Multiplex Analysis of Serum Cytokines in Melanoma Patients Treated with Interferon-α2b

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Abstract

Purpose: Interferon (IFN)-α2b is the only Food and Drug Administration–approved treatment for operable high-risk melanoma that has been shown to significantly and durably prolong relapse-free survival (RFS) of patients with stage IIB-III melanoma. Development of reliable serum assays may contribute to the development of methods for earlier detection of melanoma and the selection of patients who may be most susceptible to current available interventions with IFNα.

Experimental Design: A powerful high-throughput xMAP multiplex immunobead assay technology (Luminex Corp., Austin, TX) was used to simultaneously test 29 cytokines, chemokines, angiogenic as well as growth factors, and soluble receptors in the sera of 179 patients with high-risk melanoma and 378 healthy individuals.

Results: Serum concentrations of interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-12p40, IL-13, granulocyte colony-stimulating factor, monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, IFNα, tumor necrosis factor (TNF)-α, epidermal growth factor, vascular endothelial growth factor (VEGF), and TNF receptor II were found to be significantly higher in patients with resected high-risk melanoma compared with healthy controls. Bayesian Network algorithm classification of the data offered 90% sensitivity at 98% specificity with 96.5% of melanoma patients distinguished from healthy individuals. IFN-α2b therapy resulted in a significant decrease of serum levels of immunosuppressive and tumor angiogenic/growth stimulatory factors (VEGF, epidermal growth factor, and hepatocyte growth factor) and increased levels of antiangiogenic IFN-γ inducible protein 10 (IP-10) and IFN-α. Pretreatment levels of proinflammatory cytokines IL-1β, IL-1α, IL-6, TNF-α, and chemokines MIP-1α and MIP-1β were found to be significantly higher in the serum of patients with longer RFS values of 1 to 5 and >5 years when compared with patients with shorter RFS of <1 year.

Conclusion: These data show that multiplexed analysis of serum biomarkers is useful for the evaluation of prognostic markers of clinical outcome and potential predictive markers of response to IFN-α2b in patients with high-risk operable melanoma.

Melanoma is a potentially lethal malignancy that has shown an increase in incidence that exceeds all other solid tumors. It accounts for >79% of skin cancer–related deaths. Melanoma is surgically curable when discovered at early stages; however, once regional and systemic spread of the disease occurs, the prognosis is more ominous. Patients with localized nonulcerated primary melanoma of <1 mm Breslow depth (stage IA) at the time of diagnosis have an excellent prognosis, but those with primary melanoma of a Breslow depth >4 mm (stage IIB) or regional lymph node metastasis (stage III) have intermediate-to-high risk of relapse, and those with distant metastases have a median survival of 5 to 11 months (1–6). Thus, the early detection and treatment of melanoma is of high importance, and among patients with intermediate to high risk, the ability to predict disease outcome is critically important.

Tumor progression involves the expression of oncogenes and inactivation of tumor-suppressor genes, leading to malignant transformation in which increased production of multiple growth factors and cytokines seem to enable autonomous melanoma growth. Cultured melanoma cell lines and melanoma cells derived from primary melanoma and metastases have been shown to produce basic fibroblast growth factor (bFGF), interleukin (IL)-1α, IL-1β, IL-6, vascular endothelial growth factor (VEGF), platelet-derived growth factor, and IL-8 (7–9). These factors seem to promote tumor cell growth and increase the capacity of tumor cells to survive. In addition, they have paracrine effects and may stimulate endothelial cell proliferation, migration, and angiogenesis that are important for melanoma growth and metastasis (10). Increased production

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Note: J.M. Kirkwood, E. Gorelik, and A.E. Lokshin contributed equally to this work.

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of proangiogenic and growth factors by melanoma and stromal cells *in vivo* might result in elevation of these factors in the circulation. Indeed, numerous studies have shown significantly increased serum IL-8 and IL-6 levels in melanoma patients (7, 11–13). In addition, serum epidermal growth factor (EGF) receptor and IL-1, which modulate the microenvironment to stimulate tumor growth and invasion, have also been reported to be elevated (10, 13).

Increased concentrations of various cytokines, angiogenic factors, and growth factors might serve as useful biomarkers for early diagnosis and prognosis, and also may permit the monitoring of disease and response to therapy in patients over time. Numerous studies have evaluated the prognostic relevance and diagnostic potential of a large number of serum biomarkers for melanoma (14). Specifically, elevated level of IL-6 has been linked to poor prognosis in patients with stage IV melanoma (15) and is a predictive factor of overall survival (16, 17). Elevated levels of IL-10 have been associated with advanced stage III and IV melanoma (18). Several other biomarkers, such as melanoma-associated S100 protein, EGF, insulin-like growth factors, matrix metalloproteinases, lactate dehydrogenase, VEGF, IL-2R, tumor necrosis factor (TNF)-α, soluble TNF receptor 55 (sTNF-R55) and melanoma inhibitory activity protein were found to be of prognostic significance in melanoma (reviewed in refs. 19–21). Based on these studies, it may be concluded that no single marker provides reliable prognostic value for all melanoma patients. It is possible that a test combining several prognostic markers would prove to have higher value in predicting responses to therapy. However, such testing is difficult to perform using ELISA, in which each biomarker has to be tested and analyzed individually. In the present study, we used Luminex (Luminex Corp., Austin, TX) xMAP technology to simultaneously test multiple factors in sera of melanoma patients. We compared the concentrations of 29 cytokines, chemokines, proangiogenic factor, and growth factors in the sera of 179 melanoma patients and 378 healthy individuals to identify a profile of serum biomarkers that might be associated with melanoma.

At present, the only Food and Drug Administration–approved treatment with a durable effect upon relapse and mortality of advanced melanoma is high-dose IL-2, and the only Food and Drug Administration–approved adjuvant therapy for operable deep primary (stage IIB) and regional nodal (stage III) melanoma, is high-dose IFN-α2b (HDI). HDI has been shown to have a durable effect upon relapse-free survival (RFS) of melanoma with a 27% to 33% reduction of the hazard for relapse among patients at high risk of relapse in several multicenter randomized clinical trials of the U.S. Cooperative Groups, whereas this agent induces objective clinical response in 16% of patients with advanced inoperable melanoma. It remains unclear why some patients respond and some are nonresponsive to IFN-α2b therapy, and the molecular basis of the antitumor effects of IFN-α2b administered in the adjuvant setting have been conjectured to range from direct cytotoxic effects, to antiangiogenic and immunologic modulation of the host. Recent evidence (22) has implicated immunologic mechanisms more strongly than other potential mechanisms for the adjuvant benefit of this modality, in studies recently reported using a neoadjuvant design. We have therefore evaluated the blood samples of patients who have participated in two prospective cooperative group trials to test (*a*) whether IFN-α2b therapy altered the serum levels of a number of candidate proinflammatory cytokines and growth factors; and (*b*) whether the evaluation of combinations of multiple serum markers could better serve as a prognostic index of disease or a predictor of benefit from adjuvant therapy with IFN-α2b in patients with resected high-risk melanoma assigned to receive HDI or alternative therapies in the context of prospective cooperative group trials for which long-term follow-up is now available.

Materials and Methods

Patient populations. This study was done using the extensive serum bank that has been prospectively collected in the course of the E1694 Eastern Cooperative Oncology Group–led intergroup clinical trial. E1694 was a phase III randomized study of vaccination with ganglioside GM2 conjugated to keyhole limpet hemocyanin and administered with the saponin adjuvant QS-21 (GM2-KLH/QS-21, or GMK, Progeics, Inc., Tarrytown, NY) versus high-dose IFN-α2b for resected high-risk melanoma patients (defined using the tumor-node-metastasis system as $T_4 > 4$ mm primary lesions, or any primary lesion accompanied by regional lymph node metastasis). Only patients with melanoma of cutaneous origin were eligible for this study. Patients who were assigned to the vaccine group received GMK vaccination 12 times over a 2-year period. Patients in the HDI group received IFN-α2b 20 MIU/m^2/d i.v. 5 days in a row (Monday-Friday) per week for 4 weeks, and then 10 MIU/m^2/d s.c. 3 days per week (Monday, Wednesday, Friday) for 48 weeks. In addition, patients were required to return for follow-up visits every 3 to 6 months for 5 years. The study accrued 880 patients between June 1996 and October 1999. For the present study, we tested sera from 179 patients taken from the two trial arms according to disease status (nonrelapsed, relapsed within 1-5 years, and relapsed within 1 year). Of these, 93 patients received GMK vaccination and 86 were treated with HDI. Currently, clinical data from E1694 are available with a median of 4.6 years of follow-up for E1694. Clinical results of the trial within which these sera were collected have previously been summarized elsewhere (23–25). In addition, serum samples from 378 healthy normal subjects were evaluated (matched by sex and gender) as collected by Drs. Joel Weissfeld and Francesmary Modugno at the University of Pittsburgh Cancer Institute, the Gynecologic Oncology Group (Cleveland, OH), and by Dr. Jeffrey Marks at Duke University (Durham, NC). Written informed consent for serum collection and evaluation for the presence of serum markers was obtained from each subject. Sample collection was done after approval by the institutional review board and in accord with an assurance filed with and approved by the U.S. Department of Health and Human Services. No data allowing the identification of patients were provided.

Collection and storage of blood serum. Ten or 20 mL of peripheral blood were drawn from each of the subjects using standardized phlebotomy procedures. Handling and processing was similar for all patients. Samples were obtained from patients diagnosed with melanoma before treatment and at 12 weeks after initiation of a therapy (either GMK or HDI) and from healthy individuals. Blood samples were collected without anticoagulant into red top vacutainers and allowed to coagulate for 20 to 30 min at room temperature. Sera were separated by centrifugation, and all specimens were immediately aliquoted, frozen, and stored in a dedicated −80°C freezer. No more than two freeze-thaw cycles were allowed before testing for each sample.

Multiplex analysis. The xMAP technology (Luminex Corp.) combines the principle of a sandwich immunoassay with fluorescent bead–based technology, allowing individual and multiplex analysis of up to 100 different analytes in a single microtiter well (26). The xMAP serum assay for 29 cytokines [IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-13, IL-15, IL-17, IL-18, TNF-α, TNF-RI, TNF-RII, IFN-α, IFN-γ, granulocyte macrophage colony-stimulating factor,

(GM-CSF) EGF, IP-10, granulocyte colony-stimulating factor, bFGF, HGF, macrophage inflammatory protein (MIP)-1α, MIP-1β, MCP-1, and VEGF] was done in 96-well microplate format according to the protocol of BioSource International (Camarillo, CA) as previously described (11). Samples were analyzed using the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA). Analysis of experimental data was done using five-parametric curve fitting. Interassay variabilities for individual cytokines were in the range of 1.0% to 9.8% and intra-assay variabilities were in the range of 3.6% to 12.6% (information provided by Biosource International and validation done in our laboratory). Each assay was validated against appropriate ELISA demonstrating 95% to 99% correlations (information provided by Biosource International).

Statistical analysis of data. Concentrations of serum biomarkers were analyzed for their normality of distribution using a software package SigmaStat (SPSS, Inc., Chicago, IL). Tested biomarkers in control and melanoma groups failed to exhibit a normal distribution. Therefore, the Mann-Whitney rank sum test was used to evaluate the significance of differences in the median of the value for each marker expressed between cancer patients and healthy controls. Signed rank test was used to quantify the correlation of each marker, before and after therapy. For all statistical analysis, the level of significance was set at $P < 0.05$. Bioinformatic classification was done using the open source Bayesian Network Java class library (part of WEKA v.3.4, Waikato University, NZ) adapted for automatic generation of receiver operating characteristic curve.

Results

Analysis of serum markers in melanoma patients and healthy controls. To analyze differences in serum concentrations of different cytokines, angiogenic and growth factors in melanoma patients and healthy controls, serum samples from 179 patients before either treatments (HDI or vaccine) and 378 age- and sex-method healthy controls were screened using a 29-plex multiplexed cytokine assay. Serum levels of IL-2, IL-4, IL-5, IL-10, IL-15, IL-17, IL-18, TNF-RI, GM-CSF, INF-γ, bFGF, HGF, and IP-10 were detectable but not statistically different in the tested groups. A statistically significant increase in concentrations of IL-1α, IL-1β, IL-6, IL-8, IL-12p40, IL-13, G-CSF, MCP-1, MIP-1α, MIP-1β, IFN-α, TNF-α, EGF, VEGF, and TNF-RII was found in sera of melanoma patients compared with healthy controls ($P < 0.05$-$P < 0.001$; Table 1; Fig. 1). These data show that melanoma patients have a significantly different pattern of expression for multiple serum cytokines compared with healthy individuals.

Next, a multivariate bioinformatics Bayesian Network algorithm was used to discriminate between patients with melanoma and healthy subjects. The algorithm was first trained using a training set, and then validated using a test set. The cross-validation was done using two methods: (*a*) 45 of 55 random split, where the whole data set was repeatedly reshuffled using 55% of data as a training set and 45% as a validation set, and (*b*) 10-fold test, where first 10% of data were used as a training set and the remaining 90% as a validation set, with repeated 10-fold cyclic data shifts. Analysis using a Bayesian Network algorithm offered 90% sensitivity at 98% specificity with 96.5% of patients correctly classified. Area under the receiver operating characteristic curve was 0.985 (data not shown).

Effect of IFN-α therapy on serum levels of cytokines and growth factors. To identify specific alterations in cytokine profiles associated with IFN-α therapy, serum samples from 86 melanoma patients who were evaluated before and after 3 months of HDI therapy were analyzed.

Table 1. Cytokine expression in melanoma patients compared with healthy controls

Cytokines	Control	Melanoma	P
IL-1α	13.1 ± 3.22 0.0 (0.0-996.3)	174.4 ± 92.00 29.3 (0.0-16,630.5)	<0.01
IL-1β	40.2 ± 8.78 0.0 (0.0-2253.5)	405.7 ± 97.01 5.5 (0.0-11,815.2)	<0.001
IL-6	22.8 ± 7.00 0.0 (0.0-2,394.2)	1,045 ± 287.6 12.2 (0.0-35,459.8)	<0.001
IL-8	9.56 ± 0.40 8.6 (0.0-87.6)	2,523 ± 1,167 122.8 (2.9-98,563.7)	<0.001
IL-12p40	171.1 ± 6.52 137.5 (0.0-1,817.6)	200.5 ± 10.20 180.8 (63.4-485.8)	<0.05
IL-13	25.5 ± 2.94 0.0 (0.0-447.8)	70.6 ± 4.82 56.5 (0.0-455.2)	<0.001
G-CSF	489.2 ± 23.26 434.7 (0.0-4,511.7)	730.4 ± 36.72 708.8 (0.0-5,493.6)	<0.001
MCP-1	173.2 ± 15.04 99.8 (0.0-4,058.1)	1,842 ± 242.5 441.8 (0.0-14,390.5)	<0.001
MIP-1α	88.1 ± 14.31 19.0 (0.0-3,557.1)	1,440 ± 323.4 82.3 (0.0-24,361.9)	<0.001
MIP-1β	135.1 ± 29.22 12.9 (0.0-8,679.8)	1,664 ± 347.5 198.3 (0.0-30,180.1)	<0.001
IFNα	16.8 ± 6.59 0.0 (0.0-2,205.1)	47.2 ± 10.26 0.0 (0.0-1,286.3)	<0.01
TNF-α	34.32 ± 11.46 0.0 (0.0-3,847.6)	1,041 ± 308.6 28.1 (0.0-27,548.9)	<0.001
EGF	156.2 ± 12.42 117.7 (0.0-3,332.7)	339.3 ± 11.66 329.9 (9.6-908.9)	<0.001
VEGF	76.6 ± 6.07 34.9 (0.0-1,151.5)	215.0 ± 16.30 192.1 (0.0-783.1)	<0.05
TNF-RII	771.1 ± 29.37 697.3 (0.0-447.8)	1,007 ± 47.8 928.9 (0.0-3,537.8)	<0.001

NOTE: Serum samples from 179 patients before treatment with IFN-α or GMK vaccine and 378 samples from healthy control individuals were screened using a 29-plex multiplexed cytokine assay. Only serum biomarkers that showed differences between melanoma patients and control healthy group are presented as mean ± SE (pg/mL) and median (range).

As expected, IFN-α therapy induced significant changes in the serum concentrations of multiple cytokines. HDI therapy decreased levels of angiogenic and growth factors (VEGF, EGF, HGF; Fig. 2), whereas expression of IP-10, IFN-α, MCP-1, IL-12p40, soluble TNF-RI, TNF-RII, and IL-2R were significantly increased in the serum evaluated 3 months postinitiation of HDI therapy. The largest increase was observed in IP-10 levels, which rose from 19.68 ± 3.92 pg/mL before the treatment to 109.72 ± 13.74 pg/mL after IFNα treatment ($P < 0.001$; Fig. 2). Other data has previously indicated that IP-10 and IFN-α inhibit tumor-induced angiogenesis (27). Taken together, the new findings we report and these prior observations suggest significant changes in the levels of multiple proangiogenic and antiangiogenic markers associated with IFNα therapy. Because the trial E1694 randomly assigned patients to either HDI or to GMK vaccination, we evaluated the group of patients who were randomly assigned to receive GMK vaccine therapy, to determine whether similar changes in this group of otherwise similar patients followed in parallel showed any of the changes observed among recipients of HDI. In contrast, our analysis of serum of 93 melanoma patients 3

months after vaccination with GMK revealed no significant changes in the serum biomarkers (data not shown).

Prediction of RFS of melanoma patients based on the expression pattern of serum markers. To examine the prognostic significance of cytokine concentrations in serum specimens, we have analyzed whether serum biomarker levels are associated with therapeutic benefit (the absence of relapse, in the adjuvant setting) of melanoma patients receiving HDI. Patients were divided into three groups based on the duration of their RFS: patients with RFS < 1 year, patients with RFS between 1 and 5 years, and patients with RFS > 5 years. Our comparison of the posttherapy levels of the tested biomarkers showed no significant association with RFS (data not shown). However, we found that the expressions of IL-1β, IL-1α, IL-6, TNF-α, and chemokines (MIP-1α, and MIP-1β) before therapy were associated with RFS (Fig. 3A). High serum levels of these proinflammatory molecules measured by multiplex assay before treatment were positively correlated with the duration of RFS after initiation of HDI therapy. Sera of melanoma patients with RFS <1 year in duration had significantly lower levels of proinflammatory cytokines ($P < 0.05$) than patients with RFS 1 to 5 and >5 years. The differences in the cytokine levels in patients with RFS 1 to 5 and >5 years were not significant. Interestingly, no effect of GMK vaccination upon the RFS of melanoma patients was observed in the large randomized clinical trial E1694, and evaluation of the same biomarkers that had shown correlation to RFS among recipients of IFN, when tested in the recipients of GMK, showed no correlation with RFS (Fig. 3B). Thus, our data show that pretherapy levels of a panel of proinflammatory cytokines has potential prognostic value for prediction of RFS of melanoma patients treated with IFN-α, but not in patients receiving a specific vaccine intervention without IFN.

Discussion

This study presents the first broad multimarker screening of serum proteins for cytokines and other proinflammatory and proangiogenic proteins considered to represent the downstream intermediates relevant to the host response of patients with melanoma drawn from a large multicenter adjuvant trial, where it has been possible to evaluate these cytokines and other mediators in relation to sera from healthy age- and gender-matched controls, using a novel xMAP technology.

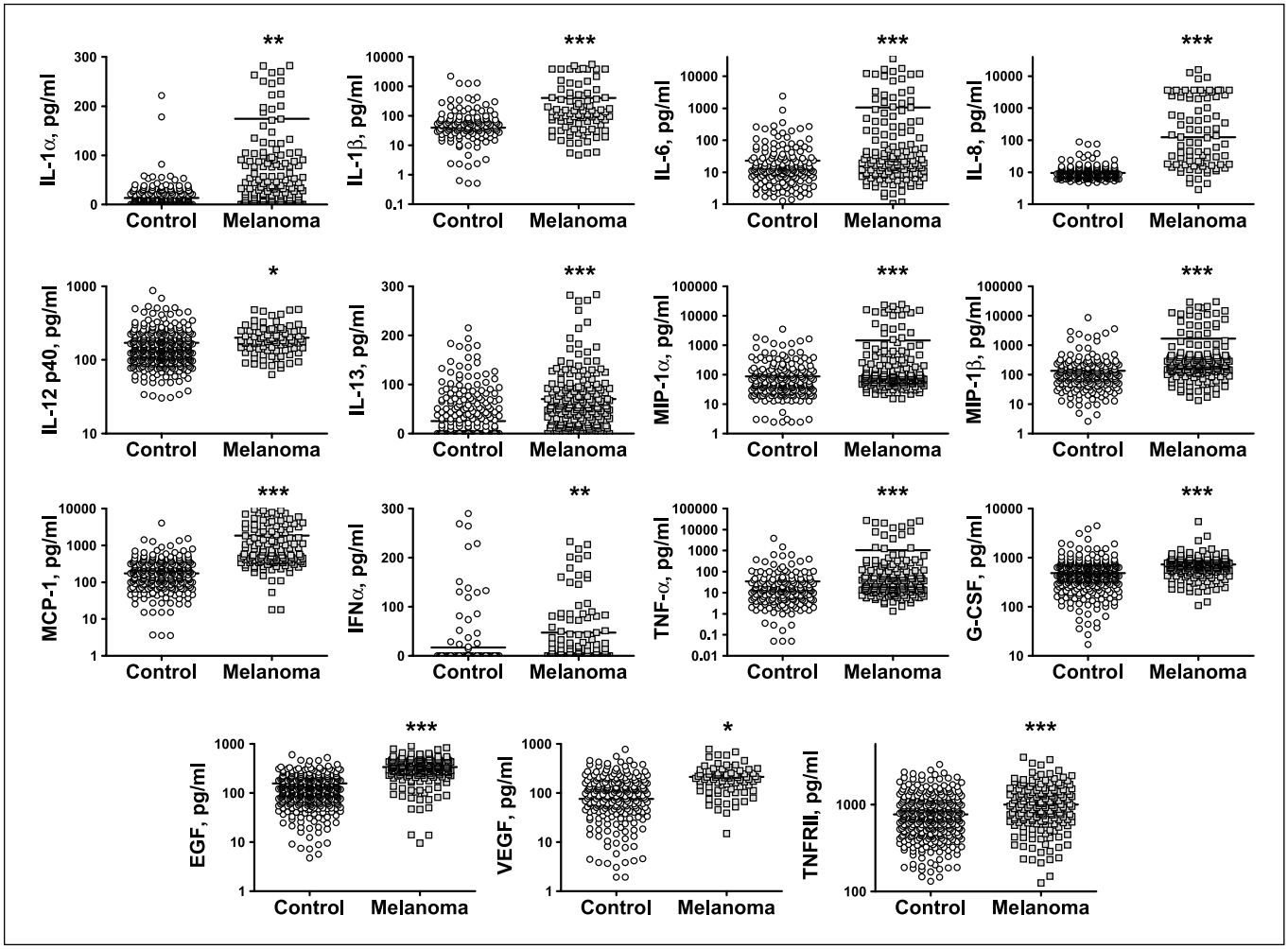

Fig. 1. Analysis of biomarkers by multiplexed xMAP assay in serum samples from melanoma patients and healthy controls. Serum samples collected from 179 patients enrolled in E1694 trial were analyzed at baseline before therapy and compared with sera from 378 healthy controls. The levels of cytokines, chemokines, angiogenic, and growth factors were determined as described in Materials and Methods. All changes are statistically significant: (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Bars indicate mean value.

The present comprehensive analysis of 29 serum biomarkers within a well-defined population of patients, who were participants in a prospective intergroup trial testing the relative benefit of a vaccine (GMK) in relation to the standard adjuvant regimen of HDI, has shown that concentrations of 15 biomarker proteins (IL-1α, IL-1b, IL-6, IL-8, IL-12p40, IL-13, G-CSF, MCP-1, MIP-1α, MIP-1β, IFNα, TNFα, EGF, VEGF, and TNF-RII) were significantly higher in the sera of patients with melanoma than among age- and sex-matched healthy controls. Some of these changes have been previously reported (12, 13, 28–32). To the best of our knowledge, we are the first to report elevated levels of IL-12 p40, IL-13, G-CSF, MCP-1, MIP-1α, MIP-1β, and TNF-RII in melanoma patients. Increased serum levels of growth stimulatory and proangiogenic IL-6, IL-8, VEGF, and EGF are probably associated with overproduction of these proteins by melanoma and/or stromal cells, and related to angiogenesis and tumor growth and survival. These findings provide additional insights into the pathobiology of melanoma and emphasize the advantage of multimarker analysis for discovery of new prognostic and predictive serum biomarkers of melanoma, where these may be readily measured and followed over time. Differences in cytokine profiles between melanoma patients and healthy subjects allowed for robust discrimination between these two groups using a Bayesian Network algorithm. High degrees of discrimination between cancer-afflicted and healthy control groups suggest that serum cytokine profiles may potentially be used for the development of blood-based diagnostic tests for melanoma, and for the prediction of therapeutic benefit among patients treated with HDI adjuvant therapy.

The mechanism(s) of IFN-α in the adjuvant therapy of melanoma has been conjectural. The difficulties that confront such investigations relate to the fact that tumor tissue is often unavailable and that mechanisms pertaining to experimental

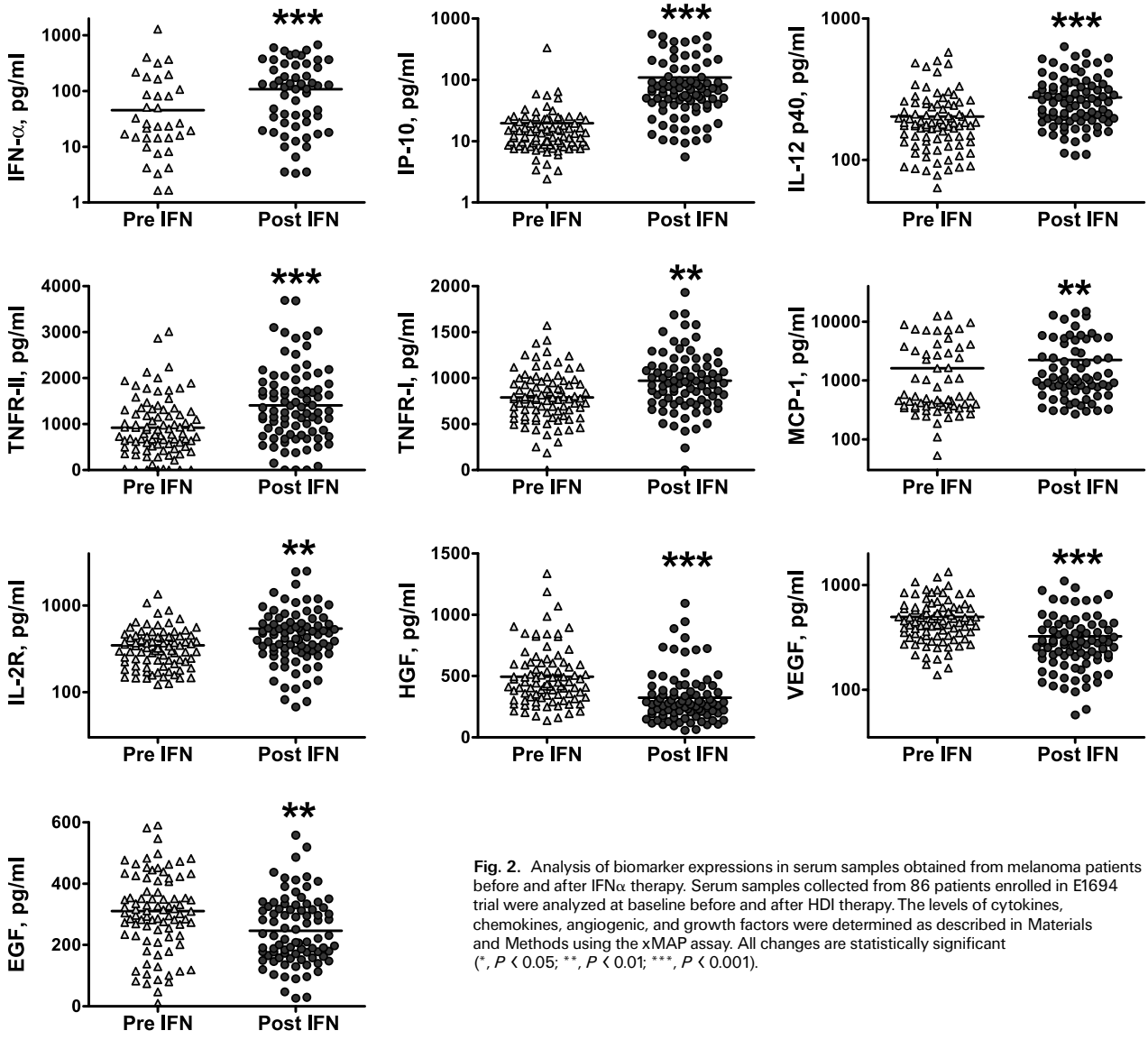

Fig. 2. Analysis of biomarker expressions in serum samples obtained from melanoma patients before and after IFNα therapy. Serum samples collected from 86 patients enrolled in E1694 trial were analyzed at baseline before and after HDI therapy. The levels of cytokines, chemokines, angiogenic, and growth factors were determined as described in Materials and Methods using the xMAP assay. All changes are statistically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

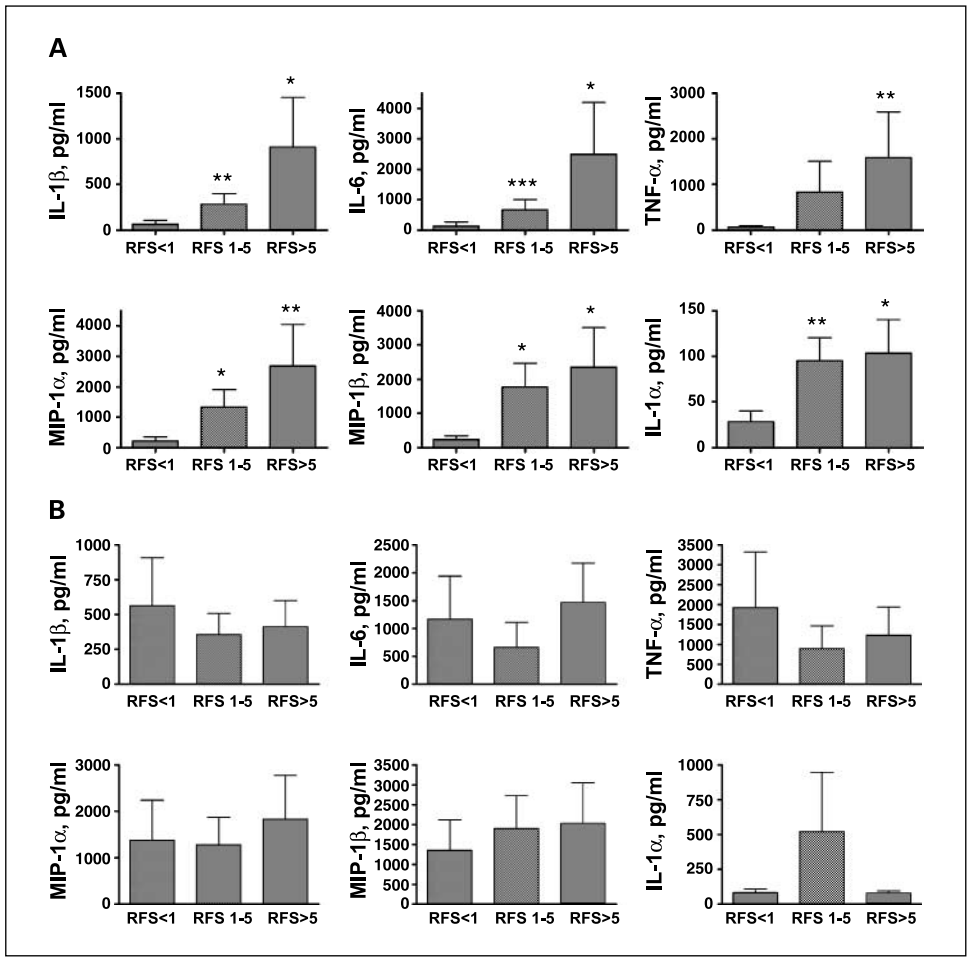

Fig. 3. Prognostic significance of serum cytokine levels for RFS. *A,* melanoma patients were divided into three groups according to RFS after IFNα treatment (RFS period <1 y, between 1 and 5 y, and >5 y). xMAP assay was done on serum collected before HDI therapy from these patients and expression of cytokines was plotted for each RFS group. *B,* analysis of proinflammatory cytokines and RFS in melanoma patients treated with GMK. Serum samples from melanoma patients were collected before GMK therapy and analyzed according to three RFS groups. Significance of differences (*, $P < 0.05$; **, $P < 0.01$; **, $P < 0.001$) were compared with the group with RFS <1. Columns, mean; bars, SE.

murine model tumor systems may not apply to human melanoma (22, 33). IFN-α adjuvant therapy has been suspected to act at the level of the tumor microenvironment and vasculature, as well as immunologically, and directly on the tumor. Our analysis of serum of melanoma patients taken 3 months after initiation of HDI therapy, in relation to pretreatment serum assays, has revealed changes in the profile of the biomarkers tested that are novel. Most of these changes were associated with reduction of the levels of proangiogenic and immunosuppressive factors such as VEGF, EGF, HGF, and increases in the level of antiangiogenic and proinflammatory IP-10 and IFN-α. However, no correlation was found between the IFN-induced reduction in angiogenic factor concentrations and RFS of melanoma patients analyzed in this study. Because the changes noted were observed 3 months after IFN treatment, it remains unclear whether the earlier or later changes in cytokine concentrations could correlate with RFS. Analysis at earlier and later periods after IFN therapy is required to address these questions. Studies reported here will be important to extend both in relation to the serum pattern of cytokine response over time and in relation to a broader array of cytokines and regulators of the cytokine response.

Remarkably, our data indicate that the pretreatment levels of the proinflammatory cytokines IL-1β, IL-1α, IL-6, TNFα, and the chemokines MIP-1α and MIP-1β positively correlate with RFS of melanoma patients treated with HDI. Melanoma patients with the highest levels of these cytokines had the highest rates of RFS at intervals of 1 to 5 and >5 years, whereas patients with the lowest levels of these cytokines tended to have RFS of <1 year. The mechanisms responsible for these correlations and the differential responses to IFNα observed in this study remain unclear, and their relationship to the induction of autoimmunity that has recently been found to be closely correlated with the RFS and overall survival benefits of HDI will also be important to evaluate. No such correlation of cytokine response with therapy was found in patients treated with GMK. It is possible that patients with high levels of proinflammatory cytokines are the most immunogenetically susceptible to the modulation of immunity by HDI or exhibit a genetic polymorphism that is associated with the capacity to develop antitumor response during therapy with IFN-α2b. Polymorphisms associated with the *CTLA4* gene that correlate with response to anti–CTLA-4 blocking antibody are one candidate for further study (34).

In conclusion, a complex network of cytokines seems to be triggered in the presence of deeply invasive primary melanoma, or melanoma that is regionally metastatic to lymph nodes, and this cytokine response seems to be involved in the regulation of melanoma cell growth and progression. Further insight into the mechanism of this host response may illuminate new strategies that may permit more effective therapy and allow a better understanding of the basis on which current approved

therapies, such as IFN-α and IL-2, achieve their effects. For example, it is plausible that the ability to induce autoimmunity that is associated with therapeutic benefit of IL-2 and IFN-α may relate to the proinflammatory cytokines that distinguish patients without relapse in the first year and those with relapse in the first year of follow-up as reported here (35). Development of reliable serum assays for the biomarkers that are critical to progression, and susceptible to modulation by available therapies, may also contribute to the development of methods for earlier detection of melanoma and the selection of patients who may be most susceptible to current available interventions with IFN-α. Our results indicate that multiplexed cytokine profiling may potentially have utility in the monitoring of adjuvant therapy of melanoma patients for efficacy, and/or for the decision whether alternative therapies would be reasonable to consider. Further investigation of the serologic biomarkers may identify a population of melanoma patients more likely to benefit from adjuvant therapies and may result in identification of novel therapeutic approaches for high-risk melanoma patients, including novel approaches to immunotherapy.

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