Safety, Correlative Markers, and Clinical Results of Adjuvant Nivolumab in Combination with Vaccine in Resected High-Risk Metastatic Melanoma

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Abstract

Purpose: The anti-programmed death-1 (PD-1) antibody nivolumab (BMS-936558) has clinical activity in patients with metastatic melanoma. Nivolumab plus vaccine was investigated as adjuvant therapy in resected stage IIIC and IV melanoma patients.

Experimental Design: HLA-A*0201 positive patients with HMB-45, NY-ESO-1, and/or MART-1 positive resected tumors received nivolumab (1 mg/kg, 3 mg/kg, or 10 mg/kg iv.) with a multi-peptide vaccine (gp100, MART-1, and NY-ESO-1 with Montanide ISA 51 VG) every 2 weeks for 12 doses followed by nivolumab maintenance every 12 weeks for 8 doses. Primary objective was safety and determination of a maximum tolerated dose (MTD). Secondary objectives included relapse-free survival (RFS), overall survival (OS), and immunologic correlative studies.

Results: Thirty-three patients were enrolled. Median age was 47 years; 55% were male. Two patients had stage IIIC disease; 31 patients had stage IV disease. Median follow-up was 32.1 months. MTD was not reached. Most common related adverse events (>40%) were vaccine injection site reaction, fatigue, rash, pruritis, nausea, and arthralgias. Five related grade 3 adverse events [hypokalemia (1), rash (1), enteritis (1), and colitis (2)] were observed. Ten of 33 patients relapsed. Estimated median RFS was 47.1 months; median OS was not reached. Increases in CTLA-4+/CD4+, CD25+Treg/CD4+, and tetramer specific CD8+ T-cell populations were observed with treatment (P < 0.05). Trends for lower baseline myeloid-derived suppressor cell and CD25+Treg/CD4+ populations were seen in nonrelapsing patients; PD-L1 tumor status was not significantly associated with RFS.

Conclusions: Nivolumab with vaccine is well tolerated as adjuvant therapy and demonstrates immunologic activity with promising survival in high-risk resected melanoma, justifying further study.

Introduction

Within the tumor microenvironment, the function of T-cells is thought to be impaired due in part to engagement of the programmed death 1 (PD-1) receptor found on T-cells with its ligand, programmed death receptor ligand (PD-L1), which is expressed by antigen-presenting cells such as dendritic cells and macrophages, as well as tumor and other cells (1–3). Tumor cells can “hijack” this pathway by ectopically expressing PD-L1 on their surface, which often is associated with a poor outcome (4–7). This interaction within the tumor microenvironment inhibits immune cell function leading to T-cell “exhaustion,” thereby inhibiting T-cell function and promoting tumor growth. A promising immunotherapy strategy being evaluated in multiple cancers is inhibition of this interaction between PD-1 and PD-L1 by the use of checkpoint to facilitate tumor cell destruction (8, 9).

Recent results from clinical trials of PD-1 and PD-L1 abrogating antibodies suggest that they can induce significant rates of tumor regression in melanoma, as well as renal cell, non–small cell lung, and bladder cancer (10–15). Objective response rates in ipilimumab-naive and ipilimumab-refractory metastatic melanoma patients treated with anti–PD-1 agents (nivolumab and pembrolizumab) range from 25% to 43%. The toxicity profile of these drugs has shown that they can induce immune-related adverse events, including hypophysitis, colitis, rash, hepatitis, and pneumonitis, with a rate of related severe (grade 3–4) adverse events that is less than 15%. Overall, anti–PD-1 and anti–PD-L1 therapies are well tolerated and toxicities are generally easily managed with supportive care and/or high-dose steroids.

Adjuvant therapy for resected high-risk melanoma continues to be an area in need of more effective strategies. Patients with resected stage IV melanoma have no FDA-approved adjuvant therapy option. Median relapse-free survival (RFS) has been reported to...
be as short as 5 months, with median overall survival (OS) ranging from 12 to 36 months (16–19). Similarly, subset analysis of resected stage IV patients on the ECOC4697 study comparing GM-CSF versus placebo demonstrated a median disease-free survival of 12 months and 6 months, respectively (20). Stage IIIC melanoma patients also have a poor prognosis, although in the United States, high-dose and pegylated interferon alfa2b are approved as adjuvant therapies for that subgroup (21–25). Because of the high relapse rate (>80%), long-term survival of less than 30% and the need for evaluation of new adjuvant treatments for these resected melanoma populations, we tested the monoclonal anti-PD-1 antibody nivolumab given with a vaccine every 2 weeks for 24 weeks, followed by maintenance therapy with nivolumab alone administered every 12 weeks for a total treatment duration of 2.3 years. Nivolumab was used in escalating doses with nivolumab alone every 12 weeks. Nivolumab (BMS-936558) is a fully human IgG4-blocking monoclonal antibody directed against PD-1 and was provided by Bristol-Myers-Squibb. The gp100 209–217 (210M) and MART-1 26–35 (27L) peptides were provided by the Cancer Therapy Evaluation Program of the National Cancer Institute. The GMP-grade gp100 260–268 (288V) and NY-ESO-1 157–165 (165V) peptides were produced by Cilinalla. All four peptides were emulsified in Montanide ISA 51 VG (Seppic), and were included to facilitate an assessment of the effects of PD-1 blockade on antigen-specific T-cell reactivity. The protocol was approved by the University of South Florida Institutional Review Board and conducted as an investigator-sponsored trial under investigator IND BB 13704.

Patients in cohort 1 were treated at 1 mg/kg, cohort 2 at 3 mg/kg, and cohort 3 at 10 mg/kg of nivolumab. Each of two induction phases consisted of intravenous nivolumab administered on the same day with subcutaneously injected multi-peptide vaccine in alternating thighs every 2 weeks for 6 doses. Patients remaining disease free by exam and scans continued on maintenance nivolumab at the same dose without vaccine, administered every 12 weeks for a total of 8 doses. Imaging studies (CT scan of the neck, chest, abdomen, and pelvis and MRI or CT of the brain) were performed at baseline and every 12 weeks during the trial (2.3 years), then every 6 months for 3 years.

The primary objective of the protocol was to determine the safety and tolerability of nivolumab in combination with a multi-peptide vaccine in resected stage IIIC and IV melanoma patients. Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 was used for event grading. Dose-limiting toxicity (DLT) was defined as a grade 3 or greater related adverse event within the first 12 weeks. Secondary endpoints included RFS, OS, and evaluation of biomarker studies to determine associations between immune assays and treatment/relapse. Sample size (10 patients per cohort) was selected based on sufficient samples for immune assessments. Patients were replaced if not evaluable for safety and leukapheresis collection after the first induction phase. Patients were followed until relapse and/or death.

Biomarker studies

Correlative studies were performed on leukapheresis blood samples collected at baseline, week 12 (postinduction cycle 1) and week 24 (postinduction cycle 2). Leukapheresis samples were processed to purify peripheral blood mononuclear cells (PBMC) and frozen at −168°C as previously described (26). After thawing, PBMC samples were batched and run blinded as to treatment time point and clinical outcome. Cells were stained with Live/Dead dye (Invitrogen) at 4°C for 20 to 30 minutes. After two washes, PBMCs were assessed for phenotypic expression by staining with fluorochrome-conjugated anti-CD3, CD8, CD4, CD25, CTLA-4, and PD-1 antibodies. T-regulatory cells (Tregs) were characterized as CD3+/CD4+ /CD127Low/FoxP3+ by staining with anti-CD3, CD4, CD8, CD127, and FoxP3 antibodies. Myeloid-derived suppressor cells (MDSC) were stained for CD3, CD19, CD56, CD11b, HLA-DR, CD14, and CD15. CD11b+/HLA-DR+/CD14+ , Lin−/CD14+ /CD11b+ /CD15+ monocytic MDSCs were assessed. For evaluation of tetramer assays, antigen-specific CD8+ T-cell populations for the gp100, MART-1, and NY-ESO-1
peptides were identified as previously described with a dump gate on CD4, CD14, CD19, and CD56 (26). Data were acquired on an LSR II flow cytometer (BD Biosciences) and analyzed with the FlowJo software (TreeStar).

Immunohistochemical staining for PD-L1 was performed as previously described (15). Briefly, an automated assay developed by Dako North America that incorporated an anti-PD-L1 rabbit monoclonal antibody (clone 28-8) was used. All sections were independently read by two pathologists and final scores were confirmed through an adjudication process. PD-L1 positivity was defined at two thresholds: ≥1% or ≥5% of tumor cells with a minimum of 100 evaluable tumor cells. Associations between PD-L1 tumor expression status and relapse event or RFS were explored.

Statistical analysis
Primary endpoints of the study were toxicity and tolerability. An adverse event was considered to be “significant” if the event required initiation of systemic steroids and/or cessation of therapy. Multiple immune parameters, relapse status, RFS, and OS were secondary endpoints. Differences in the pre- and post-treatment responses to MART-1, NY-ESO-1, NY-ESO-2, NY-ESO-3, gp100, gp209-217, gp103, gp260-275, and gp105-110 were evaluated by the Wilcoxon signed rank test. Association between categorical variables and relapse status was examined using the Fisher exact test. The Kaplan–Meier (KM) product limit method was used to estimate the distribution of a time-to-event endpoint such as RFS and OS, and the log-rank test was used to informally compare the KM curves between two groups of patients. Statistical analyses were performed using GraphPad Prism version 6.0, SPSS version 21.0, and R version 3.1. A P value of less than 0.05 was considered to be statistically significant. No multiple testing adjustments were made due to the exploratory nature of the study.

Results
Patient characteristics
A total of 33 patients were enrolled on protocol (12 patients on cohort 1, 10 patients on cohort 2, and 11 patients on cohort 3; Table 1). Three replacement patients were included (2 in cohort 1, 1 in cohort 3) to obtain leukapheresis specimens through the Wilcoxon signed rank test. Association between categorical variables and relapse status was examined using the Fisher exact test. The Kaplan–Meier (KM) product limit method was used to estimate the distribution of a time-to-event endpoint such as RFS and OS, and the log-rank test was used to informally compare the KM curves between two groups of patients. Statistical analyses were performed using GraphPad Prism version 6.0, SPSS version 21.0, and R version 3.1. A P value of less than 0.05 was considered to be statistically significant. No multiple testing adjustments were made due to the exploratory nature of the study.

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at the grade 3 level (no related grade 4 or 5) among 4 patients. These included hypokalemia (1 in cohort 2), dermatitis/rash (1 in cohort 1), enteritis (1 in cohort 3), and colitis (2 in cohorts 2 and 3). The only toxicities meeting the DLT criteria were the two colitis events. These serious adverse events responded to courses of high-dose steroids and supportive care. However, only the patient with grade 3 rash was rechallenged with study drug after recovery; there was no recurrence of serious rash. Other immune-related events of interest include grade 2 hypophysitis (n = 2) leading to adrenal insufficiency in both patients, grade 2 thyroiditis (n = 7) leading to primary hypothyroidism, and grade 1 pneumonitis (n = 1) without clinical sequelae. Adrenal insufficiency and hypothyroidism were successfully managed with hormone replacement.

### Clinical results

The median follow-up time from study enrollment was 32.1 months. Of 33 patients, 10 (30%) have had a relapse event. Six patients relapsed during induction phase I (weeks 1–12); 1 after induction phase II (weeks 12–24); 1 during the maintenance phase (weeks 24–120); and 2 patients after completing all treatment (>2.3 years). All other patients remain disease free, and 14 (42%) patients have finished all treatment. The estimated median RFS was 47.1 months (Fig. 1). Median OS has not yet been reached. The estimated 12- and 24-month OS rates are 87% and 82%, respectively. Of the 10 relapsed patients, 5 died due to metastatic melanoma; 3 were rendered free of disease surgically and remain disease-free at 2, 27, and
54 weeks after relapse. One patient (described in detail below) had spontaneous regression of disease after a biopsy-proven relapse and has been free of disease for more than 3 years. One additional relapsed patient is alive and on active therapy with dabrafenib plus trametinib.

Brain metastases subgroup
Ten patients with resected CNS disease were enrolled. This included 3 patients in cohort 1, 2 patients in cohort 2, and 6 patients in cohort 3. Only 2 of 10 patients have relapsed after a median follow-up time of 22.5 months. Both patients were in cohort 1. The first patient completed all 20 doses of study drugs and developed a new solitary lung metastasis that was resected after 47 months on protocol; she again has no clinical evidence of disease. The second patient was diagnosed with recurrent CNS disease (multiple-subdural metastases and subdural hematoma) after her first treatment with study drugs and expired 3 weeks from initiation on protocol from a CNS hemorrhage.

Postrelapse spontaneous regression
Cases of regression of melanoma after RECIST progression have been well documented for ipilimumab and anti–PD-1 antibodies, and have led to new criteria for antitumor response called immune-related response criteria (irRC; ref. 27). This may complicate the evaluation of patients on an adjuvant trial, as illustrated below. A patient with resected chest wall and pulmonary disease initiated treatment in the 3 mg/kg cohort. At the week 12 evaluation, CT scans showed new chest wall disease and a new splenic nodule of 2.7 cm, biopsy-proven by fine needle aspirate to be melanoma. While waiting to initiate treatment for metastatic disease, a repeat CT scan 4 weeks later showed shrinkage of both lesions. Over the next 24 weeks, repeat CT scans showed further regression and eventual disappearance of both lesions; he remains free of disease 38.8 months since relapse.

Correlative studies
Pharmacodynamic studies were performed to assess changes in T-cell and MDSC populations during therapy (baseline, n = 33; postinduction week 12, n = 32; and postinduction week 24, n = 25). Flow cytometric assays were used to analyze CD4+ and CD8+ T-cell populations. As shown in Fig. 2, tetramer data for antigen-specific T-cell populations demonstrated significant increases in MART-1 (27L), NY-ESO (165V), GP100 (288V), and GP100 (210M) CD8+ T-cells after each 12-week induction phase (P < 0.05 for all groups). Significant increases in CD25+ T-cell populations were also observed after 12 and 24 weeks of therapy (P < 0.001 and P = 0.013, respectively). Similar increases in CTLA4+ T-cell populations were seen (statistically significant at week 12 only). PD-1+ and PD-1+/CD8+ T-cell groups decreased during therapy (P < 0.001 for all groups). No significant differences in tetramer or flow cytometric analyses were observed between cohorts.

The identification of potential biomarkers to predict relapse was explored (Fig. 3). All cohorts were grouped together. There was a trend toward lower baseline CD25+ T-cell (P = 0.0583) and MDSC levels (P = 0.1718) in nonrelapsing patients compared with relapsing patients. No clear association was observed in other baseline T-cell populations or postinduction patient samples and relapse events. Baseline PD-L1 tumor expression was assessed on archived tumor specimens from 28 of the 33 enrolled patients. Two PD-L1 tumor positivity cutoffs were chosen to assess any association between PD-L1 status and relapse. Using a 1% PD-L1 cutoff, 18 samples were positive and 10 samples were negative. Relapse occurred in 5 of 18 (28%) PD-L1–positive patients and 4 of 10 (40%) PD-L1–negative patients (Fisher exact test = 0.677, two-sided). At a threshold of 5% PD-L1 staining, 12 samples were positive for PD-L1 and 16 were negative. Relapse occurred in 3 of 12 (25%) PD-L1–positive patients and 6 of 16 (38%) negative patients (Fisher exact test = 0.687, two-sided). As demonstrated in Fig. 3C and 3D, there was no statistically significant association between PD-L1 tumor staining and RFS in this patient population, although there is a nonsignificant trend toward better RFS in those whose tumors were PD-L1 positive using either threshold of IHC staining.

Discussion
Nivolumab in combination with a multi-peptide vaccine was well tolerated. Four of 33 patients permanently discontinued study drug due to toxicities, and only two DLTs (colitis) were observed, with no MTD defined up to 10 mg/kg of nivolumab. The common adverse events (fatigue, rash/pruritus, nausea/diarrhea, arthralgias, endocrinopathies) seen in this trial are similar to past
studies with either nivolumab or other anti–PD-1 antibodies. Only one case of grade 1 pneumonitis was seen. The addition of vaccine led to grade 1 local injection site reactions in a majority of patients. Related grade 3 events occurred in 4 of 33 patients (12%) and were manageable. This compares favorably with adverse events reported with adjuvant high-dose or pegylated interferon alpha2B, where 40% to 60% of patients experienced grade 3 events, 5% to 10% experienced a grade 4 event, and as many as

Figure 2. Correlative pharmacodynamics studies. Patient peripheral blood samples were collected by leukapheresis at baseline and after 12 weeks and 24 weeks of therapy with nivolumab plus multi-peptide vaccine. Tetramer assays showed significant increases in MART-1, NY-ESO, and GP100+/CD8 T-cell populations after 12 and 24 weeks of treatment with nivolumab and vaccine. Flow cytometry demonstrated significant increases in CD25+ Treg/CD4+ and CTLA-4+ T-cell populations with therapy. Decreased levels of PD-1+ T-cell populations were observed with therapy. Open circles, outlier values.

Figure 3. Baseline biomarker assays (pretreatment). A, lower baseline CD25+ Treg/CD4+ populations were observed in patients who remained disease free (nonrelapsed) with therapy (P = 0.0583). B, a trend for lower MDSC levels (CD11b+/DRlow/CD14+) was observed in patients who remained disease free with therapy (P = 0.1718). C and D, no statistical association was observed between PD-L1 tumor status (at either a 1% or 5% cutoff) and RFS by log-rank test.
31% of patients discontinued therapy due to toxicities (22, 23). The rate of grade 3 events in the current study is less than that of the ongoing EORTC18071 protocol with ipilimumab 10 mg/kg for resected stage III melanoma patients, where 36.5% and 5.5% of patients experienced a grade 3 or 4 adverse event, respectively, and 49% of patients discontinued ipilimumab due to toxicity (NCT00636168; ref. 28).

High-dose and pegylated interferon alpha2b have both been approved by the FDA as adjuvant therapy for resected stage III melanoma patients, but their long-term clinical benefit continues to be debated. There has been a consistent improvement in RFS with adjuvant interferon, but individual studies have largely failed to show a statistically significant improvement in OS (24, 29). Meta-analyses have demonstrated modest, but statistically significant, improvements in OS (hazard ratios of 0.85 to 0.91) with adjuvant interferon (25, 30, 31). This suggests that less toxic and more effective approaches for high-risk resected melanoma patients are clearly needed. Other adjuvant strategies have been explored, including bevacizumab (AVAST-M) and GM-CSF (ECOG4967), which have each demonstrated improvement in disease-free survival (only stage IV subset for GM-CSF) over placebo arms, but no statistical improvements in OS were seen (20, 32). Adjuvant ipilimumab for resected melanoma is currently under investigation in two large randomized phase III trials—the ECOG1609 and EORTC18071 protocols (NCT01274338, NCT00636168). The recent preliminary data presented from the EORTC18071 protocol in resected stage III patients demonstrated a median RFS of 32.2 months in the ipilimumab group compared with 17.4 months in the placebo group with a hazard ratio of 0.75 (P = 0.0013; ref. 28). However, this must be weighed against the high rate of adverse events seen in the ipilimumab group.

A number of important questions remain to be settled regarding the use of anti–PD-1 blockade in the adjuvant setting: (i) does minimal/microscopic residual disease and no effective tumor microenvironment impact efficacy, (ii) can PD-L1 expression in resected melanoma tumors serve as a biomarker for successful adjuvant treatment, and (iii) can other novel biomarkers be defined for the efficacy of PD-1 blockade in an adjuvant trial?

Our data suggest that nivolumab is clinically active in resected stage IIIC/IV melanoma patients based on the low rate of relapse (10 of 33 patients). Impressive RFS—estimated median RFS of 47.1 months, and a median OS not yet reached with more than 32 months of follow-up. Although the results cannot be compared directly with the prior adjuvant interferon and current adjuvant ipilimumab experience, these data should be placed in the context of reports on the natural history of resected stage IV melanoma. The median RFS in the SWOG S9430 protocol and Canvaxin-IV trials was 5 and 7.2 months, respectively (16–18). Median OS in these two studies was 21 months (N9430) and 32 months (control arm Canvaxin-IV trial). A retrospective study using the Surveillance, Epidemiology, and End Results (SEER) database showed a median OS of 12 months in stage IV melanoma patients undergoing metastasectomy (19). Overall, contemporary data suggest that a median RFS over 12 months would be a reasonable benchmark by which to judge the potential of an adjuvant treatment for stage IIIC/IV high risk resected melanoma. By that criterion, our data with adjuvant anti–PD-1 therapy surpasses RFS expectations and longer follow up will clarify whether superior median OS can be achieved compared with the S9430 and Canvaxin-IV studies. Furthermore, the low event rate in resected brain metastases patients suggests a potential benefit in this very high-risk population.

We demonstrated statistically significant increases in melanoma antigen-specific CD8+ T-cell populations and decreases in PD-1 expressing T-cells with exposure to nivolumab and vaccine. The former has been demonstrated in patients with active metastatic melanoma (15), whereas no change or increases in PD-1 expressing T-cell populations has been previously described in mouse models (33, 34). It is possible that the decrease in PD-1 expressing T-cells in our study is reflective of internalization of the antigen with anti–PD-1 antibody exposure rather than a true change in phenotype. Increases in CD25 Tregs/CD4+ and CTLA-4+/CD4+ T-cell populations were seen with anti–PD-1 therapy. This suggests that one adaptive mechanism that occurs with anti–PD-1 therapy and may dampen clinical activity is mediated through CTLA-4 and/or Tregs. This is supported by data from the nivolumab plus vaccine trial in unresectable stage III/IV melanoma patients where a significant increase in regulatory T-cell populations at week 12 was observed in the nonresponding patients (15). However, increases in Treg populations seen with anti–CTLA-4 therapy have been associated with improved progression-free survival in patients with melanoma with regional metastases (35, 36). Therefore, Tregs may play a different role depending on the effects of different checkpoint inhibitors. Interestingly, cotargeting CTLA-4 and PD-1 can enhance antigen-specific effector CD8+ and CD4+ T-cell function and tumor infiltration over individual checkpoint inhibition as monotherapy (33, 37). This strategy is now a promising area of clinical research with early studies of concurrent and sequential ipilimumab and nivolumab as showing high levels of durable response in patients with metastatic melanoma (38, 39). Concurrent ipilimumab and nivolumab is also under investigation in high-risk resected metastatic melanoma patients as an amendment to the current trial (NCT01176474).

Much attention has been given to tumor PD-L1 status as a biomarker for response to anti–PD-1 therapy. In a phase I study of nivolumab in advanced cancers, PD-L1 status (5% threshold on immunohistochemistry) and response data were available on 42 patients, which showed no objective responses in the 17 patients with negative PD-L1 status, whereas objective responses were seen in 9 of 25 patients with positive PD-L1 staining (14). Enthusiasm for the use of PD-L1 as a predictive biomarker has diminished as other studies have shown that patients with PD-L1 negative melanomas can still respond to anti–PD-1, albeit at lower rates (12, 15). In our study, there were slightly fewer relapses in patients with PD-L1–positive tumors (both 1% and 5% cutoffs), but this was not statistically significant on Fisher exact testing, nor was RFS by log-rank testing. Our other correlative studies showed that nonrelapsing patients tended to have lower baseline levels of CD25 Tregs/CD4+ and MDSC populations. This is supported by other data indicating the suppressive role of these immune cell populations (40, 41), which may mitigate the effect of cytotoxic T-cells (and other immune cells) expected with anti–PD-1/PD-L1 therapy, resulting in dampened antitumor activity. Prior studies by our group and others have also demonstrated associations between a 12-chemokine gene expression signature and rises in absolute lymphocyte counts with improved clinical outcomes with immunotherapy (42, 43). Further prospective investigation into the role of these findings as predictive or prognostic biomarkers will potentially help better stratify melanoma patients for systemic immune therapy.
In summary, nivolumab at doses of 1 mg/kg up to 10 mg/kg is well tolerated in patients with resected stage IIC/IV melanoma. Both RES and OS with anti–PD-1 therapy in this study were promising compared with historic data. Tumor PD-L1 expression alone does not appear to be associated with relapse after adjuvant anti–PD-1 therapy. A prospective, randomized study of nivolumab in resected high-risk melanoma patients is warranted.

Disclosures of Potential Conflicts of Interest


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