Blood mRNA Expression Profiling Predicts Survival in Patients Treated with Tremelimumab

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Abstract

Purpose: Tremelimumab (ticilimumab, Pfizer), is a monoclonal antibody (mAb) targeting cytotoxic T lymphocyte–associated antigen-4 (CTLA-4). Ipilimumab (Yervoy, BMS), another anti-CTLA-4 antibody, is approved by the U.S. Federal Drug Administration (FDA). Biomarkers are needed to identify the subset of patients who will achieve tumor control with CTLA-4 blockade.

Experimental Design: Pretreatment peripheral blood samples from 218 patients with melanoma who were refractory to prior therapy and receiving tremelimumab in a multicenter phase II study were measured for 169 mRNA transcripts using reverse transcription polymerase chain reaction (RT-PCR). A two-class latent model yielded a risk score based on four genes that were highly predictive of survival ($P < 0.001$). This signature was validated in an independent population of 260 treatment-naïve patients with melanoma enrolled in a multicenter phase III study of tremelimumab.

Results: Median follow-up was 297 days for the training population and 386 days for the test population. Expression levels of the 169 genes were closely correlated across the two populations ($r = 0.9939$). A four-gene model, including cathepsin D ($CTSD$), phospholipase A2 group VII ($PLA2G7$), thioredoxin reductase 1 ($TXNRD1$), and interleukin 1 receptor–associated kinase 3 ($IRAK3$), predicted survival in the test population ($P = 0.001$ by log-rank test). This four-gene model added to the predictive value of clinical predictors ($P < 0.0001$).

Conclusions: Expression levels of $CTSD$, $PLA2G7$, $TXNRD1$, and $IRAK3$ in peripheral blood are predictive of survival in patients with melanoma treated with tremelimumab. Blood mRNA signatures should be further explored to define patient subsets likely to benefit from immunotherapy. Clin Cancer Res; 20(12); 3310–8. ©2014 AACR.
**Translational Relevance**

It is now established that the immune system can be induced to eliminate melanoma and that immunotherapy can have a dramatic impact on survival for some patients. Biomarkers are crucial to help identify the subset of patients who can benefit from immunotherapy and may simultaneously minimize the risk for autoimmune toxicity. We report that mRNA levels in the blood predict survival of patients treated with tremelimumab (ticilimumab, Pfizer), an anti-CTLA-4 antibody with activity in melanoma. Tremelimumab is not U.S. Food and Drug Administration (FDA) approved, but it is currently in clinical trials as part of combination regimens and has an immunologic mechanism of action similar to ipilimumab (Yervoy, BMS), an FDA-approved anti-CTLA-4 antibody in wide clinical use. Blood biomarkers are particularly useful in clinical practice because they are straightforward to obtain and measure. A better understanding of biomarkers that predict response to immune treatment may change the way we treat melanoma, in particular, and cancer, in general.

IgG1 antibody directed against CTLA-4 is FDA approved on the basis of survival improvement compared with a peptide vaccine in previously treated patients with melanoma. Ipilimumab was also subsequently shown to have therapeutic efficacy combined with dacarbazine superior to dacarbazine alone in a large phase first-line III trial (9). Proposed mechanisms of action, toxicity profiles, and kinetics of response of ipilimumab and tremelimumab are similar (10, 11). Ipilimumab has not been tested alone against either chemotherapy or tremelimumab, and thus the relative activity of ipilimumab and tremelimumab is unknown (12, 13).

Response to immunotherapy may be attributed to a complex interaction between the immune system and the tumor; thus, both tumor intrinsic factors and the patient's immune system may contribute to therapeutic efficacy (14). Recent studies have identified that tumors impact the gene expression profiles of circulating leukocytes and that alterations in peripheral blood phenotype have prognostic implications for patients with cancer (15–17). It is not yet established whether specific biomarkers might determine a patient's response to immune manipulation in general and/or to a particular immunotherapy (18). Immune therapies can induce serious autoimmune disease, including colitis, pneumonitis, hypophysitis, and other toxicities. Furthermore, some patients may benefit from one immunotherapy but be refractory to another, whereas other patients may benefit from chemotherapy but not from any available immune treatments. There is thus a great need for both predictive and prognostic immune biomarkers in cancer, in general, and melanoma, in particular.

In this study, we define a gene signature in the blood predicting response to tremelimumab. Peripheral blood is a readily accessible source of biomarkers and was selected because of the ease of application to the clinical setting. Pretreatment peripheral blood was obtained from the training population (7), and mRNA expression levels of 169 inflammatory and melanoma genes measured. Four genes, CTSD, IRAK3, PLAG2, and TXNRD1, were found to correlate closely with survival, and on the basis of the level of these genes, a 4-gene score was calculated for each patient. The same 4 genes were found to predict survival in independent test population in which the 169 genes were also tested. These data show that the expression of genes in the peripheral blood correlates with clinical outcome in patients treated with tremelimumab and may be relevant to future development of biomarkers for immunotherapy in advanced melanoma.

**Methods**

**Patients and blood collection**

Whole peripheral blood samples were prospectively collected in PAXgene Blood RNA tubes (PreAnalytiX) before treatment with tremelimumab from 229 of the 246 patients with treatment-refractory unresectable melanoma enrolled in a multicenter phase II clinical trial between November 2005 and December 2006. RNA of sufficient quality for analysis was harvested from 218 of the 229 patients (Supplementary Table S1). For the validation set, peripheral blood was obtained from 268 of the 325 patients with treatment-naive unresectable patients with melanoma receiving tremelimumab as part of the treatment arm of a multicenter phase III clinical trial from March 2006 to July 2007. Adequate RNA samples were harvested in 260 of the 268 patients. Clinical and demographic information was obtained and recorded by authorized personnel after obtaining written informed consent (Table 1). Protocols and consent forms were approved by the local institutional review board.

**Gene selection**

First, 27 genes were included on the basis of data from analysis of mRNA levels in the blood of patients with melanoma as compared with normal controls conducted by Source Molecular Diagnostics (MDx) in collaboration with Dr. David Norris and colleagues at the University of Colorado (15, 19). In addition, 68 cancer-related transcripts were identified by Dr. Robert W Ross and colleagues at the Dana Farber Cancer Institute (Boston, MA) based on pilot studies using whole blood from patients with cancer (16). Next, a literature review identified 60 immune-related genes and 14 cancer-related genes, yielding a final list of 169 genes for further analysis as shown in Supplementary Table S1. The method of gene selection is shown in Table 2.

**Experimental procedures**

Whole blood samples were collected in PAXgene RNA stabilization tubes (PreAnalytiX) and RNA extracted with PAXgene Blood RNA Kit (PreAnalytiX) according to the manufacturer's instructions. Quality and integrity of the RNA was verified on an Agilent 2100 Bioanalyzer (Agilent
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Table 1. Demographics of patients in training and test populations

<table>
<thead>
<tr>
<th>Stage of disease, n (%)</th>
<th>Training set</th>
<th>Test set</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIIB</td>
<td>2 (0.9)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>IIIC</td>
<td>6 (2.8)</td>
<td>16 (6.2)</td>
</tr>
<tr>
<td>IV M1A</td>
<td>23 (10.6)</td>
<td>39 (15)</td>
</tr>
<tr>
<td>IV M1B</td>
<td>46 (21.1)</td>
<td>59 (22.7)</td>
</tr>
<tr>
<td>IV M1C</td>
<td>141 (64.7)</td>
<td>146 (56.2)</td>
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<table>
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<th>ECOG performance status</th>
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<th>Test set</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>143 (66)</td>
<td>180 (69)</td>
</tr>
<tr>
<td>1</td>
<td>73 (34)</td>
<td>80 (31)</td>
</tr>
<tr>
<td>Not recorded</td>
<td>2 (0.9)</td>
<td>0 (0)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Lives in United States</th>
<th>Training set</th>
<th>Test set</th>
</tr>
</thead>
<tbody>
<tr>
<td>U.S.</td>
<td>96 (44)</td>
<td>54 (21)</td>
</tr>
<tr>
<td>Non-U.S.</td>
<td>122 (56)</td>
<td>206 (79)</td>
</tr>
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</table>

Technologies), and the quantity of RNA was determined by NanoDrop ND-1000 spectrophotometer (Thermo Scientific). First-strand complementary DNA was synthesized using TaqMan reverse transcription reagents (Applied Biosystems, Division of Life Technologies Corporation). Quantitative PCR analysis of the 18S rRNA content was performed as a quality check of the first-strand synthesis reaction (Applied Biosystems). Target gene amplifications were performed using TaqMan Universal PCR Master Mix (Applied Biosystem, Division of Life Technologies Corporation) and Precision Profiles (Source MDx) in a quantitative PCR reaction. Individual target-gene amplifications were run in triplicate on the 7900HT fast real-time PCR system with the endogenous 18S rRNA endogenous control. For quality control, all replicate cycle threshold (Ct) values (both target gene and endogenous control) were independently checked and automatically filtered by rule. Normalized Ct values (ΔCt) for each amplified target gene replicated were calculated. Resulting triplicate ΔCt values for individual target genes were averaged yielding a final ΔCt value.

Statistical analysis

We tested the hypothesis that whole-blood RNA transcript levels could be systematically quantified to yield important prognostic information. We examined all possible 1-, 2-, and 3-gene Cox proportional hazards regression models from the 169 candidate genes. All models were then ranked from most to least significant based on the entropy $R^2$, and the relationship between gene expression and survival was examined further with the genes from the best model using stepwise regression to identify additional genes that would further improve the significance and latent class models with the selected genes used as covariates. Final gene model(s) selected for validation were those for which the partial regression coefficient for each gene was significant ($P < 0.05$). In addition, stepwise Cox regression analyses were conducted to assess the predictive power of the gene model beyond that of standard clinicopathologic variables.

For the final model that consisted of 4 genes, a 4-gene model score was computed and cutoff values were designated to stratify patients into 3 risk categories. The 4-gene score together with the predetermined cutoff points based on the training set data were used to validate the model in an independent population of patients. The ability of the risk score to predict survival in this independent test population was assessed using the log-rank test obtained from a Cox proportional hazards model where the risk score was used as a single covariate. In addition, the 4-gene score was compared with standard clinicopathologic predictors using stepwise Cox regression analyses. Patients in the test population were stratified into 3 risk categories using the predetermined cutoff values based on the training set data. Survival among risk categories was validated using the log-rank test obtained from the Cox proportional hazards model where the risk score was the single categorical covariate representing the 3 groups.

Results

Patient populations

To define a biomarker for prolonged survival in patients receiving tremelimumab, blood was harvested from 2 independent patient populations receiving tremelimumab as part of large multicenter international studies. The training population was composed of patients enrolled in a phase II clinical trial studying the efficacy, safety, tolerability, and pharmacokinetics of tremelimumab in advanced refractory or relapsed melanoma (7). The patients in the test population were enrolled in a phase III randomized trial comparing tremelimumab with chemotherapy in patients with advanced melanoma (7). In both studies, patients were treated with tremelimumab 15 mg/kg (8).

The patients in the training population had refractory disease, whereas the patients in the test population were treatment-naive. Accordingly, the training population had a significantly shorter median survival of 8.8 months compared with 13 months in the test population. The training population was younger than the test population, having a median age of 53 years as compared with 59 years. For patients living at last follow-up, time to censoring was 585 days for the training set and 469 days for the test set. Analysis of the baseline characteristics of the patients in the training and test sets shows that gender composition, disease stage,
and performance status are similar across both groups ($P > 0.05$, see Table 1).**

**Definition of a 4-gene signature predictive of overall survival but not increased autoimmune toxicity based on the training population**

We sought to define a peripheral blood biomarker predictive of enhanced survival in patients treated with tremelimumab. Thus, a panel of 169 candidate genes was selected for measurement in the peripheral blood (Table 2 and Supplementary Table S1; refs. 15, 20, 21). Of a total of 229 blood samples collected in PAXgene RNA stabilization tubes (22), RNA was successfully extracted from 218 for a success rate of 95% (Supplementary Table S3), and expression profiling by whole blood RNA detection and quantification was performed in this population. As of March 24, 2008, a total of 73 (33%) patients in the training set were alive and 145 (67%) had died. The 169 genes were entered directly as predictors in a Cox-type proportional hazards model to examine the predictive relationship between gene expression and survival. All possible 1-, 2-, and 3-gene models were separately developed to encompass possible synergy between genes. The best model consisted of the 3 genes CTSD, PLA2G7, and TXNRD1. Using stepwise Cox modeling, a fourth gene, IRAK3, was identified. The resulting coefficients for the 4 genes were found to be very similar (approximately $-1, +1, -1,$ and $+1$) in both the Cox model with the 4 genes as predictors of the log hazards rate and in a 2-class latent class model with those 4 genes as predictors in a logit model for the latent classes.

On the basis of gene expression data from the training population, the coefficients of the 4-gene Cox model were used to generate the risk score:

$$-2CTSD + PLA2G7 + 2TXNRD1 - IRAK3$$

Cutoff points of 9.6, as the upper limit of low risk, and 11.255, as the upper limit of intermediate risk, were used to stratify the 218 patients into low-, intermediate-, and high-risk mortality groups, such that the low-risk group consisted of the best quartile ($n = 54$) and the high-risk group the worst quartile ($n = 55$). Low-risk patients had a median survival of 445 days, intermediate-risk patients 322.5 days, and high-risk patients 120.5 days. Kaplan-Meier analysis of the risk groups confirms a strong prediction for overall survival time in the training population (Fig. 1A, $P < 0.0001$).

Clinical studies have suggested that patients who benefit from CTLA-4 blockade may also be prone to autoimmune toxicity (23), raising the question whether the 4-gene score correlates with autoimmune toxicity. Diarrhea is one of the most prominent autoimmune toxicities of CTLA-4 blockade (6). As shown in Fig. 1B, a low-risk score did not confer a higher rate of diarrhea, suggesting that the survival benefit observed was not associated with higher risk of autoimmune toxicity.
Validation of 4-gene signature in a second independent population of patients with advanced melanoma

The test population consisted of patients enrolled on the tremelimumab arm of a phase III study comparing tremelimumab with standard-of-care chemotherapy (8). Blood was drawn from 268 patients, and adequate RNA was extracted in 260 for a success rate of 97%. Although demographics were similar (Table 1), the test population was treatment-naive, whereas the training population was treatment-refractory. We reasoned that prior treatment might affect genomic profiles in the blood, potentially making it difficult to compare them with the patients from the phase II study; however, analysis by plotting mean values of the 169 individual genes in the training and test groups against each other showed close correlation (Fig. 2A, \( P < 0.0001 \)). Differences in the mean gene expression (\( \Delta C_t \)) of each gene were calculated between those who survived less than or equal to 1 year and those who survived more than 1 year following enrollment. Confidence intervals were then calculated using the 2-sample \( t \) test. Further analysis of the data, shown in Fig. 2B, demonstrates remarkable similarity in the genes correlating significantly with survival time longer than 1 year across both groups of patients. Thus, gene expression levels are similar between both groups of patients, and similar alterations in gene expression correlate with survival beyond 1 year in both groups.

**Figure 1.** Training set. A, four-gene panel in patient blood predicts survival in test population (\( P < 0.0001 \)); survival curves for the low-risk group are shown in light gray, intermediate-risk in black, and high-risk in dashed gray. High-, medium-, and low-risk groups are statistically significant (\( P < 0.0001 \)) by the log-rank (Mantel-Cox) test and without correcting for comparisons. B, toxicity is not significantly affected by risk score. Diarrhea is graded according to National Cancer Institute Common Toxicity Criteria (NCI CTC) and is shown for each risk group in the training population. There is no significant relationship between diarrhea toxicity and either risk categories or the 4-gene score.

**Figure 2.** Gene expression profiles are similar between training and test populations. A, mean threshold cycle (\( C_t \)) for each of the 169 genes tested in the training set (x-axis) is plotted relative to the \( C_t \) values for the same genes in the test set (y-axis). Correlation between the \( C_t \) values for the 2 data sets was (\( R = 0.9939, P < 0.0001 \)). B, plots show the value and 95% confidence interval for the mean difference in threshold cycle (\( \Delta C_t \)) for each gene between patients who survived less than 1 year and patients who survived longer than 1 year. Training population is on the left and test population is on the right. Genes are ordered according to their relative expression, with genes associated with longer survival at the top of the plot. A list of the genes in the order they are listed with values for the mean \( \Delta C_t \) in each population is included in Supplementary Table S1. Confidence intervals representing genes with significantly different expression between patients who survived less than 1 year and patients who survived longer than 1 year, without correcting for multiple comparisons, are shown in black.
The 4-gene model was then independently validated in the test population \((n = 260)\) using predefined risk groups based on gene signature score from the training population. Kaplan–Meier analysis demonstrated risk score to be a significant predictor of overall survival \((P = 0.0015, \text{Fig. 3A})\). Similar to the training population, risk score did not correlate with risk of toxicity \((\text{Fig. 3B})\). Thus, the 4-gene model predicts overall survival but not heightened toxicity in 2 patient populations treated with tremelimumab.

The 4-gene risk score enhances the predictive power of established clinical predictors

To evaluate its prognostic utility, the 4-gene score was examined in the context of clinical variables including age, sex, clinical disease stage, and Eastern Cooperative Oncology Group (ECOG) performance status. Multivariable analysis shows that the 4-gene score predicts survival in the test set with more accuracy than all the other clinical variables, including clinical staging. As shown in Table 3, the 4-gene signature was the only factor to contribute significantly to the accuracy of prediction \((P < 0.0001)\), although there was a trend toward significance for clinical stage \((P = 0.068)\). An additional analysis was performed to determine whether the addition of the 4-gene model to clinical variables would improve the predictive accuracy of these variables. The 4-gene score was entered last into a stepwise Cox regression along with the other clinical variables. Analysis showed that the 4-gene model contributes significantly to prediction, even when the clinical variables were taken into account \((P < 0.001)\).

It would be interesting to know whether the 4-gene model is a predictive marker of response to tremelimumab and/or a prognostic marker of melanoma progression. A way to address this issue is to evaluate the expression of the 4 genes identified in patients with melanoma who did not receive tremelimumab. Unfortunately, no blood was harvested from patients on the control arm of the phase III trial. However, from the University of Colorado study, mRNA levels were available from 12 patients with melanoma and 32 normal controls for whom the 4-gene score was then calculated. The 4-gene score was higher overall in patients with melanoma than in normal controls \((P = .0154, \text{Supplementary Fig. S3})\), showing that the 4-gene panel correlates with melanoma diagnosis.

Discussion

Modulation of immune checkpoints can lead to durable survival benefits in a small subset of patients with advanced melanoma. Inhibition of CTLA-4 activity by FDA-approved monoclonal antibody ipilimumab confers an overall survival benefit in patients with advanced melanoma when compared with a peptide vaccine \((24)\). However, the benefits are limited with median survival remaining under 1 year and responses rates are not high (~10%). Despite the limited improvement in median survival, there is an approximate 10% increase in survival at year 2 and

### Table 3. Multivariable analysis

<table>
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<tr>
<th>Variable</th>
<th>Regression coefficient</th>
<th>SE</th>
<th>Wald test</th>
<th>Degrees of freedom</th>
<th>(P)</th>
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<td>0.009</td>
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<td>0.925</td>
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<tr>
<td>Sex</td>
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<td>0.71</td>
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<tr>
<td>Clinical stage</td>
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<td>0.091</td>
<td>3.323</td>
<td>1</td>
<td>0.068</td>
</tr>
<tr>
<td>ECOG score</td>
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<td>0.164</td>
<td>1.915</td>
<td>1</td>
<td>0.166</td>
</tr>
<tr>
<td>4-Gene score</td>
<td>0.272</td>
<td>0.065</td>
<td>17.582</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
subsequently (25). The registration study that led to the FDA approval of ipilimumab demonstrated a response rate of about 10% and an increase in 2-year survival from 14% to 24% (24). Tremelimumab is not currently FDA-approved but has also demonstrated a response rate of about 10% that was not different from the comparator dacarbazine, although there was significantly longer duration of response (35.8 vs. 17.7 months), compared with dacarbazine, among responders to CTLA-4 blockade. Tremelimumab is still undergoing further clinical trials (8). Markers predictive of response or durable benefit to CTLA-4 blockade are not established, nor are there well-defined patient characteristics that predict response or survival benefit (14). Treatment with CTLA-4 targeted therapies can cause potentially life-threatening autoimmune toxicities. Despite having the same target, ipilimumab and tremelimumab are distinct drugs; therefore, we do not propose that the 4-gene score should be immediately applied to ipilimumab. However, this method of researching potential markers predictive of benefit is readily accessible and minimally invasive. This work highlights the need for further research on biomarkers in peripheral whole blood. Predictive biomarkers could limit the exposure of patients who are unlikely to benefit from treatment to an agent that can cause significant toxicity while also allowing these patients to benefit from other therapies earlier in their treatment course.

Studies have identified potential biomarkers, including presence of CTLA-4 polymorphisms (26), increased T-cell expression of ICOS (27), increased tumor expression of FoxP3 and IDO expression in the tumor (28), increase in ratio of effector to regulatory T cells (27), increase in the number of tumor infiltrating lymphocytes from baseline (28), the absolute lymphocyte count (29), and tumor microenvironment specific factors (30, 31). In addition, the utility of C-reactive protein and circulating myeloid-deprived suppressor cells (MDSC) and T regulatory populations have been advanced (32). The identification of these potential biomarkers is hypothesis-generating and remains in need of validation. A limitation of several of these proposed biomarkers is that they depend on changes in a patient’s blood or tumor environment following the initiation of treatment, and therefore, patients unlikely to benefit would still be exposed to treatment and potential autoimmune toxicity before assessment would be possible. The identification of predictive marker(s) that would be based upon only pretreatment patient characteristics or samples would limit these risks. This technique provides a way to study peripheral blood for biomarkers of predictive and prognostic significance in immunotherapy. It avoids unnecessary patient exposure to therapy, unlike the aforementioned markers, and the collection of blood samples is minimally invasive. Ultimately, these methods could play a pivotal role in developing predictive gene panels for a variety of immunotherapies.

Blood components flow in the circulation and therefore have exposure to the tumor microenvironment, which may modulate the expression of genes in whole blood specimens (15, 33). We used transcriptional profiling of whole blood obtained from patients with stage IV melanoma to identify and validate a 4-gene model that stratifies patients into groups at high, intermediate, and low risk for overall survival. Genes were selected using a hypothesis-driven approach to minimize artifact due to multiple testing inherent in whole genome approaches. While this introduces an element of bias, it is a practical way to identify potential targets based on available evidence in the literature. Blood samples were obtained before initiating treatment with tremelimumab, a monoclonal antibody that inhibits the activity of CTLA-4. The 4-gene model was defined from a training set of patients with refractory stage IV melanoma treated with tremelimumab as part of a multicenter phase II study (7), and the model was validated in a population of treatment-naïve stage IV patients with melanoma who enrolled in a multicenter phase III trial with tremelimumab (8).

The 4-gene model proposed here identifies patients with advanced melanoma who are more likely to benefit in terms of survival with subsequent tremelimumab treatment. However, a limitation of the study is that risk stratification based on the expression of the 4 genes in the model was performed on only the patients who received tremelimumab and not on the control patients receiving chemotherapy in the phase III study. Therefore, we cannot differentiate between the 4-gene model as a predictive biomarker for survival in the setting of tremelimumab treatment from the possibility that it is prognostic marker for survival of patients with advanced melanoma. The ability of the 4-gene model to predict survival following treatment with ipilimumab, currently the only FDA-approved anti-CTLA-4 antibody, has not been determined. We can conclude, however, that mRNA levels for biologically relevant genes were reproducible between 2 large cohorts of patients with advanced melanoma and that these levels correlated with clinical outcome in the context of tremelimumab therapy. In addition, the 4-gene score was elevated in 12 patients with melanoma compared with 32 control patients (P = 0.0154, Supplementary Fig. S3). While this is not definitive proof of the prognostic impact of the 4-gene score, it does support the hypothesis that the score is related to melanoma disease process, and consequently, that the 4-gene score may have prognostic utility independent of tremelimumab therapy.

An understanding of the function of the 4-gene products included in the model may help elucidate processes important in stratifying patients with melanoma for survival in the context of future anti-CTLA-4 and other targeted therapies. Elevated levels of 2 of the genes, IRAK3 and CTSD, were predictors of shortened survival following anti-CTLA-4 directed therapy, which is consistent with the potential immunosuppressive functions of each gene product. IRAK-3 suppresses Toll-like receptor–mediated activation of the innate immune system and expression in humans is induced during macrophage maturation (34). Induction of expression in tumor-associated macrophages has been postulated to contribute to an immunosuppressive tumor microenvironment. IRAK-3 may further regulate CTLA-4 activity by binding to and modulating the activity of CD80 (35, 36). CTSD is a lysosomal aspartate endopeptidase that...
can activate apoptotic pathways and thereby regulate CTLA-4 expression on CD4+ T cells by facilitating the secretion of lysosomal intracellular CTLA-4 upon T-cell activation (37, 38). Furthermore, expression of both of these gene products has been associated with increased potential to develop malignancy. IRAK-3 knockout mice are resistant to the growth of melanoma cells following tumor inoculation (34, 39). Expression of CTSD correlates with poorer differentiation of lung adenocarcinoma cells (40), poorer prognosis, and increased likelihood of developing metastases in breast cancer models (41) and helps promote the development of malignancy in benign prostatic epithelium (42).

The ability of PLAG2 and TXNRD1 to enhance the predictive power of the signature is likely related to regulation of the tumor microenvironment and of immune function. The protein product of TXNRD1 gene protects cells from oxidative stress, replenishes the deoxynucleotide triphosphate pool allowing for DNA synthesis and replication, and modulates the expression on macrophages of VSIG4, a B7 protein family protein member that regulates T-cell activation (43–45). The protein product of PLAG2 regulates the ability of tumor cells to undergo apoptosis and alters inflammatory responses through the metabolism of platelet-activating factor (46, 47).

Biomarkers are greatly needed to identify the subset of patients with cancer who may respond to immune therapies. Tremelimumab continues to be used in clinical trials but is unlikely to be FDA approved as a single agent for the treatment of melanoma. Nonetheless, an understanding of gene expression patterns in the blood of treated patients is likely to have implications for other immunotherapies as similarities have been shown between predictors of response to interleukin-2, ipilimumab, and other immune therapies suggesting that the tumor microenvironment as well as the activation state of blood leukocytes may have implications for the success of immune therapies in general.

In summary, we have developed and validated a pretreatment whole blood–based 4-gene model that correlates with the overall survival of patients with melanoma who were subsequently treated with tremelimumab. Components of the model represent gene products that can alter both immune activity and tumor microenvironment. The 4-gene model predicted survival better than clinical variables including stage IV substage that have been adopted by the American Joint Committee on Cancer (AJCC). Use of such a model can limit exposure of patients unlikely to benefit from treatment to a therapy that can cause significant autoimmune toxicity. Further studies to explore the prognostic significance of RNA-based blood biomarkers in patients with advanced melanoma receiving ipilimumab and chemotherapy will be important to dissect the ability of the model to predict for benefit to other CTLA-4 targeted and immunomodulatory agents to develop tools necessary for more informed and efficacious treatment decisions.

**Disclosure of Potential Conflicts of Interest**

J. Magidson is a consultant/advisory board member for Pfizer. J. Kirkwood is a consultant/advisory board member for Bristol-Myers Squibb, GlaxoSmithKline, and Merck. P. Friedlander is a consultant/advisory board member of BMS and Genentech. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: Y. Saenger, J. Magidson, K. Wassmann, J. Kirkwood

Development of methodology: Y. Saenger, J. Magidson, K. Wassmann

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Kirkwood

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Saenger, J. Magidson, B. Liaw, S. Harcharik, Y. Fu, K. Wassmann, J. Kirkwood, P. Friedlander

Writing, review, and/or revision of the manuscript: Y. Saenger, J. Magidson, B. Liaw, E. de Moll, S. Harcharik, Y. Fu, K. Wassmann, D. Fisher, J. Kirkwood, W.K. Oh, P. Friedlander

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Saenger, B. Liaw, E. de Moll, S. Harcharik, Y. Fu, P. Friedlander

Study supervision: Y. Saenger, J. Kirkwood, W.K. Oh

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