Diagnostic Pathology

RESEARCH Open Access

Combined immunohistochemistry of PRAME and p16 in the differentiation of melanocytic neoplasms, with a detailed focus on acral lesions

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Abstract

Background Isolated immunohistochemical indicators are limited to diagnose melanocytic neoplasms. This retrospective study is to assess the diagnostic value of combined immunohistochemical analysis targeting preferentially expressed antigen in melanoma (PRAME) and p16 in melanocytic neoplasms, with a detailed focus on arcal lesions.

Methods This was a single center cohort study from January 2022 to June 2023. A total of 165 identified cases were collected, including 112 melanomas (MMs) and 53 melanocytic nevi, which were composed of 122 acral samples and 43 non-acral samples. Immunohistochemistry(IHC) for both PRAME and p16 was performed in these cases, which was subsequently statistically analyzed to assess the diagnosis ability of PRAME and p16.

Results In total samples, the sensitivity and specificity of PRAME(+) for MM are 82.1% and 94.3% (AUC = 0.882, 95%CI:0.827–0.938), while of p16(-) for MM are 31.25% and 94.3% (AUC = 0.628, 95%CI:0.542–0.714); PRAME(+)/p16(-) (meaning as PRAME(+) *or* p16(-)) displayed a sensitivity and specificity of 85.7% and 88.7% for MM (AUC = 0.872, 95%CI:0.810–0.934), while PRAME(+) &p16(-) (meaning as PRAME(+) *and* p16(-)) revealed a sensitivity and specificity of 27.7% and 100% in MM (AUC = 0.638, 95%CI:0.555–0.722). In acral samples, PRAME(+)/p16(-) exhibited a specificity of 94.7% and a sensitivity of 86.9% for MM (AUC = 0.908, 95%CI: 0.849–0.968), with sensitivities of 90.9% for invasive MM and 82.5% for preinvasive MM, respectively; The sensitivity and specificity of PRAME(+) &p16(-) for MM is 22.6% and 100% (AUC = 0.613, 95%CI: 0.513–0.714) respectively. In non-acral samples, the sensitivity and specificity of PRAME(+)/p16(-) for MM are 82.1% and 73.3% (AUC = 0.777, 95%CI: 0.622–0.933), while of PRAME(+) &p16(-) are 42.9% and 100% (AUC = 0.714, 95%CI:0.564–0.864).

Conclusion Combined IHC of PRAME and p16 contributes to discriminating melanocytic neoplasms, especially for in situ acral MM.

Keywords Melanoma (MM), Melanocytic nevus, PRAME, p16, Immunohistochemistry(IHC)

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Introduction

Melanoma (MM) stands out as a highly aggressive skin neoplasm, with its incidence showing a relentless upward trend. Regrettably, the therapeutic outcomes achieved through conventional modalities like radiotherapy, chemotherapy, and immunotherapy in patients with advanced MM remain far from satisfactory [1, 2]. Within the realm of diagnostic pathology, the timely identification of MM continues to pose a formidable challenge, particularly with the limited utility of traditional immunohistochemical markers in distinguishing between benign lesions and MM. Despite notable advancements in molecular pathology techniques, such as fluorescence in situ hybridization and comparative genomic hybridization, for characterizing malignant melanocytic neoplasms, the widespread adoption of these approaches in clinical settings is impeded by their prohibitive cost implications [3, 4].

PRAME, initially identified as a CUL2 ubiquitin ligase subunit in reactive T cells from MM patients [5], exhibits a physiological expression pattern in tissues like the testis, ovaries, placenta, adrenal glands and endometrium [6, 7]. However, its expression becomes dysregulated in different cancer types, including lung, breast, kidney, ovarian, leukemia, synovial sarcoma, and mucinous liposarcoma, often correlating with aneuploidy and metastasis [8, 9]. Recent study has elucidated PRAME's role in inducing genomic instability and augmenting reliance on the alternative base excision repair pathway, rendering cells susceptible to PRAP1/2 inhibition in uveal melanoma [10]. These findings have catapulted PRAME into the limelight as a pivotal oncogenic driver and a promising target for immunotherapeutic interventions and diagnostic biomarkers [10–12].

In the authentic milestone of PRAME evaluation in surgical pathology by Lezcano C et al. in 2018 [13], the IHC assessment of PRAME expression across primary MMs, metastatic MMs, and melanocytic nevi revealed diffuse positivity in 87% of metastatic and 83.2% of primary MM cases, while melanocytic nevi exhibited negative PRAME expression in 86.4% of cases. Some scholars have used PRAME IHC to distinguish MM that are more difficult to diagnose, such as mucosal MM of the head and neck region [14], and subungual and non-subungual acral melanocytic lesions [15].

Nevertheless, PRAME positivity was also observed in some arcal nevi, dysplastic nevi, recurrent nevi, and Spitz nevi [16]. Similarly, our prior investigations documented PRAME positivity rates of 89.9% and 93.1% in primary and metastatic MM, respectively [17], alongside positive expression in 6 out of 317 melanocytic nevi. Additionally, in 91 cases of in situ acral lentiginous melanoma (ALM) and 18 cases of in situ subungual MM, PRAME exhibited sensitivities of 75.8% and 77.8%, respectively, with 2 out of 40 cases of recurrent nevi of the ALM displaying positive staining [3]. Collectively, these studies underscore PRAME as a relatively sensitive marker to differentiate melanocytic neoplasms, albeit with certain limitations particularly in situ acral melanoma (AM).

The protein p16, encoded by CDKN2a situated on chromosome 9's long arm, assumes significance in MM diagnosis [4]. Studies showed a frequent absence of p16 expression in primary and metastatic MM, in stark contrast to its consistent expression in melanocytic nevi [2, 18]. In a clinicopathologic study of 50 acral Spitz nevus cases, acral Spitz nevi were characterized by strong and diffuse p16 expression, which differed from acral nevi and ALMs [19]. Nevertheless, the utility of p16 immunostaining in discerning benign from malignant melanocytic lesions remains contentious.

Since PRAME and p16 had their own strengths and weaknesses in the differentiation of melanocytic tumors, combined PRAME and p16 or other biomarkers (e.g., SOX10, HMB-45, Ki-67) IHC were utilized for better discrimination [20–22]. Bahmad and co-workers had applied combinated PRAME and p16 IHC to distinguish melanocytic nevi(51 cases) from MMs(77 cases), and found that PRAME(+)& p16(-) melanocytic lesion was unlikely to be a nevus, and most nevi exhibited PRAME (-)&p16(+) pattern.

In this study, we retrospectively analyzed the PRAME and p16 IHC results of confirmed 112 MMs and 53 melanocytic nevi, with the view to be able to improve the diagnostic rate of MM in the clinic. Since AM has poor survival and accounts for a considerable share of MM-associated morbidity and mortality worldwide which requires early diagnosis urgently, we specifically focused on acral samples(84 AMs and 38 acral nevi) and preinvasive ones.

Materials and methods

Study subjects

112 unequivocally diagnosed cases of MM were meticulously selected from the archives of our hospital, spanning from January 2022 and June 2023, including MM typically associated with CSD Pathway: 17 cases(11 superficial spreading MMs and 6 lentigo maligna MMs); MM not consistently associated with cumulative solar damage (no CSD) Pathway: 90 cases(84 AMs, 4 MMs arising in congenital nevus,1 MM arising in blue nevus and 1 mucosal MM); and Nodular MM: 5 cases. Concurrently, 53 cases of melanocytic nevus from the corresponding time-frame served as controls, including 38 acral nevi, 10 Spitz nevi, 2 dysplastic nevi, 2 recurrent nevi and 1 mucous nevus. Each case underwent meticulous, independent scrutiny by two seasoned dermato-pathologists (Qiuju Miao and Hao Chen), ensuring adherence to the diagnostic criteria delineated in the

WHO-2018 Classification of Skin Tumors (4th edition) [23]. Comprehensive clinical data of the patients were meticulously compiled and encapsulated within Table 1.

IHC

Immunohistochemical assessment targeting PRAME and p16 was conducted on all aforementioned specimens. Formalin-fixed paraffin-embedded tissue sections (4 μm thick) were performed according to our previously established method [3, 17]. The rabbit-derived monoclonal antibody PRAME (Clone number: EPR20330. Abcam, USA) was applied at a dilution of 1:400 and incubated for 30 min at ambient temperature. The chromogenic substrates 3,3'-diaminobenzidine or FAST RED were utilized for visualization, with hematoxylin employed for counterstaining. For the analysis of protein p16, an automated IHC platform (Auto-stainer link45 System, Dako, Denmark) was employed, utilizing a mouse-derived anti-human monoclonal antibody (Clone number: MX007, Fuzhou Maixin Biotechnology Development Co., LTD, China) as the first antibody.

Result interpretation

PRAME expression was interpreted as positive when its cumulative score was greater than four points, otherwise interpreted as negative. The cumulative score was made up of PRAME expression intensity score (weak: 1, medium: 2 and strong: 3) and positive percentage score (None: 0, 1-25%: 1, 26-50%: 2, 51-75%: 3 and >75%: 4) in tumor samples according to our previous study [3] (Fig. 1). The expression of p16 could be divided into reserved expression (uniform or checkerboard expression) or deficient expression (none or partial expression: <2%), which was interpreted as negative with deficient expression, otherwise interpreted as positive (Fig. 1). The result interpretation was done double-blind by two dermatopathologists (Qiuju Miao and Hao Chen). In the event of disagreement, a discussion was held to reach a consensus on the IHC expression intensity and positive percentage score.

Table 1 Data of clinical information and the IHC of PRAME and p16 in patients with MM or melanocytic nevus

	Age (Average, Year)	Gender (M/W)	PRAME (*P*/*N*)	p16 (*P*/*N*)	*n*
MM	58.3	44/68	92/20	77/35	112
Invasive MM	59.5	26/38	58/6	46/18	64
Non-acral MM	56.2	5/15	18/2	12/8	20
AM	61.1	21/23	40/4	34/10	44
In situ MM	56.6	18/30	34/14	31/17	48
Non-acral MM	56.9	5/3	5/3	5/3	8
AM	56.5	13/27	29/11	27/13	40
Melanocytic nevus	29.7	15/38	3/50	50/3	53
Acral nevus	30.7	11/27	1/37	37/1	38
Spitz nevus	25.6	3/7	2/8	9/1	10
Dysplastic nevus	37.5	0/2	0/2	2/0	2
Recurrent nevus	33	1/1	0/2	1/1	2
Mucous nevus	10	0/1	0/1	1/0	1

IHC, immunohistochemistry; MM, melanoma; AM, acral melanoma; M, man; W, woman; P, positive; N, negative

Statistical analysis

Statistical analysis was performed using SPSS (26.0 version; IBM Corp., SPSS Statistics for Windows Version 26.0, Armonk, NY, USA). The χ2 test was used to assess whether PRAME, p16, and their combined patterns have a significant association with MM or melanocytic nevus. Their positivity and negativity, sensitivity and specificity, and ROC and AUC in MM are obtained simultaneously. $P<0.05$ was considered to be statistically significant.

Results

For total samples

In patients diagnosed with MM, the prevalence of PRAME expression was 82.1% (92/112), with 17.9% (20/112) exhibiting negative expression. Additionally, the positivity rate for p16 was 68.75% (77/112), while 31.25% (35/112) showed negative expression. Conversely, melanocytic nevi demonstrated a PRAME positivity rate of 5.7% (3/53) and a negativity rate of 94.3% (50/53). The positivity rate for p16 in melanocytic nevi was 94.3% (50/53), with a negativity rate of 5.7% (3/53).

The sensitivity of PRAME(+) for diagnosing MM was 82.1%, with a specificity of 94.3% (AUC=0.882; 95% CI: 0.827–0.938, $P<0.001$). Conversely, the sensitivity of p16(-) for MM diagnosis was 31.25%, with a specificity of 94.3% (AUC=0.628; 95% CI: 0.542–0.714, $P=0.008$). Statistical analysis via the χ2-test underscored the significance of PRAME ($P<0.001$) and p16 ($P<0.001$) in distinguishing MM from melanocytic nevus (Fig. 2).

Of the 112 MM cases, 96 exhibited the immunophenotype PRAME(+)/p16(-), while 31 cases displayed PRAME(+) &p16(-). In contrast, among the 53 melanocytic nevi cases, PRAME(+)/p16(-) was expressed in only 6 cases (3 spitz nevi, 2 acral nevi and 1 recurrent nevus), while PRAME(+) &p16(-) in 0 cases. The sensitivity and specificity of PRAME(+)/p16(-) for MM were 85.7% and 88.7% (AUC=0.872; 95%CI: 0.810–0.934, $P<0.001$), while for PRAME (+) &p16(-), the values were 27.7% and 100% (AUC=0.638; 95%CI: 0.555–0.722, $P=0.004$).

For acral samples and non-acral samples

In 122 acral samples, PRAME(+) exhibited a specificity of 97.4% and a sensitivity of 82.1% for MM (AUC=0.898; 95%CI: 0.839–0.956, $P<0.001$), with sensitivities of 90.9% and 72.5% for invasive and preinvasive MM, respectively. Besides, the sensitivity and specificity of p16(-) for MM were 27.4% and 97.4% (AUC=0.624; 95%CI: 0.524–0.724, $P=0.029$). PRAME(+)/p16(-) demonstrated a specificity

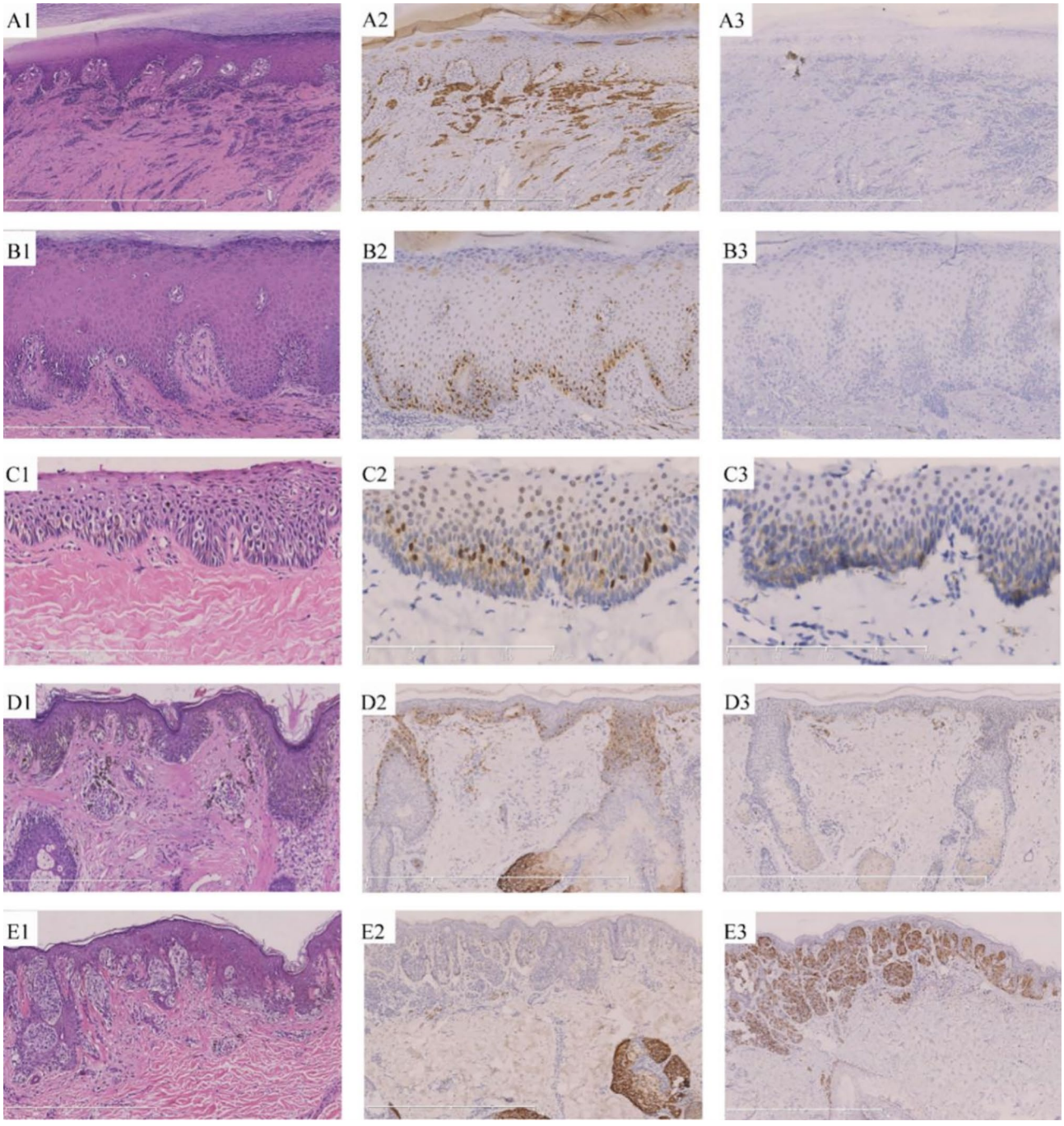

Fig. 1 The results of H&E and IHC staining of PRAME and p16 in melanocytic neoplasms. **A**, Invasive AM; **B**, AM in situ; **C**, subungual MM in situ; **D**, lentigo maligna MM; **E**, dysplastic nevus. 1, H&E staining; 2, IHC of PRAME; 3, IHC of p16. ABDE:10×10 magnification; C:10×40 magnification.IHC, immunohistochemistry; MM, melanoma; AM, acral melanoma

of 94.7% and a sensitivity of 86.9% for MM(AUC=0.908; 95%CI: 0.849–0.968, $P<0.001$), with sensitivities of 90.9% and 82.5% for invasive and preinvasive MM, respectively. PRAME(+)/p16(-) exhibited a sensitivity of 22.6% and a specificity of 100% for MM (AUC=0.613; 95%CI: 0.513–0.714, $P=0.046$) (Fig. 2).

In non-acral samples, the sensitivity and specificity of PRAME(+)/p16(-) for MM were 82.1% and 73.3% (AUC=0.777; 95%CI: 0.622–0.933; $P=0.003$), while for PRAME(+) &p16(-), the values were 42.9% and 100% (AUC=0.714; 95%CI: 0.564–0.864; $P=0.022$) (Fig. 2). Tables 2 and 3 compared additional diagnostic indicators

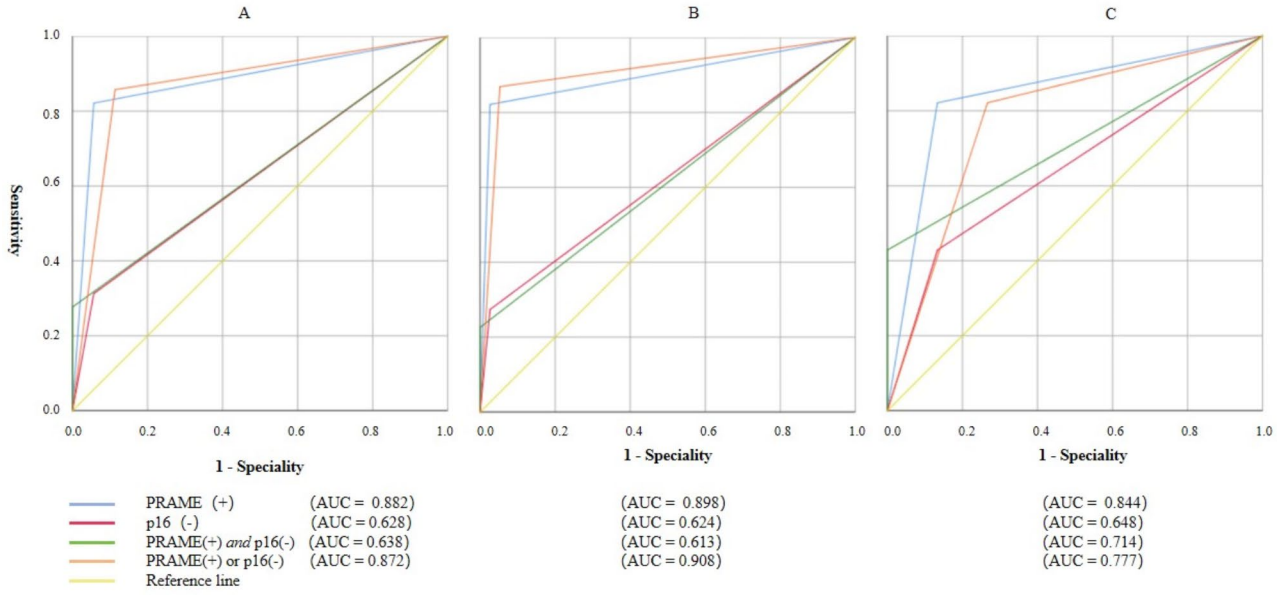

Fig. 2 The ROC for the total samples (**A**), acral samples (**B**) and non-arcal samples (**C**)

in acral and non-acral samples and in invasive and preinvasive cases, respectively.

Discussion

The immunohistochemical diagnosis of MM presents a formidable challenge, as it necessitates discrimination from common melanocytic nevi, Spitz tumors, and atypical nevi while exhibiting poor diagnostic consistency [24, 25]. Notably, a study involving 187 pathologists (113 general pathologists and 74 dermatopathologists) analyzing 24 melanocytic nevi inclusive mild dysplaia(class I from MPATH-Dx classifictaion) found a consistency less than 70% even in comparatively easy diagnoses [26]. This lack of uniformity underscores the considerable risk of misdiagnosis and subsequent adverse outcomes, a concern shared among clinicians and pathologists alike. IHC stands as a primary ancillary method for distinguishing between benign and malignant melanocytic lesions. However, conventional markers like HMB45, Melan-A, S100, and Sox10 primarily affirm melanocytic lineage rather than differentiate benign and malignant tumors.

Recent attention has turned to PRAME as a promising marker for MM and melanocytic nevus identification due to its relatively robust sensitivity and specificity [13]. Nevertheless, PRAME's reduced expression in preinvasive MM and partial expression in melanocytic nevi still contribute to diagnostic challenges [3]. PRAME appears as nuclear staining in MM. There are two main criteria used for PRAME (+) lesions, (1) significant diffuse positivity, (2) cumulative scoring methods integrating expression intensity and positive percentage [3, 16, 17]. The criteria have helped diagnosis, for instance, an arbitrary PRAME IHC score of <5 versus ≥ 5 adopted by Santandrea, et al. could accurately lead to a differentiation of 82.5% of benign and 87.1% of malignant lesions [16], while Ricci and co-colleagues showed that the first-rank cut-off ($<60\%$ versus $\geq 60\%$) of PRAME(+) cells to make a distinction between benign nevus and mucosal MM of the head and neck region [14]. Thus, the consensus cut-off value of PRAME for MM and its subtypes requires further investigation.

Similarly, p16 expression fluctuates widely in melanocytic lesions due to gene deletions or copy number alterations (CNA) [27]. This variability makes p16 unreliable as a standalone identifier. For instance, p16 positivity ranged from 12 to 93% in primary cutaneous invasive MMs, 0–71% in metastatic MMs, and 61–100% in melanocytic nevi [18].

In our study encompassing 165 melanocytic neoplasms, PRAME exhibited a sensitivity of 82.1% and specificity of 94.3% for MM identification (AUC=0.882). However, a notable portion of MM cases remained challenging to diagnose. PRAME's positivity in MM in situ was only 70.8% (34/48), in AM in situ was only 72.5%(29/40). In a study on diagnostic utility of PRAME IHC for subungual and non-subungual acral melanocytic lesions, any PRAME expression (1+to 4+) was identified in 73% (16/22) subungual MMs and 95%(19/20) AMs, respectively. One of 14 (7%) acral nevi expressed PRAME [15]. Therefore, PRAME is not a specicial biomarker for

Table 2 Comparison of diagnosis indicators among the total, acral and non-acral samples

	Total sample($n=165$)				Acral samples($n=122$)				Non-acral samples($n=43$)			
	Sensitivity	Specificity	*P*	AUC	Sensitivity	Specificity	*P*	AUC	Sensitivity	Specificity	*P*	AUC
PRAME(+)	82.1%	94.3%	<0.001	0.882	82.1%	97.4%	<0.001	0.898	82.1%	86.7%	<0.001	0.844
p16(-)	31.25%	94.3%	<0.001	0.628	27.4%	97.4%	0.001	0.624	-	-	0.104	0.648
PRAME(+) &p16(-)	27.7%	100%	<0.001	0.638	22.6%	100%	0.001	0.613	42.9%	100%	0.009	0.714
PRAME(+) /p16(-)	85.7%	88.7%	<0.001	0.872	86.9%	94.7%	<0.001	0.908	82.1%	73.3%	<0.001	0.777

PRAME(+) &p16(-), means as PRAME(+) *and* p16(-); PRAME(+)/p16(-), means as PRAME(+) *or* p16(-). *P* refers to the result of $X2$ test. AUC, the area under the receiver operating characteristic curve

Table 3 Comparative analysis of sensitivity and specificity in acral samples of invasive and preinvasive MM

	Invasive AM ($n=44$)		AM in situ ($n=40$)	
	Sensitivity	Specificity	Sensitivity	Specificity
PRAME(+)	90.9%	97.4%	72.5%	97.4%
p16(-)	22.7%	97.4%	32.5%	97.4%
PRAME(+) &p16(-)	22.7%	100%	22.5%	100%
PRAME(+) /p16+(-)	90.9%	94.7%	82.5%	94.7%

PRAME(+) &p16(-), means as PRAME(+) *and* p16(-); PRAME(+)/p16(-), means as PRAME(+) *or* p16(-). AM, acral melanoma

MM, emphasizing its limitations in differentiating benign and malignant tumors.

Conversely, p16 showed high positivity in melanocytic nevi (94.3%) but unexpectedly appeared in 68.75% of MM cases, far exceeding anticipated levels. This may be hemozygous or heterozygous deletion and alterations via DNA methylation and CNV of CDKN2A [28, 29], and indicates that p16 IHC alone has a limited capacity in differentiating melanocytic nevi from MM. Therefore, there is a requirement to explore the diagnostic and differential ability of combined IHC marker patterns.

It is currently believed that PRAME is a proto-oncogene and p16 is a tumor suppressor. In addition, PRAME(+) was significantly related to MM, and p16(+) was closely connected with melanocytic nevi [2, 3, 17, 20, 21, 30]. Combining PRAME and p16 IHC could enhance diagnostic accuracy.

In a single-center retrospective cohort study-reported by Bahmad, et al., PRAME(+) and PRAME(+)&p16(-) had a sensitivity and specificity of 89.6% and 96.1%(AUC=0.928; 95% CI: 0.878–0.979; P=0.009), 27.3% and 100%(AUC=0.636; 95% CI: 0.542–0.731; P=0.009), respectively, for MM versus melanocytic nevus; most melanocytic nevi (48/51, 94.1%) were PRAME(-)&p16(+) with a minority of MM [21].

In this study, our findings showed that PRAME(+) &p16(-) pattern achieved 100% specificity for MM but with lower sensitivity (27.7%), almost consistent with the result of PRAME(+) &p16(-) in Bahmad et al.' study. As for PRAME and p16, our result also had a similar positivity of MM and melanocytic nevus compared with that of Bahmad, et al. ' study [21]. Unsimilarly, PRAME(+)/p16(-) pattern offered higher sensitivity (85.7%) and specificity (88.7%) in total samples (AUC=0.872; 95%CI:0.810–0.934), with exceptional performance in AM(sensitivity 86.9%, specificity 94.7%, AUC=0.908; 95%CI: 0.849–0.968) prevalent in Asia, and increase the sensitivity for preinvasive AM by 10% compared with PRAME(+) alone. Thus, either PRAME-positive or p16-negative (PRAME(+)/p16(-)) balanced the

requirements of diagnostic sensitivity and specificity, and also demonstrated good predictive ability in MM, especially in AM in situ.

Despite these promising findings, our study had several limitations. Firstly, there were some biases in the statistical analysis of this study due to the uneven sample sizes of MM and melanocytic nevus, with the implication that additional PRAME combinated p16 IHC investigations are still required. Secondly, our selection of these clearly diagnosed specimens was a "priori " and also a bias as it eliminated "difficult and suspicious" cases. Thirdly, the sample size was relatively small, particularly for some MM subtypes (e.g. mucosal MM, MM arising in blue nevus) and staging, which may have limited the precision of our results. Fourthly, establishing a cut-off value for new patterns may require a larger number of samples and quantitative analysis. Lastly, our study was not multicenter studies which are warranted to further validate our findings and assess their generalizability.

Conclusions

Our study demonstrates that PRAME exhibits a sensitivity of 82.1% and a specificity of 94.3% in diagnosing MM, while p16 lacks specificity in discerning benign lesions from MM. Combining PRAME and p16, PRAME(+) & p16(-) showed 27.7% sensitivity and 100% specificity for MM diagnosis, significantly reducing the misdiagnosis rate. The combination of PRAME(+) or p16(-) improved the overall sensitivity and specificity for MM, particularly in preinvasive AM. Furthermore, the combined IHC of PRAME and p16 outperformed its application in acral lesions compared to non-acral lesions, thus contributing significantly to the differentiation of melanocytic neoplasms.

Abbreviations

PRAME	Preferentially expressed antigen in melanoma
MM	Melanoma
AM	Acral melanoma
ROC	Receiver-operator characteristic curves
AUC	Area under the receiver operating characteristic curve
IHC	Immunohistochemistry
ALM	Acral lentiginous melanoma
CNA	Copy number alterations

Acknowledgements

Not applicable.

Author contributions

J.Z. wrote the paper and analyzed the data. J. Z., Q. M., X. S., H. S. and X. W. collected data, did HE staining and immunohistochemical staining and interpret results; Y. Z. and H. C. designed the research, and reviewed & edited the manuscript. All authors reviewed the manuscript.

Funding

This work was supported by grants from CAMS Innovation Fund for Medical Sciences (CIFMS) (2023-12M-C&T-B-110).

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Ethical clearance for the study was obtained from the Institutional Review Board of Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College (Ethical Approval: No. 2013-LC/KY-033). Moreover, written informed consent was conscientiously acquired from all patients or their legal guardians.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 5 July 2024 / Accepted: 6 December 2024
Published online: 27 December 2024

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