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 \( \text{BRAF} \) and \( \text{NRAS} \) 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. \( \text{BRAF} \) mutation was associated with lymph node metastasis (adjusted RRR 2.46 CI 1.07–5.69, \( P = 0.04 \)) and sentinel lymph node positivity (adjusted odds ratio [aOR] OR 1.55, CI 1.14–2.10, \( P = 0.005 \)). \( \text{BRAF} \) mutation and \( \text{NRAS} \) mutation were associated with increased odds of developing liver metastasis (aOR 3.09 CI 1.49–6.42, \( P = 0.003 \); aOR 3.17, CI 1.32–7.58, \( P = 0.01 \)) and central nervous system (CNS) metastasis (aOR 4.65, CI 2.23–9.69, \( P < 0.001 \); aOR 4.03, CI 1.72–9.44, \( P = 0.001 \)). \( \text{NRAS} \) mutation was associated with lung metastasis (aOR 2.44, CI 1.21–4.93, \( P = 0.01 \)).

Conclusions: \( \text{BRAF} \) mutation was found to be associated with lymph node metastasis as first metastasis and sentinel lymph node positivity. \( \text{BRAF} \) and \( \text{NRAS} \) mutations were associated with CNS and liver metastasis and \( \text{NRAS} \) 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
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 enroled 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 phenotype) 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/mm²) 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 BRAF/NRAS mutant tumour (aOR 2.48, 95% CI 1.24–4.22, P = 0.007) and having a high mitotic rate (aOR 3.59, 95% CI 2.39–5.41, P < 0.001, respectively).

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.

When adjusted for 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 1.14–4.01, 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 V600K 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 1. Clinical and pathological characteristics according to BRAF and NRAS mutation status

| Clinicopathological variable | BRAF mutant (%) | NRAS 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 (nl/mm²)       |                 |                 |           |
| <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.

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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<sup>a</sup> | BRAF mutant vs NRAS mutant<sup>b</sup> |
|------------------------------|-----------------------------------------------|--------------------------------------|
|                              | Univariate analysis | Multivariate analysis | Univariate analysis | Multivariate analysis |
|                              | RRR               | 95% CI   | P-value | RRR               | 95% CI   | P-value | RRR               | 95% CI   | P-value |
| Patient sex                  |                   |          |        |                   |          |        |                   |          |        |
| Male                         | 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   |
|                              |                   |          |        |                   |          |        |                   |          |        |
| Patient age                  |                   |          |        |                   |          |        |                   |          |        |
| <50 years                    | 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 |
|                              |                   |          |        |                   |          |        |                   |          |        |
| Anatomical location          |                   |          |        |                   |          |        |                   |          |        |
| Head & neck                  | 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   |
| 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 |
| 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  |
| Breslow thickness            |                   |          |        |                   |          |        |                   |          |        |
| <1.0 mm                      | 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 |
| 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 |
| >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  |
| Histologic subtype           |                   |          |        |                   |          |        |                   |          |        |
| SSM                          | 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   |
| 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   |
| Mitotic rate (n/mm<sup>2</sup>)|                   |          |        |                   |          |        |                   |          |        |
| <5                           | 1.00              |          |        | 1.00              |          |        | 1.00              |          |        |
| 5–9                          | 1.15              | 0.79–1.67| 0.5    | 0.88              | 0.60–1.31| 0.5    | 0.82              | 0.56–1.19| 0.3   |
| >10                          | 1.03              | 0.70–1.52| 0.9    | 0.73              | 0.48–1.10| 0.13   | 0.87              | 0.55–1.36| 0.6   |
| Ulceration                   |                   |          |        |                   |          |        |                   |          |        |
| No                           | 1.00              |          |        | 1.00              |          |        | 1.00              |          |        |
| Yes                          | 1.20              | 0.88–1.64| 0.3    | 1.00              |          |        | 1.00              |          |        |

Abbreviations: CI = confidence interval; LMM = lentigo maligna melanoma; NM = nodular melanoma; RRR = relative risk ratio; SSM = superficial spreading melanoma.

<sup>a</sup>BRAF/NRAS wild-type as the reference category.

<sup>b</sup>NRAS 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 53%, 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 increased risk was not statistically significant (RRR 1.32, 95% CI 0.4–7.52, P = 0.04)
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 (\( P = 0.7 \)).

DISCUSSION

Of the 1048 patients included in our analysis, 306 (29%) developed metastasis during the study period and among these, 24%, 63% and
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-Vaquerizo 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-Vaquerizo 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; Buchet et al, 2013; Carlino et al, 2014).

Among BRAF mutant tumours, the frequency (24%) of BRAF V600K mutation 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; Buchet et al, 2013; Greaves et al, 2013; Heinzlring 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,

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

Abbreviation: IQR = interquartile range.
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; Halai et al, 2012; Heinzlerling et al, 2013). Moreover, mutations in V600K may be more frequent in Australian populations due to ultraviolet exposure, given the higher proportion of melanomas arising in chronic sun-damaged skin (Menzies et al, 2012; Vokoboykin 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; Buchet 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 on multivariate analysis with nodal disease, whether detected clinically or by 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).

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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