Novel Genes Associated with Malignant Melanoma but not Benign Melanocytic Lesions

Dmitri Talantov, Abhijit Mazumder, Jack X. Yu, Thomas Briggs, Yuqiu Jiang
John Backus, David Atkins, and Yixin Wang

Abstract

Purpose: Cutaneous melanoma is a common, aggressive cancer with increasing incidence. The identification of melanoma-specific deregulated genes could provide molecular markers for lymph node staging assays and further insight into melanoma tumorigenesis.

Experimental Design: Total RNA isolated from 45 primary melanoma, 18 benign skin nevi, and 7 normal skin tissue specimens were analyzed on an Affymetrix Hu133A microarray containing 22,000 probe sets.

Results: Hierarchical clustering revealed a distinct separation of the melanoma samples from the benign and normal specimens. Novel genes associated with malignant melanoma were identified. Differential gene expression of two melanoma-specific genes, *PLAB* and *L1CAM*, were tested by a one-step quantitative reverse transcription-PCR assay on primary malignant melanoma, benign nevi, and normal skin samples, as well as on malignant melanoma lymph node metastasis and melanoma-free lymph nodes. The performance of the markers was compared with conventional melanoma markers such as tyrosinase, gp100, and MART1.

Conclusion: Our study systematically identified novel melanoma-specific genes and showed the feasibility of using a combination of *PLAB* and *L1CAM* in a reverse transcription-PCR assay to differentiate clinically relevant samples containing benign or malignant melanocytes.

Cutaneous malignant melanoma is a serious healthcare problem with >55,100 new cases anticipated in 2004 in the U.S., with a mortality rate of about 14.5% (1). The incidence of melanoma continues to increase faster than that of any other malignancy (2). Although the prognosis for early local melanoma is favorable with 5-year overall survival of >90%, regional lymph node involvement decreases the overall survival rate by 10% to 46% (3). Therefore, regional lymph node status becomes the most significant prognostic factor for a melanoma patient's survival. Introduction of the sentinel lymph nodes technique (4) has increased the sensitivity of melanoma micrometastasis detection compared to H&E staining alone (5, 6). Nevertheless, even with enhanced by immunohistochemistry, histologic analysis is limited by the ability of light microscopy to recognize the tumor cells. Reverse transcription-PCR (RT-PCR) analysis has recently been proposed for a more sensitive detection of melanoma cells in lymph nodes. Many studies, when using well-characterized melanocyte-specific markers, such as tyrosinase and MART-1, have shown the presence of these gene transcripts in the lymph node, which were negative when using routine histologic and immunohistochemical methods (7, 8). However, these genes are not specific to tumor cells and cannot be used to discriminate between benign and malignant tissue. In fact, they cause false-positive results in the presence of benign capsular nevi (9–11). Considering that benign nevi are not rare events in the melanoma sentinel lymph nodes, the current RT-PCR assays are not clinically useful for the diagnostics of melanoma micrometastasis. Hoon et al. proposed a multimarker panel, including cancer-specific markers, for RT-PCR assay in order to increase assay specificity (11). The identification of novel melanoma-specific markers remains one of the key objectives in melanoma research.

High-density microarrays have been applied to simultaneously monitor the expression of thousands of genes in biological samples. Studies have resulted in the identification of genes differentially expressed in benign and malignant lesions (12), as well as genes that might be of prognostic value (13). In their pioneering work, Bittner et al. (14) did gene expression profiling of malignant melanoma, using a microarray containing probes for 8,150 cDNAs. These researchers identified several genes that might be associated with aggressive tumor behavior. In the recent work of Hoek et al. (15), comparison of gene expression profiles of a few melanoma and normal melanocyte cell lines led to the identification of differentially expressed genes and pathways modulated in melanoma. In this study, we report the gene expression profiling of an extensive set of clinically relevant tissue samples. Forty-five primary malignant melanomas, 18

Authors' Affiliation: Veridex, LLC, a Johnson & Johnson Company, San Diego, California

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Requests for reprints: Yixin Wang, Veridex, LLC, a Johnson & Johnson Company, 3210 Merryfield Row, San Diego, CA 92121. Phone: 858-784-3295; Fax: 858-450-2070; E-mail: ywang44@vrxus.jnj.com.

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benign skin nevi, and 7 normal skin tissues were hybridized on an Affymetrix Hu133A microarray (Santa Clara, CA) containing 22,000 probe sets. Differentially expressed genes in malignant melanoma, as compared with benign tissue, were identified. A one-step quantitative RT-PCR assay was used to test a combination of two melanoma-specific genes, *PLAB* and *L1CAM*, in a panel of clinically relevant samples that included primary malignant melanoma, benign nevi, melanoma lymph node metastasis and melanoma-free lymph node samples.

Materials and Methods

Tissue samples. Fresh-frozen malignant melanoma, benign skin nevi, normal skin, melanoma lymph node metastasis, and melanoma-free lymph node samples were obtained from Genomics Collaborative, Inc. (Cambridge, MA), Asterand (Detroit, MI), Clinomics (Pittsfield, MA), Proteogenex (Los Angeles, CA), Ardais (Lexington, MA), and Impath (Westborough, MA). All tissue vendors declared that tissue specimens used in the presented study were collected according to an Institutional Review Board–approved protocol of corresponding hospitals and principles of bioethics. Patient demographics and pathology information were also collected. The histopathologic features of each sample were reviewed to confirm diagnosis and to estimate sample preservation and tumor content. Melanoma and benign nevi primary tissues chosen for microarray analysis had melanocyte content of >50% with no mixed histology. Melanoma-positive lymph nodes were collected from malignant melanoma patients and the diagnosis of melanoma was confirmed by H&E in combination with immunohistochemistry (S100 and HMB45). Melanoma-free lymph nodes derived from patients that did not have melanoma in their clinical history and absence of melanoma was confirmed by H&E and immunohistochemistry using antibodies for S100 and HMB45.

RNA isolation and microarray hybridization. Qiagen RNeasy mini kit (Qiagen Inc., Valencia, CA) was used with a modified protocol to minimize the residue melanin in the RNA sample. For melanocyte-containing tissues, four replicate tissue samples derived from individual patients, each weighed ~5 mg, were used and processed separately. Tissue samples were homogenized in 1.0 mL RLT buffer (Qiagen) containing 10 μL of β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) by a mechanical homogenizer (UltraTurrex T8, IKA-Werke, Staufen, Germany). Following homogenization, samples were loaded on Qiagen RNeasy columns and followed by centrifugation. After discarding the flow-through, 700 mL of RW1 buffer was added. The column was kept at room temperature for 5 minutes and then centrifuged; this step was repeated thrice. Then we followed the standard Qiagen RNeasy mini kit protocol. To remove RNA from the silica gel membrane, a two-step elution was done. The total RNA derived from the same individual patient tissue was pooled and used for further analysis.

Standard Trizol protocol was used for RNA isolation from tissues that do not contain a significant proportion of melanocytes. Tissue was homogenized in Trizol reagent (Invitrogen, Carlsbad, CA). After centrifugation, the top liquid phase was collected and total RNA was precipitated with isopropyl alcohol at −20°C. RNA pellets were washed with 75% ethanol, resolved in water and stored at −80°C until use. The RNA quality was examined with an Agilent 2100 Bioanalyzer RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA).

Labeled cRNA was prepared and hybridized with the high-density oligonucleotide array Hu133A Gene Chip (Affymetrix) containing a total of 22,000 probe sets according to standard manufacturer protocols. Arrays were scanned using Affymetrix protocols and scanners. For subsequent analysis, each probe set was considered as a separate gene. Expression values for each gene were calculated by using Affymetrix Gene Chip analysis software MAS 5.0. Arrays that met three quality control standards were used: "present" call was >35%, scale factor was <12 when scaled to a target intensity of 600, and background level was <150. A lower than usual percentage of "present" calls cutoff was chosen because it is difficult to isolate undegraded RNA from the skin samples (16), resulting in lower overall gene expression levels.

Data analysis. Gene expression data were filtered to include only genes that are called "present" in two or more samples. This filter was used to remove genes that did not change expression in the samples. Of the 22,000 genes presented on the array, 15,795 passed this filter and were used for hierarchical clustering. Prior to clustering, each gene expression signal was divided by the median expression in all the samples in the data set. This standardization step minimized the effect of the magnitude of gene expression and grouped together genes with similar expression patterns in the clustering analysis. Average linkage hierarchical clustering using Pearson correlation was done on both the genes and the samples using GeneSpring 6.1 software.

In order to identify differentially expressed genes, we compared the melanoma samples to the benign nevi and the normal skin samples separately. The first analysis consisted of the 45 melanoma and 7 normal skin samples; the second analysis consisted of 45 melanoma and 18 nevi samples. Significance analysis of microarrays (17), percentile analysis, and *t* test were used in gene selection (Supplementary Fig. S1). We selected a short list of genes with at least 10-fold overexpression in melanoma as compared with the benign specimens. The complete array data set has been submitted to the National Center for Biotechnology Information/Genbank GEO database (series entry GSE3189).

Reverse transcription-PCR validation of microarray results. Ten micrograms of total RNA from each sample were treated with DNase I and reverse-transcribed with oligo (dT) primer using Superscript II reverse transcriptase according to the manufacturer's instructions (Invitrogen). A control gene *PBGD* was previously tested and reported as a housekeeping gene (18). Primers and MGB-probes for *me20m* (gp100), *L1CAM*, *NTRK3*, and the control gene *PBGD* were designed using Primer Express software (Applied Biosystems, Foster City, CA). The *PLAB* (MIC1) gene probe was FAM-TAMRA-based because sequences were inadequate to design MGB based probes. Primer/probe sequences were as follows: *me20m* (gp100) forward, 5′-TGTGTCTCTGGCTGATACCAACA-3′; *me20m* (gp100) reverse, 5′-TTCTTGACCAGGCATGATAAGCT-3′; *me20m* (gp100) probe, 5′-(6-FAM) CTGGCAGTGGTCAGC-3′; *L1CAM* forward, 5′-GCTGGGACTGGGAACAGAACT-3′; *L1CAM* reverse, 5′-GGAGCAGAGATGGCAAAGAAA-3′; *L1CAM* probe, 5′-(6-FAM) TCCCCACCATCTGCTGT-3′; *NTRK3* forward, 5′-GCCCCGGCACCCTTTA-3′; *NTRK3* reverse, 5′-AACCCTGCCAGTGGTGGAT-3′; *NTRK3* probe, 5′-(6-FAM) CAGATGGGTGTTTTC-3′; *PLAB* (MIC1) forward, 5′-GGCAGAATCTTCGTCCGCA-3′; *PLAB* (MIC1) reverse, 5′-GGACAGTGGTCCCCGTTG-3′; *PLAB* (MIC1) probe, 5′-(6-FAM) CCCAGCTGGAGTTGCACTTGCGGCC(TAMRA)-3′; *PBGD* forward, 5′-CTGCTTCGCTGCATCGCTGAAA-3′; *PBGD* reverse, 5′-CAGACTCCTCCAGTCAGGTACA-3′; *PBGD* probe, 5′-(6-FAM) CCTGAGGCACCTGGAAGGAGGCTGCAGTGT(TAMRA)-3′. All primers and probes were tested for optimal amplification efficiency above 90%. The standard curve was composed of six 10-fold dilutions of target gene PCR product with copy numbers ranging from 10 to 10^6. RT-PCR amplification was carried out in a 20 μL reaction mix containing 50 ng template cDNA, 2× TaqMan universal PCR master mix (12.5 μL; Applied Biosystems), 500 nmol/L forward and reverse primers, and 250 nmol/L probe. Reactions were run on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The cycling conditions were: 2 minutes of AmpErase UNG activation at 50°C, 10 minutes of polymerase activation at 95°C and 50 cycles at 95°C for 15 seconds and annealing temperature (60°C) for 60 seconds. In each assay, a standard curve and a no-template control along with template cDNA were included in duplicate for both the gene of

interest and the control gene. The relative quantity of each target gene was represented as ΔCt, which is equal to Ct of the target gene subtracted by Ct of the control gene.

One-step quantitative reverse transcription-PCR assay on primary and lymph node samples. Evaluation of expression of selected genes was carried out with one-step RT-PCR with RNA from primary melanoma, benign nevi, normal skin, melanoma lymph node metastasis, and melanoma-free lymph nodes. *β-Actin* was used as a housekeeping gene to control for the input quantity and quality of RNA in the reactions. DNase treatment was not used. Instead, primers or probes were designed to span an intron so they would not report on genomic DNA. Eight nanograms of total RNA were used for the RT-PCR. The total RNA was reverse-transcribed using 40× Multiscribe and RNase inhibitor mix contained in the TaqMan one-step PCR Master Mix reagents kit (Applied Biosystems). The cDNA was then subjected to the 2× Master Mix without UNG, and PCR amplification was carried out on the ABI 7900HT sequence detection system (Applied Biosystems) in the 384-well block format using a 10 μL reaction size. The primer and probe concentrations were 4 and 2.5 μmol/L, respectively. The reaction mixture was incubated at 48°C for 30 minutes for the reverse transcription, followed by a Amplitaq activation step of 95°C for 10 minutes and finally 40 cycles of 95°C for 15 seconds denaturing and 60°C for 1 minute annealing and extension. On each plate, a standard curve is generated from 8 pg to 80 ng, and when the R^2 value was >0.99, the cycle threshold (Ct) values were accepted. Sequences used in the reactions were as follows, each written in the 5′ to 3′ direction: *L1CAM* forward, CCACAGATGACATCAGCCTCAA; *L1CAM* reverse, GGTCACACCCAGCTCTTCCTT; *L1CAM* probe, TGGCAAGCCCGAAGTGCAGTTCC; *tyrosinase* forward, CTTTAGAAATACACTGGAAGGATTTGCTA; *tyrosinase* reverse, CATTGTGCATGCTGCTTTGA; *tyrosinase* probe, TCCACTTACTGGGATAGCGGATGCCTC; *MART1* forward, ACTTCATCTATGGTTACCCCAAGAA; *MART1* reverse, TCCCAGCGGCCTCTTCA; *MART1* probe, CACGGCCACTCTTACACCACGGC; *me20m* forward, CTTAAGGCTGGTGAAGAGACAAGTC; *me20m* reverse, CAGGATCTCGGCACTTTCAATAC; *me20m* probe, TCGATATGGTTCCTTTTCCGTCACCCTG; *PLAB* forward, ATTCGAACACCGACCTCGTC; *PLAB* reverse, CGCAGGTGCAGGTGGC; *PLAB* probe, GATACTCACGCCAGAAGTGCGGCT. All primers and probes were optimized towards the same amplification efficiency.

For each sample ΔCt = Ct (target gene) − Ct *β-actin* was calculated. ΔCt normalization has been widely used in clinical RT-PCR assays and was chosen as a straightforward method (19). For each gene marker, *t* test was done on ΔCt between the melanoma and nonmelanoma samples, including both primary and lymph node samples. We then used the ΔCt to construct two scores for each patient. One score was derived from a combination of two melanoma-specific genes, *PLAB* and *L1CAM*; and the other score was derived from a combination of three conventional melanoma markers, *tyrosinase, me20m,* and *MART1*. The score was defined as the weighted sum of ΔCt values of the tested genes with the corresponding *t* statistics as the weight. The two scores were normalized to have the same mean in order to compare them on the same scale.

Results

Patient clinical and pathologic characteristics. The majority of primary melanoma included in the microarray analysis represent the early stage of disease and have thicknesses of <4 mm, which is consistent with the standard melanoma patient population (20). Patient demographic, clinical, and pathology characteristics are presented in Table 1. In addition, 77 malignant melanoma lymph node metastasis and 18 melanoma-free lymph node tissue samples were used for one-step quantitative PCR assay. Melanoma-positive lymph nodes included axillary, cervical, and inguinal lymph nodes with metastasis derived from epithelioid and spindle cell primary melanomas. Out of 18 melanoma-free lymph nodes, 10 were collected from other cancer patients but no cancer cells were found in these nodes by pathologists, and 8 lymph node were from nonmalignant lesions.

Identification of genes differentially expressed in melanoma. A total of 70 gene expression profiles were used for analysis. The median percentages of "present" calls for melanoma, benign and normal sample groups were 43.8%, 46.9%, and 41.7%. Sixty microarrays (86%) had scaling factors within 3-fold range of the minimum value. Ten chips with the scaling factors >3 were equally distributed between the sample categories, melanoma, benign, and normal.

Unsupervised hierarchical clustering results revealed a distinct separation of the melanoma, benign nevi, and normal skin samples (Fig. 1). We observed four clusters, including two clusters consisting of the majority of the melanoma samples (43 out of 45), the third cluster contained all 7 normal skin, 3 benign nevi, and 2 melanoma specimens and the fourth cluster, that included 14 of the 18 benign nevi samples. The source of the samples didn't affect clustering. Specimens originating from different sources were clustered together according to the sample type (melanoma, benign, or normal). To further test the stability of the clustering patterns, we used an alternative cutoff on gene filtering prior to the cluster analysis. Specifically, we retained genes that have at least 10% "present" calls in each of the melanoma, benign nevi, and skin samples. With this cutoff, we obtained 15,306 genes and repeated hierarchical clustering. The cluster pattern on the patient samples was the same as the one from the 15,795 from the two "present" calls, confirming clustering stability.

The single nevi sample that clustered with the melanoma samples is an atypical nevi (moderate degree) sample with no melanoma *in situ* present. All three nevi samples that clustered with normal skin are compound nevi samples and one of them has a melanocyte content lower than the other nevi specimens. The melanoma samples themselves formed two clusters with 34 samples in the large and 9 samples in the smaller cluster. Samples that formed the small cluster represented epithelioid melanoma only and visually contained less melanin. The few stage III and IV tumors used in our study were all grouped in the small cluster. The large cluster was composed of specimens with epithelioid, spindle cell, and melanoma of mixed histology with more significant presence of melanin. The large cluster included stage I and II specimens only.

Distinct gene clusters were found in association with melanoma. This can be characterized by up-regulated (Fig. 1A, B, and C) and down-regulated (Fig. 1E) genes in the melanoma samples. At the same time, melanoma and benign nevi samples showed high expression of known melanocyte markers, such as *MART-1* (Fig. 2D), confirming the comparable content of melanocytes in these samples and the inability of melanocyte-specific markers to differentiate them. Our data indicates that melanoma, benign nevi, and normal skin samples have distinct gene expression profiles and can be separated on a molecular basis.

In order to identify genes up-regulated in malignant melanoma, we applied significance analysis of microarrays, in combination with *t* test with Bonferroni correction and

Table 1. Summary of patient information

Characteristics	Melanoma patients (%)	Nevi patients (%)	Normal skin donors (%)
Mean age	65.51 ± 14.55	33.17 ± 15.60	n/a
Gender			
Female	22 (48.9)	9 (50)	6
Male	23 (51.1)	9 (50)	1
Anatomic location			
Face	5 (11.1)	3 (17)	
Scalp and neck	4 (8.9)	1 (6)	
Trunk	9 (20)	10 (55)	6 (86)
Upper limb and shoulder	17 (37.8)		
Lower limb and hip	6 (13.3)	4 (22)	
Skin, NOS	4 (8.9)		1 (14)
Histologic diagnosis			
Epithelioid cell	20 (44.4)		
Spindle cell	7 (15.6)		
Malignant melanoma, NOS	18 (40)		
Compound nevus		8 (44)	
Intradermal nevus		5 (28)	
Atypical nevus		2 (11)	
Benign nevus, NOS		3 (17)	
Normal skin			7 (100)
Tstage (thickness)			
T_1	11 (24.4)		
T_2	14 (31.1)		
T_3	16 (35.6)		
T_4	4 (8.9)		
N stage			
N_0	42 (93.3)		
N_1	3 (6.7)		
M stage			
M_0	44 (97.8)		
M_1	1 (2.2)		
Clark level			
2	9 (20)		
3	16 (35.6)		
4	12 (26.7)		
5	2 (4.4)		
n/a	6 (13.3)		

percentile analysis (Supplementary Fig. S1). Bonferroni-adjusted *t* test and percentile analyses were used to address the multiple testing issue and the heterogeneity of the tumor samples, respectively. As the result of these analyses, 439 genes were selected (Supplementary Table S1). Out of 439 genes up-regulated in melanoma, we selected a short list of 33 genes that had >10-fold overexpression in the melanoma samples than that of the benign specimens. These include many genes with known association with malignant melanoma such as neurotrophic tyrosine kinase receptor, type 3 (*NTRK3;* ref. 21), L1 cell adhesion molecule (*L1CAM;* refs. 22, 23), silver homologue (*me20m*; ref. 24), as well as novel genes. Genes with >10-fold overexpression in melanoma are presented in Table 2.

We further selected three genes overexpressed in melanoma, including *NTRK3*, *PLAB*, and *L1CAM*, for quantitative real-time RT-PCR validation of the microarray results (Fig. 2). *PLAB* is a novel gene whose differential expression in melanoma, to the best of our knowledge, has not been previously reported. For *L1CAM* and *NTRK3*, differential expression in melanoma was shown at the protein level only (21–23). Moreover, we identified *PLAB* and *L1CAM* as the best combination, on a complementary basis, to separate melanoma from benign/normal tissues in our study. *me20m* is known as a melanoma-specific marker and was selected as a positive control. For the RT-PCR assay, we used a panel of 14 primary melanoma, 7 benign nevi, and 5 normal skin samples, isolated from the same tissues which were used for the microarray study. The expression value of each gene was normalized to the housekeeping control gene *PBGD*. The correlation coefficients between the RT-PCR and the microarray results for *L1CAM*, *NTRK3*, *PLAB*, and *me20m* are 0.79, 0.86, 0.87, and 0.88, respectively. This result indicates that the RT-PCR results are highly consistent with the microarray data.

One-step quantitative reverse transcription-PCR assay for malignant melanoma. We examined a combination of two genes highly overexpressed in melanoma, *PLAB* and *L1CAM*, in a variety of clinical tissue samples containing malignant melanocytes (primary melanoma and melanoma lymph node metastasis), benign melanocytes (benign skin nevi), and normal samples (normal skin and melanoma-free lymph node) by RT-PCR. The primary tissues were the same as those used for the microarray study whereas all the lymph node specimens were derived from independent patients. Conventional melanoma markers, such as tyrosinase, gp100, and MART1 were also tested on the same samples as the controls because they are the most commonly used markers for the melanoma molecular assays in current clinical studies (25–27). Calculated scores were presented for *PLAB* and *L1CAM* (Fig. 3A) and for *tyrosinase*, *me20m*, and *MART1* (Fig. 3B). The results showed a significant difference in the expression of *PLAB* and *L1CAM* between malignant melanoma samples (primary and lymph node metastasis) and benign nevi and normal lymph node. In contrast, three conventional markers showed similar expression levels in benign and melanoma samples. To further show the ability of these gene markers to separate benign and malignant tissues, we tested two cutoffs; the first was set-up as the highest score in primary normal samples, and the second as the highest score in benign nevi samples. For each cutoff, we estimated the sensitivity and specificity of the assay in the lymph node samples. With the cutoff determined on the normal samples, the new markers and the conventional markers gave sensitivities of 90% and 83%, respectively. Using of the cutoff determined on the benign samples, the sensitivities for the new and conventional markers were 88% and 42%. The results indicated that the new markers potentially have much better abilities to differentiate tissues containing benign and malignant melanocytes.

Discussion

We did gene expression profiling analysis of primary melanoma, benign nevi, and normal skin tissue specimens in order to find melanoma-specific gene markers for potential use in the lymph node–based molecular staging assay. Novel genes that are highly and differentially expressed in malignant melanoma samples were identified. The inclusion of benign nevi in the experimental design was key to our study. In contrast to normal skin, melanocyte content in benign nevi is close to that in melanoma. This was confirmed, in addition to histologic assessment, by the equally high expression levels of conventional melanoma markers such as tyrosinase and MART1 in both melanoma and nevi tissue specimens. Similar cellular compositions allowed us to monitor gene expression changes specifically associated with melanocyte malignant transformation, not just with melanocyte lineage differentiation. As a result, we identified novel genes specifically overexpressed in melanoma. One of the novel highly overexpressed in melanoma genes, prostate differentiation factor (*PLAB*, *MIC1*), is a member of the transforming growth factor-β superfamily and is also known to be associated with other malignancies

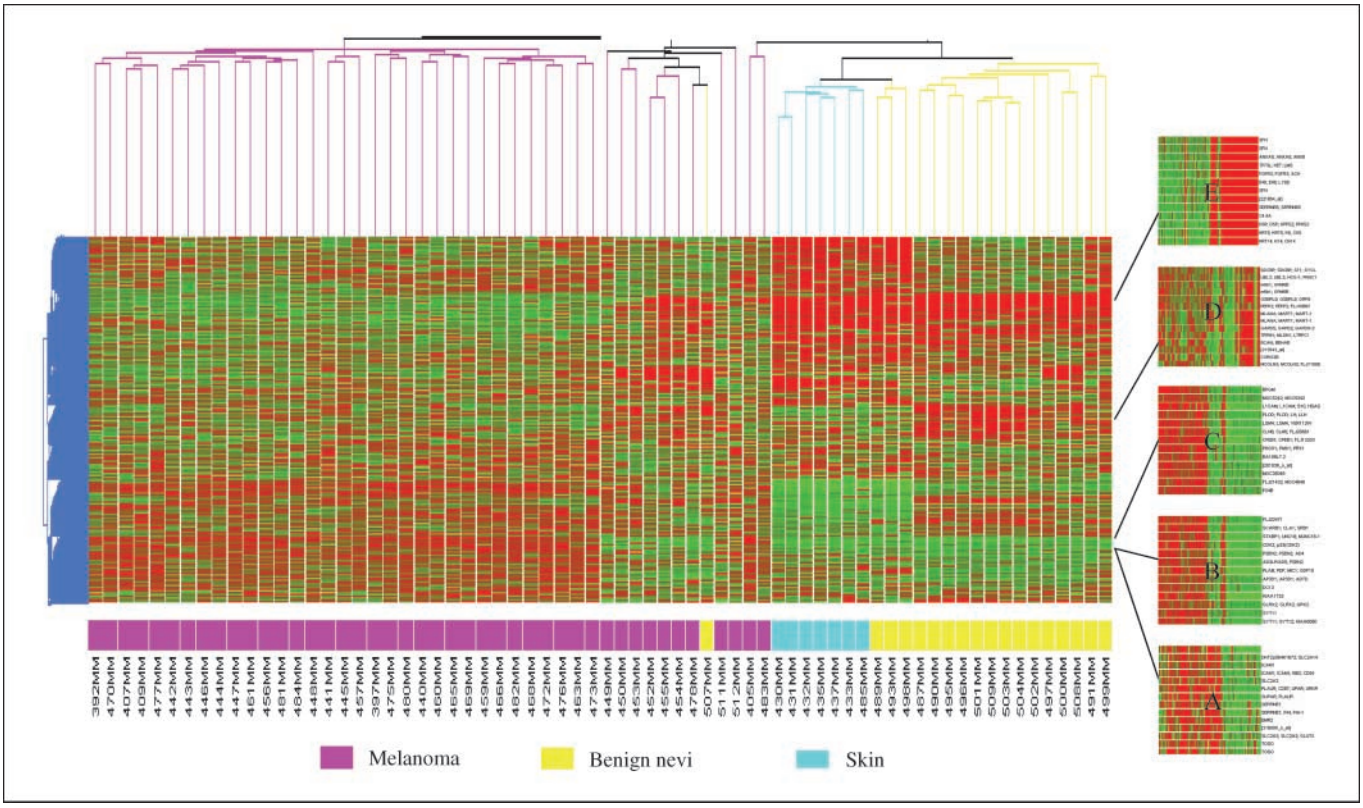

Fig. 1. Hierarchical clustering on the 15,795 genes that have at least two "present" calls in all samples. Each column is a sample and each row is a gene. Red, up-regulation; green, down-regulation; purple, melanoma samples; yellow, benign nevi; blue, normal skin.

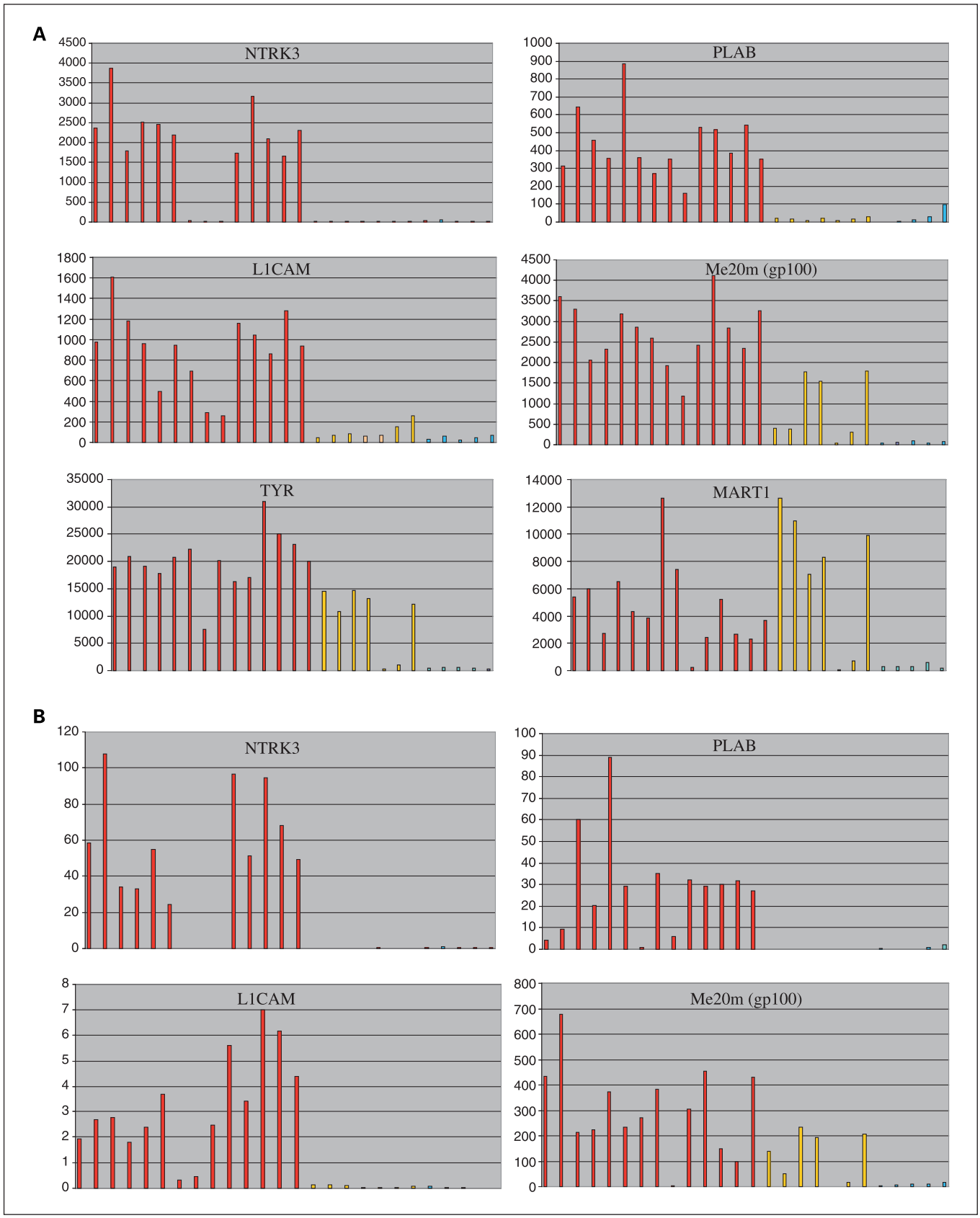

Fig. 2. Microarray expression (*A*) and real-time RT-PCR validation data (*B*) of the selected genes. The first 14 samples from the left are the melanoma tissue samples (*red*); the next seven are benign nevi samples (*yellow*), and the last five are normal skin (*blue*). *x*-axis, intensity values for microarray plots; for PCR plots, *x*-axis is $2^{\Delta CT}$, where ΔCt is Ct (target gene) − Ct *PBGD*.

(28, 29). Among the genes overexpressed in melanoma, we observed many genes previously associated with the function and development of neural tissue, such as *HEY1*, *L1CAM*, *SEMA3B*, and *NES*. This suggests that dedifferentiation of melanocytes and activation of the processes related to a pluripotent progenitor cell, common to neural tissue cells and melanocytes, might be important for melanoma development and progression.

We have compared our results to the recent study of Haqq et al. (30). In their work, a cDNA microarray containing 20,862 probes was used to profile benign nevi, primary melanoma, and metastatic melanoma specimens. The sample set included metastatic and primary melanoma and benign nevi. Similar clustering results that separated the benign nevi and the primary malignant melanoma tissues were found in their study. Common genes were reported in both studies which could discriminate melanoma from benign nevi including kinesin-like 5 (*KNSL5*), prostate differentiation factor (*PLAB*), *CITED1*, osteopontin (*SPP1*), cathepsin B (*CSTB*), cadherin 3 (*CDH3*), presenilin 2 (*PSEN2*).

The results of our one-step RT-PCR assay showed that novel melanoma-specific genes, *PLAB* and *L1CAM*, were expressed not only in primary melanoma tissues but also in melanoma lymph node metastasis. Moreover, their ability to differentiate malignant melanoma from benign nevi made them better candidates than the conventional markers for the molecular test of melanoma diagnostics. With further validation in clinical studies, these genes could be developed as specific markers for a molecular staging assay to detect melanoma micrometastasis during a sentinel lymph node biopsy procedure. Another

Table 2. Selected genes overexpressed in melanoma

PSID	Gene accession	Description	Median expression in melanoma	Fold change (cancerous versus benign)	Fold change (cancerous versus skin)
215025_at	S76476	trkC (alternatively spliced) human, brain (NTRK3)	2,365	149	93
215311_at	AL109696	full-length insert cDNA clone, EUROIMAGE 21920	10,093	86	30
213960_at	T87225	full-length insert cDNA clone, EUROIMAGE 51358	11,768	80	29
219478_at	NM_021197	WAP four-disulfide core domain 1 (WFDC1)	5,485	80	18
218839_at	NM_012258	hairy enhancer-of-split related with YRPW motif 1 (HEY1)	1,996	38	7
215115_x_at	AI613045	ets variant gene 6 (TEL oncogene)	12,421	34	15
221577_x_at	AF003934.	prostate differentiation factor (*PLAB*)	4,897	28	38
217377_x_at	AF041811	ETV6-NTRK3 fusion	12,402	27	18
213638_at	AW054711	phosphatase and actin regulator 1 (PHACTR1)	1,827	26	102
204709_s_at	NM_004856	kinesin-like 5 (KNSL5)	312	24	16
221909_at	AW299700	hypothetical protein FLJ14627	243	22	17
204584_at	AI653981	L1 cell adhesion molecule (*L1CAM*)	9,677	21	15
209875_s_at	M83248	osteopontin (SPP1)	3,038	21	12
217624_at	AA464753	PDGFA associated protein 1 (PDAP1)	349	21	20
203071_at	NM_004636	semaphorin 3B (SEMA3B)	958	19	3
213587_s_at	AI884867	chromosome 7, open reading frame 32	10,416	18	9
221815_at	BE671816	abhydrolase domain containing 2 (ABHD2)	3,293	17	144
219578_s_at	NM_030594	similar to cytoplasmic polyadenylation element binding protein (CPEB1)	1,419	17	26
207144_s_at	NM_004143	bpp300-interacting transactivator, 1 (CITED1)	593	17	24
203069_at	NM_014849	synaptic vesicle glycoprotein 2A (SV2A)	560	15	13
218678_at	NM_024609	nestin (NES)	11,356	14	20
219152_at	NM_015720	podocalyxin-like 2 (PODXL2)	365	13	10
205447_s_at	BE222201	mitogen-activated protein kinase kinase kinase 12 (MAP3K12)	567	13	5
213274_s_at	AA020826	cathepsin B (CTSB)	18,263	12	26
219555_s_at	NM_018455	uncharacterized bone marrow protein BM039	402	12	21
203827_at	NM_017983	WD40 repeat protein Interacting with phosphoinositides of 49 kDa (WIPI49)	3,380	11	8
205813_s_at	NM_000429	methionine adenosyltransferase I, α (MAT1A)	430	11	9
201850_at	NM_001747	capping protein, gelsolin-like (CAPG)	11,103	10	20
205373_at	NM_004389	catenin, α2 (CTNNA2)	579	10	8
214614_at	AI738662	homeobox HB9 (HLXB9)	542	10	9
213217_at	AU149572	adenylate cyclase 2 (brain; ADCY2)	5,848	10	6
204014_at	NM_001394	dual specificity phosphatase 4 (DUSP4)	7,184	10	46
214893_x_at	AI421964	hyperpolarization activated cyclic nucleotide-gated potassium channel 2 (HCN2)	214	10	9

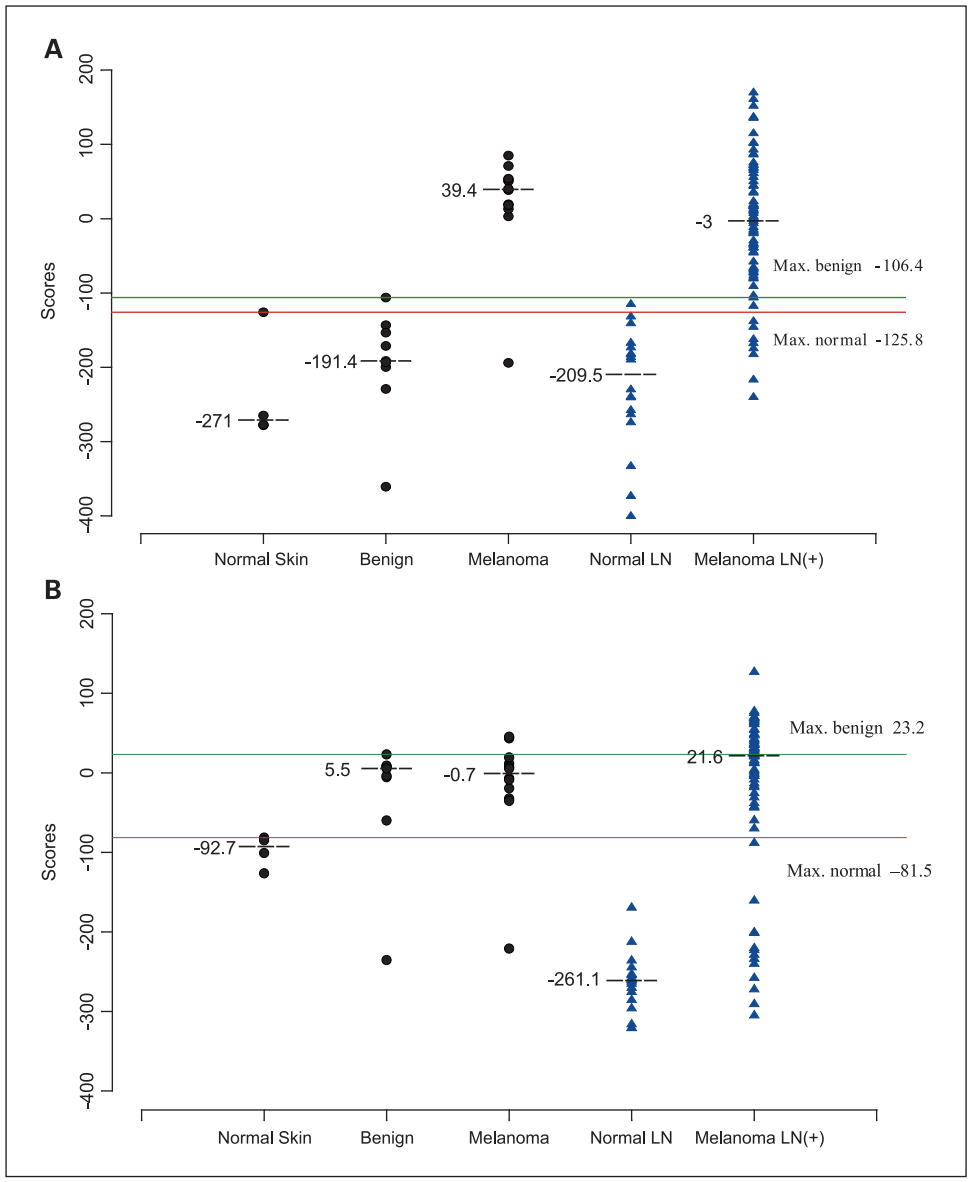

Fig. 3. One-step quantitative RT-PCR assay of *PLAB* and *L1CAM* (*A*) and conventional melanoma markers, *me20m, tyrosinase,* and *MART1* (*B*). *x*-axis, score for the new markers or the conventional markers for each plot. Median scores for each samples category are labeled. Two cutoff levels based on normal (*green*) and benign (*red*) samples are labeled on each plot.

potential application of the genes is for the diagnosis of melanocyte lesions with uncertain pathologic features. We are evaluating the markers for use in sentinel lymph node biopsies determining the presence of melanoma disease and the prognosis of tumors.

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