Research Article

Cancer Immunology Research

Impact of NRAS Mutations for Patients with Advanced Melanoma Treated with Immune Therapies

Douglas B. Johnson[1], Christine M. Lovly[1], Marisa Flavin[2], Katherine S. Panageas[2], Gregory D. Ayers[3], Zhiguo Zhao[3], Wade T. Iams[1], Marta Colgan[2], Sarah DeNoble[4], Charles R. Terry[1], Elizabeth G. Berry[1], A. John Iafrate[4], Ryan J. Sullivan[4], Richard D. Carvajal[5], and Jeffrey A. Sosman[1]

Abstract

Activating *NRAS* mutations are found in 15% to 20% of melanomas. Immune therapies have become a mainstay in advanced melanoma treatment. We sought to evaluate whether tumor genotype (e.g., *NRAS* mutations) correlates with benefit from immune therapy in melanoma. We identified 229 patients with melanoma treated with immune therapies [IL2, ipilimumab, or anti-programmed cell death-1/ligand-1 (PD-1/PD-L1)] at three centers and compared clinical outcomes following immune therapy for patients with or without *NRAS* mutations. Of the 229 patients with melanoma, 60 had *NRAS* mutation, 53 had *BRAF* mutation, and 116 had *NRAS*/*BRAF* wild type. The *NRAS*-mutant cohort had superior or a trend to superior outcomes compared with the other cohorts in terms of response to first-line immune therapy (28% vs. 16%, $P = 0.04$), response to any line of immune therapy (32% vs. 20%, $P = 0.07$), clinical benefit (response + stable disease lasting $\geq$24 weeks; 50% vs. 31%, $P < 0.01$), and progression-free survival (median, 4.1 vs. 2.9 months, $P = 0.09$). Benefit from anti–PD-1/PD-L1 was particularly marked in the NRAS cohort (clinical benefit rate 73% vs. 35%). In an independent group of patient samples, *NRAS*-mutant melanoma had higher PD-L1 expression (although not statistically significant) compared with other genotypes (8/12 vs. 9/20 samples with $\geq$1% expression; 6/12 vs. 6/20 samples with $\geq$5% expression), suggesting a potential mechanism for the clinical results. This retrospective study suggests that *NRAS* mutations in advanced melanoma correlate with increased benefit from immune-based therapies compared with other genetic subtypes. If confirmed by prospective studies, this may be explained in part by high rates of PD-L1 expression. *Cancer Immunol Res; 3(3); 288–95. ©2015 AACR.*

Introduction

The advent of molecular genetics has enabled classification of melanoma into clinically relevant subsets defined by the presence of specific "driver" mutations, each with unique clinical and genetic features. These "driver" mutations occur in multiple oncogenes, including *BRAF*, *NRAS*, and *CKIT*, and may serve as potential therapeutic targets. *NRAS*-mutant melanoma is a distinct cohort of this disease that comprises 15% to 20% of all melanomas and appears to confer a poor prognosis (1, 2). In contrast with *BRAF*-mutant melanoma, no effective small-molecule inhibitors have been approved that specifically target NRAS, although MEK inhibitors have demonstrated modest clinical activity in a phase II trial (3). In addition, melanomas without driver mutations in *BRAF* or *NRAS* (which comprise ~35% of all melanomas—hereafter referred to as "WT" for "wild type") represent another challenging subgroup without genotype-directed treatments (4, 5). More effective therapeutic strategies both for *NRAS*-mutant and WT melanomas are urgently needed.

Immune therapies are playing an increasing role in the treatment of patients with metastatic melanoma, particularly when there is no specific targeted therapy available. IL2 was a mainstay of melanoma therapy for many years, resulting in durable remissions in 5% to 10% of patients despite severe acute toxicities (6). More recently, therapeutic approaches aimed at activating antitumor immunity through blockade of immune checkpoints have shown promise. Ipilimumab, a monoclonal antibody directed at cytotoxic T lymphocyte antigen-4 (CTLA-4), demonstrated a survival advantage in metastatic melanoma (7, 8). Newer checkpoint inhibitors targeting the programmed cell death-1/ligand (PD-1/PD-L1) axis [nivolumab, pembrolizumab (MK-3475), MPDL3280A, etc.] have induced durable objective responses in 25% to 50% of

[1]Vanderbilt University Medical Center and Vanderbilt-Ingram Cancer Center, Nashville, Tennessee. [2]Memorial Sloan-Kettering Cancer Center, New York, New York. [3]Vanderbilt University School of Medicine Center for Quantitative Science, Nashville, Tennessee. [4]Massachusetts General Hospital, Boston, Massachusetts. [5]Columbia University Medical Center, New York, New York.

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D.B. Johnson and C.M. Lovly contributed equally to this article.

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Corresponding Author: Douglas B. Johnson, Vanderbilt University Medical Center, 777 Preston Research Building, Nashville, TN 37232. Phone: 205-317-3791; Fax: 615-343-7602; E-mail: douglas.b.johnson@vanderbilt.edu

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patients in early trials (9–12). These novel immune-based therapies are better tolerated than IL2, although potentially severe autoimmune side effects still occur in some patients (13). Currently, no validated biomarkers have consistently predicted clinical responses to the immune therapies, although tumor expression of PD-L1 is likely associated with response to PD-1/PD-L1–directed therapies (9, 14). At present, it is unclear whether specific "driver" mutations, such as $NRAS^{G12/G13/Q61}$ mutations, influence immune therapy outcomes. Preclinical studies have recently suggested that specific tumor driver mutations may affect the antitumor immune response through changes in expression of tumor antigens or checkpoint molecules, or production of immune-suppressive cytokines (15–18). In addition, several studies have suggested that although mutations in *BRAF* did not correlate consistently with response rates to immune therapy, *NRAS* mutations were associated with more frequent responses in patients treated with IL2 (19–21).

While we were assessing the clinical, pathologic, and therapeutic features affected by genotype in our database at Vanderbilt Ingram Cancer Center (VICC), we observed an association between *NRAS* mutations and response to immune therapy. Based on this finding, we hypothesized that *NRAS* mutations may affect the clinical outcome of patients with melanoma treated with immune therapies. We further hypothesized that *NRAS*-mutant melanoma may be associated with increased expression of PD-L1, potentially contributing to its responsiveness to immune therapies. To investigate, we performed a retrospective study reviewing clinical information from patients with melanoma treated at VICC, Memorial Sloan Kettering Cancer Center (MSKCC), and Massachusetts General Hospital (MGH) and assessed PD-L1 expression in an independent cohort of tumor samples. Our primary endpoints were response rate to immune therapy and clinical benefit rate (CBR; defined as response rate plus stable disease for $\geq$ 24 weeks); secondary endpoints were overall survival (OS), progression-free survival (PFS), and expression of PD-L1.

Patients and Methods

Study population/design

After Institutional Review Board approval was obtained, the electronic medical records were reviewed for patients with advanced melanoma seen at VICC, MSKCC, and MGH. Patients were included if they had biopsy-confirmed advanced melanoma, underwent molecular profiling for *BRAF* and *NRAS* mutations between July 1, 2010, and October 1, 2012, and were treated with immune therapies. Immune therapies included in this study were limited to high-dose IL2, ipilimumab, anti–PD-1 [nivolumab (BMS-936558) or pembrolizumab (MK-3475)], and anti–PD-L1 (MPDL3280A). Only patients who received $\geq$1 week of high-dose IL2 or >1 dose of ipilimumab or anti–PD-1/PD-L1 were included. The study population included patients treated with immune therapies between January 1, 2005, and November 1, 2012. Results (OS and PFS) were updated through February 1, 2014. All patients underwent genotyping for "hotspot" mutations in *BRAF* and *NRAS*; most patients also underwent "hotspot" testing of other genes (i.e., *CKIT*, *GNAQ*, *GNA11*, *MEK1*, etc.), although this was not required. Melanomas with mutations identified in genes other than $BRAF^{V600}$ or *NRAS* were included within the WT group. Our initial comparison was between *NRAS*-mutant and WT melanomas, although we subsequently collected clinical data from patients with *BRAF*-mutant melanoma from two centers (VICC and MGH).

Objective tumor responses were retrospectively assessed by the investigators using the RECIST 1.1 criteria as documented in radiographic study reports (PET or CT scans), provider notes, and/or tumor measurement forms (patients on experimental protocols had prospectively evaluated responses; ref. 22). We evaluated whether patients experienced complete or partial response (CR/PR) to first-line immune therapy or to subsequent lines of immune therapy during their clinical course. Clinical benefit (CB) was also assessed, defined as CR, PR, or stable disease for $\geq$24 weeks. Any patient without a radiographically evaluable response was classified as a nonresponder. Responses to cytotoxic chemotherapy, molecularly targeted therapy, or additional experimental immune therapies were not assessed. All clinical data were obtained and maintained according to Health Insurance Portability and Accountability Act standards.

Genetic analysis

Molecular profiling was performed by SNaPshot analysis (VICC and MGH) and Sequenom (MSKCC) on formalin-fixed paraffin-embedded tissue (FFPE). The SNaPshot process utilizes multiplex PCR, multiplex primer extension, and capillary electrophoresis, and has been extensively validated and described previously (4). The gene profiles performed for this study at VICC, MSKCC, and MGH are listed in Supplementary Tables S1, S2, and S3. Patients without identified mutations in *NRAS* or *BRAF* were classified as "WT."

Immunohistochemistry analysis

Melanoma samples from patients with advanced melanoma naïve to immune checkpoint inhibitor therapy were selected based on genotype (*NRAS*-mutant, $BRAF^{V600}$-mutant, WT). Expression of PD-L1 was measured by immunohistochemical testing in FFPE tumor specimens with a rabbit monoclonal antihuman PD-L1 antibody and an assay developed by Dako. The development of this assay has been previously described (12). Unstained slides were sent from VICC to the outside facility where laboratory personnel performed PD-L1 staining. A pathologist blinded to genotype and patient characteristics determined scores for clinical specimens. Samples were defined as positive at two thresholds: (i) if at least 1% or (ii) if at least 5% of tumor cells exhibited membrane PD-L1 staining of any intensity in a section containing at least 100 cells that could be evaluated, as has been previously described (9, 12, 23).

Statistical analysis

All statistical analyses were performed using SAS version 9.2. Comparisons were considered statistically significant for two-sided P values <0.05. Categorical variables were summarized by frequencies in each study group; comparisons between the NRAS and non-NRAS groups (BRAF-mutant and WT) were performed using the Pearson χ^2 test. The proportions of patients with best response of CR or PR, or stable disease, were compared between the NRAS and non-NRAS cohorts by the Pearson χ^2 test. Differences in response rates and clinical benefit rates between groups for each individual therapy are displayed descriptively; we chose to avoid P values due to small numbers per group and multiple comparisons. The proportion

Table 1. Summary of clinical characteristics and treatment selection for NRAS-mutant, BRAF-mutant, and WT (NRAS/BRAF wild-type) cohorts

	NRAS-mutant ($n = 60$)	BRAF-mutant ($n = 53$)	WT ($n = 116$)	*P* value[a]
Gender				0.29
Female	21 (35)	18 (34)	29 (25)	
Male	39 (65)	35 (66)	87 (75)	
Age				0.11
<60 years	22 (37)	35 (66)	47 (41)	
≥60 years	38 (63)	18 (34)	69 (59)	
Stage				0.78
IIIc	4 (7)	1 (2)	11 (10)	
M1a	8 (13)	8 (15)	8 (6)	
M1b	10 (17)	5 (9)	19 (16)	
M1c	38 (63)	39 (74)	79 (68)	
Location of primary tumor				0.02
Head and neck	7 (11)	8 (15)	30 (26)	
Torso	20 (33)	22 (42)	17 (15)	
Extremities	20 (33)	12 (23)	19 (16)	
Uveal	0 (0)	0 (0)	5 (4)	
Acral	3 (5)	0 (0)	15 (13)	
Mucosal	5 (8)	0 (0)	10 (9)	
Unknown	5 (8)	11 (21)	20 (17)	
LDH[b]				
<ULN[c]	38 (76)	20 (54)	49 (65)	0.07
>ULN	12 (24)	17 (46)	26 (35)	
Mutation detected				
NRAS^{Q61R}	28 (47)	*	*	*
NRAS^{Q61L}	5 (8)	*	*	
NRAS^{Q61K}	15 (25)	*	*	
NRAS^{Q61H}	3 (5)	*	*	
NRAS^{G13R}	3 (5)	*	*	
NRAS^{G13D}	1 (2)	*	*	
NRAS^{G13C}	1 (2)	*	*	
NRAS^{G12D}	2 (3)	*	*	
NRAS^{G12C}	2 (3)	*	*	
BRAF^{V600E}	*	47 (89)	*	
BRAF^{V600K}	*	4 (8)	*	
BRAF^{V600R}	*	1 (2)	*	
BRAF^{V600D}	*	1 (2)	*	
Institution				
VICC	26 (43)	29 (55)	58 (50)	
MSKCC	24 (41)	0	51 (46)	
MGH	10 (17)	24 (45)	7 (6)	
Therapy[c] (first-line)				0.89
IL2	14 (23)	27 (51)	17 (15)	
Ipilimumab	38 (63)	19 (36)	86 (74)	
Anti–PD-1/PD-L1	8 (13)	7 (13)	13 (11)	
Therapy (second-line)				0.86
IL2	1 (10)	2 (9)	1 (4)	
Ipilimumab	6 (70)	13 (59)	12 (52)	
Anti–PD-1/PD-L1	3 (20)	7 (32)	10 (43)	
Therapy (third-line)				*
IL2	0	0	0	
Ipilimumab	0	0	2	
Anti–PD-1/PD-L1	0	1	0	

NOTE: Asterisk (*) indicates "not applicable" or "none."
Abbreviations: LDH, lactic dehydrogenase; ULN, upper limit of normal.
[a]Pearson test between NRAS and non-NRAS genotypes.
[b]Before initiation of immune-based therapy, missing for some patients.
[c]Only includes immune-based therapies.

of patients with PD-L1 expression ≥1% or ≥5% within *NRAS*-mutant melanoma was compared with the proportions of those with *BRAF*-mutant and WT melanomas using the Fisher exact test. PFS was defined as the time from first immune therapy to first progression or death. OS was calculated by date of first immune therapy to date of death for any reason. Patients alive at the last date of follow-up were censored for OS; patients alive and progression free were censored for PFS. PFS and OS distributions were estimated using the method of Kaplan and Meier and compared using the log-rank test.

Results

Demographics

A total of 229 patients with advanced melanoma were included. Sixty (26%) melanomas harbored *NRAS*$^{G12/G13/Q61}$

Table 2. Response rate and clinical benefit by NRAS status

	NRAS-mutant	BRAF-mutant	WT	*P* value[a]
Best response to any line of immune therapy	$n = 60$	$n = 53$	$n = 116$	
CR/PR	19 (32%)	12 (23%)	22 (19%)	0.068
SD/PD	41 (68%)	41 (77%)	94 (81%)	
CR/PR/SD	30 (50%)	16 (30%)	34 (29%)	0.004
PD	30 (50%)	37 (70%)	82 (71%)	
Response to first-line immune therapy	$n = 60$	$n = 53$	$n = 116$	
CR/PR	17 (28%)	8 (15%)	19 (16%)	0.037
SD/PD	43 (72%)	45 (85%)	97 (84%)	
CR/PR/SD	27 (45%)	13 (25%)	31 (27%)	0.006
PD	33 (55%)	40 (75%)	85 (73%)	

Abbreviations: PD, progressive disease; SD, stable disease.
[a]Pearson χ^2 test *P* value for NRAS-mutant versus non–NRAS-mutant patients.

mutations, 53 (23%) had $BRAF^{V600}$ mutations, and 116 (51%) were WT for *NRAS* and *BRAF* by SNaPshot or Sequenom. Patient characteristics are shown in Table 1. The most common mutation identified was $NRAS^{Q61R}$ in 28 cases (47%); 85% of *NRAS* mutations occurred in codon 61. Age, gender, elevated lactate dehydrogenase, and disease stage were not related to *NRAS* mutation status, although location of primary tumors differed significantly between the NRAS and non-NRAS groups as previously described (5). Among the 53 patients with $BRAF^{V600}$ mutations, 16 had received prior BRAF- and/or MEK-directed targeted therapies and 25 received these agents following the failure of first-line immune therapy.

All 229 patients received one or more immune therapy regimens with 55 (24%) receiving a second line of immune therapy and 3 receiving two additional regimens (only including immune agents). First-line therapy consisted of high-dose IL2 in 25%, ipilimumab in 62%, and anti–PD-1/PD-L1 in 12% (Table 1). For those who received second-line immune therapy, IL2 was administered in 7% of patients, ipilimumab in 56%, and anti–PD-1/PD-L1 in 36%. Regimen selection did not differ by *NRAS* mutation status for first- ($P = 0.89$) or second-line immunotherapy ($P = 0.86$); the average number of different lines of immune therapy received per patient was 1.17 for the NRAS cohort, 1.22 for the WT group, and 1.44 in the BRAF group. Five patients (3 in the WT group and 2 in the NRAS group) received combination ipilimumab and nivolumab (BMS-936558) and were categorized in the anti–PD-1 group for the subgroup analysis. Nine patients (3 in each group) also received ipilimumab in combination with other agents (temozolomide, dacarbazine, fotemustine, GM-CSF, bevacizumab, imiquimod) on experimental protocols; these patients were classified as having received ipilimumab.

Patient outcomes

We assessed the association of *NRAS* mutation and response to therapy. We compared the proportion of patients in each group who experienced a CR or PR with immune therapy at any time during their clinical course (Table 2). In the NRAS group, 19 of 60 patients (32%) had a CR or PR compared with 34 of 169 (20%) in the non-NRAS groups ($P = 0.07$). We then compared the proportion of patients who achieved clinical benefit; this comparison more strongly favored the NRAS group (50% vs. 30%, $P < 0.01$). Assessing first-line immune therapy only, we observed increased benefit for the *NRAS*-mutant cohort in terms of overall response rate (ORR; 28% vs. 16%, $P = 0.04$) and CBR (45% vs. 26%, $P < 0.01$). Of note, patients in the BRAF cohort treated with BRAF and/or MEK inhibitors before immune therapy had a seemingly lower, although not statistically significant, ORR than those naïve to BRAF or MEK inhibitors (13% vs. 27%, $P = 0.25$), consistent with previous studies (24).

We then examined whether *NRAS* mutation status affected the ORR and CBR for different types of immune-based therapy (Table 3). We observed that patients with *NRAS*-mutant melanoma who received anti–PD-1 or anti–PD-L1 agents had markedly increased benefit compared with WT and *BRAF*-mutant patients (ORR 64% vs. 30%, CBR 73% vs. 35%; $n = 48$). Increased incidence of clinical benefit was also demonstrated for patients in the *NRAS*-mutant cohort who received ipilimumab (ORR 19% vs. 11%, CBR 42% vs. 19%; $n = 169$). In patients receiving IL2, the response rate and clinical benefit appeared similar between groups. Because many patients received multiple lines of therapy, we did not use formal analysis to compare ORR between groups.

We evaluated PFS and OS for all patients with *NRAS* mutations compared with the non-NRAS cohorts from initiation of first-line immune therapy using the Kaplan–Meier analysis. We noted a trend toward improved PFS for patients with *NRAS* mutations (Fig. 1A) and equivalent OS (Fig. 1B). The median duration of PFS was 4.1 months for NRAS-mutant patients versus 2.9 months for the non-NRAS cohort (log-rank $P = 0.08$); the median OS was 19.5 versus 15.2 months (log-rank $P = 0.51$). When examining the non-NRAS cohort further, median PFS was 3.3 months for the WT group and 2.4 months for patients with *BRAF* mutations; median OS was 13.9 months in the WT cohort and 16 months in the BRAF group. In the BRAF cohort, PFS among those who previously received BRAF and/or MEK inhibitors was equivalent to PFS in those who had not received these therapies (2.0 vs. 2.7 months, log-rank $P = 0.28$), but OS strongly favored treatment-naïve patients (7.9 months vs. 25.7 months, log-rank $P = 0.01$).

Because *NRAS* mutations have been previously associated with inferior OS (1, 2), we hypothesized that patients in the NRAS cohort who did not benefit from immune therapies would have rapid progression and death. In an exploratory, descriptive

Table 3. Response rate and clinical benefit by immune therapy type

	NRAS mutant $n = 11$	BRAF mutant $n = 14$	WT $n = 23$
Anti–PD-1/PD-L1			
Objective response	7 (64%)	3 (21%)	8 (35%)
Clinical benefit	8 (73%)	3 (21%)	10 (43%)
Ipilimumab	$n = 43$	$n = 31$	$n = 95$
Objective response	8 (19%)	4 (13%)	10 (11%)
Clinical benefit	18 (42%)	5 (16%)	19 (20%)
IL2	$n = 15$	$n = 29$	$n = 19$
Objective response	5 (33%)	6 (21%)	5 (26%)
Clinical benefit	5 (33%)	11 (34%)	7 (37%)

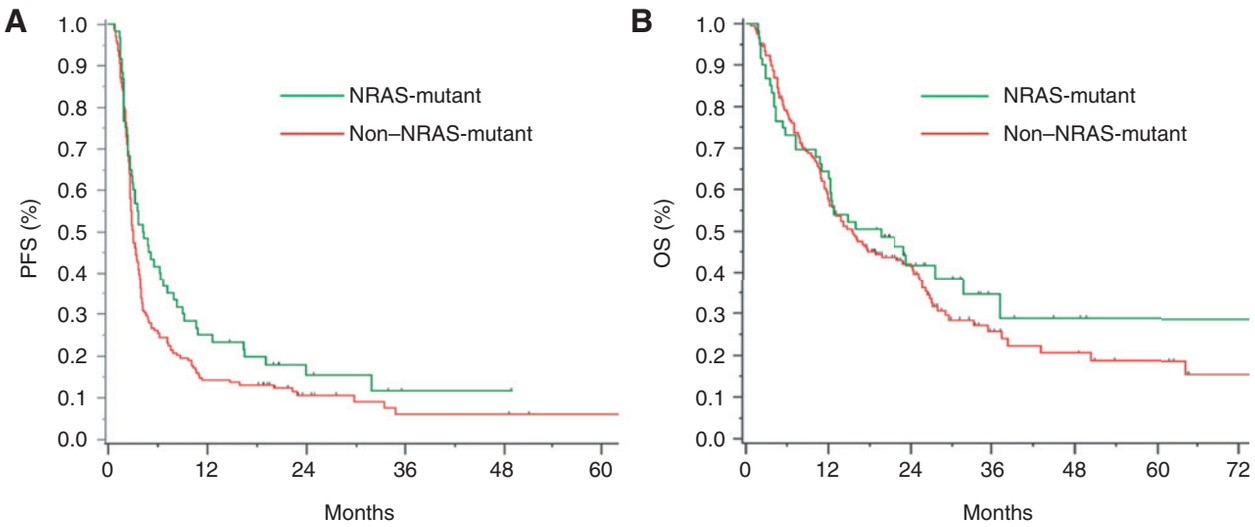

Figure 1.
Kaplan–Meier curves of (A) PFS and (B) OS from first-line immune-based therapy for NRAS-mutant and non–*NRAS*-mutant (*BRAF*-mutant and WT) cohorts.

analysis, we assessed the outcome for patients who did not experience clinical benefit from immune-based therapies (poor responders) with evaluable follow-up 6 months after the start of therapy. Among *NRAS*-mutant poor responders, only 46% (13 of 28) were alive at 6 months compared with 67% (78 of 117) of non–*NRAS*-mutant poor responders. This finding suggests that patients with *NRAS*-mutant melanoma who do not benefit from immune therapy retain a poor prognosis.

PD-L1 expression

To investigate the potential mechanisms underlying our clinical observation, we selected an independent cohort of 39 archived samples from patients with advanced melanoma (*NRAS*-mutant = 15, WT = 14, *BRAF*-mutant = 10). Patients were naïve to systemic therapy in most cases and had not received immune checkpoint inhibitor therapy (see Supplementary Table S4 for prior therapies and clinical characteristics). Seven samples were not evaluable due to low tumor content or excessive pigmentation precluding assessment. Using a 1% cutoff, *NRAS*-mutant samples appeared to have a non–statistically significant trend toward higher expression of PD-L1 (8 of 12) compared with WT (4 of 11) and *BRAF*-mutant (5 of 9).Using a cutoff of $\geq$5% expression of PD-L1, a potentially higher proportion of *NRAS*-mutant samples were PD-L1–positive (6 of 12), compared with WT (3 of 11) and *BRAF*-mutant (3 of 9; Fig. 2 and Supplementary Table S4). Differences in PD-L1 expression between the *NRAS*-mutant cohort and non-NRAS groups were not statistically significant ($P = 0.23$, 1% cutoff; $P = 0.26$, 5% cutoff; Fisher exact test).

Discussion

We hypothesized that "driver mutation" status may influence response to immune therapies, specifically examining the cohort of patients with melanoma harboring activating *NRAS* mutations. Data from our multi-institutional retrospective analysis suggest that patients with *NRAS*-mutant melanoma experience higher rates of objective response or prolonged stable disease from immune therapy compared with those with *BRAF*-mutant and *NRAS*/*BRAF* WT melanoma. This benefit was particularly notable for the novel immune checkpoint inhibitors (ipilimumab and anti–PD-1/PD-L1). Although only small numbers were treated, the clinical benefit rate was unexpectedly high with anti–PD-1 or anti–PD-L1, occurring in 8 of 11 patients with *NRAS*-mutant melanoma compared with only 13 of 37 patients in the non–*NRAS*-mutant cohorts. This finding could have implications for molecular testing and treatment decision making, and it provides early insights into the complex relationship between tumor genetics and the immune response.

In contrast with *BRAF*-mutant melanoma, no effective molecularly targeted therapeutic strategies have yet been approved for *NRAS*-mutant or WT melanoma. Immune therapies, therefore, are the cornerstones of therapy for these subtypes. A recent study by Joseph and colleagues (19) showed that IL2 treatment provided superior efficacy for patients with *NRAS*-mutant compared with *BRAF*/*NRAS* WT melanoma (ORR of 47% vs. 15%), although this analysis included only 15 patients with *NRAS*-mutant melanoma. Our study is much larger and extends to the immune checkpoint inhibitors. It should be noted that although several clinical endpoints strongly favored the *NRAS* population (ORR to first-line therapy, CBR to any and first-line therapy), only a trend was observed for several others (ORR to any therapy, PFS, OS).

We identified a potential partial explanation for this clinical observation. Because previous studies have shown that PD-L1 expression correlates with response to anti–PD-1, we hypothesized that PD-L1 may be differentially expressed in *NRAS*-mutant melanoma (9, 25). We attempted to answer this question using a relatively small, separate cohort of samples from patients who were naïve to treatment with immune checkpoint inhibitors. We observed that PD-L1 expression appeared to be modestly higher in *NRAS*-mutant–resected tumor samples compared with *BRAF*-mutant or WT melanoma. This result did not reach statistical significance and needs additional confirmation.

Of interest, a recent study demonstrated that PD-L1 expression did not differ between genotypes in melanoma cell lines (26), suggesting that mutant *NRAS* does not induce constitutive

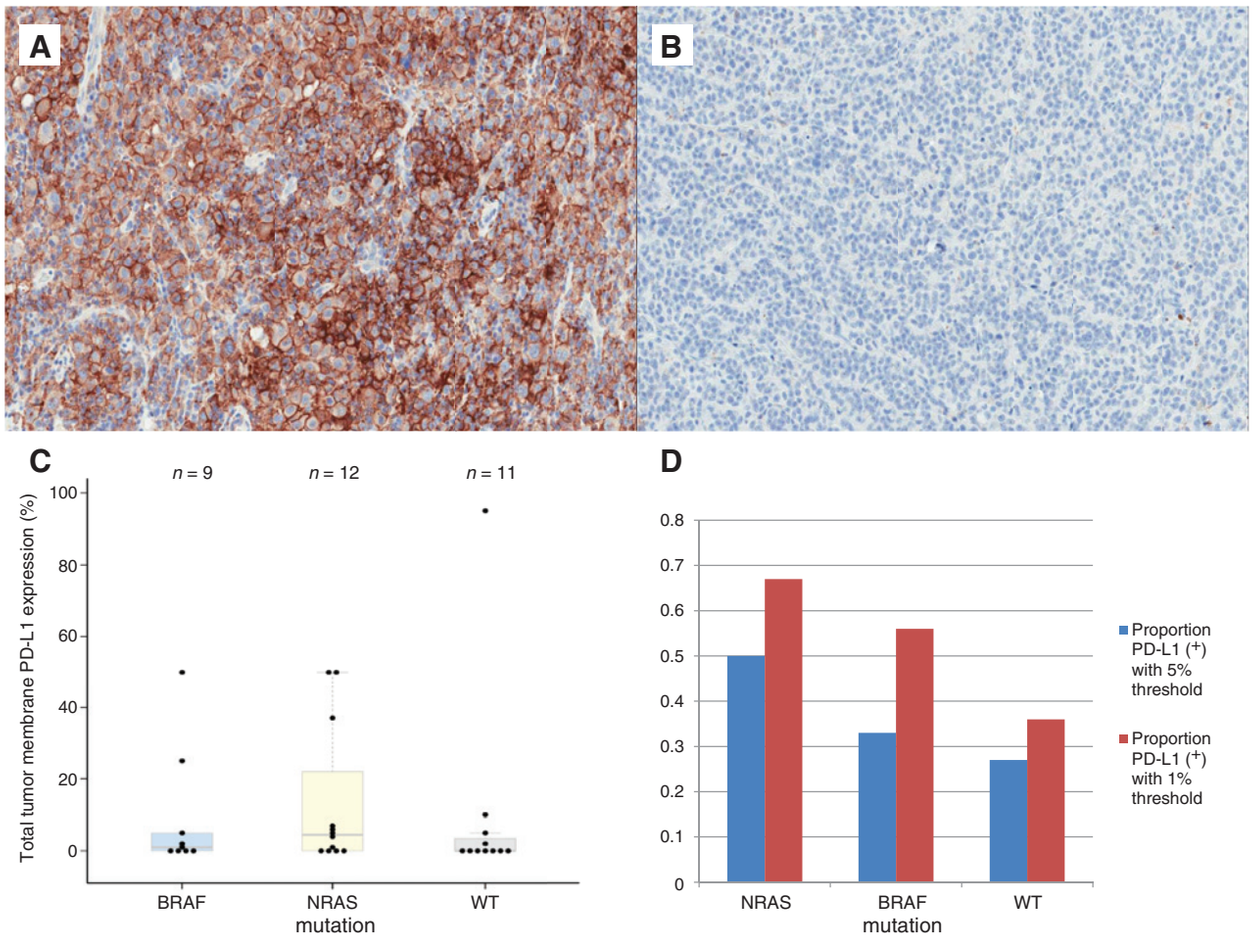

Figure 2.
Immunohistochemical analysis of tumor cell-surface expression of PD-L1 from representative samples (×20). A, *NRAS*-mutant melanoma sample with strongly positive expression (~50% of cells). B, WT melanoma with <1% of cells with PD-L1 expression. C, distribution of PD-L1 staining by genotype. D, number of samples evaluated by genotype and whether they were positive for PD-L1 expression (≥5%) or negative (<5%).

expression of PD-L1 but may be associated with enhanced immunogenicity *in vivo*. One potential hypothesis is that *NRAS* mutations may occur in melanomas with higher mutational burden, a factor linked with higher response rates to immune therapy (27, 28). Results from a large next-generation sequencing study showed that although not universally elevated, the total mutational burden appeared to be higher in *NRAS*-mutant melanoma than in melanomas of other subtypes (29). Profiling in larger populations of total somatic mutational burden, mutationally generated neoepitopes, tumor-infiltrating lymphocytes, and melanoma lineage antigens is needed to provide additional insight. Understanding the link between *NRAS* mutations and other genetic alterations in melanoma with the antitumor immune response may also provide a better rationale for approaching combination strategies of molecularly targeted and immune-based therapy. In addition, PD-L1 expression in melanoma and other cancers has been linked to poor prognosis, although this remains controversial (30–34). Based on our observation, it could be speculated that elevated PD-L1 expression may contribute both to the inferior prognosis of *NRAS*-mutant melanoma and improved response rates to anti–PD-1.

We observed only a trend toward improvements in OS and PFS in our study. Because previous studies have indicated that unselected patients with *NRAS* mutations have an inferior overall prognosis, we hypothesized that rapid progression and death in nonresponding patients may explain this finding (1, 2). This was supported by our observation that "poor responding" patients in the *NRAS* cohort (patients without clinical benefit from immune-based therapies) tended to have rapid disease progression and death. Presumably, the lack of approved molecularly targeted agents and aggressive natural history of *NRAS*-mutant melanoma has a negative impact on survival. This analysis was exploratory only; the influence of *NRAS* mutations on OS will need additional follow-up in larger cohorts treated with immune therapy.

Of note, this study included a relatively low percentage of patients harboring *BRAF* mutations (23%, $n = 53$), likely due in part to physician preference for BRAF-targeted therapy during the study time period. Interestingly, response rates and PFS were not significantly inferior among patients previously treated with BRAF or MEK inhibitors. OS, on the other hand, was vastly superior in the BRAF/MEK inhibitor–naïve group. The

population of BRAF/MEK inhibitor pretreated patients could adversely influence clinical outcomes compared with the NRAS and WT cohorts. Conversely, the availability of these therapies upon immune therapy progression may actually skew OS in favor of the *BRAF*-mutant group, because no such therapies were available to the other subgroups. The low patient numbers and variable pretreatment, therefore, limit the conclusions for the *BRAF*-mutant cohort, and additional study is needed to define the activity of immune therapy for this genetic subtype.

Our study has several other limitations. Patients identified for this study had received several different immune therapies at three centers across the United States spanning a period of approximately 8 years. In addition, genotyping for *BRAF* and especially for *NRAS* mutations was not widely available before 2010; therefore, for patients treated before that time, analysis was largely limited to those surviving to obtain genotyping. Genotyping was also performed with three different assays that could also introduce heterogeneity, although each platform has been extensively validated. In addition, somewhat distinct results were identified between immune therapies (no difference with IL2, more clinical benefit with ipilimumab, and higher responses with anti–PD-1/PD-L1); this may reflect divergent interactions of mutant *NRAS* with the immune response or may be a consequence of small sample size in each group. Finally, there was heterogeneity in the manner that responses to immune therapies were monitored, as some patients were enrolled in clinical trials and others were receiving treatment as standard of care. Therefore, prospective studies will be needed to confirm our observations.

In conclusion, we suggest that immune therapies, particularly immune checkpoint inhibitors, may be particularly effective treatment options for *NRAS*-mutant melanoma, a challenging cohort of patients with a poor prognosis. Mechanistic support for this assertion is suggested by high levels of PD-L1 expression in *NRAS*-mutant melanoma in a small cohort (although no statistically significant difference was noted). Prospective analyses and further mechanistic studies are needed to validate this finding. We are hopeful that these types of studies bring us a step closer to the goal of identifying predictive markers for immune therapy.

Disclosure of Potential Conflicts of Interest

C.M. Lovly reports receiving commercial research grants from AstraZeneca and Novartis. A.J. Iafrate has ownership interest (including patents) in ArcherDx and is a consultant/advisory board member for Bioreference Labs. No potential conflicts of interest were disclosed by the other authors.

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Authors' Contributions

Conception and design: D.B. Johnson, C.M. Lovly, M. Flavin, Z. Zhao, A.J. Iafrate, R.J. Sullivan, J.A. Sosman

Development of methodology: D.B. Johnson, C.M. Lovly, M. Flavin, G.D. Ayers, Z. Zhao, R.D. Carvajal

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.B. Johnson, C.M. Lovly, M. Flavin, W.T. Iams, M. Colgan, S. DeNoble, C.R. Terry, E.G. Berry, A.J. Iafrate, R.J. Sullivan, R.D. Carvajal, J.A. Sosman

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.B. Johnson, C.M. Lovly, K.S. Panageas, G.D. Ayers, Z. Zhao, A.J. Iafrate, R.D. Carvajal

Writing, review, and/or revision of the manuscript: D.B. Johnson, C.M. Lovly, K.S. Panageas, G.D. Ayers, Z. Zhao, W.T. Iams, A.J. Iafrate, R.J. Sullivan, R.D. Carvajal, J.A. Sosman

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.B. Johnson, C.M. Lovly, W.T. Iams, M. Colgan, S. DeNoble

Study supervision: D.B. Johnson

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