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Title: Clinical and pathological associations of the activating *RAC1* P29S mutation in primary cutaneous melanoma

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Key words: *RAC1* mutation, RAC1 immunoreactivity, RAC1 expression, melanoma metastasis, melanoma progression, *BRAF*

Running title: *RAC1* mutations in primary melanoma

Summary: Activating mutations in the GTPase *RAC1* are a recurrent event in cutaneous melanoma. We investigated the clinical and pathological associations of $RAC1^{P29S}$ in a cohort of 814 primary cutaneous melanomas with known *BRAF* and *NRAS* mutation status. The $RAC1^{P29S}$ mutation had a prevalence of 3.3% and was associated with increased thickness (OR=1.6 p=0.001), increased mitotic rate (OR=1.3 p=0.03), ulceration (OR=2.4 p=0.04), nodular subtype (OR=3.4 p=0.004) and nodal disease at diagnosis (OR=3.3 p=0.006). *BRAF* mutant tumors were also associated with nodal metastases (OR=1.9 p=0.004), despite being thinner at diagnosis than *BRAF* WT (median 1.2mm versus 1.6mm, p<0.001). Immunohistochemical analysis of 51 melanomas revealed that 47% were immunoreactive for RAC1. Melanomas were more likely to show RAC1 immunoreactivity if they were *BRAF* mutant (OR=5.2 p=0.01). *RAC1* may therefore be important in regulating the early migration of *BRAF* mutant tumors. *RAC1* mutations are infrequent in primary melanomas but may accelerate disease progression.

Significance:

This study provides further histological and clinical evidence for a role of RAC1 in the progression of primary melanoma. The association of mutations in *RAC1* and *BRAF* with regional nodal metastases, together with the association of strong RAC1 immunoreactivity in *BRAF* mutant tumors implicate RAC1 as a regulator of metastasis. This study suggests that signaling mediated by RAC1 may mediate migration and progression of disease in both *BRAF* mutant and wild-type melanoma.

Introduction

Activating mutations in the oncogene *RAC1* have recently been described in up to 9% of primary melanomas(Hodis et al., 2012; Krauthammer et al., 2012) making it a common recurrent mutation in cutaneous melanoma together with *BRAF* and *NRAS*. The proline to serine substitution at codon 29 ($RAC1^{P29S}$) is a C>T transition (CCT>TCT), which is consistent with a molecular signature associated with ultra-violet damage. The mutation has been reported to be more frequent in *BRAF/NRAS* wild-type tumors, which generally have a higher mutation burden

associated with UV damage(Hodis et al., 2012; Krauthammer et al., 2012). Currently, effective targeted inhibitor therapies for patients with *BRAF*-wild type tumors are lacking. Although direct pharmacological targeting of *RAC1* presents significant challenges, downstream effectors, such as *PAK,* are under investigation as therapeutic targets in this group(Ong et al., 2013) and Rho kinase inhibitors have been described(Patel et al., 2014).

RAC1 is a well characterized member of the Rho subfamily of small Ras-like GTPases with important roles in cell motility. It is associated with the assembly of actin filaments to form protrusions (lamellipodia) at the leading edge of cells(Jaffe and Hall, 2002) and has recently been shown to enhance ERK phosphorylation leading to cell proliferation and migration in normal melanocytes(Krauthammer et al., 2012). Although mutations in the Ras family are found in approximately 30% of all cancers, mutations in the Rho family are rare(Davis et al., 2013). Overexpression of RAC1 has been associated with progression of pancreatic(Wang et al., 2014), urinary tract(Kamai et al., 2010), colorectal(Jordan et al., 1999), testicular(Kamai et al., 2004), gastric cancers(Pan et al., 2004) and glioma(Fortin Ensign et al., 2013). Activation of guanine nucleotide exchange proteins (GEFs) that act as upstream regulators of *RAC1*, (such as Tiam-1, PREX1, Vav-1,2, Dock3) have been implicated in melanoma progression(Campbell et al., 2013; Lindsay et al., 2011; Machesky and Sansom, 2012; Sanz-Moreno et al., 2008). Cell culture studies have shown that *RAC1* is required for *NRAS*-induced tumor growth and invasion(Li et al., 2012). It has also been suggested that *BRAF* may regulate a *RAC*-dependent cadherin switch(Monaghan-Benson and Burridge, 2012) important for establishment of vertical growth of the tumor and progression, though the precise signaling pathways linking *BRAF* and *RAC1* are not fully understood.

Whilst clinical associations of mutations in *BRAF* and *NRAS* have been well studied in melanoma, there is relatively little known about the associations of mutations in *RAC1*. This study aims to characterize clinical and histological features associated with both $RAC1^{P29S}$ mutations and *RAC1* protein expression in a cohort of primary melanomas with known *BRAF* and *NRAS* status. This may provide further insight into the clinical relevance of *RAC1* in melanoma progression and the potential effectiveness of therapies targeting this signaling cascade.

Results

In a prospective cohort of 820 patients, DNA was extracted from primary melanoma samples for mutation testing, with 6 cases failing to amplify due to poor DNA quality and/or quantity. Of the remaining 814 invasive primary cutaneous melanomas, 27 (3.3%) were *RAC1* P29S mutant and 1 had a *RAC1* P29L mutation. Only those with the P29S mutation were included in the analysis due to the unknown biological significance of the P29L variant.

The median age of patients with *RAC1* mutant primary melanoma was 63 years, compared to 58 years for patients with *RAC1* wild-type (WT) tumors. Fifty-nine percent of patients harboring *RAC1* mutant primary melanomas were male (Table 1).

RAC1 mutant melanomas were thicker than *RAC1* WT tumors (median thickness 2.4mm [range=1.0, 11mm] versus 1.4mm [range=0.1, 47mm] respectively, p=0.001). The median mitotic rate also displayed some dissimilarity between groups (3 per mm^2 and 2 per mm2 for *RAC1* mutant and WT cases; p=0.03). Over 50% of *RAC1* mutant tumors were nodular melanoma (NM) subtype, and *RAC1* mutant cases were more commonly ulcerated (Table 1).

***RAC1* mutations and association with UV-damage**

Thirty percent of *RAC1* tumors were found on the head and neck, with others evenly distributed between trunk, upper and lower limbs. A total of 326 cases in the cohort were graded for the presence of solar elastosis in skin surrounding the melanoma. *RAC1* mutant tumors were just as likely to arise in non-severely sun damaged (non-SSD) skin as SSD skin (p=0.8, Table 1).

Other clinical indicators of UV induced damage were known for 24 of the 27 *RAC1* mutant cases. Evidence was lacking that presence of a *RAC1* mutation was associated with a history of blistering sunburn, a history of solar keratoses or a history of non-melanoma skin cancer (Table 1).

The co-existence of *BRAF* or *NRAS* canonical mutations in *RAC1* mutant melanomas

The *BRAF* mutation status was known for 812 of the 814 patients. Thirty-five percent had a *BRAF* mutation (28% *BRAF* V600E and 7% *BRAF* V600K). Of the *BRAF* WT cases, 199 were

tested for the presence of an *NRAS* mutation. Of these, 42 (21%) were *NRAS* mutant, with 15% of the entire cohort predicted to harbor an NRAS mutation on weighted analysis.

Of the *RAC1* mutant cases, 7 (26%) had a co-existing *BRAF* mutation and 8 (30%) had a mutation in *NRAS*. The proportion of *RAC1* mutant cases harboring an *NRAS* mutation was higher than expected given only 13% of *RAC1* WT cases were predicted to be *NRAS* mutant (Table 2).

BRAF mutant tumors were significantly thinner at diagnosis compared to *BRAF* WT tumors (median thickness 1.2mm versus 1.6mm, p<0.001). The median thickness of *NRAS* mutant tumors at diagnosis tended to be greater than that for *NRAS* WT tumors (median thickness 1.6mm versus 1.4mm, p=0.1), though this was not statistically significant.

RAC1 mutations and nodal status

Patients with *RAC1* mutant melanoma had 3-fold increased odds of presenting with involvement of regional lymph nodes at diagnosis (as determined by sentinel node biopsy or synchronous stage 3B or 3C disease) (OR=3.3 95%CI 1.4, 7.8, p=0.006) compared to RAC1 wild-type cases. Patients with *BRAF* mutant melanomas were more likely to present with regional nodal disease (OR=1.9 95%CI 1.2, 3.0, 0.004). There was no evidence of an association between *NRAS* mutant melanoma and nodal status (Table 3). Of patients with *RAC1* mutant melanomas and nodal involvement, 3 had a concomitant *BRAF* mutation, 2 were *NRAS* mutant and 3 were *BRAF/NRAS* wild-type.

Taking thickness, mitotic rate and ulceration into account in a multivariate analysis, the association between mutation status and nodal disease remained strong for *RAC1* mutant tumors, though of borderline statistical significance (OR=2.5, 95%CI 1.0, 6.3, p=0.06). The association between *BRAF* mutant tumors and nodal disease exhibited a similar strength of association when the same prognostic factors were considered (OR=2.6 95%CI 1.5, 4.3, p<0.001, Table 3).

RAC1 immunoreactivity expression

In order to investigate the pattern of RAC protein immunoreactivity in primary melanomas and its biological significance, all *RAC1* mutant melanomas with tissue available and an approximately

equal number of *RAC1* WT melanomas were selected randomly from the larger cohort for immunohistochemical analysis of RAC1 immunoreactivity.

Whilst the RAC antibody used in this study may not reflect of the activation state of RAC1, it has been shown to be highly specific for total RAC1 levels demonstrated through immunofluorescent staining of GFP-tagged RAC1 (Woodcock et al.,2010). Immunohistochemical staining of either total RAC1 or RAC1-GTP (i.e. active RAC1) have been reported to be analogous (Dalton et al., 2013).

RAC1 immunoreactivity was detectable in 24 (47%) of the 51 primary melanomas tested. Only 6 (24%) of 25 *RAC1* mutant primary melanomas had detectable RAC1 expression by this method, compared to 18 (69%) *RAC1* wild-type cases (p=0.001). *BRAF* mutant melanomas were more likely to be immunoreactive for RAC1 than *BRAF* wild-type tumors (OR=5.2 95%CI 1.5, 18.3 p=0.01) (Table 4).

There was some evidence that RAC1 immunoreactive tumors are thinner (median 1.7mm vs 2.6mm, p=0.05) and tend to be less mitotically active (median 1.8/mm^2 vs 6/mm^2, p=0.09) than those that are not immunoreactive, and that superficial spreading melanomas are more likely to be immunoreactive for RAC1 than either NM or LMM (p=0.06); however it is possible that these observed differences arose by chance, see Table 4.

RAC1 immunoreactivity expression in tissue sections varied depending on the melanoma subtype. In nodular melanoma where an intraepidermal component was inconsistently present, RAC1 expression dominated in the vertically invading melanocytes in the most superficial aspect of the dermis (Figure 1A), showing loss of RAC1 expression in the deeper placed invading melanocytes. In SSM cases, 65% showed strong RAC expression in the melanocytes at the leading edge of the horizontal intra-epidermal growth phase (see Figure 1B) and of these cases, 90% also showed a localised upregulation of a strong perinuclear (golgi) pattern of RAC expression in neighbouring keratinocytes (see Figure 1C and Supplementary figure 1) to suggest increased RAC protein packaging within the keratinocytes adjacent to the invading melanocytes.

Discussion

This study shows that *RAC1* mutant melanomas are more likely to have spread to regional lymph nodes at presentation compared to *RAC1* WT tumors, though this is explained in part by their

increased thickness and mitotic rate. *BRAF* mutant tumors were thinner, but were also more likely to present with nodal disease, even after adjustment for other known prognostic factors. *BRAF* mutant tumors have been reported to metastasize more frequently to regional lymph nodes (Broekaert et al., 2010). Our results support findings from other studies that *RAC1* has an important role in melanoma progression(Dalton et al., 2013; Krauthammer et al., 2012; Sanz-Moreno et al., 2008), as in other malignancies(Fortin Ensign et al., 2013; Kamai et al., 2010; Kamai et al., 2004; Wang et al., 2014).

RAC1 mutations occur at a frequency of 3.3% in this large cohort of patients from an area of high melanoma incidence and UV exposure. This is slightly less than the 5.3% of melanoma cell lines from Queensland and the 9.2% of melanomas in the extended Yale cohort of 217 'sun-exposed' cases reported by Krauthammer et al.(Krauthammer et al., 2012). *RAC1* mutations were found to be associated with male gender, though we did not find strong evidence for an association with sex. *RAC1* mutations were also found to occur in melanomas arising in 'sun-exposed' (defined as non-glabrous) sites rather than acral, mucosal or uveal melanomas(Krauthammer et al., 2012). To investigate this further, we assessed whether the degree of sun exposure was associated with the acquisition of this mutation, but found no association with markers of severe sun exposure (SSD skin, history of sunburns or previous non-melanoma skin cancer). As the molecular signature of this mutation is consistent with UV damage, these results would suggest that both intermittent and chronic sun exposure play a role in the development of *RAC1* mutations, and that the mutation is no more prevalent in geographical regions with greater UV exposure.

Interestingly, there was an association between *RAC1* mutant tumors and nodular subtype in this cohort. This suggests that the activating *RAC1* mutation plays an important role in tumor proliferation and early invasion (vertical growth) of the primary tumor. The acquisition of a new driver mutation is likely to trigger clonal expansion of the subpopulation carrying that driver and hence a period of rapid growth(McFarland et al., 2013). Nodular melanomas have been shown to grow more rapidly on average compared to other subtypes(Liu et al., 2006). It has been suggested that the *RAC*-specific GEF Tiam-1 may activate *RAC1* in nodular melanomas to induce vertical growth(Dalton et al., 2013).

Thirty percent of *RAC1* mutant tumors in this cohort harbored an *NRAS* mutation, which was disproportionate to the remainder of the cohort (13%). *NRAS* mutations have been associated with nodular subtype in some studies(Devitt et al., 2011; Edlundh-Rose et al., 2006; Jakob et al., 2012). Contrary to the report by Krauthammer et al. we did not find evidence for an association with *BRAF/NRAS* WT tumors(Krauthammer et al., 2012). Our results suggest that the mutation burden in *RAC1* mutant tumors is likely to be in the mid-range, similar to other tumors with dominant driver mutations (*BRAF* and *NRAS*), which also have mutation loads within the low-to-mid range(Krauthammer et al., 2012; Mar et al., 2013). This is in keeping with the lack of association with SSD skin, which tends to give rise to tumors with large numbers of passenger mutations. It has been suggested that a greater burden of passenger mutations may actually slow tumor progression(McFarland et al., 2013).

While the relatively small numbers tested preclude strong conclusions being made, our results indicated that melanomas immunoreactive for *RAC1* are thinner and tend to be SSM subtype rather than NM. This, together with the pattern of staining predominantly within the leading edge of the intra-epidermal component of SSM cases and superficial dermis of NM cases, would suggest that *RAC1* protein expression is upregulated as an early event to stimulate tumor migration and possibly an epithelial-mesenchymal transition that has been suggested to play a role in the early shift from horizontal to vertical growth phase.

Recent studies have demonstrated that oncogenes signaling through the MEK/ERK pathway induce epithelial-mesenchymal-transcription factor (EMT-TF) reprogramming, whereby transcription factors associated with normal melanocyte growth (ZEB2 and SNAIL) are downregulated and those associated with increased proliferation and invasion (ZEB1 and TWIST1) are upregulated(Caramel et al., 2013). This switch in transcription factors is thought to be orchestrated by FRA-1, and favors downregulation of E-cadherin, particularly at the invasive edge of the tumor(Caramel et al., 2013). RAC1 has been shown to co-localise with E-cadherin in epidermal keratinocytes(Akhtar et al., 2000). In this study, immunohistochemical reactivity showed localized upregulation of RAC1 in epidermal keratinocytes adjacent to melanocytes at the leading edge of the tumor (Figure 1C and Supplementary figure 1). While speculative at this stage, activation of *RAC1* or presence of high RAC1 protein expression could lead to endocytosis of E-cadherin and disruption of cell-cell adhesion allowing for cell migration(Akhtar and

Hotchin, 2001). As a melanoma progresses, switching from E-cadherin to N-cadherin expression could allow melanocytes to communicate with cells in the dermis and invade.

In this study, *BRAF* mutant tumors were considerably more likely to be immunoreactive for RAC1 compared to *BRAF* wild-type tumors. This might explain why *BRAF* mutant tumors were more likely to present with nodal disease despite being significantly thinner at diagnosis. This finding is supported by a study in gastrointestinal epithelia, whereby *BRAF* driven MEK/ERK activation induced *RAC1* activation(Ray et al., 2007). Another study in a melanoma cell line showed that *BRAF* mutant signaling through MEK initiated a RAC-dependent cadherin switch, which was not observed in *NRAS* mutant cells(Monaghan-Benson and Burridge, 2012). Intriguingly, *RAC1* mutant tumors were less likely to have detectable immunoreactivity expression. This is possibly due to proteasome-dependent negative feedback mediating rapid degradation of activated RAC1 protein(Nethe and Hordijk, 2010). Consistent with this proteasome inhibitors have been shown to increase constitutively activated mutant RAC1 protein levels(Kovacic et al., 2001). An alternative explanation may be that the RAC antibody, although specific(Woodcock et al., 2010) and reported as analogous to active RAC(Dalton et al., 2013), is not detecting active RAC-GTP.

The exact mechanisms of *RAC1* activation and interactions with Ras and MAPK pathways in melanoma are likely complex. Activating mutations in the Ras / MAP-MEK-ERK pathway are common in melanocytic lesions, including benign naevi, however negative feedback loops and bypass mechanisms modulate signaling strength. This negative feedback may be disabled in *BRAF* mutant tumors, resulting in increased transcriptional output(Pratilas et al., 2009) (Figure 2). *RAC1* has been shown to have increased binding to *PAK1* and *MLK3*, which in turn allows activated *RAF* to interact with *MEK*(Chadee et al., 2006; Krauthammer et al., 2012; Wang et al., 2010) (Figure 2). Both of these mechanisms may work to increase activity of *ERK* and activation of *FRA1* with subsequent effects on EMT-TFs. Tiam1 has been shown to associate with activated Ras via a specific Ras-binding domain, thereby mediating Ras dependent activation of *RAC1*(Lambert et al., 2002). DOCK3 is another Rac-GEF expressed in melanoma cells and activates *RAC1* to initiate mesenchymal-type tumor cell movement, whereas amoeboid movement is more dependent on Rho signaling(Sanz-Moreno et al., 2008). The balance between *RAC1* and Rho signaling is thought to be important in determining the ability to migrate through different tissue microenvironments(Sanz-Moreno et al., 2008). The ERK-MAPK signaling pathways may

have an important role in regulating this balance in melanoma, as has been shown in colorectal cancer(Vial et al., 2003). Epigenetic modifications may also play a role in activation of *RAC1*, such as hypermethylation of miR-124, as recently described in pancreatic cancer(Wang et al., 2014). Further investigation is required to fully understand *BRAF* and *RAC1* crosstalk in melanoma.

This study provides further evidence that *RAC1* plays an important role in primary melanoma progression. *RAC1* mutations occur at relatively low frequencies and are associated with nodular phenotype and nodal involvement in this cohort. *RAC1* protein expression is more common, particularly amongst *BRAF* mutant melanomas, and may explain their early migration to regional nodes. Targeting of *RAC1* signaling pathways may therefore be useful in slowing the migration and progression of disease.

Methods

Study participants and data collection

Patients referred to one of three tertiary referral centres (The Victorian Melanoma Service (VMS), Peter MacCallum Cancer Centre or the Austin Hospital) with primary cutaneous melanoma were eligible for enrolment in the Melbourne Melanoma Project (MMP), which commenced recruiting patients in 2009. The primary melanomas of all patients enrolled were tested for the presence of a *BRAF* mutation for research purposes. Patients enrolled to June 2013, with excess DNA available following extraction from *BRAF* testing were included in this cohort. Patients with in-situ melanoma, uveal, mucosal melanoma or melanoma of unknown primary site were excluded. Human Research Ethics Committee approval was granted at all three institutions involved in the MMP (Project number 07/38).

Data collection

Patient information including date of birth, date of diagnosis, gender and site of the primary melanoma was collected from the patient by their treating Physician. Details on history of blistering sunburns, history of non-melanoma skin cancers and previous melanomas were

collected at initial presentation. The treating doctor assessed evidence of solar keratoses. The same data sheets were used across all three institutions. The histopathology of all primary melanomas treated at the VMS (n=286) and all RAC1 mutant cases were assessed by one dermatopathologist (CM). The histological variables: Breslow thickness (mm), mitotic rate (no./mm^2), ulceration and tumor subtype, were assessed. Melanoma subtype was classified according to WHO criteria into nodular melanoma (NM), superficial spreading melanoma (SSM) and lentigo maligna melanoma (LMM) or other(LeBoit et al., 2006). Solar elastosis was graded by a single investigator (VM) according to the amount of elastotic fibres in normal skin adjacent to the melanoma in the excision specimen (0, none; 1, mild; 2, moderate; 3, severe)(Viros et al., 2008) and dichotomized as non-severely sun damaged (non-SSD) (solar elastosis scores of 0 or 1) and SSD (solar elastosis scores of 2 or 3). Nodal status was defined as either sentinel node biopsy positive or synchronous stage 3B or 3C disease.

BRAF and _NRAS_ mutation detection by high resolution melting PCR analysis.

Genomic DNA was extracted from formalin fixed, paraffin-embedded tumor tissue and tested for *BRAF* mutations using methods described previously(Richter et al., 2013) If samples were negative for *BRAF* mutations, samples were subsequently screened for the presence of mutations within the region spanning codon 61 in exon 3 of the *NRAS* gene by high resolution melting (HRM) analysis with mutant samples confirmed by pyrosequencing. HRM for *NRAS* was performed using the LightCycler 480 (Roche Diagnostics). A set of two HRM reactions were performed for each sample to allow pyrosequening of in both directions. The primers for both reactions were: NRAS set1 For 5'-biotin-GATGGTGAAACCTGTTTGTTG-3', *NRAS* set1 Rev 5'-'GCCTTCGCCTGTCCTCATGTAT-3' and *NRAS* set2 For 5'-GATGGTGAAACCTGTTTGTTGGAC-3', *NRAS* set2 Rev 5'- biotin-GCCTTCGCCTGTCCTCATGTA-3' giving amplicon size of 93bp each. Each PCR mix contained 1x PCR buffer, 2.5 µM $MgCl_2$, 200 nM of forward primer, 400nm reverse primer, 200 µM of dNTPs, 5 µM of SYTO 9 (Invitrogen, Carlsbad, CA), 0.5U of HotStarTaq polymerase (Qiagen), 10 ng DNA and PCR grade water in a total volume of 20 µl. PCR conditions included an activation step of 15 min at 95°C followed by 55 amplification cycles: 95°C for 10 sec (ramp rate 4.4°C/s) followed by 56°C for 10 sec (ramp 2.2°C/s) and 72°C for 20 seconds(ramp 4.4°C/s). This was followed by the HRM step which included 95°C (hold 1min, ramp rate 4.4°C/s), 45°C (hold 1min, ramp rate 2.2°C/s), 70°C, (hold 1sec, ramp rate 1°C/s), 99°C, (ramp rate 0.02°C/s,

30 acquisitions per °C). Each run had four wildtype controls and two positive controls with the Q61K and Q61L mutation. The mutation status of samples showing aberrant melt curves was confirmed through pyrosequencing using Pyromark 96 ID system according to manufacturer's instructions using following sequencing primers: *NRAS*_pyro set1 5'-CCTGTTTGTTGGACATA-3' and *NRAS* pyro set2 5'-TCCTCATGTATTGGTCT-3'. Nucleotide dispensation order for the forward and the reverse sequence were respectively: CTGATACTAGCTGACGATAGAGAGTACATGTGCATGAGAGACA and CTCATGCACTAGTACTCTCATACGTCAGCTGATATCAGTAT.

***RAC1* mutation detection by high resolution melting analysis.**

PCR and HRM were performed using the LightCycler 480 (Roche Diagnostics). The primer sequences used were 5'- TTAGAGCTGTAGGTAAAACTTGCC -3' and 5'- cttacACAGTAGGGATATATTCTCC -3', giving an amplicon size of 96 bp. The reaction mixture included 1x PCR buffer, 2.5 µM $MgCl_2$, 200 nM of each primer, 200 µM of dNTPs, 5 µM of SYTO 9 (Invitrogen, Carlsbad, CA), 0.5U of HotStarTaq polymerase (Qiagen), 10 ng DNA and PCR grade water in a total volume of 10 µl. PCR conditions included an activation step of 15 min at 95°C followed by 55 cycles of 95°C for 10 sec, annealing for 10 sec comprising 10 cycles of a touchdown from 65°C to 55°C at 1°C/cycle followed by 35 cycles at 55°C, and extension at 72°C for 30 sec. All analyses were performed in duplicate. At least three different normal controls were included in each run. A positive control sample with the *RAC1* P29S mutation (CO21-M1) was included for each run. Samples showing an aberrant melt profile compared to normal controls via HRM were directly sequenced from a 1/10 dilution of the HRM product using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

RAC1 immunohistochemistry

Immunohistochemical analysis of RAC1 protein expression was performed on formalin-fixed, paraffin embedded tissue of 25 *RAC1* mutant melanomas as well as a random sample of 26 *RAC1* wild-type melanomas. Tissue was exhausted or unavailable for 2 *RAC1* mutant cases, which were therefore excluded from the analysis. Tissue sections were cut at 3µm thickness, dewaxed and rehydrated through graded alcohols to water. Pretreatment was done with Proteinase K enzyme

(Dako Cat# S3020) for 6 minutes at room temperature. The RAC1 antibody (BD BioScience Cat#610651) was a mouse monoclonal (clone 102/Rac1) used at 1:200 dilution for 60mins at room temperature. The Ventana (ultraView Universal Alkaline Phosphatase Red Detection Kit Cat#760-501) detection kit was used according to the manufacturer's specifications. The preferred IHC protocol was alkaline phosphatase based, utilizing Fast Red as the chromogen because of the presence of brown endogenous melanin pigment within the sections. The sections were then counterstained with haematoxylin and coverslipped. The intensity of staining was scored on a scale of 0 (lowest intensity) to 3 (highest intensity). Scores of 2 or 3 were considered positive.

Statistical Analysis

Univariate logistic regression was used to assess associations between *RAC1* mutation status and other variables. Univariate and multivariate logistic regression models for the risk of presentation with nodal disease were used for analyses of associations with mutation status with adjustment for known prognostic variables in the multivariate models. As thickness and mitotic rate data were skewed, values were log transformed to follow approximately normal distributions. A p-value ≤0.05 was considered significant. As not all samples were tested for *NRAS* mutations, weights were applied in the relevant analyses and calculated as the inverse probability of selection for testing. All analyses were performed using Stata statistical software version 12.1.

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Table 1. Associations between *RAC1* mutation and other clinical and histological variables

	***RAC1* mutant**		***RAC1* WT**				
Continuous Variables	**Median**	**[IQR]**	**Median**	**[IQR]**	**OR**	**95%CI**	**p-value**
Age (years)	63	[54, 73]	58	[46, 69]	1.0	1.0, 1.0	0.5
Thickness (mm)	2.4	[0.75, 2.8]	1.5	[1.6, 3.8]	1.6	1.2, 2.2	0.001
Mitotic rate (mm^2)	3	[1, 6]	2	[1, 7]	1.3	1.0, 1.6	0.03

Categorical variables	**n**	**%**	**n**	**%**	**OR**	**95%CI**	**p-value**
Total cohort	27	3	801	97			
Gender							
Male	16	59	464	59	1.0	0.4, 2.1	1.0
Female	11	41	325	41			
Site							
HN	8	30	155	21			
UL	7	26	197	26	0.7	0.2, 1.9	0.5
T	5	19	224	30	0.4	0.1, 1.3	0.1
LL	7	26	148	20	0.9	0.3, 2.6	0.9
Other	0	0	29	4	-	-	-
Subtype							
SSM	10	37	446	61			
NM	14	52	186	25	3.4	1.5, 7.7	0.004
LMM	3	11	52	7	2.6	0.7, 9.7	0.2
Other	0	0	47	6	-	-	-
Ulceration							
No	15	58	563	77			
Yes	11	42	169	23	2.4	1.1, 5.2	0.04
Solar elastosis							
Non-SSD	11	41	127	42			
SSD	16	59	172	58	1.1	0.5, 2.5	0.8
Solar keratoses							
No	11	48	394	57			
Yes	12	52	300	43	1.4	0.6, 3.3	0.4

History of NMSC							
No	20	83	539	71			
Yes	4	17	219	29	0.5	0.2, 1.4	0.2
History of blistering sunburn							
No	9	38	263	36			
Yes	15	62	475	64	0.9	0.4, 2.1	0.9

WT wild type; IQR interquartile range; SSM superficial spreading melanoma; NM nodular melanoma; LMM lentigo maligna melanoma; HN head and neck; UL upper limb; T trunk; LL lower limb; SSD severely sun-damaged skin; NMSC non-melanoma skin cancer.

Table 2. The association of *RAC1* mutant melanoma with *BRAF* and *NRAS* mutations

	***RAC1* mutant**		***RAC1* WT**				
Mutation	**n**	**%**	**n**	**%**	OR	95%CI	p-value
BRAF							
WT	20	74	508	65	1		
V600E	4	15	222	28	0.5	0.2, 1.4	0.2
V600K	3	11	55	7	1.4	0.4, 4.8	0.6
NRAS*							
WT	19	70	687* (422)	87*	1		
Mutant	8	30	98* (34)	13*	2.9*	1.2, 7.3	0.01

WT wild type.

*Analysis weighted by sample selection probabilities to account for absence of *NRAS* measurement in some patients. Number of *RAC1* WT cases with known *NRAS* status shown in brackets.

Table 3. Univariate and multivariate logistic regression analyses for mutation status and nodal disease

Mutation	Nodal status				Univariate			Multivariate^		
	Positive(%)		Negative(%)		**OR**	[95%CI]	**p**	**OR**	[95%CI]	**p**
RAC1										
WT	83	(11)	648	(89)	3.3	[1.4, 7.8]	0.006	2.5	[1.0, 6.3]	0.06
Mutant	8	(30)	19	(70)						
BRAF										
WT	46	(10)	426	(90)	1.9	[1.2, 3.0]	0.004	2.6	[1.5, 4.3]	<0.001
Mutant	42	(17)	206	(83)						
NRAS*										
WT		(11)		(89)	1.0	[0.3, 2.8]	0.9	0.7	[0.2, 2.6]	0.6
Mutant		(13)		(87)						

WT wild type.

***NRAS* weighted to account for missing data in whole cohort

^Multivariate analysis included adjustment for thickness, ulceration and mitotic rate

Table 4. RAC1 protein immunoreactivity expression

	Total cases tested	IHC expression (Score 2-3)	%	No IHC expression (Score 0-1)	%	OR	95%CI	p
RAC1								
WT	26	18	69	8	31	1		
Mutant	25	6	24	19	76	0.1	[0.04, 0.5]	0.002
BRAF								
WT	33	11	33	22	67	1		
Mutant	18	13	72	5	28	5.2	[1.5, 18.3]	0.01
NRAS								
WT	40	21	53	19	48	1		
Mutant	11	3	27	8	73	0.7	[0.1, 3.5]	0.7
Median thickness								
mm [IQR]		1.7 [1.2-3.0]		2.6 [1.8-5.2]		0.6	[0.3, 1.0]	0.05
Median MR								
mm^2 [IQR]		1.8 [1-3]		6 [3-10]		0.7	[0.4, 1.1]	0.09
Subtype								
SSM	25	16	67	9	33	1		
NM	22	8	33	14	52	0.3	[0.07, 1.1]	0.06
LMM	4	0	0	4	15	NA		

WT wild-type; MR mitotic rate; IQR interquartile range; SSM superficial spreading melanoma; NM nodular melanoma; LMM lentigo maligna melanoma; NA not available due to low frequency