data are described as percentages. Quantitative data are described as means ± standard deviations. Pearson's chi-square tests, Fisher's exact tests, and Mann-Whitney U tests were used to assess correlations between the qualitative variables. Survival time was assessed using disease-free survival (DFS), progression-free survival (PFS), and OS. DFS and PFS were calculated separately as the duration from the time of initial surgery to the diagnosis of a recurrence and the time of targeted therapy to the diagnosis of progression. OS time was assessed for prognostic analysis. We used Kaplan-Meier and log-rank tests to analyze survival differences between groups. Univariate Cox regression analyses were performed to examine correlations between ctDNA levels and clinicopathologic measures and PFS. These tests were bilateral and the significance level was defined as 0.05, so that P<0.05 was considered to indicate a statistically significant difference.

Results

Concordance between BRAF[V600E] in ctDNA (3D dPCR) and in tumor tissue (PCR). We used the maximum concentration of BRAF[V600E] in ctDNA from 4 healthy individuals (0.097-0.133 copies/ml) to establish our criteria and divided the 58 patients with melanoma into two groups: undetectable type (UT), and mutant type (MT). 43 (74.1%) of 58 plasma samples had the BRAF[V600E] mutation, while 13 (25.9%) did not. A total of 50 tissue samples had the BRAF[V600E] mutation, as assessed using PCR. The concordance and sensitivity of BRAF[V600E] between ctDNA and tumor tissue were 70.2% and 76%, respectively. The results are presented in Table I. Fluorescent amplitude plots of 3D dPCR in ctDNA samples and Sanger Sequencing in tumor tissues from melanoma patients are presented in Fig. 1. The plot of 3D dPCR contains the negative droplets, positive droplets for wild-type, mutant type and both. And the plot of Sanger Sequencing shows the location and variation of nucleotides in BRAF gene.

Clinical correlations between BRAF[V600E] in ctDNA and clinical characteristics of patients. The presence of the BRAF[V600E] mutation in ctDNA was correlated with LDH concentration (P=0.04) and ECOG performance status (P=0.04). The levels of BRAF[V600E] mutation in ctDNA levels were markedly higher in patients with high LDH levels compared to those with normal LDH levels [UT: ctDNA median, 0.08 copies/ml (0-0.094 copies/ml); LDH median, 197 IU/l (125-269 IU/l); MT: ctDNA median, 0.37 copies/ml (0.149-456.9 copies/ml); LDH median, 206 IU/l (129-1371 IU/l)]. Worse ECOG performance status was associated with higher BRAF[V600E] mutation levels in plasma. There were no significant associations between BRAF[V600E] mutation in ctDNA and other clinical characteristics, such as sex, age, subtype, stage, thickness, or ulcer. The details of our findings are presented in Table II.

Clinical correlations between BRAF[V600E] in ctDNA and clinical benefits after targeted therapy. A total of 30 out of 58 patients with melanoma were treated with BRAF inhibitors. This accounted for 20% (6/30), 66.7% (20/30), and 13.3% (4/30) of patients with first-, second-, and third-line targeted therapy, respectively. The median follow-up time was 34.9 months (range, 30.3-39.6 months). There was no correlation between BRAF[V600E] in ctDNA and response, PFS, or OS after targeted therapy with BRAF inhibitors. The subset of patients undergoing targeted therapy included 13 patients with partial responses (PR), 13 with stable disease (SD), and 1 progressive disease (PD), as assessed using RECIST criteria. We also had 3 unassessable patients (Table III). Objective response rate (ORR), PFS, and OS stratified by BRAF[V600E] in ctDNA were 30.0% vs. 56.7% (P=0.3) (Table III), 8.1 months vs. 6.7 months (P=0.38) (Fig. 2), and 65.6 months vs. 42.3 months (P=0.52), respectively for the UT and MT groups. Quantitative and

qualitative data of response are presented in Fig. 3 and Table III, respectively.

Discussion

In the present study we used 3D dPCR to detect ctDNA with BRAF^{V600E} in 58 Chinese patients with melanoma and statistically analyzed the correlations between ctDNA level and clinical characteristics, treatment response, and survival. The aim of our study is to know whether ctDNA is a great alternative to biopsy and whether 3D dPCR is a suitable detection method for ctDNA. Furthermore, we want to explore if BRAF^{V600E} ctDNA is a candidated biomarker in patients with melanoma or even a predictive marker after treatment of BRAF inhibitors.

In prior reports, the concordance between BRAF^{V600E} in ctDNA and BRAF^{V600E} in tissue samples was ~70% and the sensitivity was 38-79% (9,11-13). The concordance and sensitivity between ctDNA and tumor tissue in our study were 70.2 and 76%, which were higher than those of prior reports. The main reason for this observation is that our study uniquely used 3D dPCR, which is highly sensitive, quantitative, and real-time. Unlike other detection methods, such as Beads Emulsion Amplification Magnetics (BEAMing), Amplification Refractory Mutation System (ARMS), and Next Generation Sequencing (NGS) (9,11-13), each reaction of 3D dPCR has 20,000 wells and each well is isolated from its neighbors. This ensures the high sensitivity and accuracy of 3D dPCR. In addition, 3D dPCR is simple to perform. In fact we were able to obtain our results within 2 h. The use of 3D dPCR prevents mistakes due to complex operation and calculation errors. Although BRAF^{V600E} in ctDNA was identified by 3D dPCR, which is the most sensitive among all of the detection tools use thus far, 29.8% of the patients had plasmic BRAF statuses that were distinct from those of their tumor tissues. This may have been owing to the mechanisms underlying the transfer of ctDNA into the blood, which are as yet not completely clear. It is also known that the levels of ctDNA vary at different times of the day. In addition, the heterogeneity of the tumors may have led to differences between the ctDNA and the tissue. CtDNA derived from apoptotic or necrotic cell debris of primary tumors, metastatic tumors, or CTCs may better reflect the entire picture of the tumor when compared to using one site to obtain tumor tissue (18). Finally, genetic alterations may appear after different treatments (13). This is important, as ~80% of the patients with melanoma in this study had not been treated with the first-line therapy.

Our results are in agreement with those of previous studies that have shown that BRAF^{V600E} mutant ctDNA correlates with tumor burden (19). Patients with lower or undetectable amounts of BRAF^{V600E} mutant ctDNA tend to be those with less disease burden as measured by LDH, RECIST sum of diameters, and ECOG performance (12). LDH is the only blood-based biomarker incorporated into the staging system, as elevated levels of LDH are associated with significantly decreased survival (19). Both BRAF^{V600E} in ctDNA and LDH in the blood are good prognostic markers and are thought to be useful in the follow-up of patients with melanoma (20). LDH is neither sensitive nor specific, and other studies have shown that ctDNA is more consistent and informative

Table III. Associations between baseline ctDNA concentrations and response to targeted treatment.

BRAF^{V600E} mutation	PR	SD	PD	Total	P-value
UT	3	6	0	9	0.30
MT	10	7	1	18	
Total	13	13	1	27	

Qualitative data were statistically analyzed using Mann-Whitney U tests. UT, undetectable type; MT, mutant type; PR, partial responses; SD, stable disease; PD, progressive disease.
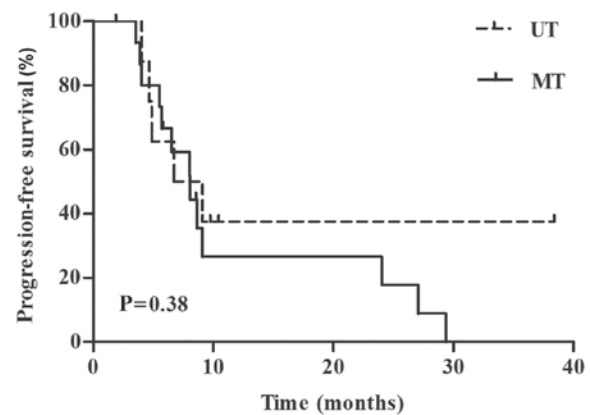

Figure 2. Kaplan-Meier plots of PFS probabilities according to baseline ctDNA BRAF^{V600E} status in cases treated with targeted therapy (n=26). PFS stratified by BRAF^{V600E} in ctDNA were 8.1 months vs. 6.7 months (P=0.38) for the undetectable type and mutant type. The P-value are indicated below the graph. PFS, progression free survival.
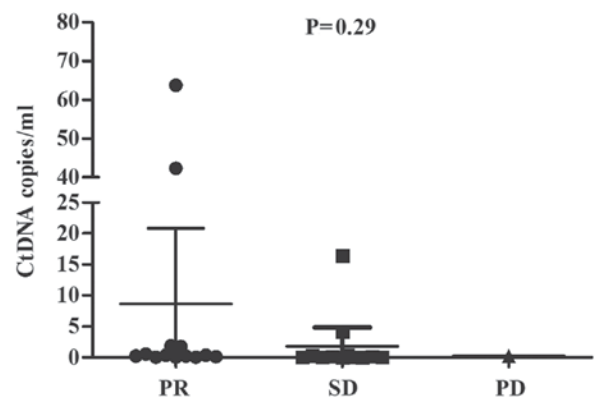

Figure 3. Associations between baseline ctDNA concentrations and response to targeted treatment. Quantitative data were statistically analyzed using Mann-Whitney U tests. The median concentrations of baseline ctDNA for PR, SD and PD groups refered to 0.37, 0.12, 0.24 copies/ml respectively. The P-value are indicated above the graph. PR, partial responses; SD, stable disease; PD, progressive disease.

than LDH (21,22). In addition, ctDNA is significantly more accurate for tracking disease status than traditional serum markers, such as carcinoembryonic antigen (CEA) and cancer antigen 15-3 (CA15-3), which are used to diagnose colorectal

and breast cancer, respectively (23,24). In the future, we will monitor the dynamic status of ctDNA and compare the results thus obtained with those of LDH in a large scale study. Here, 18 patients that had surgery before in our study collected their blood samples during the period between the metastatic lesions determined and the first-line therapy. Of note, DFS stratified by BRAF^{V600E} in ctDNA was 26.4 months vs. 9.1 months (P=0.013) for UT and MT, respectively. This indicates that patients with longer DFS before recurrences tended to have lower levels of BRAF^{V600E} mutant ctDNA. Some experts in the breast cancer field have also found that level of ctDNA are reduced or even disappeared after surgery, although some patients have remaining ctDNA (25). This highlights a new direction for ctDNA research. Specifically, it will be interesting to determine whether ctDNA detected after surgery identifies patients at risk for recurrence. This may then guide adjuvant therapy decisions for individual patients.

Another objective of our study was to determine whether BRAF^{V600E} in ctDNA can be used as criteria to select patients for BRAF-targeted therapy. Some previous reports have shown that low baseline ctDNA is a good predictor of response to treatment, longer PFS, and even OS in targeted therapy (12,13,20). Although there was no statistical significance between BRAF^{V600E} in ctDNA and clinical benefit, patients with wild-type BRAF ctDNA have longer PFS and OS than mutant type which supports the findings of previous reports. Three factors may explain the reason of no statistical significance. First, the major factors influenced the effect of BRAF inhibitor is unknown. A decrease of BRAF^{V600E} in ctDNA indicated response to therapy (13,26). Also the increased concentration of mutant copies observed following disease progression (26) and the state of a secondary resistance to the treatment may associated with effect of therapy (13). The effect of BRAF inhibitors would be affected by many factors such as a secondary resistance, so baseline ctDNA of BRAF^{V600E} maybe not the best predictive marker to target therapy, but the variation of this mutation or other gene associated with resistance. Second, it will take time for the PFS and OS to mature. Since only 75% of the patients have reached their endpoints, mortality only accounts for 43.1% of the outcomes. Finally, our study was carried out on a small scale. In fact, there was only one patient who did not have a response after targeted therapy. Therefore, it remains to determine the realistic relationship between clinical benefit and BRAF^{V600E} in ctDNA.

Due to the limitation of our study, we hope a large scale research in the future which includes multiple plasma samples during the treatment and progression. CtDNA from different periods can reflect different conditions of disease and predict clinical benefits of treatment. In addition, experts can make comparison between ctDNA and traditional biomarkers.

In conclusion, our data confirm that 3D dPCR is suitable for ctDNA detection and that BRAF^{V600E} ctDNA is a non-invasive prognostic marker in patients with melanoma.

Acknowledgements

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