Mutations in BRAF codons 594 and 596 predict good prognosis in melanoma

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Abstract. B-Raf proto-oncogene serine/threonine kinase (BRAF) V600E is the most common kinase-activating mutation and is associated with poor prognosis in melanoma. However, the clinical significance of kinase-impairing mutations remains unclear. The present study aimed to analyze kinase-impairing mutations in BRAF codons 594 and 596 in non-Caucasian patients with melanoma and to investigate their possible clinical significance. To detect hotspot mutations, exon 15 of the BRAF gene was amplified using polymerase chain reaction in samples from 1,554 patients with melanoma. Among these patients, a total of 912 valid follow-up data were obtained. These patients were divided into three groups according to their BRAF activation status: BRAF wild-type (n=752), BRAF V600E (n=147); and BRAF D594/G596 (n=13). Then the correlation between BRAF activation status, and the clinicopathological features and overall survival (OS) of the patients were analyzed. The prevalence of BRAF mutations in non-Caucasian patients with melanoma was 24.3% (377/1554). Three patients carried two mutations simultaneously. The overall mutation frequencies of kinase-activating mutations, kinase-impairing mutations, and mutations with unknown effects were 93.4 (355/380), 3.4 (13/380), and 3.2% (12/380), respectively. BRAF V600E was identified to be associated with a poor prognosis. Patients with BRAF mutations in codons 594 and 596 had a longer OS time compared with those with a BRAF V600E mutation [median OS, 45 vs. 25 months; HR, 0.45 (95% confidence interval, 0.31-0.97); P=0.043]. To the best of our knowledge, this is the first study to examine a large number of samples from non-Caucasian patients with melanoma and report the characteristics of BRAF mutations according to mutant kinase activity. Melanoma arising from a mutation in BRAF codon 594 or 596 can be differentiated from BRAF V600E-induced melanoma, and mutations in these codons may be good prognostic factors for melanoma. The results of the present study are thus of significance for the development of accurate personalized medicine to treat melanoma.

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Introduction

Melanoma is an aggressive cancer with an extremely poor prognosis. In 2015, the number of newly diagnosed cases of melanoma in the United States was 73,870, and the number of deaths was approximately 9,940 (1). In China, the number of newly diagnosed cases of melanoma in 2015 was 8,000, and the number of deaths was approximately 3200 (2).

BRAF is an important serine/threonine kinase in the mitogen-activated protein kinase (MAPK) signaling pathway, which plays a critical role in cell proliferation and apoptosis (3,4). Abnormal expression or activation of BRAF has been found in a variety of tumors (5). In melanoma, BRAF mutations have been detected in 66% of Caucasian patients (6) and 25.5% of non-Caucasian patients (7). Moreover, BRAF p.V600E (1799T>A) is a hotspot mutation that is detected in melanoma, and this mutation can increase BRAF kinase activity by 10-20-fold (8) and accounts for 80-90% of all detected BRAF mutations (7,9). Patients can be treated effectively because the selective inhibitors vemurafenib plus cobimetinib (10) or dabrafenib plus trametinib (11) have been classified as the primary first-line therapy by the National Comprehensive Cancer Network (NCCN) for the treatment of advanced BRAF V600-mutant melanoma. However, the clinical significance of other BRAF mutations, which account for approximately 10 to 20% of cases, is largely unknown.

Recent studies suggest that the effects of BRAF mutations can be divided into kinase-activating (e.g., p.V600E) and kinase-impairing (e.g., p.D594G or G596N) mutations (12). In contrast to BRAF V600E, which causes hyperactivation of downstream kinase pathways, kinase-impairing mutations lead to a reduction in BRAF kinase activity or alternatively activation of silent and wild-type CRAF to elevate MEK activity (13,14).

Because BRAF kinase-impairing mutations are different from kinase-activating (e.g., p.V600E) mutations, we retrospectively collected samples and clinical data from a non-Caucasian patient population and compared the clinical and pathological characteristics as well as clinical outcomes of patients bearing BRAF kinase-activating mutated melanoma with those of patients with BRAF wild-type melanoma. Our goal was to shed light on different therapeutic strategies for treating BRAF-mutated tumors.

Materials and methods

Patients and tissue samples. This study used samples from 1,554 melanoma patients who were hospitalized between July 2012 and July 2015 at Beijing Cancer Hospital and Institute. All samples were analyzed by hematoxylin and eosin (H&E) staining and by immunohistochemistry to confirm the diagnosis of melanoma. Clinical data, including age, sex, stage, thickness (Breslow), ulceration, and survival status (follow-up was continued until December 2016), were collected. A total of 912 valid follow-up datas were obtained. This 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.

Mutation screening. Genomic DNA was extracted from melanoma tissue sample sections using DNA FFPE Tissue kit (Qiagen, Hilden, Germany). To detect hotspot mutations, we amplified exon 15 of the BRAF gene by Nested PCR. The primer sequences are listed in Table I. We purified PCR products with QIAquick (Qiagen) and sequenced them on an ABI3130 automated sequencer (Applied Biosystems, Foster City, CA, USA). All mutations were confirmed by repeat bidirectional sequencing.

Statistical analysis. All statistical analyses were performed with a significance level of 0.05 (two-sided) using SPSS 20.0 software. Fisher's exact test or the χ^2 test was used when comparing clinical and pathological characteristics according to BRAF mutational status. Overall survival (OS) analysis was performed according to the Kaplan-Meier method, and survival curves were compared using the log-rank test. A Cox proportional hazard model was adopted in the multivariate analysis.

Results

Prevalence of BRAF mutations in Chinese melanoma patients. Of the 1,554 melanoma samples analyzed, 380 BRAF mutations were detected in 377 patients (3 patients carried two mutations simultaneously). The prevalence of BRAF mutations in this group of Chinese melanoma patients was 24.3% (377/1554). BRAF p.V600E (1799T>A) accounted for 87.5% (330/377) of the detected mutations, which was consistent with previous studies. The remaining 12.5% (47/377) of patients harbored non-p.V600E mutations, of which p.V600K was the most frequent [5.3% (20/377)], and 7 patients harbored p.D594G [1.9% (7/377)]. Twenty other mutation types were also found, such as p.K601E, p.D594N, and p.G596R.

BRAF mutations categorized by serine-threonine kinase activity. To better understand the BRAF mutation spectrum in melanomas, we categorized the BRAF mutations according to the reported effects of the mutation on serine-threonine kinase activity (Table II) (12). As showed in Table II, the overall mutation frequency was 93.4% (355/380) for kinase-activating mutations, 3.4% (13/380) for kinase-impairing mutations, and 3.2% (12/380) for mutations with unknown effects. Interestingly, the kinase-impairing mutations detected in our cohort were all focused on BRAF codon 594 or 596, which is of potential therapeutic importance.

Correlation of BRAF mutational status with the clinicopathologic features of melanoma. To better compare the clinical and pathological characteristics of patients bearing different BRAF kinase activation status, we conducted a long-term follow-up to these patients. A total of 912 valid follow-up datas were obtained. These patients were divided into three groups: the BRAF wild-type group (n=752), BRAF V600E group (n=147), and BRAF D594/G596 group (n=13) (Table III). In our cohort, the median age was significantly different among the three groups (P<0.001). The median age of patients with BRAF V600E was 48 years old, whereas that of patients with BRAF D594/G596 was 57 years old; the difference between these two groups was significant (P<0.0001; Table III).

We found that BRAF V600E mutations were more frequent in non-acral cutaneous melanoma (51.0%), whereas BRAF D594/G596-mutated tumors occurred more frequently in mucosal melanomas (53.8%); the difference between these two groups was significant (P<0.001; Table III). Clinical stage is an important clinical feature of melanoma. Among the 147 patients with BRAF V600E mutations, the percentages of patients at stages I, II, III, and IV were 1.4 (2 cases), 26.5 (39 cases), 19.7 (29 cases), and 52.4% (77 cases), respectively, which was not significantly different from the respective percentages for BRAF D594/G596 mutations (P=0.801; Table III). In our cohort, the overall ulceration rate was 57.1% (521/912). The frequencies of ulceration in the BRAF wild-type group, BRAF V600E group, and BRAF D594/G596 group were approximately 56.6, 66.7, and 38.5%, respectively. The ulceration rate in patients with BRAF V600E mutations was significantly higher than that in patients with BRAF D594/G596 mutations (P=0.043).

Prognostic significance of BRAF mutational status for OS in melanoma. We further analyzed the correlation between BRAF mutational status and OS time with a median follow-up period of 32.2 (range: 3.0-124.0) months. BRAF V600E-mutated patients had a shorter median survival time (median OS =25.0 months) than patients with wild-type BRAF (median OS =31.0 months) or with BRAF D594/G596 mutations (median OS =45 months). The median OS was significantly different among these three groups (P<0.0001; Fig. 1). BRAF V600E was again associated with a poor prognosis. Patients with BRAF D594/G596 mutations had longer OS times than patients with BRAF V600E mutations (median OS: 45 vs. 25 months; HR: 0.45 (95% CI: 0.31-0.97), P=0.043; Table IV). In the Cox regression analysis, BRAF mutational status, stage, thickness and pathological subtype had a combined effect on the patients' prognosis.

Table I. Primers used in Nested PCR.

Gene	Exon	Primer set 1	Primer set 2
BRAF	15	F: 5'-TTATTGACTCTAAGAGGAAAGATGAAG-3' R: 5'-TGATTTTTGTGAATACTGGGAAC-3'	F:5'-TTATTGACTCTAAGAGGAAAGATGAAG-3' R: 5'-GGCCAAAAATTTAATCAGTGGA-3'

F, forward; R, reverse.

These data suggest that BRAF D594/G596 mutations may have positive prognostic significance for melanoma patients, whereas BRAF V600E mutations have a negative association with the OS.

Discussion

Targeted therapy and immunotherapy have shown promising clinical efficacy in treating melanoma (9-11,15,16). Selective inhibitor combination therapy has been approved to treat melanoma patients harboring the BRAF activation mutation p.V600E (9-11). However, mutations in the BRAF gene can result in activation or impairment of downstream kinases, leading to entirely different active states of the MAPK pathway through different molecular mechanisms. BRAF V600E is a kinase-activating mutation that stimulates downstream kinase pathways. Whereas BRAF kinase-impairing mutations increase MEK activity by activating CRAF (17).

However, the clinical significance of BRAF kinase-impairing mutations is largely unknown. Selecting treatment options for patients with non-V600E/K mutations including these kinase-impairing mutations is difficult. The BRAF mutations that these patients carry are rare, and there is a lack of retrospective studies or clinical guidelines to determine the impact of these mutations on disease progression and survival. These reasons prompted us to hypothesize that kinase-impairing mutations were of potential importance in targeted therapies. In this study, we examined the largest number of samples from non-Caucasian patients to date to describe the characteristics of BRAF mutations. Moreover, we categorized patients according to BRAF kinase activity and compared the clinical characteristics among groups. This approach differs from previous studies, making our results important for identifying therapeutic strategies for treating melanoma.

Consistent with other studies (7), BRAF mutations were detected in 24.3% (377/1,554) of malignant melanomas in non-Caucasian patients, which was less than the rate of 60% reported in a Caucasian population (18), again demonstrating that there is a large difference in the genetic features of melanoma between non-Caucasians and Caucasians. In this study, most of the BRAF gene mutations were concentrated among three mutation types, i.e., BRAF V600E, BRAF V600K, and BRAF D594G, which accounted for 86.8, 5.3, and 1.8% of all detected mutations, respectively. Mutations in codon 594 also appeared in other forms, such as D594E (n=1) and D594 N (n=2). In our study, the mutation probability for codon 594 was 0.64% (10/1554), which was lower than that reported in a previous study (12). The reason for this difference may be differences in the sample size (our sample size was 1,554, and that of the previous study was 152).

Several previous studies have identified a variety of kinase-inactivating mutations, such as T598I, D593V, and others (19). In our cohort, kinase-impairing mutations were found in 13 non-Caucasian patients, and these mutations were all in BRAF codon 594 or 596. Kinase-inactivating mutations

Table II. BRAF mutations categorized by serine-threonine kinase activity.

Kinase activity	BRAF mutations	No.
Kinase-activating mutations	L597R	1
	A598V	1
	V600E	330
	V600K	20
	V600R	1
	K601E	2
	Total	355 (93.4%)
Kinase-impairing mutations	D594E	1
	D594G	7
	D594N	2
	G596R	2
	G596D	1
	Total	13 (3.4%)
Mutations with unknown effects	V590A	1
	I592T	1
	V599 ins	1
	S602P	2
	S602F	1
	S605N	1
	S607F	1
	Q612stop	1
	S616F	1
	1795 ins ACT	1
	1793 ins CTA	1
	Total	12 (3.2%)
Total		380[a]

[a]Three patients harbered 2 BRAF mutations,including A598V, S616F; V600E, S602F; V600E, S607F, respectively.

Table III. Association of BRAF gene mutation with clinicopathological features.

Characteristics	BRAF wide-type (n=752) n (%)	BRAF V600E mut (n=147) n (%)	BRAF 594 or 596 mut (n=13) n (%)	P-value[a]	P-value[b]	P-value[c]
Sex				0.091	0.109	0.303
Male	397 (52.8)	67 (45.6)	4 (30.8)			
Female	355 (47.2)	80 (54.4)	9 (69.2)			
Age (years)				<0.001	<0.001	0.017
Median	55	49	58			
Range	7-92	7-84	25-75			
Subtype				<0.001	<0.001	<0.001
Acral	395 (52.5)	58 (39.5)	3 (23.1)			
Mucosal	208 (27.7)	14 (9.5)	7 (53.8)			
Non-acral cutaneous	149 (19.8)	75 (51)	3 (23.1)			
Clinical stage				<0.001	<0.001	0.801
I	8 (1.1)	2 (1.4)	0 (0)			
II	439 (58.4)	39 (26.5)	5 (38.5)			
III	165 (21.9)	29 (19.7)	2 (15.4)			
IV	140 (18.6)	77 (52.4)	6 (46.2)			
Ulceration				0.034	0.030	0.043
Yes	426 (56.6)	90 (66.7)	5 (38.5)			
No	326 (43.4)	45 (33.3)	8 (61.5)			
Thickness (mm)				0.017	0.008	0.124
≤1.0	71 (9.4)	12 (8.2)	0 (0)			
1.1-2.0	86 (11.4)	9 (6.1)	3 (23.1)			
2.1-4.0	196 (26.1)	26 (17.7)	2 (15.4)			
>4.0	399 (53.1)	100 (6.8)	8 (61.5)			

[a]Comparison of the three groups (BRAF wild-type versus BRAF V600E mut versus BRAF 594 or 596 mut). [b]Comparison of BRAF wild-type versus BRAF V600E mut. [c]Comparison of BRAF V600E mut versus BRAF 594 or 596 mut.

Table IV. HR for death in relation to BRAF mutation status.

Groups	HR (95% CI of ratio)	P-value
BRAF V600E vs. BRAF WT	1.45 (1.27-1.92)	<0.0001
BRAF 594/596 mut vs. BRAF WT	0.61 (0.35-1.25)	0.214
BRAF 594/596 mut vs. BRAF V600E	0.45 (0.31-0.97)	0.043

HR, hazard ratio.

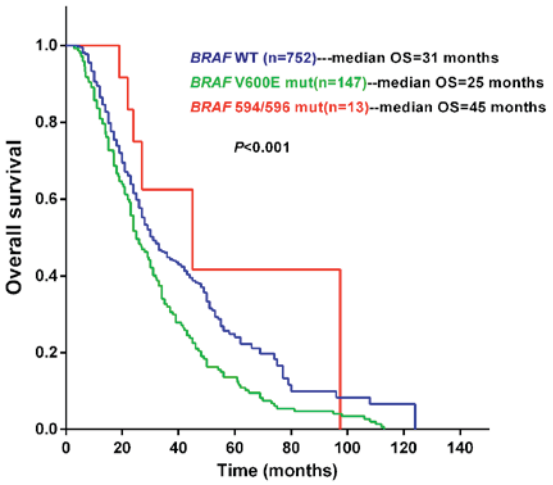

Figure 1. Overall survival of melanoma patients in relation to BRAF mutation status. According to the log-rank (Mantel-Cox) test, the median age was significantly different among the three groups (P<0.001).

are concentrated in these loci, and these may be characteristics of non-Caucasian melanoma patients.

Patients with BRAF V600E mutations were younger, and the proportion of patients with non-acral skin melanoma was higher than that of other subtypes. These results are consistent with those of Bauer's group (20). We also found that patients with BRAF D594/596 mutations were older and that these mutations were more common in mucosal melanoma patients. In addition, patients with BRAF V600E mutations were more likely to ulcerate, whereas the incidence of ulceration in the

BRAF D594/G596 group was significantly lower than that of the BRAF V600E mutation group. Thus, there were significant differences in pathological characteristics between BRAF V600E-mutated melanomas and BRAF D594/G596-mutated melanomas. A recent study reported a similar conclusion for mCRC patients (21), but such a finding has not been reported in melanoma research.

The evidence from the clinical outcomes analysis suggests that BRAF D594/G596 mutations may predict a longer survival time. However, standardized treatment options are not available for these types of mutations. BRAF D594/G596-mutated melanomas may be not sensitive to specificity BRAF inhibitors such as Vemurafenib due to the reduction in BRAF kinase activity (13,14). *In vitro* studies, D594 G mutated melanoma lines were highly resistant to the MEK inhibitor U0126 (17). This indicated that BRAF D594/G596-mutated melanomas may be insensitive to either BRAF or MEK inhibitors. Smalley *et al* reported that sorafenib (BAY 43-9006, Nexxavar), a multi-kinase inhibitor including inhibit CRAF, was better at reducing the growth of melanoma xenografts with D594G mutation than those with V600E mutation (17). These indicated that CRAF inhibitors including but not limited to sorafenib may be a possible treatment strategy for BRAF D594/G596-mutated melanomas. In future studies, we will further study possible mechanisms and therapeutic strategies in melanoma cell models and animal models.

In conclusion, we identified a rare and unexplored subtype of melanoma with different molecular features, pathological characteristics, and clinical outcomes compared with BRAF V600E-mutated melanomas. Our work is thus of significance for the development of accurate personalized medicine to treat melanoma.

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