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ORIGINAL ARTICLE

Prevalence of *BRAF* and *NRAS* mutations in cutaneous melanoma patients in Taiwan

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Background/Purpose: *BRAF* and *NRAS* mutations have been described in melanomas among Caucasians and some Asian populations. However, few large-scale studies have investigated the status and clinical significance of *BRAF* and *NRAS* mutations in a Taiwanese population.
Methods: Melanoma samples ($n = 119$) were analyzed for mutations in exons 11 and 15 of the *BRAF* gene, and in exons 1 and 2 of the *NRAS* gene. The samples were studied in genomic DNA, using polymerase chain reaction amplification and Sanger sequencing. Mutations of the *BRAF* and *NRAS* genes were then correlated with clinicopathological features and patients' prognosis.
Results: The incidence of somatic mutations within the *BRAF* and *NRAS* genes was 14.3% (17/119 patients) and 10.1% (12/119 patients), respectively. Among the 17 patients with *BRAF* mutations, 15 (88.2%) had V600E mutations. *BRAF* mutation was frequently detected in younger patients ($p = 0.0035$), in thin melanomas ($p = 0.0181$), and in melanomas with less ulceration ($p = 0.0089$). *NRAS* mutation was more often seen in patients with lymph node metastasis ($p = 0.0332$). Both *BRAF* and *NRAS* mutations were not significantly correlated with overall survival and disease-free survival.
Conclusion: As *BRAF* and *NRAS* mutations are rare in Taiwan, *BRAF*- or *NRAS*-targeted therapies may be effective only for selected Taiwanese melanoma patients.

Conflicts of interest: The authors have no conflicts of interest relevant to this article.

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Introduction

Cutaneous melanomas are categorized by the World Health Organization into the following four subtypes: acral lentiginous melanomas (ALMs), superficial spreading melanomas (SSMs), lentigo maligna melanomas (LMMs), and nodular melanomas (NMs).[1] *BRAF* and *NRAS* are the most frequently altered oncogenes in cutaneous melanomas. *BRAF* mutations are detected in approximately 50% and *NRAS* mutations in approximately 20% of tumors.[2] Genetic mutations in *BRAF* and *NRAS* have been correlated to the clinicopathological features and prognosis of patients with melanomas.[1–4] Of note, in a meta-analysis of 36 studies of different melanoma subtypes, *BRAF* mutation was frequently detected in SSMs and in melanomas arising in nonchronic sun-damaged (non-CSD) skin.[2,5] By contrast, *NRAS* mutation was frequently evident in NMs and in melanomas arising in skin with chronic sun damage (CSD).[2] These observations have been made based mainly on studies among Caucasian populations. Few similar large series of studies have been conducted to correlate the mutation status of *BRAF* and *NRAS* to clinicopathological features of melanoma in Taiwan.

The aim of the current study was to establish the frequency of *BRAF* and *NRAS* mutations in a series of melanomas from Taiwanese patients, and to correlate mutation status with various clinicopathological features and prognosis of these patients.

Materials and methods

Patients and tissues

A total of 119 patients with primary cutaneous melanomas and nine paired metastases diagnosed at the National Taiwan University Hospital, Taipei, Taiwan between January 1995 and November 2009 were enrolled in the study. All melanoma patients enrolled in the study provided written informed consent to use their resected tissues. This study was approved by the Research Ethics Committee of the National Taiwan University Hospital and was conducted according to the Declaration of Helsinki principles. The overall survival (OS) data were collected from chart reviews and the Taiwan Cancer Registry (follow-up persisted until December 2013 or until missed follow-up or death of the patient).

Mutation analysis of *BRAF* and *NRAS* genes in melanoma tissues

DNA was isolated from three consecutive 10-μm sections of each formalin-fixed, paraffin-embedded tissue sample. Genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocols. DNA concentration was quantified using an A 260 absorbance with an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany). Genomic DNA (50–100 ng/sample) was used as a template. The isolated DNA was used for real-time LightCycler polymerase chain reaction (LC PCR), with LightMix kit *BRAF* V600E (Roche Diagnostics, Indianapolis, IN, USA), a new assay method for *BRAF* mutation detection, and methylation-specific PCR analyses.[6] For *NRAS* mutation detection, exons 1 and 2 of the *NRAS* gene were amplified by PCR in at least two separate preparations of genomic DNA, as described previously.[3]

Statistical analysis

The data were summarized using descriptive statistics. Continuous data, such as age, were described using the mean ± standard deviation of the median (range) for normally distributed data. Pearson's Chi-square test was used to differentiate the rates of different groups. Survival probabilities were estimated using the Kaplan–Meier method and analyzed by log-rank tests. The influence of each variable on survival was assessed using a multivariate Cox proportional hazard model. All statistical tests were two sided, and $p \leq 0.05$ was considered statistically significant. All statistical analyses were carried out using SAS 9.2 software (SAS Inc., Cary, NC, USA).

Results

A total of 119 patients with primary cutaneous melanomas were recruited for this study. The participants included 66 men and 53 women, with a mean age of 62.1 years (median: 65 years; range: 1–89 years). The most common melanoma subtypes observed were ALMs (74.8%, 89/119), whereas NMs (12.6%, 15/119), SSMs (10.1%, 12/119), and LMMs (2.5%, 3/119) were less common. Ulceration was present in 33.6% of patients. The average thickness of the 119 samples was 3.94 mm, which was much thinner than the previous reports of samples from Chinese patients.[3] One hundred and five patients (88.2%) were followed for > 5 years or until their death. The median follow-up duration after diagnosis was 4.4 years (range: 0.1–18.6 years). The 5-year OS rate among the 119 melanoma patients was 52.5%.

BRAF mutations were found in 17 (14.3%) of the 119 primary cutaneous melanomas (Table 1). The mutation rate in men was 16.7% and that in women was 11.3%, suggesting no sex difference in the mutation rates. When mutations

Table 1 *BRAF* and *NRAS* mutations identified in 119 primary melanomas.

Gene	Exon	Nucleotide change	Amino acid change	No.	Subtype (*n*)
BRAF	15	1799T>A	V600E	15	ALM (6), SSM (6), NM (1), LMM (2)
	15	1798GT>AA	V600K	2	SSM (1), LMM (1)
NRAS	1	G38>A	G13D	2	ALM (2)
	2	A183>C	Q61H	2	ALM (1), NM (1)
	2	A182>T	Q61L	7	ALM (6), LMM (1)
	2	A821>G	Q61R	1	ALM (1)

ALM = acral lentiginous melanoma; LMM = lentigo maligna melanoma; NM = nodular melanoma; SD = standard deviation; SSM = superficial spreading melanoma.

were stratified by melanoma subtype, *BRAF* mutations were detected in 58.3% (7/12) of the SSM samples, 6.7% (6/89) of the ALM samples, 6.7% (1/14) of the NM samples, and 100% (3/3) of the LMM samples. Of *BRAF* mutation cases, 15 (88.2%) were found to harbor V600E and two (11.8%) V600K. In our cohort, patients with *BRAF* mutation were significantly younger than those without *BRAF* mutations at the time their melanoma was diagnosed ($p = 0.0035$, Table 2). Patients with *BRAF* mutation had thinner tumors and less ulceration at presentation than patients without *BRAF* mutation. *BRAF* mutation was observed in 47.1% of melanomas on non-CSD skin, 37.5% of melanomas on CSD skin, and 6.4% of ALMs. There were no differences in lymph node metastasis status and stage of melanoma between the patients with and without *BRAF* mutations.

NRAS mutations were detected in 12 patients (10.1%). Of *NRAS*-mutant cases, seven (58.3%) had Q61L, two (16.7%) Q61H, two (16.7%) had G13D, and one (8.3%) had Q61R mutations. *NRAS* Q61 was the predominant genetic alteration among patients with *NRAS* mutations (83.3%; 10/12), which is consistent with previously reported results.[7] Age was not significantly different between patients with and without *NRAS* mutations ($p = 0.2352$). No relationship was found between sex and *NRAS* mutations ($p = 0.6881$). The prevalence of > 4 mm thick tumors in *NRAS*-mutated melanomas was not significantly different from that in *NRAS* wild-type tumors (50% vs. 28%; $p = 0.1813$). The rate of ulceration was not significantly different between *NRAS* mutated and *NRAS* wild-type melanomas (58.3% vs. 30.8%; $p = 0.1025$). *NRAS* mutations were most frequently found in LMMs (1/3, 33.3%), followed by in NMs (2/15, 13.3%) and ALMs (9/89, 10.1%) (Table 2). *NRAS* mutations were not detected in SSMs. The difference in the incidence of *NRAS* mutations between the subtypes was not statistically significant. The incidence of *NRAS* mutations according to tumor site was highest in the extremities (11/12, 91.7%), followed by in the head and neck (1/12, 8.3%). *NRAS* mutations were not detected in melanomas located on the

Table 2 Association of *BRAF* and *NRAS* mutation status with patients and melanoma characteristics.

Clinicopathological factor	*BRAF* genotype, *n* (%)			*NRAS* genotype, *n* (%)		
	Mutation	Wild type	*p*	Mutation	Wild type	*p*
Age (y)			0.0035			0.2352
≥65	3 (5)	57 (95)		8 (13.3)	52 (86.7)	
<65	14 (23.7)	45 (76.3)		4 (6.8)	55 (93.2)	
Sex			0.4075			0.6881
Male	11 (16.7)	55 (83.3)		6 (9.1)	60 (90.9)	
Female	6 (11.3)	47 (88.7)		6 (11.3)	47 (88.7)	
Thickness (mm)			0.0181			0.1813
>4	1 (2.8)	35 (97.2)		6 (16.7)	30 (83.3)	
≤4	16 (19.3)	67 (80.7)		6 (7.2)	77 (92.8)	
Ulceration,			0.0089			0.1025
Present	1 (2.5)	39 (97.5)		7 (17.5)	33 (82.5)	
Absent	16 (20.3)	63 (79.8)		5 (6.3)	74 (93.7)	
Location			0.0365			1
Trunk	2 (33.3)	4 (66.7)		0 (0)	6 (100)	
Head and neck	3 (37.5)	5 (62.5)		1 (12.5)	7 (87.5)	
Extremity	12 (25)	93 (75)		11 (10.5)	94 (89.5)	
Subtypes			<0.0001			0.2526
ALM	6 (6.7)	83 (93.3)		9 (10.1)	80 (89.9)	
SSM	7 (58.3)	5 (41.7)		0 (0)	12 (100)	
NM	1 (6.7)	14 (93.3)		2 (13.3)	13 (86.7)	
LMM	3 (100)	0 (0)		1 (33.3)	2 (66.7)	
Sun-exposure pattern						
CSD	3 (37.5)	5 (62.5)	<0.0001	1 (12.5)	7 (87.5)	1
Non-CSD	8 (47.1)	9 (52.9)		1 (5.9)	16 (94.1)	
Acral	6 (6.4)	88 (93.6)		10 (10.6)	84 (89.4)	
Lymph node metastasis			0.5445			0.0332
Present	5 (17.9)	23 (82.1)		6 (21.4)	22 (78.6)	
Absent	12 (13.2)	79 (86.8)		6 (6.6)	85 (93.4)	
AJCC stage			0.3074			0.0598
I	7 (18)	32 (82.1)		1 (2.6)	38 (97.4)	
II	5 (10.6)	42 (89.4)		5 (10.6)	42 (89.4)	
III	5 (21.7)	18 (78.3)		3 (13)	20 (87)	
IV	0 (0)	10 (100)		3 (30)	7 (70)	

Data are presented as *n* (%).
ALM = acral lentiginous melanoma; CSD = melanoma of skin with chronic sun-induced damage; LMM = lentigo maligna melanoma; NM = nodular melanoma; Non-CSD = melanoma on skin without chronic sun-induced damage; SSM = superficial spreading melanoma.

Table 3 Univariate and multivariate analyses of risk factors associated with overall survival.

Clinicopathological factor	Univariate risk ratio (95% CI)	*p*	Multivariate risk ratio (95% CI)	*p*
Age (≥65 y)	1.01 (1–1.03)	0.1286		
Male sex	2.14 (1.25–3.63)	0.0052	2.00 (1.74–3.41)	0.0107
Thickness (>4 mm)	2.03 (1.32–3.36)	0.0055		
Ulceration	1.52 (0.91–2.53)	0.1101		
Lymph node metastasis	4.1 (2.43–6.93)	<.0001		
Stages III, IV	5.42 (3.23–9.09)	<.0001	5.22 (3.11–8.76)	<0.0001
BRAF mutation	0.69 (0.31–1.51)	0.3464		
NRAS mutation	1.67 (0.82–3.39)	0.1564		

CI = confidence interval.

trunk. *NRAS*-mutated melanomas show a propensity for developing on the extremities.[2,7] The *NRAS* mutation was found in 12.5% of CSD melanomas, 10.6% of cases with ALMs, and 5.88% of cases with non-CSD melanomas ($p = 1$). The lymph node metastasis rate in patients with *NRAS* mutations (50.0%) was significant higher ($p = 0.0332$) than that in patients without *NRAS* mutations (20.6%). These data suggest that patients harboring *NRAS* mutations were prone to having lymph node metastasis.

Two tumors carried mutations in both *BRAF* exon 15 and *NRAS* exon 2 (V600E in *BRAF* plus Q61H in *NRAS* of NM on the calf; V600K in *BRAF* plus Q61L in *NRAS* of LMM on the scalp), which was consistent with the recent reports, but contrary to the notion that *BRAF* and *NRAS* mutations are mutually exclusive.[3,8] One patient was found to have regional nodal metastasis on presentation and died after 1 year. Another patient died after 7.7 years.

The *BRAF* and *NRAS* genotypes of primary tumors and metastases were compared in donor-matched paired samples. All nine paired primary and metastatic melanomas (8 ALMs and 1 NM) were wild-type melanomas in this study.

Overall, the combined frequency of *BRAF* and *NRAS* mutations was 22.7% (27/119), with the highest mutation frequency being observed within the LMM subgroup (100%, 3/3). In the SSM, ALM, and NM subtypes, the combined frequencies of *BRAF* and *NRAS* mutations were 58.3% (7/12), 16.9% (15/89), and 13.3% (2/15), respectively.

This cohort included 56 (47.0%) patients who relapsed, six of whom (6/17; 35.3%) were in the *BRAF*-mutant group, nine (9/12; 75%) were in the *NRAS*-mutant group, and 42 (42/92, 45.7%) had no mutation. Sixty-three patients died: seven in the *BRAF*-mutant group (7/17, 41.2%) and nine in the *NRAS*-mutant group (9/12, 75%), and 49 had no mutation (49/92, 53.3%). As expected, the previously established prognostic factors in melanoma, such as male gender, tumor thickness, lymph node metastasis and stage, were significantly associated with the OS and disease-free survival (DFS) in melanomas in the univariate analysis (Tables 3 and 4). Multivariate Cox regression analysis showed that male gender and tumor stage were independent prognostic factors in OS (Table 3) and DFS (Table 4). *BRAF* or *NRAS* mutations showed no significant association with OS and DFS in our cohorts (Fig. 1). However, the number of samples with either mutation is too small to allow a firm conclusion about survival and mutation status.

Discussion

BRAF and *NRAS* mutations have been documented in all subtypes of melanomas.[3] However, most studies have been conducted in Caucasian populations. Thus, there are few guidelines for deciphering oncogenic differences and establishing proper treatment of melanomas in Asian populations.[7–9] In Caucasian populations, the major subtype of melanoma is SSM on intermittently sun-exposed areas.[2] By contrast, acral melanomas, which constitute a small proportion of melanomas in Caucasians, are the most prevalent melanoma subtypes in non-Caucasians, especially among Asians.[2,3,9–11] In Taiwan, ALMs comprise between 50% and 58% of cutaneous melanomas.[12] However, ALMs accounted for 74.9% (89/119) of all melanomas in this study. Since the study consisted of patients who were referred to a single medical center for treatment and had a relatively small sample size, it might have the possibility of referral bias

Table 4 Univariate and multivariate analyses of risk factors associated with disease-free survival.

Clinicopathological factor	Univariate risk ratio (95% CI)	*p*	Multivariate risk ratio (95% CI)	*p*
Age (≥65 y)	1.01 (1–1.03)	0.1591		
Male sex	2.04 (1.26–3.31)	0.0038	1.77 (1.09–2.89)	0.0213
Thickness (>4 mm)	2.05 (1.27–3.36)	0.0032		
Ulceration	1.19 (0.74–1.93)	0.4772		
Lymph node metastasis	4.76 (2.85–7.95)	<0.0001		
Stages III, IV	6.17 (3.71–10.25)	<0.0001	5.72 (3.44–9.52)	<0.0001
BRAF mutation	0.71 (0.35–1.43)	0.34		
NRAS mutation	1.84 (0.94–3.59)	0.0739		

CI = confidence interval.

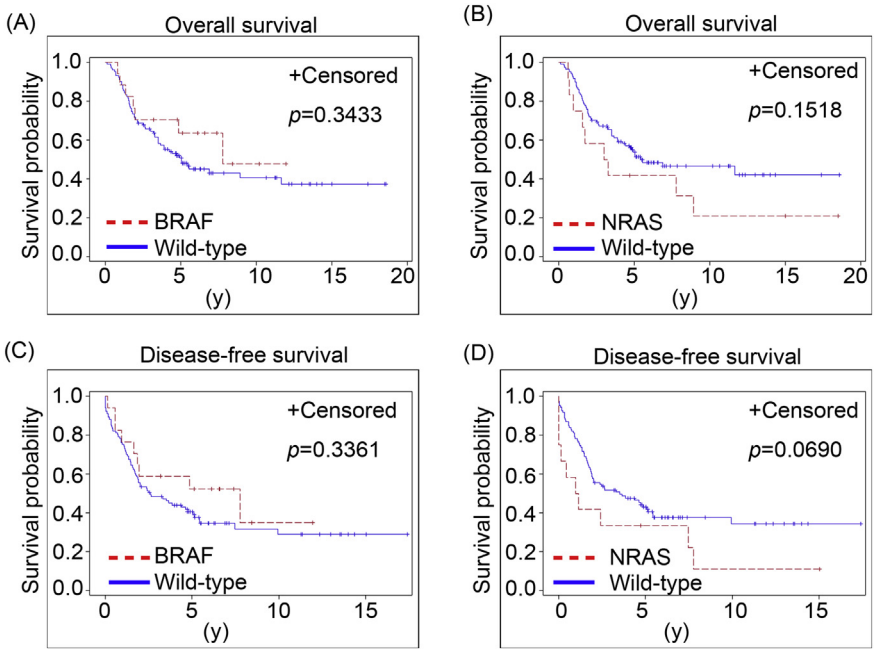

Figure 1 (A) Overall survival of patients according to the presence or absence of *BRAF* mutation (log-rank test, $p = 0.3433$). (B) Disease-free survival of patients according to the presence or absence of *BRAF* mutation (log-rank test, $p = 0.3361$). (C) Overall survival of patients according to the presence or absence of *NRAS* mutation (log-rank test, $p = 0.1518$). (D) Disease-free survival of patients according to the presence or absence of *NRAS* mutation (log-rank test, $p = 0.0690$).

and not be reflective of Taiwanese population as a whole, possibly explaining why the frequencies of ALM was higher than previous report.

BRAF mutations were detected in 14.3% (17/119) of melanoma in this study, a mutation rate similar to some previous reports from China, Korea, and Taiwan but lower than that observed in Japanese and Chinese studies (Table 5).[1,3,9,13–18] This may be due to different sample sizes, differences in the distribution of melanoma subtypes, and genetic predisposition.[3,9,14,15] The *BRAF* mutation rate in Yamazaki et al's[1] study was higher than that in other studies of Asian melanoma patients, which may be due to higher proportion of SSMs in their study population (43%). A number of studies have shown that ALMs have a lower *BRAF* mutation frequency than SSMs.[1] Recent studies have revealed that there exist site-specific genetic alterations in melanoma.[2,5,16] In this study, *BRAF* mutation was more often seen in the melanomas arising on the head, neck, and trunk than in those arising on the extremities. *BRAF* mutations are significantly more common in melanomas located on the skin with intermittent sun exposure than on the skin with chronic exposure or with relatively low or no sun exposure.[2,5] We also found that the incidence of *BRAF* mutation was lower in CSD and acral melanomas, as compared with that in non-CSD melanomas. Furthermore, *BRAF* mutation was detected in all LMMs (100%, 3/3), which was in disagreement with a previous study that showed that melanomas with *BRAF* mutations occur frequently on non-CSD skin.[2] The rate of NM seems to be lower than that in a previous report[12] and the *BRAF* mutation rate of NM is again much lower than that in other studies.[1] These disagreements may be due to relatively small sample size, and these results should be confirmed by future studies using larger numbers of patients. Bauer et al[19] have recently confirmed that patient age is independently associated with *BRAF* mutation frequency.[8,10] We can also observe that *BRAF* mutation inversely correlated with age, as demonstrated by the younger age in *BRAF*-mutation-positive patients. Some reports have shown that the *BRAF* mutation is associated with thinner tumor thickness and a lower rate of proliferation.[2,8,10,16,17] In this study, the proportion of thickness of $>$ 4 mm was lower in *BRAF*-mutant melanomas

Table 5 Summary of *BRAF* and *NRAS* mutations in primary cutaneous melanoma in Asians.

Refs	Total melanomas	*BRAF* mutation, % (*n*)	*NRAS* mutation, % (*n*)
Sasaki et al[15]	35	25.7 (9/35)	—
Qi et al[14]	180	15 (27/180)	—
Ashida et al[9]	79	25.3 (20/79)	—
Yamazaki et al[1]	79	41.8 (33/79)	—
Si et al[3]	432	25.5 (110/432)	7.2 (31/432)
Zhou et al[13]	86	16.3 (14/86)	10.5 (9/86)
Jin et al[17]	202	11.9 (24/202)	—
Hong et al[16]	36	19.4 (7/36)	—
Uhara et al[7]	102	—	7.8 (8/102)
Shen et al[18]	108	18.5 (20/108)	—
Current study	119	14.3 (17/119)	10.1 (12/119)
Total	1458	20.7 (281/1356)	8.1 (60/739)

— = not determined.

than that in wild-type tumors. Specifically, the ulceration rate was higher in melanomas without *BRAF* mutation in this study, which may be attributable to a particular aggressiveness or higher proportion of ALMs with later diagnosis.[20] We could not find any association of *BRAF* mutation with lymph node metastasis or stage of melanoma. A recent study showed that *BRAF* mutations were more frequent in late-stage ALMs than in early-stage ALMs.[1] However, there was little difference in the *BRAF* mutation frequency between late-stage (8.3%, 2/24) and early-stage (6.2%, 4/65) ALMs in this study. DFS and OS were not statistically different among *BRAF*-mutant and *BRAF*-wild-type melanomas in this study, which was comparable to the results of previous studies.[2,8,16,17]

The prevalence of *NRAS* mutations in melanomas of Taiwanese patients detected in our study was comparable to that of other East Asian populations (7.2—10.5%, Table 5).[3,7] However, the frequency of *NRAS* mutations was slightly lower than that of Caucasian patients (15—25%).[7] *NRAS* mutations were detected in 10.1% of ALMs, which was similar to the frequency reported in the Caucasian population (10%).[8] *NRAS* mutations were more common in the melanomas arising in the extremities, which was similar to the findings of previous reports, suggesting a possible relationship between *NRAS* mutations and exogenous skin stimuli.[2,7] Even though statistically insignificant, patients with *NRAS* mutation had a tendency to have thicker tumors, a higher ulceration rate, and older age at diagnosis. *NRAS* mutation was more frequently observed in melanomas with CSD.[2] However, the frequency of *NRAS* mutation was not associated with the sun-exposure pattern in this study. This result might be explained by the small sample size.

Results of one recent study stated that there was a trend for patients with *NRAS* mutation to present with a higher stage of tumor than patients with wild-type tumors.[8] In this study, patients presenting with late-stage disease also tend to carry *NRAS* mutations ($p = 0.0598$, Table 2). Furthermore, we noted that *NRAS*-mutation-positive patients show a significantly higher lymph node metastasis rate compared to patients without *NRAS* mutation ($p = 0.0332$, Table 2).[21] Oncogenic RAS induces alterations in cell—cell and cell—matrix interactions and the acquisition of a migratory phenotype that ultimately contributes to the metastatic process.[22] In addition, oncogenic RAS protects tumor cells from matrix-deprivation-induced apoptosis, or anoikis, thereby contributing to the cells' capacity to migrate through the circulatory system.[15,22,23] It is interesting to note, however, that in our study population, survival did not differ between patients whose primary tumors carried or did not carry *NRAS* mutations, even though the mutated tumors tended to show lymph node metastasis. However, we observed a trend for *NRAS*-mutant patients to have worse DFS than patients with wild-type melanomas. The statistical power was limited by a low prevalence of patients with *NRAS* mutations, and we had few relevant events for analysis in this study.

Our studies suggest that *BRAF* as well as *NRAS* mutations play a lesser role in the carcinogenesis of malignant melanoma in Taiwanese people than in Western patients[2] and that other genetic abnormalities might be involved in the development of malignant melanoma.[14] Furthermore, *BRAF* mutation was not associated with lymph node metastasis or stage of melanoma, while *NRAS* mutation was associated with higher lymph node metastasis.

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