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Tumour mutation status and sites of metastasis in patients with cutaneous melanoma

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Background: Cutaneous melanoma can metastasise haematogenously and/or lymphogenously to form satellite/in-transit, lymph node or distant metastasis. This study aimed to determine if *BRAF* and *NRAS* mutant and wild-type tumours differ in their site of first tumour metastasis and anatomical metastatic pathway.

Methods: Prospective cohort of patients with a histologically confirmed primary cutaneous melanoma at three tertiary referral centres in Melbourne, Australia from 2010 to 2015. Multinomial regression determined clinical, histological and mutational factors associated with the site of first metastasis and metastatic pathway.

Results: Of 1048 patients, 306 (29%) developed metastasis over a median 4.7 year follow-up period. 73 (24%), 192 (63%) and 41 (13%) developed distant, regional lymph node and satellite/in-transit metastasis as the first site of metastasis, respectively. *BRAF* mutation was associated with lymph node metastasis (adjusted RRR 2.46 95% CI 1.07–5.69, $P=0.04$) and sentinel lymph node positivity (adjusted odds ratio [aOR] OR 1.55, 95% CI 1.14–2.10, $P=0.005$). *BRAF* mutation and *NRAS* mutation were associated with increased odds of developing liver metastasis (aOR 3.09, 95% CI 1.49–6.42, $P=0.003$; aOR 3.17, 95% CI 1.32–7.58, $P=0.01$) and central nervous system (CNS) metastasis (aOR 4.65, 95% CI 2.23–9.69, $P<0.001$; aOR 4.03, 95% CI 1.72–9.44, $P=0.001$). *NRAS* mutation was associated with lung metastasis (aOR 2.44, 95% CI 1.21–4.93, $P=0.01$).

Conclusions: *BRAF* mutation was found to be associated with lymph node metastasis as first metastasis and sentinel lymph node positivity. *BRAF* and *NRAS* mutations were associated with CNS and liver metastasis and *NRAS* mutation with lung metastasis. If these findings are validated in additional prospective studies, a role for heightened visceral organ surveillance may be warranted in patients with tumours harbouring these somatic mutations.

Recent advances in melanoma treatment have led to more intensive surveillance of high-risk patients as there is evidence to suggest that certain treatments are more effective in patients with

low volume metastatic disease (Hodi *et al*, 2010; Sosman *et al*, 2012; Ribas *et al*, 2016). An improved understanding of the pathways of metastatic disease, including the clinicopathological

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factors that influence these pathways, is important to improve surveillance strategies and to individualise follow-up of high-risk patients.

Cutaneous melanoma can metastasise haematogenously and/or by the lymphatic system (Mervic, 2012). Cutaneous melanoma can metastasise as satellite or in-transit metastasis, lymph node metastasis or distant metastasis (Meier *et al*, 2002; Leiter *et al*, 2004). Satellite metastasis has been defined as the development of metastatic nodules within two centimetres of the primary tumour, while in-transit metastasis has been defined as the development of metastasis within the dermal and subdermal lymphatics in the drainage area before the first regional lymph node basin (Meier *et al*, 2002; Leiter *et al*, 2004). Satellite, in-transit and regional lymph node metastasis represents locoregional disease. Approximately two-thirds of patients who develop metastasis initially present with locoregional disease, while one-third present with distant metastasis (Reintgen *et al*, 1992; Soong *et al*, 1998; Cohn-Cedermark *et al*, 1999; Meier *et al*, 2002; Tejera-Vaquero *et al*, 2007). Nonetheless, there has been limited research investigating the clinicopathological factors associated with the pathways of progression in patients with primary cutaneous melanoma.

It is well-recognised that 40–50% and 15% of cutaneous melanomas harbour activating mutations of *BRAF* and *NRAS*, respectively (Smalley, 2003; Hocker & Tsao, 2007; Liu *et al*, 2007; Devitt *et al*, 2011; Long *et al*, 2011). Mutations in *BRAF* and *NRAS* oncogenes are associated with distinct phenotypic and histopathological characteristics (Ellerhorst *et al*, 2011; Long *et al*, 2011; Colombino *et al*, 2012; Hodis *et al*, 2012; Barbour *et al*, 2014). The relationship between tumour mutation status and the metastatic pathways of progression is not yet understood.

The primary aim of this study was to determine if *BRAF* and *NRAS* mutant tumours compared to wild-type tumours have a propensity to metastasise as satellite/in-transit, regional lymph node or distant metastasis as the first site of metastasis and if these tumours behave differently in their anatomical metastatic pathways. We also aimed to investigate the relationship between clinicopathological characteristics and the anatomical pathways of disease progression. A secondary aim was to determine if the time course to the development of distant metastasis depends on these anatomical metastatic pathways.

MATERIALS AND METHODS

This was a prospective cohort study of participants in the Melbourne Melanoma Project (MMP). Patients referred to one of three tertiary referral centres in Melbourne, Australia (Victorian Melanoma Service at The Alfred Hospital, Peter MacCallum Cancer Centre and the Olivia Newton-John Cancer Research Institute at the Austin Hospital) with a histologically confirmed primary cutaneous melanoma were eligible for enrolment in the MMP. Patients were enrolled within 6 months of presentation to the abovementioned institutions between 2010 and 2015. The majority of patients (84%) had stage I/II disease at diagnosis. Patients with uveal melanoma, mucosal melanoma or melanoma of unknown primary site were excluded. Patients with multiple invasive primary melanomas and patients with *in situ* melanoma were excluded. Institutional ethics approval was obtained from the contributing sites (project number 07/38). Written and verbal consent was obtained from all patients.

Clinical, pathological and molecular characteristics were prospectively recorded. The primary melanomas of 73% of all patients enrolled in MMP were tested for the presence of a *BRAF* and *NRAS* mutation. Patients without *BRAF* and *NRAS* mutation testing were excluded. Mutational testing was performed at the Department of Anatomical Pathology, Alfred Hospital, Melbourne, Australia or

the Department of Diagnostic Molecular Pathology, Peter MacCallum Cancer Centre, Melbourne, Australia. Hematoxylin and eosin-stained sections of formalin-fixed, paraffin-embedded tissue were reviewed by a pathologist, followed by macrodissection to ensure the percentage of tumour cells was enriched to at least 30%. DNA was then extracted from each sample and checked for adequate concentration. Matrix assisted laser desorption ionisation time-of-flight mass spectrometry was used for mutational analyses. DNA quality was evaluated via Eppendorf spectrophotometer. The sample was checked for multiple known mutations in *BRAF* (exon 11 and 15), *NRAS* (exon 2, 3 and 4) and *KIT* (exon 11, 13 and 17) using Sequenom (Agena) Mass ARRAY OncoFocus panel (Version 3).

Clinical variables recorded by the treating doctor at the patients' initial presentation included: age, sex, phenotypic markers (eye colour, hair colour and skin phototype) and personal history of melanoma.

The tumour characteristics that were collected in MMP included: date of diagnosis, anatomical location of the primary tumour, Breslow thickness (mm), Clark level, histologic subtype, mitotic rate (n/mm^2) and ulceration. Tumour histologic subtype was classified according to the current World Health Organization classification system. We included patients with superficial spreading melanoma (SSM), nodular melanoma (NM) and lentigo maligna melanoma (LMM). Patients with acral lentiginous melanoma, desmoplastic melanoma and other less common subtypes, including naevoid, balloon cell, spindle cell and Spitzoid melanoma, were excluded. The anatomical location of the primary tumour was classified as upper extremity, lower extremity, head and neck region, or trunk.

Patients were followed up as per routine care by one of the tertiary institutions listed above or by community doctors (i.e., general practitioners or specialists), depending on their stage and disease progression. Postal questionnaires seeking information on disease recurrence were sent to community doctors annually. Patients' disease progression was prospectively recorded. The date and site of detected metastasis and the mode of detection (clinical or radiological) were recorded. Radiologic detection included computed tomography, positron emission tomography, magnetic resonance imaging or ultrasound. The details were recorded for the initial site of metastasis as well as for all subsequent recurrences. The date of death was recorded for all participant deaths and the cause of death was recorded as either due to melanoma, another malignancy or other cause. Notification of death was from community doctors, hospital medical records, 'deceased, return to sender' letters or family correspondence.

Four distinct metastatic routes to the development of distant metastasis were used in our analysis, which have been described by Meier *et al* (2002). These included: 1-development of satellite or in-transit metastases followed by regional lymph node metastases and distant metastases, 2-development of satellite or in-transit metastases followed by distant metastases, 3-development of regional lymph node metastases followed by distant metastases and 4-development of distant metastases as first tumour recurrence (Meier *et al*, 2002).

All statistical analyses were performed using Stata version 14.2 (StataCorp LP, College Station, TX, USA) statistical software. Breslow thickness was analysed as a categorical variable (<1.00 mm, 1.0–2.0 mm, 2.01–4.0 mm, >4.01 mm) and age was dichotomised as less than or greater than 50 years. Univariate and multivariate multinomial regression analyses were conducted to compare various clinical and pathological variables between patients with *BRAF* mutations, *NRAS* mutations and *BRAF*/*NRAS* wild-type tumours with the associations summarised as relative risk ratios (RRR) and 95% confidence intervals (CI). Univariate and multivariate multinomial regression analyses were conducted to describe associations of clinical, histological and mutational

factors with the site of first metastasis and the anatomical pathways of progression. Logistic regression was used to assess associations of various clinicopathological characteristics with *BRAF* V600E and V600K mutational subtypes and with sentinel lymph node positivity, summarised as odds ratios (OR). Statistical significance was defined as a *p*-value less than 0.05. Melanoma-specific survival (MSS) was compared between patients with *BRAF* mutant, *NRAS* mutant and wild-type tumours. Multivariate Cox proportional hazards regression was performed to estimate associations with survival.

RESULTS

The MMP cohort included 1955 patients with clinical and histologic data who had a new diagnosis of cutaneous melanoma between 2010 and 2015. We excluded 192 patients with a single primary *in situ* melanoma, 219 patients with multiple invasive primary melanomas and 106 patients with less common melanoma subtypes. Of the remaining 1438 patients, 390 patients were excluded as they did not have *BRAF* and *NRAS* mutation testing of their tumours. Thus, 1048 patients were included in the analyses for this study.

Descriptive statistics. The median age at diagnosis was 58 years (range 20–90 years) and 58% of participants were male. Further, 360 (34.7%) melanomas were located the trunk, 251 (24.2%) on the upper extremity, 220 (21.2%) on the head and neck and 206 (19.9%) on the lower extremity. The median Breslow thickness was 1.6 mm (IQR 0.8–3.0 mm). The median mitotic rate was 2 mitoses per mm² (IQR 0–6 per mm²) and 28% of tumours were ulcerated.

Tumour mutation frequencies. Among the 1048 primary melanomas, 48.6% were *BRAF* mutant, 19.0% were *NRAS* mutant and 32.4% were *BRAF/NRAS* wild type. Among the *BRAF* mutant tumours, the most common genotype was V600E (70.0%), followed by V600K (24.2%) and less common genotypes (5.8%). The majority (93.2%) of *NRAS* mutant tumours had an *NRAS* codon 61 mutation.

Tumour mutation status and clinicopathological correlations. Clinical and pathological characteristics are described by *BRAF* and *NRAS* mutation status in Table 1 with corresponding estimates of associations presented in Table 2. Median age differed between patients with *BRAF* mutant, *NRAS* mutant and *BRAF/NRAS* wild-type tumours (53 vs 62 vs 61 years, respectively). Even when adjusted for other factors, compared to those aged >50 years, patients aged <50 years had 2.48-fold higher relative risk of having a *BRAF* mutant tumour than a *BRAF/NRAS* wild-type tumour and a 3.59-fold higher relative risk of having a *BRAF* mutant tumour than a *NRAS* mutant tumour (RRR 2.48, 95% CI 1.82–3.38, *P*<0.001; RRR 3.59, 95% CI 2.39–5.41, *P*<0.001, respectively). Compared to *BRAF/NRAS* wild-type tumours, *BRAF* mutation was associated with truncal location and superficial spreading subtype after adjustment for other variables (Tables 1 and 2).

Compared to *BRAF* mutations, *NRAS* mutations were more common in tumours on the upper extremities (adjusted RRR (aRRR) 2.38, 95% CI 1.38–4.10, *P*=0.002) and lower extremities (aRRR 1.77 95% CI 1.02–3.09, *P*=0.04) than the head and neck region.

Compared to patients with *BRAF* V600E mutant tumours, patients with *BRAF* V600K mutant tumours had an increased odds of being male (OR 2.04, 95% CI 1.33–3.19, *P*<0.001), older (OR ≥50 years 6.38, 95% CI 3.78–10.79, *P*<0.001) and having tumours located on the head and neck region compared to the trunk (RR 2.45, 95% CI 1.42–4.22, *P*=0.001). When adjusted for

Table 1. Clinical and pathological characteristics according to *BRAF* and *NRAS* mutation status

Clinicopathological variable	<i>BRAF</i> mutant (%)	<i>NRAS</i> mutant (%)	WT/WT (%)
Total number	509 (48.6)	199 (19.0)	340 (32.4)
Patient sex			
Males	278 (45.4)	118 (19.3)	216 (35.3)
Females	231 (53.0)	81 (18.6)	124 (28.4)
Patient age			
<50 years	216 (65.8)	34 (10.4)	78 (23.8)
≥50 years	290 (40.6)	164 (23.0)	260 (36.4)
Anatomical location			
Head & neck	94 (42.7)	25 (11.4)	101 (45.9)
Trunk	207 (57.5)	60 (16.7)	93 (25.8)
Upper extremity	98 (39.0)	62 (24.7)	91 (36.3)
Lower extremity	104 (50.5)	49 (23.8)	53 (25.7)
Breslow thickness			
<1.0 mm	175 (50.7)	37 (10.7)	133 (38.6)
1.00–2.0 mm	142 (51.1)	71 (25.5)	65 (23.4)
2.01–4.0 mm	113 (43.6)	62 (23.9)	84 (32.4)
>4.01 mm	73 (47.7)	26 (17.0)	54 (35.3)
Histologic subtype			
SSM	346 (52.3)	125 (18.9)	190 (38.7)
NM	129 (46.6)	57 (20.6)	91 (32.9)
LMM	14 (23.3)	5 (8.3)	41 (68.3)
Mitotic rate (n/mm ²)			
<5	334 (48.9)	119 (17.4)	230 (33.7)
5–9	92 (47.9)	45 (23.4)	55 (28.7)
≥10	78 (48.2)	32 (19.8)	52 (32.1)
Ulceration			
No	346 (48.3)	130 (18.2)	240 (33.5)
Yes	147 (50.3)	60 (20.6)	85 (29.1)

Abbreviations: LMM = lentigo maligna melanoma; NM = nodular melanoma; SSM = superficial spreading melanoma; WT = wild type.

confounders, the relationship between *BRAF* V600K mutation and older age (aOR 5.93, 95% CI 3.43–10.27, *P*<0.001) and with head and neck location (aOR 2.16, 95% CI 1.16–4.01, *P*=0.015) remained. The association between V600K mutation and male sex was partially accounted for by other factors (aOR 1.41, 95% CI 0.86–2.33, *P*=0.17).

BRAF V600K mutant tumours, compared to V600E tumours, had an increased odds of being thick tumours than thin tumours (OR 2.39, 95% CI 1.27–4.50, *P*=0.007) and having a high mitotic rate (OR ≥10/mm² compared to <5/mm² 2.05 95% CI 1.19–3.54, *P*=0.01). The relationship between V600K mutation and greater tumour thickness (aOR thick compared to thin tumours 1.69, 95% CI 0.74–3.84, *P*=0.2) and mitotic rate (aOR ≥10/mm² compared to <5/mm² 1.41 95% CI 0.71–2.80, *P*=0.3) were partially explained by other factors. There was no evidence to suggest that ulceration was related to *BRAF* V600 subtype (univariate OR V600K 1.18, 95% CI 0.75–1.86, *P*=0.5).

Sentinel lymph node and tumour mutation status. Among the 690 patients who were eligible for sentinel lymph node biopsy (SLNB), 426 (62%) underwent this procedure and 119 (28%) were positive for metastatic melanoma. Among patients with a positive SLNB, 62% were *BRAF* mutant. After adjusting for age, sex, mitotic rate, ulceration, Breslow thickness, histologic subtype and anatomical site of primary tumour, *BRAF* mutant tumours had 1.55 times increased odds (aOR 1.55, 95% CI 1.14–2.10, *P*=0.005) of having a positive SLNB compared to *BRAF/NRAS* wild-type tumours. There were similar odds of SLNB positivity between *NRAS* mutant and *BRAF/NRAS* wild-type tumours (aOR 1.06, 95% CI 0.73–1.56, *P*=0.8).

Table 2. RRR describing clinical and pathological correlates of BRAF mutation estimated in univariate and multivariate multinomial regression analyses

Clinicopathological variable	BRAF mutant vs BRAF/NRAS wild-type ^a						BRAF mutant vs NRAS mutant ^b					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	RRR	95% CI	P-value	RRR	95% CI	P-value	RRR	95% CI	P-value	RRR	95% CI	P-value
Patient sex												
Male	1.00			1.00			1.00			1.00		
Female	1.45	1.09–1.92	0.01	1.21	0.88–1.67	0.24	1.21	0.87–1.69	0.3	1.30	0.89–1.91	0.18
Patient age												
<50 years	1.00			1.00			1.00			1.00		
≥50 years	0.40	0.30–0.55	<0.001	0.48	0.34–0.67	<0.001	0.28	0.18–0.42	<0.001	0.29	0.19–0.45	<0.001
Anatomical location												
Head & neck	1.00			1.00			1.00			1.00		
Trunk	2.39	1.65–3.47	<0.001	2.14	1.41–3.24	<0.001	0.92	0.54–1.55	0.8	0.77	0.43–1.36	0.4
Upper extremity	1.16	0.78–1.73	0.5	1.06	0.68–1.66	0.8	0.42	0.24–0.72	0.002	0.38	0.21–0.69	0.002
Lower extremity	2.11	1.37–3.26	0.001	1.66	1.02–2.71	0.04	0.56	0.32–0.98	0.04	0.41	0.22–0.77	0.005
Breslow thickness												
<1.0 mm	1.00			1.00			1.00			1.00		
1.00–2.0 mm	1.66	1.15–2.40	0.007	1.69	1.12–2.53	0.011	0.42	0.27–0.67	<0.001	0.36	0.22–0.59	<0.001
2.01–4.0 mm	1.02	0.71–1.47	0.9	1.23	0.80–1.88	0.3	0.39	0.24–0.62	<0.001	0.39	0.23–0.66	<0.001
>4.0 mm	1.03	0.68–1.56	0.9	1.39	0.83–2.35	0.22	0.59	0.34–1.05	0.07	0.58	0.31–1.13	0.11
Histologic subtype												
SSM	1.00			1.00			1.00			1.00		
NM	0.78	0.56–1.07	0.13	0.88	0.60–1.31	0.5	0.82	0.56–1.19	0.3	1.15	0.75–1.78	0.5
LMM	0.19	0.10–0.35	<0.001	0.33	0.17–0.65	0.001	1.01	0.36–2.87	1.0	1.18	0.40–3.49	0.8
Mitotic rate (n/mm²)												
<5	1.00						1.00					
5–9	1.15	0.79–1.67	0.5				0.73	0.48–1.10	0.13			
≥10	1.03	0.70–1.52	0.9				0.87	0.55–1.38	0.6			
Ulceration												
No	1.00						1.00					
Yes	1.20	0.88–1.64	0.3				0.92	0.64–1.32	0.7			

Abbreviations: CI = confidence interval; LMM = lentigo maligna melanoma; NM = nodular melanoma; RRR = relative risk ratio; SSM = superficial spreading melanoma.
^aBRAF/NRAS wild-type as the reference category.
^bNRAS mutant as the reference category.
 Multivariate analysis adjusted for patient sex, patient age, anatomical location of the primary tumour, Breslow thickness and histologic subtype. Mitotic rate and ulceration were excluded as there were not found to be associated with mutation status in univariate analyses.

Site of first detected metastasis. Over a median 4.7 year follow-up period (IQR 3.6–5.9 years), 306 (29%) patients developed metastasis. Of these, 73 (24%), 192 (63%) and 41 (13%) patients developed distant, regional lymph node and satellite/in-transit metastasis as the first site of metastasis, respectively. Among the 192 patients who developed lymph node metastasis as the site of first metastasis, 119 (62%) were detected by a positive SLNB and the remainder were detected either clinically or radiologically. Excluding lymph node metastases detected by a tumour-positive SLNB, the mode of detection for first metastasis was radiological in 47% and clinical in 53%. The mode of detection was more commonly radiological than clinical for distant metastases (78% vs 22%, respectively) and vice versa for satellite/in-transit metastases (15% vs 85%, respectively).

Clinicopathological characteristics associated with the site of first metastasis. Table 3 displays the site of first metastasis by various patient- and tumour-related characteristics. In the multivariate regression model, patients with tumours located on the head and neck region had a 2.80 times increased risk compared to patients with truncal tumours of developing satellite/in-transit metastasis rather than lymph node metastasis (RRR 2.80, 95% CI 1.04–7.52, *P* = 0.04). In multivariate analysis, there was weak evidence to suggest that females were more likely than males to develop satellite/in-transit metastasis rather than distant metastasis as first metastasis (RRR 2.09, 95% CI 0.88–4.98, *P* = 0.1).

Tumour mutation status and the site of first detected metastasis. The site of first metastasis was similar in both NRAS mutant and BRAF/NRAS wild-type mutant groups (Table 4). BRAF mutant tumours, compared to BRAF/NRAS wild-type tumours, had increased risk of developing regional lymph node metastasis rather than satellite/in-transit metastasis as first metastasis (univariate RRR 3.24, 95% CI 1.49–7.08, *P* = 0.003). This increased risk remained statistically significant after adjustments (RRR 2.46 95% CI 1.07–5.69, *P* = 0.04; Table 4). BRAF mutant tumours had increased risk of developing regional lymph node metastasis rather than distant metastasis as the site of first metastasis; however, this increased risk was not statistically significant (RRR 1.32, 95% CI 0.70–2.49, *P* = 0.4).

Metastatic pathways and mortality in patients with primary cutaneous melanoma. Among the 306 patients who developed metastasis, 174 developed distant metastatic disease. The proportions of patients who followed each of the four distinct metastatic pathways are displayed in Figure 1.

During the study period, 134 patients died. Of these, 103 died from metastatic melanoma, 3 from another malignancy, 10 from other causes and 18 from unknown causes. Of the 103 patients who died from metastatic melanoma, 24.3% of tumours had an NRAS mutation, 59.2% had a BRAF mutation and 16.5% had BRAF/NRAS wild-type tumours.

In univariate analysis, both patients with BRAF mutant and NRAS mutant melanomas had a worse MSS than patients with

BRAF/*NRAS* wild-type tumours (hazard ratio (HR) 2.46 95% CI 1.43–4.20, $P=0.001$; HR 2.70, 95% CI 1.46–5.00, $P=0.002$, respectively). When adjusted for age, sex, ulceration, Breslow thickness, histologic subtype and mitotic rate, the relationship between *BRAF* mutation and MSS was slightly strengthened (HR 2.95, 95% CI 1.64–5.29, $P<0.001$). After adjustments, patients with *NRAS* mutant melanomas had a worse MSS than patients with *BRAF*/*NRAS* wild-type melanomas (HR 3.08, 95% CI 1.56–6.08, $P=0.001$).

Clinicopathological and molecular characteristics and the anatomical metastatic pathways. There was little evidence to suggest that the pathways of progression to distant disease differed by mutation status. Similarly, patient age and sex, ulceration, Breslow thickness, mitotic rate, histologic subtype and tumour

location were not associated with the four metastatic pathways (Supplementary analyses).

Tumour mutation status and the patterns of organ involvement. With respect to patients who developed central nervous system (CNS) metastasis, 50/77 (65%) had a *BRAF* mutant tumour and 17/77 (22%) had a *NRAS* mutant tumour. *BRAF* mutation and *NRAS* mutation were associated with increased odds of developing CNS metastasis compared to *BRAF*/*NRAS* wild-type tumours (*BRAF* aOR 4.65, 95% CI 2.23–9.69, $P<0.001$; *NRAS* aOR 4.03, 95% CI 1.72–9.44, $P=0.001$).

Among patients who developed liver metastasis, 40/65 (62%) had a *BRAF* mutant tumour and 14/65 (21%) had an *NRAS* mutant tumour. There was evidence to suggest that the presence of either a *BRAF* or *NRAS* mutation was associated with an increased odds of developing liver metastasis compared to *BRAF*/*NRAS* wild-type tumours (*BRAF* aOR 3.09, 95% CI 1.49–6.42, $P=0.003$; *NRAS* aOR 3.17, 95% CI 1.32–7.58, $P=0.01$). Among patients who developed lung metastasis, 50/98 (51%) had a *BRAF* mutant tumour and 23/98 (24%) had a *NRAS* mutant tumour. While there was evidence to suggest that *NRAS* mutation was associated with lung metastasis (aOR 2.44, 95% CI 1.21–4.93, $P=0.013$), there was uncertainty regarding an association between *BRAF* mutation and the development of lung metastasis (aOR 1.78, 95% CI 0.98–3.25 $P=0.06$).

Time course to the development of detected first metastasis. The median time to satellite/in-transit, regional lymph node (excluding patients with a tumour-positive SLNB) and distant metastasis as the site of first detected metastasis was 17.0 months (IQR 5.3–30.3 months), 16.1 months (IQR 7.1–27.7 months) and 14.9 months (IQR 6.5–26.9 months), respectively ($P=0.09$). Excluding patients with a tumour-positive SLNB, the overall median time to first metastasis was 14.7 months (IQR 5.9–25.4 months). Excluding patients with a tumour-positive SLNB, the median time to first metastasis was shorter in patients with *BRAF* mutant tumours (12.5 months, IQR 5.0–22.5 months) compared to *NRAS* mutant (13.4 months, IQR 5.6–21.7) and *BRAF*/*NRAS* wild-type tumours (18.1 months, IQR 7.4–32.3 months) ($P=0.14$).

Time course to the development of distant metastasis by the different metastatic pathways. The median time to distant metastasis was similar among the four metastatic pathways (Table 5). The median time to distant metastasis was similar among patients with *BRAF* mutant (15.0 months (IQR 8.5–26.4)) *NRAS* mutant (16.2 months (IQR 10.8–25.1)) and *BRAF*/*NRAS* wild-type (17.2 months [IQR 11.5–29.2]) tumours ($P=0.7$).

Table 3. Patient- and tumour-related characteristics and the site of first tumour metastasis

Patient and tumour-related characteristics	Site of first tumour metastasis (%)				P-value
	Distant metastasis	Lymph node metastasis	Satellite/in-transit metastasis		
Patient sex					
Males	52 (25.9)	126 (62.7)	23 (11.4)		0.3
Females	21 (20.0)	66 (62.9)	18 (17.1)		
Patient age					
< 50 years	16 (19.8)	59 (72.8)	6 (7.4)		0.07
≥ 50 years	56 (25.0)	133 (59.4)	35 (15.6)		
Anatomical location					
Head & neck	27 (32.5)	42 (50.6)	14 (16.9)		0.04
Trunk	25 (22.7)	77 (70.0)	8 (7.3)		
Upper extremity	7 (14.3)	32 (65.3)	10 (20.4)		
Lower extremity	12 (20.3)	38 (64.4)	9 (15.3)		
Breslow thickness					
< 1.0 mm	7 (22.6)	18 (58.1)	3 (19.4)		0.6
1.0–2.0 mm	20 (24.4)	56 (68.3)	6 (7.3)		
2.01–4.0 mm	24 (21.6)	71 (64.0)	16 (14.4)		
> 4.0 mm	20 (25.6)	45 (57.7)	13 (16.7)		
Histologic subtype					
SSM	36 (21.8)	110 (66.7)	19 (11.5)		0.11
NM	26 (23.9)	65 (59.6)	18 (16.5)		
LMM	6 (40.0)	5 (33.3)	4 (26.7)		
Mitotic rate (n/mm²)					
< 5	31 (21.7)	91 (63.4)	21 (14.7)		0.6
5–9	17 (22.7)	51 (68.0)	7 (9.3)		
> 10	23 (28.1)	47 (57.3)	12 (14.6)		
Ulceration					
Yes	36 (21.4)	108 (64.3)	24 (14.3)		0.8
No	32 (24.8)	80 (62.0)	17 (13.2)		

Abbreviations: LMM = lentigo maligna melanoma; NM = nodular melanoma; SSM = superficial spreading melanoma.

DISCUSSION

Of the 1048 patients included in our analysis, 306 (29%) developed metastasis during the study period and among these, 24%, 63% and

Table 4. Univariate and multivariate multinomial regression analyses of tumour mutation status and the site of first metastasis

Mutation status	Site of first metastasis		Univariate analysis						Multivariate analysis ^a					
	S/IT		LN		D		LN vs S/IT ^b		D vs S/IT ^b		LN vs S/IT ^b		D vs S/IT ^b	
	n	%	n	%	n	%	RRR (95% CI)	P-value	RRR (95% CI)	P-value	RRR (95% CI)	P-value	RR (95% CI)	P-value
<i>BRAF</i> mutant	14	8	111	67	40	24	3.24 (1.49–7.08)	0.003	2.45 (1.02–5.88)	0.05	2.46 (1.07–5.69)	0.04	2.14 (0.84–5.48)	0.11
<i>NRAS</i> mutant	9	16	37	64	12	21	1.68 (0.68–4.19)	0.3	1.14 (0.39–3.33)	0.8	1.39 (0.52–3.72)	0.5	1.28 (0.41–4.03)	0.7
<i>BRAF</i> / <i>NRAS</i> WT	18	22	44	53	21	25	1.00		1.00		1.00		1.00	

Abbreviations: CI = confidence interval; D = distant; LN = lymph node; RRR = relative risk ratio; S/IT = satellite/in-transit; WT = wild type.
^aMultivariate analysis was adjusted for patient sex, patient age, anatomical location of the primary tumour and histologic subtype.
^bSatellite/in-transit metastasis as the reference category.

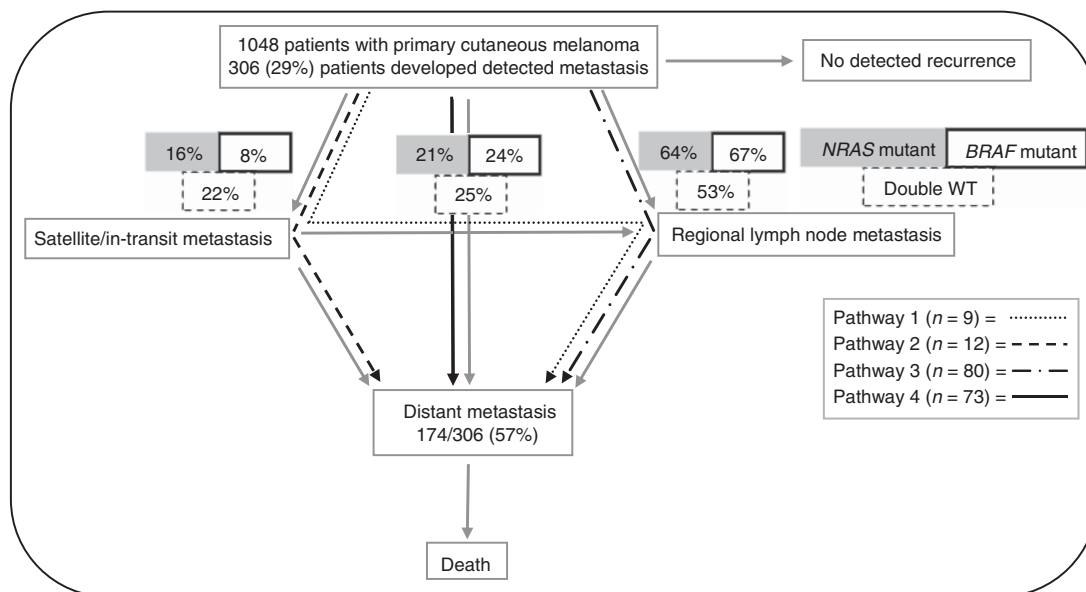


Figure 1. Metastatic pathways in patients with primary cutaneous melanoma. Figure adapted from Meier *et al* (2002). The metastatic pathways of progression: 1-development of satellite or in-transit metastases followed by regional lymph node metastases and distant metastases, 2-development of satellite or in-transit metastases followed by distant metastases, 3-development of regional lymph node metastases followed by distant metastases and 4-development of distant metastases as first tumour recurrence.

Table 5. Time to distant metastasis in patients with primary cutaneous melanoma by four different metastatic pathways

Metastatic pathway	Median time to distant metastasis (months)	IQR (months)	P-value
Pathway 1 Development of satellite or in-transit metastases followed by regional lymph node metastases and distant metastases	15	(9, 17)	0.4
Pathway 2 Development of satellite or in-transit metastases followed by distant metastases	16	(7, 26)	-
Pathway 3 Development of regional lymph node metastases followed by distant metastases	18	(10, 27)	-
Pathway 4 Development of distant metastases as first tumour recurrence	15	(7, 28)	-
Total	16	(9, 26)	-

Abbreviation: IQR = interquartile range.

13% developed distant, regional lymph node and satellite/in-transit metastasis as the site of first metastasis, respectively. These findings are consistent with the limited available literature, which suggests that 15–35% of patients with primary cutaneous melanoma exhibit disease progression and of these patients, approximately two-thirds initially present with loco-regional disease and one-third present with distant metastasis (Reintgen *et al*, 1992; Soong *et al*, 1998; Cohn-Cedermark *et al*, 1999; Meier *et al*, 2002; Tejera-Vaquero *et al*, 2007).

Meier *et al*'s (2002) landmark study traced the metastatic pathways of 3001 patients with primary cutaneous melanoma from 1976 to 1996. Among these patients, 466 developed recurrence and

of these, 28%, 50% and 22% developed distant, lymph node and satellite/in-transit metastasis as the site of primary recurrence (Meier *et al*, 2002). In our cohort, the proportion of patients who developed regional lymph metastasis as the site of first metastasis was higher than previously described, likely due to the increased use of SLNB in a substantial fraction of patients at the institutions included in our study. The use of SLNB was not routine at the time of Meier *et al*'s study (Meier *et al*, 2002) or at the time of other previous studies investigating the metastatic pathways of patients with cutaneous melanoma (Reintgen *et al*, 1992; Soong *et al*, 1998; Cohn-Cedermark *et al*, 1999; Tejera-Vaquero *et al*, 2007).

Consistent with the existing literature (Maldonado *et al*, 2003; Lang & MacKie, 2005; Edlundh-Rose *et al*, 2006; Poynter *et al*, 2006; Liu *et al*, 2007; Thomas *et al*, 2007; Viros *et al*, 2008; Broekaert *et al*, 2010; Hacker *et al*, 2010; Bauer *et al*, 2011; Devitt *et al*, 2011; Lee *et al*, 2011; Long *et al*, 2011; Menzies *et al*, 2012; Ekedahl *et al*, 2013; Barbour *et al*, 2014; Carlino *et al*, 2014; Kim *et al*, 2015; Thomas *et al*, 2015), our study found that *BRAF* positivity is associated with younger age and superficial spreading subtype. In the multivariate regression analysis, *BRAF* mutation was not associated with Breslow thickness. Previous work on a subset of this cohort suggested that *BRAF* mutant tumours were thinner at diagnosis compared to *BRAF* wild-type tumours (Mar *et al*, 2014). However, other studies have found no relationship between Breslow thickness and *BRAF* mutation (Shinozaki *et al*, 2004; Edlundh-Rose *et al*, 2006; Bauer *et al*, 2011; Lee *et al*, 2011; Long *et al*, 2011; Bucheit *et al*, 2013; Carlino *et al*, 2014).

Among *BRAF* mutant tumours, the frequency (24%) of *BRAF* V600K in our cohort was somewhat higher than expected. The frequency of *BRAF* V600K mutation has been reported to range between 6 and 30% (Willmore-Payne *et al*, 2005; Spittle *et al*, 2007; Ugurel *et al*, 2007; Halaban *et al*, 2010; Rubinstein *et al*, 2010; Long *et al*, 2011; Jewell *et al*, 2012; Lovly *et al*, 2012; Menzies *et al*, 2012; Bucheit *et al*, 2013; Greaves *et al*, 2013; Heinzerling *et al*, 2013). Of note, Long *et al*'s Australian study determined that 20% of tumours had *BRAF* V600K oncogenic mutations (Long *et al*, 2011). The broad range for the reported V600K frequency may be explained by differences in methods used for mutation analysis. For instance,

studies using methods with lower sensitivities for reliably detecting non-V600E mutations (such as Sanger sequencing) or with lower specificities in distinguishing variant mutations may underestimate the frequency of mutations in V600K (Long *et al*, 2011; Anderson *et al*, 2012; Halait *et al*, 2012; Heinzerling *et al*, 2013). Moreover, mutations in V600K may be more frequent in Australian populations due to high ultraviolet exposure given the higher proportion of melanomas arising in chronic sun-damaged skin (Menzies *et al*, 2012; Voskoboynik *et al*, 2016).

In our study, compared to patients with *BRAF* V600E mutant tumours, patients with *BRAF* V600K mutant tumours were more likely to be older and have tumours located on the head and neck region. Previous studies have similarly demonstrated associations between V600K genotype with older patient age and head and neck location (Jewell *et al*, 2012; Menzies *et al*, 2012; Bucheit *et al*, 2013). These findings suggest that different genotypes exist within *BRAF* mutant melanoma, whereby V600K and V600E genotypes may represent biologically and clinically distinct entities.

In our study, *NRAS* positivity was more common in older patients and in tumour located on the extremities compared to the head and neck region. Several other studies have demonstrated an association between *NRAS* mutation and older patient age (Goel *et al*, 2006; Devitt *et al*, 2011; Ekedahl *et al*, 2013); however, a meta-analysis did not demonstrate this association (Lee *et al*, 2011). Other studies have similarly revealed that *NRAS* mutant melanomas have a propensity to develop on the extremities compared to the head and neck region (Edlundh-Rose *et al*, 2006; Ellerhorst *et al*, 2011; Jakob *et al*, 2012). Thomas *et al*'s cohort study demonstrated an inverse relationship between *NRAS* mutant melanomas and scalp/neck location (Thomas *et al*, 2015).

Furthermore, even when adjusted for other factors, *BRAF* mutant tumours had an increased risk of regional lymph node metastasis compared to satellite/in-transit metastasis as the site of first metastasis. Our study has also demonstrated that patients with melanomas harbouring a *BRAF* mutation had increased odds of a tumour-positive SLNB. The fact that *BRAF* mutation was associated on multivariate analysis with nodal disease, whether detected clinically or by a tumour-positive SLNB, suggests that this is likely to be a true association. We have previously reported an association between *BRAF* mutation status and nodal involvement at diagnosis in a subset of the MMP cohort (Mar *et al*, 2014). Broekaert *et al* (2010) suggested that *BRAF* mutant tumours metastasise more frequently to regional lymph nodes, with *BRAF* wild-type tumours more likely to metastasise to non-nodal sites. In contrast, another study found that *BRAF* mutant melanomas were most likely to recur with distant metastasis as the site of first recurrence and that isolated regional lymph node metastasis was rare (Barbour *et al*, 2014).

Several studies that have investigated the relationship between mutation status and the patterns of visceral involvement in metastatic disease (Chang *et al*, 2004; Jakob *et al*, 2012; Carlino *et al*, 2014; Maxwell *et al*, 2016). Our study indicated that *BRAF* mutation and *NRAS* mutation were associated with the subsequent development of CNS metastasis (aOR 4.65, 95% CI 2.23–9.69 $P < 0.001$; aOR 4.03, 95% CI 1.72–9.44, $P = 0.001$, respectively) and liver metastasis (aOR 3.09, 95% CI 1.49–6.42 $P = 0.003$; aOR 3.17, 95% CI 1.32–7.58, $P = 0.01$, respectively). Compared to *BRAF/ NRAS* wild-type tumours, *NRAS* mutation was also associated with lung metastasis (aOR 2.44, 95% CI 1.21–4.93, $P = 0.013$). These wide CIs indicate some uncertainty in our findings and further prospective studies are required to validate our results. Consistent with our finding of an association between liver metastasis and *BRAF* mutation, a small retrospective study found that melanomas harbouring *BRAF* mutations were more likely than *BRAF* wild-type tumours to metastasise to the liver (Chang *et al*, 2004). In contrast, Jakob *et al* (2012) retrospective cohort study concluded that there was no association between mutation status and liver metastasis.

Jakob *et al* (2012) study revealed that tumour mutation was associated with an increased risk of CNS involvement at diagnosis of stage IV disease ($P = 0.008$), with melanomas harbouring *BRAF* and *NRAS* mutations more likely to have CNS involvement compared to *BRAF/ NRAS* wild-type patients (24.1%, 23.1% and 12.4%, respectively). Carlino *et al*'s more recent study demonstrated a trend towards higher rates of brain metastasis at initial stage IV diagnosis, in keeping with Jakob and colleagues' study (Jakob *et al*, 2012); however, the risk of brain metastasis at any time was comparable irrespective of *BRAF/ NRAS* mutation status (Carlino *et al*, 2014). A recent, single-institution, retrospective cohort study demonstrated that *BRAF*-V600 patients, who were not treated with a selective *BRAF* inhibitor, compared to *BRAF* wild-type patients, had an increased risk of brain metastasis ($P = 0.027$; Maxwell *et al*, 2016).

In our study, patients with tumours located on the head and neck region had an increased risk of developing satellite/in-transit metastasis compared to nodal metastasis. In accordance with this finding, in Meier *et al*'s study, satellite or in-transit metastases were more likely to occur in patients with tumours located on the head and neck ($P < 0.001$; Meier *et al*, 2002).

In our study, Breslow thickness was not related to the site of first metastasis. Consistent with this, Cohn-Cedermark and colleagues' study demonstrated that the type of primary recurrence was unrelated to the Breslow thickness of the primary tumour (Cohn-Cedermark *et al*, 1999). Contrary to their findings and ours, in Meier *et al*'s study, tumours between 0.75 and 1.5 mm in thickness demonstrated the highest frequency of direct distant metastases, while tumours < 0.76 mm and > 1.5 mm in thickness had increased rates of satellite or in-transit metastasis (Meier *et al*, 2002). These findings have not been validated elsewhere and we are unable to provide a meaningful explanation for their results. Tejera-Vaquerizo and colleagues' study reported that melanomas greater than 4 mm in thickness had an increased risk of developing distant metastasis compared to locoregional metastasis as the first site of recurrence (Tejera-Vaquerizo *et al*, 2007). In view of their small sample size, it is difficult to draw any firm conclusions.

Excluding patients with positive SLNB, the median time course to the first detected metastasis was similar among those who developed satellite/in-transit, regional lymph node and distant metastasis ($P = 0.14$). In Meier and colleagues' study, the median time to first tumour recurrence as distant, lymph node and satellite/in-transit metastasis was 25 months, 16 months and 17 months, respectively (Meier *et al*, 2002). Excluding patients with a tumour-positive SLNB, the decreased median time to first detected metastasis (15 months) in our study is likely due to referral bias as our patient cohort may have had more aggressive disease due to the fact that our study was conducted at three major tertiary referral centres and the increasing use of radiological surveillance in high-risk patients. Indeed, among patients with distant metastasis as the site of first metastasis, the mode of detection was radiological in 78% of patients. At the time of Meier *et al*'s study, routine radiological surveillance of high-risk patients was likely not performed (Meier *et al*, 2002). Furthermore, it is important to consider lead time bias in the time course to the development of metastases. That is, small in-transit and lymph node metastases are more likely to be detected clinically, leading to earlier detection, compared to distant visceral metastases of the same size, which may be asymptomatic.

In our study, the time course to the development of distant metastatic disease was established to be independent of the anatomical metastatic pathway. Other studies have similarly demonstrated that the time to distant metastasis is similar across the various metastatic pathways, irrespective of the site of first metastasis (Dong *et al*, 2000; Meier *et al*, 2002; Tejera-Vaquerizo *et al*, 2007).

The median time to the development of distant metastatic disease in patients with *BRAF* mutant tumours was shorter than in patients with *BRAF/NRAS* wild-type tumours; however, this difference was not statistically significant. Other studies have also failed to demonstrate a significant difference in the time to distant metastatic disease according to mutation status (Chang *et al*, 2004; Long *et al*, 2011; Carlino *et al*, 2014).

Strengths of our study included the large sample size and the multicentre and prospective nature of the study design. In addition, the MMP database includes comprehensive information on disease recurrence and high-quality longitudinal follow-up data, which compares favourably to other databases. It also contains a rich data set of phenotypic and tumour-related variables, including mutational data. Therefore, the MMP database provided a clinically, histologically and molecularly well-characterised cohort of patients.

Nonetheless, referral bias discussed above is a limitation of our study. Furthermore, many of the new systemic agents used in melanoma treatment may alter the natural course of the disease (Hodi *et al*, 2010; Flaherty *et al*, 2012; Hauschild *et al*, 2012); therefore, the relationship between disease progression and mutation status may be potentially confounded by these therapeutic agents. Another limitation of our study is that the median follow-up time was 4.7 years; thus, our study would not have captured patients with slow tempo disease. However, in view of the fact that the IQR for the time to distant metastatic disease among all the metastatic pathways was between 9 and 26 months, our study likely captured the vast majority of recurrences and therefore, our median follow-up period is considered to be reasonable. When our findings are interpreted within this context, our results provide information on the clinical, pathological and mutational characteristics related to the routes of metastasis in a large Australian cohort. Nonetheless, further studies with a comparatively longer observational period are warranted in order to definitively capture patients with slow tempo disease.

To conclude, patients with *BRAF* mutant tumours have an increased risk of regional lymph node metastasis as the site of first metastasis and sentinel lymph node positivity. The presence of either a *BRAF* or *NRAS* mutation was associated with the development of CNS and liver metastasis and the presence of an *NRAS* mutation was associated with the development of lung metastasis. If these findings are validated in additional prospective studies, a role for heightened visceral organ surveillance may be warranted in patients with tumours harbouring these somatic mutations.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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