Distinct Clinical and Pathological Features Are Associated with the *BRAF*$^{T1799A(V600E)}$ Mutation in Primary Melanoma

Wendy Liu[1,2,3], John W. Kelly[2], Melanie Trivett[1], William K. Murray[1], John P. Dowling[2], Rory Wolfe[4], Graham Mason[5], Jill Magee[6], Christopher Angel[7], Alexander Dobrovic[1] and Grant A. McArthur[1,3]

The *BRAF*^{T1799A} mutation encodes BRAF^{V600E} that leads to activation of the mitogen-activated protein kinase pathway. This study aimed to assess the clinico-pathological features of primary invasive melanomas containing the *BRAF*^{T1799A} mutation. Patients ($n=251$) with invasive primary melanomas from Australia were interviewed and examined with respect to their melanoma characteristics and risk factors. Independent review of pathology, allele-specific PCR for the *BRAF*^{T1799A} mutation, immunohistochemical staining with Ki67, and phospho-histone-H3 (PH3) were performed. The *BRAF*^{T1799A} mutation was found in 112 (45%) of the primary melanomas. Associations with the *BRAF*^{T1799A} mutation ($P<0.05$) were as follows: low tumor thickness (odds ratio (OR) = 3.3); low mitotic rate (OR = 2.0); low Ki67 score (OR = 5.0); low PH3 score (OR = 3.3); superficial spreading melanoma (OR = 10.0); pigmented melanoma (OR = 3.7); a lack of history of solar keratoses (OR = 2.7); a location on the trunk (OR = 3.4) or extremity (OR = 2.0); a high level of self-reported childhood sun exposure (OR = 2.0); $\leqslant$50 years of age (OR = 2.5); and fewer freckles (OR = 2.5). We conclude that the *BRAF*^{T1799A} mutation has associations with host phenotype, tumor location, and pigmentation. Although implicated in the control of the cell cycle, the *BRAF*^{T1799A} mutation is associated with a lower rate of tumor proliferation.

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INTRODUCTION

The *BRAF* proto-oncogene encodes the BRAF kinase, which is a member of the RAF family of serine/threonine cytoplasmic kinases. The RAF kinases are part of the mitogenic-activated protein kinase (MAPK) pathway, which is a RAS activated protein kinase cascade involved in the control of cell growth, proliferation, and differentiation (Robinson and Cobb, 1997).

The high incidence of *BRAF* mutations throughout melanoma progression suggests that BRAF may be an attractive therapeutic target (Karasarides *et al.*, 2004; Sharkey et al., 2004; Sumimoto *et al.*, 2004). Despite a large number of studies, the clinical and pathologic associations of the *BRAF*^{T1799A} mutation in primary human melanoma remain poorly understood. A better understanding of the biological role of *BRAF* in human melanoma will assist in evaluating this oncogene as a therapeutic target. The aim of this study was to examine the clinical and pathological associations of the *BRAF*^{T1799A} mutation in invasive primary melanoma.

[1]*Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia;* [2]*Victorian Melanoma Service, The Alfred Hospital, Melbourne, Victoria, Australia;* [3]*Department of Medicine, St Vincent's Hospital, The University of Melbourne, Melbourne, Victoria, Australia;* [4]*Department of Epidemiology and Preventive Medicine, Monash University, Melbourne, Victoria, Australia;* [5]*Melbourne Pathology, Collingwood, Victoria, Australia;* [6]*Dorevitch Pathology, Heidelberg, Victoria, Australia and* [7]*Gribbles Pathology, Clayton, Victoria, Australia*

Correspondence: Associate Professor Grant A. McArthur, Division of Clinical Haematology and Medical Oncology, Peter MacCallum Cancer Centre, St Andrew's Place, East Melbourne, Victoria 3002, Australia.
E-mail: grant.mcarthur@petermac.org

Abbreviations: MAPK, mitogenic-activated protein kinase
MITF, microphthalmia-associated transcription factor; OR, odds ratio
PH3, phospho-histone-H3

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RESULTS

A total of 251 patients with invasive primary melanomas were included in the final analyses. The male to female ratio was 1.4. The median and mean ages were 56 and 55 years, respectively (range 7–91 years). The median and mean tumor thicknesses were 1.1 and 2.1 mm, respectively (range 0.1–18 mm). The *BRAF*^{T1799A} mutation was found in 112 (45%) of the primary melanomas. Of the 251 patients with BRAF status available, 209 patients had sufficient tumor tissue to perform immunohistochemical staining with Ki67 and phospho-histone-H3 (PH3).

BRAF and rate of tumor proliferation

The percentage of melanomas with the *BRAF*^{T1799A} mutation decreased with increasing tumor thickness (P_{trend}, a test of trend for ordered categories = 0.001) and mitotic rate ($P_{trend}=0.038$). A total of 55% of tumors that were <1 mm in Breslow thickness contained the *BRAF*^{T1799A} mutation, whereas only 27% of those $\geqslant$4 mm contained the mutation.

A total of 53.9% of tumors that were less mitotically active (<1/mm^2) contained the *BRAF*^{T1799A} mutation, compared to a mutation frequency of 35.5% among tumors that were highly mitotically active (⩾10/mm^2). To further examine the relationship between the *BRAF*^{T1799A} mutation and cell proliferation, staining with PH3 (a sensitive marker of mitosis (Goto *et al.*, 1999)) and Ki67 (a marker of actively cycling cells (Gerdes *et al.*, 1984)) was performed. The rate of *BRAF*^{T1799A} mutation decreased with increasing PH3 ($P_{trend}=0.031$) and Ki67 labelling ($P_{trend}=0.026$). An example of staining for PH3 in a primary melanoma with a high mitotic count is shown in Figure S1.

Clinical associations of BRAF

When compared to superficial spreading melanoma, the frequency of the *BRAF*^{T1799A} mutation was lower among nodular melanomas (superficial spreading melanoma, 55.3%; nodular melanoma, 39.1%; $P=0.055$) and lowest among lentigo maligna melanoma (15.4%, $P=0.001$). No acral lentiginous melanomas ($n=6$) or desmoplastic melanomas ($n=7$) harbored the *BRAF*^{T1799A} mutation.

Tumor pigmentation was also strongly associated with the *BRAF*^{T1799A} mutation ($P=0.001$). There was a substantial level of agreement (Landis and Koch, 1977) between the presence of pigmentation as assessed by macroscopic assessment of the pathology specimen and the presence of pigmentation as detected by patient ($\kappa=0.61$) and both correlated with the presence of the *BRAF*^{T1799A} mutation ($P=0.001$).

The *BRAF*^{T1799A} mutation occurred more frequently among patients who were 50 years of age or younger when compared to those over 50 years ($P<0.001$). Melanomas from patients with few freckles more frequently had the *BRAF*^{T1799A} mutation than those with moderate number of freckles ($P=0.002$) (Table 1).

The *BRAF*^{T1799A} mutation in melanomas was negatively associated with a history of solar keratoses ($P<0.001$) and positively associated with melanomas arising on intermittently

Table 1. Associations of the *BRAF*^{T1799A} mutation

	n	T1799A	WT	% T1799A	% WT	OR	95% CI	*P*	P_{trend}
Thickness									
<1 mm	120	66	54	55.0	45.0	—			
1–4 mm	94	36	58	38.3	61.7	0.5	0.3, 0.9	0.016	
>4 mm	37	10	27	27.0	73.0	0.3	0.1, 0.7	0.004	0.001
Mitosis									
<1/mm^2	104	56	48	53.9	45.2	—			
1–4/mm^2	86	33	53	38.4	61.6	0.5	0.3, 1.0	0.034	
5–9/mm^2	30	12	18	40.0	60.0	0.6	0.2, 1.3	0.184	
⩾10/mm^2	31	11	20	35.5	64.5	0.5	0.2, 1.0	0.076	0.038
Ph3									
<1/mm^2	63	33	30	52.4	47.6	—			
1–4/mm^2	65	34	31	52.3	47.7	1.0	0.5, 2.0	0.993	
5–9/mm^2	26	10	16	38.5	61.5	0.6	0.2, 1.4	0.235	
10–20/mm^2	25	6	19	24.0	76.0	0.3	0.1, 0.8	0.019	
>20/mm^2	30	12	18	40.0	60.0	0.6	0.3, 1.5	0.266	0.031
Ki67									
⩽10%	131	68	63	51.9	48.1	—			
11–20%	32	11	21	34.4	65.6	0.5	0.2, 1.1	0.079	
21–40%	27	12	15	44.4	55.6	0.7	0.3, 1.7	0.481	
>40%	19	4	15	21.1	78.9	0.2	0.1, 0.8	0.018	0.026
Type									
SSM	161	89	72	55.3	44.7	—			
NM	46	18	28	39.1	60.9	0.5	0.3, 1.0	0.055	
LMM	26	4	22	15.4	84.6	0.1	0.0, 0.4	0.001	<0.001*
ALM	6	0	6	0.0	100.0				
DM	7	0	7	0.0	100.0				
Others	5	1	4	20.0	80.0				

Table 1 continued on the following page

Table 1. continued

	n	T1799A	WT	% T1799A	% WT	OR	95% CI	*P*	P_{trend}
Amelanosis									
Yes	46	10	36	21.7	78.3	—			
No	183	93	90	50.8	49.2	3.7	1.7, 7.9	0.001	
Age									
⩽50	92	55	37	59.8	40.2	—			
>50	159	57	102	35.9	64.1	0.4	0.2, 0.6	<0.001	
Freckles[1]									
Few	55	30	25	54.6	45.4	—			
Moderate	42	13	29	30.9	69.1	0.4	0.2, 0.9	0.002	
Many	23	10	13	43.5	56.5	0.6	0.2, 1.7	0.374	0.159
History of solar keratoses									
Yes	89	27	62	30.3	69.7	—			
No	156	84	72	53.9	46.1	2.7	1.5, 4.6	<0.001	
Sites									
Head and neck	58	17	41	29.3	70.7	—			
Trunk	84	49	35	58.3	41.7	3.4	1.7, 6.9	0.001	
Extremities	102	46	56	45.1	54.9	2.0	1.0, 3.9	0.051	0.003*
Volar/subungual	7	0	7	0.0	100.0				
Self-reported childhood sun exposure									
High	196	94	102	48.0	52.0	—			
Low	42	13	29	31.0	69.0	0.5	0.2, 1.0	0.047	

ALM, acral lentiginous melanoma; CI, confidence interval; DM, desmoplastic melanoma; LMM, lentigo maligna melanoma; *n*, number; NM, nodular melanoma; OR, odds ratio; *P*, *P*-value; PH3, phospho-histone-H3; P_{trend}, *P*-value for test of trend; SSM, superficial spreading melanoma; T1799A, *BRAF*^{T1799A}; WT, *BRAF*$^{wild\text{-}type}$.
*Unordered categories hence *P*-value is for the global test of all pairwise comparisons of categories.
[1]Examined patients only (*n*=125).
—, reference point.
Note: variation in total numbers of patients in each category is due to some data being unobtainable.

sun-exposed sites (trunk, odds ratio (OR) = 3.4, $P = 0.001$; extremities, OR = 2.0, $P = 0.051$).

A high level of self-reported childhood sun exposure was associated with melanomas harboring the *BRAF*^{T1799A} mutation (48.0 *vs* 31.0%, $P = 0.047$).

Although mutations in BRAF are found in a high proportion of melanocytic nevi (Pollock *et al.*, 2003), the *BRAF*^{T1799A} mutation was associated with neither the total number of melanocytic nevi ($P = 0.499$), nor the number of dysplastic nevi ($P = 0.527$) as assessed by clinical examination in our patient cohort. The *BRAF*^{T1799A} mutation was not associated with a history of melanoma ($P = 0.641$) or a family history of melanoma ($P = 0.402$). Other clinical variables assessed that were not associated with the *BRAF*^{T1799A} mutation were: the presence of ulceration on histology, gender, skin phototype, eye color, degrees of solar lentigines, a history of blistering sunburns, self-reported level of recreational or occupational sun exposure.

Multivariate analyses

In a multivariate model, fewer freckles (OR = 5.0, $P = 0.004$), pigmented melanomas (OR = 8.6, $P = 0.013$), a lack of history of solar keratoses (OR = 4.2, $P = 0.012$), a location on the trunk (OR = 9.0, $P = 0.004$), a location on the extremity (OR = 6.2, $P = 0.013$), and a high level of self-reported childhood sun exposure (OR = 5.0, $P = 0.020$) were found to independently associate with melanomas containing the *BRAF*^{T1799A} mutation (Table 2).

DISCUSSION

Findings from our study suggest that the *BRAF*^{T1799A} mutation is associated with distinct clinical, phenotypic, and pathological features. The data demonstrated that fewer freckles, pigmented melanomas, a location on the trunk or extremity, a lack of history of solar keratoses are independently associated with the *BRAF*^{T1799A} mutation, as well as melanomas with lower thickness and lower rate of proliferation.

A novel finding derived from our study was that fewer freckles were independently associated with the *BRAF*^{T1799A} mutation. The term freckle used here refer to ephelides, a clinical entity characterized by multiple small, pale brown macular lesions that occur in fair skinned individual that darken with sun exposure and fade with sun protection. Freckles are known to associate with sun sensitivity (Bastiaens *et al.*, 1999) and the presence of melanocortin-1-receptor variants (Bastiaens *et al.*, 2001). We speculate that there may be an interaction between the melanocyte-stimulating hormone–melanocortin-1-receptor pathway and the RAS–RAF–MAPK pathway, through mediators such as cAMP (Busca *et al.*, 2000).

The pattern of sun exposure was associated with melanomas containing the *BRAF*^{T1799A} mutation. Markers of cumulative sun exposure, including a history of solar keratoses and the location on the head and neck, were both found to be inversely related to the *BRAF*^{T1799A} mutation. Solar keratoses are considered as a personal indicator of cumulative sun exposure (Frost and Green, 1994; Kennedy *et al.*, 2003). These inverse associations with the *BRAF*^{T1799A} mutation argue against cumulative sun exposure being important in the induction of *BRAF*^{T1799A} mutation in melanocytic cells. In contrast, the association between the *BRAF*^{T1799A} mutation and the intermittently sun exposed body sites such as the trunk or extremities suggests that intense UV exposure on sun-sensitive skin may contribute to the DNA damage in the skin and to the development of the *BRAF*^{T1799A} mutation. Our findings were consistent with previous findings of Maldonado *et al.* (2003) and in this respect, support the hypothesis of a ''dual pathway'' to melanoma genesis (Rivers, 2004).

Table 2. The multivariate analyses of independent associations of the *BRAF*^{T1799A} mutation

	Multivariate		
	OR	**95% CI**	***P***
Amelanosis			
Yes	1.0	—	
No	8.6	1.6 46.6	0.013
Site			
Head/neck	1.0	—	
Trunk	9.0	2.0, 40.6	0.004
Extremities	6.2	1.5, 26.1	0.013
Self-reported childhood sun exposure			
High	1.0		
Low	0.2	0.0, 0.8	0.020
Freckles			
Few	1.0	—	
Moderate	0.2	0.0, 0.6	0.004
Many	0.5	0.1, 2.1	0.383
Solar keratoses			
Yes	1.0	—	
No	4.2	1.4, 13.0	0.012

CI, confidence interval; OR, odds ratio; *P*, *P*-value.
—, reference point.

Another novel finding from this study was the association of the *BRAF*^{T1799A} mutation with tumor pigmentation. Pigmentation in a melanoma is related to the amount of melanin production. Melanin is produced from the amino acid tyrosine by a biosynthetic pathway that includes the enzyme tyrosinase. One of the key regulators of tyrosinase production is microphthalmia-associated transcription factor (MITF) (Goding, 2000). MITF is critical for melanocyte lineage commitment (Widlund and Fisher, 2003) and is considered essential for melanocyte survival (Tassabehji *et al.*, 1994). MITF activity is modulated by a number of mechanisms (Goding, 2000), including the MAPK-dependent phosphorylation (Hemesath *et al.*, 1998). In our study, the observation of more frequent pigment production in primary melanomas containing the *BRAF*^{T1799A} mutation supports previous evidence of a close link between MAPK and MITF in both cell culture and animal models (Hemesath *et al.*, 1998). Mutant BRAF^{V600E} may activate the MAPK pathway and modulate MITF function and melanocyte differentiation.

It is interesting to note that the *BRAF*^{T1799A} was significantly correlated with favorable prognostic indicators such as lower thickness ($P_{trend} = 0.001$), lower mitotic rate ($P_{trend} = 0.038$), lower expression of the mitotic marker PH3 ($P_{trend} = 0.031$), and lower expression of the cell proliferation marker (Ki67, $P_{trend} = 0.026$). This observation indicates that *BRAF*^{T1799A}, when compared to melanomas that are wild-type for *BRAF*, may be associated with a more differentiated form of melanoma with a slower cell proliferation rate. Although considered an oncogene, RAF activation can also induce cell senescence in non-immortalized human lung fibroblasts (Zhu *et al.*, 1998) and in melanocytic nevi (Michaloglou *et al.*, 2005). Moreover, a study has shown that MITF functions downstream of BRAF to regulate differentiation of melanocytes and can activate p21^{Cip1}, hypo-phosphorylate the retinoblastoma protein and drive cells into senescence (Carreira *et al.*, 2005). Therefore in some contexts, activation of BRAF may promote differentiation, senescence and reduced proliferation, providing one possible explanation for the inverse relationship between the BRAF^{V600E} mutation and markers of aggressive melanoma.

In summary, this prospective study on the clinical and pathologic associations of the *BRAF*^{T1799A} mutation in primary melanoma provided evidence of distinct associations with host phenotype, environmental sun exposure pattern, tumor histologic characteristics, and pigment production. Although part of the MAPK pathway that regulates cell proliferation, the *BRAF*^{T1799A} was associated with lower tumor thickness and lower rate of tumor proliferation. Inhibition of BRAF in human melanoma, therefore, may have additional biological affects to reduced cell

proliferation, such as induction of apoptosis, altered cell motility or differentiation. Investigations into the genetic changes in melanoma have suggested that a number of proteins that interact with MAPK-pathway such as RAS, MITF, phosphatase and tensin homolog deleted on chromosome ten, retinoblastoma protein, cyclin-dependent kinase 4, and cyclin D1 (Curtin *et al*., 2005) may play a role in melanoma biology. Further studies of how these proteins relate to mutations in the BRAF oncogene including examination of clinicopathologic features will enhance our understanding of their biological relevance and the rationale for targeted therapy of melanoma.

MATERIALS AND METHODS

From May 2003 to September 2004, 251 patients with primary cutaneous melanoma tissue available were recruited from two tertiary melanoma referral centers in Melbourne. Study investigations were performed with adherence to the Declaration of Helsinki Principles after approval by the local Human Research Ethics Committees. Informed consent was obtained from all participants. The age, gender, and the site of the melanoma for each patient were recorded. An amelanotic melanoma as determined by pathology was defined as a melanoma which lacked pigmentation, based on macroscopic examination of the excised tissue by a pathologist.

All patients were interviewed by a medically trained study investigator. The questions explored were as follows: a history of melanoma, a family history of melanoma, a history of solar keratoses, a history of blistering sunburn, skin phototypes, eye color, and the pigmentary characteristics of the melanoma. Each patient was asked to estimate his or her level of occupational, recreational sun exposure and childhood sun exposure as high or low. An amelanotic melanoma as recognized by patient was defined as a melanoma that was predominantly pink, red, or white in color as reported by the patient.

Those patients attending the Victorian Melanoma Service had their skin examined by one of two dermatologists with respect to total number of melanocytic nevi, number of clinically dysplastic nevi, number of freckles, and solar lentigines. To look for potential selection bias, we analyzed the characteristics of the examined patients ($n=125$) and non-examined patients ($n=126$) and the results were presented in the Table S1.

Independent review of pathology was performed on all specimens with respect to: Breslow thickness, Clark's level, ulceration, mitotic rate, and tumor subtype (Clark *et al*., 1969).

Genomic DNA was extracted from paraffin-embedded tumor tissue derived from microdissection, using a Qiagen DNeasy extraction kit (Qiagen Ltd, Valencia, CA). All primary melanomas were screened with duplex allele-specific-PCR for the $BRAF^{T1799A}$ mutation using primers design and PCR conditions as outlined by Pollock *et al*. (2003). Duplex allele-specific-PCRs were repeated in a random subset of samples ($n=68$, 27, 100% concordance). The positive control used was DNA from the SKMEL28 cell lines and the negative control used was DNA extracted from a blood sample of a normal donor. Serial dilution tests of mixing DNA known to harbor the $BRAF^{T1799A}$ mutation with control genomic DNA revealed that the sensitivity of the allele-specific-PCR was 2%.

Sections were stained with a mAb to Ki67 (clone MIB1, 1:200 dilution, Dako, Glostrup, Denmark) to detect proliferating cells (Gerdes *et al*., 1984) and a polyclonal antibody to PH3 (1:800 dilution, Upstate, Charlottsville, VA) to detect mitotic cells (Goto *et al*., 1999). The antigens were unmasked by pressure cooking and all incubations were performed at room temperature.

All slides were assessed independently by two investigators. The Ki67 staining (% of staining cells in the dermis) and the PH3 staining (numbers of staining cells/mm^2 in the dermis) were assessed, beginning in the most immunoreactive area. The interclass correlations of the two independent assessors were 0.91 for PH3 scores (95% confidence interval = 0.89–0.93) and 0.89 for Ki67 (95% confidence interval = 0.87–0.92), indicating an excellent degree of agreement (Fleiss, 1981). The final score of Ki67 and the final score of PH3 were defined as the average of scores from the two assessors.

Statistical analyses

Univariate logistic regression models were used to relate variables with the presence of the $BRAF^{T1799A}$ mutation. For variables with ordered categories, we performed tests of trend to assess whether there was evidence of a constant increment in prevalence of the $BRAF^{T1799A}$ mutation for each increasing category. Within the logistic regression models, tests of trend were calculated by considering ordered categories to have increasing integer scores and analyzing the variable as though these were continuous. All variables that had a significant (defined as $P<0.05$) likelihood-ratio test on univariate analysis were entered into a multivariate logistic regression model. A backward stepwise selection procedure based on likelihood-ratio tests was used to select an optimal multivariate logistic regression model.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Characteristics of the examined group and the non-examined group.

Figure S1. Identification of cells in mitosis by immunohistochemistry using antibodies to phosphorylated-histone H3.

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