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ORIGINAL ARTICLE

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Circulating Melanoma Cell Subpopulations: Their Heterogeneity and Differential Responses to Treatment

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Metastatic melanoma is a highly heterogeneous tumor; thus, methods to analyze tumor-derived cells circulating in blood should address this diversity. Taking this into account, we analyzed, using multiparametric flow cytometry, the co-expression of the melanoma markers melanoma cell adhesion molecule and melanoma-associated chondroitin sulphate proteoglycan and the tumor-initiating markers ATP-binding cassette sub-family B member 5 (ABCB5), CD271, and receptor activator of NF-κβ (RANK) in individual circulating tumor cells (CTCs) from 40 late-stage (III–IV) and 16 early-stage (I–II) melanoma patients. CTCs were heterogeneous within and between patients, with limited co-expression between the five markers analyzed. Analysis of patient matched blood and metastatic tumors revealed that ABCB5 and RANK subpopulations are more common among CTCs than in the solid tumors, suggesting a preferential selection for these cells in circulation. Pairwise comparison of CTC subpopulations longitudinally before and 6–13 weeks after treatment initiation showed that the percentage of $RANK^+$ CTCs significantly increased in the patients undergoing targeted therapy ($N=16$, $P<0.01$). Moreover, the presence of $\geqslant 5$ $RANK^+$ CTCs in the blood of patients undergoing targeted therapies was prognostic of shorter progression-free survival (hazards ratio 8.73, 95% confidence interval 1.82–41.75, $P<0.01$). Taken together, our results provide evidence of the heterogeneity among CTC subpopulations in melanoma and the differential response of these subpopulations to targeted therapy.

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INTRODUCTION

Melanoma is an aggressive cancer, which is responsible for 80% of skin cancer–related deaths. Most melanoma patients are cured after surgical excision of the primary tumor, but 10–20% of patients progress to develop metastatic disease. Metastatic melanoma has a tendency to spread rapidly and be intrinsically resistant to chemotherapy. Over the last 3 years, the implementation of targeted therapies and immunotherapies has slowed down the progression of metastatic melanoma in many patients. However, patients on targeted therapies develop drug resistance within months after treatment, and immunotherapies are only effective in a proportion of patients (Homet and Ribas, 2014). Currently, our ability to monitor recurrence and to predict treatment efficacy is limited. Future treatment decisions might be improved by the use of biomarkers capable of early prediction of treatment failure, so that patients could be switched earlier to a different modality.

There is increasing evidence that circulating tumor cells (CTCs) in blood are an important indicator of the potential for metastatic disease, poor prognosis, treatment response, and disease recurrence in breast, colon, prostate, and lung cancers (de Bono *et al.*, 2008; Krebs *et al.*, 2011; Hou *et al.*, 2012; Lucci *et al.*, 2012). Similarly, the number of CTCs has been shown to be prognostic of the overall survival in metastatic melanoma patients (Khoja *et al.*, 2013).

In melanoma, the most common marker used for CTC enrichment is the melanoma-associated chondroitin sulphate proteoglycan (MCSP, HMW-MAA, CSPG4, and NG2; Faye *et al.*, 2004; Ulmer *et al.*, 2004). In addition, the CellSearch detection of melanoma CTCs involves the capture of melanoma cell adhesion molecule (MCAM, CD146, and MUC18)-expressing cells and immunofluorescence-based

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Abbreviations: ABCB5, ATP-binding cassette sub-family B member 5; CTC, circulating tumor cell; MCAM, melanoma cell adhesion molecule; MCSP, melanoma-associated chondroitin sulphate proteoglycan; RANK, receptor activator of NF-κβ; PFS, progression-free survival

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detection of MCSP-expressing cells (Rao *et al.*, 2011). However, it has long been appreciated that tumors are composed of heterogeneous populations, with a pool of cells that display stem cell properties and drive evolution of the tumor to a gradually more aggressive phenotype. Recent studies have shown that CTCs isolated from breast, colon, and hepatic cancer patient blood are similarly composed of a heterogeneous pool of tumor cells, some of which have tumor-initiating/stem cell properties and display epithelial–mesenchymal transition features and low apoptotic propensity (Pilati *et al.*, 2012; Baccelli *et al.*, 2013; Sun *et al.*, 2013). In melanoma, subpopulations of tumor cells with tumor-initiating/stem cell properties are marked by Nestin (Grichnik *et al.*, 2006), ATP-binding cassette sub-family B member 5 (ABCB5; Schatton *et al.*, 2008), CD133 (Monzani *et al.*, 2007), CD20 (Fang *et al.*, 2005), CD271 (Boiko *et al.*, 2010), JARID1B (Roesch *et al.*, 2008), and the receptor activator of NF-κβ (RANK, CD265; Kupas *et al.*, 2011).

We previously demonstrated that immunomagnetic capture of circulating melanoma cells, using a combination of antibodies to MCAM, MCSP, ABCB5, and CD271, resulted in the enrichment of larger numbers of cells than when individual markers were targeted (Freeman *et al.*, 2012). However, using immunomagnetic beads to isolate CTCs, we could not identify cells that co-expressed multiple markers, nor could we determine the proportion of cells expressing particular marker combinations. To expand our understanding of CTC expression profiles and to identify different CTC subpopulations, here we analyzed whole-blood samples using multiparametric flow cytometry. Using the melanoma-associated markers MCAM and MCSP, and the melanoma-initiating cell markers ABCB5, CD271, and RANK, we identified previously unreported CTC subtypes. These subtypes were compared between patients at early and late disease stages, as were the dynamics of different CTC subtypes before and after initiation of treatment with targeted therapies or immunotherapy. Moreover, we performed a preliminary evaluation of the potential prognostic value of these CTC subpopulations.

RESULTS

Marker expression in melanoma cell lines

We first evaluated, in several melanoma cell lines, the expression of the previously identified CTC markers MCAM, MCSP, ABCB5, CD271, and RANK (Kupas *et al.*, 2011; Freeman *et al.*, 2012). A total of nine melanoma cell lines were analyzed by flow cytometry: seven derived from metastatic melanoma (C8161, A2058, SK-MEL-2, SK-MEL-5, UACC62, SK-MEL-28, and MM229) and two derived from primary melanomas (MM540, MM200). We detected the five markers at varying frequencies; MCAM and MCSP were expressed in 100% of the cells for most cell lines (Figure 1a). However, MCSP was expressed in only 68% of SK-MEL-5 cells and at lower mean fluorescence intensity than in A2058 cells (Figure 1b). Most cell lines expressed the melanoma stem cell markers CD271, ABCB5, and RANK at low frequencies. CD271 was expressed in 70%, 32%, 24%, and 17% of A2058, SK-MEL-2, SK-MEL-28, and MM229 cells, respectively, but in $<3\%$ of the other five cell lines. ABCB5 expression was low ($<3\%$) in most cell lines with only A2058 and MM540 expressing ABCB5 in ~13% of cells. RANK was expressed in both primary melanoma cell lines, 90% of MM540 (Figure 1c) but in only 23% of MM200. Among metastatic melanoma cell lines, only MM229 and SK-MEL-2 expressed RANK at 30 and 28%, respectively, whereas it was expressed at low frequencies ($<7\%$) in all other metastatic cell lines (Figure 1a).

Spiking experiments

Prior to performing the analysis of CTCs in the blood from melanoma patients, we determined the efficiency of the flow cytometric detection method by adding increasing numbers—i.e., 6, 60, 600, or 6,000 cells of the melanoma cell line A2058 to 4 ml of whole blood from healthy volunteers. Given that expression of MCAM and MCSP can be readily detected in 100% of A2058 cells, spiked whole-blood samples were stained with antibodies specific for these antigens. After exclusion of $CD45^+$ and $CD34^+$ cells, melanoma cells were counted as double-positive for MCAM and MCSP. No double-positive cells were detected in control blood without melanoma cells. An average recovery of 85% was obtained using a direct staining method (Supplementary Figure S1A and S1B online). The addition of a pre-enrichment step to deplete CD45-positive cells using the EasySep kit (StemCell) substantially decreased the recovery rate to $<20\%$ (Supplementary Figure S1C online). Given the low numbers of CTCs found in the blood of most melanoma patients (Freeman *et al.*, 2012), the direct staining method was selected as the method of choice.

Identification of CTCs by flow cytometry

Blood samples from melanoma patients at early (TNM stages I–II, $N=16$) and late clinical disease stages (TNM stages III–IV, $N=40$), and from healthy controls ($N=15$) were tested by multiparameter flow cytometry for the presence of cells expressing MCSP, MCAM, RANK, ABCB5, or CD271. Demographic data for the three groups are described in Table 1. Cells negative for CD45 and CD34 and positive for any of the five chosen markers were identified as CTCs. A maximum of 10 cells positive for any of the markers were found among healthy control samples and defined as background. Blood samples from melanoma patients (all stages combined) contained a significantly larger number of marker-positive cells ($P<0.01$). In particular, late-stage patients had significantly more cells compared with healthy controls; however, this difference was not as apparent for early-stage patients (Figure 2a).

Co-expression of surface markers on CTCs

We next analyzed the number of CTCs that expressed different combinations of the five markers and determined the co-expression of these markers. The level of background, defined as the number of marker-positive cells in healthy controls, varied depending on marker combinations from 0 to 4 cells. Accordingly, the maximum cell number detected in controls for each marker combination (background) was subtracted from the respective cell numbers detected in

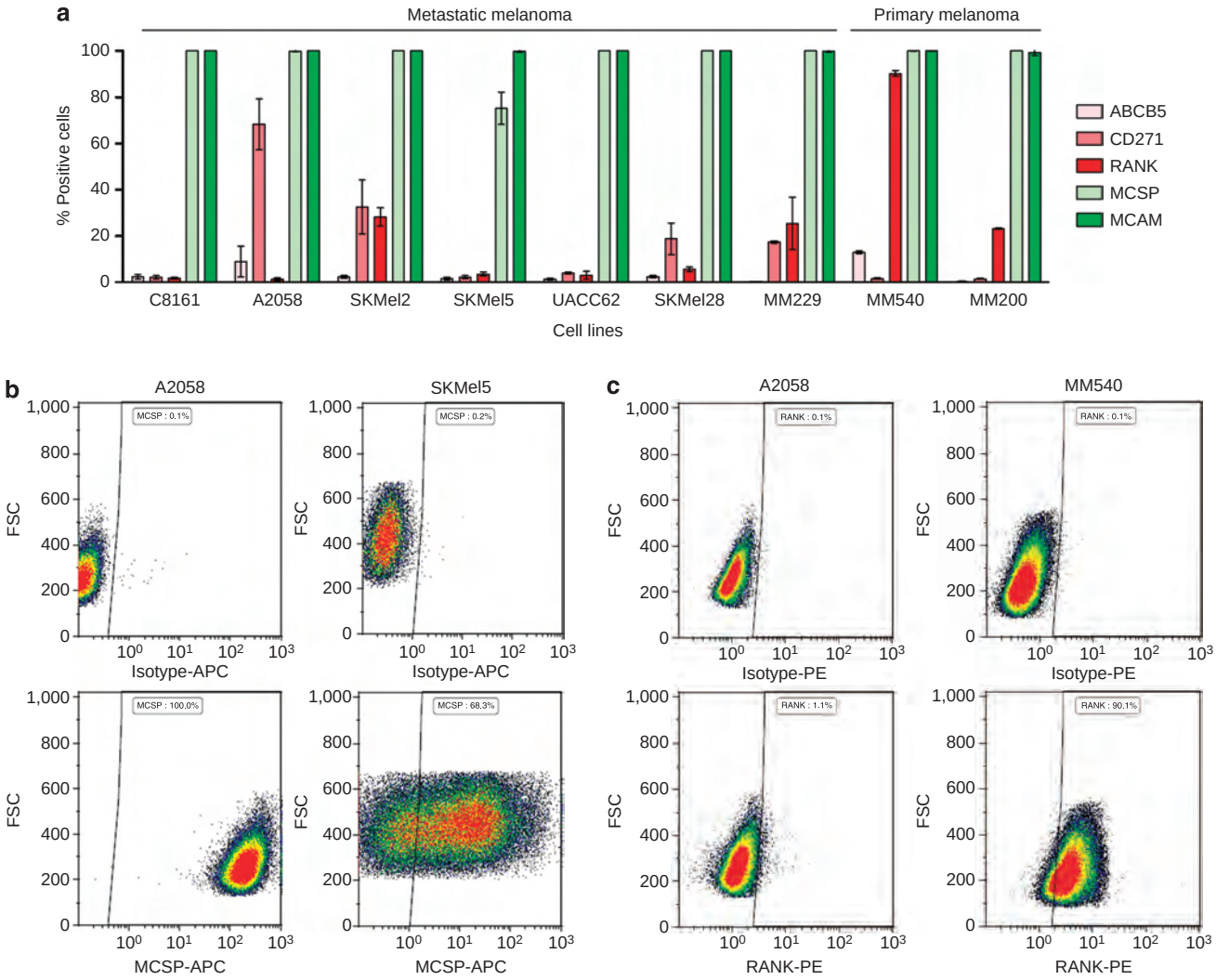

Figure 1. Marker expression on the surface of melanoma cell lines. (**a**) Metastatic and primary melanoma cell lines were tested for the expression of ABCB5, CD271, RANK, MCSP, and MCAM by flow cytometry. The percentage (mean ± SEM) of positive cells obtained in three independent experiments was determined for each marker. Representative dot plot of flow cytometric staining of (**b**) MCSP on A2058 and SK-MEL-5 cells or (**c**) RANK on A2058 and MM540 cells. The corresponding isotype controls are also depicted. ABCB5, ATP-binding cassette sub-family B member 5; MCAM, melanoma cell adhesion molecule; MCSP, melanoma-associated chondroitin sulphate proteoglycan; RANK, receptor activator of NF-κβ.

melanoma patients. Of the 31 possible marker combinations, only 9 were identified among melanoma patients and were further analyzed in detail. Graphical representation of the different CTC subtypes present in each patient illustrates the heterogeneity between the cells found within and between patients (Figure 2b). CTCs in early-stage patients showed positivity mainly for a single marker, whereas late-stage patients had a large number of CTCs and/or their CTCs co-expressed several markers.

Interestingly, six of the nine marker combinations found in patients involved the presence of ABCB5, a melanoma multidrug resistance mediator recently shown to maintain melanoma-initiating cells (Wilson *et al.*, 2014); however, the number of cells, as well as the number of patients with those cells, was low for each independent combination. Taking together all 6 combinations (including ABCB5 alone), $ABCB5^+$ cells were found in 13 out of 40 late-stage and 3 out of 16 early-stage patients, accounting for 52% of patients with detectable CTCs or 29% of all melanoma patients tested.

The $RANK^+$-only subpopulation was the most commonly detected being found in 46% of all patients. Including all combinations, $RANK^+$ cells were detected in 22 out of 40 late-stage and 4 out of 16 early-stage patients, accounting for 84% of patients with detectable CTCs and 46% of all melanoma patients tested.

MCSP-expressing subpopulations were detected in 10 out of 40 late-stage and 3 out of 16 early-stage patients, accounting for 42% of patients with detectable CTCs and 23% of all melanoma patients tested. Samples with $MCAM^+MCSP^+$ cells, either with or without other markers, were not found in early-stage patients but were present in 6 of 40 late-stage patients (15%).

Table 1. Study participants demographics and clinical characteristics

		Melanoma patients	
	Healthy controls	Stages I–II	Stages III–IV
N	15	16	40
Age (Range)	54 (26–69)	68 (36–90)	59 (30–85)
Male/female	9/6	13/3	24/16
Stage			
I		10	
II		6	
III			2
IV			38
Number of metastatic sites			
0		16	
1–2			11
>2			29
BRAF status			
V600E			13
V600K			7
V600R			1
K601E			1
Wild type			16
Unknown			2
Treatment			
Vemurafenib			4
Dabrafenib			2
Dabrafenib/trametinib			14
Ipilimumab			11
Pembrolizumab			4
Nivolumab			1
Others			4

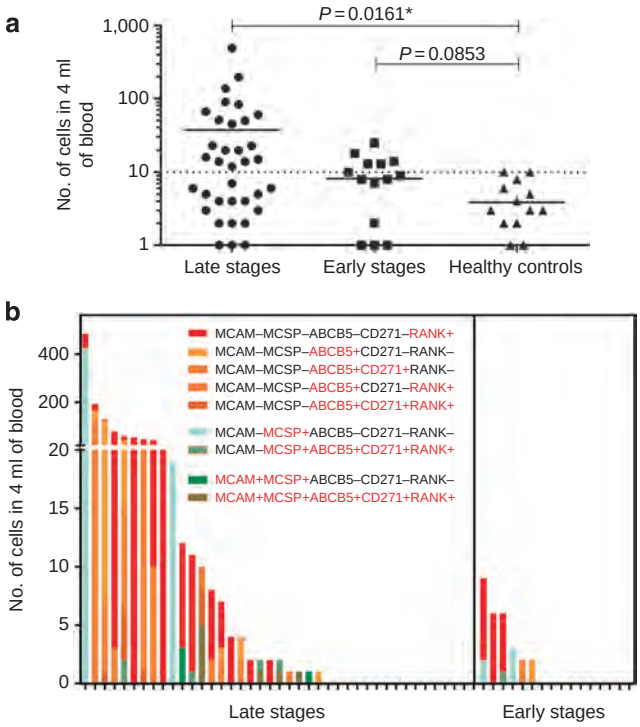

Figure 2. Analysis of circulating tumor cells (CTCs) in melanoma patients and healthy controls (**a**) Total number of cells detected in 4 ml of whole blood expressing any of the markers in melanoma patients at late ($N = 40$) and early ($N = 16$) stages of the disease and in healthy controls ($N = 15$). The mean is indicated for each group. An omnibus Kruskal–Wallis test produced a P-value = 0.07. Mann–Whitney tests were performed and the P-value indicated for each comparison. (**b**) Number of cells in 4 ml of blood corresponding to each CTC subpopulations identified. Each bar represents a single melanoma patient. Absent bars represent patients in which CTCs were not detected. ABCB5, ATP-binding cassette sub-family B member 5; MCAM, melanoma cell adhesion molecule; MCSP, melanoma-associated chondroitin sulphate proteoglycan; RANK, receptor activator of NF-κβ.

Among the metastatic melanoma patients analyzed, 22 had tumors with a mutated BRAF and 16 patients were found to be wild type for BRAF (Table 1). We analyzed the presence of CTCs or of a specific marker combination in relation to the BRAF status of the patient's tumor but failed to find any association between these two parameters.

RANK and ABCB5 expression in match tumor tissues

Next we analyzed the expression of RANK and ABCB5 in metastatic tissue from two patients in which CTCs expressing these two markers could be detected: patient MM15, in which 100% of detected CTCs were RANK$^+$ and 25% were ABCB5$^+$, and patient MM26, in which 75% of CTCs were RANK+ but no ABCB5$^+$ CTCs were detected. The metastases analyzed were removed from these patients within 1 month prior to blood collection for CTC analysis. Immunofluorescence staining of the tissue was performed using antibodies to MART-1 to define the tumor cells together with either RANK or ABCB5 (Figure 3). In contrast with the large percentage found among CTCs, the number of RANK (~2%) and ABCB5 (6–7.5%)-expressing cells was sparse within the tumor.

Analysis of CTCs during BRAF/MEK-targeted therapy or immune checkpoint blockade

Of the 40 patients with unresectable metastatic melanoma enrolled in the study, 29 were tested for the presence of CTCs before treatment and within 6–13 weeks after therapy initiation with BRAF/MEK-targeted therapy or immune checkpoint blockade. Of these, four individuals were treated with vemurafenib, ten with a combination of dabrafenib and trametinib, two with dabrafenib monotherapy, ten with ipilimumab, two with pembrolizumab, and one with nivolumab. We found no significant change in the total number of CTCs after treatment initiation (Figure 4a). On the basis of our analysis of CTC subpopulations, we examined each marker combination as a percentage of total CTCs before and after therapy. For most populations, no statistically significant

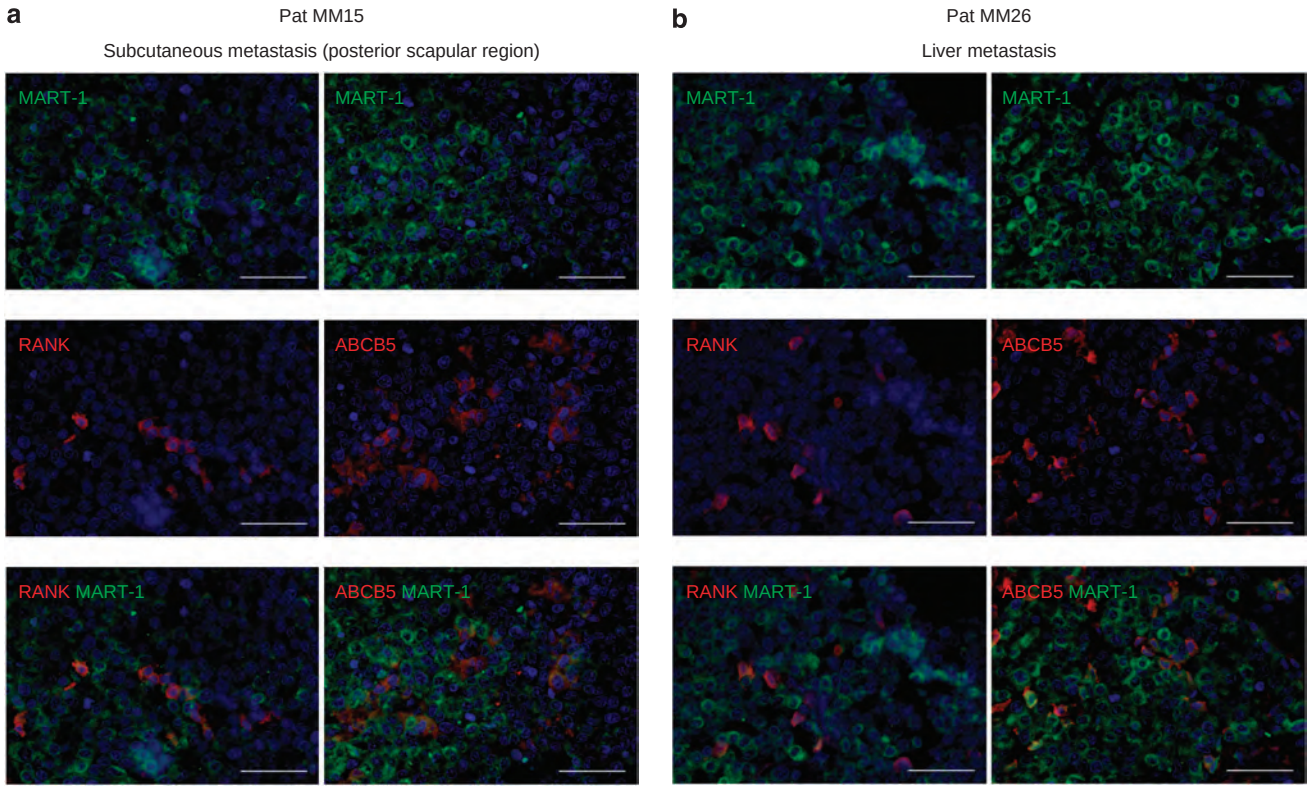

Figure 3. Receptor activator of NF-κβ (RANK) and ATP-binding cassette sub-family B member 5 (ABCB5) expression in metastases from patients with circulating tumor cells (CTCs) expressing these markers. Immunofluorescence staining of metastatic melanoma tissue corresponding to patients MM15 (**a**) and MM26 (**b**). MART-1 staining was used to identify melanoma cells. Original magnification ×400. Scale bar = 20 μm.

increase or decrease in their percentage was noticed (Supplementary Figure S2 online). Interestingly, a statistically significant increase in the percentage of $RANK^+$ cells was noticed after therapy initiation in patients treated with BRAF- or BRAF/MEK-targeted therapy (vemurafenib, dabrafenib/trametinib; $P<0.01$, Figure 4b). However, this increase was not apparent among patients treated with checkpoint inhibitors, suggesting that this effect might be specific to the targeted therapy treatment.

Prognostic significance of specific CTC subtypes

Of 16 patients treated with targeted therapies (4 vemurafenib, 10 dabrafenib/trametinib, and 2 dabrafenib), 14 individuals had a reported size reduction in their tumors by Positron Emission Tomography or Computer Tomography scan at first assessment at around 6 weeks; 12 showed partial responses and 2 complete responses as per Response Evaluation Criteria in Solid Tumours (RECIST) 1.1. Of those responding, nine had controlled tumor growth for 6 months or more, whereas four experienced tumor relapse within the first 6 months after treatment initiation.

A Kaplan–Meier analysis was performed to determine the association between CTC presence and prognostic factors such as response to treatment and progression-free survival (PFS). Given that the median follow-up time of these patients was 30 weeks, it is too early to evaluate the predictive value of the CTC subtypes on overall survival. Exploratory analyses were performed repeatedly using different cut-off values to define a favorable or an unfavorable CTC number, at 1–10 cells (Supplementary Table S1 and S2 online). PFS was not associated with total CTC counts at baseline or after treatment initiation (data not shown). Potential association with PFS was also analyzed in a log-rank test for each of the nine CTC subtypes (Supplementary Figure S3 online). The presence of $RANK^+$-only CTCs at baseline did not show statistical significance at any of the cut-off values tested (Figure 4c, Supplementary Table S1 online). Interestingly, the presence of RANK+ cells before or after treatment initiation was significantly associated with shorter PFS. The confidence intervals for the hazard ratios were qualitatively similar at various cut-off values (Supplementary Table S2 online), with the difference in PFS between patients with five or more RANK-expressing CTCs and those with <5 cells illustrated in Figure 4d (hazards ratio 8.73, 95% confidence interval 1.82–41.75, $P<0.01$).

RANKL/RANK stimulation of melanoma cells affects sensitivity to vemurafenib

To explore the mechanism of RANK-mediated resistance to BRAF inhibitors, we selected two melanoma cell lines, A2058 and 1205Lu, and stimulated them with recombinant RANKL prior to vemurafenib inhibition. A2058 cells express RANK,

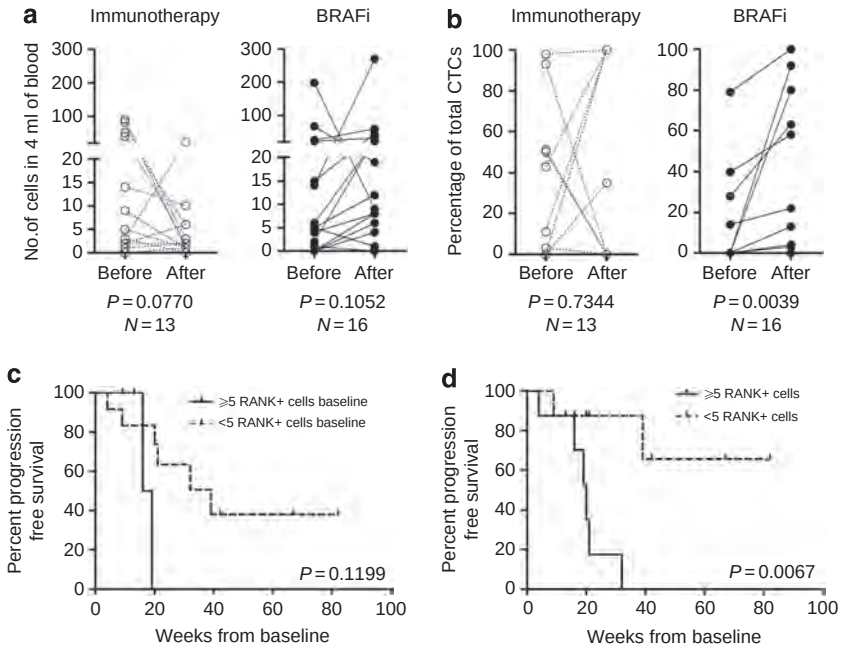

Figure 4. Prognostic significance of RANK$^+$ cells. (**a**) Total CTC counts and (**b**) the percentage of CTCs expressing MCAM-MCSP-CD271-ABCB5-RANK+ were quantified in 29 patients with unresectable metastatic melanoma at baseline and 6–13 weeks after treatment initiation. Open circles indicate patients treated with immunotherapy ($N = 13$); ipilimumab ($N = 10$), pembrolizumab ($N = 2$), and nivolumab ($N = 1$). Black circles indicate patients treated with targeted therapies ($N = 16$); vemurafenib ($N = 4$), dabrafenib/trametinib ($N = 10$), and dabrafenib ($N = 2$). The *P*-values of Wilcoxon matched-pairs signed rank tests are indicated for each comparison. (**c** and **d**) Kaplan–Meier curves for progression-free survival of metastatic melanoma patients undergoing targeted therapies ($N = 16$). Patients were stratified as those with ⩾5 and <5 RANK$^+$ cells in 4 ml of blood at (**c**) baseline (before therapy) and (**d**) after therapy initiation. The long rank *P*-values are indicated. ABCB5, ATP-binding cassette sub-family B member 5; CTC, circulating tumor cell; MCAM, melanoma cell adhesion molecule; MCSP, melanoma-associated chondroitin sulphate proteoglycan; RANK, receptor activator of NF-κβ.

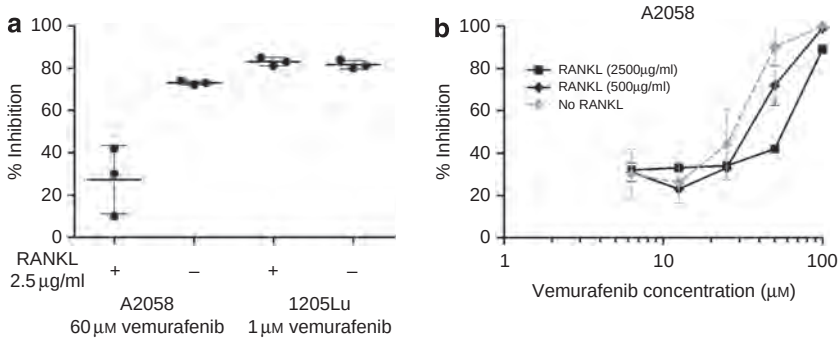

Figure 5. RANKL stimulation effect in vemurafenib sensitivity. (**a**) Sensitivity to vemurafenib/PLX4032 of A2058 and 1205Lu melanoma cells pre-incubated with or without RANKL at 2.5 μg ml^{-1} for 12 hours prior to treatment. Cells were treated with approximately two times their respective 50% inhibitory concentration (IC_{50}), 60 μM for A2058, and 1 μM for 1205Lu. Percentage of inhibition was calculated relative to cells not exposed to vemurafenib. **(b)** Dose-response curve of vemurafenib growth inhibition of cells pre-incubated with RANKL at 2.5 and 0.5 μg ml^{-1}. Experiments were performed in triplicate and the mean and SD are indicated for each set. RANK, receptor activator of NF-κβ.

albeit at low frequency. On the other hand, 1205Lu cells do not express RANK by flow cytometry or by reverse transcriptase–PCR (data not shown). Pre-incubation with RANKL did not affect the sensitivity to vemurafenib of 1205Lu cells (Figure 5a). Conversely, A2058 cell stimulation with 2.5 μg ml^{-1} RANKL, but not 0.5 μg ml^{-1} RANKL, showed less sensitivity to vemurafenib than unstimulated cells (Figure 5a and b).

DISCUSSION

We previously demonstrated that a multi-marker approach enhances the isolation of CTCs in melanoma (Freeman *et al.*, 2012). Here we further support this multi-targeted CTC detection approach by identifying for the first time cellular heterogeneity of CTCs within and among melanoma patients. Moreover, we provide evidence that distinct CTC subpopulations are differentially affected by alternate melanoma therapies.

Here we found MCAM$^+$MCSP$^+$ CTCs in 15% of patients, exclusively in those with late-stage melanoma. This is consistent with the report of Khoja *et al.* (2013) using the CellSearch system for identification of CTCs. The addition of tumor initiation markers to the panel increased the frequency (62%) and the number of cells detected in late-stage melanoma. Moreover, CTCs were also detected in a proportion of early-stage cases. The prognostic relevance of the presence of these markers in patients with localized melanoma needs to be explored in large prospective studies powered to address this important question.

A relevant observation derived from our study is the large proportion of detected CTCs expressing stem cell markers. This is consistent with our previous observations that addition of stem cell markers for immunocapture of CTCs increases the number of detected cells (Freeman *et al.*, 2012) and that BRAF V600E mutations can be detected in CTCs enriched with antibodies to ABCB5 and RANK (Reid *et al.*, 2014). The frequency of cells expressing ABCB5 and RANK is rather low in the tumor tissue, as shown here as well as in previous studies (Schatton *et al.*, 2008; Kupas *et al.*, 2011). This is consistent with the observations by Ma *et al.* (Ma *et al.*, 2010) in which a larger proportion of the CTCs found in mouse blood expressed ABCB5 in comparison with the correspondent tumor tissue. This enrichment of stem-like cells among the CTC pool suggests a selective process through which melanoma-initiating cells preferentially reach the circulation. Alternatively, environmental conditions within the blood induce the expression of these markers. This enrichment in the blood of cancer cells with higher tumor initiation potential is a critical phenomenon for metastatic spreading and warrants further investigation.

Interestingly, the percentage of RANK$^+$ cells increased after therapy initiation in patients treated with BRAF inhibitors. Moreover, the presence of these RANK$^+$ cells was associated with shorter PFS in patients treated with BRAF inhibitors. Comparison with a similar cohort of patients treated with immune checkpoint blockade indicates that this increase in RANK$^+$ CTCs is specific to the treatment with MAPK inhibitors. Nevertheless, larger studies are needed to corroborate this observation. Moreover, it is important to elucidate the mechanism through which the RANKL/RANK axis affects MAPK inhibition in melanoma.

The relevance of RANK expression in melanoma has not been studied in detail. Kupas *et al.* (2011) identified heterogeneous expression of RANK in cells from tumors and peripheral blood from melanoma patients. The authors found a large number of RANK$^+$ cells in the blood of some melanoma patients consistent with our observation. Moreover, they demonstrated that RANK$^+$ CTCs had an enhanced tumor-initiating capacity in immunodeficient mice. This is consistent with reports in breast cancer where RANK overexpression and RANKL stimulation induce epithelial-mesenchymal-transition and stemness in human mammary epithelial cells and promote tumorigenesis and metastasis (Palafox *et al.*, 2012).

Our preliminary results into the role of RANK in BRAF inhibition suggest that stimulation through the RANK/RANKL axis results in a decreased sensitivity to vemurafenib. A recent study showed that activation of NF-κβ is associated with a distinct melanoma cell state with an intrinsic resistance to MAPK inhibitors (Konieczkowski *et al.*, 2014); thus, RANK-mediated activation of NF-κβ could lead the cells to a similar state. Moreover, RANKL/RANK stimulation via TRAF6 is known to activate JNK1, AKT/PKB, and p44/42 ERK, (Palafox *et al.*, 2012), all of which could bypass BRAF inhibition resulting in decreased drug sensitivity. Further studies into the mechanism behind this RANK-mediated resistance in melanoma cells warrant further studies.

Kupas *et al.* showed that most RANK-expressing cells also expressed the melanoma stem cell markers ABCB5 and CD133 (Kupas *et al.*, 2011). Previous research has shown that treatment of three melanoma cell lines with vemurafenib and to lesser extent dacarbazine resulted in an increase in ABCB5-positive cells (Chartrain *et al.*, 2012). Across our study, we found RANK$^+$ CTCs also expressing ABCB5 in 14 out of 40 metastatic patients with RANK$^+$ CTCs. It is known that chemotherapies preferentially eliminate rapidly dividing cells (Blagosklonny, 2005). Indeed, survival of a subpopulation of slow-cycling melanoma cells expressing JARID1B after vemurafenib treatment has been recently described (Roesch *et al.*, 2013). Future studies are needed to assert whether RANK is an alternative marker of slow cycling cells and reduced sensitivity to the MAPK pathway inhibitors.

Our results underscore the importance of determining the prognostic value of different CTC subpopulations. In particular, the observed increase in a CTC subpopulation upon treatment further supports a multi-marker approach for capturing melanoma CTCs in order to monitor treatment responses. The analysis of CTCs may provide a suitable strategy to study, in real time, the pharmacokinetics of resistance in metastatic melanoma and evaluate therapeutic strategies to overcome drug resistance.

MATERIALS AND METHODS

Patient blood samples

Patients, recruited from 3 clinics in Perth, Western Australia, were diagnosed and staged according to guidelines of the American Joint Committee on Cancer. Patients were recruited between October 2012 and May 2014. Peripheral blood samples were obtained from 16 non-metastatic patients (TNM stages I and II) within 2 weeks of removal of the primary melanoma lesion. Blood from 40 metastatic melanoma patients (TNM stages III and IV) was obtained prior to commencement of treatment. In addition, blood samples were analyzed during treatment for 22 metastatic melanoma patients. In addition, blood samples were obtained from 15 healthy volunteers. Blood was drawn by phlebotomists into BD Vacutainer K2 EDTA tubes (BD Biosciences, San Jose, CA), after the first few milliliters were discarded to avoid epithelial contamination, and refrigerated at 4 °C until used. Samples were processed within 48 hours from collection time. Participants signed informed consent with the clinician in accordance with the protocols safeguarding patient rights. All procedures have been accepted by the Human Research Ethics Committees at Edith Cowan University (No. 2932) and Sir Charles Gairdner Hospital (No. 2007-123).

Flow cytometric staining

For detection of CTCs, two vials of 4 mls of blood samples were treated with an isotonic solution (140 mM NH4Cl, 17 mM Tris, pH 7.65) for red blood cell lysis, followed by a wash in FACS buffer (0.1% bovine serum albumin, 100 mM EDTA, 10 mM HEPES, phosphate-buffered saline). One sample was stained with antibodies to CD45-APC.AF750, CD34-AF700, MCSP-APC, MCAM-PE.Cy7, CD271-PerCPCy5.5, ABCB5-PE.TxRed, and RANK-PE, and the second sample was stained with the CD45 and CD34 antibodies and isotype controls for APC, PE.Cy7, PerCPCy5.5, PE.TxRed, and PE. Antibodies were incubated with the cells for 30 minutes at 4 °C. After washing out unbound antibodies, dead cells were stained using the Green Dead Cell Stain Kit (LifeTechnologies, Carlsbad, CA) for 15 minutes at 4 °C. After two washes with FACS buffer, cells were immediately acquired in a Gallios flow cytometer (Beckman Coulter, Miami, FL). Data were processed using Kaluza Analysis Software (Beckman Coulter). Gates were delineated using the isotype controls to obtain a minimum background signal.

Melanoma cell lines were removed from the culture flask at 80% confluence using 2 mM EDTA and 2×10^5 cells were stained for flow cytometric analysis as described above, except for the CD45 and CD34 staining.

The recovery of CTCs after a pre-enrichment of CD45-negative cells was evaluated using spiked blood. CD45-positive cells were depleted using the EasySep kit (StemCell Technologies, Vancouver, BC) following the manufacturer's instructions. Peripheral blood mononuclear cells were isolated in Ficoll-Paque plus (GE healthcare, Milwaukee, WI) and washed once with red blood cell lysing buffer and twice with 2% fetal bovine serum/phosphate-buffered saline, prior to incubation with CD45 depletion cocktail.

Statistical analysis

As data did not pass the Kolmogorov-Smirnov test for normal distribution, the non-parametric Mann–Whitney *U*-test and the Wilcoxon matched-pairs signed-rank test were used where appropriate. The number of CTCs within each subtype was dichotomized in order to determine the best cut-off value to discriminate between groups with a favorable and an unfavorable PFS. Survival curves were plotted using the Kaplan–Meier method and the log rank test using GraphPad Prism 5 for Windows (La Jolla, CA), and univariate Cox proportional hazards regression analyses were performed to evaluate the prognostic value of CTC subpopulations for PFS using the Statistical Package for Social Sciences for Window version 22 (SPSS, Chicago, IL). The reported *P*-values have not been adjusted for multiple comparisons. Tumor responses were assessed radiologically at 2 to 3 monthly intervals. CT scans were assessed by the RECIST 1.1 criteria and classified as having a complete response, partial response, stable disease, or progressive disease. Response time was calculated from the date at baseline to the date of first reported partial response or complete response. PFS time was calculated from baseline date to the date of first reported progressive disease.

Drug sensitivity and RANKL stimulation

Cell proliferation inhibition experiments were performed in a 96-well plate in triplicate. Cells were seeded at 10^4 cells per well and stimulated for 24 hours with rRANKL at 2.5 μg ml^{-1}, 0.5 μg ml^{-1} or left unstimulated. After 12 hours of stimulation, cells were treated with serial dilutions of vemurafenib/PLX4032 for 72 hours. Viable cells were qualified using the CellTiter-GLO Luminescence Kit (Promega, Madison, WI) following the manufacturer's instructions.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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