

Prospective Molecular Profiling of Melanoma Metastases Suggests Classifiers of Immune Responsiveness

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Abstract

We amplified RNAs from 63 fine needle aspiration (FNA) samples from 37 s.c. melanoma metastases from 25 patients undergoing immunotherapy for hybridization to a 6108-gene human cDNA chip. By prospectively following the history of the lesions, we could correlate transcript patterns with clinical outcome. Cluster analysis revealed a tight relationship among autologous synchronously sampled tumors compared with unrelated lesions (average Pearson's $r = 0.83$ and 0.7 , respectively, $P < 0.0003$). As reported previously, two subgroups of metastatic melanoma lesions were identified that, however, had no predictive correlation with clinical outcome. Ranking of gene expression data from pretreatment samples identified ~30 genes predictive of clinical response ($P < 0.001$). Analysis of their annotations denoted that approximately half of them were related to T-cell regulation, suggesting that immune responsiveness might be predetermined by a tumor microenvironment conducive to immune recognition.

Introduction

Efforts aimed at the discovery of independent predictors of clinical outcome have identified molecular subsets of cancer based on mathematical analyses of their gene expression profiles (1–3). Subcategories of lymphomas (1) and breast carcinomas (3) with distinct prognostic and/or clinical behavior were recognized. Bittner *et al.* (2) suggested two biologically distinct molecular profiles of cutaneous melanoma lesions with divergent metastatic potential *in vitro* but unknown clinical relevance. In addition, one subclass was characterized by the enhanced expression of MART-1, a classic melanoma differentiation antigen. This triggered the hypothesis that perhaps two different disease taxonomies, both classified according to visual methods as melanoma, could be identified by total transcript analysis and might be characterized by different immune responsiveness. We, therefore, wondered whether direct *ex vivo* documentation of molecular portraits of metastatic melanoma lesions could prospectively identify taxonomically different entities of this disease or subsets related to its natural progression that could, in either case, be of clinical relevance (4). To account for experimental variance attributable to the intrinsic heterogeneity of different tumor deposits and/or the evolving genetic profiles of individual lesions with time, we directly correlated clinical information pertaining to each lesion with its biological profile. Thus, we collected serial FNAs² (23-gauge) of

individual lesions that allowed prospective documentation of their natural history and/or therapeutic outcome. Limitations attributable to the small amount of total RNA obtainable by FNA were circumvented by a recently validated amplification method (5). Because melanoma is a disease predisposed to different forms of immune modulation, in this exploratory study, emphasis was put on the intrinsic biology of individual lesions rather than the specificity of the immunotherapy administered. Thus, FNA samples obtained before conceptually similar but not identical forms of immunotherapy were studied with the goal of identifying candidate predictors of immune responsiveness based on the working hypothesis that common effector pathways may ultimately determine immune rejection of cancer.

Materials and Methods

FNAs were obtained before treatment in a period spanning from January 5, 1999 through December 16, 1999 from 37 metastases in 25 patients with metastatic melanoma referred to the Surgery Branch, National Cancer Institute for various immunotherapy treatments. The size of each metastasis was serially documented as two perpendicular diameters. The same operator (G. A. O.) performed the FNA by aspirating four quadrants of each lesion. To minimize RNA metabolism and degradation, the material was immediately placed at the bedside in ice-cold RPMI 1640 culture (Biofluids, Rockville, MD) without serum and carried on ice to the laboratory for processing. Over this period, ~300 FNAs of s.c. melanoma metastases were accrued. From those, thirty-seven s.c. lesions were selected from 25 patients based on quality of material available and relevant clinical outcome (Table 1). A second FNA was obtained, when possible after at least one course of treatment. Paired pre- and posttreatment samples were obtainable in 25 lesions (median follow-up, 11 weeks). Metastases were separated into groups: group 1, cr; group 2, pr (>50% reduction in the product of two perpendicular diameters); group 3, stable disease (<50% reduction and <25% increase); and group 4, no response (>25% increase). All responses lasted >30 days. Thirteen pretreatment FNAs were available from cr lesions. Of those, 4 (P3-a0-cr, P18-a0-cr, P18-b0-cr, and P21-a0-cr) regressed before follow-up FNAs could be obtained. Two pr, 8 sd, and 11 nr pretreatment FNAs were also available.

Microarrays. Total RNA extracted from FNA and control samples was transcribed *in vitro* into aRNA and reverse-transcribed into fluorescently labeled cDNA for hybridization to 6108 gene cDNA-based microarrays as described previously (5). Control samples consisted of NHEMs derived from neonatal foreskin and grown in melanocyte culture medium (Clonetics, San Diego, CA). The RCC, the P11-Mel melanoma cell line, and the fibroblast strain (FB) were expanded in standard RPMI 1640 containing 10% human AB serum from FNAs of metastatic renal cell (RCC) and a melanoma metastasis (P11-Mel and FB) cell lines. All three were used for the analysis before they reached the fifth passage in culture. Pooled peripheral blood mononuclear cells from six donors were used to prepare reference aRNA to be cohybridized in all

Received 11/26/01; accepted 5/6/02.

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²The abbreviations used are: FNA, fine needle aspiration; cr, complete regression; pr, partial regression; sd, stable disease; nr, no response; aRNA, antisense RNA; NHEM,

normal human epithelial melanocyte; RCC, renal cell carcinoma; TNF, tumor necrosis factor; IRF, IFN regulatory factor; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein.

Table 1 *Metastases that regressed completely in response to therapy*

The number following P refers to patient's identity. The following letter (a, b, . . .) identifies individual metastasis. The following number codes the relationship between FNA and treatment (0, pretreatment; 1, first and 2, second biopsy after treatment. Size of tumor, two orthogonal diameters. Time of FNA in relation to treatment (pre *versus* post). Treatment type, melanoma antigen targeted by the immunization. Systemic IL-2 corresponds to 720,000 IU/kg every 8 h to limit toxicity.

Case no.	Sex/Age	FNA site	Date of FNA	Size of metastasis	Time of FNA	Treatment type	Systemic IL-2 administration	Date of last follow-up	Size at last follow-up	Source total RNA (μg)	aRNA yield (μg)
P1-a0-cr	M/34	R Groin	10/7/1999	1 × 1	Pre	MART-1/GP	yes	2/29/2000	0	0	87
P1-a1-cr	M/34	R Groin	12/2/1999	1.5 × 1.5	Post	MART-1/GP	yes	2/29/2000	0	0	48
P2-a0-cr	M/52	L Parotid	6/22/1999	1.5 × 1.5	Pre	TRP-1	yes	10/15/1999	0	17	149
P2-a1-cr	M/52	L Parotid	8/6/1999	2 × 2	Post	TRP-1	yes	10/15/1999	0	14	140
P3-a0-cr	M/31	L Axilla	2/5/1999	2 × 2	Pre	TRP-1	yes	4/1/1999	0	10	73
P6-a0-cr	M/49	L Lat Knee	2/23/1999	2.5 × 2.5	Pre	GP	yes	7/29/1999	0	2	125
P6-a1-cr	M/49	L Lat Knee	6/29/1999	3 × 2.5	Post	GP	yes	7/29/1999	0	4	145
P6-b0-cr	M/49	L Med Knee	2/23/1999	1.5 × 1.5	Pre	GP	yes	7/29/2000	0	3	70
P6-b1-cr	M/49	L Med Knee	6/29/1999	1 × 1	Post	GP	yes	7/29/2000	0	3	195
P10-a0-cr	M/67	L Neck	3/17/1999	2 × 2	Pre	GP	no	8/31/1999	0	27	103
P10-a1-cr	M/67	L Neck	7/1/1999	2 × 3	Post	GP	no	8/31/1999	0	1	96
P14-a0-cr	M/49	R Face Inf	4/6/1999	1 × 2	Pre	GP	yes	8/11/1999	0	5	115
P14-a1-cr	M/49	R Face Inf	7/1/1999	2 × 2	Post	GP	yes	8/11/1999	0	1	77
P14-b0-cr	M/49	R Face Sup	3/19/1999	2 × 2	Pre	GP	yes	8/11/1999	0	1	92
P14-b1-cr	M/49	R Face Sup	7/1/1999	2 × 2	Post	GP	yes	8/11/1999	0	1	20
P16-a0-cr	M/62	R Chest	4/27/1999	1 × 1.5	Pre	MART-1	yes	9/1/1999	0	7	134
P16-a1-cr	M/62	R Chest	6/10/1999	1.5 × 1.5	Post	MART-1	yes	9/1/1999	0	12	143
P18-a0-cr	F/65	L Neck	6/8/1999	2 × 2	Pre	GP	yes	8/31/1999	0	4	51
P18-b0-cr	F/65	R Neck	6/8/1999	3 × 3	Pre	GP	yes	8/31/1999	0	5	37
P21-a0-cr	F/35	L Temple	9/21/1999	1 × 1.5	Pre	GP (cells)	no	11/3/1999	0	90	11
P23-a0-cr	M/53	R Thigh Dist	6/22/1999	2.5 × 2.5	Pre	MART-1	yes	1/6/2000	0	3	52
P23-a1-cr	M/53	R Thigh Low	9/30/1999	1 × 1.5	Post	MART-1	yes	1/6/2000	0	3	84
P23-b1-cr	M/53	R Thigh Prox	9/30/1999	1.5 × 2	Post	MART-1	yes	1/6/2000	0	2	9
Metastases that regressed partially in response to therapy											
P4-b0-pr	M/67	R Knee	3/11/1999	1 × 1	Pre	GP	yes	7/7/1999	0.5 × 0.5	23	147
P4-b1-pr	M/67	R Knee	6/22/1999	0.5 × 0.5	Post	GP	yes	7/7/1999	0.5 × 0.5	51	16
P20-a0-pr	M/54	R Groin	7/27/1999	4 × 4	Pre	MART-1/GP	no	9/7/1999	2 × 3	43	41
P20-a1-pr	M/54	R Groin	9/7/1999	2 × 3	Post	MART-1/GP	no	9/7/1999	2 × 3	8	25
Metastases that remained stable during the observation period											
P4-a0-sd	M/67	R Prox Leg	3/11/1999	2 × 2	Pre	GP	yes	7/7/1999	1.5 × 1.5	5	116
P4-a1-sd	M/67	R Prox Leg	6/22/1999	1.5 × 1.5	Post	GP	yes	7/7/1999	1.5 × 1.5	5	92
P5-a0-sd	F/58	L Groin	7/28/1999	1 × 1	Pre	GP-T cells	no	10/14/1999	1.5 × 1.0	0	118
P5-a1-sd	F/58	L Groin	8/23/1999	1.5 × 1.5	Post	GP-T cells	no	10/14/1999	1.5 × 1.0	0	33
P5-b0-sd	F/58	L Leg	7/28/1999	1 × 2	Pre	GP-T cells	no	10/14/1999	2 × 2	2	150
P5-b1-sd	F/58	L Leg	8/23/1999	2 × 2	Post	GP-T cells	no	10/14/1999	2 × 2	2	85
P8-b0-sd	M/74	R Arm	6/29/1999	3 × 3	Pre	GP	no	8/17/1999	3 × 3.5	2	147
P8-b1-sd	M/74	R Arm	8/17/1999	3 × 3.5	Post	GP	no	8/17/1999	3 × 3.5	14	166
P13-a0-sd	M/58	R Arm Inf	9/9/1999	2 × 2	Pre	GP	no	12/7/1999	2 × 2.5	3	101
P13-a1-sd	M/58	R Arm Inf	12/7/1999	2 × 2.5	Post	GP	no	12/7/1999	2 × 2.5	10	115
P13-b0-sd	M/58	R Arm Sup	9/9/1999	2 × 2	Pre	GP	no	12/7/1999	2.5 × 3.5	18	138
P21-b0-sd	F/35	R Occipital	9/21/1999	2.5 × 3	Pre	GP (cells)	no	11/3/1999	3 × 3.5	30	124
P21-b1-sd	F/35	R Occipital	11/3/1999	3 × 3.5	Post	GP (cells)	no	11/3/1999	3 × 3.5	37	102
P22-a0-sd	M/32	R Leg	4/29/1999	2 × 2	Pre	MART-1/GP	no	7/1/1999	2 × 2	8	62
Metastases that did not respond to therapy											
P7-a0-nr	F/76	L Breast	6/10/1999	1 × 2	Pre	GP	no	7/27/1999	2 × 2	0	116
P8-a0-nr	M/74	Abdomen	6/29/1999	4 × 6	Pre	GP	no	8/17/1999	7 × 7	2	65
P8-a1-nr	M/74	Abdomen	8/17/1999	7 × 7	Post	GP	no	8/17/1999	7 × 7	14	57
P9-a0-nr	F/59	L Scalp	1/5/1999	1 × 1	Pre	GP	yes	3/1/9399	3 × 3	3	241
P11-a0-nr	M/48	R Neck	5/27/1999	2.5 × 3	Pre	GP	yes	10/14/1999	9 × 10	9	41
P11-a1-nr	M/48	R Neck	7/13/1999	6 × 10	Post	GP	yes	10/14/1999	9 × 10	56	149
P12-a0-nr	F/58	R Thigh Dist	5/27/1999	1 × 1	Pre	GP	yes	9/3/1999	2 × 2	0	102
P12-a1-nr	F/58	R Thigh Dist	9/3/1999	2 × 2	Post	GP	yes	9/3/1999	2 × 2	1	98
P12-b0-nr	F/58	Epigastric	5/27/1999	1.5 × 1.5	Pre	GP	yes	9/3/1999	4 × 4	7	129
P12-b1-nr	F/58	Epigastric	9/3/1999	4 × 4	Post	GP	yes	9/3/1999	4 × 4	14	102
P12-c0-nr	F/58	R Thigh Prox	5/27/1999	3 × 3	Pre	GP	yes	12/15/1999	11.5 × 13	13	149
P12-c1-nr	F/58	R Thigh Prox	9/3/1999	10 × 13	Post	GP	yes	12/15/1999	11.5 × 13	13	128
P15-a1-nr	M/55	L Chest	1/21/1999	1 × 1	Post	GP	yes	3/4/1999	2 × 2	0	16
P17-a0-nr	M/52	L Supraclav	6/15/1999	1 × 2	Pre	GP	yes	9/30/1999	3 × 3.5	2	149
P17-a1-nr	M/52	L Supraclav	9/30/1999	3.0 × 3.5	Post	GP	yes	9/30/1999	3 × 3.5	3	111
P19-a0-nr	M/50	T Chest	3/11/1999	3 × 4	Pre	GP	yes	5/28/1999	8 × 8	2	16
P24-a0-nr	F/32	R Back	6/28/1999	2 × 2	Pre	none	yes	8/31/1999	4 × 5	19	113
P24-a1-nr	F/32	R Back	8/31/1999	4 × 4	Post	none	yes	8/31/1999	4 × 5	28	202
P25-a1-nr	F/51	L Abdomen	8/24/1999	2 × 2.5	Post	none	yes	12/16/1999	4 × 5	2	77
P25-a2-nr	F/51	L Abdomen	12/16/1999	4 × 5	Post	none	yes	12/16/1999	4 × 5	5	91
P25-b0-nr	F/51	R Abdomen	3/30/1999	2 × 3	Pre	none	yes	12/16/1999	6 × 8	7	118
P25-b1-nr	F/51	R Abdomen	12/16/1999	6 × 8	Post	none	yes	12/16/1999	6 × 8	27	130

experiments with test aRNA. cDNA targets were labeled with aRNA using Cy3 (green) for reference material and Cy5 (red) for test material. A $16 \times 20 \times 20$ (6400-spot) human cDNA microarray printed at Advanced Technology Center, NCI has 6108 sequence-verified clones representing 5492 unique genes and 537 expressed sequence tag clusters.

Statistical Methods. All statistical analyses were performed with SPLUS package. The log₁₀-based ratios were normalized by making the median value in an array equal to zero. Pearson correlation coefficients of log-ratios of two expression profiles were used to quantify the similarity between the samples. We visualized relationships among expression profiles by performing average linkage hierarchical clustering and multidimensional scaling analyses (6). In these analyses, we used one-correlation coefficient as the distance between pairs of samples. We performed these analyses both including all genes and including only genes showing high variation in log expression ratios across the entire set of samples. The results were similar, and the latter analyses are shown in this report. The variance of each gene across the entire set of samples was computed, and the median was determined. The genes with high variation were defined as those with variance significantly ($P < 0.001$ by χ^2 test) greater than this median. Two-sample *t*-statistics and Wilcoxon rank statistics were

used to identify the genes that are differentially expressed between two groups (such as cr versus other lesions, melanoma versus melanocytes, and others). We used relatively stringent cutoff levels for significance because of the number of genes being tested. To determine whether the number of differentially expressed genes is higher than expected because of chance, we randomly shuffled the phenotype labels (e.g., cr versus non-cr) and recomputed the two-sample *t*-statistics for each gene. This analysis was repeated 10,000 times, and the proportions of the random replications that resulted in as many significant genes as seen in the actual data were reported as the significance levels for the number of genes.

Paired value *t*-statistics and Wilcoxon rank statistics were used to identify the genes that have significant changes between pre- and posttreatment samples in each group. For the paired value *t* test, the labels of paired pre- and posttreatment samples were switched randomly, and such analysis was also repeated 1000 times to generate permutation-based statistical significance for the number of genes significantly changed between pre- and posttreatment samples ($P < 0.001$ by *t* test). To determine whether the association between pairs of synchronous lesions of the same patient is stronger than expected because of chance, we randomly shuffled the patient identifier labels for the

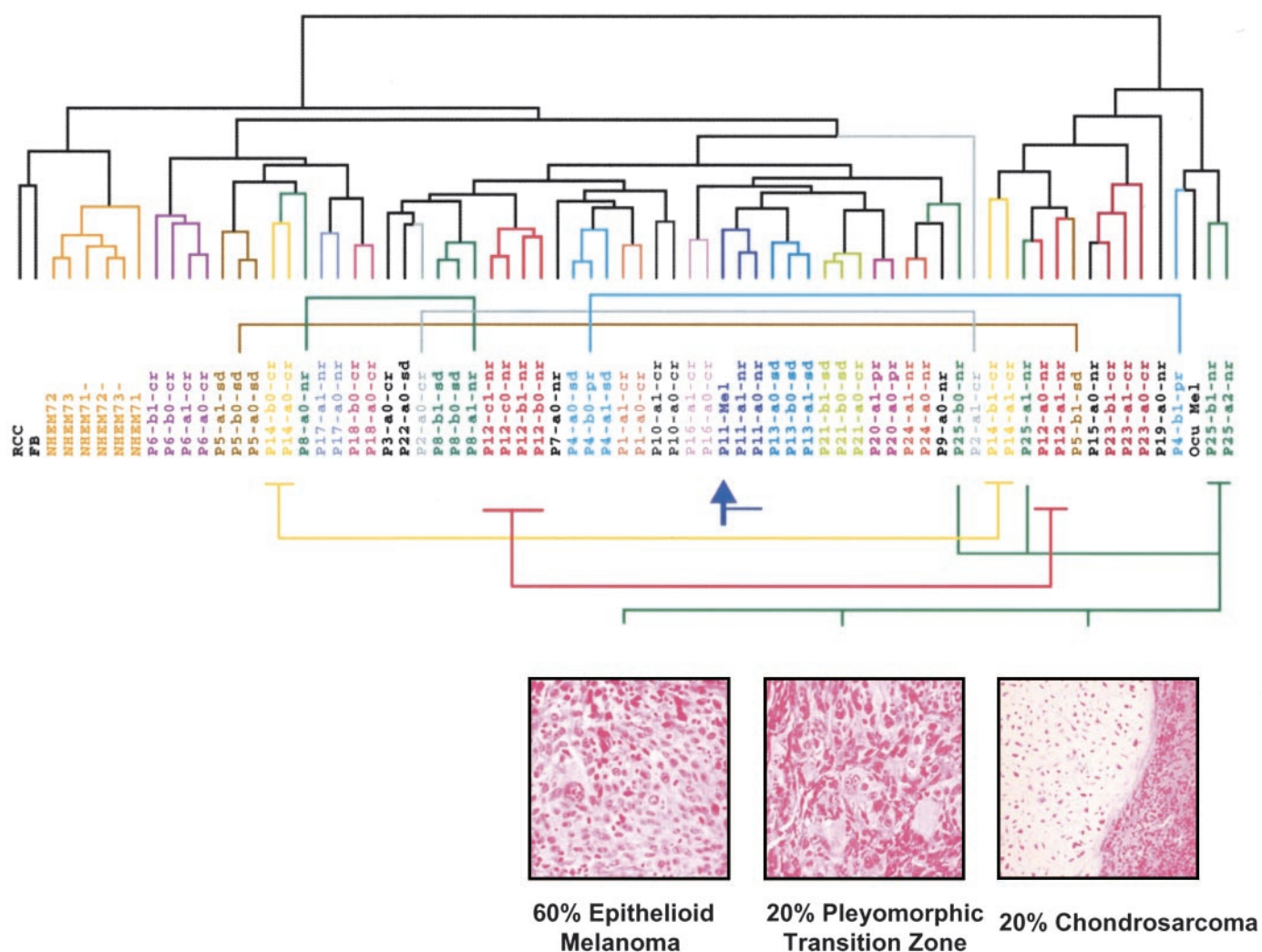


Fig. 1. Evolving molecular portraits of metastatic melanoma. Eisen's hierarchical clustering dendrogram of all samples studied applied to 4293 genes allowed by high-stringency filtering (Cy5: Cy3 ratios with a 3-fold change, signal intensity >500 unless other channel >3000 and 75% of the target pixel >1 SD above background). Control samples included: FB, fibroblast cell line derived from a melanoma metastasis; RCC, renal cancer cell line). The minus sign denotes that the melanocyte strain was cultured without growth factors for 48 h. P11-Mel, melanoma cell line derived from P11-a1 FNA; Ocu Mel, FNA of a hepatic metastasis from an ocular melanoma. FNA samples are color coded and numbered according to the identity of the patient (P). The letter after the number refers to lesion identity, whereas the following number refers to the order in which the FNA of that lesion was obtained (0, pretreatment; 1, first FNA after treatment; 2, second). The blue arrow points to the tight clustering of the melanoma cell line with the lesion from which it originated and underlines the ability of FNA-based global transcript analysis to identify clonal relatedness independently of infiltrating normal cells. Most often autologous FNAs clustered together; exceptions are underlined by colored lines consistent with the color assigned to the patient. In particular, the green line at the bottom of the cluster points shows molecular portrait changes in FNAs from P25 in a period spanning 9 months. P25-b1-nr and P25-a2-nr were excised shortly after the FNAs, and representative H&E stains are shown below for P25-b1-nr. Three different histological phenotypes were identified: epithelioid cell melanoma (60% of the lesion); pleiomorphic transition zone (20%); and chondrosarcomatous metaplasia (20%). This neoplastic metaplasia (19) could not be identified in previous samples from the same or other lesions.

synchronized pairs. The average correlation of log-ratios for synchronous pairs in the data set was compared with the distribution of average correlations based on 10,000 sets of randomly paired lesions.

Results and Discussion

Overview of Melanoma Metastases. The data set was globally screened by applying a high-stringency filter to minimize labeling or random hybridization bias (Ref. 5; Fig. 1). Clustering algorithms based on the resulting 4293 genes suggested a high degree of relatedness among simultaneously biopsied (synchronous) autologous lesions. Most metastases did not significantly change with time; however, exceptions were noted, as outlined by the color lines in Fig. 1. Two clusters were identified that included 49 and 14 samples, respectively. The larger cluster (cluster I) appeared closely associated with NHEMs. Correlation with clinical information did not segregate the two clusters into biologically distinct categories because metastases from the same patients biopsied at different time points were observed in either group. Instead, the smaller cluster (cluster II) appeared to portray a late/progression expression profile because its members included an inordinate proportion of later FNAs (16 of 49 pretreatment samples in cluster I *versus* 11 of 14 in cluster II; Fisher test $p_2 = 0.003$). Serially sampled lesions of some patients demonstrated a shift toward this subset with time, *i.e.*, later samples of FNA pairs shifted in cluster II (P4-b1-pr, P5-b1-sd, P14-a1, cr, P14-b1-cr, and P25-b1-nr). Thus, global transcript analysis failed to identify subsets of melanoma metastases with predictive value with regard to immune responsiveness but rather suggested a progressive drift in time of melanoma metastases away from NHEMs.

Treatment-dependent Adaptation in Expression of Individual Genes. Because the previous analyses indicated that immune responsiveness could not be predicted by a specific subset of melanoma metastases, we turned our attention to individual gene expression. By comparing individual gene expression in paired pre- and posttreatment samples, we observed that the number of genes differentially expressed in response to immunotherapy was greater in cr lesions. In particular, 17 genes were differentially expressed with a high degree

of statistical significance (t test, $P < 0.001$) in 9 pairs of cr lesions (Fig. 2A). Because when this large number of tests is performed a low P can simply be obtained by chance, we then performed a permutation analysis to address the frequency in which such a number of significant ($P < 0.001$) observations would occur by pure chance (see "Materials and Methods"). This was a significantly greater number than would be expected by chance as confirmed by permutation analysis ($P < 0.015$). This permutation test P gives a general estimate of the significance of the findings based on the complete data set and does not represent a correction for individual P s. When a similar analysis was performed on 14 pairs of lesions that did not undergo clinical regression, only 6 genes were identified to such degree of significance, a number close to the one expected by chance ($P < 0.2$). This observation suggests that clinical regression is associated with significant alterations in the transcriptional profile of tumors, whereas lack of response is associated with an indolent intratumor microenvironment rather than a turbulent reaction to a brisk immune response through the adoption of escape mechanisms (7). Analysis of the functional annotations of the genes identified by this analysis depicted a general pattern associated with increased tissue metabolism because it could be expected during tissue destruction and reactive repair mechanism. However, *IRF1* up-regulation implicated strong immune stimulatory conditions (see below).

Statistical Ranking of Individual Genes Differentially Expressed in Pretreatment Samples Suggests Classifiers of Immune Responsiveness. To avoid disturbances attributable to the effect of time and/or treatment, only pretreatment samples were analyzed further to test whether subsets with clinically divergent behavior could be identified. Lesions synchronously sampled before treatment from the same patients were significantly closer to each other than unrelated lesions (average $R = 0.83$ and 0.7 , respectively; permutation test, $P < 0.0001$). However, clustering of pretreatment samples could not identify clear subsets of melanoma. Nonparametric comparison of individual gene expression between 13 pretreatment cr samples and all other pretreatment lesions ($n = 21$) identified 14 genes differentially expressed to a $P < 0.001$ level of significance (Wilcoxon test;

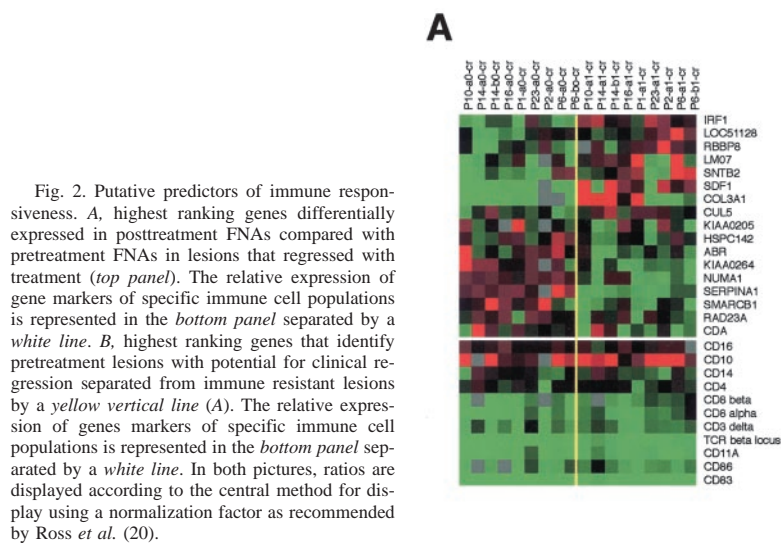


Fig. 2. Putative predictors of immune responsiveness. A, highest ranking genes differentially expressed in posttreatment FNAs compared with pretreatment FNAs in lesions that regressed with treatment (*top panel*). The relative expression of gene markers of specific immune cell populations is represented in the *bottom panel* separated by a white line. B, highest ranking genes that identify pretreatment lesions with potential for clinical regression separated from immune resistant lesions by a yellow vertical line (A). The relative expression of genes markers of specific immune cell populations is represented in the *bottom panel* separated by a white line. In both pictures, ratios are displayed according to the central method for display using a normalization factor as recommended by Ross *et al.* (20).

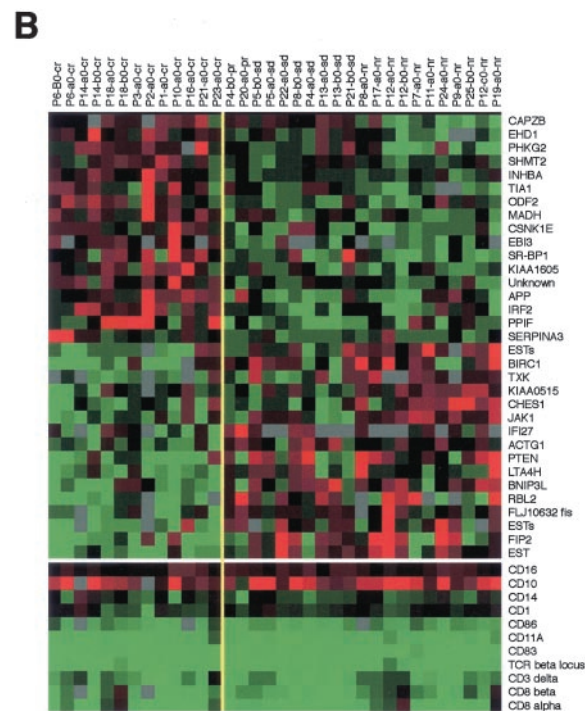


Table 2; Fig. 2B). Similarly, parametric analysis identified 18 genes with levels of expression significantly different at $P < 0.001$ (t test), mostly overlapping those identified by the Wilcoxon test. Permutation analysis established that the number of differentially expressed genes was significantly greater than that expected by chance ($P < 0.05$). To further explore the significance of these findings, we compared differences between 13 cr versus 11 nr pretreatment FNAs characterized by most dramatically divergent clinical behavior. Nonparametric analysis identified 13 differentially expressed genes (Wilcoxon $P < 0.001$) and parametric analysis identified 9 (t test $P < 0.001$) several overlapping with the previous data set. In total, the combined analysis identified 33 genes of potential interest. Seven genes had no annotated function. Review of available literature suggested that 12 of the remaining 26 genes were associated with immune/inflammatory function (Table 2, boldface entries). This rate was significantly higher than the frequency of genes with known immune regulatory function present in the cDNA chip (10% of 100 randomly selected clones, Fisher test $p_2 < 0.0001$). All of the statistical analyses described here (with the exception of the random sampling of 100 genes) were done *a priori* and independently of the characterization of the genes.

Among the genes preferentially expressed in responding lesions, *TIA-1* is a cytolytic granule component responsible for killing of CTL targets (8). *EBI3* has a facilitator role in the secretion of IL-12 (9) and is strongly associated with activation of antigen-presenting cells (10). Other genes are associated with transforming growth factor- β -like function (*MADH3*, *INHBA*; Ref. 11), IFN (IRF-2 and IF127), or TNF regulation (*FIP2*). Similarly, *JAK-1* and *Txk*, relatively suppressed in responding lesions, regulate T-cell signaling and differentiation. In addition, *IRF-2* expression coincided with the expression of a casein kinase belonging to a family of kinases possible associated with IRFs transcriptional activity (12). Of particular interest was the enhanced expression of IRF2 in immune-responsive lesions. Both *IRF1* (up-regulated in responding lesions) and *IRF2* may act as functional agonists and can modulate genital wart immune responsiveness through the *JAK/STAT* pathway (13) and the responsiveness of carcinoid tumors (14) and chronic myeloid leukemia (15) to IFN- α treatment and, in general, immune-mediated tumor suppression (16). The differential expression of immune-regulatory genes between immune-responsive and resistant lesions was not associated with significant differences in density of different immune cell populations, as

Table 2 Classifiers of immune responsiveness

Clone ID	Abbrev.	Title	Wilcoxon	t test	Putative function ^a	Wilcoxon	t test	Relative expression ^b
Genes that discriminate between pretreatment melanoma lesions that regressed completely or did not (13 versus 21)						13 cr vs. 11 nr		
195458	EST	EST	0.00004	0.00003	Unknown	0.00040	0.00031	Suppressed
768496	EBI3	EBI3	0.00004	0.00051	IL-12 facilitator	0.00005	0.00138	Enhanced
269815	INHBA	Inhibin, β A	0.00008	0.00013	TGF-β family	0.00312	0.00344	Enhanced
280752	RBL2	Retinoblastoma-like 2 (p130)	0.00018	0.00010	Putative tumor suppressor	0.02035	0.00500	Suppressed
811116	EST	EST	0.00029	0.00022	Unknown	0.00479	0.00520	Enhanced
504469	ODF2	Outer dense fibre of sperm tails 2	0.00040	0.00019	Cell migration	0.00885	0.00606	Enhanced
588637	ACTG1	Actin-cytoskeleton γ actin	0.00040	0.00022	Cell migration	0.00389	0.00184	Suppressed
323917	EHD1	EH domain containing 1	0.00044	0.00554	Ligand-induced endocytosis	0.00056	0.00397	Enhanced
365098	BNIP3L	BCL2/adenovirus E1B 19kD-interacting protein 3-like	0.00047	0.00013	Putative tumor suppressor	0.00092	0.00138	Suppressed
814095	LTA4H	Leukotriene A4 hydrolase	0.00047	0.00146	Immediate hypersensitivity	0.00385	0.00743	Suppressed
321739	IRF2	IFN- γ regulatory factor 2	0.00055	0.00019	IFN expression regulation	0.00727	0.00289	Enhanced
109316	Serpina3	α 1-antichymotrypsin	0.00055	0.00872	Proteinase inhibitor	0.01075	NS ^c	Enhanced
1457955	EST	EST	0.00076	0.00019	Unknown	0.00385	0.00175	Suppressed
51463	BIRC1	Neuronal apoptosis inhibitory protein	0.00095	0.00129	Apoptosis regulator	0.00562	0.01252	Suppressed
323371	APP	Amyloid β (A4) precursor protein	0.00100	0.00099	Intracellular signaling	0.00594	0.00402	Enhanced
142259	FIP2	Tumor necrosis factor α -inducible cellular protein	0.00118	0.00041	TNF pathway	0.00727	0.00441	Suppressed
854138	CSNK1E	Casein kinase 1, epsilon	0.00118	0.01792	DNA replication repair	0.00030	0.00258	Enhanced
322160	PTEN	MMAC1-PTEN-Tumor suppressor gene	0.00118	0.00067	Putative tumor suppressor	0.00482	0.00338	Suppressed
796297	KIAA1605	KIAA1605	0.00136	0.00051	Unknown	0.00092	0.00055	Enhanced
345935	MADH	MADH3	0.00157	0.00044	TGF-β response regulator	0.00053	0.00030	Enhanced
588915	IFI27	IFN, α -inducible protein 27	0.00176	0.01109	IFN-induced tumor suppressor	0.00098	NS	Suppressed
344080	SHMT2	Serine hydroxymethyltransferase mitochondrial precursor	0.00206	0.00097	Cell metabolism	0.00474	0.02665	Enhanced
240109	FLJ10632	<i>Homo sapiens</i> cDNA FLJ10632	0.00216	0.00065	Unknown	0.01587	0.01430	Suppressed
725284	PHKG2	Phosphorylase kinase, gamma 2	0.00238	0.00301	Kinase	0.00219	0.00091	Enhanced
240099	EST	EST	0.00327	0.01459	Unknown	0.00086	0.00514	Suppressed
324210	SR-BP1	Sigma receptor (SR31747 binding protein 1)	0.00354	0.00271	Cell proliferation regulation	0.00197	0.00085	Enhanced
43884	PPIF	Peptidylprolyl isomerase F (cyclophilin F)	0.00402	0.00099	Cell metabolism	NS	0.04096	Enhanced
563423	JAK1	JAK1	0.00830	0.01792	IL-2 receptor regulation	0.00008	0.00007	Suppressed
136218	TIA1	TIA1 cytotoxic granule-associated RNA-binding protein	0.01111	0.00642	CTL-mediated cytotoxicity	0.00056	0.00168	Enhanced
1103633	KIAA0515	KIAA0515	0.02179	0.03866	Unknown	0.00053	NS	Suppressed
322961	CAPZB	Capping protein (actin filament) muscle Z-line, beta	0.03756	0.02295	Cell migration	0.00055	0.00027	Enhanced
221846	CHES1	Checkpoint suppressor 1	0.04612	0.04285	DNA-damage checkpoint	0.00154	0.00071	Suppressed
148421	TXK	TXK tyrosine kinase	NS	NS	T-cell regulation	0.00156	0.00012	Suppressed
Abundance of immune cell marker genes in pretreatment melanoma lesions that regressed completely or did not (13 versus 21)						13 cr vs. 11 nr		
377560	CD3 delta	CD3 δ	ND	0.46798	T-cell marker	ND	0.50000	Equal
306841	TCR	T cell receptor beta locus	ND	0.68111	T-cell marker	ND	0.75879	Equal
771258	CD8 alpha	CD8 α chain	ND	0.85709	Cytotoxic T-cell marker	ND	0.39163	Equal
86189	CD4	CD4 (p55)	ND	0.09511	Helper T-cell marker	ND	0.34623	Equal
282679	CD16	CD16-Fc γ receptor IIIa	ND	0.62066	NK cell marker	ND	0.95314	Equal
154015	CD11A	CD11A-Integrin, α L-LFA-1 α chain	ND	0.84775	Leukocyte-associated marker	ND	0.80301	Equal
435434	CD14	CD14	ND	0.42577	Macrophage marker	ND	0.56275	Equal
50214	CD86	CD86, B72, CD28/CTLA-4 ligand	ND	0.37608	Macrophage/B-cell marker	ND	0.97991	Equal
564503	CD83	CD83-B-G antigen IgV domain homolog-HB15	ND	0.38766	Activated B cells and dendritic cells	ND	0.69852	Equal

^a Boldface entries show genes associated with immune/inflammatory function.

^b Relative expression of individual genes in responding compared with nonresponding lesions.

^c NS, not significant; ND, not done.

suggested by various differentiation markers present in the array (Table 2 and Fig. 2B). In addition, quantitative real-time PCR measurement demonstrated similar expression of lymphoid cell markers, such as T-cell receptor V β chain constant domain, CD3 and CD8 in *cr versus nr* lesions, while confirming the differential expression of immune-regulatory genes (data not shown).

This explorative study was designed to test the potential of using FNA-derived material to follow, by global transcript analysis, the progression of events occurring in the tumor microenvironment. Our results show that FNA sampling can allow direct documentation of evolving biological processes marking the natural course of a disease or its response to treatment. Subsets of melanoma metastases were identified that could be best explained by temporal changes in the transcriptional pattern of the disease. Because the lesions consisted of a relatively homogeneous collection of *s.c.* metastases, broader molecular diversity predictive of metastatic potential and tissue localization suggested by others (2) might have been missed.

Contrary to other studies in which global transcript analysis identified clinically relevant subsets of lymphomas and breast carcinomas (1, 3), we did not observe subsets of melanoma predictive of clinical outcome. Individual gene analysis, however, suggested that immune responsiveness may be predetermined and not solely dependent upon the extent of the immune responses elicited by a given treatment. In the same set of data, we have observed that melanoma metastases express a heterogeneous array of cytokines, growth factors, and metalloproteinases (17, 18). Among them, several have chemotactic properties on nucleated blood cells such as BLC, eotaxin, IL-1, IL-8, IL-16, lymphotactin, MCP-1, MCP-3, MCP-4, and RANTES whereas others display potent inflammatory activity such as IL-6, MIP-1 α , MIP-1 β , MIP-2 α (GRO 1/2), and TNF- γ . In addition, the expression profile was found to be surprisingly similar among various cytokines and correlated in most metastases with that of a subset of IFN-responsive elements including IRFs. Thus, it is possible that immune-stimulatory and/or inflammatory stimuli occurring at the tumor site may induce coordinate expression of several immune modulators in some tumors and predispose to immune rejection an otherwise dormant host's immune system tolerant of poorly immunogenic tumor cells. Our observations yield a novel hypothesis suggesting that responsiveness of melanoma metastases to immunotherapy is predetermined. Because the study was based on a patient population receiving conceptually similar yet heterogeneous therapy, these findings have only exploratory significance, and future studies should address their predictive significance in the context of different therapies.

Acknowledgments

We acknowledge Douglas E. Kesselring for the expert preparation of the visual graphics.

References

- Alizadeh, A. A., Eisen, M. B., Davis, R. E., Ma, C., Lossos, I. S., Rosenwald, A., Bedrick, J. C., Sabet, H., Tran, T., Xin, Y., Powell, J. I., Yang, L., Marti, G. E., Moore, T., Hudson, J., Jr., Lisheng, L., Lewis, D. B., Tibshirani, R., Sherlock, G., Chan, W. C., Greiner, T. C., Weisenburger, D. D., Armitage, J. O., Warnke, R., Levy, R., Wilson, W., Grever, M. R., Byrd, J. C., Botstein, D., Brown, P. O., and Staudt, L. M. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature (Lond.)*, *403*: 467–578, 2000.
- Bittner, M., Meltzer, P., Chen, Y., Jiang, E., Seftor, E., Hendrix, M., Radmacher, M., Simon, R., Yakhini, Z., Ben-Dor, A., Dougherty, E., Wang, E., Marincola, F. M., Gooden, C., Lueders, J., Glatfelter, A., Pollock, P., Gillanders, E., Dietrich, K., Alberts, D., Sondak, V. K., Hayward, N., and Trent, J. M. Molecular classification of cutaneous malignant melanoma by gene expression: shifting from a continuous spectrum to distinct biologic entities. *Nature (Lond.)*, *406*: 536–540, 2000.
- Perou, C. M., Sertle, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., al-Katib, A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S. X., Lenning, P. E., Berresen-Dale, A.-L., Brown, P. O., and Botstein, D. Molecular portraits of human breast tumors. *Nature (Lond.)*, *406*: 747–752, 2000.
- Wang, E., and Marincola, F. M. A natural history of melanoma: serial gene expression analysis. *Immunol. Today*, *21*: 619–623, 2000.
- Wang, E., Miller, L., Ohnmacht, G. A., Liu, E., and Marincola, F. M. High fidelity mRNA amplification for gene profiling using cDNA microarrays. *Nat. Biotech.*, *17*: 457–459, 2000.
- Khan, J., Simon, R., Bittner, M., Chen, Y., Leighton, S. B., Pohida, T., Smith, P. D., Jiang, Y., Gooden, G. C., Trent, J. M., and Meltzer, P. S. Gene expression profiling of alveolar rhabdomyosarcoma with cDNA microarrays. *Cancer Res.*, *58*: 5009–5013, 1998.
- Marincola, F. M., Jaffe, E. M., Hicklin, D. J., and Ferrone, S. Escape of human solid tumors from T cell recognition: molecular mechanisms and functional significance. *Adv. Immunol.*, *74*: 181–273, 2000.
- Tian, Q., Streuli, M., Saito, H., Schlossman, S. F., and Anderson, P. A polyadenylate binding protein localized to the granules of cytolytic lymphocytes induces DNA fragmentation in target cells. *Cell*, *67*: 629–639, 1991.
- Devergne, O., Birkenbach, M., and Kieff, E. Epstein-Barr virus-induced gene 3 and the p35 subunit of interleukin-12 form a novel heterodimeric hematopoietin. *Proc. Natl. Acad. Sci. USA*, *94*: 12041–12046, 1997.
- Hashimoto, S.-I., Suzuki, E., Nagai, S., Yamashita, T., Toyoda, N., and Matsushima, K. Identification of genes specifically expressed in human activated and mature dendritic cells through serial analysis of gene expression. *Blood*, *96*: 2206–2214, 2000.
- Zhang, Y., Feng, X., and Derynck, R. Receptor-associated Mad homologues synergize as effectors of the TGF- β response. *Nature (Lond.)*, *383*: 168–172, 1996.
- Lin, R., and Hiscott, J. A role for casein kinase II phosphorylation in the regulation of IRF-1 transcriptional activity. *Mol. Cell. Biol.*, *19*: 169–180, 1999.
- Arany, I., Tyring, S. K., Brysk, M. M., Stanley, M. A., Tomai, M. A., Miller, R. L., Smith, M. H., McDermott, D. J., and Slade, H. B. Correlation between pretreatment levels of interferon response genes and clinical responses to an immune response modifier (Imiquimod) in genital warts. *Antimicrob. Agents Chemother.*, *44*: 1869–1873, 2000.
- Zhuo, Y., Wang, S., Gobl, A., and Oberg, K. The interferon- α regulation of interferon regulatory factor 1 (IRF-1) and IRF-2 has therapeutic implications in carcinoid tumors. *Ann. Oncol.*, *11*: 707–714, 2000.
- Hochhaus, A., Yan, X. H., Willer, A., Hehlmann, R., Gordon, M. Y., Goldman, J. M., and Melo, J. V. Expression of interferon regulatory factor (IRF) genes and response to interferon- α in chronic myeloid leukemia. *Leukemia (Baltimore)*, *11*: 933–939, 1997.
- Taniguchi, T. Transcription factors IRF-1 and IRF-2: linking the immune responses and tumor suppression. *J. Cell. Physiol.*, *173*: 128–130, 1997.
- Mocellin, S., Wang, E., and Marincola, F. M. Cytokine and immune response in the tumor microenvironment. *J. Immunother.*, *24*: 392–407, 2001.
- Marincola, F. M., Wang, E., Herlyn, M., Seliger, B., and Ferrone, S. Tumors as elusive targets for immunotherapy. *Lancet Oncol.*, in press, 2002.
- Banerjee, S. S., Coyne, J. D., Menasce, L. P., Lobo, C. J., and Hirsch, P. J. Diagnostic lessons of mucosal melanoma with osteocartilagenous differentiation. *Histopathology*, *33*: 255–260, 1998.
- Ross, D. T., Scherf, U., Eisen, M. B., Perou, C. M., Rees, C. A., Spellman, P. T., Iyer, V., Jeffrey, S. S., van de Rijn, M., Waltham, M., Pergamenschikov, A., Lee, J. C., Lashkari, D., Shalon, D., Myers, T. G., Weinstein, J. N., Botstein, D., and Brown, P. O. Systematic variation in gene expression patterns in human cancer cell lines. *Nat. Genet.*, *24*: 227–235, 2000.

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Cancer Res 2002;62:3581-3586.

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