Noninvasive Analysis of High-Risk Driver Mutations and Gene Expression Profiles in Primary Cutaneous Melanoma

JID Open

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Tools that help reduce the number of surgical biopsies performed on benign lesions have the potential to improve patient care. The pigmented lesion assay (PLA) is a noninvasive tool validated against histopathology that helps rule out melanoma and the need for surgical biopsies of atypical pigmented skin lesions. Genetic information is collected using adhesive patches and the expression of two genes, *LINC* and *PRAME*, is measured. By using genetic material collected noninvasively and to further validate the PLA, somatic hotspot mutations in genes known to be drivers of early melanoma development (BRAF other than V600E, NRAS, and the *TERT* promoter) can also be identified. The frequency of these hotspot mutations in samples of early melanoma was 77%, which is higher than the 14% found in nonmelanoma samples ($P < 0.0001$). *TERT* promoter mutations were the most prevalent mutation type in PLA-positive melanomas; 82% of PLA-negative lesions had no mutations, and 97% of histopathologically confirmed melanomas were PLA and/or mutation positive (cohort 1, n = 103). Mutation frequencies were similar in prospectively collected real-world PLA samples (cohort 2, n = 519), in which 88% of PLA-negative samples had no mutations. Combining gene expression and mutation analyses enhances the ability to noninvasively detect early cutaneous melanoma.

Journal of Investigative Dermatology (2018) ■, ■–■; doi:10.1016/j.jid.2018.10.041

INTRODUCTION

Currently, cutaneous melanoma is typically diagnosed based on histologic examination of lesions identified as suspicious based on clinical appearance (Bichakjian et al., 2011; High, 2017). As our understanding of the genetic basis of melanoma unfolds, the assumption is that genetic mutations and other early changes responsible for tumor promotion will result in morphologic changes that pathologists describe and use to make the histopathologic diagnoses upon which clinical decision making is currently based. Histology can thus be seen as a surrogate marker of the mutational burden and genetic changes of a melanocytic lesion, and there are several disadvantages to this more indirect approach. First, to acquire tissue for evaluation, a surgical biopsy must be performed, which carries the risk of scarring and infection. Second, about 90% of about 3,000,000 biopsies performed annually in the United States to rule out melanoma are found to be benign or low-risk lesions and thus are surgically biopsied without clear benefit to the patient (Lott et al., 2018; Nufer et al., 2018). Third, histology is subjective, and consensus agreement among pathologists is lower than expected by many, as corroborated by a recent landmark study analyzing performance data from 10 US states (Elmore et al., 2017). The calculated sensitivity of histopathology for early melanomas (Melanocytic Pathology Assessment Tool and Hierarchy for Diagnosis [MPATH-Dx] class III and IV, melanoma in situ and early invasive pT1a melanoma cases), based on data by Elmore et al. (2017) was 65%. These numbers highlight the fact that the current criterion standard leaves room for improvement (Elmore et al., 2017). Based on this and other key studies, the negative predictive value for the current surgical biopsy pathway is around 83%, reflecting the fact that in addition to the low specificity of the current approach, there is also a 17% probability of missing melanomas (Malvehy et al., 2014; Elmore et al., 2017).

The recently described pigmented lesion assay (PLA), is a molecular tool for clinicians that uses gene expression profiling to aid in making biopsy decisions to rule out melanoma (Childs, 2018; Ferris et al., 2017, 2018; Gerami et al., 2017; Hornberger and Siegel, 2018; Rivers et al., 2018; Yao et al., 2016, 2017). Using adhesive patches for noninvasive sample collection, the PLA analyzes the expression of two genes, *LINC* and *PRAME*, both of which are often increased in melanoma (Gerami et al., 2017; Yao et al., 2017). *PRAME* is a cancer biomarker used in two other commercial melanoma tests, and *LINC* is part of a new class of regulatory RNAs that play an important role in cancer biology (Clarke, et al., 2015; Gerami et al., 2017; Haqq et al., 2005).

The performance metrics of the PLA were validated against histopathologic consensus reads (by three

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Abbreviations: gDNA, genomic DNA; LINC, long intergenic non-coding RNA 518; PLA, pigmented lesion assay; PRAME, preferentially expressed antigen in melanoma; qPCR, quantitative PCR

Received 11 April 2018; revised 27 October 2018; accepted 29 October 2018; accepted manuscript published online 27 November 2018; corrected proof published online XXX

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dermatopathologists specializing in pigmented lesions) (Gerami et al., 2017). Compared with the current standard of visual assessment followed by histopathology, the PLA has a lower false negative rate (9% vs. 16%), a higher negative predictive value (99% vs. 83%), and a higher specificity (<32% vs. >69%) (Ferris et al., 2017, 2018).

Somatic mutations in *BRAF* (other than V600E), *NRAS*, and the *TERT* promoter have recently been characterized as key drivers of early stage melanoma. The biology of early cutaneous melanoma is generally driven by initial UV damage events that lead to somatic hotspot mutations in these relatively small numbers of key target genes that alter gene expression and ultimately result in functional changes and morphological changes of melanocytic lesions (Chiba et al., 2017; Horn et al., 2013; Huang et al., 2013; Liu et al., 2016; Mar et al., 2013; Menzies et al., 2012; Shain et al., 2015, 2016; Shay, 2017; Tsao et al., 2012). These early hotspot driver mutations in *BRAF*, *NRAS*, and especially *TERT*, not only correlate with adverse histopathologic criteria, but are found in early and persist in late stage disease, identifying risk of aggressive tumor behavior with metastasis and poor prognosis (Griewank et al., 2014; Liu et al., 2016; Nagore et al., 2016; Poynter et al., 2006; Prior et al., 2012; Roh et al., 2017; Shain et al., 2016; Vinagre et al., 2013; Yeh et al., 2013).

We recently optimized the ability to reliably extract both DNA and RNA from adhesive patch skin samples, which made it possible to noninvasively analyze pigmented lesions suspicious for melanoma, not only for gene expression via PLA, but also for the presence of somatic mutations. In this study, we correlated the presence of hotspot driver mutations described in early melanoma and lesions transitioning toward early melanoma with PLA results. Our objective was to determine if histology and expression of *LINC* and *PRAME*, the roles of which in melanoma progression are less well characterized, correlate with the presence of somatic mutations in the *TERT* promoter, in *NRAS*, and in *BRAF* (other than V600E, also frequently found in nevi), allowing mutations to serve as an additional PLA validation platform. We also attempted to determine if a combined strategy based on gene expression and mutation analyses affects the sensitivity and specificity of the PLA.

RESULTS

The study designs for patient cohorts 1 and 2 are summarized in Figure 1. Cohort 1 (n = 103, archival) consisted of patients for whom both PLA and histopathology results were obtained, and cohort 2 (n = 519, prospective) consisted of patients with real-world routine-use PLA (histopathology not available). We identified somatic mutations in three genes (*BRAF* other than V600E, *NRAS*, and the *TERT* promoter) known to be drivers of early melanoma development in a total of 622 PLA-tested pigmented lesions clinically suspicious for melanoma.

Table 1 summarizes cohort 1 results of histopathologic consensus diagnoses, gene expression (PLA), and mutation analyses. Of 103 cohort 1 patients, 30 had melanomas (six melanomas in situ and 24 invasive melanomas with a median tumor thickness of 0.58 mm), and 73 had nonmelanoma lesions, including 61 nevi (46 atypical and 15 conventional nevi) and 12 lesions with nonmelanocytic consensus diagnoses (two seborrheic keratoses, eight solar lentigines, and two squamous cell carcinomas). The PLA result was positive (*LINC* and/or *PRAME* gene expression detected) in 28 (93%) of 30 patients, with a histopathologic consensus diagnosis of melanoma in 31 (42%) of 73 nonmelanoma patients. Mutation analysis showed hotspot driver mutations (*BRAF* other than V600E, *NRAS*, or *TERT* promoter mutations) in 23 (77%) of the 30 melanoma patients but only in 10 (14%) of the 73 nonmelanoma patients. The frequency of the assessed early hotspot driver mutations in histopathologically confirmed melanomas (77%, 23/30) is statistically higher than the frequency in nonmelanomas (14%, 10/73; $P < 0.0001$). Ninety-seven percent of patients (29/30) with a histopathologic consensus diagnosis of melanoma were either PLA gene expression or mutation positive, and 48% (35/73) of nonmelanomas were negative for expression of *LINC*, *PRAME*, and driver mutations, highlighting the allure of an approach that looks at both RNA and DNA risk factors in a single noninvasively obtained sample. In addition, Table 1 shows that *TERT* promoter mutations were the most prevalent mutation type both in melanomas confirmed by histopathologic consensus diagnosis (73%, 22/30) and in PLA-positive melanomas (79%, 22/28). The *TERT* promoter mutations observed most often were −124G>A and −146G>A mutations (each present in 11 patients, and single patients also harbored −126G>A, −132G>A, and −138G>A mutations). *BRAF* V600E mutations were present at similar frequencies in melanoma and nonmelanoma samples (in 10% [3/30] and 8% [6/73] patients, respectively). Conversely, *BRAF* V600K mutations (6%, 2/30) and *NRAS* G61R and K5E (10%, 3/30) mutations were found in melanomas only. Thirty-eight percent (9/24) of invasive and 17% of (1/6) in situ melanomas harbored multiple hotspot mutations.

Table 2 provides details on two PLA-negative patients with histopathologically diagnosed melanomas. One of the patients harbored two *TERT* mutations, and the other patient showed no assessed molecular risk factors. Table 3 expands the data presented and correlates histopathologic diagnoses with mutation analyses and PLA results (double positive with *LINC* and *PRAME* detected and single positive with either *LINC* or *PRAME* gene expression detected). Ninety-three percent (28/30) of all melanomas confirmed by histopathologic consensus diagnoses were PLA positive (95% [23/24] invasive and 83% [5/6] in situ melanomas). PLA positive results were observed in 42% (31/73) of nonmelanoma patients in cohort 1. The highest percentage of the PLA-positive nonmelanoma samples (68%, 15/22)) occurred in nevi read as severely dysplastic by at least one of the three consensus panel dermatopathologists. The presence of hotspot driver mutations other than BRAF V600E followed the same pattern as PLA gene expression results. These hotspot driver mutations were detected in 83% (20/24) invasive and 50% (3/6) in situ melanomas but only in 14% of nonmelanoma patients (11% [5/46] in atypical nevi, 13% [2/15] in conventional nevi, and 17% [2/12] in nonmelanocytic lesions). Figure 2 shows how the presence of different hotspot driver mutations (excluding *BRAF* V600E), gene expression consistent with melanoma (*LINC* and/or *PRAME* detected), and histopathology (consensus diagnosis established by three expert

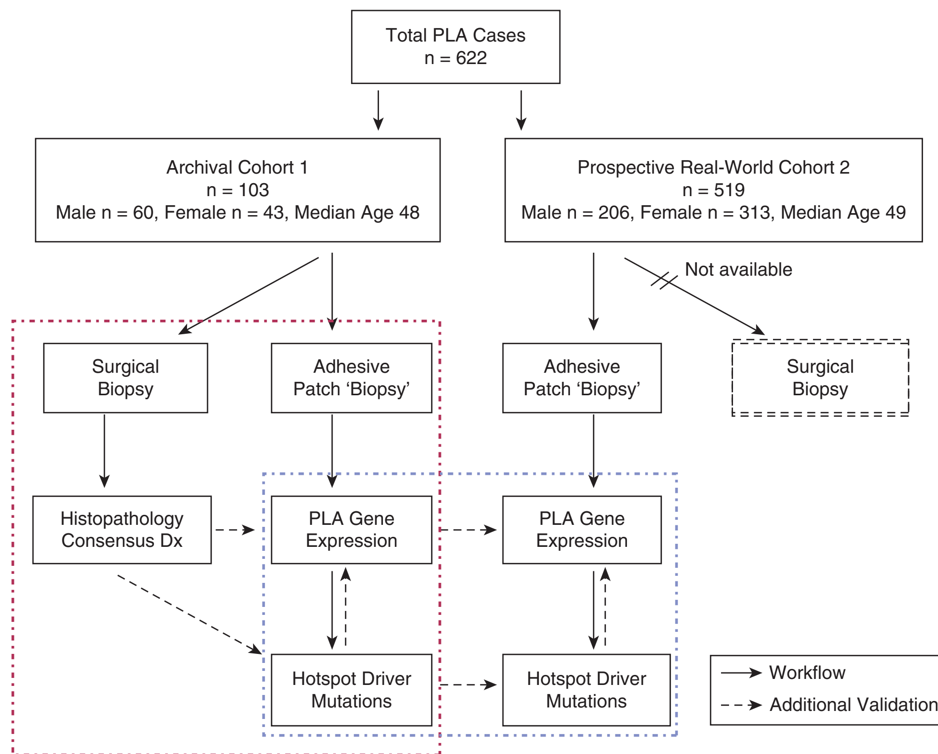

Figure 1. Study design and patient information. Cohort 1 (n = 103) included patients with fully annotated archival PLA results with histopathology based on consensus diagnosis by three dermatopathology experts. Cohort 2 (n = 519) included US patients with prospectively collected, real-world routine-use PLA. PLA, pigmented lesion assay.

dermatopathologists) correlate. Overall, the presence of hotspot driver mutations overlaps with positive gene expression and histopathology with 79% (22/28) of PLA-positive melanoma patients harboring at least one driver mutation, whereas only 10% (3/31) of PLA-positive nonmelanoma patients harbored hotspot mutations. The frequency of hotspot driver mutations other than *BRAF* V600E in melanomas was the highest in lesions located on extremities (93%, 13/14) followed by lesions on the face (67%, 6/9) and the trunk (57%, 4/7). The same pattern was maintained for patients with nonmelanoma consensus diagnoses, of whom 33% (6/18) with lesions on extremities, 23% (3/13) with lesions on the face, and 2% (1/42) with lesions on the trunk had detectable mutations in *BRAF* (other than V600E), *NRAS*, or the *TERT* promoter.

After establishing in well-annotated cohort 1 samples that the hotspot driver mutations in *BRAF* other than V600E, *NRAS*, and the *TERT* promoter investigated here and known to arise during the development of early-stage melanoma are present in about 80% of PLA-positive adhesive patch samples diagnosed histopathologically as melanomas but are absent in more than 80% of nonmelanoma samples, we studied the presence or absence of these mutations in more than 500 routine-use PLA samples (Figure 1 summarizes the study design). A total of 519 prospectively collected real-world PLA samples from cohort 2 (387 PLA-negative and 132-PLA positive samples) were analyzed for these same mutations, and a similar difference in the frequency of hotspot driver mutations was found (Table 4). Eighty-eight percent (342/387) of real-world PLA-negative samples were also negative for any of these melanoma-related mutations, similar to the 82% (36/44) in cohort 1 (Figure 3). The mutation frequencies in cohort 1 and cohort 2 were not statistically different.

DISCUSSION

We showed that expression of *LINC* and *PRAME* determined noninvasively via PLA is highly correlated with the presence of somatic mutations in three genes (*BRAF* non-V600E, *NRAS*, and *TERT*) known to be important in melanoma development and progression. Mutations detected by PLA are highly correlated to those found in surgical biopsy tissue blocks, showing that both RNA expression and somatic mutations can be detected noninvasively using the PLA. These findings are important for two reasons. First, assessing the genetic profile of a pigmented lesion provides insight into its malignant potential with a biologic basis that is consistent with the known role of these genes in melanoma progression and with an objectivity that histology alone cannot provide. Second, by analyzing both gene expression and somatic mutations, the sensitivity of noninvasive PLA testing further improves to a level at which 97% of melanomas can be identified based on a reference standard that uses consensus diagnoses from three expert dermatopathologists. These findings help further validate the importance of *LINC* and *PRAME* genes in melanoma and expand the depth of molecular risk factor analysis in noninvasively obtained samples to help clinicians make more informed biopsy decisions and limit the number of benign biopsies performed. These findings were further evaluated in a large, real-world, US dermatology office cohort of 519 patients with lesions clinically suspicious for melanoma, corroborating the differences in mutation frequency between PLA-positive and PLA-negative results.

In addition to providing an option for situations in which a surgical biopsy is undesirable, there are other advantages to using molecular risk factor data to aid in diagnosing melanoma. Although melanoma has traditionally been diagnosed based on histology, several studies have shown significant intrarater and interrater variation in the classification of melanocytic lesions.

Table 1. Hotspot mutations in cohort 1[1]

Consensus Pathology Diagnosis (Patients)	PLA Gene Expression Analysis	Mutation Analysis	Combined PLA and Mutation Analysis
Melanoma (n=30) pT1a (n=24, median tumor thickness 0.58mm) Melanoma in situ/lentigo maligna (n=6)	PLA(+): 28/30 (93%)	Mut(+): 23/30 (77%), includes *TERT*(+): 22/30 (73%) *NRAS*(+): 3/30 (10%) *BRAF*(+), non-V600E: 2/30 (6%)	PLA(+)/Mut(+): 29/30 (97%) Patients with at least one gene expression or hotspot mutation risk factor
	PLA(−): 2/30 (7%)	Mut(−) 7/30 (23%)	
Nonmelanoma (n=73) Nevus (n=61) Nonmelanocytic (n=12)	PLA(+): 31/73 (42%)	Mut(+): 10/73 (14%), includes TERT(+): 5/73 (7%) NRAS(+): 2/73 (3%) BRAF(+) non-V600E: 3/73 (4%)	
	PLA(−): 42/73 (58%)	Mut(−): 63/73 (86%)	PLA(−)/Mut(−): 35/73 (48%) Patients without gene expression or hotspot mutation risk factors

Abbreviations: Mut, mutation; PLA, pigmented lesion assay; pT1a, primary tumor less than 0.8 mm and without ulceration.

[1]Overview of somatic hotspot driver mutations (*BRAF* other than V600E, *NRAS*, and *TERT*) in fully annotated archival cohort 1 pigmented lesion samples clinically suspicious for melanoma (n = 103). PLA gene expression results and histopathologic consensus diagnoses are available for all patients. Tables 2 and 3 further characterize the cases studied.

In many cases, these variations are not subtle grading of atypia but would result in differential patient treatment plans (Elmore et al., 2017; Malvehy et al., 2014). The evolution of melanoma is now better understood, and adding mutations and gene expression changes present in early stage melanoma to an improved toolbox may lead to a more rational and accurate way to diagnose melanoma at its earliest stages when morphological alterations may not yet be present.

Our findings are in agreement with those of others who have studied mutations in melanoma and precursor nevi. The progression of melanocytic skin lesions is generally initiated by *BRAF* and *NRAS* mutations known to activate the MAPK pathway, followed by telomerase activation and disruption of G1-S checkpoints (Chiba et al., 2017; Shain et al., 2015, 2016; Shay, 2017; Tsao et al., 2012). Because somatic mutations in melanoma are frequently consequences of UV damage events and early occurrences in the establishment of melanoma, this study confirms PLA's ability to identify at-risk lesions early and may also allow the identification of a subset of early disease with potentially indeterminate morphological findings described as *intermediate neoplasms* (Shain et al., 2015, 2016). In addition, these hotspot driver mutations have prognostic implications. In our study, invasive melanomas carried multiple hotspot mutations over twice as often as in situ melanomas; multiple mutations have also been reported to be associated with adverse histopathology (higher mitotic index and tumor thickness), disease progression, and worse prognosis by others (Nagore et al., 2016). Although TERT promoter mutations, among other mutations, have also been described in lesions other than melanoma (Heidenreich et al., 2017), they are independently associated with poor overall survival in patients with nonacral melanomas (median survival = 80 months vs. 291 months for wild type, $P = 0.006$), and the coexistence of *TERT* promoter and *BRAF* mutations in cutaneous melanoma is associated with clinicopathological features of tumor aggressiveness (Griewank et al., 2014; Macerola et al., 2015). Additionally, *TERT* promoter mutations in combination with *BRAF*/*NRAS* mutations can be used to identify patients at risk for aggressive disease (Nagore et al., 2016). As our understanding improves further, the type of observed hotspot mutations may carry additional prognostic information.

Not all *TERT* promoter mutations, the mutation type observed in 79% of melanomas confirmed by histopathologic consensus diagnosis, may be created equal. Borah et al. (2015) found *TERT* promoter mutations at position −124 in most of their urothelial cancer cell lines studies, and this mutation may confer tumor aggressiveness and facilitate the

Table 2. Two of the studied patients with histopathologically diagnosed melanomas were PLA negative[1]

Patient	Pathology Diagnosis 1	Pathology Diagnosis 2	Pathology Diagnosis 3	PLA Analysis	Mutation Analysis
1	Melanoma 0.4 mm	Melanoma 0.3 mm	Melanoma 0.2 mm	PLA(−)	Mut(−)
2	Melanoma in situ	Lentigo maligna	Lentigo maligna	PLA(−)	Mut(+): *TERT* (−146 and −148 G>A)

Abbreviations: Mut, mutation; PLA, pigmented lesion assay.

[1]Diagnosis based on histopathologic consensus diagnosis. *PRAME* and *LINC* not detected. Patient 1: male, age 78 years, Fitzpatrick skin type I, location on left upper arm, new lesion not present before, lesion size = 12 × 9 mm, no ulceration, ABCDE 5/6, ugly duckling, regression, personal history of melanoma, several dermoscopy criteria present. Patient 2: male, age 65 years, Fitzpatrick skin type II, location on right side of face, new lesion not present before, lesion size = 15 × 7 mm, no ulceration, ABCDE 5/6, ugly duckling, no regression, no personal history of melanoma, most dermoscopy criteria absent.

Table 3. Further details on the PLA gene expression and mutation status (without BRAF V600E mutations) within cohort 1

Pathology Diagnosis	Patients, n	Lesion Type (n)	PLA Gene Expression[1], n (%)	Hotspot Mutations (*BRAF* except V600E, *NRAS, TERT*), n (%)
Melanoma	30	Invasive (24)	PLA(L&P): 21 (70)	Mut(+): 18 (60)
			PLA(L/P): 2 (7)	Mut(+): 2 (7)
			PLA(−): 1 (3)	Mut(+): 0 (0)
		In situ (6)	PLA(L&P): 2 (7)	Mut(+): 1 (3)
			PLA(L/P): 3 (10)	Mut(+): 1 (3)
			PLA(−): 1 (3)	Mut(+): 1 (3)
Nonmelanoma	73	Atypical nevi (46)[2]	PLA(L&P): 8 (11)	Mut(+): 1 (1)
			PLA(L/P): 14 (19)	Mut(+): 0 (0)
			PLA(−): 24 (33)	Mut(+): 4 (5)
		Conventional nevi (15)	PLA(L&P): 2 (3)	Mut(+): 1 (1)
			PLA(L/P): 6 (8)	Mut(+): 0 (0)
			PLA(−): 7 (10)	Mut(+): 1 (1)
		Nonmelanocytic (12)[3]	PLA(L&P): 0 (0)	Mut(+:) 0 (0)
			PLA(L/P): 1 (1)	Mut(+): 0 (0)
			PLA(−): 11 (15)	Mut(+): 2 (3)

Abbreviations: L, *LINC;* Mut, mutation; PLA, pigmented lesion assay; P, *PRAME.*
[1]L/P indicates *LINC* or *PRAME* detected. L&P indicates *LINC* and *PRAME* detected. PLA(−) indicates neither *LINC* nor *PRAME* detected.
[2]Overall, 15/22 (68%) of PLA(+) atypical nevi were read as severely atypical by at least one of three consensus panel dermatopathologists.
[3]Overall,11/12 (92%) of the nonmelanocytic lesions studied were PLA negative (a single solar lentigo showed detectable levels of *LINC* gene expression). Two of 11 of the PLA-negative samples, nonmelanocytic by histopathology (one solar lentigo and one seborrheic keratosis), showed *TERT* mutations.

establishment of cell lines. Although further studies are needed to elucidate the roles that different *TERT* promoter mutations may play in the progression of melanocytic lesions, it is of interest to note that −124 mutations were the mutation type most often observed in melanomas positive for both *LINC* and *PRAME*.

Our growing understanding of the genetic evolution of cutaneous melanomas also creates opportunities for improving the PLA assay. In this study, 97% of histopathologically confirmed melanomas were PLA and/or mutation positive, with a specificity of 48%, highlighting the potential of combined mutation and gene expression analyses to improve the accuracy of diagnosing melanoma noninvasively. Also, although most other image-based diagnostic aids frequently misclassify seborrheic keratoses as higher grade melanocytic lesions, we showed that using *LINC* and *PRAME* expression analysis accurately classified all seborrheic keratoses as benign (Malvehy et al., 2014). This is important, because these lesions are frequently the cause of referrals to dermatology specialists by primary care providers, and providing them with such a tool could reduce avoidable referrals.

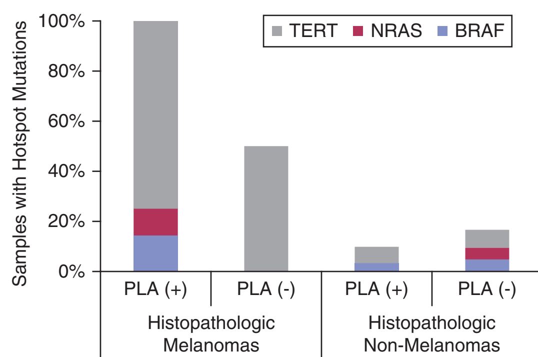

Figure 2. Correlation of mutation analyses (at least one *BRAF* non-V600E, *NRAS*, or *TERT* promoter hotspot mutation detected), PLA gene expression analyses with PLA(+) or PLA(−) samples, and histopathologic analyses (histopathologic consensus diagnoses) in cohort 1 samples (n = 103). Differences between melanoma and nonmelanoma groups were highly statistically significant ($P < 0.0001$). Tables 1−3 provide further details on this visual overview. PLA, pigmented lesion assay.

Based on the findings presented here and a growing body of evidence form other groups (Chiba et al., 2017; Griewank et al., 2014; Macerola et al., 2015; Nagore et al., 2016; Shain et al., 2015, 2016; Shay, 2017; Tsao et al., 2012), we believe that the assessment of early stage melanoma should ultimately shift away from morphologic assessment of disease risk to a molecular assessment of disease risk based on gene expression and mutations. We present data showing that this information can be obtained noninvasively and with high sensitivity and specificity.

MATERIALS AND METHODS

Study subjects and sample collection

This study was conducted in accordance with the Declaration of Helsinki principles and was approved by the Western-Copernicus Group (Santee, CA) independent review board. Figure 1 outlines the study design and summarizes key patient information. The study included a total of 622 epidermal skin samples from two patient cohorts with pigmented skin lesions clinically suspicious for melanoma and collected noninvasively from the patients by using an adhesive patch sample collection kit according to package insert instructions (DermTech, La Jolla, CA). Written informed patient consent was obtained for cohort 1 as required by the

Table 4. Comparison of the gene expression and mutation findings in cohort 1 and cohort 2[1]

Study Cohort	Number of Patients	Histopathologic Diagnosis	PLA Gene Expression Test Result, n		Mutation Test Result, n (%)
1	103	Yes	PLA(+): 59 28 Mel(+) 31 Mel(−)	PLA(L&P): 33	Mut(+): 21 (64)
				PLA(L/P): 26	Mut(+): 4 (15)
			PLA(−): 44 2 Mel(+) 42 Mel(−)		Mut(+): 8 (18)
					Mut(−): 36 (82)
2	519	No	PLA(+): 132	PLA(L&P): 54	Mut(+): 35 (65)
				PLA(L/P): 78	Mut(+): 14 (18)
			PLA(−): 387		Mut(+): 45 (12)
					Mut(−): 342 (88)

Abbreviations: L, *LINC;* Mel, melanoma; Mut, mutation; P, *PRAME*; PLA, pigmented lesion assay.

[1]Mel(+) indicates melanoma by histopathologic consensus diagnosis. Mel(−) indicates nonmelanoma by histopathologic consensus diagnosis. PLA(+) indicates PLA positive result with *LINC* and/or *PRAME* gene expression detected. L&P indicates both *LINC* and *PRAME* gene expression detected. L/P indicates *LINC* or *PRAME* gene expression detected. Mut(+) indicates one or more melanoma hotspot driver mutations (*BRAF* other than V600E, *NRAS*, *TERT* promoter) detected. Mut(−) indicates no melanoma hotspot driver mutation detected.

independent review board. The need for written informed consent was waived by the independent review board for cohort 2 real-world use subjects.

Nucleic acid extraction and quantification

Adhesive patches with pigmented lesion epidermal skin samples were macrodissected via CO_2 laser (GCC, New Taipei City, Taiwan) to separate pigmented lesion material from surrounding normal skin tissue. Cells from the macrodissected area were lysed in a modified lysis buffer obtained from Norgen (Thorold, Ontario, Canada), and both RNA and DNA were co-extracted from the cell lysate using silica-coated magnetic beads on a KingFisher Duo Primer System (Thermo Fisher Scientific, Waltham, MA).

Total human RNA and human genomic DNA (gDNA) in bead extraction eluents were quantified by quantitative real-time reverse transcriptase−PCR (rt-qPCR) (for RNA) and quantitative PCR (qPCR) (for gDNA) on an ABI7900 PCR system (Life Technologies, Carlsbad, CA), with qScript cDNA SuperMix (Quanta Biosciences, Beverly, MA, for RNA reverse transcription to cDNA) and Takyon Rox Probe Mastermix UNG (Eurogentec, Liege, Belgium, for qPCR on cDNA and gDNA) following the manufacturers' instructions. Total human RNA in bead eluents was quantified by using a human β-actin (*ACTB*) gene transcript (mRNA) as a quantification marker and a TaqMan gene expression assay (Hs01060665_g1, probe spans exons), and human gDNA in the same bead eluents was quantified by using the same *ACTB* gene as a quantification marker but a separate TaqMan *ACTB* gene copy number assay (Hs03023880_g1, primers and probe within the same exon). Both TaqMan *ACTB* assays (one for RNA and one for gDNA quantification) were purchased from Life Technologies (Waltham, MA) and used on an ABI 7900 PCR (Life Technologies) system, following the manufacturer's instructions. Details of the process have been reported previously (Gerami et al., 2017; Yao et al., 2016, 2017).

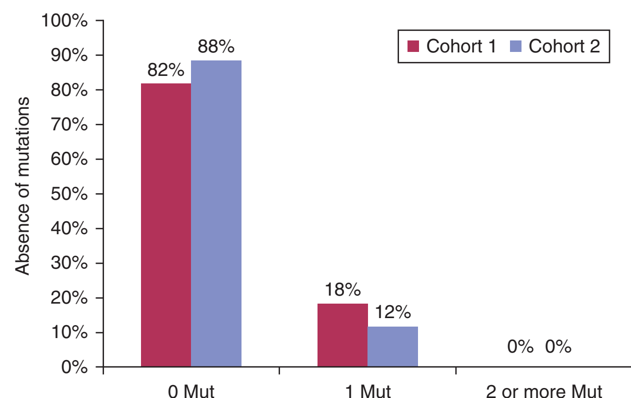

Figure 3. Comparison of hotspot driver mutations in PLA-negative samples of cohort 1 and cohort 2. PLA-negative samples were assessed for the absence of *BRAF* (non-V600E), *NRAS*, and *TERT* promoter hotspot mutations. There were no statistically significant differences between cohort 1 and cohort 2. Details on the mutation status of PLA-positive cohort 1 and cohort 2 samples are provided in Table 4. PLA, pigmented lesion assay.

Target gene expression

After quantification, a normalized quantity of total human RNA from the bead eluents was used for gene expression analysis of the two melanoma-associated genes, *LINC* and *PRAME*, through quantitative real-time reverse transcriptase−PCR on an ABI 7900 PCR system (Life Technologies). In brief, total RNA from the bead eluent was first reverse transcribed to cDNA, which was then used in two biplex qPCR reactions, one containing TaqMan probes for *LINC* (Hs00332749_m1, probe spans exons) and *ACTB* (Hs01060665_g1, probe spans exons, where *ACTB* serves as a control) and one containing TaqMan probes for *PRAME* (Hs01022301_m1, probe spans exons) and *PPIA* (Hs04194521_s1, probe spans exons, where *PPIA* serves as another control). Each biplex qPCR reaction received an amount of the cDNA equivalent to 3 pg of total human RNA in the bead eluents (normalized through the *ACTB* gene expression assay) and was run in duplicate. All TaqMan probes were purchased from Life Technologies. Any qPCR reactions that produced a detectable amplification (or measurable Ct count) of *LINC*, *PRAME*, or both *LINC* and *PRAME* were considered PLA detection positive and consistent with a gene expression pattern observed in melanoma.

Target gene mutation analysis

Sanger sequencing was used to detect gene mutations in human gDNA from the same bead eluents that were tested on *LINC* and *PRAME* gene

expression with PLA. Bead eluents contain both human total RNA and human gDNA from the skin samples collected with the adhesive patch sampling platform described. PCR amplicons were generated from each gDNA sample from five gene regions known to accumulate UV damage-induced somatic mutations of the three genes, *BRAF*, *NRAS*, and *TERT* promoter. These included two amplicons for the two *BRAF* gene exons that cover the hotspot mutations at the 600th and 469th amino acids; two amplicons for the two *NRAS* gene exons that cover hotspot mutations at the 12th, 13th, and 61st amino acids; and one amplicon from the promoter region of *TERT* that covers described key hotspot mutations at −124G>A, −124/125GG>AA, −138/139GG>AA, −146G>A, and −149G>A.

The four amplicons from *BRAF* and *NRAS* were co-amplified in a multiplex PCR reaction, and the amplicon on the *TERT* promoter region was amplified in a monoplex PCR reaction. Both PCR reactions were run in 25 μL final volume of 1× AccuStart II GelTrack PCR SuperMix from Quanta Biosciences (Gaithersburg, MD) that contained 200 nmol/L of each primer (forward and reverse for each exon) and 100 pg of human gDNA from the bead eluent, with the exception of the *TERT* monoplex PCR, which also contained 3% DMSO. The multiplex PCR reactions (for *BRAF* and *NRAS*) were cycled 40 times at 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 60 seconds after the initial 120-second denaturation at 94 °C, and the monoplex PCR reactions (for *TERT*) were cycled 50 times at 94 °C for 10 seconds, 70 °C for 10 seconds, and 72 °C for 60 seconds after the initial 120-second denaturation at 94 °C. Primer information is provided in Supplementary Table S1 online.

After thermal cycling, all PCR products were incubated with ExoSap-It (Thermo Fisher Scientific), following the manufacturer's instruction to enzymatically remove unconsumed deoxynucleotides and primers, as well as any other single-stranded DNA present in the PCR products. These cleaned PCR products were then used for Sanger sequencing of individual gene regions in separate tubes, one per gene region, with a nested primer specific to that gene region. Chromatograms from the Sanger sequencing were reviewed, and sequence data from all samples were aligned to a reference sequence with a multisequence alignment analysis software, Sequencher, version 5.4.6 (GeneCodes, Ann Arbor, MI) to determine the presence of mutations (sequence mismatches) in amplicons from each gDNA sample.

Statistical analysis

Statistical analyses were performed using Excel (Microsoft, Redmond, WA) or R (R Core Team, Vienna, Austria) for which the null hypothesis was no difference among procedures or conditions; analyses were also performed with Student *t*-test. *P* values of less than 0.05 were considered significant.

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CONFLICT OF INTEREST

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ACKNOWLEDGMENTS

We thank Michael Walker (Stanford University, Stanford, CA) for help with statistical analyses and Kamaryn Peters and Jim Rock (DermTech, La Jolla, CA) for support with study logistics.

This study was partially funded by DermTech.

This work was also shared as a late-breaking presentation at the 2018 Annual Meeting of the American Academy of Dermatology in San Diego (February 16–20, 2018) and at the 2018 International Investigative Dermatology/Society for Investigative Dermatology Meeting in Orlando (May 16–19, 2018).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.10.041.

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