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Improved prognosis and evidence of enhanced immunogenicity in tumor and circulation of high-risk melanoma patients with unknown primary

Ahmad A Tarhini [ORCID],[1] Sandra J Lee,[2,3] Aik-Choon Tan,[4] Issam M El Naqa,[5] F Stephen Hodi,[3] Lisa H Butterfield [ORCID],[6,7] William A LaFramboise,[8] Walter J Storkus [ORCID],[9] Arivarasan D Karunamurthy,[10,11] Jose R Conejo-Garcia,[12] Patrick Hwu,[13] Howard Streicher,[14] Vernon K Sondak,[15] John M Kirkwood[10,16]

Correspondence to
Professor Ahmad A Tarhini; ahmad.tarhini@moffitt.org

ABSTRACT

Background Melanoma of unknown primary (MUP) represents a poorly understood group of patients both clinically and immunologically. We investigated differences in prognosis and candidate immune biomarkers in patients with unknown compared with those with known primary melanoma enrolled in the E1609 adjuvant trial that tested ipilimumab at 3 and 10 mg/kg vs high-dose interferon-alfa (HDI).

Patients and methods MUP status was defined as initial presentation with cutaneous, nodal or distant metastasis without a known primary. Relapse-free survival (RFS) and overall survival (OS) rates were estimated by the Kaplan-Meier method. Stratified (by stage) log-rank test was used to compare RFS and OS by primary tumor status. Gene expression profiling (GEP) was performed on the tumor biopsies of a subset of patients. Similarly, peripheral blood samples were tested for candidate soluble and cellular immune biomarkers.

Results MUP cases represented 12.8% of the total population (N=1699) including 11.7% on the ipilimumab arms and 14.7% on the HDI arm. Stratifying by stage, RFS (p=0.001) and overall survival (OS) (p=0.009) showed outcomes significantly better for patients with unknown primary. The primary tumor status remained prognostically significant after adjusting for treatment and stage in multivariate Cox proportional hazards models. Including only ipilimumab-treated patients, RFS (p=0.005) and OS (p=0.023) were significantly better in favor of those with unknown primary. Among patients with GEP data (n=718; 102 MUP, 616 known), GEP identified pathways and genes related to autoimmunity, inflammation, immune cell infiltration and immune activation that were significantly enriched in the MUP tumors compared with known primaries. Further investigation into infiltrating immune cell types estimated significant enrichment with CD8 +and CD4+T cells, B cells and NK cells as well as significantly higher major histocompatibility complex (MHC)-I and MHC-II scores in MUP compared with known primary. Among patients tested for circulating biomarkers (n=321; 66 unknown and 255 known), patients with MUP had significantly higher circulating levels of IL-2R (p=0.04).

Conclusion Patients with MUP and high-risk melanoma had significantly better prognosis and evidence of significantly enhanced immune activation within the TME and the circulation, supporting the designation of MUP as a distinct prognostic marker in patients with high-risk melanoma.

INTRODUCTION

The incidence of melanoma continues to rise annually with 106 110 new invasive melanoma cases and 7180 deaths estimated in the USA in the year 2021.[1] This increase in incidence similarly applies to high-risk melanoma including patients with resectable regional nodal (stage III) or distant (stage IV) metastatic disease, who can be rendered disease free surgically, but continue to be at a high risk for disease relapse and death from melanoma that rises with stage.[2 3] Unknown primary melanoma represents a significant proportion of the disease, and unknown primary is typically attributed to the phenomenon of complete regression at the primary melanoma site.[4] The American Joint Committee on Cancer (AJCC) staging system divides cutaneous melanoma into four stages.[4] Stages I and II include patients where the detectable primary melanoma is confined to the skin and where prognosis is primarily defined by the depth of the tumor and its ulceration status. Stage III includes patients with regional nodal metastases where the primary tumor depth and ulceration status continue to impact prognosis and staging. Stage IV is defined by the spread of metastasis into distant sites. When it comes to melanoma of unknown primary (MUP), there are limited data about prognosis and how these patients should be optimally staged.[4] Retrospective single institutional analyses have suggested improved survival compared with similarly staged patients with known primary,

based on the sites of metastases.[5–7] However, the AJCC staging system recommends that patients with MUP (T0) who present with lymph node metastases be assigned to the corresponding N category based on the number of metastatic lymph nodes and the presence or absence of intransit metastases while acknowledging that the proposed staging should be used until additional data are available.[4] Clearly, additional data are needed in order to better define the prognosis of this patient population and better understand the underlying biology and immunogenicity of unknown primary melanomas. Such data may have implications related to staging and the design of future adjuvant trials where unknown primary status could be accounted for as a stratification factor.

The North American Intergroup trial E1609 tested ipilimumab at 3 mg/kg (ipi3) or 10 mg/kg (ipi10) vs high-dose interferon-α (HDI) and demonstrated significant overall survival (OS) benefits with ipi3 compared with HDI and less toxicity compared with ipi10.[8] This trial is the second largest adjuvant trial reported to date in melanoma with participation from 1673 patients.[8] E1609 allowed the enrolment of melanoma patients with unknown primary that were randomized across the three arms of the study. We observed that a significant proportion of E1609's adult patients had an unknown primary melanoma and have, therefore, conducted an in-depth analysis to better understand the clinical and prognostic features of this patient population as compared with similarly staged patients with a known primary. Furthermore, we conducted tumor tissue and blood based immune monitoring studies in consenting patients with available biospecimens. This report presents the findings from our clinical and laboratory immune monitoring analyses.

Patients and methods
Patients
The phase III E1609 randomized patients with melanoma of cutaneous or unknown primary origin that was histologically confirmed and had AJCC seventh edition stages IIIB, IIIC or IV (M1a or M1b) and were rendered disease free surgically within 12 weeks of registration on the trial.[8] An Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1 and safety laboratory test criteria were mandated at screening. Patients were excluded if they had a history of autoimmune disorders or conditions of immunosuppression that necessitated the use of systemic corticosteroids or other immunosuppressants.E

Trial design and treatments
E1609 was an open-label phase III trial where patients were randomized to adjuvant therapy with either ipi3, ipi10 or HDI. Patients were stratified by stage (AJCC seventh edition IIIB, IIIC, M1a, M1b).[8] Details of the treatment regimens, randomization procedures, clinical trial endpoint points and trial oversight were previously published.[8] Patient disposition is described in the consort diagram included in online supplemental figure S1.

Methods and statistical analysis
Unknown primary melanoma status was defined as initial presentation with cutaneous, nodal or lung metastasis that was completely surgically resected without a history of known primary melanoma as registered on the study. All pathology reports were required to be submitted for each surgical intervention related to melanoma management and centrally reviewed by the study's principal investigator. Central histology review was conducted on a subset of cases by the study's pathology cochair (UR). Individual records and pathology reports were reviewed by study chair (AAT) in order to verify patient designated AJCC stage working closely with the study's pathology cochair (UR) and the study team. Patient distribution by the initial site of metastasis was analyzed. Further, we evaluated the risk of relapse and death by the primary tumor status and by the AJCC stage group. Five-year relapse-free survival (RFS) and overall survival (OS) rates and 95% CIs were estimated by the Kaplan-Meier method. Stratified (by stage) log-rank test was used to compare RFS and OS by primary tumor status in the overall study population and among the different study arms. Two-sided p values were reported.

Gene expression profiling
Gene expression profiling (GEP) was performed on the tumor biopsies of 718 (102 unknown, 616 known primary) melanoma patients. Only metastatic tumors were included that were resected to render patients' disease free prior to clinical trial enrolment. Microdissection of FFPE tumor specimens was performed manually using an inverted microscope (Nikon Eclipse TE200) as needed to obtain a minimum of 90% tumor cells for RNA purification. Dissection involved scraping cells from unstained sections of 5 μm thickness on slides aligned in register with serially cut H&E-stained specimens including tumor domains demarcated by a surgical pathologist (ADK). RNA purification was performed using the Qiagen miRNeasy FFPE Kit and protocol (Qiagen, Valencia, California, USA) with isolated RNA suspended in nuclease-free water. Inclusion in subsequent in vitro amplification assays was determined both by spectrophotometric absorption ratio (260/280>1.8 (NanoDrop, Wilmington, Delaware, USA)) and RIN values (RNA Integrity Index) determined via microchip electrophoretic analysis (Agilent Bioanalyzer 2100, Agilent Technologies, Santa Clara, California, USA). We previously established that RIN values ranging from 5.0 to 8.0 in RNA from FFPE specimens can undergo successful in vitro transcription and amplification using a multiplex primer approach. Amplification was performed using the NuGen whole transcription method comprizing the Ovation FFPE WTA assay (NuGEN, San Carlos, California, USA) employing random and 3′ primers to eliminate amplification bias beginning with 100 ng total RNA. Confirmation of cDNA diversity was obtained using the Bioanalyzer 2100 to generate an electrophoretogram for each amplification reaction regarding sample yield, integrity and size diversity compared with a laboratory human

RNA standard and a Universal Human Reference RNA (Stratagene, La Jolla, California, USA). A 5 µg of purified cDNA was incubated with fragmentation buffer (NuGEN, San Carlos, CA) at 37°C for 30 min, then 95°C for 2 min. All cDNA samples underwent hybridization on Affymetrix GeneChip HG U133A 2.0 arrays which contain overlapping probe sets for transcripts comprehensively representing the functionally characterized human genome. Briefly, fragmented cDNAs were mixed in a hybridization cocktail with water to a final volume of 220 µL. 130 µL of hybridization cocktail is hybridized on each array at 45°C for 18 hours. The arrays were then washed and stained with streptavidin-phycoerythrin in a GeneChip Fluidics Station 450 (Affymetrix) and scanned using a GeneChip Scanner 3000 (Affymetrix). Raw microarray data sets were normalized by using the Robust Multi-array Average (RMA) method using Affymetrix Power Tools (APT) as previously published.[9] Multiple probe sets representing the same genes were collapsed by using the probe with maximum gene expression. Gene set enrichment analysis (GSEA) was performed by comparing the unknown and known primary tumor samples.[10] For this purpose, KEGG pathways gene sets were obtained from MSigDB (PMID: 21546393) to interrogate the enrichment of pathways in the unknown versus known primary samples.[11] In order to further to deconvolute the cell types in the bulk transcriptomics, we used gene sets obtained from CIBER-SORT[12 13] and TIMEx[14] in comparing the unknown vs known primary samples. Gene sets with a false discovery rate q-value <0.1 were deemed as significant. Major histocompatibility complex (MHC)-I and MHC-II scores were generated from bulk gene expression profiles by comparing unknown primary tumors and known primaries. For each sample, the scores were computed by averaging the standardized z-score of 6 MHC-I genes (HLA-A, HLA-B, HLA-C, B2M, TAP1, TAP2) and 13 MHC-II genes (HLA-DMA, HLA-DMB, HLA-DOA, HLA-DOB, HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQA2, HLA-DQB1, HLA-DQB2, HLA-DRA, HLA-DRB1, HLA-DRB5) as previously described.[15] Mann-Whitney U test was performed between the two groups and $p < 0.05$ was deemed as statistically significant.

Similarly, peripheral blood mononuclear cells (serum and PBMC) samples collected at baseline (prior to initiating systemic therapy) were tested for soluble and cellular immune biomarkers in a subset of patients (N=321; 66 unknown and 255 known primary).

Blood processing
Red top vacutainer tubes (BD, no anticoagulant) were used for serum collection and all samples were processed within 24 hours of collection (samples received before 17:00 hour were processed on receipt, those arriving after 17:00 hour were processed the following morning). Serum samples were centrifuged at 2500 rpm for 10 min at 4°C according to laboratory standard operating procedures (SOPs) and single use aliquots of each patient's sera were then stored at −80°C. The laboratory freezers were monitored continuously for any temperature fluctuations and maintained the samples at −80°C.

Multiplex serum cytokine analysis
Twenty-one serum cytokines were selected for analysis based on function. These included Th1 type cytokines (IL-12p70, IL-17, IL-2, IP-10), proinflammatory (IL-1α, IL-1β, IL-6, TNF-α, TNF-RII, IL-2R, IL-8, C reactive Protein (CRP), IL-17, IFN-α), immunoregulatory (TGF-α, IL-10, TIMP1), growth factor (VEGF-A), and other/chemokines (CCL3/MIP-1α, CCL4/MIP-1β, CXCL9/MIG, CXCL11/I-TAC). The xMAP Luminex serum assay for these cytokines was performed according to the manufacturer's protocol (BioSource International (Camarillo, California, USA) as previously described,[16] and laboratory SOPs, and analyzed on the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, California, USA). Experimental data were analyzed using five-parametric curve fitting and assay controls included kit standards and multiplex QC controls (R&D Systems). Interassay variabilities for individual cytokines were 1.0%–9.8% and intra-assay variabilities were 3.6%–12.6% (information provided by Biosource International and validation performed in our laboratory). CRP was run singly as it requires different dilutions.
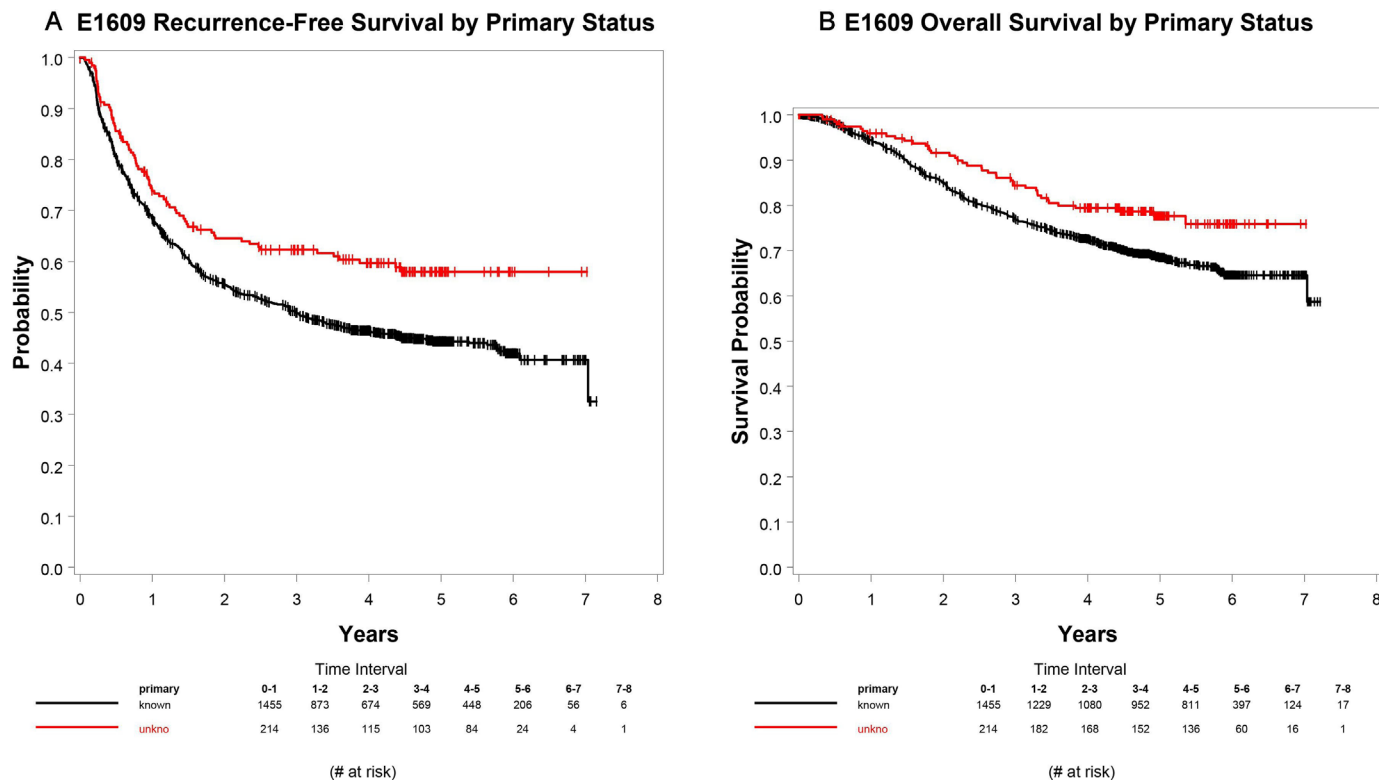
Multicolor flow cytometry
Multicolor flow cytometry was used to compare cell subset phenotypes on thawed patient PBMC, with healthy donor controls, run according to laboratory SOPs. Regulatory T cells (Treg) were defined as CD4 +CD25+FOXP3+or CD4+CD25hi+CD39+cells, to incorporate the candidate functional marker CD39 as previously described.[17] Myeloid-derived-suppressor cells were defined as cells expressing Lin-neg/HLA-DR−/CD33+/CD11b+in either a 'lymphocyte' (small FSCxSSC) gate, or in a 'monocyte' (larger FSCxSSC) gate, and as HLA-DR+/lo CD14 +cells in a large gate. We also tested the frequencies of CD4 +and CD8+T cells specific to shared tumor-associated antigens (Gp-100, MART-1, NY-ESO-1) using overlapping peptide libraries (15-mer peptides overlapping by 4) and a short (4–5 hours) in vitro culture to identify activated (CD69+) and cytokine producing (intracellular IFNγ+) T cells. Detailed methods were described previously.[17]

RESULTS
Patient characteristics and treatment details were previously published.[8] Unknown primary melanoma cases represented 12.8% of the total study population included in this analysis (214/1669) including 11.7% (121/1034) on the ipilimumab arms and 14.6% (93/635) on the HDI arm. Site of metastasis at initial presentation among the unknown primary cases included cutaneous and nodal (97%; 202/208) and lung (3%; 6/208). Among the overall E1609 study population, the proportion of patients with unknown primary was 10.5% (92/879) for

A E1609 Recurrence-Free Survival by Primary Status

B E1609 Overall Survival by Primary Status

Figure 1 Kaplan-Meier curves for relapse-free survival (RFS) (A) and overall survival (OS) (B) for patients with unknown primary melanoma compared with those with known primary. Stratifying by treatment and stage, RFS (p=0.001) and OS (p=0.008) were significantly better for patients with unknown primary tumor compared with known primary.

IIIB, 11.6% (77/662) for IIIC, 42.2% (38/90) for M1a and 18.4% (7/38) for M1b across all treatment arms.

Stratifying by treatment and stage, RFS (p=0.001) and OS (p=0.008) were significantly better for patients with unknown primary tumor compared with known primary. Figure 1A,B shows the Kaplan-Meier curves for RFS and OS, respectively for all patients. Five-year RFS and OS rates by primary tumor status and stage group among all patients enrolled on the study are summarized in online supplemental table S1. Improvements in RFS and OS were consistently in favor of the unknown primary status across the stage groups (IIIB, IIIC and M1a/M1b). Furthermore, the primary tumor status remained significant after adjusting for treatment and stage in multivariate Cox proportional hazards models.

Including only ipi3 and ipi10 patients, RFS (p=0.005) and OS (p=0.024) were consistently significantly better in favor of patients with unknown primary status. Figure 2A,B shows the Kaplan-Meier curves for RFS and OS, respectively for ipilimumab-treated patients. Five-year RFS and OS rates by primary tumor status and stage group among patients treated with ipilimumab (ipi3 and ipi10 arms) are summarized in online supplemental table S1.

Including only HDI patients, RFS (p=0.055) and OS (p=0.129) were marginally better in favor of the unknown primary status. Figure 3A,B shows the Kaplan-Meier curves for RFS and OS, respectively for HDI-treated patients. Five-year RFS and OS rates by primary tumor

status and stage group among patients treated with HDI are summarized in online supplemental table S1.

GSEA identified pathways and genes related to autoimmunity, inflammation, immune cell infiltration and immune activation that were significantly enriched in the unknown primary tumors compared with known primaries (table 1). TIMEx revealed that unknown primary tumors were enriched with immune cell types infiltrated in the bulk transcriptomics, especially CD8 +and CD4+T cells, B cells and NK cells (TIMEx, table 2). Similar results were also identified by interrogating the CIBERSORT signatures (CIBERSORT, online supplemental table S2). On the other hand, known primaries were enriched with stromal fibroblasts and myofibroblasts (table 2).

MHC-I and MHC-II scores were computed from bulk gene expression profiles. MUP tumors had significantly higher MHC-I (p=0.004) and MHC-II (p=0.023) scores as compared with known primary tumors (figure 4).

Among the subset of patients tested for circulating biomarkers, patients with unknown primary had significantly higher levels of IL-2R than those with known primary (p=0.04) as shown in figure 5.

DISCUSSION

MUP appears to represent a unique patient population with improved prognosis compared with similarly staged melanoma patients with known primary. This study was
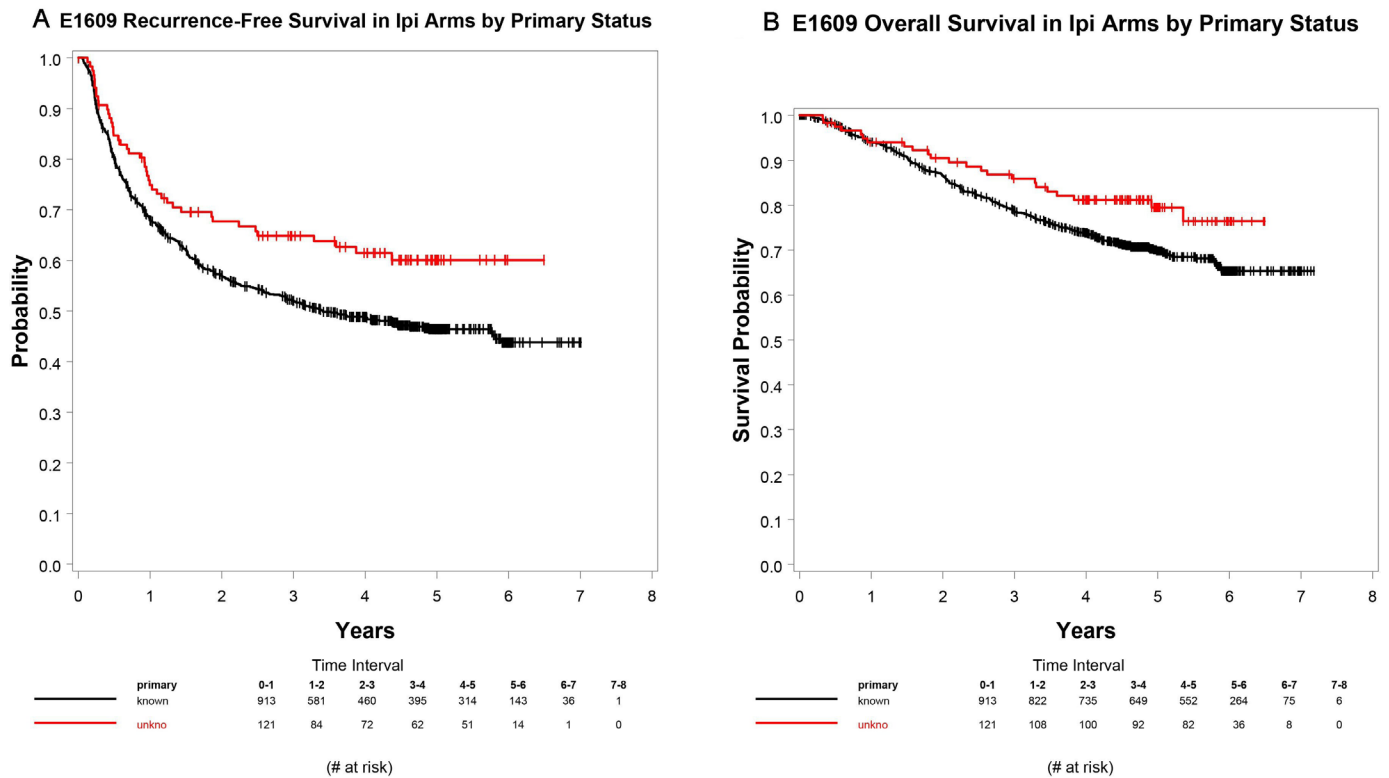
Figure 2 Kaplan-Meier curves for relapse-free survival (RFS) (A) and overall survival (OS) (B) for ipilimumab-treated patients with unknown primary melanoma compared with those with known primary. RFS (p=0.005) and OS (p=0.024) were consistently significantly better in favor of the unknown primary status.
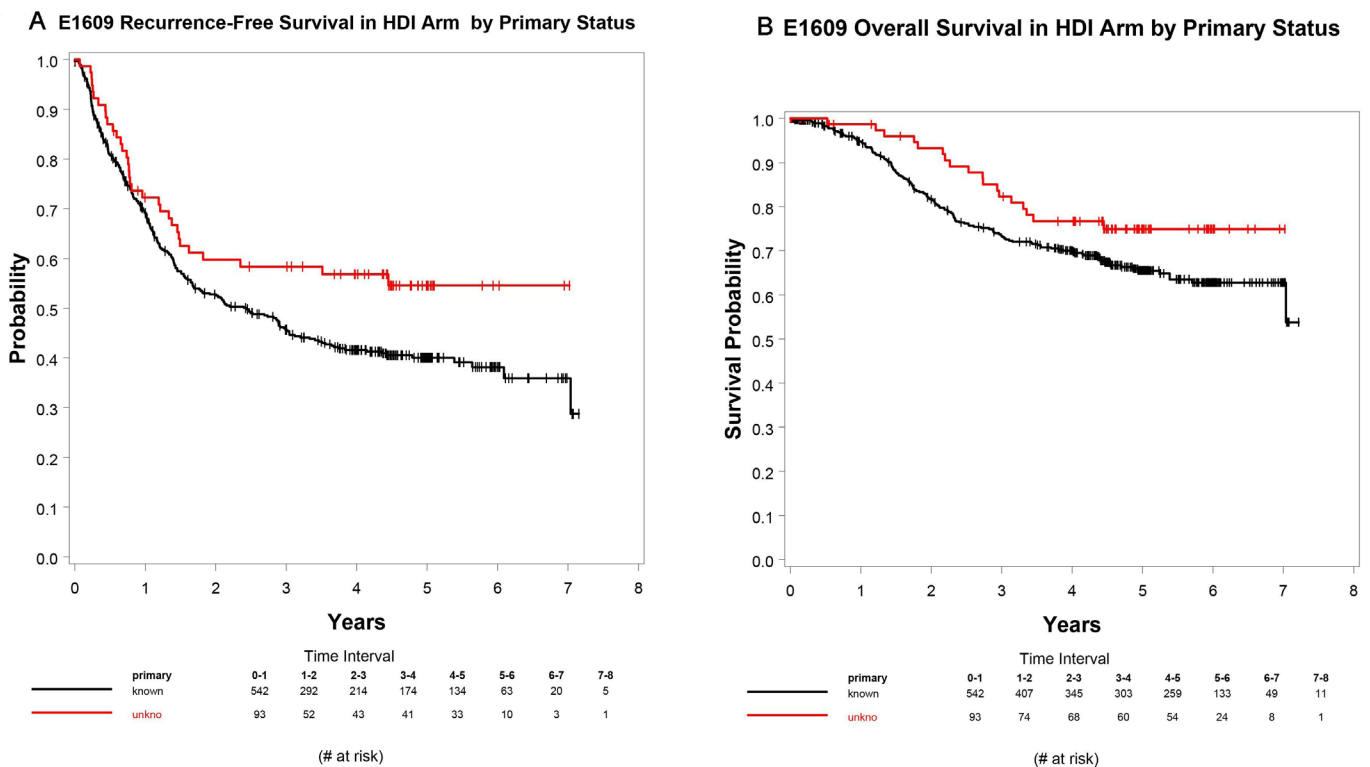
Figure 3 Kaplan-Meier curves for relapse free survival (RFS) (A) and overall survival (OS) (B) for high dose interferon-alfa (HDI)-treated patients with unknown primary melanoma compared with those with known primary. RFS (p=0.055) and OS (p=0.129) were marginally better in favor of the unknown primary status.

Table 1 Immune related pathways found to be significantly enriched in unknown primary melanomas compared with known primary as computed by gene set enrichment analysis (using KEGG pathways gene sets) (NES: Normalized Enrichment Score)

KEGG pathways	NES	NOM p value	FDR q value
Antigen processing and presentation	2.28	0.0000	0.0000
Autoimmune thyroid disease	2.25	0.0000	0.0000
Allograft rejection	2.22	0.0000	0.0000
Systemic lupus erythematosus	2.21	0.0000	0.0000
Intestinal immune network for iga production	2.11	0.0000	0.0002
Graft versus host disease	2.07	0.0000	0.0001
Leishmania infection	1.98	0.0000	0.0007
Primary immunodeficiency	1.96	0.0000	0.0011
Type I diabetes mellitus	1.94	0.0000	0.0011
DNA Replication	1.70	0.0097	0.0386
Toll like receptor signaling pathway	1.69	0.0000	0.0364
Viral myocarditis	1.69	0.0031	0.0342
Pantothenate and Coa Biosynthesis	1.68	0.0203	0.0358
Prion diseases	1.63	0.0084	0.0564
Natural killer cell mediated cytotoxicity	1.63	0.0030	0.0531
Protein export	1.62	0.0188	0.0529
Asthma	1.59	0.0223	0.0633
Starch and sucrose metabolism	1.57	0.0161	0.0776
Complement and coagulation cascades	1.56	0.0142	0.0774
Cell adhesion molecules cams	1.55	0.0031	0.0777

FDR, false discovery rate; NOM p-val, Nominal p value.

nested within the E1609 randomized controlled trial that enrolled patients with AJCC seventh edition stages IIIB, IIIC, M1a and M1b operable melanoma that remain at high risk for recurrence and death following surgical resection. The E1609 study allowed MUP as part of the eligibility criteria allowing for the enrolment of a relatively large sample size that represented 12.8% of the total study population. While MUP was not a stratification factor in E1609, MUP cases were similarly distributed between the ipilimumab arms and the HDI arm (11.7% and 14.7%, respectively). Although the study enrolled patients with resected lung metastases, these cases represented only 3% of the MUP population and the overwhelming majority of MUP were cutaneous and nodal metastases. In terms of RFS and OS, patients with MUP had significantly better outcomes in the overall study population after adjusting for treatment and stage. The significant improvements in RFS and OS in favor of MUP persisted when the analysis included only ipilimumab treated cases, but were only marginally better when including HDI cases alone. This may be explained in terms of ipilimumab's superior adjuvant efficacy in melanoma especially when considering a more immunogenic TME of MUP, which may potentially be predictive of response to immune checkpoint inhibitors.[18] These data support the hypothesis that MUP represents a distinct patient population with significantly better prognosis compared with

similarly staged patients according to the AJCC staging system. To date, evidence in the literature to support this hypothesis has been limited to retrospective case series.[19] A retrospective analysis of melanoma patients diagnosed between 1990 and 2001 who underwent surgical resection of regional lymph nodes compared two cohorts including patients with MUP and control patients with a median follow-up of 7.7 years.[20] OS rates at 5 and 10 years were 55% and 44%, respectively, for patients with MUP, compared with 42% and 32% for the control group with known primary (p=0.04). Moreover, in multivariate analyses, MUP was found to be a favorable prognostic factor for OS, with HR 0.61, 95% CI (0.42 to 0.86; p=0.006) apart from therapy. Similarly, a retrospective study of a single institution melanoma database reported significantly higher overall survival rates after surgical resection for melanoma patients with lymphadenopathy from MUP. Five-year OS rates 55% compared with 44% in favor of MUP; p=0.00210.[6] Another retrospective study also reported similar observations where patients with MUP had significantly better survival outcomes compared with those with a known primary.[7] Interestingly, case series that performed molecular characterization of unknown primary cases have suggested a similar incidence of *BRAF* and *NRAS* mutations to that expected in known cutaneous primaries.[21]

Table 2 Tumor immune microenvironment cell types estimated by TIMEx signatures found to be significantly enriched in unknown primary melanomas compared with known primary and vice versa as computed by GSEA (NES: Normalized Enrichment Score)

	NES	NOM p value	FDR q value
Enriched in unknown primary tumors			
IMMUNE_CD8TEX	2.53	0.0000	0.0000
IMMUNE_B	2.34	0.0000	0.0000
IMMUNE_NK	2.22	0.0000	0.0000
IMMUNE_PLASMA	2.21	0.0000	0.0000
IMMUNE_CD4TCONV	2.17	0.0000	0.0000
IMMUNE_CD8T	2.15	0.0000	0.0000
MINOR_CD8TCM	2.13	0.0000	0.0000
MINOR_CD4TN	2.11	0.0000	0.0000
IMMUNE_TMKI67	1.98	0.0000	0.0000
MINOR_TH1	1.95	0.0000	0.0000
IMMUNE_DC	1.94	0.0000	0.0000
MINOR_CDC2	1.91	0.0000	0.0001
MINOR_CD8TEFF	1.90	0.0000	0.0001
MINOR_CD4TEFF	1.89	0.0000	0.0001
MINOR_M1	1.87	0.0000	0.0001
IMMUNE_PDC	1.82	0.0000	0.0004
IMMUNE_TREG	1.82	0.0000	0.0003
MINOR_MONOCYTE	1.51	0.0079	0.0168
MINOR_TH17	1.50	0.0361	0.0184
IMMUNE_NEUTROPHILS	1.50	0.0162	0.0184
IMMUNE_ILC	1.38	0.0666	0.0467
Enriched in known primaries			
STROMAL_FIBROBLASTS	−1.51	0.0000	0.0799
STROMAL_MYOFIBROBLASTS	−1.48	0.0000	0.0484

FDR, false discovery rate; GSEA, gene set enrichment analysis.

In order to investigate the underlying molecular pathways in MUP that contribute to improved outcomes, we performed GSEA by comparing MUP tumors to known primaries in a subset of patients using gene expression profiles. For this purpose, we used KEGG pathway gene sets, a reference database for pathway mapping representing knowledge of molecular interaction, reaction and relation networks for metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, human diseases and drug development.[22] Our GSEA findings supported evidence of enhanced expression of immune-related genes and the activation of immune related pathways in MUP. The top pathways identified via GSEA and associated molecules were notably immune related and highly statistically significantly associated with MUP tumors over those with known primary. Next, we used CIBERSORT and TIMEx in order to deconvolute the cell types in the bulk transcriptomics.[12 14] TIMEx, our recently developed portal of tumor immune microenvironment

cell type signatures collected from pan-cancer single cell RNA sequencing data sets, is a tumor immune microenvironment deconvolution method that emphasizes estimating infiltrating immune cell types.[14] It includes 37 tumor immune microenvironment cell type signatures. Using TIMEx, the infiltrating immune cell types in the unknown primaries were enriched with T cells, B cells and NK cells. These findings were also validated by using CIBERSORT leukocytes gene signatures that includes 22 human hematopoietic cell phenotypes. Immune-related gene expression profiles were found to be prognostic of improved outcome in melanoma and other malignancies and potentially predictive of clinical benefit in patients treated with immune checkpoint inhibitors.[18] We previously reported an immune related 22-gene expression signature detected in the tumors of patients treated with neoadjuvant ipilimumab that was significantly associated with clinical benefit.[18] These genes constituted a proinflammatory gene expression profile of chemokines and other immune-related genes associated with a Type I

MHC-I Score

p = 0.004

MHC-II Score

p = 0.023
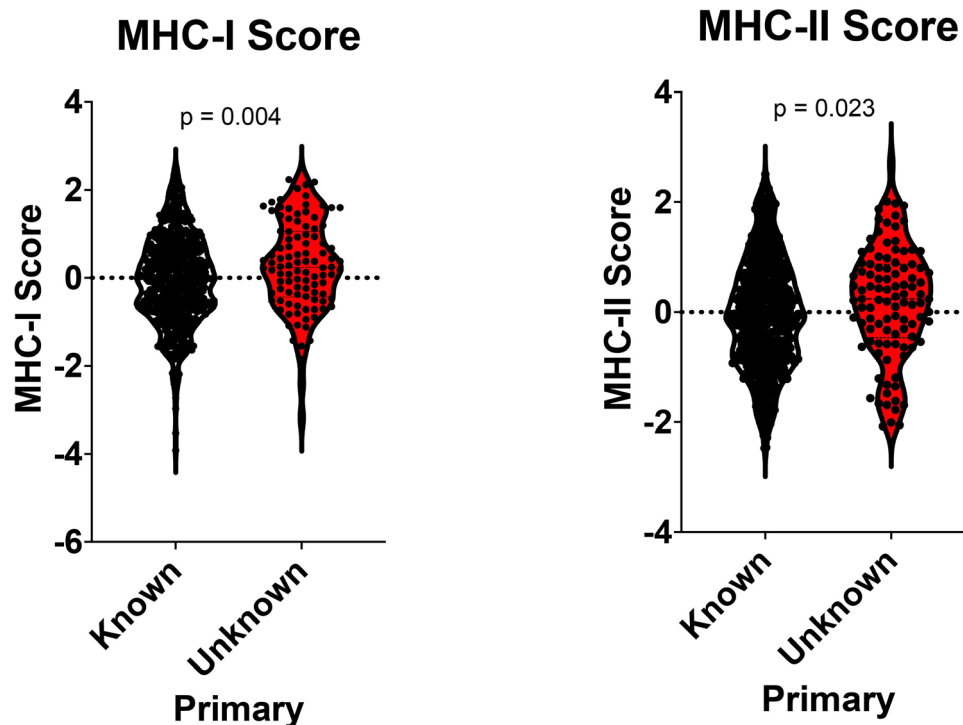
Figure 4 Major histocompatibility complex (MHC)-I and MHC–II scores were computed from bulk gene expression profiles by comparing unknown primary melanoma (MUP) tumors and known primaries. MUP had significantly higher MHC-I (p=0.004) and MHC-II (p=0.023) scores as compared with known primary.

p-value = 0.0426
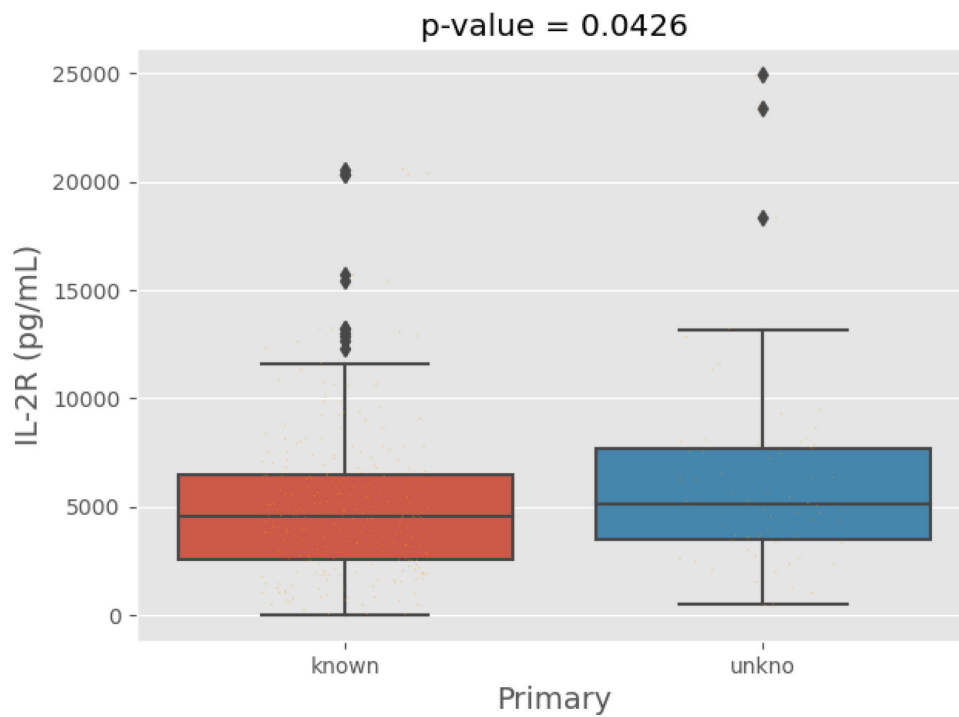
Mann-Whitney U test

Figure 5 Patients with unknown primary melanoma had higher serum levels of IL-2R compared with those with known primary (p=0.04).

immune tumor microenvironment, antigen presentation and cytotoxic and helper T cell activity.[18] Furthermore, immune-related gene expression profiles were found to be predictive of response following PD-1 blockade therapy in patients with advanced NSCLC, head and neck squamous cell carcinoma, and melanoma.[23] This is in addition to a T cell-inflamed gene expression profile tested in melanoma and nine other malignancies that is currently being evaluated in clinical trials testing pembrolizumab.[24]

Antigen presentation by MHC proteins is essential for adaptive immunity. We were interested in exploring MHC-I and MHC-II gene expression in unknown primary tumors compared with known primaries and used recently reported MHC-I and MHC-II scores in our analysis.[15] MUP tumors had significantly higher MHC-I and MHC-II scores further supporting enhanced immunogenicity in MUP and a higher likelihood of response to immune checkpoint blockade.[25] Overall, our findings and related literature support the hypothesis that interactive immune-related pathways related to tumor biology and host immunology may explain the improved prognosis in patients with MUP.

Although we tested a broad panel of candidate circulating and soluble biomarkers, only IL-2R was found to be significantly higher in MUP patients. This could be explained by the smaller sample size included in this analysis (N=321; 66 unknown and 255 known primary). These findings support the hypothesis that MUP patients are more likely to have immunogenic tumor microenvironments, and evidence of peripheral proinflammatory immune responses that drive immune resistance to melanoma and make MUP more susceptible to adjuvant immune checkpoint blockade interventions. Unknown primary is typically attributed to the phenomenon of complete regression at the primary melanoma site.[4] Evidence of immunity to melanoma is known to be essential for disease control and improved prognosis in the adjuvant, neoadjuvant and inoperable disease settings. Melanoma spontaneous regression has been reported, suggesting a role for host immunity, that is also indirectly supported histologically by findings of tumor infiltrating lymphocytes in primary melanoma associated with tumor regression.[26] Furthermore, lymphoid immune infiltrates within the tumor have been shown to be prognostic in primary melanoma,[27] and melanoma metastatic to regional lymph nodes.[28–30] T cell infiltrates within regional nodal metastasis were associated with response following neoadjuvant interferon-α and ipilimumab.[17 28 31 32] The presence of these immune features in melanoma have been reported to be associated with benefit from systemic immunotherapy including cytokine therapy and immune checkpoint inhibitors.[33] Therefore, a completely regressed primary in MUP may represent a consequence of prior host immune recognition and development of melanoma immune resistance that benefits from immunotherapeutic interventions. We propose that future adjuvant trials consider stratifying for MUP and we support the AJCC efforts in further investigating the prognostic value of MUP and its contributions to the melanoma staging system.

Conclusions

Unknown primary high-risk melanoma patients had a significantly better prognosis and showed evidence of significantly enhanced immune activation within the TME and the circulation, supporting the designation of unknown primary melanoma as a distinct favorable prognostic marker among patients with high-risk melanoma.

Author affiliations
[1]Cutaneous Oncology, Immunology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, USA
[2]Biostatistics, Harvard Medical School, Boston, Massachusetts, USA
[3]Dana Farber Cancer Institute, Boston, Massachusetts, USA
[4]Biostatistics and Bioinformatics, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, USA
[5]Machine Learning, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, USA
[6]The Parker Institute for Cancer Immunotherapy, San Francisco, California, USA
[7]Microbiology, Immunology, University of California San Francisco, San Francisco, California, USA
[8]Pathology and Laboratory Medicine, Allegheny Cancer Institute, Allegheny Health Network, Pittsburgh, Pennsylvania, USA
[9]Immunology, Dermatology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA
[10]UPMC Hillman Cancer Center, Pittsburgh, Pennsylvania, USA
[11]Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
[12]Immunology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, USA
[13]Administration, Cutaneous Oncology, Immunology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, USA
[14]Cancer Therapy Evaluation Program, National Cancer Institute, Rockville, Maryland, USA
[15]Cutaneous Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, USA
[16]Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

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ORCID iDs
Ahmad A Tarhini http://orcid.org/0000-0002-3193-9702
Lisa H Butterfield http://orcid.org/0000-0002-3439-9844
Walter J Storkus http://orcid.org/0000-0001-8961-4444

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