STAT3 as a Biomarker of Progression in Atypical Nevi of Patients with Melanoma: Dose–Response Effects of Systemic IFNα Therapy

Wenjun Wang[1], Howard D. Edington[2], Uma N.M. Rao[3], Drazen M. Jukic[3,4], Hong Wang[5], Janice M. Shipe-Spotloe[1] and John M. Kirkwood[1]

Signal transducer and activator of transcription (STAT3) plays a pivotal role in tumor progression. Atypical nevi are nonobligate risk markers of melanoma, affording investigators a target for evaluation of progression biomarkers *in vivo*. pSTAT1tyr701 (pSTAT1) and pSTAT3tyr705 (pSTAT3) oppose one another in biological function. Therefore, an analysis of phosphorylated STAT1 (pSTAT1) and pSTAT3 signaling was performed simultaneously using double-immunohistochemistry in biopsies of 168 atypical nevi from 42 patients receiving high- or low-dose IFNα (HDI and LDI). With maturation of melanocytes from junctional into dermal components of nevi, pSTAT1 expression increased, whereas pSTAT3 expression decreased. The percentage of pSTAT3-positive melanocytes was positively associated with the atypical degree of nevi ($P<0.0001$). In the junctional component of nevomelanocytic lesions, HDI and LDI downregulated the percentage of pSTAT3-positive melanocytes ($P=0.008$ and $P=0.0003$, respectively) while upregulating the percentage of pSTAT1-positive melanocytes ($P=0.016$ and $P=0.0059$, respectively) and augmented the pSTAT1/pSTAT3 ratio ($P=0.008$ and $P=0.0040$, respectively). It is suggested that the relative balance of pSTAT1/pSTAT3 may be associated with melanocyte differentiation *in vivo*. pSTAT3 is a potential biomarker of melanocytic transformation and progression and is modulated by IFNα dose-dependently. STAT3 is a potential target for chemoprevention of melanoma.

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INTRODUCTION

Melanoma incidence is rising at a rate exceeding that of all other solid tumors, and to date, no therapy has shown a significant impact on the overall survival of advanced melanoma. Interest has, therefore, turned to earlier diagnosis and prevention. However, biomarkers suitable for targeting on prevention remain elusive. IFNα is the only Food and Drug Administration (FDA)-approved adjuvant therapy of melanoma. All trials of high-dose IFNα (HDI) have demonstrated significant reduction in relapse frequency, and two independent multicenter trials of this regimen have also demonstrated significant prolongation of survival (Kirkwood *et al.*, 1996, 2000, 2001, 2004). In this setting, IFNα was evaluated as a probe in relation to biomarkers of progression in melanoma.

Clinically atypical and histopathologically dysplastic nevi have been demonstrated to be nonobligate precursors and biomarkers of melanoma risk, as reviewed by Hussein (2005). The effects of IFNα on these lesions have not been documented, nor have reliable biomarkers of melanoma risk been identified. This study has evaluated the effects of IFNα on markers of progression in atypical (dysplastic) melanocytic nevi to examine the clinical and pathological effects of modulation of the signal transducer and activator of transcription (STAT)1 and STAT3 signaling pathways. In previous studies, it has been demonstrated that the STAT1 and STAT3 signaling pathways are closely related to the impact of HDI upon metastatic melanoma (Wang *et al.*, 2007). STAT3 has been shown to be constitutively activated in melanoma, and the pSTAT1tyr701 (pSTAT1)/pSTAT3-tyr705 (pSTAT3) ratio has been evaluated as a potential prognostic index, with higher pSTAT1/pSTAT3 ratios in tumor tissue predicting improved overall survival of patients

[1]*Division of Hematology/Oncology, Department of Medicine, University of Pittsburgh, Melanoma and Skin Cancer Program, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania, USA;* [2]*Department of Surgery, Magee Women's Hospital, Pittsburgh, Pennsylvania, USA;* [3]*Department of Pathology, University of Pittsburgh Medical Center-Shadyside, Pittsburgh, Pennsylvania, USA;* [4]*Department of Dermatology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania, USA and* [5]*Department of Biostatistics, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania, USA*

Correspondence: Dr John M. Kirkwood, Department of Medicine, University of Pittsburgh, Melanoma and Skin Cancer Program, University of Pittsburgh Cancer Institute, Hillman Cancer Center, Research Pavilion, Suite 1.32, 5117 Centre Avenue, Pittsburgh, Pennsylvania 15213-2584, USA.
E-mail: kirkwoodjm@upmc.edu

Abbreviations: HDI, high-dose IFNα; LDI, low-dose IFNα; pSTAT, phosphorylated signal transducer and activator of transcription; STAT, signal transducer and activator of transcription

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(Wang *et al.*, 2007). IFNα upregulates the pSTAT1/pSTAT3 ratio (Wang *et al.*, 2007). Therefore, the balance of pSTAT1 and pSTAT3 was evaluated as an index of melanoma progression.

The JAK/STAT signaling pathway of type I IFN action is associated with cell growth, differentiation, and immunoregulation as reviewed recently by Schindler *et al.* (2007). STAT1 is a tumor suppressor important for growth restraint as well as a regulator of host tumor immune response that mediates effects of IFNα in murine models. STAT3 is a mediator of neoplastic progression. STAT3-induced immunosuppression is an important component of melanoma progression. The relevance of changes in STAT1 and STAT3 signaling was therefore evaluated in the context of two prospective IFNα trials (HDI and low-dose IFNα (LDI)) focusing on patients with multiple (>4) clinically atypical nevi and a history of prior cutaneous melanoma as depicted in Figure 1. We report that pSTAT3 is a potential biomarker of melanoma risk and that STAT signaling is regulated in a dose-dependant manner by IFNα *in vivo*.

RESULTS

Baseline level of pSTAT3 expression is positively associated with the degree of histological atypia of nevi

One hundred and sixty-eight nevi obtained from 42 patients were studied. Thirty-two patients were evaluated from UPCI 96-043 and 10 patients were recruited from UPCI 95-071. First, it was noted that with maturation of melanocytes from the uppermost junctional elements to the deepest dermal elements, there was progressively diminished expression of pSTAT3 (denoted in red), whereas the expression level of pSTAT1 (denoted in blue) was observed to increase, as shown in Figure 2. Figure 2a was taken using a ×10 objective, whereas Figure 2b was the amplification of the outer junctional melanocytes and Figure 2c was the amplification of the deeper dermal melanocytes. In Figure 2, the level of pSTAT3 expression is higher in junctional melanocytes than in dermal melanocytes, both in regard to intensity and percentage of positive cells, whereas pSTAT1 expression level is higher in dermal melanocytes than that in junctional melanocytes. The baseline percentages of melanocytes expressing pSTAT1 or pSTAT3 are shown in Table 1. The percentages of melanocytes positive for pSTAT1 or pSTAT3 are different (pSTAT1: $P<0.0001$; pSTAT3: $P=0.011$) between junctional (pSTAT1: 6.18 ± 1.2; pSTAT3: 37.74 ± 3.66) and dermal (pSTAT1: 24.79 ± 3.22; pSTAT3: 28.93 ± 3.3) compartments. The pSTAT1/pSTAT3 ratios are different ($P<0.0001$) between junctional (0.36 ± 0.16) and dermal (2.71 ± 1.50) compartments. We further describe the pattern of pSTAT1 and pSTAT3 expression among nonatypical

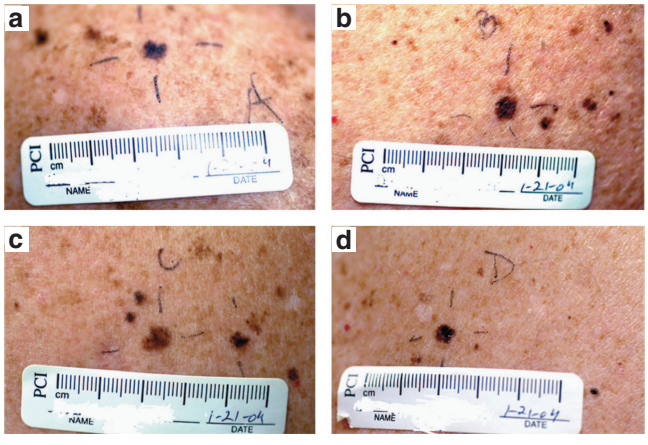

Figure 1. Photographic documentation for the study of multiple nevi. Patients with multiple nevi larger than 4 mm diameter and a history of primary cutaneous melanoma are eligible for two separate studies. Four nevus lesions (**a**–**d**) were selected randomly for study. Two of them were chosen randomly to be resected before LDI or HDI treatment. The remaining two nevi were resected post-LDI or HDI therapy.

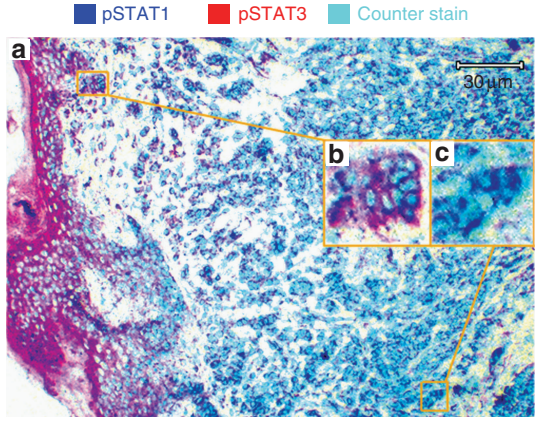

Figure 2. The pattern of expression of pSTAT1 and pSTAT3 in nevus. The baseline expression levels of pSTAT1 and pSTAT3 were observed in this prospective study. With the maturing of melanocytes from the epidermal junction to the deep dermis, the expression of pSTAT3 (red chromogen) was decreased, whereas the expression of pSTAT1 (blue chromogen) was increased. (**a**) Taken by ×10 objective. (**b**) The amplification of junctional melanocytes and (**c**) the amplification of dermal melanocytes. The pSTAT3 expression level is higher in junctional melanocytes than that in deep dermal melanocytes, whereas pSTAT1 expression level is higher in deep dermal melanocytes than that in junctional melanocytes.

Table 1. pSTAT1 and pSTAT3 of junctional and dermal melanocytes

Biomarkers	Melanocyte	*n*	Mean	SE	*P*-value
pSTAT1	Junctional	34	6.18	1.2	<0.0001
	Dermal	34	24.79	3.22	
pSTAT3	Junctional	34	37.74	3.66	0.011
	Dermal	34	28.93	3.3	
pSTAT1/pSTAT3	Junctional	33	0.36	0.16	<0.0001
	Dermal	33	2.71	1.5	

n, number of patients; pSTAT, phosphorylated signal transducer and activator of transcription.
Data are presented as mean ± SE. $P<0.05$ is considered significant.

nevi, atypical nevi, as well as in primary melanoma (three cases) and metastatic melanoma with regional lymph node involvement. The pattern of our observation is that the baseline expression levels of pSTAT3 increased progressively for lesions that lie in the sequence of progression from benign melanocytes, atypical melanocytes, primary melanoma cells, and up to metastatic melanoma cells of regional lymph nodes, both in terms of intensity and percentage of positive cells. However, melanoma cells metastatic to regional lymph nodes exhibited both high levels of pSTAT1 and pSTAT3.

We analyzed the correlation between pSTAT3 expression and the severity of histopathological atypia and architectural disorder in the atypical nevi sampled in this prospective trial. We assessed the severity of histopathological atypia from the formal pathology reports, using a formal synoptic algorithm that has been in place for more than a decade in the UPCI Melanoma and Skin Cancer Program. According to the cytomorphology, atypical nevi were grouped into three categories (Arumi-Uria *et al.*, 2003; Shapiro *et al.*, 2004; Elder, 2006): mild atypia (melanocytes are slightly larger than adjacent keratinocytes, with small nucleoli); moderate atypia (junctional melanocytes increased slightly in amount of cytoplasm with larger nucleoli and have two or more junctional nests); and severe atypia (large melanocytes with abundant cytoplasm, 1.5–2 times the size of adjacent keratinocytes, with large nucleoli, and lentiginous proliferation at the shoulder or between nests). Data analysis used Spearman's rank correlation coefficient "*r*" to demonstrate that the baseline percentage of pSTAT3-positive melanocytes is positively associated with the degree of atypia judged formally and recorded in the pathology reports for the sampled lesions (Figure 3, $n=57$, $r=0.50$, $P<0.0001$). In Figure 3, a score of atypia $=0$ signifies no evidence of atypia; a score $=1$ signifies mild atypia; a score $\geqslant 2$ signifies moderate or more severe atypia. Wilcoxon rank sum tests further demonstrated that the percentage of pSTAT3-positive melanocytes was significantly different in terms of no atypia (19.58 ± 5.13, $n=12$) *versus* mild atypia (40.29 ± 3.93, $n=38$, $P=0.0090$), mild atypia *versus* moderate atypia (67.86 ± 9.12, $n=7$, $P=0.0096$), and no atypia *versus* atypia (44.58 ± 3.87, $n=45$, $P=0.0024$). The original data relating to baseline levels of junctional pSTAT1 and pSTAT3, the grade of atypia, and the thickness of the primary melanoma are listed in Table 1 of the Appendix.

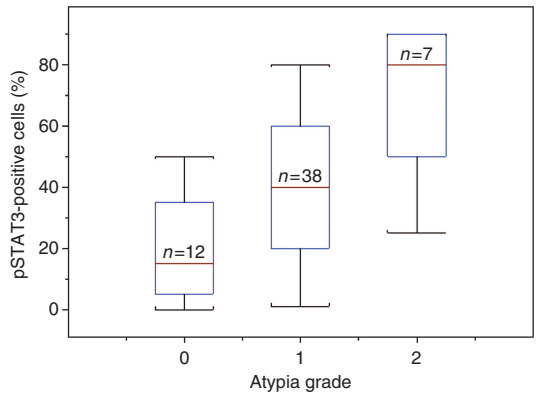

Figure 3. The expression of pSTAT3 is positively associated with the severity of atypia. The baseline expression level of pSTAT3 is positively associated with the degree of atypia in nevi, $n=57$, $r=0.50$, $P<0.0001$. Zero represents no atypia ($n=12$); 1, mild atypia ($n=38$); and 2, moderate atypia ($n=7$). The bar inside the box represents the median.

LDI and HDI downregulate pSTAT3tyr705 and upregulate pSTAT1tyr701 and the pSTAT1/pSTAT3 ratio in nevomelanocytic lesions dose-dependently

On the basis of the above observations, pSTAT1 and pSTAT3 were considered as potential biomarkers of nevomelanocytic progression. As HDI is the only approved adjuvant therapy for high-risk melanoma (Kirkwood *et al.*, 1996, 1999, 2001, 2004; Kirkwood and Tarhini, 2003), we asked what effect this therapy had on the potential biomarkers of progression in dysplastic nevi. IFNα2b was selected as a positive control and reference point for the evaluation of the impact of alternative regimens of potential utility, to assess changes in the marker(s) that may correlate with clinical outcome in potential precursors, such as atypical nevi. We analyzed the effects of LDI (28 cases) and HDI (8 cases) on pSTAT1 and pSTAT3 in atypical melanocytic lesions, as these lesions are currently the best understood histopathologic risk markers of melanoma and represent nonobligate precursor lesions. As shown in Table 2, junctional melanocytes demonstrated downregulation of the percentage of pSTAT3-positive melanocytes before ($40.91 \pm 4.19\%$) and after ($26.00 \pm 3.07\%$, $P=0.0003$) treatment with LDI. The corresponding distributions of the percentage of pSTAT3-positive melanocytes before and after HDI were observed to be $40.31 \pm 4.94\%$ and $18.13 \pm 2.83\%$ ($P=0.008$), respectively. The percentage of pSTAT1-positive melanocytes measured in biopsy samples

Table 2. pSTAT1 and pSTAT3 of junctional melanocytes

IFNα2b	Biomarker	Time point	*n*	Mean	SE	*P*-value
High-dose	STAT1	Pre	8	2.88	0.99	0.016
		Post	8	25.06	9.88	
	STAT3	Pre	8	40.31	4.94	0.008
		Post	8	18.13	2.83	
	STAT1/STAT3	Pre	8	0.073	0.03	0.008
		Post	8	1.51	0.6	
Low-dose	STAT1	Pre	28	6.04	1.25	0.0059
		Post	28	12.27	2.23	
	STAT3	Pre	28	40.91	4.19	0.0003
		Post	28	26	3.07	
	STAT1/STAT3	Pre	27	0.24	0.08	0.004
		Post	27	0.71	0.19	

n, number of patients; STAT, signal transducer and activator of transcription.
Data are presented as mean ± SE. $P<0.05$ is considered significant.

of atypical nevi was found to be upregulated from pretreatment values of 6.04 ± 1.25% rising to 12.27 ± 2.23% ($P = 0.0059$) with LDI, and from 2.88 ± 0.99% rising to 25.06 ± 9.88% ($P = 0.016$) with HDI. The pSTAT1/pSTAT3 ratio was upregulated from a baseline value of 0.24 ± 0.08 to 0.71 ± 0.19 ($P = 0.0040$) with LDI, and from 0.073 ± 0.03 to 1.51 ± 0.60 ($P = 0.008$) with HDI, respectively. Table 3 demonstrates changes in pSTAT3, which is downregulated 36% from baseline with LDI and 55% from baseline with HDI. Conversely, pSTAT1 expression was found to be upregulated by LDI (100%) and HDI (770%) over baseline. The ratio of pSTAT1/pSTAT3 was upregulated 2- and 20-fold from baseline ratios by LDI and HDI, respectively. Figure 4 demonstrates that the regulation of STAT signaling by IFNα is strongly dose-dependent. Figure 4a and b represent pre- and post-treatment images from subjects receiving LDI illustrating that LDI downregulates pSTAT3 and upregulates pSTAT1 significantly. Figure 4c and d represent pre- and post-treatment images obtained from subjects receiving HDI, showing downregulation of pSTAT3 and upregulation of pSTAT1 by HDI exceeding that observed with LDI.

Table 3. The fold of post-treatment pSTAT1 and pSTAT3 against baseline

	Fold against baseline		
	pSTAT1	pSTAT3	pSTAT1/pSTAT3
High-dose IFNα2b	7.7	−0.55	20.0
Low-dose IFNα2b	1.0	−0.36	2.0

pSTAT, phosphorylated signal transducer and activator of transcription. Data are presented as (post-treatment mean – pretreatment mean)/pretreatment mean.

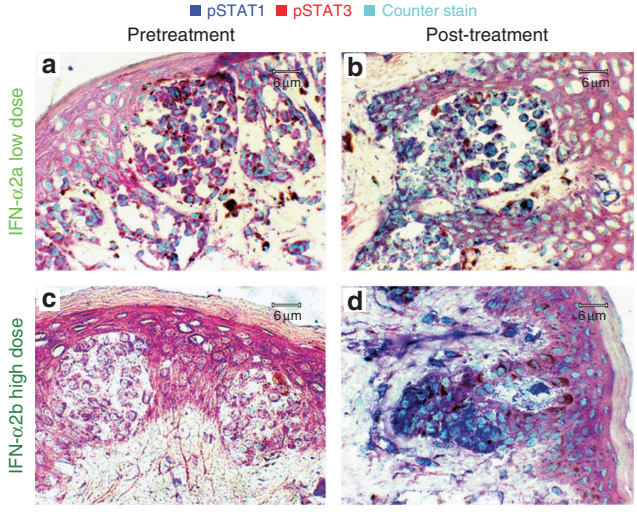

Figure 4. pSTAT1 and pSTAT3 immunohistochemistry of nevi. (**a** and **b**) The pre- and post-treatment with LDI, show that LDI downregulates pSTAT3 (red color) and upregulates pSTAT1 (blue color). (**c** and **d**) The pre- and post-treatment with HDI, show that HDI downregulates pSTAT3 (red color) and upregulates pSTAT1 (blue color) to a greater extent than LDI.

DISCUSSION

A defect in the IFNα promoter observed in early melanoma supports the consideration of IFN signaling as one component of the etiology of melanoma (Price *et al.*, 2005). A range of treatment regimens with IFN at various dosages has been explored for therapy of melanoma in multiple trials conducted worldwide over the past 20 years. At low dosages, IFNα2a delays recurrence of intermediate-risk melanoma patients (Grob *et al.*, 1998). At high dosages, we have previously demonstrated improvement in both relapse-free and overall survival of patients assessed at median intervals ranging from 2 to more than 16 years of follow-up (Kirkwood *et al.*, 1996, 2001, 2002). This study evaluated the effects of LDI and HDI on pSTAT1tyr701 and on the ratio of pSTAT1/pSTAT3 expression in nevi and demonstrated augmentation of both of these parameters as well as downregulation of pSTAT3tyr705 expression in the atypical nevi of melanoma patients who participated in the two clinical trials of IFNα evaluated here. This study provides data that are consistent with the results obtained in earlier biopsy studies of lymph node metastases we have conducted in the neoadjuvant setting (trial UPCI 00-008) (Moschos *et al.*, 2005; Wang *et al.*, 2007). This study documents the dose-dependent modulation of STAT signaling molecules in atypical/dysplastic melanocytic nevi of patients with melanoma. Analysis of dose–response effects have demonstrated greater magnitude of STAT modulation by HDI than LDI, with effects on pSTAT1, pSTAT3, and the pSTAT1/pSTAT3 ratio that are consistent with the results of clinical trials that have explored these regimens in the adjuvant therapy of intermediate- and high-risk melanoma, as already discussed. The impact of IFNα on the STAT1/STAT3 signaling pathways appears to predict the clinical outcome of patients treated in this series.

It has been documented by others that the antitumor effects of IFNα are abrogated in STAT1-deficient knockout mice (Lesinski *et al.*, 2003). Deficient IFN-induced STAT1 phosphorylation *in vitro* and *in vivo* has also been associated with melanoma (Kovarik *et al.*, 2003). Most recently, it has been demonstrated that deficient STAT1 phosphorylation in T cells of patients with melanoma can be corrected by exposure to high, but not low, concentrations of IFNα (Critchley-Thorne *et al.*, 2007). Those previous studies support the conclusion drawn from these data that IFN-mediated upregulation of pSTAT1 is important in relation to the effects of IFNα on melanoma and its precursors. Upregulation of pSTAT1 in melanoma cells associated with the antimelanoma effects of IFN has been documented *in vitro* (Gollob *et al.*, 2005). With melanocyte maturation from the dermoepidermal junction to the deeper dermis, we have observed that expression of pSTAT1 increases, whereas expression of pSTAT3 decreases. This observation is consistent with the previous studies, suggesting that pSTAT1 is associated with growth restraint and antagonizes the effects of STAT3 (Schindler *et al.*, 2007). We have noted that expression of pSTAT1 in metastatic tumor cells of regional lymph nodes is much higher than that in nevi and primary melanoma. We hypothesize that the host immune factors in

the lymph node lead to upregulation of pSTAT1 in melanoma cells and restraint of melanoma proliferation.

Constitutive expression of STAT3 is associated with melanoma and other solid tumors, and STAT3 has therefore been evaluated here in the melanocytes of clinically atypical and histopathologically dysplastic nevi. The role of STAT3 as a biomarker of progression has been evaluated in two prospective studies in which the impact of IFNα was evaluated at low or high dosages. The degree of pSTAT3 expression has been shown to be associated with the degree of severity of melanocytic atypia judged pathologically, and IFNα has been shown to downregulate pSTAT3 expression in the junctional melanocytes of atypical nevi, much as we have previously documented that it modulates STAT3 expression in regional nodal metastases of melanoma. It has been documented that activated pSTAT3 is a potential biomarker of cancer progression (Huang, 2007). STAT3 and microphthalmia-associated transcription factor cooperatively induce c-*fos*, resulting in cellular transformation *in vitro* (Joo *et al.*, 2004). STAT3 is a pivotal transcription factor in the regulation of cell motility and tumor progression (Gao and Bromberg, 2006; Yang *et al.*, 2006). STAT3 signaling, along with mitogen-activated protein kinase signaling, appear to be essential for immune evasion by human melanoma (Sumimoto *et al.*, 2006). We conclude that pSTAT3 is a promising biomarker of melanocytic progression, is associated with the histopathological dysplasia of melanocytic nevi, and merits further evaluation as a biomarker of melanoma progression in prospective adjuvant therapy trials as well as in prevention trials.

MATERIALS AND METHODS

Surgical specimens and patient treatment

Institutional review board-approved clinical protocols UPCI 96-043 and UPCI 95-071 were the basis of this study. Patients (42) with multiple ($\geqslant 4$) nevi of a diameter $\geqslant 4$ mm and a history of primary cutaneous melanoma were eligible for these studies. Patients (32) with low or intermediate risk of relapse (defined as a melanoma of less than stage IIB or III) were candidates for UPCI 96-043. The protocols of the Declaration of Helsinki Principles were followed, and all of the participating patients of the described research provided written informed consent. After providing written informed consent and having clinical photographic documentation, baseline biopsies were obtained during the course of this study; patients then received low-dose IFNα2a (LDI) therapy for 12 weeks. Patients (10) with a high-risk melanoma (defined as a melanoma of stage IIB or III) were candidates for UPCI 95-071, and following similar informed consent, these patients had clinical, photographic, and biopsy documentation and received high-dose IFNα2b as standard adjuvant therapy (HDI). Treatment with LDI used IFNα2a provided by Hoffmann La-Roche Pharmaceuticals (Nutley, NJ) (Roferon A), administered at a dosage of 3×10^6 U subcutaneously three times weekly for 12 weeks. Treatment with HDI used IFNα2b obtained commercially and was administered according to the FDA-approved E1684 regimen in which treatment was given intravenously daily for 5 days per week for 4 weeks at $20 \times 10^6\,\mathrm{U\,m^{-2}}$, followed by treatment given subcutaneously at $10 \times 10^6\,\mathrm{U\,m^{-2}}$ three times per week for 48 weeks. Each patient was examined by members of the dermatology and/or medical oncology faculty of the Melanoma and Skin Cancer Program of the UPCI and by allied health professionals in the Melanoma and Skin Cancer Program who have expertise in the clinical assessment of nevocellular nevi. Lesions were scored by consensus to select the four most atypical nevi of a diameter exceeding 4 mm in each participant. Nevi were then marked and denoted alphabetically A–D in descending order of clinical atypia, and photographically documented. The four nevi selected as the most atypical were required not to exhibit features that were regarded as suspicious of overt melanoma, and they were then assigned a random order for biopsy under the direction of the study statistician, through the Office of Clinical Research of the Melanoma and Skin Cancer Program. For assignment of biopsy order, a table of random numbers provided by the study statistician was used. The photographic documentation for this study is shown in Figure 1. Two atypical nevi chosen in this manner at random were scheduled for excisional biopsy performed before initiation of any treatment with LDI or HDI. The remaining two nevi were followed photographically through the course of 3 months of treatment at monthly intervals and resected post-treatment with LDI or at the third month of treatment with HDI. Nevi (168) were evaluated initially and prosected by the dermatopathologist prior to any research laboratory procedures. A portion of the biopsies was evaluated to confirm the diagnosis, and a portion of the remaining sample was then released by the Pathology Department for use in research purposes described in protocols UPCI 96-043 and UPCI 95-071.

Immunohistochemistry

Rabbit polyclonal anti-human pSTAT1tyr701 and anti-human pSTAT3tyr705 were purchased from Cell Signaling Technology (Beverly, MA). Unconjugated goat anti-rabbit IgG (H + L) antibody, which is used to block the first primary antibody in the double immunostain, was purchased from Vector Laboratories (Burlingame, CA).

Snap-frozen tissues were fixed in ice-cold acetone for 10 minutes. Indirect immunohistochemistry was performed to detect the specific antigens of concern with Vector Laboratories' Vectastain ABC System (alkaline phosphatase system), and double immunostains were performed according to the manufacturer's instructions.

Data analysis and statistics

The $\times 20$ objective was used to evaluate the whole section by two pathologists who were blinded in regard to treatment assignment. Quantitation was decided by consensus of the two pathologists and a research faculty member. The percentage of cells with positive staining was evaluated for all samples. Cells with staining intensity 1+ to 4+ were considered positive; cells without staining (intensity 0) were considered negative. If the percentage of stained cells was less than 1%, it was considered zero. Statistical analysis was performed by the UPCI Biostatistical Facility. Mean values of the percentage of positive cells were presented with SE. Comparisons between pre- and post-therapy samples were performed with Wilcoxon signed rank tests, in terms of the fraction of cells expressing the biomarkers studied and the ratios calculated. Wilcoxon rank sum tests were used to compare the percentage of cells expressing the biomarkers between different atypical degrees of nevi in the pretreatment biopsies. Spearman's rank correlation coefficients were computed to analyze the correlation between the

grades of atypia and the percentage of positive staining cells. Two-sided *P*-values were calculated and $P<0.05$ was considered significant.

CONFLICT OF INTEREST

John M. Kirkwood, MD, serves on the Speakers Bureau for Schering Plough Corp. and has received research funding from Schering Plough Corp. to the University of Pittsburgh.

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