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A novel method to assess copy number variations in melanocytic neoplasms: Droplet digital PCR for precise quantitation of *MYC* and *MYB* genes

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Abstract

Introduction: While most melanocytic neoplasms can be classified as either benign or malignant by histopathology alone, ancillary molecular diagnostic tests can be necessary to establish the correct diagnosis in challenging cases. Currently, the detection of copy number variations (CNVs) by fluorescence in situ hybridization and chromosomal microarray (CMA) are the most popular methods, but remain expensive and inaccessible. We aim to develop a relatively inexpensive, fast, and accessible molecular assay to detect CNVs relevant to melanoma using droplet digital polymerase chain reaction (ddPCR) technology.

Methods: In this proof-of-concept study, we evaluated CNVs in *MYC* and *MYB* genes from 73 cases of benign nevi, borderline melanocytic lesions, and primary and metastatic melanoma at our institution from 2015 to 2022. A multiplexed ddPCR assay and CMA were performed on each sample, and the results were compared.

Results: Concordance analysis of ddPCR with CMA for quantification of *MYC* and *MYB* CNVs revealed a sensitivity and specificity of 89% and 86% for *MYC* and 83% and 74% for *MYB*, respectively.

Conclusion: We demonstrate the first use of a multiplexed ddPCR assay to identify CNVs in melanocytic neoplasms. With further improvement and validation, ddPCR may represent a low-cost and rapid tool to aid in the diagnosis of histopathologically ambiguous melanocytic tumors.

KEYWORDS

ambiguous melanocytic neoplasms, chromosomal microarray, copy number variations, droplet digital polymerase chain reaction, melanoma, molecular assays, multiplexed dddPCR assay, quantitation of *MYC* and *MYB* genes

1 | INTRODUCTION

While light microscopy remains the gold standard for diagnosing melanoma, a subset of melanocytic neoplasms has ambiguous features, resulting in poor diagnostic concordance, even among highly experienced dermatopathologists.[1–3] While underdiagnosis can be associated with greater morbidity and mortality, overdiagnosis can also result in undesirable outcomes, especially when lesions are surgically treated in cosmetically sensitive areas (i.e., genitals, centro-facial, acral). Furthermore, overdiagnosis of melanoma can be a source of significant anxiety for patients. Thus, in the past two decades, there has been a collective effort to develop ancillary molecular technologies to aid in the diagnosis of these challenging cases.[1–4]

The ongoing discovery and understanding of genetic mutations and pathways in melanocytic lesions have led to the successful development of powerful molecular diagnostic assays based on DNA and RNA.[2,3] Currently, the detection of DNA copy number variations (CNVs) by fluorescence in situ hybridization (FISH) and chromosomal microarray analysis (CMA), namely array comparative genomic hybridization and single-nucleotide polymorphisms array, are the most widely used technologies.[2–4] Although many academic institutions have incorporated these molecular techniques in the diagnostic workup of challenging melanocytic lesions, they have not been widely adopted by many dermatopathologists outside of academia.

The current discrepancy in practice is largely attributed to the high costs, extended turnaround times, and restricted accessibility of these tests, which are predominantly limited to a small number of referral laboratories in the United States.

Droplet digital polymerase chain reaction (ddPCR) is a new technology that allows for rapid, relatively simple, and cost-effective evaluation of gene copy numbers. Using ddPCR, we aim to develop an accessible DNA-based assay that detects CNVs in melanocytic tumors with comparable efficacy to CMA. In our preliminary pilot studies, we established a successful correlation between digital PCR and CMA for the *RREB1* and *CDKN2A* genes using a singlicate ddPCR assay.[5,6] Building upon this foundation, our current proof-of-concept investigation aimed to develop a multiplex ddPCR assay capable of simultaneous detecting chromosomal alterations on two genes within a single well/reaction. We specifically focused on targeting the MYC and MYB genes, two well-established contributors to the pathogenesis of melanoma.[7,8]

2 | METHODS

This proof-of-concept study protocol was approved by the institutional review board and was conducted following standard operating procedures.

2.1 | Patient cohort

We included all patients from our institution diagnosed with benign nevi, borderline melanocytic lesions, primary melanoma, and metastatic melanoma from 2015 to 2022, for which CMA was performed as part of clinical care or research. The borderline cohort consisted of histopathologically ambiguous lesions diagnosed as atypical Spitz tumor, pigmented epithelioid melanocytoma, dysplastic nevus with severe atypia, and melanocytic tumor of uncertain malignant potential (MELTUMP). All cases were diagnosed by at least one board-certified dermatopathologist (A.S., R.E.L., S.Y., S.M.) from our institution. Clinical and histopathologic information was abstracted from the medical records, including patient age, sex, location and size of the lesion, Breslow depth, and ulceration status (where appropriate).

2.2 | CMA and ddPCR

All cases were assessed using CMA and ddPCR for MYC and MYB copy number status. To ensure assay comparability, both technologies used the same DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue using the QIAGEN QIAamp FFPE Tissue Kit. We diluted the extracted DNA down to a concentration of 2.5 ng/μL. Then, we used 4 μL of this diluted DNA in the reaction mixes. DNA concentration was measured using a Qubit Fluorometer 3.0 and the Qubit dsDNA High-Sensitivity Assay Kit (Thermo Fisher Scientific), and samples with a DNA amount less than 14 ng were excluded. For ddPCR, DNA was pretreated with the HAEIII restriction enzyme (New England BioLabs) for 1 h at 37°C, followed by 5 min at 95°C.

CMA was performed using the OncoScan® Assay Kit (Affymetrix, a Thermo Fisher Scientific Company) following the manufacturer's instructions. Multiplexed ddPCR was done with three main PCR master mix solutions, which were prepared using targeted probes for *MYC*, *MYB*, and four reference genes obtained from Bio-Rad (*MYC*: *THNSL2*, *EIF2C1*, *LIPI*, *SLAIN2*; *MYB*: *THNSL2*, *EIF2C1*, *EFTUD2*, *RPLPO*). These reference genes were selected from genomic loci that show relatively infrequent copy number changes in dermatological neoplasms and evaluated in a preliminary dataset.[9]

The first ddPCR mix was prepared in a 22 μL solution containing 1.1 μL of HEX-labeled *MYC* probes, 0.66 μL of HEX-labeled *MYB* probes, 1.1 μL of FAM-labeled *THNSL2* probes, 0.66 μL of FAM-labeled *EIF2C1* probes, 5.5 μL of ddPCR multiplex supermix, 4 μL of DNA, 0.30 μL of 300 nM DTT, and 8.68 μL of molecular-grade water. The second ddPCR mix was prepared in a 22 μL solution containing 1.1 μL of HEX-labeled *MYC* probes, 1.1 μL of FAM-labeled *LIPI* probes, 0.66 μL of FAM-labeled *SLAIN2* probes, 5.5 μL of ddPCR multiplex supermix, 4 μL of DNA, 0.30 μL of 300 nM DTT and 9.34 μL of molecular-grade water. The third ddPCR mix was similar to the second, except the target gene was *MYB*, and the two reference genes were *EFTUD2* and *RPLPO*.

PCR solutions were then placed into the Bio-Rad automated droplet generator system (Bio-Rad), which produced approximately 20 000 oil droplets/reactions. All emulsified PCR reactions were run in a 96-well plate on the C1000 Touch™ Thermal Cycler, starting with incubation at 95°C for 10 min, followed by 40 cycles of 94°C for 30 s, 60°C for 60 s, 10 min incubation at 98°C and a final hold at 4°C for

1 h. Once finalized, the droplet PCR products were read on the QX200TM ddPCR system and analyzed using the QuantaSoft™ software (Bio-Rad). To ensure consistency of the results, ddPCR reactions were carried out in duplicate. After DNA extraction, the cost for each ddPCR run was approximately $150 per FFPE sample and the final results were available within 72 h.

2.3 | Statistical analysis

We randomly split our data into two cohorts: training data ($n = 36$) and testing data ($n = 37$). The training data were used to calculate the optimal *MYC* and *MYB* copy number cut-off thresholds for separating positive and negative ddPCR samples, whereas the testing data were used to validate those thresholds and assess their performance with "unseen" ddPCR samples. First, a receiver operating characteristic curve analysis was performed to determine *MYC* and *MYB* optimal cut-off thresholds using mean and median ddPCR values across all respective reference genes (Figure 1). Then, the area under the curve, sensitivity, and specificity were used to assess concordance between CMA and ddPCR. To establish the number of additional samples necessary to validate the *MYC* and *MYB* optimal cut-off thresholds, a sample size calculation was performed following the method described by Obuchowski et al.[10] We ensured the method was appropriate and ddPCR values followed binormal distributions by using the Shapiro–Wilk normality test and quantile–quantile plots, respectively. After confirming that the testing data cohort contained an appropriate number of samples, we classified those 37 observations based on the calculated *MYC* and *MYB* cut-off thresholds. Confusion matrices were used to examine the number of correctly and incorrectly classified samples. All statistical analyses were performed using R software (R Core Team).

3 | RESULTS

A total of 73 FFPE skin specimens from 71 patients were included. Forty-seven patients were male (66%) and 24 were female (34%), with an average age of 59 years (range: 7–98). The final cohort consisted of 7 benign nevi (9.6%), 16 borderline lesions (21.9%), 38 primary melanomas (52.1%), and 12 metastatic melanomas (16.4%). In the melanoma subgroup, there were nine superficial spreading melanomas (23.7%), eight nodular melanomas (21.1%), six melanomas not otherwise specified (15.7%), five nevoid melanomas (13.1%), three lentigo maligna melanomas (7.9%), three Spitz melanomas (7.9%), two spindle cell melanomas (5.3%), and two melanomas arising from blue nevus (5.3%). The most common location was the head and neck area ($n = 23$, 32%), followed by the trunk ($n = 19$, 26%) and extremities ($n = 16$, 22%). Clinically measured lesion size ranged from 0.2 to 5.6 cm, with an average of 1.2 cm. The median Breslow depth for primary cutaneous melanomas ($n = 38$) was 2.5 mm, and 12 cases were ulcerated (Table 1).

The optimal ddPCR copy number cut-off thresholds based on the mean values of the training data were 2.52 for *MYC* gain and 1.34 for *MYB* loss (Figure 1). When applied to the 37 observations used for testing, we obtained an accuracy of 87%, sensitivity of 89%, and specificity of 86% for MYC. For MYB, accuracy, sensitivity, and specificity were 76%, 83%, and 74%, respectively.

In the *MYC* CNV analysis, there were five discordant results (6.8%) between ddPCR and the gold-standard CMA, consisting of four false positives and one false negative. The four false positive results involved 1 benign nevus, one borderline lesion (MELTUMP), and two melanomas. The false negative result was a metastatic melanoma of the gastrointestinal tract (Table 1). In the *MYB* CNV analysis, there were 11 discordant results between ddPCR and CMA (15%), of which 9 were false positives and 2 were false negatives (Figure 2). The nine

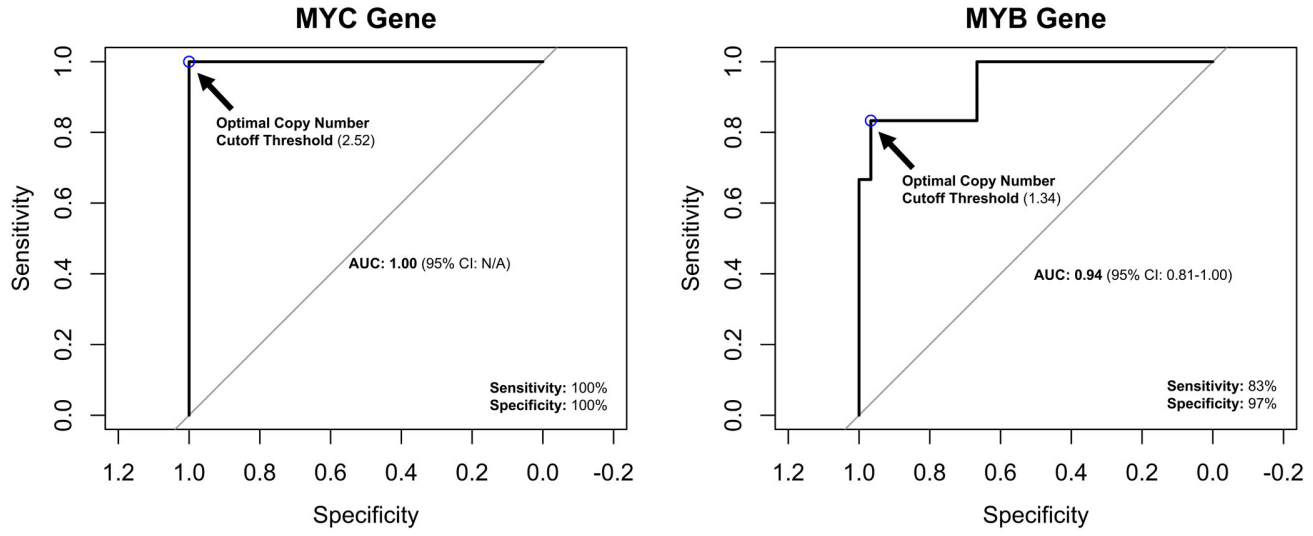

FIGURE 1 Receiver operating characteristic (ROC) curve analysis to determine optical *MYC* and *MYB* copy number cut-off thresholds using training data mean values.

TABLE 1 Summary of clinical characteristics, histological diagnosis, and molecular assay results.

Classification	Age (years)	Sex	Lesion location	Size[a]	BD[b]/ulceration	Histologic diagnosis	ddPCR MYC (+/−)	CMA MYC (+/−)	ddPCR MYB (+/−)	CMA MYB (+/−)
Benign nevus	45	F	Head/neck	0.7	–	Compound melanocytic nevus	(+)	(−)	(+)	(−)
Benign nevus	38	F	Lower extremity	0.8	–	Compound melanocytic nevus	(−)	(−)	(−)	(−)
Benign nevus	23	F	Lower extremity	1.6	–	Compound melanocytic nevus	(−)	(−)	(−)	(−)
Benign nevus	76	M	Trunk	0.7	–	Compound melanocytic nevus	(−)	(−)	(+)	(−)
Benign nevus	50	F	Head/neck	0.3	–	Junctional melanocytic nevus	(−)	(−)	(−)	(−)
Benign nevus	74	M	Head/neck	1.7	–	Intradermal melanocytic nevus	(−)	(−)	(−)	(−)
Benign nevus	20	F	Trunk	0.6	–	Spitz nevus	(−)	(−)	(−)	(−)
Borderline lesion	41	M	Upper extremity	1.1	–	Dysplastic nevus with severe atypia	(−)	(−)	(−)	(−)
Borderline lesion	84	F	Upper extremity	0.9	–	Dysplastic nevus with severe atypia	(−)	(−)	(−)	(−)
Borderline lesion	19	M	Head/neck	0.7	–	Atypical Spitz tumor	(−)	(−)	(−)	(−)
Borderline lesion	20	M	Trunk	1.0	–	Atypical Spitz tumor	(−)	(−)	(+)	(−)
Borderline lesion	33	F	Upper extremity	0.9	–	Atypical Spitz tumor	(−)	(−)	(−)	(−)
Borderline lesion	7	F	Trunk	0.7	–	Atypical Spitz tumor	(−)	(−)	(−)	(−)
Borderline lesion	12	M	Head/neck	0.6	–	Atypical Spitz tumor	(−)	(−)	(−)	(−)
Borderline lesion	18	F	Special site	0.7	–	Atypical Spitz tumor	(−)	(−)	(−)	(−)
Borderline lesion	33	F	Head/neck	0.7	–	Melanocytoma	(−)	(−)	(−)	(−)
Borderline lesion	47	F	Head/neck	1.0	–	Melanocytoma	(−)	(−)	(−)	(−)
Borderline lesion	34	F	Trunk	0.6	–	Melanocytoma	(−)	(−)	(−)	(−)
Borderline lesion	39	F	Trunk	0.4	–	Melanocytoma	(−)	(−)	(−)	(−)
Borderline lesion	47	F	Head/neck	1.0	–	Melanocytoma	(−)	(−)	(−)	(−)
Borderline lesion	61	F	Upper extremity	0.4	–	MELTUMP	(−)	(−)	(−)	(−)
Borderline lesion	48	F	Upper extremity	1.3	–	MELTUMP	(−)	(−)	(−)	(−)
Borderline lesion	43	M	Head/neck	0.7	–	MELTUMP	(+)	(−)	(−)	(−)
Melanoma	75	M	Head/neck	1.5	–	Lentigo maligna	(−)	(−)	(+)	(−)
Melanoma	67	M	Head/neck	1.0	0.2 mm/no	Lentigo maligna melanoma	(−)	(−)	(−)	(−)
Melanoma	80	M	Trunk	4.0	0.4 mm/no	Lentigo maligna melanoma	(−)	(−)	(+)	(−)
Melanoma	65	M	Trunk	0.6	0.3 mm/no	Superficial spreading melanoma	(−)	(−)	(−)	(−)
Melanoma	69	F	Trunk	1.5	0.7 mm/no	Superficial spreading melanoma	(−)	(−)	(−)	(−)
Melanoma	75	M	Head/Neck	1.5	3.0 mm/yes	Superficial spreading melanoma	(−)	(−)	(−)	(−)
Melanoma	58	M	Trunk	1.1	0.3 mm/no	Superficial spreading melanoma	(−)	(−)	(−)	(−)
Melanoma	71	M	Trunk	1.4	0.7 mm/no	Superficial spreading melanoma	(−)	(−)	(+)	(−)
Melanoma	84	M	Head/neck	0.4	1.2 mm/no	Superficial spreading melanoma	(−)	(−)	(−)	(−)
Melanoma	65	M	Trunk	1.9	0.9 mm/no	Superficial spreading melanoma	(−)	(−)	(−)	(−)

TABLE 1 (Continued)

Classification	Age (years)	Sex	Lesion location	Size[a]	BD[b]/ulceration	Histologic diagnosis	ddPCR MYC (+/−)	CMA MYC (+/−)	ddPCR MYB (+/−)	CMA MYB (+/−)
Melanoma	75	M	Trunk	1.7	2.9 mm/no	Superficial spreading melanoma	(+)	(+)	(+)	(+)
Melanoma	70	M	Upper extremity	0.7	2.2 mm/no	Superficial spreading melanoma	(+)	(+)	(−)	(−)
Melanoma	61	M	Trunk	2.3	5.0 mm/yes	Nodular melanoma	(+)	(+)	(−)	(−)
Melanoma	65	M	Lower extremity	1.6	1.6 mm/no	Nodular melanoma	(−)	(−)	(+)	(+)
Melanoma	64	M	Trunk	1.1	3.2 mm/no	Nodular melanoma	(−)	(−)	(−)	(−)
Melanoma	62	M	Trunk	2.5	3.3 mm/no	Nodular melanoma	(−)	(−)	(−)	(−)
Melanoma	71	M	Head/Neck	0.2	2.8 mm/no	Nodular melanoma	(−)	(−)	(−)	(−)
Melanoma	60	M	Trunk	1.2	4.2 mm/yes	Nodular melanoma	(+)	(+)	(−)	(−)
Melanoma	76	M	Trunk	1.5	2.5 mm/yes	Nodular melanoma	(+)	(+)	(−)	(+)
Melanoma	98	M	Upper extremity	4.2	9.8 mm/yes	Nodular melanoma	(+)	(−)	(−)	(−)
Melanoma	80	M	Head/neck	1.6	0.7 mm/no	Nevoid melanoma	(−)	(−)	(−)	(−)
Melanoma	84	F	Special site	1.3	4.8 mm/yes	Nevoid melanoma	(−)	(−)	(+)	(+)
Melanoma	58	M	Head/neck	0.7	1.6 mm/no	Nevoid melanoma	(+)	(+)	(−)	(−)
Melanoma	49	M	Upper extremity	0.9	2.1 mm/no	Nevoid melanoma	(+)	(+)	(+)	(+)
Melanoma	69	M	Head/neck	0.7	1.2 mm/no	Nevoid melanoma	(−)	(−)	(−)	(−)
Melanoma	84	M	Head/neck	4.0	4.8 mm/yes	Spitz melanoma	(−)	(−)	(−)	(−)
Melanoma	59	M	Upper extremity	0.6	0.6 mm/no	Spitz melanoma	(−)	(−)	(−)	(−)
Melanoma	41	F	Upper extremity	0.5	0.3 mm/no	Spitz melanoma	(+)	(−)	(+)	(−)
Melanoma	73	M	Head/neck	5.6	6.4 mm/yes	Spindle cell melanoma	(−)	(−)	(−)	(−)
Melanoma	70	M	Head/neck	5.5	15.0 mm/yes	Spindle cell melanoma	(−)	(−)	(−)	(−)
Melanoma	39	M	Trunk	2.5	6.6 mm/yes	Melanoma arising in blue nevus	(+)	(+)	(+)	(+)
Melanoma	45	M	Head/neck	4.8	16.0 mm/no	Melanoma arising in blue nevus	(+)	(+)	(+)	(−)
Melanoma	98	M	Special site	0.3	2.7 mm/yes	Not otherwise specified	(−)	(−)	(+)	(+)
Melanoma	74	M	Head/neck	1.5	4.1 mm/yes	Not otherwise specified	(−)	(−)	(+)	(−)
Melanoma	67	F	Upper extremity	1.1	1.3 mm/no	Not otherwise specified	(−)	(−)	(−)	(−)
Melanoma	84	M	Head/neck	0.3	4.9 mm/no	Not otherwise specified	(+)	(+)	(−)	(−)
Melanoma	63	F	Upper extremity	0.7	1.9 mm/no	Not otherwise specified	(+)	(+)	(−)	(−)
Melanoma	69	M	Lower extremity	1.4	3.8 mm/no	Not otherwise specified	(+)	(+)	(−)	(−)
Metastatic melanoma	54	M	Lymph node	1.0	–	Metastatic melanoma	(−)	(−)	(−)	(+)
Metastatic melanoma	57	M	Lymph node	0.4	–	Metastatic melanoma	(+)	(+)	(−)	(−)
Metastatic melanoma	63	M	Lymph node	1.5	–	Metastatic melanoma	(−)	(−)	(−)	(−)
Metastatic melanoma	68	M	Cutaneous	1.7	–	Metastatic melanoma	(−)	(−)	(+)	(+)
Metastatic melanoma			Cutaneous	1.4	–	Metastatic melanoma	(−)	(−)	(+)	(+)

(Continues)

TABLE 1 (Continued)

Classification	Age (years)	Sex	Lesion location	Size[a]	BD[b]/ulceration	Histologic diagnosis	ddPCR MYC (+/−)	CMA MYC (+/−)	ddPCR MYB (+/−)	CMA MYB (+/−)
Metastatic melanoma	88	M	Cutaneous	4.0	-	Metastatic melanoma	(−)	(−)	(−)	(−)
Metastatic melanoma	55	F	Gastrointestinal	4.7	-	Metastatic melanoma	(−)	(+)	(−)	(−)
Metastatic melanoma	71	F	Gastrointestinal	-	-	Metastatic melanoma	(+)	(+)	(−)	(−)
Metastatic melanoma	67	M	Liver	0.6	-	Metastatic melanoma	(+)	(+)	(−)	(−)
Metastatic melanoma			Liver	0.7	-	Metastatic melanoma	(+)	(+)	(−)	(−)
Metastatic melanoma	82	F	Breast	2.4	-	Metastatic melanoma	(+)	(+)	(+)	(+)
Metastatic melanoma	89	M	Brain	3.0	-	Metastatic melanoma	(−)	(−)	(+)	(+)

Abbreviation: ddPCR, droplet digital polymerase chain reaction.
[a]Clinical size (cm).
[b]Breslow depth.

false positive results consisted of two benign nevi, one borderline lesion (atypical Spitz tumor), and six melanomas, while the two false negatives were one primary melanoma and one metastatic melanoma to the lymph node (Table 1).

4 | DISCUSSION

Melanomas are characterized by the presence of well-known specific chromosomal imbalances, with gain of copies of oncogenes and loss of tumor suppressor genes. On the other hand, with very few exceptions, such alterations are rare or absent in benign nevi. The most widely recognized CNVs in melanoma occur on chromosomal loci 6q23 (*MYB*), 6p24 (*RREB1*), 8q24 (*MYC*), 9q21 (*CDKN2A*), 11q13 (*CCND1*), and 12q13 (*CDK4*).[2,4–8,11,12] The exact pathophysiologic mechanisms by which these genetic alterations contribute to melanomagenesis are not completely understood, but their implications in the evolution of melanoma are well-established. They constitute the basis of our current advances in the field of molecular genomics in pigmented skin lesions.[2–4,11,12]

The development of CMA and FISH to detect CNVs in melanocytic lesions was a breakthrough discovery. These excellent ancillary molecular tools can aid a pathologist in rendering a final diagnosis in challenging histopathologically ambiguous melanocytic lesions, ultimately improving patient care. The accuracy of CMA and FISH vary between studies, nonetheless, they have satisfactory sensitivities and specificities ranging from 80% to 90% and 80% to 100%, respectively.[11,12] Perhaps the biggest issue with these molecular tests is the lack of accessibility to many dermatopathologists.[11]

As the field of molecular diagnostics progresses, there is a clear need for more accessible, faster, and less costly molecular assays, with comparable reliability to CMA and FISH. Digital PCR has been used successfully to detect CNVs in different malignancies, including melanocytic neoplasms.[5,6,13–15] In our current study, we observed an acceptable concordance between ddPCR and CMA, with MYC and MYB exhibiting concordant results in 93.2% and 85.0% of cases, respectively. Similarly, studies have reported concordance rates ranging from approximately 70%–90% between FISH and CMA.[4,16]

Currently, FISH and CMA are considered the gold-standard diagnostic approaches for histopathologically ambiguous melanocytic lesions. Despite being excellent technologies, they have certain limitations. CMA is a complex assay, requiring highly specialized devices and multiple labor-intensive sequential steps that may take days to complete.[11,17] Although this technology provides valuable genome-wide information, the chromosomal data needs to be interpreted by specialized personnel to get accurate final results.[18] Finally, CMA requires a high proportion of neoplastic cellular DNA content in the tissue block to yield precise results.[16] Some of these factors may potentially contribute to challenges related to accessibility and longer turnaround times experienced with CMA.

FISH is a targeted assay that assesses a limited number of genes of interest, depending on the panel used. To obtain accurate results, FISH requires highly trained personnel to manually count nuclei,

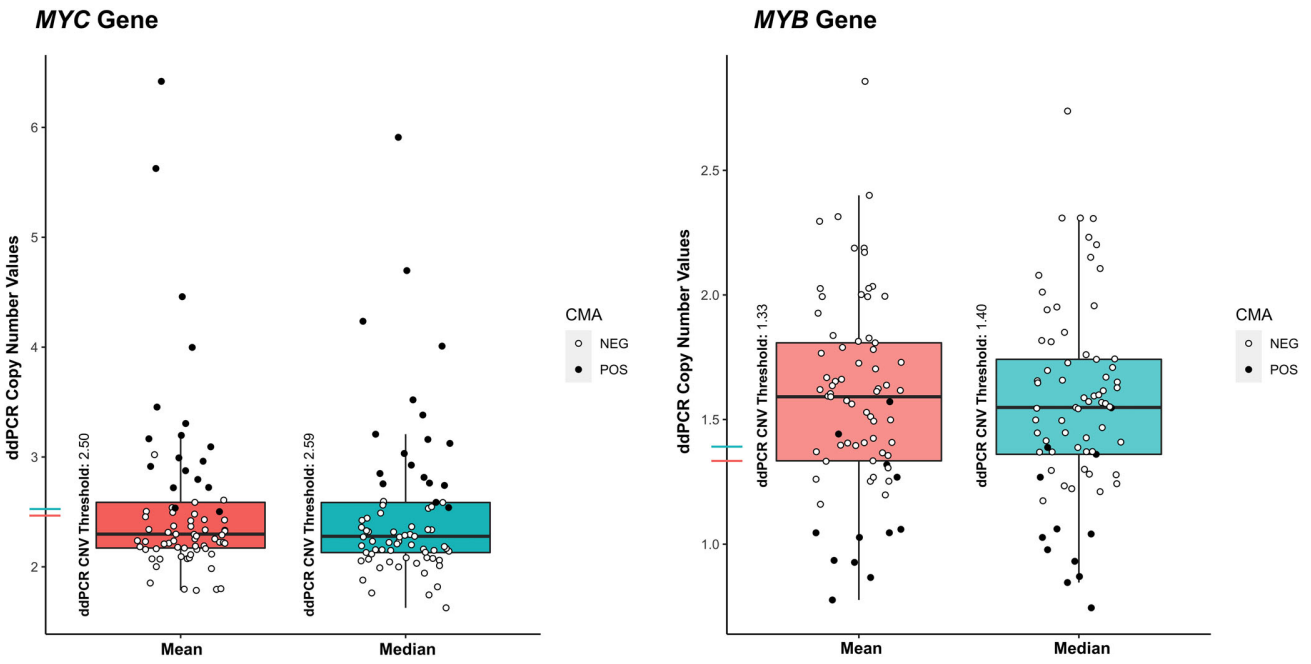

FIGURE 2 *MYC* and *MYB* droplet digital polymerase chain reaction (ddPCR) and chromosomal microarray analysis (CMA) whole dataset results.

record the number of individual hybridizations, and determine whether they exceed established cutoffs. Cut-off values can vary substantially across laboratories due to the desired sensitivity and specificity profiles of the test.[19] Furthermore, technical challenges, such as distinguishing tumor cells from background nevus or polyploidy, can lead to interobserver variability and misinterpretation. Because of this, competence in evaluating FISH requires both a substantial case volume and molecular pathology experience. In addition to the aforementioned limitations, both CMA and FISH can be expensive.[11]

Digital PCR offers a streamlined, targeted CNV detection technology that can be completed within hours by a trained technician. This technology offers high sensitivity, minimal sample volume requirements, the capacity to process multiple samples simultaneously and is unaffected by cellular polyploidy.[19,20] The process involves three main steps: (1) Combining the extracted target DNA with ddPCR mix and introducing it into the automated droplet generator to produce the PCR droplets; (2) transferring the reaction plate containing the droplets to a thermal cycler for PCR amplification; (3) the droplet solution is introduced to the ddPCR reader to obtain an easy-to-read count of target DNA copies. The simplified workflow, coupled with the low cost of ddPCR consumables and reagents, holds promise for reducing expenses and enhancing accessibility.

4.1 | Future applications of ddPCR in melanocytic neoplasms

Digital PCR shows potential for a variety of future research applications. In contrast to our prior studies, where we employed singlicate ddPCR assays to identify chromosomal alterations in the *RREB1* and *CDKN2A* genes, the current study demonstrates the utilization of a multiplexed ddPCR assay capable of simultaneously detecting MYC and MYB CNVs. The diagnostic utility of this emerging technology may be further enhanced with the development of a more comprehensive gene panel, similar to FISH (e.g., *RREB1*, *CCND1*, *MYC*, *MYB*, *CDKN2A*).[2,4,11] The versatility of digital PCR may even allow the possibility of customized gene panels depending on the lesion being examined (e.g., a different gene panel when evaluating Spitzoid tumors versus a suspected nevoid melanoma). In addition, one could theoretically analyze targeted genomic abnormalities in primary melanomas and metastatic lesions to assess possible genetic relationships.

4.2 | ddPCR limitations and discordant results

Digital PCR has certain limitations. As a targeted molecular assay, like FISH, it can only detect chromosomal alterations in a restricted set of chromosomal loci of interest.[19] In contrast, FISH offers the unique advantage of directly visualizing chromosomal abnormalities in specific lesional areas of interest, which is not feasible with digital PCR. Moreover, digital PCR can solely identify known CNVs, unlike CMA, which has the capability to reveal previously unknown CNVs that may possess crucial pathological significance.[19]

In preliminary data, we found that cases with more than a month between embedding and DNA extraction sometimes yielded spurious results. These variabilities may be due to formalin-induced DNA fragmentation and practices related to tissue fixation. Hence, we restricted our cases to only those for which DNA was extracted within a month of embedding. In terms of performance, there were a

total of 16 ddPCR discordant *MYC* and *MYB* results, leading to 13 false positives and 3 false negatives. These results are likely multifactorial and we did not determine the exact cause of each. We noted that two of the three false negative cases were associated with dense inflammation, which could theoretically skew genomic results as inflammatory cell's DNA can dilute tumor DNA. Therefore, it is plausible that ddPCR results may be influenced by tumor purity. Interestingly, 8 of the 13 false positive results were melanoma cases.

5 | CONCLUSION

To our knowledge, this is the first use of a multiplexed digital PCR assay to accurately quantitate *MYC* and *MYB* chromosomal abnormalities in melanocytic lesions. While digital PCR is not a replacement for FISH and CMA, it shows potential as an affordable and rapid alternative. We believe that this novel molecular assay can be refined into a precise diagnostic tool, ultimately leading to its widespread accessibility among dermatopathologists and patients alike.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ETHICS STATEMENT

Reviewed and approved by the institutional review board at Dartmouth Health System (Study number: 00031828).

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