Outcome-Related Signatures Identified by Whole Transcriptome Sequencing of Resectable Stage III/IV Melanoma Evaluated after Starting Hu14.18-IL2

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ABSTRACT

Purpose: We analyzed whole transcriptome sequencing in tumors from 23 patients with stage III or IV melanoma from a pilot trial of the anti-GD2 immunocytokine, hu14.18-IL2, to identify predictive immune and/or tumor biomarkers in patients with melanoma at high risk for recurrence.

Experimental Design: Patients were randomly assigned to receive the first of three monthly courses of hu14.18-IL2 immunotherapy either before (Group A) or after (Group B) complete surgical resection of all known diseases. Tumors were evaluated by histology and whole transcriptome sequencing.

Results: Tumor-infiltrating lymphocyte (TIL) levels directly associated with relapse-free survival (RFS) and overall survival (OS) in resected tumors from Group A, where early responses to the immunotherapy agent could be assessed. TIL levels directly associated with a previously reported immune signature, which is associated with RFS and OS, particularly in Group A tumors. In Group A tumors, there were decreased cell-cycling gene RNA transcripts, but increased RNA transcripts for repair and growth genes. We found that outcome (RFS and OS) was directly associated with several immune signatures and immune-related RNA transcripts and inversely associated with several tumor growth–associated transcripts, particularly in Group A tumors. Most of these associations were not seen in Group B tumors.

Conclusions: We interpret these data to signify that both immunologic and tumoral cell processes, as measured by RNA-sequencing analyses detected shortly after initiation of hu14.18-IL2 therapy, are associated with long-term survival and could potentially be used as prognostic biomarkers in tumor resection specimens obtained after initiating neoadjuvant immunotherapy.

Introduction

Melanoma is considered an immunogenic and relatively immunoresponsive tumor with a relatively high tumor mutation burden (1, 2), often harboring many tumor-infiltrating lymphocytes (TIL), which have prognostic value (3, 4). Improved biomarkers of outcome following melanoma immunotherapy are needed. Immune checkpoint inhibition is an effective therapy for some patients with melanoma (5–8). The mechanisms of melanoma response to checkpoint inhibition immunotherapy are complex and multifactorial (9). Previous transcriptomic analyses have found adaptive immune signatures in melanoma tumor biopsies soon after checkpoint blockade, which are predictive of response (10). Recently, whole transcriptomic interrogation of MYCN nonamplified pediatric neuroblastoma has shown immune signatures, which are associated with outcomes in children (11). In contrast, melanoma-derived driver mutations (12–14) as well as antiimmunity signaling pathways (15, 16) also contribute to decreased long-term survival (17). The relative contribution of tumor versus immunologic parameters to cancer survival is unclear; in colorectal carcinoma each side contributes approximately 50% (18).

The hu14.18-IL2 immunocytokine (IC) is a humanized mAb that binds to GD2 and is linked, as a fusion protein, to IL-2 at its Fc region (19, 20). GD2 is a cell membrane disialoganglioside found in neuroectodermal tumors (melanoma, neuroblastoma, and sarcomas), but demonstrates only low expression in select normal tissues (cerebellum, peripheral nerves; refs. 21, 22). Hu14.18-IL2 has been studied in vitro and in mouse models against melanoma and neuroblastoma (23–25). In mice, the antitumor effects of hu14.18-
IL2 involve cytotoxic T cells and NK cells (26–28). In addition, mice with smaller tumors or with minimal residual disease (MRD) at treatment initiation elicit the most robust responses to single-agent IC therapy (29, 30). Hu14.18-IL2 has undergone phase I and II testing in adults with metastatic melanoma and children with neuroblastoma (31–35), and shows reproducible antitumor activity, particularly in the MRD setting.

Here we present tumor analyses from a clinical trial (CO05601), wherein 21 of 23 patients with advanced melanoma received surgical resection of all evident disease to attain a complete response (CR) together with systemic IC administration (36). Patients were randomized to have their first of three monthly courses of hu14.18-IL2 started either just before (Group A, n = 13) or just after (Group B, n = 8) their complete surgical resection. Our primary goal for this report is to identify the differences in gene expression in these tumors prior to and after treatment with hu14.18-IL2; our secondary goal is to determine whether gene expression patterns before treatment, or induced by the treatment, are associated with outcome. Published data from this trial reported prolonged tumor-free survival in some patients at high risk for recurrence (36). In this report, we analyze whole transcriptome sequencing from these resected recurrent/refractory melanoma tumors and compare results for tumors obtained before versus after one course of hu14.18-IL2. This is the first transcriptomic analysis of tumors from human patients receiving hu14.18-IL2. The literature is becoming rich in transcriptomic studies in the context of checkpoint inhibition, but it is relatively poor for experimental treatment outside this category of drugs. The data presented extend prior work evaluating the predictive ability of immune signatures for long-term outcome associated with other forms of immunotherapy (particularly checkpoint blockade), and indicates the greater predictive power of transcriptomic analyses of tumor tissue obtained just after starting this form of immunotherapy over that obtained prior to initiating the therapy.

Translational Relevance

This study further advances our understanding of the clinical and immunologic activity of a novel immunocytokine and discusses plans for subsequent clinical testing. Our data suggest that both immune and tumor cell processes, as measured by RNA-sequencing (RNA-seq) analyses, are associated with OS and RFS when evaluated in tumors resected approximately 2 weeks after starting the first of three scheduled monthly courses of hu14.18-IL2 immunotherapy. We identify specific immune- and tumor response–related molecular pathways associated with improved outcome after starting immunotherapy. We provide new insights into immunologic processes involved in effective melanoma immunotherapy as well as prognostic information derived from the RNA-seq data that can facilitate patient management. These findings may be utilized for functionally similar forms of immunotherapy for other malignancies.

Materials and Methods

Clinical trial design

Twenty-three patients with advanced melanoma participated in this trial (UWCCC Protocol CO05601). The UW Human Subjects Committee and the FDA approved the study (ClinicalTrials.gov: NCT00590824; IND-12220). This study was conducted in accordance with the Declaration of Helsinki. Informed written consent was obtained from each subject. These human investigations were performed after approval by the UW institutional review board and in accordance with an assurance filed with and approved by the U.S. Department of Health and Human Services. Details of study design, conduct, and clinical results have been reported previously (36). All patients had recurrent stage III (recurrent regional metastasis), or stage IV (any distant metastasis) melanoma with biopsy-proven (current or previous) stage III or stage IV disease (36). Eligibility criteria required that at study entry, all patients have recurrent melanoma involving $\leq 3$ sites, judged to be totally resectable, where resection would be clinically recommended. Patients signed informed consent forms and were randomized into Group A or B prior to treatment. In addition to hu14.18-IL2 treatment, 3 of these 23 patients also received cilengitide, an antiangiogenic RGD-pentapeptide, as part of the original study (36). After enrolling those 3 patients, the study was amended, removing cilengitide treatment because of toxicity. The three cilengitide-treated patients were excluded from the previously reported clinical report (36). Two of 10 patients randomized to Group B were not treated with hu14.18-IL2 as they did not achieve the required CR status following surgery. One of these 2 patients left the protocol due to extensive residual disease after surgery; the second was taken off protocol because melanoma was detected by PET/CT prior to the first hu14.18-IL2 course. The remaining 8 patients were designated as Group B patients. All 13 Group A patients received one cycle of hu14.18-IL2 prior to surgery and remained on study after resection.

Our previous report focused on clinical tolerance and treatment results for patients receiving only hu14.18-IL2 (36); this article focuses on the biological analyses of the tumors and differences in the tumors for patients in Group A versus Group B. To augment statistical power in this relatively small study, we have included tumors from the 3 cilengitide-treated patients. Therefore, this study includes histologic and transcriptomic analyses for all tumors that were resected from all 23 patients. Associations with relapse-free survival (RFS) and overall survival (OS) were performed for patients that received any amount (1 to 3 cycles) of hu14.18-IL2 and included the 3 patients (2 patients from Group A and 1 patient from Group B) also given cilengitide.

Tumor processing and histologic analyses

Each patient’s surgical resection specimen (obtained following IC course-1 for Group A and prior to IC course-1 for Group B) was processed by the UWHC Surgical Pathology Unit (Madison, WI). Two representative 1 mm punch cores were taken from intratumoral regions within one representative formalin-fixed paraffin-embedded (FFPE) block of each patient’s resected melanoma. FFPE cores were coded and shipped to NCI (Bethesda, MD) on dry ice. For histologic assessment, representative sections of each patient’s tumor were coded and analyzed by our board-certified anatomic pathologist (E.A. Ranheim) for diagnosis confirmation and TIL assessment on hematoxylin and eosin slides that were masked for treatment. Quantitation of TILs (%) was defined as (cross-sectional area of TILs/area of tumor) × 100 (see Supplementary Methods).

RNA extraction and transcriptome sequencing

RNA was extracted from FFPE tumor cores using RNaseasy FFPE kits according to the manufacturer’s protocol (Qiagen). RNA-sequencing (RNA-seq) libraries were generated using >50 ng of RNA and TruSeq RNA Access Library Prep Kits according to the manufacturer’s protocol (TruSeq RNA Exome kits; Illumina) and sequenced on NextSeq500 sequencers using 80bp paired-end sequencing method (Illumina). On average, >50 million aligned sequencing reads were
generated from each RNA-seq library (>100 x coverage of the coding regions in human genome). Quality control data are shown in Supplementary Table ST1. Gene expression level was performed as described previously (11).

Gene signature analysis
We determined the immune and stromal scores for each sample by using the single-sample gene set enrichment analysis (ssGSEA) and the ESTIMATE gene sets (37; Supplementary Tables ST2–ST3) as described previously (11). We also utilized 22 CIBERSORT immune cell–specific gene sets (38) and a cytolytic score (average expression of GZMA, GZMB, GZMH, GZMK, GZMM, and PRF1) to assess the immune infiltrates in each sample. These signatures underwent validation in 3,809 The Cancer Genome Atlas transcriptional profiles (37), 3,061 human transcriptomes (38), and a cohort of 150 patients with neuroblastoma (11), respectively.

Regarding tumor-associated biomarkers, a more exploratory analysis interrogating the data on an individual gene level was undertaken based on the catalog of “cancer-related” genes under the list of protein classes on the Human Protein Atlas website (https://www.proteinatlas.org/). A total of 1,713 cancer-related genes were screened against OS and RFS with the top pathologically relevant (recognized as a neoplastic molecular biomarker aberration in molecular pathologic diagnosis) hits listed in Fig. 4 and Supplementary Fig. SF15. Molecular pathologic relevance was cross-referenced against the known genomic and epigenomic landscape of cutaneous melanoma (12, 14).

Statistical analysis
RFS and OS were defined in the prior report (36), as detailed in Supplementary Methods. Gene- and gene signature–level survival analyses were performed using the univariable Cox proportional hazard regression with the likelihood ratio test (39). This study assumes the proportional hazards requirement holds. The Cox regression was implemented in R version 3.2.3 (https://www.R-project.org/). Estimated HRs and P values from the Cox regression model were used for screening in gene- and gene signature–level survival analyses. Survival data were also analyzed using the Kaplan–Meier methodology with the log-rank test. Descriptive tumor statistics, TIL data analysis, Kaplan–Meier analysis, correlation analysis, and significance tests were performed, and HR plots generated using Prism 8, version 8.01, software (GraphPad). Student t tests, Fisher exact tests, Spearman rank and Pearson product-moment correlation coefficients, and corresponding P values were calculated in Microsoft Excel and results confirmed with GraphPad Prism software. Due to the small sample sizes and exploratory nature of the statistical inference for the TIL data focused primarily on comparing Groups A and B across the same set of genes/signatures, we report the nominal P values, rather than P values corrected for multiple comparisons. To mitigate the issue of multiple comparisons, we use a P value of <0.01 to indicate significance in this study. Given small sample size for Group B (n = 8), power calculations are provided in Supplementary Methods.

Naive tumors refer to an aggregate of resected tumors from 8 Group B patients as well as from 2 never-treated patients (these two were randomized to Group B, but nonevaluable for outcome as they did not achieve CR following resection).

Network- and pathway-level analysis of genes whose expression is correlated with survival
The ConsensusPathDB web-server (http://cpdb.molgen.mpg.de; ref. 40) was used to perform two types of analyses: (i) network-level analysis and (ii) pathway-level enrichment analysis (41, 42) on genes whose expression levels are correlated with OS time. These are detailed in Supplementary Methods and data are presented in Supplementary Fig. S16.

Data and materials availability
The RNA sequencing data are uploaded on the NCBI’s GEO website with Accession Number GSE133713 and with a release date set to be July 1, 2020. (NCBI tracking system no.20118519) The raw sequencing data have been deposited in the dbGaP under the accession phs001947. The processed RNA-seq data can be visualized and explored at https://clinomics.ccr.cancer.gov/clinomics/public/login using the database named “Melanoma_NCT00590824_CCR.”

Results

Description of patient treatment timelines tumor characteristics
The study treatment schematic is found in Supplementary Fig. SI. Specific patient treatment timelines and tumor characteristics are found in Supplementary Table ST4. There was no significant difference in age, sex distribution, largest tumor diameter, or tumor burden between Groups A and B (Supplementary Table ST5). Group A patients had tumor resection 12.5 days (mean, range 9–17 days) after starting hu14.18-IL2 and Group B patients had their resection 28.6 days (mean, range 18–43 days) before starting hu14.18-IL2 (Supplementary Table ST6). The median diameter of largest tumor focus for Groups A and B were 0.7 cm and 1.1 cm, respectively (Supplementary Table ST7). The median estimated tumor burden for Groups A and B were 0.25 cm³ and 0.46 cm³, respectively (Supplementary Table ST8). None of these parameters were significantly different between Groups A and B.

TIL levels and stromal signature are prognostic of RFS and OS in Group A, but not Group B patients
We found a significant prognostic effect of TILs on survival endpoints (RFS, P = 0.0088; OS, P = 0.0307) when separated by median values, irrespective of treatment group, with low TILs associated with worse outcomes (Fig. 1A). When stratified by treatment groups, however, these effects were only seen in Group A patients (RFS, P = 0.0030; OS P = 0.0417), but not in Group B patients (Fig. 1B and C). In addition, the stromal signature (SS; refs. 37, 11) demonstrated significant prognostic value for survival endpoints (RFS, P = 0.0072; OS, P = 0.0036) when separated by the median, irrespective of treatment group, with low SS demonstrating worse outcomes (Fig. 1D). When stratified by treatment group, however, these effects were seen in Group A patients (RFS, P = 0.0082; OS, P = 0.0145), but not in Group B patients (Fig. 1E and F). In addition, these relationships are somewhat similar for the cytolytic and immune signature (Supplementary Fig. S2).

We applied Cox regression analyses to assess whether the gene expression–based signatures provide adjunct prognostic value when combined with TILs. In this analysis, the immune and SSs were handled as continuous variables, while TILs were handled as a categorical variable with “low”/“high” categories (as in Fig. 1). In the case of RFS time, the univariable Cox regression shows that in Group A both TILs and the SS are significantly associated with survival (TILs: HR = 0.073 and P = 0.018, SS: HR = 0.097 and P = 0.006). The immune signature in this univariable model shows a trend toward statistical significance (HR = 0.38 and P = 0.051). The bivariate Cox regression that combines TILs and the SS shows that the overall model is significant (P = 0.01), with both covariates also being significant.
Figure 1.
Morphologic TILs assessment and the stromal gene signature prognosticate RFS and OS after hu14.18-IL2 IC treatment, but not before IC treatment. A–C, Kaplan-Meier curves of patients stratified by TIL assessment are shown. A, Stratification by median TIL assessment is prognostic for RFS and OS within all patients. Patients with TIL levels below the median demonstrate worse outcomes. B and C, This prognostic effect on RFS and OS, respectively, is retained in treatment Group A, but not in treatment Group B. D–F, Kaplan-Meier curves of patients stratified by the stromal signature (37). D demonstrates that the stromal signature is also prognostic of RFS and OS within all patients. Patients with stromal signature below the median demonstrate worse outcomes. Similarly, E and F show this prognostic effect on RFS and OS, respectively, retained in treatment Group A, but not in treatment Group B. H&E, hematoxylin and eosin.
The bivariate Cox regression that combines TILs and the immune signature shows that the overall model is significant ($P = 0.02$), with TILs being significant ($HR = 0.095$ and $P = 0.031$) and the immune signature not significant ($HR = 0.405$ and $P = 0.117$). Thus, the results of this analysis indicate that, in the case of RFS time in Group A, both TILs and the SS are significantly associated with survival, even after controlling for the other variable (i.e., the SS provides an adjunct prognostic value). The results of a similar analysis performed for OS time in Group A indicate that only the SS is significantly associated with survival after controlling for the other variable ($HR = 0.027$ and $P = 0.04$). In Group B, for both OS and RFS time, no significant associations are observed in either univariate or bivariate Cox regression models.

**Positive correlations between myeloid and lymphoid cells gene signatures in Group A (pretreatment) tumors**

Spearman correlation matrices of the 25 whole transcriptomic-based immunologic gene signatures were created on treatment-naïve tumors ($n = 10$; Supplementary Fig. S3A) as well as on Group A tumors previously treated with hu14.18-IL2 ($n = 13$; Supplementary Fig. S3B). Significance levels in the form of log10 (1/P) estimates are shown in Supplementary Fig. S3A and S3B. While naïve tumors demonstrated high levels of correlation between lymphocytic signatures (B, T, and NK cells) in Supplementary Fig. S3A, these were less pronounced correlations than seen with treated tumors between lymphocytic and myeloid signatures (monocytes, macrophages, dendritic cells, eosinophils, and neutrophils) in Supplementary Fig. S3B. Subtraction of these Spearman correlation coefficients between hu14.18-IL2 treated and naïve tumors demonstrates a marked increase in correlation of myeloid signatures with lymphoid signatures (Supplementary Fig. S3C) in tumors treated with hu14.18-IL2; this implies that the hu14.18-IL2 treatment caused parallel changes in lymphoid and myeloid compartments. A comparison using alternative methods of determining correlation (Spearman and Pearson) confirmed these findings (Supplementary Fig. S4C–S4D).

**Immunologic gene signatures prognosticate favorable patient survival in hu14.18-IL2–treated (Group A) tumors, but not in untreated (Group B) tumors**

The 25 immunologic gene signatures consistently demonstrated favorable HRs for RFS and OS ($Fig. 2A$ and $B$). For Group A patients, 10 (40%) of these signatures showed statistically significant favorable HRs of RFS (and 10 others showed a trend, $0.01 < P \leq 0.1$); in addition, 6 (24%) were statistically significant favorable HRs of OS (and 8 others showed a trend, $0.01 < P \leq 0.1$). In contrast, none of these relationships between gene signatures and survival demonstrated statistical significance (or showed a trend, $0.01 < P \leq 0.1$) within Group B patients (RFS in $Fig. 2A$ and OS in $Fig. 2B$). The sample size of Group B is smaller than Group A ($n = 8$ vs. $n = 13$, respectively), but the difference in $P$ values between Groups A and B is large, and thus is unlikely to only be attributed to the smaller sample size. Another observation emphasizing the key difference between Groups A and B independently of sample size is that in Group A the HRs for all 25 immunologic signatures in both the OS and RFS analyses are favorable ($<1$, $Fig. 2$), whereas in Group B in the OS analysis, the HRs for 20 of 25 signatures are unfavorable ($>1$) and in the RFS analysis the HRs for 9 of 25 signatures are unfavorable for OS. Thus, the immunologic signatures do not show significant associations with improved outcome in Group B because the HRs for Group B are larger than those for Group A patients.
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Group A, not because of a smaller sample size. A more granular representation of the data as a heatmap with patient-level tumor gene signatures is found in Supplementary Fig. S5.

Immunologic gene expression levels demonstrate favorable survival and increased significance in Hu14.18-IL2–treated (Group A) tumors, but not in untreated (Group B) tumors

The transcriptomic data were interrogated on a more granular level by analyzing individual expression levels of 644 immune-related genes (Supplementary Table ST9) within Groups A and B. These relationships are represented in volcano plots between Groups A and B for associations with RFS and OS (Fig. 3). We found several immune-related genes that demonstrated significantly favorable prognostic values for both OS and RFS in Group A tumors (Fig. 3A and C). In Fig. 3A, there were 59 genes associated with RFS versus 4 genes associated with decreased RFS in Group A patients (Supplementary Tables ST10–ST11). In Fig. 3C, there were 67 genes associated with OS versus 6 genes associated with decreased OS in Group A patients (Supplementary Tables ST12–ST13). Interestingly, expression levels of CCR3 (highlighted in red), a chemokine receptor on eosinophils and Th cells, stood out as highly significant and associated with a very skewed HR, prognostic of favorable RFS and OS in Group A (Fig. 3A and 3C). While not as significant or potentially influencing HR, expression of VNN1 (a membrane protein influencing hematopoietic and T-cell trafficking, highlighted in red) is also significantly associated with a favorable HR for both RFS and OS in Group A (Fig. 3A and C). In contrast, we found very few immunologic genes that demonstrated statistically significant favorable prognostic values for OS or RFS in Group B tumors (Fig. 3B and D). Associations between expression levels of individual immunologic genes and RFS and OS are presented (Supplementary Fig. S6). Again, these relationships were most robustly seen in Group A, but not in Group B patients.

We also examined the association of HR with the gene expression of class-based immune molecules and RFS and OS times. Heatmaps of HR and P values for RFS and OS times for TLRs (Toll-like receptors), KIRs (Killer-like Ig receptors), FcRs (antibody fragment constant region receptors), TNFSFs (tumor necrosis factor superfamily proteins and receptors), ILs (interleukins), and immune-related CD (clusters of differentiation) markers are shown in Supplementary Figs. S7–S12, respectively. The above gene sets were taken from existing literature, showing these classes of genes having significant contribution toward antitumor immunity (43). Consistent with our observations described above, the gene expression of many of these were more significantly associated with improved HR (green) in Group A tumors, but not in Group B tumors.

Melanoma-related gene expression increases HR and apoptosis-related gene expression decreases HR of RFS and OS in Group A, but not Group B patients

Next, we interrogated transcriptomic data of 1,713 cancer-related genes (found at https://www.proteinatlas.org/search/protein_class:Cancer-related+genes), which may have an influence on survival times. We found a number of clinically relevant melanoma-related genes that demonstrated significantly increased (unfavorable) HRs for patients whose tumors express these genes (Fig. 4, top) in Group A patients but not in Group B. These include MLANA (Melan A), SOX10, S100A, S100B, MITF, and PMEL (HMB-45). These markers are used regularly as melanoma markers for clinical pathologic identification and assessment by IHC. In contrast, apoptosis-related genes (including: BCL10, CASP8 (Caspase 8), CASP10 (Caspase 10), FAS, TNFSF10 (TRAIL), and others shown) revealed significantly decreased (favorable) HR in Group A patients, but not Group B (Fig. 4, bottom). A more granular, individual patient level breakdown of these melanoma-related and apoptosis-related genes may be found in Supplementary Fig. S13.
Hu14.18-IL2-treated tumors demonstrate increased levels of growth factors and repair, overall gene expression, but decreased levels of melanoma-related genes

Next, we examined the global gene expression alterations resulting from one course of Hu14.18-IL2 treatment by comparing genes significantly different in Group A versus Group B and untreated (Naïve) tumors; such individual genes may shed light on hu14.18-IL2 effects on the tumor microenvironment (Fig. 5). There are 52 genes (at $P < 0.01$) and 610 genes (at $P < 0.05$) that show significant fold difference between treated and hu14.18-IL2 naïve tumors (Supplementary Table ST14). In general, the data demonstrate a marked preponderance of genes that are significantly increased in expression in 13 Group A versus 10 naïve (8 Group B and 2 never-treated) tumors, indicating an overall increase in gene expression of many genes after treatment with hu14.18-IL2. A more granular assessment indicates that Hu14.18-IL2-treated tumors show increased levels of repair (growth factor and DNA damage repair) genes and immunologic genes, but decreased levels of melanoma, Warburg metabolism, and cell cycling–related genes (Supplementary Fig. S14).

Pathway-level analysis reveals overrepresentation of genes involved in immunologic pathways in Group A, but not in Group B

A more integrative pathway-level enrichment analysis performed by using the ConsensusPathDB web-server indicates that the elevated expression of genes involved in innate immune response pathways is linked to favorable survival in the hu14.18-IL2–treated tumors (Group A), but not in the yet untreated tumors (Group B, Fig. 6, green left-sided panels). A more extensive catalogue of 53 pathways associated
IC treatment reveals a predominance of increased rather than deceased gene expression. A, Gene expression for individual genes are plotted with log2 fold changes from naive (B in Group B and 2 untreated (No IC)) patients to 13 Group A hu14.18-IL2–treated patients (y-axis) against significance levels (x-axis). There is a marked preponderance of genes that are significantly increased (top right quadrant), indicating an overall increase in gene expression of transcripts after treatment with hu14.18-IL2. B, Gene expression plot for individual cancer-related genes (annotated from the Human Protein Atlas) with log2 fold changes from 10 naive patients to 13 Group A patients demonstrates a preponderance of with favorable OS (HR < 1) also demonstrated innate immunologic processes (Supplementary Fig. S15). Conversely, elevated expression of genes involved in glycolysis pathways was linked to unfavorable survival in the hu14.18-IL2–treated tumors (Group A), but not in the treatment naïve tumors (Group B) (Fig. 6, pink right-sided panels). In addition, the elevated expression of genes involved in deubiquitination and processing of ubiquitinated proteins appears to be linked to unfavorable survival in Group B (Fig. 6, pink right-sided panels), but not in Group A.

**Network-level analysis of genes correlated with the OS time in Group A indicates that ELAVL1 is a hub protein that interacts with the products of many of these genes**

Intriguingly, the network-level analysis of genes whose expression is correlated with the OS time in Group A revealed a complex network of physical interactions (Supplementary Fig. S16A) in which ELAVL1 is a hub protein that interacts with the products of 25 of these genes. The majority of these genes (20 of 25) were genes whose expression was positively correlated with the OS time in Group A (at this study). In contrast to Group A, the network-level analysis of genes whose expression was correlated with the OS time in Group B produced a very limited network of physical interactions that did not include ELAVL1 and did not contain any hub proteins (Supplementary Fig. S16B). This implies that hu14.18-IL2 treatment may result in a coordinated immune response centered with ELAVL1 which may impact on patient’s outcome.

**Discussion**

We analyzed histology and tumor mRNA whole transcriptomes from stage III and IV patients with melanoma to screen for predictive biomarkers for patients participating in this hu14.18-IL2 immunotherapy trial. Patients were scheduled to receive three courses of hu14.18-IL2 and were randomized to have course 1 of the hu14.18-IL2 begin just before (Group A) or just after (Group B) surgical resection of all disease. No significant differences in RFS or OS between Groups A and B were seen in the OS (36). However, when evaluating tumor immune microenvironment using immune scores generated from ssGSEA (11), we found that TIL levels, as measured by histologic assessment, strongly and directly correlate with immune scores for all patients. Patients’ TIL levels in their resected tumors directly correlated with RFS and OS in the tumors from Group A patients who had been significantly increased (top right quadrant), but not decreased, expression of genes in Group A versus naïve tumors. C, Gene expression plot for individual ssGSEA CIBERSORT immune genes with log2 fold changes from 10 naive patients to 13 Group A patients demonstrates a preponderance of significantly increased, but not decreased, expression of genes in Group A versus naïve tumors. There are 52 genes (at P < 0.01) and 610 genes (at P < 0.05) that show significant fold difference between treated and hu14.18-IL2 naïve tumors. The vast majority of these were upregulated rather than downregulated. Fold change is log2 transformed such that a point estimate of 1.0 denotes a fold change of 2.0 and a point estimate of −1.0 denotes a fold change of 0.5. In A, B, and C, the vertical dotted lines represent P values of 0.05 and 0.01. The horizontal dotted line represents a ratio of 1.0 for expression of the individual gene in the tumors from 13 Group A patients versus in the 10 naïve tumors. Immunologic genes were taken from all 645 immunologically related genes that are included in our ssGSEA analysis. For A–C, to the right of the dot plots for gene expression ratio versus P value are box plots and density histograms of those points that are significant (P < 0.01), together with those that have 0.01 < P < 0.05.
Figure 6.
Pathway-level enrichment analysis of gene sets whose expression correlates with survival. The pathway-level enrichment analysis indicates that genes involved in innate immune response are significantly overrepresented in patients with improved OS (HR < 1) in the IC-treated tumors (n = 13, Group A, green top left panels), but not in the untreated tumors (n = 8, Group B). A complete list of 53 pathways significantly associated with favorable OS in Group A patients may be found in Supplementary Fig. S15. In contrast, only a few pathways including ectodermal differentiation and activation of SMO were found to associate with Group A patients with decreased OS (HR > 1). Pathways associated with improved RFS (HR < 1) in Group A patients include chemokine and cytokine signaling (green top right panel). In Group B patients, pathways associated with focal adhesion associated with favorable OS in Group B (green bottom left panel). In contrast, genes involved in deubiquitination and processing of ubiquitinated proteins are overrepresented in patients with unfavorable OS (HR > 1) in Group B (pink bottom right panel). Pathways are ranked by their log (1/q-value) values and colored blue when the q-value < 0.05, with darkening blue representing increasing statistical significance. The q-value is the FDR-adjusted P-value and provides a correction for multiple tests (i.e., takes into account the total number of pathways used in the analysis). The q-value is commonly used in genome-wide studies.

treated with hu14.18-IL2 prior to resection but no such correlation was seen in Group B patients who had not yet received hu14.18-IL2 prior to resection (Group B). In addition, SS is associated with improved HR of RFS and OS, in tumors that were resected after the first course of hu14.18-IL2 treatment (Group A), but not observed in tumors that were resected before initiating the hu14.18-IL2 (Group B).

We found a number of other immune signatures associated with decreased (favorable) HR of RFS and OS for Group A patients, but these findings were not found in Group B tumors. Specifically, several individual immune gene transcripts were associated with decreased HR of RFS and OS for Group A patients (but not for Group B patients). Among these transcripts associated with improved outcome for Group A patients were those related to cytotoxic T cells (Granzymes), NK cells (KIRs), and innate immune cells (TLRs). In addition, there appeared to be a number of tumor-related genes where increased gene expression was significantly associated with increased HR of RFS/OS (melanoma marker transcripts) or decreased HR of survival (apoptosis transcripts) in Group A patients; no similar associations were seen for Group B. These findings suggest that the associations with outcome for these tumor transcripts were the result of changes in transcript expression induced by the hu14.18-IL2 therapy that were associated with RFS and OS. The inability of the immunotherapeutic intervention to induce these changes in a portion of the evaluable patients in Group A strongly predicted poorer clinical outcomes in these patients.

In addition, the data revealed that compared with naive (untreated) tumors, hu14.18-IL2–treated tumors (Group A) demonstrated decreased levels of cell-cycling transcripts (MKI67, CDK1), melanoma markers [MLANA (Melan A), MAGED1], and glycoysis transcripts [LDHA, HK1 (Supplementary Fig. S14)]. Conversely, Group A tumors demonstrated increased levels of growth factor [PDGFRA, FGFR1, PDGFB, TGFβ3 (Supplementary Figs. S14)] and repair transcripts (XPC) compared with naive tumors. We take relationships between tumor-related transcripts as evidence of differences in tumor biology (or differences in tumor biology due to immune effects) that may indicate a change from a tumor replicative phenotype to an inflamed/healing-wound phenotype following hu14.18-IL2 therapy.

When evaluating Group A tumors, we found that expression of a number of individual genes were strongly and significantly associated with outcome (Fig. 3; Supplementary Figs. S7–S12). Some of these may provide clues to mechanisms involved in improved outcome using hu14.18-IL2 like therapy and could potentially indicate additional molecules/pathways for targeting effective immunotherapy. We

<table>
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<tr>
<th>q-value Log (1 / q-value)</th>
<th>Pathway Source</th>
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<th>q-value Log (1 / q-value)</th>
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<td>0.00031 4.505</td>
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<td>0.015880 1.795</td>
<td>Activation of SMO</td>
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RNA-seq of Melanoma after Hu14.18-IL2 Predicts Outcome

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believe that the differences in transcript expression reflect changes in tumor-biology induced by the antitumor immune effects of hu14.18-IL2 treatment that was initiated only 12.5 days (mean) prior to the tumor resection for Group A. These changes in tumor biology are somewhat similar to that seen after a subacute (~2 weeks prior) injurious event (44).

Recently, an immuno-predictive score (IMPRES), a predictor of immune checkpoint blockade (ICB) response in melanoma which encompasses 15 pairwise transcriptomics parameters between immune checkpoint genes, has been described (45). The authors developed this gene expression predictor to indicate whether melanoma in a specific patient is likely to respond to treatment with ICB. We have done an analysis of IMPRES scores for these 13 Group A and 8 Group B patients; all patients have somewhat similar scores (ranging from 9 to 13). We see no statistical association of RFS or OS with IMPRES score within Group A or within Group B (data not shown). In contrast, we demonstrate the utility of the immune score as a predictor for response to hu14.18-IL2 when evaluated after treatment in this study of hu14.18-IL2. Checkpoint blockade is considered a mechanism to "release the brakes" on an immune response that is already underway. Thus, one would hypothesize that pretreatment scores indicating preexisting immune-activation (like the IMPRES) might be associated with clinical benefit from checkpoint blockade. In contrast, the induction of innate immune recognition by antibody-dependent cell-mediated cytotoxicity, together with IL2 induced activation-proliferation of immune cells, as shown for hu14.18-IL2 (46), might be considered an approach toward "starting the engine and providing some gas" to the immune response (47). From this perspective, one might expect that increased expression of immune genes seen following treatment initiation with this form of therapy, rather than gene expression seen before treatment, would be a better predictor of outcome, as observed in this report.

Finally, a network level analysis of genes associated with OS in this study revealed a complex interaction of many molecules associated with outcome for Group A (Supplementary Fig. S16A), with no similar complex network identified for Group B (Supplementary Fig. S16B). The network for Group A indicates a key position for ELAVL1, which is known to bind 3′-UTR regions of mRNAs with A-U rich elements and stabilize them, thus augmenting gene expression (48). ELAVL1 expression is known to be associated with better prognosis in stage II melanoma, increased C-reactive protein and inflammation, increased TNF and TLR4, as well as regulation of GATA-3 and Th2 cytokine genes (49). These functions may put ELAVL1 in a key position for further analyses and possible targeting.

In summary, these data suggest that both immune and tumor cell processes, as measured by RNA-seq analyses, are associated with OS and RFS when evaluated in tumors resected approximately 2 weeks after starting the first of three scheduled monthly courses of hu14.18-IL2 immunotherapy. These results may help point to specific immune-related and tumor response-related molecular pathways associated with improved outcome after immunotherapy. Furthermore, if this approach is validated, it may enable analyses of RNA-seq immune and tumor cell signatures obtained after initiating immune therapy to potentially predict which patients should continue their current regimen and which might need a change or addition to their therapy, due to the absence of a favorable histologic or RNA-seq response.

Disclosure of Potential Conflicts of Interest
Z.S. Morris is an employee/paid consultant for Archeus Technologies and Seneca Therapeutics, reports receiving speakers bureau honoraria from ViewRay, and holds ownership interest (including patents) in WARF, Archeus Technologies, and Seneca Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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Study supervision: E.A. Ranheim, J. Khan, P.M. Sondel
Other (hypothesis generation for RNA-seq): J.B. Kuznetsov

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References


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