Development of a novel noninvasive adhesive patch test for the evaluation of pigmented lesions of the skin

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Background: The accurate clinical assessment of melanocytic neoplasms is a challenge for clinicians. Currently, obtaining a biopsy specimen and conducting a histologic examination is the standard of care. The incidence of melanoma in white populations is high, resulting in a large number of biopsy specimens.

Objective: The objective of this study is to develop a noninvasive genomic method using mRNA to classify pigmented skin lesions as either benign or malignant.

Methods: An adhesive patch method was used to obtain cells from the surface of melanocytic lesions. mRNA was extracted and a genomic signature was formulated in a training set of benign and malignant melanocytic neoplasms and subsequently tested in a validation set.

Results: A 2-gene signature assessing the expression levels of CMIP and LINC00518 was able to differentiate melanomas from nevi in an independent validation set of 42 melanomas and 22 nevi with a sensitivity of 97.6% and specificity of 72.7%.

Limitations: Larger and more diverse sets of melanomas and nevi are needed for additional validation of the molecular expression profiling in various subsets of melanocytic neoplasms.

Conclusion: Our data suggest that mRNA molecular signatures can serve as a highly useful noninvasive method of differentiating melanoma from nevi and decrease the number of unnecessary biopsies. (J Am Acad Dermatol 2014;71:237-44.)

Key words: dysplastic nevi; melanocytic neoplasms; melanoma; molecular signatures; nevi.

The incidence of melanoma is increasing at a rate of 3% to 7% per year for fair-skinned white populations, faster than any other major cancer.[1,2] While mortality from almost all preventable cancers has markedly decreased since 1975,[3,4] melanoma-related mortality remains steady, increasing up to 6.5% in certain patient populations annually.[4] The American Cancer Society (ACS) estimates that approximately 76,100 new cases of melanoma will be diagnosed, resulting in 9710 deaths caused by melanoma in 2014.[2-5]

Abbreviations used:

ACS:	American Cancer Society
AUC:	area under the curve
DTI:	DermTech International
NF-κB:	nuclear factor kappaB
NNT:	number needed to treat
NPV:	negative predictive value
PPV:	positive predictive value
qRT-PCR:	quantitative real-time polymerase chain reaction
ROC:	receiver operator characteristic

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Thin melanomas have an excellent prognosis. In situ melanoma has a cure rate of virtually 100%, and early invasive melanomas with a Breslow depth of <1 mm have a 10-year survival rate of >90%.[6,7] Conversely, tumors with a Breslow depth >4 mm have a 10-year survival rate of <50%.[6] A number of studies have shown that early detection through a skin examination allows for diagnosis at an earlier and more curable stage of disease.[8-11] In 1 study, screening skin examinations reduced mortality by 63%.[8]

Currently, obtaining a biopsy specimen and conducting a histologic examination is the standard of care for the evaluation of clinically suspicious pigmented skin lesions. A measure known as number needed to treat (NNT) is used to measure physician accuracy in assessing pigmented skin lesions. This value is determined by dividing the total number of biopsy specimens obtained to exclude melanoma by the total number of melanomas according to histopathologic assessment. Various studies analyzing this value have reported NNT values between 8 for experienced dermoscopy users and >30 for other health care professionals.[12-14] To diagnose the 76,690 invasive melanomas and 60,000 in situ melanomas predicted by the ACS using a conservative NNT between 8 to 15 would require 1 to 2 million skin biopsy specimens.[2]

Histopathology, the criterion standard for the diagnosis of melanocytic neoplasms, has limitations.[15-18] The histopathologic examination consists of a 2-dimensional morphologic assessment from a single moment in time of 3-μm–thick sections of tumors that are often >4 mm. Investigators performing dermoscopic evaluations of melanocytic neoplasms have noted that some lesions that are likely early melanomas based on their clinical behavior may not meet the full histologic criteria for melanoma.[19] In addition, the discrepancy between the increased incidence of melanoma and death from melanoma has led many investigators to speculate that a subset of indolent melanomas may exist. These tumors may meet the histologic criteria for melanoma but have an indolent clinical behavior.[20]

In this study, we evaluated an alternative method of assessing melanocytic neoplasms using mRNA obtained from the surface of lesions using a noninvasive adhesive patch technology. Our data suggest that mRNA signatures can improve physicians' NNT value and the overall quality of patient care.

CAPSULE SUMMARY

- Developmental studies show that molecular signatures may be an accurate noninvasive method of identifying melanomas.
- This study shows that our previous mRNA signature could be reduced to 2 genes (CMIP and LINC00518) while still accurately discriminating melanomas and nevi.
- This method has the potential to reduce unnecessary biopsy procedures.

METHODS

Between 2004 and 2010, DermTech International (DTI) created a prospectively ascertained biorepository of both normal and diseased skin specimens obtained by noninvasive adhesive patch sampling, with subsequent surgical biopsy and dermatopathologic evaluation. The biorepository contained >4700 adhesive patch–sampled specimens. All study protocols were reviewed and approved by a central institutional review board (ie, the Western Institutional Review Board) along with the institutional review boards at the sites that submitted specimens (a full site list is provided at the end of this article). The study was conducted according to the principles of the Declaration of the Helsinki, and all subjects provided informed consent.

Inclusion criteria

Subjects were eligible if they were at least 18 years of age and had a clinically suspicious pigmented lesion that was $\geq$4 mm in diameter. If the patient had multiple clinically suspicious lesions, they had to be at least 4 mm apart.

Exclusion criteria

Subjects were excluded if they had used topical medications or systemic steroids within 30 days of beginning the study; if the subject had a generalized skin disorder not related to skin cancer; if the pigmented lesion was in the acral or vulvar region; if the subject was allergic to the adhesive patch or latex rubber; if in the past 30 days they had participated in an investigational study; or if the patient had clinical findings suggestive of an advanced stage lesion.

Adhesive patch procedure

Pigmented lesions were sampled by adhesive patch before biopsy specimens were obtained. The patch was applied to the site and briskly rubbed at least 15 times in a circular motion with a 1-cm diameter rounded device or plastic tube before the patch was removed. The border of the lesion was demarcated on the patch. A total of 4 patches were

used per lesion. After sampling, the patches were stored at −20°C or below within 10 minutes of stripping. The patches were shipped to DTI on dry ice, by express mail, for RNA extraction within 1 week.

Biopsy

After the adhesive patch procedure, the lesion was excised according to standard clinical practice. All tissues removed were formalin fixed and embedded in paraffin and sectioned for histopathologic analysis. The slides were sent to a central dermatopathologist. Three separate dermatopathologists (the site pathologist and 2 central pathologists contracted by DTI) were involved in the reading of each case. Inclusion criteria required agreement among all 3 dermatopathologists. All were blinded to the molecular results.

Quantitation of gene expression

All laboratory personnel were blinded to the pathology diagnoses. The outlined lesional portion of each patch was manually dissected, and the 4 patches for each lesion were pooled for additional processing. Total RNA was isolated for each pigmented lesion and used for cDNA synthesis; cDNA was subsequently used for preamplification and quantitative real-time polymerase chain reaction (qRT-PCR) studies using standard Taqman chemistry. RNA was isolated using Ambion MELT (Life Technologies, Foster City, CA), cDNA synthesis was performed using SuperScript VILO (Life Technologies), and preamplification was performed using a custom pool of primers and probes for all genes assayed (Life Technologies). Quantitation of gene expression levels was performed using the QuantStudio OpenArray system (Life Technologies). Expression levels of candidate signature genes for each specimen were normalized to the geometric mean of expression levels of 3 housekeeping genes (eg, ACTB [beta-actin], B2M [beta-2-microglobulin], and PPIA [peptidylprolyl isomerase A]) for that specimen.

Statistical analysis

An optimal classification signature was created separately for each of 2 independently ascertained datasets. The selection of genes for inclusion in the classification signature was performed using stochastic gradient boosting analysis coupled with bootstrap logistic decision tree modeling (TreeNet; Salford Systems, San Diego, CA) and logistic regression (R; R Foundation for Statistical Computing)[21] on a previously determined gene list.[22,23] TreeNet was used to create the classification algorithm and to determine each gene's relative importance. A TreeNet battery incorporating 10 independently derived models was constructed using random resampling and replacement from 140 specimens to select sets of 91 for training and 49 for testing in each model, with each set comprised of approximately 50% melanoma and 50% nevi. Dichotomous disease status was the dependent variable, and the gene expression values were independent variables. Logistic regression was similarly used to assess the performance of each possible gene pair taken from the previously determined gene list. All specimens used had complete data for gene expression values. The classification algorithm computes a score between 0 and 1 for each specimen based on the measured gene expression inputs. Sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV), receiver operator characteristic (ROC) curves, and area under the ROC curve (AUC) were calculated using R software.

Table I. Discovery cohort, set A

	Invasive melanoma	Melanoma in situ	Nevi	Other (ie, NMSC, seborrheic keratosis, and lentigo)
Site				
Trunk	20	10	46	7
Extremity	12	7	3	7
Head and neck	13	7	3	5
Age (y)				
Range	23-87	36-92	20-73	20-91
Mean	55.9	65.3	43.2	63.0
Sex				
Male	29	17	28	12
Female	15	7	24	7
Total	45*	24	52	19

NMSC, Nonmelanoma skin cancer.
*The sex of 1 patient with invasive melanoma was not indicated.

RESULTS

Gene expression classification of melanoma

A previously discovered, high accuracy 15-gene classification signature was used as a starting point for validation.[23] The relative importance of each gene within the total profile was assessed. Most of the 15 genes did not make large contributions, with a relative importance of $\leq 30\%$. This served as an impetus to investigate reducing the number of genes in the signature.

All 105 pairwise combinations from within the previous 15-gene signature were investigated for accuracy by bootstrap multivariate logistic regression using a previous 140-specimen set (Table I, set A) including 69 melanomas, 52 nevi, and 19 other

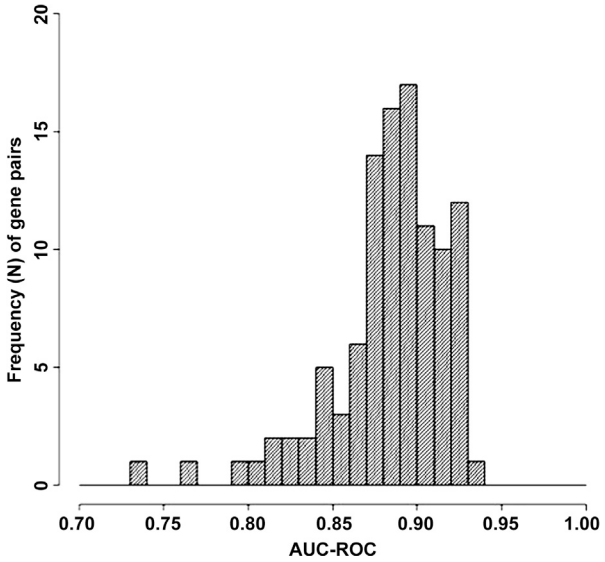

Fig 1. Performance of gene pairs from the 15-gene classifier. Histogram of all 105 pairwise combinations from the 15 genes of the previously reported melanoma signature, binned by the area under the curve of the receiver operating characteristic (*AUC-ROC*) for each pair. The width on the x axis of each column represents the lower and upper limits of the AUC-ROC for that bin. The number of genes in each bin is represented by the height of the column on the y axis.

nonmelanocytic neoplasms of the skin.[23,24] This set was created by randomly sampling the biorepository log using a balanced design of 100 melanoma and 100 nonmelanoma subjects. Case attrition from insufficient quantity of stored material, specimen signals below the limit of detection, exclusion because of use in earlier studies, or major discordance among the 3 expert dermatopathologists resulted in a set of 140 specimens (Table I, set A). As shown in Fig 1, the mean AUC of each gene pair's iterations ranged from a low of 0.72 to a high of 0.94 (an AUC of 1.00 is a perfect classification system). Additional analysis of the AUC for larger combinations of the genes revealed that optimal classification is given by 2 genes; the inclusion of additional genes did not improve accuracy. The gene pairs with average AUCs above 0.90 were further investigated using TreeNet analysis (Fig 1).

CMIP and LINC00518 was the best performing pair in both logistic regression and TreeNet. The signature using those 2 genes was then validated in a newly tested set of 64 cases consisting of 42 melanomas and 22 nevi (Table II, set B). This set was created in the same fashion as set A and resulted in 64 specimens (Table II, set B). Table III gives the 2 × 2 table of disease status versus expression signature classification; Fig 2 shows a graphic distribution of the cases' scores. The resulting sensitivity was

Table II. Validation cohort, set B

	Invasive melanoma	Melanoma in situ	Nevi	Other (ie, NMSC, seborrheic keratosis, and lentigo)
Site				
Trunk	13	2	16	1
Extremity	12	5	3	—
Head and neck	3	7	2	—
Age (y)				
Range	20-83	40-83	18-82	35
Mean	54.19	63.14	41.14	35
Sex				
Male	16	11	15	0
Female	12	3	6	1
Total	28	14	21	1

NMSC, Nonmelanoma skin cancer.

Table III. Pathology diagnosis versus expression signature classification, classifier A on set B

Gene expression classification	Pathology diagnosis	
	Melanoma	Nevi
Melanoma	41	6
Nonmelanoma	1	16
Sensitivity	41/42 (97.6%)	—
Specificity	—	16/22 (72.7%)

97.6% and specificity was 72.7%. The NPV was 94% at the study prevalence of 67% melanoma, and was 99.6% at an adjusted melanoma prevalence of 10% to more closely reflect what is reported for general dermatology practices (the ratio of nevi to melanoma was numerically adjusted to 9:1 while maintaining the sensitivity and specificity values).

A cross-validation was subsequently performed by similarly using the same 2 genes to create a classification algorithm from set B and applying it to the independent set A. Comparable validation accuracies were seen, with sensitivity of 97% and specificity of 68%. Table IV gives the 2 × 2 table of disease status versus expression signature classification; Fig 3 shows a graphic distribution of the cases' scores. The NPV was 88% at the study prevalence of 73% melanoma, and was 99.4% at an adjusted melanoma prevalence of 10% to more closely reflect what is reported for general dermatology practices (Table III).

Limitations

A significant proportion of cases were lost because of insufficient mRNA or mRNA signal below

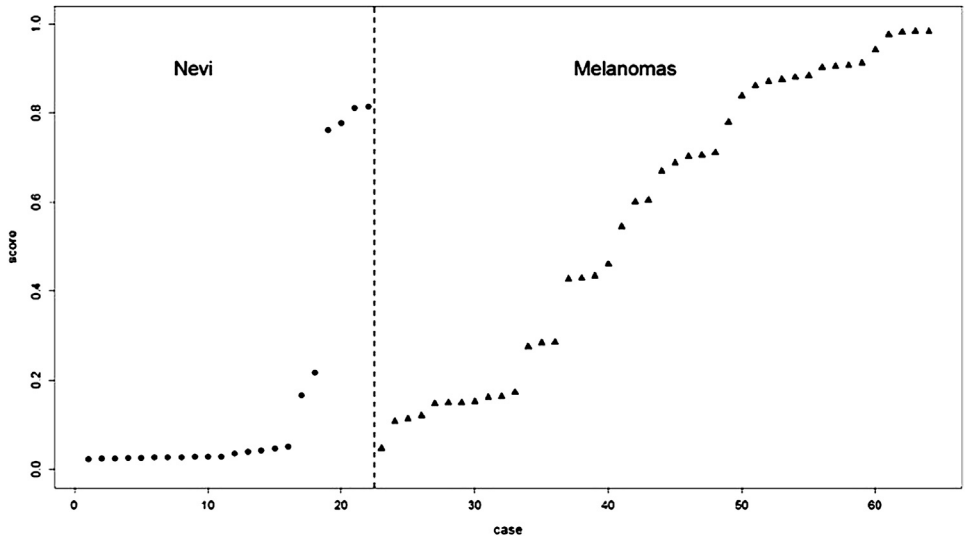

Fig 2. Case distribution of classifier scores, set B. Distribution of independent set B cases scored by the set A classifier. The x axis represents the case number. The y axis represents the algorithmic score based on expression levels of a 2-gene classifier. Nevi are plotted on the *left* as *black circles*; melanomas are plotted on the *right* as *black triangles*.

Table IV. Pathology diagnosis versus expression signature classification, classifier B on set A

Gene expression classification	Pathology diagnosis	
	Melanoma	Nevi
Melanoma	67	23
Nonmelanoma	2	48
Sensitivity	67/69 (97.1%)	—
Specificity	—	48/71 (67.6%)

the limit of detection or because of use in previous studies. The loss of cases related to mRNA insufficiency could limit clinical use and/or require repeated sampling attempts in order to obtain an adequate specimen; process improvements are underway that will address this important issue. It is not clear what bias that may have introduced. The 1 case in set B diagnosed as melanoma by histopathology but not by the gene classifier was a lentigo maligna melanoma in situ. It would be worthwhile in future studies using larger datasets to determine if there is a different level of accuracy when assessing the various subtypes of melanoma—including melanomas from acral or vulvar skin, which were not included in this study. Cases were also limited to a minimum diameter of 4 mm.

CONCLUSIONS

A previous report[25] has described gene expression signatures that accurately discriminated melanomas from nevi and other nonmelanoma specimens. These gene sets were derived from the results of an initial genome-wide microarray experiment and further refined to a 15-gene set by computational analyses. We now report that 2 genes, CMIP and LINC00518, have an equivalent classification ability. The 2 genes' combined ability to accurately discriminate between melanoma and nonmelanoma cases represents a novel biomarker discovery arrived at via top-down genomic data mining. This approach was agnostic to existing biologic knowledge of disease states and processes, and did not involve the biases of a priori knowledge. Therefore, any assessment of the roles of CMIP and LINC00518 in the pathophysiology of melanoma must be constructed from our current understanding of those genes' functions.

The CMIP gene is involved in oncogenesis via the modulation of key signaling pathways. CMIP can promote apoptosis by downregulating nuclear factor kappa B (NF-κB),[25,26] a transcription factor that responds to abnormal cellular stimuli and is a key component in cell proliferation, survival, and inflammatory and immune responses. In this study, CMIP mRNA was downregulated in melanoma relative to nonmelanoma lesions; the CMIP reduction may decrease its inhibitory effect on NF-κB, leading to decreased control of proliferation with subsequent melanomagenesis. NF-κB is dysregulated in many tumors.[27]

LINC00518 mRNA was upregulated in melanoma relative to nonmelanoma lesions. Its chromosomal location is 6p24.3, immediately adjacent to the 6p25 region which has frequent copy number gains in melanoma.[28] It is a member of the large class of

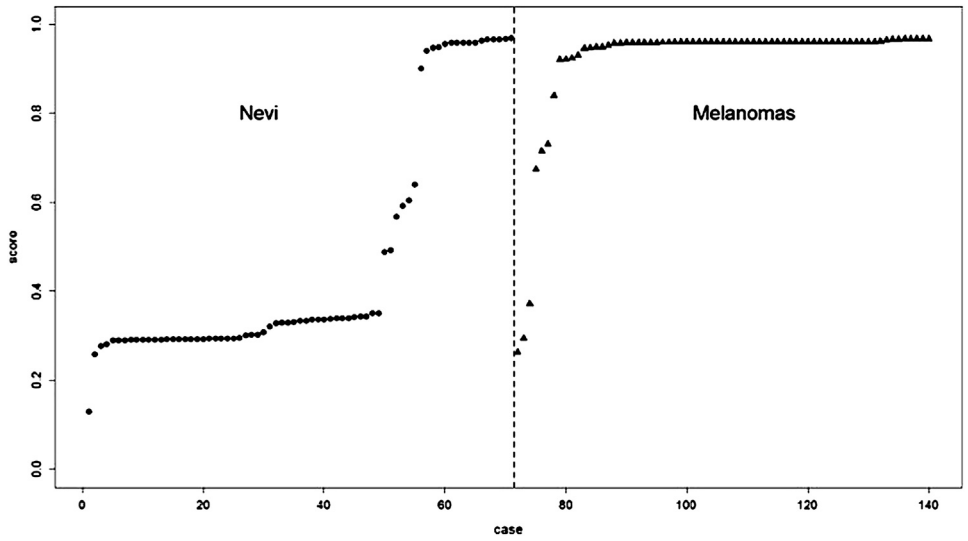

Fig 3. Case distribution of classifier scores, set A. Distribution of independent set A cases scored by the set B classifier. The x axis represents the case number. The y axis represents the algorithmic score based on expression levels of a 2-gene classifier. Nevi are plotted on the *left* as *black circles*; melanomas are plotted on the *right* as *black triangles*.

regulatory molecules known as long noncoding RNAs (lncRNAs), which do not code for proteins. Recent studies on melanoma tissue specimens and cell lines have shown the importance of lncRNAs as regulatory molecules influencing proliferation, differentiation, and cellular processes. A number of lncRNAs are differentially expressed in melanoma cell lines versus melanocytes and keratinocytes in culture.[29] One study of primary melanomas and lymph node metastases identified a lncRNA gene that may promote metastatic potential when highly expressed in primary tumors.[30] The authors postulated that the overexpression of certain lncRNAs plays a role in the development of human melanoma. A recent study of uveal melanomas revealed alternative splicing of protein coding genes associated with upregulation of a lncRNA, and indicated that a possible function of lncRNA is to rearrange and control protein coding regions of the human genome.[31]

In vivo, mRNA produced by melanocytes is carried in organelles and/or vesicles that are phagocytized by epithelial cells.[32] The adhesive patch method is noninvasive, only removing a superficial layer of epithelial cells, and does not result in any wound or require any down time from physical activity or work. While the overall morbidity associated with obtaining a skin biopsy specimen is quite low, each specimen obtained may result in decreased physical activity, time off from work, anxiety, and scarring. In patients with dysplastic nevus syndrome, this procedure would allow for the simultaneous evaluation of a greater number of atypical nevi, whereas obtaining more than 3 or 4 excisional biopsies at one time could result in considerable discomfort and significant wound care.

Currently, the NNT for pigmented lesions is between 10 and 30; a single patient diagnosed with 2 melanomas in their lifetime may require 20 to 60 biopsy specimens to diagnose melanoma. In addition, obtaining biopsy specimens from some pigmented lesions on the face, genitalia, or other sensitive anatomic locations may result in unsightly scars. The high sensitivity (97.6%) of the assay accompanied with a specificity of 72.7% could significantly decrease the number of unnecessary biopsy specimens, resulting in a significant improvement in quality of life.

Six of 22 cases with a histologic diagnosis of nevus had a molecular score consistent with a diagnosis of melanoma. Interestingly, among those 6 cases, 3 had a histologic reading of dysplastic nevus with severe atypia. Unfortunately, it is impossible to determine whether these 3 cases were truly false positives or actually early melanomas.

In summary, this procedure represents a novel noninvasive molecular approach to evaluating melanocytic neoplasms when coupled with expression analysis of the genes described in this report.

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