Aberrations and clinical significance of BRAF in malignant melanoma
A series of 60 cases in Chinese Uyghur

Xiaojing Kang, MD, PhD[a,∗], Ying Zeng, MM[a], Junqin Liang, MD[a], Jing Li, MM[a], Danyang Ren, MM[a], Li Chai, PhD[a], Zhenzhu Sun, MD[b], Shirong Yu, MD[a], Xiujuan Wu, PhD[a], Wen Han, MM[b], Weijia Wang, MM[a]

Abstract
Malignant melanoma (MM) is a highly malignant melanocytic tumor, it occurs mostly in the skin, the mucosal membrane close to the skin, but also in the tunicae rhagoides and the pia mater. The Uyghur is the largest ethnic group living in the Xinjiang Uyghur Autonomous Region of China, accounting for 46% of the total population of 20 million. Large-scale studies on MMs in Asian countries are limited. This study aimed to investigate BRAF mRNA expression and mutations in Chinese Uyghur patients with MMs and to identify the clinical features associated with these parameters.

Formalin-fixed, paraffin wax-embedded tumor sections from 60 MMs were analyzed for BRAF expression using reverse transcription polymerase chain reaction (RT-PCR). Exons 11 and 15 of BRAF were analyzed for the presence of mutations using PCR and DNA sequencing. Sixty MMs were followed by mobile phone for survival analysis. BRAF mRNA expression was higher in MMs than in pigmented moles and normal skin tissues. Fourteen of 60 MMs had BRAF mutations. The frequency of BRAF mutations was significantly higher in patients younger than 60 years (10/28, 4/32, P = .02). A significant difference was observed in the frequency of BRAF mutations among specimens of mucosal, acral, chronic sun-induced damage (CSD), and non-CSD MMs (2/10, 3/19, 8/25, 1/6, P = .002). No significant association was found among BRAF mutations, sex, ulceration, or lymph node metastasis. MMs lymph node metastasis (hazard ratio 2.54 [95% confidence interval 1.062–6.066], P = .01) affected survival.

This study indicated that BRAF mutations and expression might serve as independent adverse prognostic factors in melanoma.

Abbreviations: CI = confidence interval, CSD = chronic sun-induced damage, HR = hazard ratio, MM = malignant melanoma, MST = median survival times, RT-PCR = reverse transcription polymerase chain reaction.

Keywords: BRAF, Chinese Uyghur, melanoma, mRNA expression, mutation

1. Introduction
Malignant melanoma (MM), a common type of skin cancer, originates in melanocytes. It has the clinical features of high metastatic rate, rapid development, poor prognosis, and high mortality rate. Its incidence is also rising globally. Based on the chronic sun-induced damage (non-CSD); melanomas on skin with metastatic rate, rapid development, poor prognosis, and high mortality rate. It occurs mostly in the skin, the mucous membrane close to the skin, but also in the tunicae rhagoides and the pia mater. The Uyghur is the largest ethnic group living in the Xinjiang Uyghur Autonomous Region of China, accounting for 46% of the total population of 20 million. Large-scale studies on MMs in Asian countries are limited. This study aimed to investigate BRAF mRNA expression and mutations in Chinese Uyghur patients with MMs and to identify the clinical features associated with these parameters.

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Kinase inhibitors for BRAF, especially the BRAF V600E-specific inhibitors PLX4032 and GSK2118436, which have been demonstrated to be effective in clinical trials on Caucasian populations.\(^{17–19}\) Identification of mutations in BRAF may be a great translational relevance for future clinical practice. Therefore, all ongoing trials require documenting any BRAF mutation in the tumor tissue before a patient is treated. However, these correlations and translations of melanomas are currently conducted in Caucasian populations. Thus, it is clinically significant to detect whether same aberrations of BRAF and clinical features might be present in Chinese populations, particularly the unique ethic group of Uyghur.

The aim of the present study was to investigate the clinicopathology of melanomas in Chinese Uyghur patients in Xinjiang, and to analyze BRAF mutations and mRNA expression in these patients to determine whether either of them correlated with the clinical features of melanoma, to discuss whether the BRAF mutation is associated with the survival time of melanoma patients.

2. Methods

The present study was approved by the ethics committee of the People’s Hospital of the Xinjiang Uyghur Autonomous Region (PHXUAR) and conducted according to the principles of the Declaration of Helsinki. All patients provided written informed consent.

2.1. Patient selection

In total, 60 patients (31 men, 29 women) with histologically confirmed diagnosis of MM, 20 patients (7 face, 7 acrals, 6 trunks, 10 men, 10 women) with histologically confirmed diagnosis of pigmented nevi, the median age of the patients was (60 ± 10.2) years, and 10 patients (5 prepuces, 5 traumas, 5 men, 5 women) with normal skin, the median age of the patients was (61 ± 10.7) years at PHXUAR between January 2011 and December 2015 were enrolled. All patients were Chinese Uyghur. The demographic and clinicopathological characteristics included age, sex, MM subtype, ulceration, and regional lymph node metastasis.

2.2. BRAF mRNA expression

Stored samples of formalin-fixed, paraffin wax-embedded tumors were obtained from the Departments of Dermatology and Pathology, People’s Hospital of Xinjiang. The tissue was cut into serial 3-μm-thick sections, and then 10 sections of tumor-rich areas were collected. Genomic DNA was extracted from the samples using a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. The genomic DNA was used as template for separate PCR amplifications of the DNA sequences, encompassing exons 11 and 15 of BRAF. The primers used for the PCR amplification are shown here (Exon 11 For 5'-AGGAATGTAATCTTTGAGGGTGA-3’, Exon 11 Rev 5'-TTGTGAAACACTTTTGGAGGAG-3’, The Length is 350 bp. Exon 15 For 5'-TCATAATTGCTGCTGGA-TAGGA-3’, Exon 15 Rev 5'-GGCCTTAAATTATTTAGTCTGCAG-3’. The Length is 224bp). PCR was performed using 2 μL of genomic DNA, 1 μL of each primer (20 μmol/L), and 25 μL of Master Mix (TaKaRa, Shiga, Japan) in a total volume of 50 μL. Thermal cycling for the amplification of exon 11 was performed at 95°C for 5 minutes, followed by 40 cycles of 95°C for 40 seconds, 58°C for 40 seconds, and 72°C for 40 seconds, with a final extension at 72°C for 10 minutes. Conditions for amplification of exon 15 were 95°C for 5 minutes, followed by 40 cycles of 95°C for 40 seconds, 57°C for 40 seconds, and 72°C for 40 seconds, with a final extension at 72°C for 10 minutes. The PCR products were stored at 4°C. The PCR products were separated by electrophoresis in a 1.5% agarose gel (Sangon Biotech, Shanghai, China), and the PCR products were confirmed by DNA sequencing (Sangon).

2.4. Follow-up

The patients were followed by mobile phone from January 2011 to December 2015. No patients were lost at follow-up and all deaths were tumor related in our patients.

2.5. Statistical analysis

Statistical analysis was performed using the SPSS software (v17.0; IBM, NY). The level of BRAF mRNA expression was evaluated by the 2^(-ΔΔct) method. The measurement data were expressed as mean ± standard deviation, and the 2 groups were compared by using the t test. The single factor analysis of variance was used. Associations between the demographic and clinicopathological characterics and the BRAF mutation status were evaluated using the Chi-squared test or Fisher exact test.
The analysis provided descriptive statistics estimated with a 95% confidence interval (CI). Median survival times (MST) was estimated using the Kaplan–Meier product limit estimator. Overall survival times were stratified according to the clinical variables that potentially affect survival. Log-rank tests were used to assess the significance of the differences between the groups. Hazard ratios were estimated using a proportional hazard Cox regression model. A P value < .05 was considered statistically significant.

3. Results

3.1. Demographic and clinicopathological characteristics

The patients’ demographic and clinicopathological characteristics were listed in Table 1. The median age of the Uyghur patients was (62±11.8) years. Ulceration was present in 24 patients, while 22 patients (37.1%) had regional lymph node metastases. As shown in Fig. 1, CSD MMs were the most common tumor subtype (n=25), followed by acral (n=19), mucosal (n=10), and non-CSD (n=6) MMs.

3.2. BRAF mutation status

Of the 60 patients, 14 with BRAF mutations and 46 with wide-type BRAF, giving an overall mutation rate of 23.2%. Ten of the patients with BRAF mutations were aged ≥60 years at diagnosis, and the other 4 were younger than 60 years. The male:female ratio was 0.75 (6 men, 8 women) (Table 2). Of the 14 patients, 12 had exon 15 mutations (10 with V600E mutations, 1 with an L597Q mutation, and 1 with a G593C mutation), and 2 had exon 11 mutations (1 with an S465F mutation and 1 with a P453H mutation) (Fig. 2). BRAF mutations were more common in CSD (n=7) and non-CSD MMs (n=3).

3.3. Analysis of BRAF mRNA expression

The level of BRAF mRNA expression was measured by quantitative real-time PCR in samples with high-quality RNA. No difference was found in groups with BRAF mutation and with wide-type BRAF in MMs (P=.0903). In pigmented nevi, it had no difference between groups with BRAF mutation and with wide-type BRAF (P=.6275). No difference was found in terms of

| Variable | n | Positive | Negative | p* |
|----------|---|----------|----------|----|
| Age (y)  |   |          |          |    |
| <60      | 28| 10       | 18       | .02|
| ≥60      | 32| 4        | 28       |    |
| Sex      |   |          |          |    |
| Male     | 31| 7        | 24       | .097|
| Female   | 29| 7        | 22       |    |
| Tumor subtype | | | | |
| Acral    | 19| 3        | 16       | .002|
| Mucosal  | 10| 2        | 8        |    |
| CSD      | 25| 8        | 17       |    |
| Non-CSD  | 6 | 1        | 1        |    |
| Ulceration |   | | | |
| Yes      | 24| 5        | 19       | .587|
| No       | 36| 9        | 27       |    |
| Lymph node metastases |   | | | |
| Yes      | 22| 5        | 17       | .735|
| No       | 38| 9        | 29       |    |

* Chi-squared test or Fisher exact test.
patient’s age or sex for both nevi and MM samples, but BRAF mRNA expression levels were significantly higher in MM (0.377 ± 0.167) than in pigmented nevi (0.159 ± 0.167; \( P = .0093 \)). In addition, the BRAF mRNA expression levels were also significantly higher in MM tissues than in their corresponding normal skin tissues (0.134 ± 0.050; \( P = .0087 \)) (Fig. 3).

### 3.4. Follow-up status and survival analysis

The median follow-up duration was 36 months (range, 10–47 months), 14 of 60 patients died because of melanoma. The median survival time was 32 months (range, 11–38 months). The 1-year, 3-year, and 5-year survival rates were 70.3%, 22.7%, and 9.2%, respectively. MMs regional lymph node metastasis (hazard ratio 2.54 [95% CI 1.062–6.066], \( P = .01 \)) affected survival, whereas age, sex, MM subtype, ulceration, and BRAF status did not (Table 3).

### Table 3

Survival according to age, sex, MM subtype, ulceration, BRAF status, regional lymph node metastasis.

| Variable                        | n  | Log-rank \( \chi^2 \) | \( P \) value | Cox HR          |
|---------------------------------|----|-----------------------|---------------|-----------------|
| Age (y)                         |    |                       |               |                 |
| <60                             | 28 | 1.21                  | .272          |                 |
| ≥60                             | 32 |                       |               |                 |
| Sex                             |    |                       |               |                 |
| Male                            | 31 | 2.61                  | .106          |                 |
| Female                          | 29 |                       |               |                 |
| Tumor subtype                   |    |                       |               |                 |
| Acral                           | 19 | 1.65                  | .199          |                 |
| Mucosal                         | 10 |                       |               |                 |
| CSD                             | 25 |                       |               |                 |
| Non-CSD                         | 6  |                       |               |                 |
| Ulceration                      |    |                       |               |                 |
| Yes                             | 24 | 3.67                  | .07           |                 |
| No                              | 36 |                       |               |                 |
| BRAF status                     |    |                       |               |                 |
| Wild type                       | 46 | 0.27                  | .590          |                 |
| Mutation                        | 14 |                       |               |                 |
| Lymph node metastases           |    |                       |               |                 |
| Yes                             | 22 | 6.60                  | .01           | 2.54 (1.062–6.066) |
| No                              | 38 |                       |               |                 |

CSD = chronic sun-induced damage, HR = hazard ratio, MM = malignant melanoma.

\( P < .05. \)
4. Discussion

CSD has been documented as the major subtype of MM in Caucasian populations.\[1,2,20,21]\] Acral and mucosal types only account for a small proportion of MM, but these 2 are the most common subtypes in Asian populations, especially in Chinese.\[15,22]\] Chinese Han patients are different from Chinese Uyghur patients. CSD MM is the most prevalent MM among Chinese Uyghur patients, whereas acral and mucosal MMs are the most prevalent in Chinese Han patients.\[17,18]\] CSD (25/60) was found to be the most common type of MM in Uyghur patients in Xinjiang, which was reported by a previous study.\[1,17]\] The incidence of BRAF mutations was only 32% (8/25). The present study was the first to examine the Uyghur patients with MM. Thus, this study is of significance to understand melanoma tumorigenesis.

Approximately, 90% of BRAF mutations occur at V600E, which is located in the activation domain of BRAF kinase.\[22,23]\] Consistent with previous studies, this study further confirmed that BRAF mutations concentrated in exons 11 and 15. Twelve cases of mutations were found in exon 15 (rate 80%), of which 10 were V600E heterozygous missense mutations and the other 2 were L597Q and G593C mutations; S465F and P453H mutations were found in exon 11. A most recent report, which examined the BRAF mutational status in a Chinese Han population, suggested that 15.0% of MM harbored the BRAF V600E mutation while BRAF mutation might not be related to the melanocyte transformation.\[24]\] The present study found that the frequency of BRAF V600E mutation in Uyghur patients with MM (23.2%) was slightly higher. But the BRAF V600E mutation was lower than that (25.5%) reported by Long et al\[18]\] who studied with Chinese Han patients. Previous studies have found correlations of BRAF mutations with age, sex, tumor, ulceration, and lymph node metastases at diagnosis.\[25]\] These notions were further confirmed in this study, which found that patients with CSD MMs had higher BRAF mutation rates compared with other subtypes. But in Chinese Han patients who with non-CSD MMs had higher BRAF mutation rates. And they found others BRAF mutations which we did not find.\[14]\] The frequency of BRAF mutations was significantly higher in patients younger than 60 years than in those older than 60 years. However, this study did not find any relevance of BRAF mutations to patient’s sex and ulceration. But Long et al found it has a relevance of BRAF mutations to patient’s ulceration in Chinese Han patients. The tissues from the metastatic sites were excluded in this study, indicating that the present results might be more relevant to the primary melanomas.

BRAF mRNA expression was significantly higher in MM than in pigmented nevi and normal skin tissues (Fig. 2).\[26]\] In the study, using immunohistochemistry, phosphorylated (active) MAPK and BRAF expression was studied in 24 common nevi, and 26 cutaneous melanomas. BRAF mutations at codon 600 were assessed by PCR-RFLP. Active MAPK was detected in 29% of common nevi, and 85% of cutaneous melanomas. In all, 23% of common nevi, and 93% of cutaneous melanomas with BRAF mutation have activated MAPK. BRAF mutation does not seem to be sufficient to produce MAPK activation in melanoctye nevi, and it is suggested that other events are needed to induce MAPK activation, that is, BRAF overexpression, inhibition of MAPK phosphatases, or suppression of RAF kinase inhibitors.\[27]\] In melanoma, the gene amplification and mutation of BRAF can lead to overexpression of BRAF. The overexpression of BRAF can activate the MAPK pathway, then stimulate the growth of melanoma cells. It might be reasonable to speculate that BRAF mRNA expression is correlated with malignancy, regardless of its mutational status. Recent research also shows that BRAF mutations occur in a high proportion of nevi, indicating that measuring the mRNA expression may be more effective to identify MM, than detecting BRAF mutations. Using larger cohorts and collecting mRNA samples by fine-needle aspiration may be necessary in the future to further confirm the connection of BRAF mRNA expression with malignancy.

The 1-year, 3-year, and 5-year survival rates were 70.3%, 22.7%, and 9.2%, respectively. MMs regional lymph node metastasis affected survival, but BRAF mutation did not.\[28]\] The 5-year survival rate is low. Stage III and VI melanoma affected survival. Stage III and VI melanoma always had lymph node metastasis. So in MMs lymph node metastasis may be one of the indicators of poor prognosis.

BRAF may be an important oncogene causing MM, which regulates proliferation, survival, and invasion/metastasis.\[29]\] It has been established that BRAF is a valid and important therapeutic target. BRAF mutations may have a great clinical significance in identifying patients who may benefit from small-molecule inhibitors.

Selective BRAF inhibitors, such as PLX4032 and GSK2118436, have already been proved to be clinically promising, with the overall response rate of about 63% to 80%.\[17,19]\] The prevalence of BRAF V600E mutation in Chinese patients with melanoma may indicate that clinical trials of PLX4032 or GSK2118436 may be reasonable and ideal in Asian patients with MM, particularly the Uyghur patients.

In conclusion, this study confirmed that CSD MM is the most prevalent subtype of melanoma in Uyghur patients. It indicated that BRAF mutations and expression might serve as independent adverse prognostic factors in melanoma. Future studies can improve the diagnosis and prognosis of melanoma, hence benefiting the design of personalized treatment for patients.

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