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Prevalence of BRAF V600E mutation in Chinese melanoma patients: Large scale analysis of BRAF and NRAS mutations in a 432-case cohort

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ABSTRACT

Background: Mutations of *NRAS* and *BRAF* have been described in Caucasian melanomas. However, the status and the clinical significance of *BRAF* and *NRAS* mutations in the Asian population have not been investigated on a large scale.

Methods: Melanoma samples ($n = 432$) were analysed for mutations in exons 11 and 15 of the *BRAF* gene, and exons 1 and 2 of the *NRAS* gene in genomic DNA by polymerase chain reaction (PCR) amplification and Sanger sequencing. Mutations of *BRAF* and *NRAS* genes were correlated to clinicopathologic features and prognosis of the patients.

Results: The incidence of somatic mutations within the *BRAF* and *NRAS* genes was 25.5% (110/432) and 7.2% (31/432), respectively. Among the 110 patients with *BRAF* mutations, 98 patients (89.1%) had V600E mutations. Melanomas without chronic sun-induced damage (Non-CSD) were more likely ($P < 0.01$) to show *BRAF* mutations while *NRAS* mutation frequency was unbiased between melanoma subtypes. Patients with genetic mutations in *BRAF* ($P < 0.01$) or *NRAS* ($P = 0.04$) gene are more likely to have ulceration as compared to patients without *BRAF* or *NRAS* mutations, respectively. Both *BRAF* ($P = 0.003$) and *NRAS* mutations ($P = 0.031$) are inversely correlated to overall survival.

Conclusions: *BRAF* mutation is frequent while mutations in *NRAS* gene are rare. The most prevalent *BRAF* mutation type is V600E. Patients with mutations in *BRAF* or *NRAS* gene are frequently present with ulceration, and mutation in *BRAF* or *NRAS* gene is indicator for poor prognosis. Our study may warrant a clinical trial of kinase inhibitors targeting BRAF V600E in Chinese and Asian melanoma patients.

1. Introduction

RAS mutations typically occur at codons 12, 13 or 61 and maintain RAS in a constitutively active state.[1,2] Mutations K-RAS of are the most frequent in many human malignancies,[1,2] mutations of *NRAS* are rare in melanomas.[3–10] Meanwhile, *BRAF* somatic missense mutations are detected in 66% of malignant melanomas, with a single sub-

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stitution (V600E) accounting for 80% of all *BRAF* mutations.[9–22]

The genetic mutations in *BRAF* and *NRAS* genes have been correlated to clinicopathologic features and prognosis of melanomas.[5,6,8,11–16,19,21,22] However, these observations are conducted in Caucasian populations, and the mutation status of *BRAF* and *NRAS* genes and the clinical significance of these mutations have not been established in other populations (e.g. Asians). It has been demonstrated that acral melanomas account for only 1–7% of all cutaneous melanomas in Caucasians, whereas the percentage is significantly higher in Asian.[23–27] Curtin et al. reported that mutations of *KIT* gene were detected in about 29% of melanomas while our study in a Chinese cohort suggested that the incidence was about 11%, indicating that oncogene mutation status may also be different between Caucasians and Asians.[28,29]

Generally, studies regarding examination of *NRAS* and *BRAF* mutations have garnered intense attention. Given that kinase inhibitors for BRAF, especially the BRAF V600E-specific inhibitors PLX4032 and GSK2118436, have been demonstrated to be effective in clinical trials in Caucasian population,[30–32] identification of mutations in *BRAF* and *NRAS* mutations may be of great translational relevance for future clinical trails targeting *BRAF* or *NRAS*. To further explore the potential targets for melanoma treatments, we evaluated genetic mutations of *BRAF* and *NRAS* in a non-Caucasian, Chinese patient population, and correlated the *BRAF*/*NRAS* mutations to clinicopathologic features and prognosis of Chinese melanoma patients.

2. Patients and methods

2.1. Patients and tumour tissue samples

This study involved samples from primary lesions of 432 melanoma patients, hospitalised during January 2007 and January 2010 at the Peking University Cancer Hospital & Institute (Supplementary Table S1). These samples were confirmed for the diagnosis of melanoma as described previously.[29] Clinical data, including age, sex, TNM (tumour-node-metastases) stage, thickness (Breslow), ulceration and survival (follow-up persisted until December 2011 or until the missing of follow-up or death of patients) were collected. This study was approved by the medical ethics committee of the Beijing Cancer Hospital & Institute and was conducted according to the Declaration of Helsinki Principles.

2.2. DNA preparation and mutation screening

Genomic DNA was extracted from formalin-fixed, paraffin-embedded sections using a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). To detect hotspot mutations, we amplified exons 11 and 15 of the *BRAF* gene, and exons 1 and 2 of *NRAS* gene, by PCR in at least two separate preparations of genomic DNA as described previously.[9,21] The primer sequences are listed in Supplementary Table S2.

2.3. Statistical analysis

All the statistical analyses were performed using SPSS 13.0 software. Categorical data are described using frequencies and percentages. Continuous data such as age are described using means ± standard deviations or median (range) for normally distributed data. The χ^2 test, Fisher's exact test or Kruskal–Wallis test was used to differentiate the rates of different groups, and differences in measurement data of two groups were evaluated by unpaired *t* test or mann–whitney test. Survival curves were established using the Kaplan–Meier method and compared by the log-rank test. All statistical analyses were two sided and significance was assigned at $P < 0.05$.

3. Results

3.1. Genetic mutations of BRAF and NRAS genes in melanoma

Typical sequencing results for mutations are shown in Supplementary Fig. S1–S4. The overall mutation frequency was 25.5% (110/432) for *BRAF* gene and 7.2% (31/432) for *NRAS* gene, respectively (Table 1). The highest mutation frequency was detected in Non-CSD melanoma (57.1%), which was in consistent with the recent reports showing that *BRAF*-mutated melanomas occur on skin without marked solar elastosis.[21,33] However, the high frequency of *BRAF* mutation in Non-CSD melanoma may not necessarily indicate that *BRAF* mutation was unrelated to chronic sun exposure, given that *BRAF* mutation could also be detected in CSD melanomas (18.2%) and that *BRAF* mutations in melanocytic lesions could arise from DNA damage induced by ultraviolet radiation.[34] Of note, the frequencies of *BRAF* mutations detected in acral (15.5%) and mucosal (12.5%) melanomas were also comparable with the corresponding rates previously reported in Caucasian patients.[17] On the contrary, *NRAS* mutation was generally rare, with the lowest mutation incidence detected in Non-CSD melanomas (2.0%).

Considering that the primary sites of mucosal melanoma are usually variable, we analysed the *BRAF* mutation frequencies in 120 cases of mucosal melanomas. We found that the highest frequency (46.7%) of *BRAF* mutation was detected in mucosal melanomas located in sinonasal mucosa among the 15 mucosal melanomas bearing *BRAF* mutation (Supplementary Table S3).

Table 1 – Genetic mutations of *BRAF* and *NRAS* in melanoma subtype.

Subtype	*BRAF* mutation			*NRAS* mutation		
	No.	Positive	%	No.	Positive	%
Acral	148	23	15.5	148	13	8.8
Mucosal	120	15	12.5	120	11	9.2
CSD	22	4	18.2	22	1	4.5
Non-CSD	98	56	57.1	98	2	2.0
UP	44	12	27.3	44	4	9.1
Total	432	110	25.5	432	31	7.2

Abbreviations: CSD, melanomas on skin with chronic sun-induced damage; Non-CSD, melanomas on skin without chronic sun-induced damage; UP, melanoma of unknown primary.

Table 2 – Mutation types of *BRAF* gene in melanoma.

DNA mutation	Protein mutation	No.	%[a]	Exon affected	Subtype
G1324A	G442S	1	0.9	11	Acral
G1349A	W450Stop	1	0.9	11	Acral
T1370C	I457T	1	0.9	11	Mucosal
G1396A	G466R	1	0.9	11	Acral
T1782G	D594E	1	0.9	15	Mucosal
A1781G	D594G	1	0.9	15	Acral
T1799A	V600E	98	89.1	15	Acral; mucosal; CSD; Non-CSD; UP
G1798A;T1799A	V600K	3	2.7	15	Mucosal; Non-CSD
A1801G	K601E	1	0.9	15	Mucosal
G1811A	W604Stop	1	0.9	15	Acral
T1846C	S616F	1	0.9	15	Mucosal

Abbreviations: CSD, melanomas on skin with chronic sun-induced damage; Non-CSD, melanomas on skin without chronic sun-induced damage; UP, melanoma of unknown primary.

[a] The percentage indicates for the cases showing the indicated mutation type to the total of 110 cases bearing *BRAF* mutations.

3.2. *Mutation types of BRAF and NRAS in melanoma*

In our study, 11 different types of *BRAF* mutations and 12 different *NRAS* mutations were detected (Table 2). We found that, among the detected *BRAF* mutations, three novel mutations in exon 11 (G442S, W450Stop and I457T) were detected in addition to eight previously reported *BRAF* mutation types.[9–21] Acral and mucosal melanomas demonstrated more variable mutations while CSD and Non-CSD melanomas demonstrated either V600E or V600K mutation (Table 2). One novel mutation in exon 1 (Y4C) and one novel mutation in exon 2 (A59D) of *NRAS* were detected in addition to 10 previously reported *NRAS* mutations.[3,8–10,15,18,21] Acral and mucosal melanomas demonstrated more variable *NRAS* mutations while Non-CSD melanomas mainly demonstrated mutations in codon 61 (Table 3).

The BRAF V600E mutation was the single most common genetic alteration in this cohort, detected in 98 samples (89.1%) among the 110 samples positive for *BRAF* somatic mutations (Table 2). Another common mutation of *BRAF* is V600K, which was detected in three samples (2.7%). Notably, both V600E and V600K are sensitive to specific BRAF inhibitor GSK2118436.[30–32] For the patients containing *NRAS* mutations, 58.1% of them demonstrated mutations in codon 61, with Q61R as the most frequent mutation in *NRAS* (35.5%, Table 3). Moreover, we detected two cases of mucosal melanomas that simultaneously harbour mutations in both *BRAF* and *NRAS* genes (D594E in BRAF plus G13R in NRAS; V600E in BRAF plus Y4C in NRAS), which was consistent with the previous study reported by Lin et al.,[35] but was in contrary to the notion that *BRAF* and *NRAS* mutations were mutually exclusive.[8,21,36]

3.3. *Correlation of BRAF and NRAS mutations to the clinicopathologic features of melanoma*

In our cohort, the mean age ($N = 432$) was not significantly different between patients with *BRAF* mutation and those without *BRAF* mutation (Table 4). The mean age (48.9 ± 14.4) of patients bearing BRAF mutations was similar to that of a recent report on an American population.[21] On the contrary, another recent report suggests that *BRAF*-mutated melanomas

Table 3 – Mutation types of *NRAS* gene in melanoma.

DNA mutation	Protein mutation	No.	%[a]	Exon affected	Subtype
A11G	Y4C	1	3.2	1	Mucosal
G35C	G12A	1	3.2	1	Mucosal
G34T	G12C	1	3.2	1	Acral
G35A	G12D	3	9.7	1	Mucosal; CSD
G34A	G12S	1	3.2	1	Mucosal
G37C	G13R	3	9.7	1	Acral; mucosal
A50G	S17N	1	3.2	1	UP
A176G	A59D	2	6.5	2	Acral; mucosal
A183T	Q61H	1	3.2	2	Mucosal
C181A	Q61K	2	6.5	2	Acral; UP
A182T	Q61L	4	12.9	2	Acral; mucosal; Non-CSD
A182G	Q61R	11	35.5	2	Acral; Non-CSD; UP

Abbreviations: CSD, melanomas on skin with chronic sun-induced damage; Non-CSD, melanomas on skin without chronic sun-induced damage; UP, melanoma of unknown primary.

[a] The percentage indicates for the cases showing the indicated mutation type to the total of 31 cases bearing *NRAS* mutations.

Table 4 – Correlation of *BRAF* and *NRAS* genotype to clinicopathologic features of melanoma.

Clinicopathologic factor	*BRAF* genotype			*NRAS* genotype		
	Mutation	Wild type	*P* value[a]	Mutation	Wild type	*P* value[a]
Age (year)	48.9 ± 14.4	51.9 ± 14.3	0.39	56.5 ± 14.3	50.8 ± 14.5	0.02
Gender (F, %)	59 (52.6)	165 (51.2)	0.75	16 (51.6)	207 (51.6)	0.86
Thickness (mm)	5.0 (0.5–10)	4.5 (1.0–10)	0.18	5.0 (1.5–10)	5.0 (0.5–10)	0.69
Ulceration *N* (%)	58 (61.1)	117 (40.9)	<0.01	16 (64.0)	145 (40.7)	0.04
Stages *N* (%)						
I	6 (5.5)	16 (5.3)	0.90	0 (0)	22 (5.7)	0.37
II	35 (32.1)	129 (42.4)	0.13	10 (33.3)	154 (40.2)	0.19
III	36 (33.0)	77 (25.3)	0.18	14 (46.7)	99 (25.8)	0.02
IV	32 (29.4)	82 (27.0)	0.73	6 (20.0)	108 (28.2)	0.46
Subtypes *N* (%)						
Acral	23 (20.9)	125 (38.8)	<0.01	13 (41.9)	134 (33.4)	0.63
Mucosal	15 (13.6)	105 (30.6)	<0.01	11 (35.5)	105 (26.2)	0.38
CSD	4 (3.6)	18 (5.6)	0.60	1 (3.2)	21 (5.2)	0.95
Non-CSD	56 (50.9)	42 (13.0)	<0.01	2 (6.5)	92 (22.9)	0.06
UP	12 (10.9)	32 (9.9)	0.92	4 (12.9)	4 (1.0)	<0.01

Abbreviations: F, female; CSD, melanomas on skin with chronic sun-induced damage; Non-CSD, melanomas on skin without chronic sun-induced damage; UP, melanoma of unknown primary.

[a] For evaluation of age and thickness, the unpaired *t* or *t*' tests were used. For evaluation of gender, ulceration, stages and subtypes, the chi-square tests or Fisher's exact tests were used.

occur in a younger age group on skin without marked solar elastosis[33], indicating that more investigations may needed to determine the relationship between *BRAF* mutation and the age of melanoma patients. Additionally, the mean age ($N = 432$) in the group showing *NRAS* mutations was significantly older than the group showing wild type *NRAS* ($P = 0.02$).

The average thickness of all 381 available samples was more than 5 mm, which was much thicker than the previous reports but was the actual status for Chinese patients upon hospitalisation.[27,29] The median thickness of samples without *BRAF* or *NRAS* mutations was not significantly different to that of samples with *BRAF* or *NRAS* mutations, respectively (Table 4).

In our cohort, the overall ulceration rate was 59.7% (228/383). The ulceration rate in patients with *BRAF* mutations (61.1%) was significantly higher ($P < 0.01$) than that in patients without *BRAF* mutations (40.9%). Moreover, the ulceration rate in patients with *NRAS* mutations (64.0%) was significantly higher ($P = 0.04$) than that in patients without *NRAS* mutations (40.7%). These data suggest that patients harbouring either *BRAF* or *NRAS* mutations were prone to have ulceration in melanoma lesions.

Among the 110 patients with *BRAF* mutations and with the clinical stage data available, the percentages of patients at stage I, II, III and IV were 5.5% (6 cases), 32.1% (35 cases), 33.0% (36 cases) and 29.4% (32 cases), respectively, which were not significantly different from those without a *BRAF* mutation (Table 4). For the patients with *NRAS* mutations, the percentages of the four stages were 0% (0 case), 33.3% (10 cases), 46.7% (14 cases) and 20.0% (6 cases), respectively (Table 4). The frequency of *NRAS* mutations in patients at Stage III (46.7%) was significantly higher ($P = 0.02$) than that of wild type *NRAS* in patients at Stage III (25.8%), indicating that patients with *NRAS* mutations were more likely to be at disease stage III.

We went further to examine whether *BRAF* or *NRAS* mutations may be associated with particular melanoma subtypes. The percentages of acral (20.9%) and mucosal (13.6%) melanomas in patients harbouring *BRAF* mutations were significantly lower ($P < 0.01$) than those in patients showing wild type *BRAF*; the percentage of Non-CSD (50.9%) melanomas in patients harbouring *BRAF* mutations was significantly higher ($P < 0.01$) than that in patients showing wild type *BRAF* (Table 4). However, *NRAS* mutation frequency was unbiased between melanoma subtypes.

3.4. *Prognostic significance of BRAF and NRAS mutations for overall survival of melanoma*

The survival data were collected for patients ($N = 395$) who were diagnosed as primary melanoma or melanoma of unknown primary (Supplementary Table S1) from the first time of diagnosis as melanoma to December 2011. The median follow-up period was 24.0 (range: 3.0–229.0) months. We found that the median survival time for patients with *BRAF* mutations (33.0 months) was significantly shorter than that for patients with wild-type tumours (53.0 months; $P = 0.005$, Fig. 1A). In addition, patients with *NRAS* mutations had a worse survival (33.0 months) than patients with wild-type tumours (48.0 months; $P = 0.031$, Fig. 1B). These data suggest that *BRAF* and *NRAS* mutations may be of prognostic significance for melanoma patients.

We have reported previously that *KIT* mutations may be independent prognostic factors for melanoma.[29] Among the 432 cases of melanoma, 43 cases (10.0%) demonstrated *KIT* mutations, among which four cases simultaneously showed *BRAF* mutations, indicating that *KIT* mutations and *BRAF* mutations may be not mutually exclusive. When analysed with *COX* regression of survival data for this 432-case population, both *KIT* mutation ($P = 0.003$) and *BRAF* mutation ($P = 0.01$) were independent prognostic factors with the hazard ratios of 1.989 (95% confidence interval (CI): 1.236, 3.131) and 1.536 (95% CI: 1.110, 2.124), respectively.

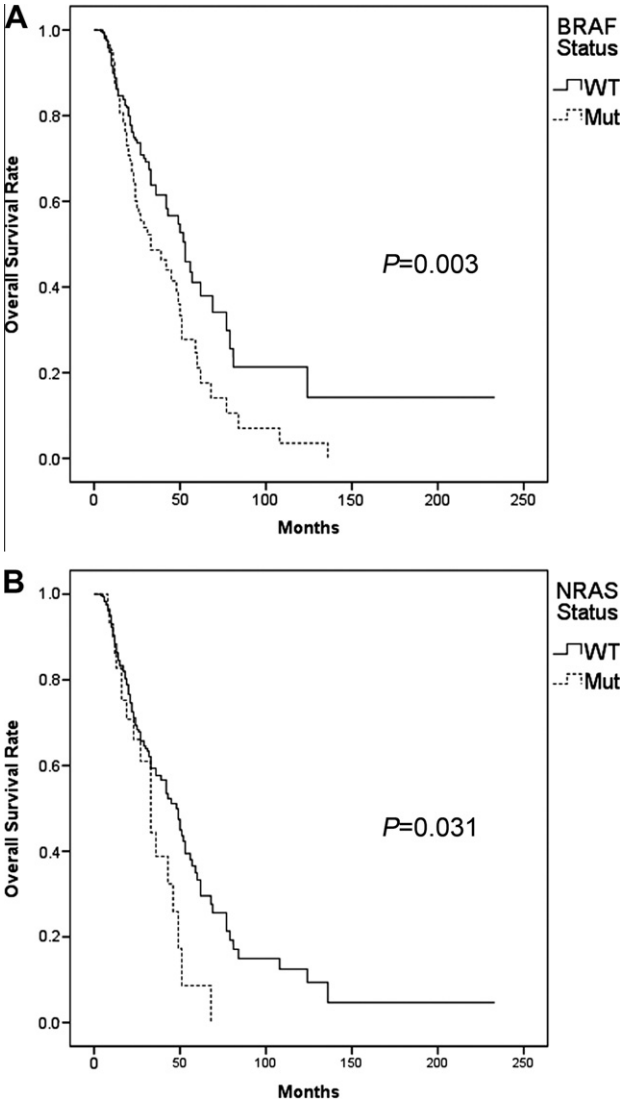

Fig. 1 – Overall survival of melanoma patients in relation to *BRAF* and *NRAS* mutations. Solid line: wild type (WT); dotted line: mutation (Mut).

4. Discussion

BRAF and *NRAS* mutations have been documented in all subtypes of melanoma.[2–22] However, most of the studies are conducted in Caucasian population, and indicate for the requirement of deciphering oncogenic differences between Caucasian and Asian populations for proper treatment of melanomas. Given that our study has examined the largest size of samples in Chinese/Asian patients, *BRAF* may be the major mutated gene in Chinese melanomas. Our study is thus of significance for the understanding of tumourigenesis of melanomas worldwide.

Codon 61 is the most common position of *NRAS* alterations in melanoma and mutations in this residue lock the RAS protein in the GTP-bound state with a subsequent continuous activation of its downstream effectors.[1,3–10] Approximately 90% of reported *BRAF* mutations occur at residue 600, which is located in the activation domain of this kinase.[1,9] Consistent with these studies, our data confirm that *BRAF* mutation in codon 600 (V600E and V600K, comprised 91.8% of all *BRAF* mutations) and *NRAS* mutations in codon 61 (Q61H, Q61K, Q61L and Q61R, comprised 58.1% of all *NRAS* mutations) are the major mutations in melanoma. A most recent report, which examined the *BRAF* mutational status in a Chinese Han population (195 cases of malignant melanoma and 379 cases of melanocytic nevi), suggested that 15.0% of malignant melanoma harboured the *BRAF* V600E mutation while *BRAF* mutation may be unrelated to melanocyte transformation.[37] The *BRAF* V600E mutation frequency in our cohort (25.5%) was higher than that (15.0%) reported by Qi et al.,[37] which may be due to different sample size and difference in regional selection (all regions of China in our cohort versus northeast region of China in Qi's cohort). Since we have not examined the *BRAF* mutational status in melanocytic nevi, we could not evaluate the significance of *BRAF* mutation in initiation of melanoma. It should be noted that mutations detected in our study may not necessarily lead to activation of BRAF and NRAS. For example, two kinds of point mutations in *BRAF* (W450Stop and W604Stop) can result in termination of BRAF translation, which may lead to loss of BRAF kinase activities. S17N mutation in NRAS may serve as a dominant negative form of NRAS and may inhibit the NRAS activity. Several novel mutations in *BRAF* and *NRAS* have also been detected by us. Further investigations of these mutations biochemically and clinically may help to establish therapies targeting BRAF or NRAS.

For therapeutic purposes, *BRAF* mutations may be of clinical importance in identifying patients who may benefit from small molecule inhibitors (e.g. BRAF V600E inhibitors). Although Sorafenib is a RAF kinase inhibitor, phase II clinical trials suggest that Sorafenib may be ineffective in melanoma treatment, with a clinical response of about 19% stable disease.[38] In contrast, selective RAF inhibitors, including PLX4032 and GSK2118436, demonstrated much better clinical response, with the overall response rate of about 80% and 63%, respectively.[30–32] The prevalence of BRAF V600E mutation in Chinese melanoma may indicate that clinical trials of PLX4032 or GSK2118436 may be reasonable and ideal for Chinese and Asian melanoma patients.

Previously, correlations of *NRAS* mutations to Clark level of invasion, Breslow thickness, ulceration rate and mean age at diagnosis have been observed.[5,8,18,21] However, other reports could not demonstrate any association of *NRAS* mutation with these clinicopathological factors.[6,39] In our study, *NRAS* mutation positive patients show higher mean age at diagnosis and higher ulceration rates as compared to patients without *NRAS* mutations. However, we did not find the association of *NRAS* mutations with gender and Breslow thickness. For *BRAF* mutations, most of the studies suggest that *BRAF* mutation may be not correlated to thickness, age, gender, ulceration and prognosis.[8,12,15,40] However, a most recent study suggests that *BRAF* mutation may be correlated to ulceration and Brelow thickness, but not overall survival.[21] It has also been reported that the presence of *BRAF* mutations in melanoma metastases was associated with shortened survival from the time point of removal of the lesions or from the diagnosis of stage IV disease.[11,13] We found that *BRAF* mutation may be correlated to ulceration rates and overall survival, with *BRAF* mutation positive patients showing higher ulcera-

tion rates and worse prognosis. However, we could not find the association of *BRAF* mutation with age, gender and thickness. The consistent findings for *BRAF* and *NRAS* mutations are the association of *BRAF* and *NRAS* mutations to ulceration and overall survival in Chinese melanoma. These differences and consistencies between our studies and others may represent the unique features of Chinese melanoma. Specifically, the samples in our cohort are mainly derived from the primary lesion sites but not metastatic sites, indicating that our data are of significance for primary melanoma.

In conclusion, our study has confirmed that the most prevalent mutation type of BRAF and NRAS is V600E and G61R, respectively. We found that *BRAF* and *NRAS* mutations are unrelated to the gender and thickness of primary melanomas. Patients with mutations in *BRAF* or *NRAS* genes are more likely to have ulceration. Most importantly, genetic mutations in *BRAF/NRAS* may be independent adverse prognostic factors in melanoma. Our study also highlights that there are numerous types of *BRAF/NRAS* mutations present in this population, with variable mutations of *BRAF/NRAS* in acral and mucosal melanomas but with limited mutation types in Non-CSD melanomas.

Contributions of authors

Jun Guo, Lu Si, Yan Kong, Xiaowei Xu and Keith T. Flaherty were involved in the conception and design of the study. Jun Guo, Lu Si and Yan Kong wrote the manuscript. Zhihong Chi, Chuanliang Cui, Xinan Sheng, Siming Li and Lili Mao provided study material and quality control. Jun Guo, Yan Kong and Lu Si collected, analysed and interpreted the data. All authors validated the report.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.06.056.

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