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Prevalence of NRAS Mutation, PD-L1 Expression and Amplification, and Overall Survival Analysis in 36 Primary Vaginal Melanomas

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Disclosures of potential conflicts of interest may be found at the end of this article.

Key Words. Primary vaginal melanoma • Ooncogenic mutations • NRAS • PD-L1 expression

Abstract

Background. Primary vaginal melanomas are uncommon and aggressive tumors with poor prognosis, and the development of new targeted therapies is essential. This study aimed to identify the molecular markers occurring in these patients and potentially improve treatment strategies.

Materials and Methods. The clinicopathological characteristics of 36 patients with primary vaginal melanomas were reviewed. Oncogenic mutations in *BRAF*, *KIT*, *NRAS*, *GNAQ* and *GNA11* and the promoter region of telomerase reverse transcriptase (*TERT*) were investigated using the Sanger sequencing. The expression and copy number of programmed death-ligand 1 (*PD-L1*) were also assessed.

Results. Mutations in *NRAS*, *KIT*, and *TERT* promoter were identified in 13.9% (5/36), 2.9% (1/34), and 5.6% (2/36) of the primary vaginal melanomas, respectively. *PD-L1* expression and amplification were observed in 27.8% (10/36) and 5.6% (2/36) of cases, respectively. *PD-L1* positive expression and/or amplification was associated with older patients (p = .008). Patients who had *NRAS* mutations had a poorer overall survival compared with those with a wild-type *NRAS* (33.5 vs. 14.0 months; hazard ratio [HR], 3.09; 95% CI, 1.08–8.83). Strikingly, two patients with/without *PD-L1* expression receiving immune checkpoint inhibitors had a satisfying outcome. Multivariate analysis demonstrated that >10 mitoses per mm^2 (HR, 2.96; 95% CI, 1.03–8.51) was an independent prognostic factor.

Conclusions. *NRAS* mutations and *PD-L1* expression were most prevalent in our cohort of primary vaginal melanomas and can be potentially considered as therapeutic targets. ***The Oncologist*** 2020;25:e291–e301

Implications for Practice: This study used the Sanger sequencing, immunohistochemistry, and fluorescence in situ hybridization methods to detect common genetic mutations and *PD-L1* expression and copy number in 36 primary vaginal melanomas. *NRAS* mutations and *PD-L1* expression were the most prevalent, but *KIT* and *TERT* mutations occurred at a lower occurrence in this rare malignancy. Two patients receiving immune checkpoint inhibitors had a satisfying outcome, signifying that the *PD-L1* expression and amplification can be a possible predictive marker of clinical response. This study highlights the possible prospects of biomarkers that can be used for patient selection in clinical trials involving treatments with novel targeted therapies based on these molecular aberrations.

Introduction

The incidence of melanoma is on a gradual rise and growing at a faster rate than other solid tumors [1]. Vaginal malignant melanoma, one of its highly aggressive subtypes and often diagnosed at an advanced stage, is an extremely rare mucosal melanoma that accounts for 2.4%–2.8% of all vaginal cancers and 0.3%–0.8% of all malignant melanomas [2]. Nowadays, there are multiple therapeutic strategies for treating vaginal melanomas, including surgery, radiotherapy, chemotherapy, targeted therapy, boron neutron capture therapy, and immunotherapy, but their prognoses are

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still dismal, with a 5-year survival rate ranging from 0% to 32.3% [3–8].

In-depth research on melanoma has shown that targeted and immunotherapies could improve the outcomes in patients with melanoma [9]. Previous studies have shown that mucosal melanomas at different anatomic locations exhibit different oncogenic aberrations [10–13]. Several studies have focused on identifying the molecular alterations of melanomas composed of specific mutations in *NRAS*, *BRAF*, *KIT*, *TERT*, and *GNAQ/GNA11*, observed in distinct subtypes of melanomas. The overall rate of *BRAF* mutation in melanoma has been found to be up to 67% [14, 15], and the mutation frequently occurs in non-chronically sun-damaged (CSD) skin. *NRAS* is mutated in 10%–25% of cutaneous melanomas and occurs most frequently at hotspots in codons 12 and 61 [16–18] and activates downstream effectors. An increase in copy number (up to 25%) and mutations (10%–20%) of *KIT* in mucosal, acral, and CSD melanomas were identified [19]. Mutations in *GNAQ/GNA1*1, a gene encoding an α subunit of heterotrimeric G proteins, are found in up to 83% of uveal melanomas [20–22]. *GNAQ* and *GNA11* mutations in melanomas affect codons 209 or 183 and result in consistent activation of the protein kinase C and *MAPK* pathways [21, 23]. *TERT*, which encodes the catalytic subunit of telomerase, is mutated in cutaneous melanoma [24–26] and has been found to be associated with aggressive behavior of the melanoma and a poorer prognosis [25, 27]. In recent years, mutant-selective *BRAF* [28], *MEK* [29–31], and *KIT* inhibitors [32] have demonstrated impressive clinical results in molecularly selected patients.

Several previous studies have demonstrated that patients wth melanoma, non-small cell lung cancer, and renal cell carcinoma could achieve a 10%–40% clinical response with immune checkpoint inhibitions [33, 34]. However, approximately 7%–34% of these cases do also experience high-grade immune-related adverse events [35, 36]. Therefore, to increase treatment compliance and outcome, appropriate biomarkers capable of predicting response are highly needed for identifying patients who would be most beneficial to these targeted therapies. Of them, the programmed death-ligand 1 (*PD-L1*) is the most broadly investigated and binds to inhibitory checkpoint molecule *PD-1*. The detection of *PD-L1* expression in tumor cells or tumor-associated stromal cells by immunohistochemistry (IHC) has enabled the identification of tumors which would response to anti-*PD-L1* blockade [34, 37, 38]. However, published correlative data for vaginal melanoma remain scarce.

In the present study, we performed an analysis of the clinicopathological features of 36 patients with primary vaginal melanoma in a single institution. Further major molecular alterations including the *PD-L1* status were characterized to improve the current understanding of altered molecular pathways and thereby explore possible strategies for their therapeutic management.

Subjects, Materials, and Methods

Study Participants

A total of 36 primary vaginal melanomas samples were collected from patients treated at the Sun Yat-sen University Cancer Center between March 2004 and February 2018. Of them, 32 had surgery as their primary treatment, including radical surgery and local excision with wide margin, 2 were treated with chemoradiotherapy or immune checkpoint inhibitors after biopsy, and 2 refused treatment after diagnosis. Of those 32 patients, 20 received chemotherapy, 12 received radiotherapy, 5 received a second-time surgical resection, 2 received interferon-α, and 1 received immune checkpoint inhibitors during the course of subsequent treatment (supplemental online Table 1). The following pathological characteristics of tumor were evaluated: presence or absence of ulceration or pigmentation, depth of invasion (DOI; measured from the outermost point of the mucosa to the deepest point of invasion), number of mitoses per mm^2, and the predominant cell type (epithelioid, spindle cell, or mixed). The tumor was staged according to the 8th edition of the American Joint Committee on Cancer staging system for vaginal melanoma [39]. The protocol was designed in accordance with the Declaration of Helsinki. This study was approved by the Research Ethics Committee of the Sun Yat-sen University Cancer Center (No. B2016–069–01).

DNA Isolation and Genetic Mutation Detection

Formalin-fixed, paraffin-embedded (FFPE) tissue blocks were reviewed for quality control, and the regions containing more than 50% of tumor cells were selected for macrodissection. Genomic DNA was extracted using the QIAamp DNA FFPE Tissue kit (QIAGEN, Hilden, Germany). Direct sequencing of *KIT* (exons 9, 11, 13, 17, and 18), *NRAS* (exons 2 and 3), *BRAF* (exon 15), *TERT* (promoter region), and *GNAQ* and *GNA11* (exons 4 and 5) were performed using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions for the 3500XL Genetic Analyzer (Applied Biosystems). The primer sequences are listed in supplemental online Table 2. Mutations in *BRAF* exon 15 V600E were identified by the 7500 real-time quantitative PCR system (Applied Biosystems) using the minor groove binder (MGB) probes. Primers and probes for the V600E assay were as follows: forward: 5'-ATGAAGACCTCACAGTAA AAATAGG-3'; reverse: 5'-AGACAACTGTTCAAACTGATGGG-3'; mutation anchor: FAM-TCTAGCTACAGAGAAA-MGB; wild anchor: HEX-TCTAGCTACAGTGAAA-MGB.

PD-L1 Expression and Copy Number Detection

The determination of *PD-L1* expression was performed with the rabbit monoclonal anti-*PD-L1* antibody (E1L3N; dilution 1:200; Cell Signaling Technology, Danvers, MA) using an Autostainer Plus (Dako; Agilent Technologies, Santa Clara, CA). *PD-L1* expressions in tumor cells and tumor-infiltrating lymphocytes (TILs) were classified as positive if moderate-to-strong membrane staining was observed in >1% of the tumor cells and/or TILs. *PD-L1* gene copy number per cell was investigated by fluorescence in situ hybridization (FISH) using a *PD-L1*/chromosome 9 centromere probe (LBP Medicine Science and Technology Co., Ltd, Guangzhou, China). FISH analysis was independently reviewed by two investigators who were blinded to the gene expression data (by X.Z. and X.-H.Y.). The copy numbers were counted in 100 non-overlapping tumor cell nuclei. As there is no consensus on a

standard approach in the *PD-L1* FISH scoring system, for this study tumors with >5 *PD-L1* copies per cell were classified as PD-L1 FISH positive (+) according to the Cappuzzo scoring system [40, 41], including *PD-L1* amplification, which was characterized by tumor cells with *PD-L1*-CEP9 ratio > 2.0 or > 10 copies per cell in >10% tumor cells. *PD-L1* positive expression and/or amplification were regarded as a *PD-L1*+. *PD-L1* loss was characterized by a *PD-L1*-CEP9 ratio of <0.8. The FISH signals were assessed under a microscope (Olympus BX61; Olympus, Tokyo, Japan) equipped with a triple-pass filter (DAPI/Green/Orange, Vysis). Images were acquired using the BioView Automated Imaging Analysis System (BioView Ltd, Rehovot, Israel).

Statistical Analysis

Differences in the distributions of baseline characteristics were investigated using the chi-squared (χ^2) or Fisher's test between subgroups. Overall survival (OS) and 95% confidence intervals (CIs) were calculated using the Kaplan-Meier method and compared using the log-rank test. A Cox proportional hazard model was initially built for a univariate analysis and then used to evaluate independent factors for each biological and clinical feature associated with survival. All statistical analyses were performed using the SPSS software version 19.0 for Windows (SPSS Inc., Chicago, IL), and statistical significance was defined as a probability level <.05.

Results

Patients' Clinical Characteristics

The main clinicopathological and molecular features of the 36 vaginal melanomas are summarized in Table 1 and supplemental online Table 2. The median age of the patients was 48 years (range, 27–77). The predominant growth pattern observed was the nodular subtype (88.9%, 32/36), followed by the superficial spreading (5.6%, 2/36). Twenty-eight cases (77.8%) showed an epithelioid morphology, and five (13.9%) displayed a spindle cell morphology. Pigmentation was observed in 33 vaginal melanomas (91.7%). The DOI of the tumors ranged from 0.4 to 62.5 mm (median, 10.0), and ulceration were observed in 52.8% of the cases (19/36). Mitotic activity was 0 to 80 per mm^2 (median, 8 per mm^2).

The Prevalence of Oncogenic Mutations

Direct sequencing was performed to identify the status of the gene mutations. *NRAS* mutations were found in 5 of the 36 (13.9%) primary melanomas, of which 4 had Q61R mutation, and one had Q61P mutation. No clinicopathological feature demonstrated any significant association with the *NRAS* mutation status (supplemental online Table 3). *KIT* sequence analysis was performed in 34 cases, of which a missense and V559D mutation in *KIT* exon 11 was detected in one patient (2.9%, 1/34). *TERT* C228T mutations were identified in two cases (5.6%), one of whom had a concurrent *NRAS* Q61R mutation. *BRAF*, *GNAQ*, and *GNA11* mutations were not detected in any cases. Samples of

Table 1. Clinicopathologic characteristics of the 36 primary vaginal melanomas

Variable	Patients, *n* (%)
Total	36
Median age (range), yr	48 (27–77)
Tumor phenotype	
Superficial spreading	2 (5.6)
Nodular	32 (88.9)
Unknown	2 (5.6)
Ulceration	
Absent	17 (47.2)
Present	19 (52.8)
Cellularity	
Epitheloid	28 (77.8)
Spindle cell	5 (13.9)
Mixed	3 (8.3)
Pigmentation	
Absent	3 (8.3)
Present	33 (91.7)
Mitotic activity, n/mm^2	
0	4 (11.1)
1–10	16 (44.4)
>10	16
Breslow thickness, mm	44.4
Median (range)	10 (0.4–62.5)
≤1.0	1 (2.8)
1.0–2.0	2 (5.6)
2.0–4.0	3 (8.3)
4.0–10.0	13 (36.1)
>10.0	14 (38.9)
Unknown	3 (8.3)
Surgery approach	
WLE	17 (47.2)
RE	15 (41.7)
Biopsy	4 (11.1)
Lymphadenectomy	
Yes	26 (72.2)
No	10 (27.8)
Lymph node metastasis	
Present	10 (27.8)
Absent	22 (61.1)
Unknown	4 (11.1)
AJCC Stage	
I	2 (5.6)
II	22 (61.1)
III	10 (27.8)
Unknown	2 (5.6)

Abbreviations: AJCC, American Joint Committee on Cancer; RE, Radical excision; WLE, wide local excision.

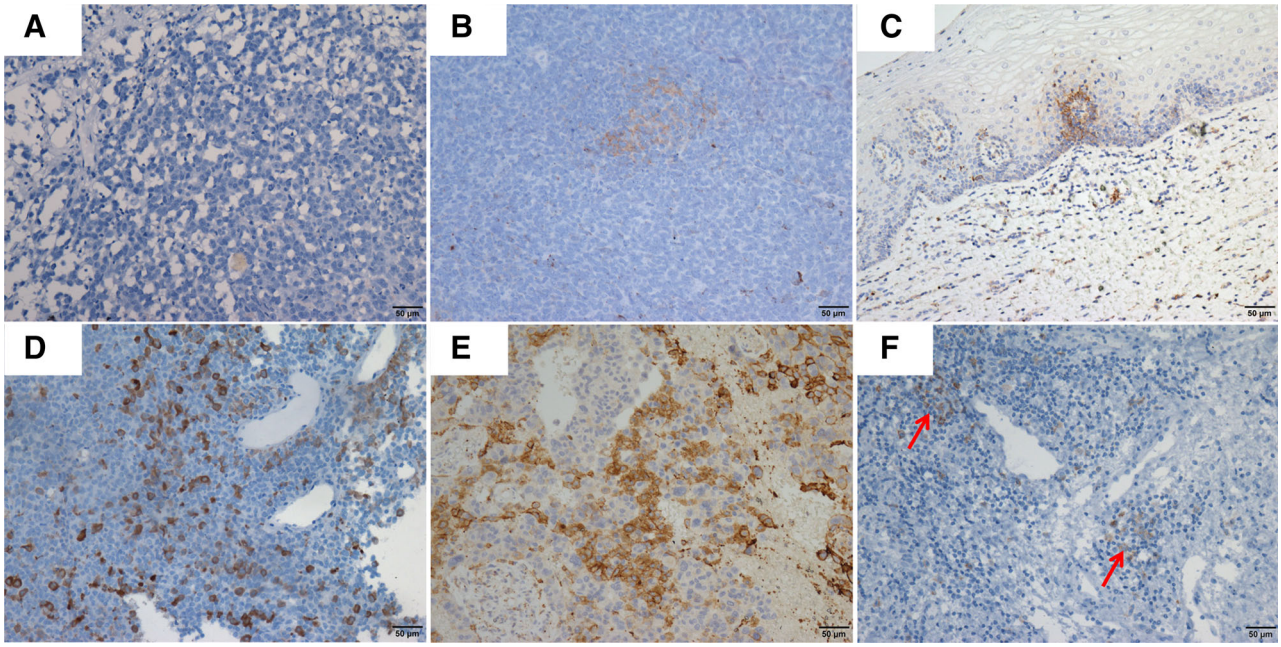

Figure 1. Photomicrographs showing immunohistochemistry staining of the programmed death-ligand 1 (*PD-L1*) expression of tumor cells and tumor-infiltrating lymphocytes (TILs) in vaginal melanoma samples. The percentage of *PD-L1* expression in tumor cells was 0% **(A)**, 3% **(B and C)**, 20% **(D)**, and 30% **(E)**. *PD-L1*-positive staining in TILs was indicated by arrows **(F)**.

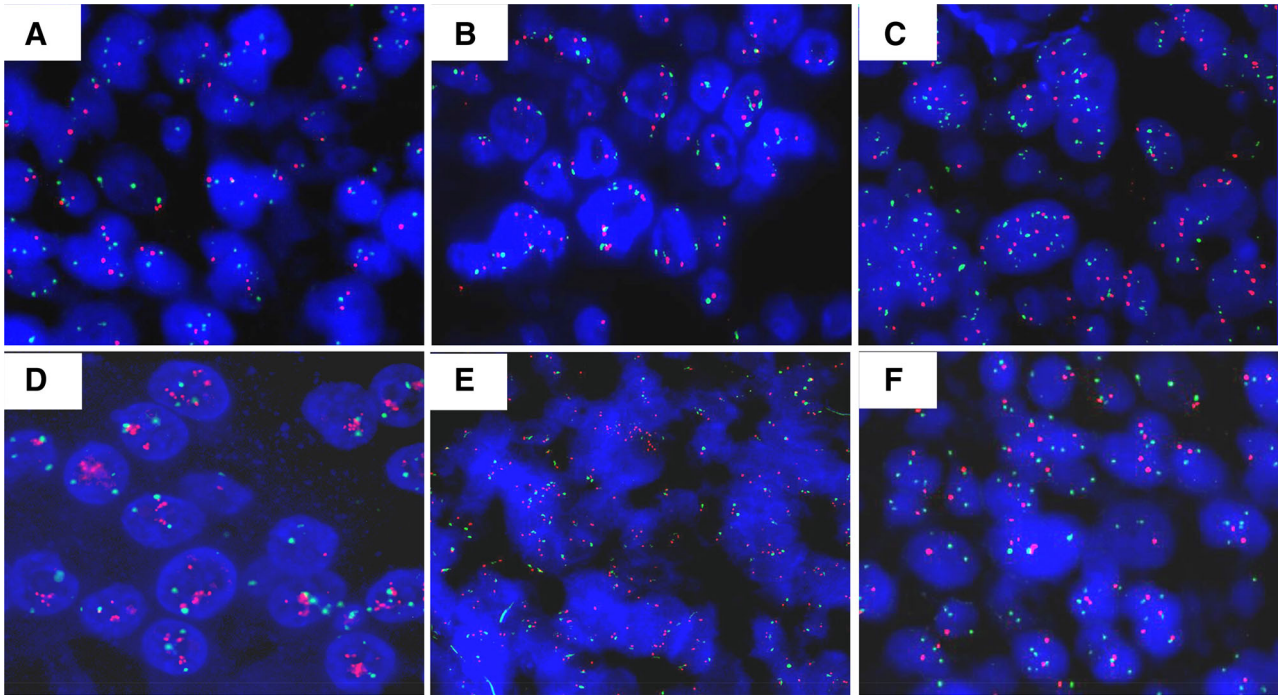

Figure 2. The representative images of the programmed death-ligand 1 (*PD-L1*) copy number changes detected by fluorescence in situ hybridization. *PD-L1* signals with less than five copies per cell were detected in patients **(A and B)**. Six *PD-L1* signals per cell were identified in case number 32 **(C)**; 4.2 copies but ratio equals to 2.23 were determined in case number 28 **(D)**; 10.7 copies and ratio equals to 2.67 were identified in case number 19 **(E)**, whereas *PD-L1* loss was found in case number 33 **(F)**.

the sequencing electropherograms of cases demonstrating *NRAS*, *KIT*, and *TERT* mutated genes are shown in supplemental online Figure 1.

PD-L1 Expression and Copy Number Alterations

The representative images for IHC and FISH are illustrated in Figures 1 and 2, respectively. The IHC method for *PD-L1* expression demonstrated that 27.8% of patients (10/36) exhibited *PD-L1* focal staining, of these, the percentage of tumor cell staining was 3% in three cases, 5% in 3, 8% in one, 20% in one, and 30% in one, whereas *PD-L1* positive expression of TILs was observed in only one patient (Fig. 1; Table 2). *PD-L1* FISH+ was found in three patients, of whom two harbored *PD-L1* amplifications defined by a ratio of

Table 2. Clinical, pathological, and molecular features of the investigated 36 patients with primary vaginal melanomas

Case no.	Age	Surgical approach	Tumor type	Ulceration	Cellularity	Pigmentation	Mitotic activity, n/mm^2	Depth of invasion, mm	AJCC Stage	Molecular findings	PD-L1 expression	PD-L1 copy numbers/ ratio	Follow-up time, mo	Follow-up status
1	36	RE	NM	Present	E	Present	8	8.2	pT4bN0M0, IIC	NRAS p.Q61R	No staining	2.1/1.06	14.0	DOD
2	72	WLE	SSM	Absent	E	Present	0	0.4	pT1aN0M0, IA	None	TC, 3%	2.1/1.13	32.2	DOD
3	47	RE	NM	Absent	E	Present	24	27.5	pT4aN1M0, IIIC	NRAS p.Q61R	No staining	2.0/1.03	8.9	DOD
4	69	WLE	NM	Present	E	Present	3	1.8	pT2bN0M0, IIA	None	No staining	2.3/1.23	17.2	DOD
5	67	RE	NM	Present	E	Present	7	3.5	pT3bN0M0, IIB	None	No staining	3.5/1.56	15.0	DOD
6	74	WLE	NM	Present	S	Present	0	15.0	pT4bN0M0, IIC	NRAS p.Q61R	No staining	2.0/1.03	42.7	DOD
7	40	Biopsy	NA	Present	E	Present	6	7.4	cT4bN0M0, IIC	KIT p.V559D	No staining	2.2/1.05	Unknown	Unknown
8	41	RE	NM	Present	E	Absent	1	35.0	pT4bN0M0, IIC	None	TC, 3%	2.0/1.06	8.2	DOD
9	42	RE	NM	Present	S	Present	4	14.3	pT4bN1M0, IIIC	None	No staining	2.0/1.04	40.3	DOD
10	46	WLE	NM	Absent	E	Present	2	9.1	pT4aN3M0, IIIC	TERT C228T	No staining	2.1/1.07	11.5	DOD
11	33	Biopsy	NM	Absent	E	Present	8	Unknown	Unknown	None	No staining	2.8/1.24	6.2	DOD
12	64	WLE	NM	Present	E	Present	0	7.9	pT4bN0M0, IIC	None	No staining	2.0/1.04	66.4	DOD
13	38	RE	NM	Present	E	Present	6	16.3	pT4bN1M0, IIIC	NRAS p.Q61R TERT C228T	No staining	3.0/1.20	6.5	DOD
14	60	WLE	NM	Absent	E	Present	13	10.7	pT4aN0M0, IIB	None	No staining	2.2/1.11	56.5	DOD
15	59	WLE	NM	Present	E	Present	8	17.8	pT4bN1M0, IIIC	None	No staining	2.0/1.05	7.5	DOD
16	43	WLE	NM	Absent	S	Present	14	7.0	pT4aN0M0, IIB	None	No staining	2.0/1.03	6.9	DOD
17	57	WLE	NM	Absent	E	Absent	0	4.0	pT3aN0M0, IIA	None	No staining	3.9/1.37	33.5	DOD
18	47	RE	NM	Present	E	Present	4	8.6	pT4bN1M0, IIIC	None	No staining	2.3/1.14	29.9	DOD
19	67	WLE	NM	Absent	E	Present	80	2.0	pT2aN0M0, IB	None	TC, 8%	10.7/2.67	14.5	A/L
20	65	RE	NM	Absent	E	Present	15	12.0	pT4aN0M0, IIB	None	TIL staining	2.0/1.03	19.6	A/L
21	68	WLE	NM	Absent	E	Present	3	9.0	pT4aN0M0, IIB	None	TC, 5%	2.2/1.05	15.0	A/L
22	34	RE	NM	Absent	E	Present	23	63.0	pT4aN2M0, IIIC	None	TC, 5%	2.0/1.04	13.9	A/L
23	44	Biopsy	NM	Absent	E	Present	5	Unknown	Unknown	None	TC, 20%	2.1/1.03	20.0	A/L
24	39	RE	NM	Absent	E	Present	7	10.0	pT4aN0M0, IIB	None	No staining	2.0/1.04	9.8	A/L
25	70	WLE	NM	Present	S	Present	29	13.0	pT4bN0M0, IIC	None	TC, 30%	3.2/1.23	7.8	A/L
26	46	RE	NM	Absent	E	Present	17	51.5	pT4aN3M0, IIIC	None	No staining	3.4/1.20	82.9	A/L
27	45	RE	NM	Present	E	Present	21	17.2	pT4bN1M0, IIIC	None	TC, 5%	2.0/1.02	12.9	A/L
28	73	WLE	NM	Present	E + S	Present	65	27.5	pT4bN0M0, IIC	None	No staining	4.2/2.23	5.4	A/L
29	52	Biopsy	NA	Present	E	Present	30	> 4.0	cT3bN0M0, IIB	None	No staining	3.7/1.24	25.4	A/L
30	50	RE	NM	Present	E	Present	17	10.0	pT4bN0M0, IIC	None	No staining	2.2/1.09	23.6	DOD

(continued)

Table 2. (continued)

Case no.	Age	Surgical approach	Tumor type	Ulceration	Cellularity	Pigmentation	Mitotic activity, n/mm^2	Depth of invasion, mm	AJCC Stage	Molecular findings	PD-L1 expression	PD-L1 copy numbers/ratio	Follow-up time, mo	Follow-up status
31	48	RE	NM	Present	E	Present	16	3.0	pT3bN0M0, IIB	None	No staining	2.0/1.04	69.0	A/L
32	47	WLE	NM	Absent	E	Present	10	9.0	pT4aN1M0, IIIC	None	No staining	6.0/1.33	70.2	DOD
33	51	WLE	SSM	Absent	E	Present	12	5.0	pT4aN0M0, IIB	NRAS p.Q61P	No staining	2.1/0.74	15.7	DOD
34	27	WLE	NM	Present	E + S	Present	8	10.0	pT4bN0M0, IIC	None	No staining	2.0/1.04	43.3	DOD
35	77	WLE	NM	Present	E + S	Present	12	15.0	pT4bN0M0, IIC	None	TC, 3%	3.8/1.22	9.5	A/L
36	48	RE	NM	Absent	S	Absent	20	5.5	pT4aN0M0, IIB	None	No staining	2.0/1.06	8.4	A/L

Abbreviations: A/L, alive or lost to follow-up; DOD, died of disease; E, epithelioid; NM, nodular melanoma; RE, radical excision; S, spindle cell morphology; SSM, superficial spreading melanoma; TC, tumor cell; TIL, tumor-infiltrating lymphocyte; WLE, wide local excision.

2.23 and 2.67 (Fig. 2D and E; Table 2). By contrast, there was one patient with *PD-L1* loss (*PD-L1*/CEP9 ratio = 0.74; Fig. 2F; Table 2). Interestingly, there were two cases (case number 28 [*PD-L1* copy numbers/ratio, 4.2/2.23] and 32 [*PD-L1* copy numbers/ratio, 6.0/1.33]) in which no *PD-L1* expression was detected but showed *PD-L1* positivity in FISH (Table 2). *PD-L1*+ was found to occur more frequently in older patients (> 60 years; p = .008) but showed no significant association with other clinicopathological features (supplemental online Table 3).

Patients' Survival

Follow-up information was available for 35 patients, among whom 27 (77.1%) developed recurrences and metastases, and 21 (60.0%) died. Furthermore, Kaplan-Meier survival analyses showed that patients with mutated *NRAS* had a worse OS compared with those with a wild-type *NRAS* (33.5 vs. 14.0 months; HR, 3.09; 95% CI, 1.08–8.83; p = .035; Fig. 3A), whereas no statistical significance in OS was observed for patients with *PD-L1* expression and/or *PD-L1* amplification compared with those without expression/amplification (Fig. 3B). Multivariate analyses demonstrated that >10 mitoses per mm^2 (HR, 2.96; 95% CI, 1.03–8.51; p = .043) was an independent prognostic factor in patients with primary vaginal melanomas (Table 3).

Two cases were prescribed immune checkpoint inhibitors as subsequent treatment after wide local excision or chemoradiotherapy. The first one, case number 21, showed a 5% positive staining for *PD-L1* and was treated by nivolumab as adjuvant therapy (1 mg/kg every 3 weeks). The patient relapsed, identified by computed tomography (CT) imaging, after 7 months but still continued the nivolumab therapy, then changed to pembrolizumab after one cycle, and at last follow-up, Dec 24, 2018, the tumor was observed to have shrunken (13 × 16 mm to 13 × 10 mm). The second case, case number 29, did not show any *PD-L1* expression. Upon completion of chemoradiotherapy, the patient was diagnosed with liver metastasis and underwent 11 cycles with pembrolizumab (10 mg/kg every 2 weeks). During the treatment, brain metastasis was identified on CT peri-treatment follow-up examination and the patient was given concurrent radiotherapy. Fourteen months later, the tumor was observed by abdominal ultrasound to have metastasized to the liver, and the patient was offered ablation therapy. During the course of the treatment, pembrolizumab was continuously given, and at last follow-up, Oct 18, 2018, the patient was in good condition, Eastern Cooperative Oncology Group (ECOG) performance status 1.

Discussion

Little information is known about the molecular characteristics in primary vaginal melanoma, although it has been demonstrated that mucosal melanoma is genetically distinct from cutaneous melanoma and more commonly exhibits *KIT* and *NRAS* mutations. We reviewed 36 cases of vaginal melanomas and found a relatively low frequency of genetic mutations. *NRAS* was the most mutated gene in primary vaginal melanomas and was associated with worse OS.

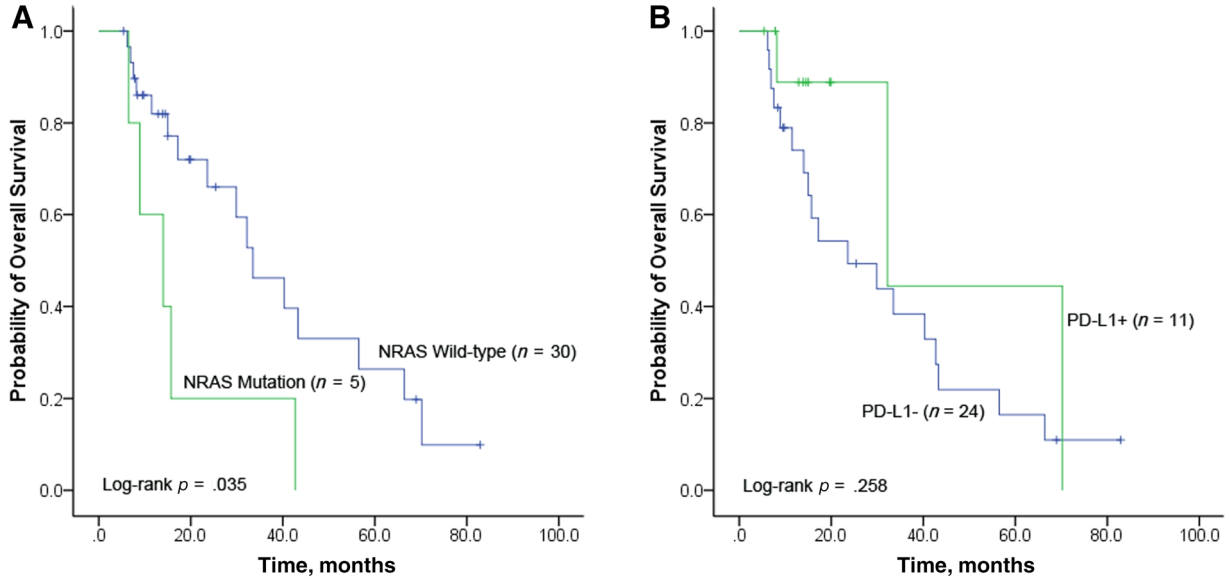

Figure 3. Kaplan-Meier curves for overall survival (OS) in the 36 investigated patients with primary vaginal melanoma. **(A):** Patients with wild-type *NRAS* had a favorable OS than those with mutated *NRAS* (p = .035). **(B):** No statistical significance for OS between patients with/without programmed death-ligand 1 (*PD-L1*) positive staining and/or amplifications was found.

Table 3. Univariate and multivariate Cox Regression analyses for overall survival in primary vaginal melanoma

Variables	Univariate HR (95% CI)	p value[a]	Multivariate HR (95% CI)	p value[a]
Age, years (≤60 vs. >60)	1.32 (0.51–3.45)	.571		
Tumor type (NM vs. SSM)	1.84 (0.41–8.27)	.426		
Ulceration (present vs. absent)	0.73 (0.30–1.79)	.488		
Cellularity (epithelioid vs. spindle)	0.97 (0.32–2.99)	.956		
Pigmentation (present vs. absent)	1.86 (0.42–8.33)	.417		
DOI (≤4 mm vs. >4 mm)	0.87 (0.29–2.61)	.796		
Mitotic activity (≥10 vs. <10)	3.24 (1.15–9.19)	.027	2.96 (1.03–8.51)	.043
LN metastasis (absent vs. present)	0.99 (0.37–2.62)	.975		
Clinical Stage (I vs. II + III)	1.02 (1.32–7.87)	.985		
NRAS status (mut vs. wt)	3.09 (1.08–8.83)	.035	2.58 (0.89–7.43)	.080
PD-L1 status (PD-L1+ vs. PD-L1–)[b]	2.05 (0.59–7.07)	.258		

[a]p values were from Cox proportional hazard regression models.
[b]PD-L1+ was included PD-L1 positive staining and/or PD-L1 amplification.
Abbreviations: CI, confidence interval; DOI, depth of invasion; HR, hazard ratio; LN, lymph node; mut, mutant; NM, nodular melanoma; SSM, superficial spreading melanoma; wt, wild-type.

PD-L1 expression was observed commonly. We also reported the patients with or without *PD-L1* expression may benefit from immune checkpoint inhibitors. These findings offer insights therapeutic targets for these patients.

In contrast to existing literature, *NRAS* mutations were frequently found in melanomas of the skin with CSD and had mutation rates of up to 24% [42]. The frequency of *NRAS* mutations varied among mucosal melanomas, with a wide range of 0%–43% mutation rates reported in previous studies [10, 43–46]. It was found that *NRAS* mutations were present in 37.5% (6/16) of esophageal mucosal melanomas [47] and 7.1%–30% of sinonasal melanomas [48–50], and none were detected until now in oral mucosal melanomas [50, 51]. In melanomas of the female urogenital tract, there were two studies showing the different mutation rates of 13.3% (2/15) [43] and 21.4% (3/14) [44]. In our series, *NRAS* mutations were detected in 13.9% of vaginal melanomas. Previously, *NRAS* mutations have been observed to be associated with some features predictive of aggressive behavior in cutaneous melanomas, such as the Clark level of invasion, Breslow thickness, ulceration rate, and adverse outcome [15, 16]. However, the prognostic value of *NRAS* status in melanoma is still a matter of intense debate. Several studies have been carried out to examine the effect of *NRAS* mutations on clinical outcomes and none of them found any impact on OS [52–54]. In contrast, Devitt et al. reported that *NRAS* mutations were an adverse prognostic factor in multivariate analysis in a prospective cohort of

249 patients with melanoma [55]. Besides, most of the observations have been conducted in white populations, with a scarce report from other geographic areas like Asia. In the present study, it was found that patients with mutated *NRAS* had inferior OS compared with those with wild-type *NRAS*, although *NRAS* mutation status but had no significant correlation with any of the investigated clinicopathological features.

In previous studies, Beadling et al. [56] found *KIT* mutations in 15.6% of mucosal melanomas, and Curtin et al. [19] found *KIT* mutations and/or an increase in the copy number of *KIT* in 39.0% of mucosal melanomas. In contrast to our present study, only one patient (2.9%) was found to harbor the *KIT* V559D mutation, which was located in the juxtamembrane domain of the *KIT* receptor. Our finding is in line with previous studies that have also reported a low frequency (0%–8.3%) of *KIT* mutations, with only one case reported in vaginal melanomas (supplemental online Table 4). Patients with melanoma with this type of mutation were reported to benefit from imatinib [57], whereas a recent ECOG phase II trial investigating the use of dasatinib in mucosal melanomas with *KIT* alterations revealed low response rate [58], demonstrating that molecularly targeted therapy may not always be effective. Advancement in the therapeutics of these patients are difficult considering the rarity of mucosal melanomas and that only few of such patients do present with *KIT* mutations, thereby hindering the launch of large clinical trials to verify the effectiveness of *KIT* inhibitors.

No patients were found with mutations in *BRAF* exon 15 in the present study, which was similar with other published data on vaginal melanoma [10, 43, 44, 46]. *BRAF* V600E mutation is the most common mutation in melanomas, with reports of up to a 40% prevalence in cutaneous melanoma [14, 15]. However, in mucosal melanoma, *BRAF* mutations occur at a lower frequency (3.0%–15.5% in unspecialized mucosal melanoma) [15, 19]. This is similar for vaginal melanoma, in which only four cases of *BRAF* mutations have been reported to date; one case was found in Aulmann et al.'s study [43], and three cases were in one study conducted by Hou et al. [45]. Mutation in *GNAQ/GNA11* exhibits tissue specificity in melanomas. No mutations were observed in the present study (vaginal melanoma) and in sinonasal and oral mucosal melanomas [50, 51], whereas it was demonstrated that the *GNAQ/GNA11* active mutation is a major contributor in the development of uveal melanoma [21, 22]

TERT promoter mutations were recently identified at high frequencies in cutaneous malignant melanoma tumor samples [24], whereas few have shown a low frequency of *TERT* promoter mutations in mucosal melanomas. Three studies have reported a relatively low frequencies of these mutations in unspecified location of mucosal melanomas: 23% (6/26) [25], 13.2% (7/53) [59], and 12.5% (1/8) [26]. In specific location of mucosal melanomas, namely sinonasal malignant melanomas, the frequencies of *TERT* mutations were separately identified as 8% (4/49) [60] and 11.5% (3/28) [50]. To our knowledge, this present study is the first investigate the *TERT* promoter mutations in vaginal melanomas. Two patients (7.7%) with primary tumors were diagnosed as stage IIIC vaginal melanomas with *TERT* promoter mutations in C228T and had an overall survival of less than 12 months. Moreover, *TERT* promoter mutations have been reported to be associated with older patients, increased Breslow thickness, and worse prognosis [25, 26]. By contrast, an investigation from Asia demonstrated that the *TERT* promoter mutations were not correlated with OS [61]. The difference in frequencies and relationship with clinicopathological features reveal that the *TERT* promoter mutations may vary depending on the melanoma subtypes and locations.

Although vulvar melanoma arises on hairy and glabrous skin of the vulva, it was described as a mucosal melanoma because of its continuity with the vaginal mucosa and its low-sunlight exposure location [62]. Several studies have characterized the molecular events of melanomas in both the vulva and vagina. Hou et al. [45] assessed all the reported cases of vulvar and vaginal melanoma with molecular detection and concluded that these two types of melanomas had distinct molecular signatures. The genes most commonly mutated in vulvar melanomas were *KIT* (26.5%, 9/34) and *BRAF* (27%, 9/33), whereas *NRAS* mutations were more prevalent than *KIT* and *BRAF* mutations in vaginal melanomas [45]. Although the varying mutation frequencies might be related to the limited number of cases analyzed and differences in methodology in all of those studies, the different frequencies of molecular alterations suggest that the development of vulvar and vaginal melanomas involves different tumorigenesis pathways. This finding is essential for patients with vaginal melanomas who harbor frequent *NRA*S mutations because they might benefit from MEK inhibition [30].

Accordingly, the potential clinical implications for testing molecular alterations and targeted treatments were further explored in this study. The regulation of immune checkpoint has been recently investigated in a variety of malignancies. In previous clinical trials, a number of monoclonal antibodies against *PD-1* and *PD-L1* with antitumor activities have been observed in some of patients with cutaneous melanoma [63, 64]. A phase Ib KEYNOTE-001 study has reported that *PD-L1* expression, assessed by IHC assay (clone, 22C3), is a potential predictive marker for anti-*PD-L1* activity in patients with advanced melanoma who were treated with pembrolizumab [65]. In the present study, we confirmed that 27.8% of the patients with vaginal melanoma were considered as having *PD-L1*-positive staining (E1L3N). From the medical records, one patient with *PD-L1*-positive staining received nivolumab treatment, whereas another patient without *PD-L1*-positive staining received pembrolizumab. Both of them were still alive with stable tumor response until the end of the follow-up period. Because of the low incidence of mucosal melanoma, only limited data have been published regarding the efficacy of immune checkpoint inhibitors in this disease subtype. Previous reports have suggested that the response rates to ipilimumab treatment in patients with advanced mucosal melanoma are much lower than in patients with cutaneous melanoma [66, 67]. However, there are few investigations on the response of pembrolizumab and nivolumab in vaginal melanoma. Because of the small number of patients, it

may be difficult to interpret the data. Nevertheless, this observation could act as a hint that patients with vaginal melanoma with *PD-L1* expression could benefit from immune checkpoint inhibitors even for those without *PD-L1* expression, which is in line with the report of a previous study [65].

Despite multiple effective treatment options for cutaneous melanoma, data on the treatment of vagina mucosa melanomas are limited, especially from Asia. Therefore, patients with vaginal melanomas should be encouraged to perform more comprehensive analysis, including that of *NRAS* and *KIT*, and *PD-L1* expression/copy number at the time of initial diagnosis to search for an effective target and participate in clinical trials involving treatments with novel targeted therapies based on molecular aberrations. Two patients had no *PD-L1* expression but harbored *PD-L1* FISH+ in our study. To our knowledge, *PD-L1* FISH might be a good complement even in the absence of *PD-L1* expression, suggesting that more people could benefit from the immune checkpoint inhibitors. This is in agreement with a recent study which showed that *PD-L1* amplified solid tumors had beneficial objective responses after administration of immune checkpoint inhibitors [68]. Further investigation is required to determine whether *PD-L1* expression in TILs and/or tumor cells predict response to immunotherapy in patients with vaginal mucosal melanoma. *PD-L1* amplification did not always correlate with *PD-L1* expression by IHC analysis. Additional large-scale, prospective studies of *PD-L1*-amplified cancers are warranted to confirm the responses to immune-checkpoint blockade prescribed, even in the absence of *PD-L1* expression. Therefore, a coordinated, collaborative effort is required to collect more samples and further progress is to be made in understanding the pathogenesis and offering optimal treatment.

There were several limitations in our study. Vaginal mucosal melanomas are rare, with few existing large retrospective studies. The sample size was small and may have limited the univariate and multivariate analyses for predicting the correlation between clinical variables and gene status with oncogenic outcome. The definitive prognostic factors are yet to be fully determined using larger cohorts of patients.

Conclusion

We provided highlights of the molecular insights within the spectrum of vaginal melanomas. In this cohort of patients with primary vaginal melanoma, we observed that *NRAS* mutations and *PD-L1* expression were most prevalent, whereas the detection rate of *KIT* and *TERT* mutations was low. Patients with *NRAS* mutations had a poorer survival outcome as compared with those with wild-type *NRAS*. No significant difference in OS was observed between those with and without *PD-L1* expression and amplification. The only clinicopathological feature identified as an independent factor for survival was mitotic activity.

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Ethic approval was given by the Ethics Committee of the Sun Yat-sen University Cancer Center (No.B2016-069-01). Informed consent was obtained from all individual participants included in this study. All procedures of this study involving human participants were performed in accordance with the ethical standards of the Sun Yat-sen University Cancer Center and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

The raw data in this paper has been successfully uploaded onto the Research Data Deposit with RDD identifier number: RDDA2019001043.

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Disclosures

The authors indicated no financial relationships.

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