The RAC1 P29S Hotspot Mutation in Melanoma Confers Resistance to Pharmacological Inhibition of RAF

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Abstract

Following mutations in BRAF and NRAS, the RAC1 c.85C>T single-nucleotide variant (SNV) encoding P29S amino acid change represents the next most frequently observed protein-coding hotspot mutation in melanoma. However, the biologic and clinical significance of the RAC1 P29S somatic mutation in approximately 4% to 9% of patients remains unclear. Here, we demonstrate that melanoma cell lines possessing the RAC1 hotspot variant are resistant to RAF inhibitors (vemurafenib and dabrafenib). Enforced expression of RAC1 P29S in sensitive BRAF-mutant melanoma cell lines confers resistance manifested by increased viability, decreased apoptosis, and enhanced tumor growth in vivo upon treatment with RAF inhibitors. Conversely, RNAi-mediated silencing of endogenous RAC1 P29S in a melanoma cell line with a co-occurring BRAF V600 mutation increased sensitivity to vemurafenib and dabrafenib. Our results suggest RAC1 P29S status may offer a predictive biomarker for RAF inhibitor resistance in melanoma patients, where it should be evaluated clinically. Cancer Res; 74(17): 4845–52. ©2014 AACR

Introduction

Hotspot mutations in BRAF and NRAS are well-established driver mutations in the MAPK pathway (RAF–MEK–ERK signal transduction cascade), which occurs in more than 50% and 20% of melanomas, respectively (1). The identification of oncogenic mutations in BRAF, predominantly at codon 600 (2), was the main driving force in the development of small-molecule inhibitors targeting MAPK kinases (MEK) and BRAF in melanoma, which includes vemurafenib and dabrafenib. Patients with BRAF-mutant melanomas treated with RAF and MEK inhibitors have shown significant improvement in progression-free and overall survival as single agents (3–6). Patient survival is further improved with the use of combination treatment of RAF and MEK inhibitors (7). However, most patients treated with vemurafenib and dabrafenib develop disease progression within 6 to 8 months (reviewed in refs. 8 and 9). In addition, some patients present with intrinsic resistance (often termed de novo) that do not respond at all or have short-lived responses to RAF inhibitors progressing on drug treatment in less than 12 weeks.

Recently, two large-scale whole-exome sequencing (WES) studies have revealed that, in addition to recurrent mutations in BRAF and NRAS, the next most frequently observed somatic protein-coding hotspot mutation in melanoma of cutaneous origin is the RAC1 c.85C>T single-nucleotide variant (SNV) encoding the P29S amino acid change found in approximately 4% to 9% of melanomas (10, 11). RAC1 is a member of the Rho family of small GTP-binding proteins whose activity has been implicated in tumorigenesis and metastasis (12). RAC1 transduces extracellular signals from growth factor, integrin, and G-protein–coupled receptors, and its best characterized function is in the regulation of cytoskeleton rearrangement (13). Although the RAC1 P29S mutation has been shown to be oncogenic and biochemically active (10, 11, 14), the biologic role and clinical relevance of the RAC1 hotspot mutation in melanoma remains unclear. Recent publications on genomic characterization of matched pre- and posttreated melanoma samples from patients who received RAF and MEK inhibitors (15–19) have confirmed many of the acquired resistance mechanisms identified in preclinical in vitro studies (20–29).

In one such study, the authors observed a significant correlation of the RAC1 P29S hotspot mutation and early resistance in patients who received vemurafenib or dabrafenib monotherapy (17). Here, we present functional data supporting a role for the RAC1 P29S mutation in resistance to RAF inhibitors by...
maintaining activation of the MAPK signaling pathway. Our results suggest that RAC1 P29S status in melanoma may be an important predictor for vemurafenib and dabrafenib resistance in patients, and consequently should be evaluated as a predictive biomarker to such therapies in the clinic.

Materials and Methods

Sequenom and Sanger sequencing

gDNA was isolated using the DNaseasy Blood and Tissue Kit (Qiagen). Mass spectrometric genotyping (Sequenom) was performed as previously described (10, 30). Sequenom was employed to test all cell lines used in this study for RAC1, BRAF, and RAS family member hostpot mutations. Confirmation of the RAC1 c.85C>T SNV encoding for amino acid change P29S was validated by amplifying exon 2 with forward (TGCTAA-CACCGGGTACCTCAAC) and reverse (TCATCCAGTCTCTG-TACCTCAC) primers. PCR products were purified by QiAqick Gel Extraction Kit (Qiagen) followed by bidirectional sequencing with forward (TTTTAATTAATATGTAAGATCTA) and reverse (TTGGTCAAGAAGATTTGGAAC) primers on ABI 3730 DNA sequencers using Big Dye terminator cycle sequencing chemistry.

Plasmids and shRNA

pDONR RAC1 plasmid was obtained from the hORFeome collection from the Center for Cancer Systems Biology at Dana-Farber Cancer Institute (Boston, MA). The c.85C>T RAC1 mutation encoding for amino acid change P29S was generated using Quick-Change Lightning Site-Directed Mutagenesis (Stratagene) according to the manufacturer's protocol. Subcloning was performed by Invitrogen Gateway Technology to a pLENTI6.3-CMV (Invitrogen) and pHAGE-EF1-IRE-RES GFP expression vector that was kindly provided by Dr. Simona Colla (The University of Texas, MD Anderson Cancer Center, Houston, TX). Inducible shRNA RAC1 and control constructs were generated using BLOCK-iT Inducible H1 RNAi Entry Vector Kit (Invitrogen). Subcloning was performed by Invitrogen Gateway Technology to a PLKO-Tet-On vector (a gift from Dr. Timothy P. Heffernan, The University of Texas MD Anderson Cancer Center, Houston, TX). The hairpin sequences were as follows: shGFP: ACAACAGCCACACGTCTAT CGAA ATA-GACGTGTGGTGTG; shRAC1 71: CGCAAACAGATGTGTG; shRAC1 72: CGTGAAGAAGAGAAGACTG; shRAC1 71: GCAGAAGACATCGTGTG; shRAC1 72: CGTGAAGAGAGAAGACATCGTGTG.

Cell culture and cell viability assays

A375, MALME-3M, 451Lu, IGR1, CP66, and HMVI melanoma cell lines were maintained in RPMI1640 medium ( Gibco, Life Technologies) and WM3060 cells in Leibovitz L-15 medium ( Gibco, Life Technologies) in 10% heat-inactivated FBS(Gibco, Life Technologies) at 37°C in a humidified 5% CO2 incubator. Selected cell lines were authenticated by short tandem repeat (STR) DNA fingerprinting (STR profiles available in Supplementary Table S4; ref. 32). Stably expressing doxycycline-inducible shRNA cells were cultured in Tet System Approved FBS (Clontech). Doxycycline-treated cells were cultured in media at a concentration of 0.4 μg/mL. CellTiter-Glo Luminescent Cell Viability Assays (Promega) were used to measure viability following cell treatment with dabrafenib (GSK2118436), vemurafenib (PLX4032), trametinib (GSK1120212), and MEK inhibitor (PD325901) treatment (Selleck Chemicals). Briefly, 5,000 cells were seeded in 96-well plates in triplicates and 12 hours later treated with the indicated drug concentrations for 72 to 96 hours. The percentage of cell viability was calculated by comparing to DMSO-treated control. Analysis and half maximal inhibitory concentrations (IC50) were calculated by GraphPad Prism 6 software.

Immunoblots and RAC1 activation assay

Cells growing in monolayers were lysed using Cell Extraction Buffer (Life Technologies) supplemented with complete protease inhibitors and PhosSTOP phosphatase inhibitor cocktail tablets (Roche). Cell lysates were cleared by centrifugation, protein concentrations were determined by DC Protein Assay (Bio-Rad), and denatured lysates were run on 4% to 12% Bis-Tris gradient gels (Invitrogen). Gels were transferred to nitrocellulose membranes (Bio-Rad) before being immuno-blotted with indicated antibodies. Cleaved PARP antibody was obtained from Cell Signaling Technology. RAC1 activation assays were performed as previously described, according to the manufacturer’s protocol (Cell Biolabs; ref. 10).

Xenograft assays

Four- to 6-week-old NCR-nude female mice were obtained from Taconic Farms. A375 isogenic cell lines overexpressing GFP, RAC1 wild-type (WT), and P29S mutant were resuspended in a solution made up of two thirds Hank Balanced Salt Solution (Life Technologies) and one third BD Matrigel Matrix (BD Biosciences). Ten million cells were injected in 100 μL volume in 15 mice per group (GFP, RAC1 WT, and the P29S mutant) and were monitored for tumor formation. An approximate 100- to 250-mm3 tumor volume was observed mutant) and were monitored for tumor formation. An approximate 100- to 250-mm3 tumor volume was observed after 1 week of injection, and all mice were given PLX4720-admixed chow (AIN-76A diet), with a dose of 417 mg/kg diet (Plexxikon and Research Diets Inc.). Body weight and chow were measured to ensure that no significant differences existed in mouse weight or intake between groups during the course of the experiment. Tumor volumes were measured biweekly and calculated by the following formula: volume = 0.5 × (length × (width)²). Survival end points were defined by the first time point when tumor length reached 15 mm or there was visible evidence of ulceration. Mann–Whitney nonparametric tests were used to determine significant differences in tumor volume. Log-rank (Mantel–Cox) tests were used to determine significant differences in survival. Twotailed Fisher exact tests were used for comparison of mouse cohorts that progressed or responded to PLX4720 treatment. Tumor growth inhibition values (TGI) for GFP, RAC1 WT, and the P29S mutant were recorded. Two-tailed Fisher exact tests were used for comparison of mouse cohorts that progressed or responded to PLX4720 treatment. Tumor growth inhibition values (TGI) for GFP, WT, and P29S groups were calculated by taking sum of tumor volumes at each time point when tumor length reached 15 mm or there was visible evidence of ulceration. Mann–Whitney nonparametric tests were used to determine significant differences in survival. Two-tailed Fisher exact tests were used for comparison of mouse cohorts that progressed or responded to PLX4720 treatment. Tumor growth inhibition values (TGI) for GFP, WT, and P29S groups were calculated by taking sum of tumor volumes at each treatment. All mouse experiments were performed with the approval of Institute Institutional Animal Care and Use Committee at MD Anderson Cancer Center.
Results

RAC1 P29S–mutant melanoma cell lines have higher levels of activated RAC1 and display differential sensitivity to RAF and MEK inhibitors

To determine the biologic role of the RAC1 c.85C>T SNV encoding for the P29S amino acid change, we probed the RAC1 P29S variant status in a panel of melanoma cell lines by mass spectrometry genotyping (Sequenom; ref. 10). We identified two melanoma cell lines with SNV encoding for S29 (IGR1 and WM3060), which were confirmed by PCR and Sanger resequencing (Supplementary Fig. S1). We documented by Sequenom genotyping and Sanger resequencing that IGR1 cells harbor a co-occurring BRAF c.1798_1799GT>AA dinucleotide variant encoding the V600K amino acid change, whereas the WM3060 melanoma possesses an NRAS Q61K mutation (data not shown).

It has been shown that RAC1 P29S is found in a higher active GTP-loaded fraction compared with the WT protein (10, 11, 33), as measured by RAC1 interaction with the p21-binding domain (PBD) of p21-activated protein kinase (PAK; ref. 34). We demonstrate that endogenous RAC1 is found in a higher active fraction in PBD-binding assays in cell lines with P29S compared with those with WT (Fig. 1A, compare lanes 7, 10, 13, and 16; Supplementary Fig. S2A), confirming that melanoma cell lines with P29S possess higher levels of activated RAC1.

To investigate the functional role of RAC1 P29S variant in melanoma, we treated IGR1 and WM3060 cell lines with inhibitors targeting RAF, MEK, PI3K, and mTOR pathways. One observation we noted consistently was that P29S-expressing cell lines were less sensitive to both RAF inhibitors (vemurafenib and dabrafenib) and MEK inhibitors (trametinib and PD325901). IGR1 cells had approximately 40- to 185-fold higher IC50 in response to RAF inhibitors and a 4- to 12-fold increase in IC50 in response to MEK inhibitors when compared with RAC1 WT BRAF V600E–mutant cell lines A375, MALME-3M, and 451Lu (Fig. 1B–E; Supplementary Table S1). Furthermore, IGR1 cells did not reach below 50% cell viability even at doses as high as 10 μmol/L in response to vemurafenib, dabrafenib, trametinib, and PD325901 after 72 hours of treatment.
72-hour treatment (Supplementary Table S1). Similarly, the NRAS Q61K–mutant WM3060 cell line with the P29S variant was less sensitive to MEK inhibitor treatment compared with two other NRAS Q61K–mutant melanoma cell lines, CP66 and HMVII (Supplementary Fig. S2 and Supplementary Table S2). These results suggested that RAC1 P29S modulates sensitivity to inhibitors of the MAPK pathway in melanoma.

**RAC1 P29S mediates resistance to RAF and MEK inhibition**

To assess whether RAC1 P29S promotes resistance to RAF and MEK inhibition, we stably infected 451Lu cells with GFP, RAC1 WT, and P29S expressing pLENTi6.3-CMV plasmids (Fig. 2A). We observed a 13- to 145-fold increase in IC_{50} of RAC1 P29S–mutant expressing cells compared with GFP controls in

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**Figure 2.** Overexpression of RAC1 P29S increases cell viability to RAF inhibitor treatment. A, immunoblot of 451Lu cells stably expressing GFP, RAC1 WT, and P29S mutant from pLENTi6.3-CMV plasmids shows active GTP-loaded RAC1 from PBD pull-down with corresponding input lysates. Log-transformed dose–response curves percentage of viability compared with DMSO-treated control) for 451Lu isogenic cell lines treated with RAF inhibitors vemurafenib (PLX4032; B) and dabrafenib (GSK2118436; C) and MEK inhibitors trametinib (GSK1120212; D) and PD325901 (E) for 96 hours with doses up to 100,000 and 10,000 nmol/L, and serial dilutions of 1,000, 333.33, 111.11, 37.04, 12.35, 4.12, 1.37, 0.46, 0.15 nmol/L. The x-axis represents the log-transformed drug dose concentration in pmol/L. Error bars, SEMs.

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response to vemurafenib and dabrafenib, and a 19- to 74-fold increase in IC50 following trametinib and PD325901 treatment (Fig. 2B–E; Supplementary Table S3). This effect was also observed in isogenic 451Lu cells stably expressing RAC1 P29S and controls from a pHAGE-EF1α plasmid possessing a weaker promoter (Supplementary Fig. S3 and Supplementary Table S3). Another independent human melanoma cell line, A375, showed a 3.5- to 7-fold increase in IC50 in response to RAF and MEK inhibitors in the presence of P29S mutation (Supplementary Fig. S4; Supplementary Table S3) and similar results were observed in MALME-3M isogenic cell lines (Supplementary Fig. S5).

We next examined the apoptotic response to RAF inhibition in our isogenic cell lines by immunoblotting for a marker of apoptosis, cleaved PARP, and β-actin from lysates of A375 (A) and 451Lu (B) cells stably expressing GFP and RAC1 P29S treated with 1.25 and 5.0 µmol/L of dabrafenib (GSK2118436). C: phospho-MEK1/2 S217/S221 and total MEK immunoblots from lysates of A375 cells following dabrafenib (GSK2118436) treatment with indicated doses for 8 hours are shown. D, immunoblot of phospho-ERK1/2 T202/Y204, phospho-MEK1/2 S217/S221, and RAC1 from lysates of stably expressing doxycycline (DOX)-inducible RAC1 shRNA IGR1 cells after 72 hours of doxycycline treatment.

Complementing the gain-of-function studies above, we generated IGR1 cells stably expressing doxycycline-inducible RAC1 shRNAs. Upon administration of doxycycline for 72 hours, we observed a decrease in RAC1 protein levels with multiple independent shRNAs compared with two controls (shGFP and shLuciferase; Fig. 3D and Supplementary Fig. S6A). Along with the knockdown of RAC1 expression, we observed concomitant decrease in MAPK signaling as measured by decreased levels of phospho-MEK1/2 S217/S221 and phospho-MAPK3/MAPK1 (ERK1/2) T202/Y204 (Fig. 3D). Accordingly, a consistent trend of increased sensitivity to RAF and MEK inhibitor treatment was also observed in IGR1 cells upon RAC1 knockdown (Supplementary Fig. S6B–S6E). Taken together, these in vitro biochemical and drug sensitivity studies suggest that the P29S mutant of RAC1 can confer resistance to RAF and MEK inhibitors by maintaining an elevated level of MAPK activity.

**RAC1 P29S decreases the effect of RAF inhibitor on tumor growth in vivo**

To evaluate how the RAC1 P29S mutant modulates the response to RAF inhibitors in vivo, we implanted the A375 isogenic cells described above in NCR-nude mice. When an...
Figure 4. RAC1 P29S decreases effect of RAF inhibitor treatment on tumor growth in vivo. A375 cells expressing GFP, RAC1 WT, and RAC1 P29S were injected subcutaneously in flanks of female NCR-nude mice. An approximate 100- to 250-mm³ tumor volume was observed after 1 week of injection for all three mouse cohorts, and all mice were given PLX4720-admixed chow with a dose of 417 mg/kg diet. A, tumor volume measurements in the presence of PLX4720 drug are shown (15 mice in each cohort) up until the first mouse within cohort reached survival end point [GFP: day 38; RAC1 WT: day 23; RAC1 P29S: day 19; day 17, Mann-Whitney test: ***; P < 0.0005 (GFP vs. P29S), P < 0.0005 (WT vs. P29S); day 19, Mann-Whitney test: ***; P < 0.0005 (GFP vs. P29S) and ***; P < 0.0005 (WT vs. P29S)]. B, Kaplan-Meier survival curves for the GFP, RAC1 WT, and P29S cohorts are shown [log-rank (Mantel-Cox) test: ***; P < 0.0005 (GFP vs. P29S); †, P < 0.05 (WT vs. P29S), not indicated on graph, P < 0.005 (GFP vs. WT)]. Survival end points were maximal one-sided tumor measurement of 15 mm or ulceration. C, percentage of cohort that progressed on PLX4720 treatment as determined by reaching twice the original tumor volume before drug administration [Fisher exact test: *; P < 0.05 (GFP vs. P29S)]. D, doubling times for mice that progressed on treatment in each cohort are shown [Mann-Whitney test: #, P < 0.005 (GFP vs. WT); ***; P < 0.0005 (GFP vs. P29S), not significant (WT vs. P29S)]. Error bars, SEMs. **, P < 0.001 (GFP vs. P29S), not significant (WT vs. P29S).
higher GTP-active fraction compared with cell lines of similar genotype. We should note that, although some melanoma oncogenes and tumor suppressors are known to be involved in familial melanoma, the RAC1 P29S somatic mutation is caused by presumptive UVB-induced DNA damage and misrepair (C>T transition; refs. 10, 11), and has not been detected as a germline mutation in the 1000 Genomes database or among over 2,500 germline exomes sequenced at Yale University (New Haven, CT; ref. 11). Interestingly, cell lines expressing RAC1 P29S are more resistant to RAF and MEK inhibitor treatment. Overexpression of the P29S mutant in BRAF-mutant melanoma cell lines conferred resistance to MAPK inhibitors in vitro. Knockdown of endogenous P29S RAC1 mutant had an additive effect in decreasing cell viability in response to RAF inhibitors. Finally, RAC1 P29S overexpression in the RAF inhibitor–sensitive cell line, A375, resulted in a decreased effect of the drug on tumor growth in vivo. Taken together, these functional and biochemical studies support RAC1 P29S as a RAF inhibitor resistance mutation in melanoma.

Our conclusion is supported by clinical data from a recent study of 45 patients that received vemurafenib and dabrafenib monotherapy where pre- and posttreatment BRAF V600–mutant metastatic samples underwent WES analysis (17). Of the 45 patient data examined, 14 acquired resistance early in the course of treatment. Interestingly, the 3 patients in this cohort of 45 with RAC1 P29S mutation were all found to belong to this early resistance subgroup. The P29S mutation was not found in any melanoma sample from patients that had a sustained response to therapy in this study (the enrichment in the early resistant group was found to be statistically significant). Together, this clinical correlation in context of our functional results would suggest that RAC1 P29S status in melanoma may not only confer vemurafenib and dabrafenib resistance, but may predict those patients with intrinsic resistance to this class of targeted therapy. However, we acknowledge that an adequately powered study in the clinic will be necessary to definitively demonstrate the utility of RAC1 hotspot mutation in predicting intrinsic resistance to RAF inhibitors.

Mechanistically, our studies suggest that RAC1 P29S may sustain MAPK signaling in the presence of RAF inhibitors. Phospho-MEK1/2 levels were elevated in A375 isogenic cell lines overexpressing RAC1 P29S in response to dabrafenib, and we observed levels of phospho-MEK1/2 S217/S221 and phospho-ERK1/2 T202/Y204 decreased following RAC1 knockdown of endogenous RAC1 P29S in IGR1 cells. This is consistent with previous studies implicating RAC1 in the regulation of MAPK signaling (reviewed in ref. 12), and a recent study demonstrating RAC1 P29S can induce ERK phosphorylation in melanocytes (11). A number of studies have identified RAF inhibitor resistance mechanisms, which can be broadly characterized into groups that include reactivation of the MAPK pathway, upregulation of the PI3K–PTEN–AKT pathway, and dysregulation of melanocytic signaling (15–29). We cannot exclude the possibility that there may be alternative mechanisms that RAC1 P29S can drive RAF inhibitor resistance, including upstream or parallel to the MAPK pathway. Investigation of the aberrant oncogenic signaling caused by the RAC1 hotspot mutation in melanoma, and potential combination therapies to target this mutation, remains an important area of investigation for future study.

Disclosure of Potential Conflicts of Interest
K. Stemke-Hale has ownership interest (including patents) in GlaxoSmithKline. M.A. Davies reports receiving commercial research grants from GlaxoSmithKline, Genentech, AstraZeneca, and Oncothyreon and is a consultant/advisory board member for GlaxoSmithKline, Genentech, Novartis, and Sanofi Aventis. G.B. Mills received commercial research grants from Adelion Medical Research Foundation, AstraZeneca, Critical Outcome Technologies, and GlaxoSmithKline, has ownership interest (including patents) in Catena Pharmaceuticals, PTV Ventures, and Spindle Top Ventures, and is a consultant/advisory board member for AstraZeneca, Blend, Tau Therapeutics, Critical Outcome Technologies, HanBi Bio Korea, Naevolution, Pfizer, Provista Diagnostics, Roche, SignalChem Lifesciences, and Symphogen. No potential conflicts of interest were disclosed by the other authors.

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