Predictive biomarkers and tumor microenvironment in female genital melanomas: a multi-institutional study of 55 cases

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Abstract

Female genital melanomas are rare. At diagnosis, most affected patients have advanced disease. Surgery remains the primary treatment, and adjuvant therapy is largely ineffective. Recently, immune checkpoints and the mitogen-activated protein kinase pathway have been explored as treatment targets. However, evaluation of these biomarkers in genital melanomas is limited. We evaluated the clinicopathological features of 20 vulvar, 32 vaginal, and three cervical melanomas and assessed programmed cell death ligand 1 (PD-L1) expression, CD8 tumor-infiltrating lymphocyte density, mismatch repair proteins, VE1 immunohistochemistry, and *KIT* and *BRAF* mutations. The median age of the patients was 66 years, and median tumor sizes were 25, 30, and 20 mm for vulvar, vaginal, and cervical tumors, respectively. Mean mitotic figures were 18, 19, and 30 per mm². Thirty-seven patients (67%) had operable tumors. After a median follow-up of 15 months, only nine patients (16%) were alive. Eight of the nine survivors did not have lymph node metastasis. Using 5% as the threshold, PD-L1 expression was observed in 55%, 50%, and 33% of vulvar, vaginal, and cervical tumors, respectively, when the Roche SP263 antibody was used and 20%, 53%, and 0%, respectively, when the Dako 28-8 antibody was used. The median CD8 tumor-infiltrating lymphocyte density was significantly higher in vulvar/vaginal than cervical melanomas and correlated with PD-L1 expression. No cases exhibited loss of mismatch repair proteins. Five cases harbored *KIT* mutations, three of which were hotspots. *BRAF* V600E mutation was not detected. Univariable analysis showed that tumor size greater than or equal to 33 mm, mitotic figures of greater than or equal to 10 per mm², lymph node metastasis, and low CD8+ tumor-infiltrating lymphocyte density were adverse prognostic factors. Thus, patients with genital melanomas have a poor prognosis, and evaluation of multiple biomarkers is necessary to identify patients who may benefit from immunotherapy or targeted therapy.

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Introduction

Malignant melanomas involving the female genital tract account for less than 3% of melanoma cases in women [1–5]. These tumors are aggressive, and affected patients usually

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have advanced disease at presentation, with overall poor prognosis [1–3, 6–9]. Even if surgically resectable, obtaining adequate surgical margins may be challenging. There is no standardized adjuvant treatment, and those available are relatively ineffective [4, 10]. Targeted therapy and immunotherapy have been shown to be successful in cutaneous melanomas [11–13]. Genital melanomas have pathogenic mechanisms different from those of other melanomas, and reports on different biomarkers for predicting treatment responses are limited [4, 9].

Immune checkpoint inhibitors that block the programmed cell death-1 (PD-1)/programmed cell death ligand-1 (PD-L1) interaction have become invaluable for treating many malignancies [14–16]. However, few reports have described PD-L1 expression in genital melanomas, and the frequencies of expression among different subtypes, the relationships between expression and treatment responses, and survival rates in patients with or without PD-L1 expression are unclear [17–21]. Other potential biomarkers, such as tumor-infiltrating lymphocytes and microsatellite instability, may help to identify patients who may benefit from immune checkpoint inhibitors [22–24]. The presence of tumor-infiltrating lymphocytes has long been recognized as a favorable prognostic factor in patients with melanoma, and their presence in the tumor has been shown to correlate with PD-L1 expression [14, 19]. Thus, combined assessment of PD-L1 and tumor-infiltrating lymphocytes is expected to be more informative as it is a better representation of the tumor microenvironment and may guide the use of immune checkpoint inhibitors [25, 26]. Regardless of origin, tumors showing high microsatellite instability or mismatch repair deficiency have been shown to respond to immune checkpoint inhibitors [27]. Although few melanomas have been reported to be high microsatellite instability [22, 28–30], the microsatellite status of genital melanomas is unknown.

The mitogen-activated protein kinase pathway is often dysregulated in melanomas, and hotspot mutations in *C-KIT*, *NRAS*, *BRAF*, and *MEK* have become targets for treatment [31–34]. Initial screening for tumors harboring the *BRAF* V600E mutation may be performed by using immunohistochemistry with a specific VE1 monoclonal antibody [35]. Nevertheless, the frequency of *BRAF* mutations in genital melanomas is low, and the usefulness of this antibody remains uncertain [36, 37].

In this study, we aimed to elucidate the clinicopathological features of a rare collection of genital tract melanomas and evaluate potential biomarkers, including PD-L1, CD8+ tumor-infiltrating lymphocytes, mismatch repair proteins, VE1 immunohistochemistry, and *KIT* hotspot and *BRAF* V600E mutations, that identify patients who may benefit from immune checkpoint inhibitors and/or anti-C-KIT and anti-BRAF targeted therapies.

Materials and methods

This was a multi-institutional study evaluating all available vulvar, vaginal, and cervical melanomas from five major hospitals in Hong Kong. Only cases with sufficient clinical information with follow-up and available formalin-fixed, paraffin-embedded blocks were included. Patients who had simultaneous or previous cutaneous melanomas were excluded. This study was approved by the Institutional Review Boards of all participating hospitals (HKU/HA HKW IRB: UW 16-1022).

Clinical features

For each case, the patient's age, presentation, gross features and size of the tumor, extent of involvement, and clinical or radiological evidence of lymph node or other metastases were noted. Mode of primary treatment and any postoperative adjuvant radiation and/or chemotherapy were recorded. Follow-up data were reviewed. For vulvar melanomas, a stage was assigned using the 8th Edition of the American Joint Commission on Cancer (AJCC) staging system, as previously recommended [38, 39]. Staging for vaginal melanoma is not recommended in the 8th Edition of the AJCC staging system and there is no existing AJCC staging system for cervical melanomas [6, 38, 39]. Therefore, staging for vaginal and cervical melanomas was not performed.

Pathological features

Histologically, the tumor cell types (epithelioid, spindle, or mixed), tumor thickness, ulceration, number of mitotic figures per mm², and any lymphovascular space and perineural invasion were determined. The presence of tumor-infiltrating lymphocytes was determined by examination of hematoxylin and eosin-stained sections. Tumor-infiltrating lymphocytes were lymphocytes which were concentrated at the tumor-stromal interface. Quantification of tumor-infiltrating lymphocytes was further confirmed using immunohistochemistry. Clearance of resection margins was determined from the pathology report or by re-examination of histological sections.

Immunohistochemistry

Immunohistochemical studies were performed on large resected specimens in 69% of cases. Two vulvar, 13 vaginal, and two cervical melanomas could only be investigated with biopsy specimens because these patients had inoperable tumors. Staining was conducted on whole-mount formalin-fixed, paraffin-embedded sections in a laboratory accredited by the College of American Pathologists. Staining with Ventana PD-L1 SP263 (Roche, Switzerland) and

PD-L1 IHC 28–8 pharmDx (SK005; Dako, Denmark) was performed using a Ventana BenchMark Ultra instrument (Roche) and Autostainer Link 48 (Agilent Dako, USA), respectively. The T-lymphocyte marker CD8 (C8/144B; Dako) was detected using a Leica Bond III stainer (Leica Camera AG, Germany), and the mismatch repair proteins mutL homolog 1 (MLH1; clone M1), mutS homolog 2 (MSH2; clone G219-1129), mutS homolog 6 (MSH6; clone SP93), and PMS1 homolog 2 (PMS2; clone A16–4) as well as anti-BRAF V600E (mouse monoclonal, clone VE1, 790-4855) were detected using a Ventana BenchMark Ultra instrument (Roche). In order to distinguish true cytoplasmic staining from melanin, we used an ultraView Universal Alkaline Phosphatase Red Detection Kit (Ventana Medical Systems, USA) to detect BRAF V600E protein expression. Two clinical specimens of melanomas harboring the *BRAF* V600E mutation were used as positive controls. For all other markers, benign tissue (a multi-tissue sausage block) and appropriate tumors were used as positive controls according to the manufacturers' recommendations.

PD-L1 evaluation was performed by pathologists who had completed training conducted by the manufacturers (for interpretation of Roche Ventana and Dako antibodies). Interpretation of immunohistochemical staining was performed under a multiheaded microscope during consensus sessions and with agreement on final results. Scoring was performed for tumor cells but not lymphoid cells. In each section, the presence of at least 100 tumor cells was confirmed. Tumor cells with circumferential or partial membranous staining were considered positive for PD-L1. Necrotic tumor cells and cytoplasmic tumor cell staining were excluded from scoring. The extent of staining was deduced by assessing the percentage of positively stained tumor cells in the entire section and stated as greater than or equal to 5% or 10% [17, 19, 20]. To facilitate meaningful comparison with other studies, analyses were performed using a threshold of greater than or equal to 5%. For tumor-infiltrating lymphocytes, nuclear staining for CD8 was considered positive. We adopted a simple, inexpensive, robust quantitative assessment for tumor-infiltrating lymphocytes count without the need for using sophisticated image analysis tools. An area with the highest density of tumor-infiltrating lymphocytes on a whole-mount tissue section was predetermined and marked. In this area, the absolute count of positive cells per high-power field ($\times 40$ objective; 0.55-mm field diameter; 0.24 mm²) was scored by three observers (YY, KLC, and PI). Counting was facilitated using a manual counter. The median scores of the three observers were calculated. A tumor was considered to have loss of mismatch repair protein expression when nuclear staining was absent for one or more antibodies targeting MLH1, PMS2, MSH2, and MSH6 in the presence of positively stained lymphoid cells in the background.

Anti-BRAF VE1 antibody staining was considered positive if there was unequivocal diffuse cytoplasmic staining in more than 66% of tumor cells. The intensity of staining for BRAF expression was determined to be negative, weak, medium, or strong. Assessment was carried out without knowledge of the mutational status.

KIT and BRAF mutation analysis

The same sections used for VE1 immunohistochemistry were used for analysis of *KIT* and *BRAF* mutations. Representative areas containing tumor cells were marked by pathologists. The target cells were microdissected from four or five 10- μ m-thick sections using a sterile needle under a microscope at $\times 10$ magnification. DNA was extracted with a Qiagen QIAamp DNA FFPE Tissue Kit (Qiagen Corp., USA), and the concentration of DNA for each sample was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., USA). All samples containing extracted DNA were determined to be of high quality (with an A260/A280 ratio of 1.8–2.0). Polymerase chain reaction was performed using primers designed to flank the exons of the *KIT* and *BRAF* genes (Supplementary Table 1). DNA (50 ng) was amplified by 100 nmol forward and reverse primers in 25- μ L reactions in the presence of HotStarTaq Plus DNA polymerase (Qiagen Corp.). Polymerase chain reaction conditions were as follows: initial denaturation at 95 °C for 10 min; 40 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. Successful amplification was confirmed by gel electrophoresis. *KIT* and *BRAF* gene mutations were analyzed by Sanger sequencing. Polymerase chain reaction products (1 μ L) corresponding to exons in the *KIT* and *BRAF* genes were used for BigDye Termination bidirectional sequencing and sequenced on an Applied Biosystems 3730xl DNA Analyzer (Thermo Fisher Scientific Inc.). Results were analyzed using Chromas sequence analysis software.

Two melanomas with known *BRAF* V600E mutations served as positive controls, and two genital melanocytic cell lines, i.e., HMVII (cat. no. RCB0777; vaginal melanoma; RIKEN Cell Bank, Japan) and HEMa-LP (Thermo Fisher Scientific Inc.), with known mutation profiles (and absence of the *BRAF* V600E mutation) served as negative controls.

Statistical analysis

Clinicopathological variables among histological subtypes were analyzed using Student's *t*-tests and Chi-squared tests. Optimal cut-off values for continuous variables, such as size, thickness, and CD8 count, were determined by choosing maximum values of Youden's index obtained from receiver operating characteristic analysis. Disease-free survival was defined as the time interval from the date of primary treatment

to the date of recurrence or last follow-up. Overall survival was defined as the time interval from the date of primary surgery to the date of death or being censored at the date of the last follow-up. Univariable and multivariable analyses were performed with Cox proportional hazards models, and *p*-values of less than 0.05 were considered to represent statistical significance. Survival curves for significant variables were generated using the Kaplan–Meier method. The inter-observer reproducibility of the tumor-infiltrating lymphocytes count was evaluated with intraclass correlation coefficients. Agreement was expressed in terms of the following threshold values: less than 0.00, no agreement; 0.00–0.20, slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; and 0.81–1.00, near-perfect agreement. The linear relationship between PD-L1 score and CD8 tumor infiltrating lymphocytes was evaluated using Pearson's correlation coefficient. Analyses were performed with SPSS Statistics for Macintosh, Version 22.0 (IBM, USA).

Results

Clinicopathological features of female genital tract melanomas

Fifty-five genital melanomas (20 vulvar, 32 vaginal, and three cervical) were collected from the pathology archives of five major hospitals from a 22-year period between 1996 and 2018. All contributing pathologists confirmed the original diagnoses based on histology review and immunohistochemistry (S-100 protein, HMB45/melan-A). At the time the biopsies were collected, none of the patients had been treated with targeted therapy drugs or immune checkpoint inhibitors.

Vulvar melanomas

The median age of the 20 patients was 64 years. At the time of presentation, two patients (10%) had inoperable tumors, and 11 patients (55%) already had lymph node metastases. Ten patients were classified as stage IIIC, three-stage IIIB, six-stage IIC, and one stage IIB. Seventeen patients (85%) underwent surgery (Table 1). The surgical margins were involved in seven of 17 (41%) patients, three of whom (43%) subsequently died of disease. Postoperative adjuvant radiation therapy was given to four patients, and adjuvant chemotherapy was given to three patients. The median tumor size was 25 mm. Fifteen (75%) tumors contained epithelioid tumor cells, and five (25%) had mixed epithelioid and spindle cells (Fig. 1a, b). Melanin deposits were observed in each case. The mean mitotic figures count was 18 per mm². The tumor thickness was 2–8 mm (median: 5

mm), and ulceration was present in 14 tumors (70%). Tumor-infiltrating lymphocytes were present in 17 cases (85%; Fig. 1c, d). Three cases had lymphovascular space invasion, but none had perineural invasion. The clinical and pathological features are summarized in Table 1.

Follow-up ranged from 4 to 208 months (median: 29 months). Fourteen patients (70%) developed recurrence and disease progression after a median of 15 months (range: 4–54 months). Metastases developed in the lungs (*n* = 4), brain (*n* = 1), brain and liver (*n* = 2), bone and liver (*n* = 2), pelvis (*n* = 1), and multiple organs (*n* = 4). From the final follow-up records, 13 patients died of disease, and one remained alive with disease. Six patients (30%) were alive and with no evidence of disease. Notably, five of these six survivors did not have lymph node metastasis at initial diagnosis.

Vaginal melanomas

The median age of the 32 patients was 68 years. The most common presentation was abnormal vaginal bleeding (34%) or masses (28%). Two (6%) had abnormal Pap smears. At the time of presentation, 13 patients (40%) had inoperable tumors, eight of whom received palliative radiotherapy. Thirteen patients (41%) already had lymph node metastases. Nineteen patients (59%) underwent surgery (Table 2). The surgical margins were involved in nine of 19 (47%) patients, seven of whom (78%) subsequently died of disease. Postoperative adjuvant radiation therapy was given to eight patients, and adjuvant chemotherapy was given to four patients. The median tumor size was 30 mm. Twenty tumors (63%) were epithelioid in nature, four (13%) contained spindle cells, and eight (25%) were mixed. Melanin deposition was present in each case. The mean mitotic figures count was 19 per mm². The assessable tumor thickness was 2–23 mm (median: 8 mm), and ulceration was present in 27 tumors (84%). Tumor-infiltrating lymphocytes were present in 10 cases (31%). In tumors that were excised and assessable, lymphovascular space invasion, and perineural invasion were not identified. The clinical and pathological features are summarized in Table 2.

Follow-up ranged from 2 to 138 months (median: 12 months). Eighteen patients (70%) developed recurrence after a median of 8 months (range: 1–102 months). Metastases developed in the pelvis (*n* = 4), lungs (*n* = 4), liver (*n* = 2), bone (*n* = 1), intestines (*n* = 1), and multiple organs (*n* = 6). At last follow-up, 30 patients (94%) died of disease. The two survivors (Va3 and Va36) had tumors that were 20 and 25 mm in size, respectively, and these had invaded through the vaginal wall but without involvement of the pelvic wall. There was also no lymph node metastasis at initial diagnosis. Resection margins were involved in each of their excisions. Patient Va3 received adjuvant radiation therapy and developed a local recurrence at

Table 1 Clinicopathological features of vulvar melanomas

Case	Age	Presentation	Treatment	Cell type	Size (mm)	Thickness (mm)	Mitotic count (per mm ²)	Adjuvant treatment	Lymph node metastasis (number)	Stage (AJCC 8 th edition)	Follow-up (months)	Status
V1	86	Mass/bleed	*RV/LND	E	35	7	50	–	–	IIC	37	DOD
V2	80	Mass	RV	Mixed	77	6	34	–	+(2)	IIIC	67	DOD
V3	54	Mass	RT/GM-CSF	E	2	3	35	–	+(7)	IIIC	13	DOD
V4	53	Mass/pruritis	WLE	E	10	2	7	RT	+(3)	IIIB	38	DOD
V5	72	Mass/bleed	WLE	E	7	7	2	–	–	IIC	128	ANED
V6	75	Mass/urinary symptoms	RV	E	10	4	4	–	–	IIB	80	ANED
V7	64	Mass/bleed	*WLE	Mixed	30	24	8	–	–	IIC	208	ANED
V8	61	Mass	WLE	E	25	2	20	Chemo	+(2)	IIIB	11	DOD
V9	80	Mass	WLE	E	40	11	27	–	+(1)	IIIC	12	DOD
V10	76	Mass/bleed	RV	E	40	3	20	RT	+(1)	IIIC	29	DOD
V11	31	Mass/bleed	WLE	E	22	5	11	–	+(1)	IIIC	29	DOD
V12	68	Mass/bleed	*WLE	Mixed	22	5	17	–	+(1)	IIIC	23	DOD
V13	84	Mass/pain	Palliative RT	E	30	>15	30	N/A	+(2)	>IIIC	33	DOD
V15	44	Mass/bleed	Palliative RT and Chemo	Mixed	50	>5	20	N/A	+(1)	>IIIB	4	DOD
V16	39	Mass	*RV/LND	E	10	6	9	RT	–	IIC	53	ANED
V17	89	PMB	*WLE	E	30	6	30	Palliative Chemo	Unknown	IIIC	12	DOD
V18	44	Mass/pruritis	WLE	E	20	4	20	Chemo	Unknown	IIIC	12	DOD
V19	44	Mass	RV/LND	Mixed	25	5	8	–	–	IIC	54	AWD
V21	57	Mass/pruritis	*RV/LND	E	30	8	5	RT	+(2)	IIIC	18	ANED
V22	64	Bleed	*WLE	E	8	4	5	–	–	IIC	9	ANED

RV radical vulvectomy, *surgical margins involved, LND lymph node dissection, E epithelioid, – absent, DOD died of disease, + present, GM-CSF granulocyte macrophage colony stimulating factor, WLE wide local excision, RT radiation therapy, ANED alive with no evidence of disease, Chemo chemotherapy, N/A not applicable, PMB postmenopausal bleeding, AWD alive with disease

Fig. 1 Microscopic features and immunohistochemistry of genital melanomas. **a** Epithelioid tumor cells exhibit enlarged, oval nuclei and prominent nucleoli (hematoxylin and eosin). **b** Malignant melanomas with spindle cells show elongated and hyperchromatic nuclei (hematoxylin and eosin). **c** Melanoma with brisk tumor-infiltrating lymphocytes (hematoxylin and eosin). **d** Quantification of tumor-infiltrating lymphocytes using immunohistochemistry (anti-CD8). **e** PD-L1 immunohistochemistry (Roche SP263). **f** PD-L1 immunohistochemistry (Dako 28-8). **g** Vulvar melanoma with weak cytoplasmic staining for VE1 antibody as assessed with an alkaline phosphatase red detection kit. This case did not have an underlying *BRAF* V600E mutation. **h** Metastatic melanoma with known *BRAF* V600E mutation with diffuse and strong cytoplasmic staining for VE1 as compared with that in (g)

102 months. After re-excision, the patient was alive and without disease at 138 months. Patient Va36 had no adjuvant therapy, and local recurrence was discovered at 6 months. The patient was given radiation therapy and had no evidence of disease at 15 months.

Cervical melanomas

One of the three patients (C1) was asymptomatic, and the diagnosis was made during a routine Pap smear. As the tumor was confined to the cervix, she was treated with surgery, and the surgical margins were clear. Six courses of postoperative adjuvant radiation therapy were administered.

Follow-up at 6 months showed local recurrence and distant metastases involving the abdomen, bone, lungs, and brain. No additional treatment was given, and the patient died at 66 months. The other two patients had abnormal vaginal bleeding and cervical masses. At diagnosis, they both had advanced, inoperable disease treated with palliative radiation therapy (Table 3).

Clinicopathological comparison between vulvar and non-vulvar melanomas

Vulvar melanomas were more likely to present as an exophytic mass ($p = 0.01$), while vaginal and cervical

Table 2 Clinicopathological features of vaginal melanomas

Case	Age	Presentation	Treatment	Cell type	Size (mm)	Thickness (mm)	Mitotic count (per mm ²)	Adjuvant treatment	Lymph node metastasis (number)	Follow-up (months)	Status
Va1	49	Mass/bleed	*Excision	Mixed	50	23	3	–	+(2)	12	DOD
Va2	62	Mass	*THBSO	E	23	18	8	RT	–	46	DOD
Va3	71	Mass	*WLE/ LND	E	20	3	5	RT	–	138	ANED
Va4	79	PMB	*WLE	E	17	2	15	–	Unknown	10	DOD
Va5	72	Mass/bleed	Palliative RT	E	30	20	44	N/A	+(4)	17	DOD
Va6	61	PMB	Palliative RT	E	50	Unknown	30	N/A	+(2)	19	DOD
Va7	41	Mass	Pelvic exent.	S	45	13	8	RT	+(1)	10	DOD
Va8	80	PMB	Palliative RT	E	30	7	22	N/A	–	15	DOD
Va9	58	PMB	None	E	10	9	14	–	Unknown	14	DOD
Va10	69	Mass/bleed	*WLE	Mixed	30	18	38	RT	–	63	DOD
Va11	58	PMB	Pelvic exent.	E	60	Unknown	4	Imatinib	+(2)	11	DOD
Va12	53	Mass	Excision	S	20	3.5	25	Chemo	+(1)	12	DOD
Va13	59	Mass	WLE/LND	E	20	Unknown	10	Chemo	+(1)	58	DOD
Va14	53	Mass	Excision	S	20	6	30	Chemo	+(1)	12	DOD
Va15	72	PMB	*WLE	E	24	10	11	Chemo	–	127	DOD
Va16	53	Mass	Excision	E	20	7	8	RT	+(2)	23	DOD
Va17	33	Vaginal discharge	Palliative RT	E	60	8	40	N/A	+(?)	2	DOD
Va18	77	PMB	Palliative RT	Mixed	15	6	20	N/A	–	6	DOD
Va19	65	Abnormal Pap smear	Pelvic exent.	Mixed	30	9	18	RT	+(1)	12	DOD
Va20	35	Vaginal discharge	None	S	40	10	21	–	Unknown	8	DOD
Va21	66	Mass/PMB	*THBSO	E	25	11	16	–	+(2)	12	DOD
Va23	80	Mass/bleed	Palliative RT	E	27	6	–	N/A	Unknown	10	DOD
Va24	87	Mass/urinary symptoms	*Excision	E	35	20	12	RT	Unknown	29	DOD
Va25	73	Abnormal Pap smear	Pelvic exent.	Mixed	6	3	30	–	+(2)	13	DOD
Va28	66	PMB/vaginal discharge	Excision	Mixed	45	15	28	RT	–	12	DOD
Va30	78	Mass/urinary symptoms	Palliative RT	E	30	Unknown	1	N/A	Unknown	28	DOD
Va31	88	Mass	None	E	50	Unknown	20	–	Unknown	6	DOD
Va32	85	Mass/PMB	Excision	E	20	Unknown	44	–	–	5	DOD
Va33	93	Mass	None	E	30	20	33	–	Unknown	6	DOD
Va34	74	Vaginal discharge	Palliative RT	Mixed	40	8	14	N/A	Unknown	9	DOD
Va35	86	PMB	Palliative RT	Mixed	30	20	19	N/A	Unknown	19	DOD
Va36	61	PMB	*WLE	E	25	13	8	–	–	15	ANED

*surgical margins involved, – absent, + present, *DOD* died of disease, *THBSO* total hysterectomy and bilateral salpingo-oophorectomy, *E* epithelioid, *RT* radiation therapy, *LND* lymph node dissection, *ANED* alive with no evidence of disease, *N/A* not applicable, *PMB* postmenopausal bleeding, *WLE* wide local excision, *Pelvic exent.* pelvic exenteration, *Chemo* chemotherapy

melanomas were significantly thicker ($p = 0.02$). Patients with vaginal and cervical melanomas were also significantly more likely to develop recurrence, exhibit disease progression with metastasis ($p = 0.012$), and die of the disease ($p = 0.017$) than patients with vulvar melanomas. The mortality rates were 65% (13/20) and 94% (33/35) for vulvar and non-vulvar melanomas, respectively. Age, gross pigmentation, tumor size, cell type, mitotic count, ulceration, necrosis, and presence of in-situ melanoma and melanin deposits were not significantly

different between vulvar and non-vulvar lesions ($p > 0.05$).

Immunohistochemistry for PD-L1

When the Roche Ventana SP263 antibody was used, greater than or equal to 5% staining was observed in 55% of vulvar, 50% of vaginal, and 33% of cervical melanomas. When the Dako 28-8 antibody was used, greater than or equal to 5% staining was observed in 20% of vulvar, 53% of vaginal,

Table 3 Clinicopathological features of cervical melanomas

Case	Age	Presentation	Treatment	Cell type	Size (mm)	Thickness (mm)	Mitotic count (per mm ²)	Adjuvant treatment	Lymph node metastasis (number)	Follow-up (months)	Status
C1	49	Abnormal Pap smear	THBSO, PLD	Mixed	15	2	2	RT	–	66	DOD
C2	63	Mass/bleed	RT	Mixed	20	>6	30	–	+(2)	26	DOD
C4	85	Mass/bleed	Palliative RT	Mixed	40	>22	40	N/A	+(1)	16	DOD

THBSO total hysterectomy and bilateral salpingo-oophorectomy, PLD pelvic lymph node dissection, RT radiation therapy, – absent, DOD died of disease, + present, N/A not applicable

Table 4 PD-L1 staining and tumor-infiltrating lymphocytes density in genital melanomas

	Vulvar (n = 20)	Vaginal (n = 32)	Cervical (n = 3)
PD-L1 (SP263), ≥5%	11 (55%)	16 (50%)	1 (33%)
PD-L1 (SP263), ≥10%	8 (40%)	14 (44%)	1 (33%)
PD-L1 (28–8), ≥5%	4 (20%)	17 (53%)	0 (0%)
PD-L1 (28–8), ≥10%	2 (10%)	14 (44%)	0 (0%)
CD8+ TIL (median)	114/hpf	127/hpf	50/hpf

SP263 Roche Ventana SP263. 28–8, Dako 28–8, TIL tumor-infiltrating lymphocytes, hpf high-power fields

and 0% of cervical melanomas (Fig. 1e, f) (Table 4). The SP263 antibody detected more cases with positive staining among vulvar melanomas.

Tumor-infiltrating lymphocytes density

The median densities of CD8+ tumor-infiltrating lymphocytes in vulvar, vaginal, and cervical melanomas were 114, 127, and 50 per high-power field, respectively. During manual scoring of tumor-infiltrating lymphocytes using immunohistochemistry, close to complete agreement was noted among the three observers (intraclass correlation coefficient: 0.96; $p < 0.05$). CD8+ tumor-infiltrating lymphocytes showed a significantly higher density among vulvar/vaginal melanomas than cervical melanomas ($p < 0.05$; Fig. 1d). Using Pearson correlation analysis, CD8+ tumor-infiltrating lymphocytes counts were moderately positively correlated with a PD-L1 score greater than or equal to 5% (Roche Ventana SP263), with a coefficient of 0.30 ($p < 0.05$). The relationships between PD-L1 expression and CD8+ tumor-infiltrating lymphocytes counts among different subtypes are shown in Table 5.

KIT mutation

Seven *KIT* mutations were found in five cases of vulvar and vaginal melanoma (Va10, Va17, Va18, Va36, and V22), but none were found in the three cervical melanoma cases. Three of the *KIT* mutations were hotspot mutations at exon 13 (Table 6).

Table 5 Tumor microenvironment according to PD-L1 expression and CD8+ tumor-infiltrating lymphocyte count (adapted from Teng et al. [25])

Type 1: PD-L1+, high TIL	Type 2: PD-L1–, low TIL
10 Vulvar	3 Vulvar
7 Vaginal	14 Vaginal
1 Cervical	
Type 4: PD-L1–, high TIL	Type 3: PD-L1+, low TIL
6 Vulvar	1 Vulvar
2 Vaginal	9 Vaginal
2 Cervical	

TIL tumor-infiltrating lymphocytes

BRAF (VE1) and mismatch repair protein expression

Five vulvar and two vaginal melanomas showed diffuse and weak cytoplasmic staining for VE1 (Fig. 1g). None of the 55 cases of female genital tract malignant melanomas had abnormal mismatch repair protein expression.

BRAF mutation

In one vaginal melanoma (Va6), a pathogenic mutation (F610I) was identified in exon 15 (COSM133631 c.1828 T>A) [40]. None of the 55 cases had *BRAF* V600E mutations, including seven cases in which immunohistochemical staining was diffuse and weak. Another vaginal melanoma (Va36) harbored four other mutations; however, these mutations were considered benign.

Table 6 *KIT* and *BRAF* mutations in genital melanomas

Gene	Sample	Position	Variation	Pathogenicity
<i>KIT</i>	Va10	Exon 13*	Rs121913512 c. 1924A>G, p.K642Q HGMD: CM002803 (associated with GIST) COSM96871	Pathogenic
	Va17	Exon 10	Novel mutation c. 1548C>T	–
	Va18	Exon 13*	c.1898A>G, p.E633G COSM 133765	Pathogenic
	Va36	Intron 1	Rs999020 IVS1+56T>C	–
		Intron 17	Rs1008658 IVS17+78T>C	–
	V22	Exon 13*	rs121913512 c.1924A>G, p.K642E COSM1304	Pathogenic
		Exon 14	c.2041T>A, p.F681I COSM5581487	Pathogenic
<i>BRAF</i>	Va6	Exon 15*	COSM133631 c. 1828 T>A, p.F610I	Pathogenic
	Va36	Exon 7	Rs41282721 IVS7+27G>A	–
		Intron 12	Rs62487918 IVS13–48C>T	–
		Exon and Intron 16	Rs9648696 c.1920A>G p.G643 Rs3789806 IVS16+16G>C	– –

*Hotspot mutation, *GIST* gastrointestinal stromal tumor

Survival analysis

The median disease-free survival was 10 months and the median overall survival was 14 months. In univariable analysis, tumor size greater than or equal to 33 mm, mitotic figures counts greater than or equal to 10 per mm², presence of lymph node metastasis at the time of initial diagnosis, and CD8+ tumor-infiltrating lymphocytes count less than 113 per high-power field were adverse prognostic factors for disease-free survival and overall survival (Fig. 2). PD-L1 expression had no prognostic significance (Table 7). In multivariable analysis, only mitotic counts greater than or equal to 10 per mm² ($p = 0.04$) was an adverse prognostic factor.

Discussion

Melanomas arising from the vulva, vagina, and cervix have a poor prognosis [1, 9, 41, 42]. In this study, we evaluated the clinicopathological features and potential biomarkers of

genital melanomas. Our results showed that 94% of patients with vaginal melanoma and 100% of patients with cervical melanoma died after median follow-up periods of 12 and 26 months, respectively, although only 65% of patients with vulvar melanoma succumbed to disease after a median follow-up of 29 months.

Diagnosis of malignant melanoma by initial Pap smear is uncommon [43, 44]. In our series, one cervical and two vaginal melanomas were detected by Pap smear. For other patients, presentation as a mass was more frequent in vulvar than in vaginal or cervical tumors (Fig. 3), whereas abnormal bleeding or discharge was a more common presentation for vaginal and cervical tumors than for vulvar tumors. Poor prognostic factors reported for genital melanomas include older age, black race, nonvulvar location, large tumor size, greater depth of invasion, presence of ulceration, high mitotic figures count, lymph node metastasis, and margin involvement [2, 3, 7, 9, 45–48]. In our series, age was not an adverse prognostic factor, but most patients were either peri- or postmenopausal (median age: 66 years), similar to an earlier Asian study [8]. We also found that tumor size greater than or equal to 33 mm, nonvulvar location, mitotic figures count of greater than or equal to 10 per mm², presence of lymph node metastasis at diagnosis, and low CD8+ tumor-infiltrating lymphocytes count were adverse prognostic factors, whereas cell type, lymphovascular space, and perineural invasion were not prognostic factors.

Because genital melanomas are rare, experience with these tumors is limited, and the primary goal is complete surgical resection. However, in clinical practice, most patients have advanced disease at presentation, and complete resection is not always possible [46, 47, 49, 50]. The choice of surgical procedure and observation of clear resection margins are thought to be important for prognosis [4]. Notably, in our study, these factors did not affect overall survival, possibly because many patients already exhibited lymph node or distant metastasis. Regional lymph node dissection has been recommended for patients with clinically apparent nodal disease [51]. Our results showed that absence of nodal metastasis at diagnosis was associated with a good prognosis [9, 10, 49, 52]. Eight of the nine survivors in our study did not have lymph node metastasis at diagnosis and were alive after a median of 54 months. Sentinel lymph node dissection is now a standardized procedure for cutaneous melanomas and is recommended for tumors with a thickness of 1–4 mm [53]. However, most genital melanomas are thicker, as in our series in which the median thickness was greater than or equal to 5 mm, and the value of sentinel lymph node mapping remains unclear. Owing to the rarity of genital melanomas, very few patients have been recruited for systemic therapy trials, and the benefits of adjuvant chemotherapy remain unclear [10, 54, 55]. Dacarbazine is often used either alone or in combination with vincristine, cisplatin/carboplatin, or nimustine [4, 45, 56]. Moreover, genital melanomas are

Fig. 2 Prognostic value of pathological parameters in genital melanomas. Kaplan–Meier plots indicating **a** disease-free survival and **b** overall survival of patients stratified by a cut-off value of 113 CD8+ tumor-infiltrating lymphocytes per high-power field. Kaplan–Meier plots indicating **c** disease-free survival and **d** overall survival of

patients stratified by mitotic count per 10 per mm². Kaplan–Meier plots indicating **e** disease-free survival and **f** overall survival of patients stratified by the presence or absence of lymph node metastasis at initial diagnosis

Table 7 Univariable analysis of clinicopathological features of female genital melanomas

Univariable	Hazard ratio	95.0% CI		<i>p</i> -value
		Lower	Upper	
Size (mm)				
<33	1.00	–	–	–
≥33	2.06	1.11	3.81	<0.05
Thickness (mm)				
<7.9	1.00	–	–	–
≥7.9	1.46	0.78	2.72	0.24
Mitotic figures (per mm ²)				
<10	1.00	–	–	–
≥10	3.88	1.77	8.51	<0.05
Lymph node*				
No	1.00	–	–	–
Yes	2.22	1.13	4.34	<0.05
PD-L1 (SP263)				
<5%	1.00	–	–	–
≥5%	0.90	0.50	1.62	0.72
PD-L1 (28–8)				
<5%	1.00	–	–	–
≥5%	1.19	0.66	2.16	0.57
CD8+ TIL count				
<113 per hpf	1.00	–	–	–
≥113 per hpf	0.31	0.14	0.67	<0.05

CI confidence interval, * presence of lymph node metastasis at initial diagnosis, TIL tumor-infiltrating lymphocytes, hpf high-power fields

typically resistant to radiation therapy [57]. Before the era of immune checkpoint inhibitors, 13 patients in our series received postoperative adjuvant radiation, but only two survived. One study showed that the combination of radiotherapy and ipilimumab, a cytotoxic T-lymphocyte-associated protein 4 antagonist, was more effective than radiotherapy alone in treating lower genital tract melanomas [58].

In cutaneous melanomas, expression of PD-L1 in tumor cells is correlated with treatment response to immune checkpoint inhibitors and serves as a prognostic marker [24, 26, 59–63]. Although several reports have described PD-L1 expression in genital melanomas, direct comparisons among these studies are difficult to conduct because of the different antibody clones used and different cut-off scores [17–19]. This lack of comparative data indicates that there is currently no consensus with regard to a clinically relevant expression threshold for genital melanomas. Nevertheless, when a cut-off greater than or equal to 5% was used, our results were comparable to those reported in two other studies using the same parameter [17, 18] (Supplementary Table 2).

Currently, there are four anti-PD-L1 antibodies registered with the US Food and Drug Administration for use on two

Fig. 3 Gross appearance of a malignant melanoma involving the female genital tract. This vaginal tumor has a polypoid exophytic surface component. Hemorrhagic fleshy areas alternate with ulceration. Black-brown pigmentation is prominent

different platforms (Dako 22C3 and 28-8 on Autostainer Link 48 and Roche SP263 and SP142 on Ventana BenchMark Ultra). Of the two clones tested, Roche SP263 identified more PD-L1-positive cases than Dako 28-8. This observation has also been noted in other studies [64]. The difference in positive staining may be related to the differences in the affinities of antibody binding to the protein (SP263 binds to the intracellular domain, and 28-8 binds to the extracellular domain) [65]. Tissue factors, such as spatial heterogeneity, may be of less relevance because consecutive sections from the same biopsy are used for both tests [65, 66]. Recent data supporting the interchangeability of Dako 22C3, Dako 28-8, and Roche SP263 PD-L1 assays have been described, mostly in non-small cell lung cancers, with only one study addressing this issue in melanomas [65, 67]. Unfortunately, the number of cases in the latter study was only 34, and whether any of the cases were of genital origin was unknown [65]. Although it is premature to draw any conclusions regarding the performance and interchangeability of these antibodies in genital melanoma, awareness of the choice of antibody used when treatment decisions are made is important.

The rarity of genital melanomas has resulted in difficulties in obtaining sufficient numbers of cases for large-scale studies, and several clinical trials have grouped genital melanomas into the mucosal melanoma category. Accordingly, the significance of PD-L1 expression and responses of genital melanomas to anti-PD-1/PD-L1 therapies remain unclear [19, 20, 38, 68–70]. Nevertheless, regardless of the PD-L1 immunohistochemistry status, some genital melanomas have been shown to respond to immune checkpoint inhibitors [21, 71]. The discordance between the absence of PD-L1 expression and drug responsiveness indicates that the use of PD-L1 immunohistochemistry as a single

predictive marker may not be sufficient. Accordingly, other potential biomarkers should be investigated in combination with PD-L1 immunohistochemistry [22–24, 28].

Understanding the tumor microenvironment has become important in the era of cancer immunotherapy [72]. The presence of tumor-infiltrating lymphocytes has long been recognized as a favorable prognostic factor in melanomas [73], and higher quantities of tumor-infiltrating lymphocytes, particularly CD8⁺ cytotoxic T cells, have been shown to correlate with improved patient survival [74]. In our study, we demonstrated that a higher CD8⁺ tumor-infiltrating lymphocytes density was associated with longer survival in patients with genital melanomas. This finding was consistent with the results of other studies [19, 20]. In future studies, digital image analysis is expected to provide a more detailed characterization of immune cell profiles and functions [75].

The combined assessment of PD-L1 and tumor-infiltrating lymphocytes has been proposed to be useful for guiding treatment decisions [14, 25]. A subgroup of melanomas demonstrating adaptive immune resistance, characterized as PD-L1⁺/high tumor-infiltrating lymphocytes, was found to be responsive to immunotherapy [15, 24]. Indeed, blocking of PD-L1 activity is thought to permit pre-existing high-density intratumoral cytotoxic T cells in the “hot” immune microenvironment to exert anticancer effects. In our study, 10 of 20 vulvar melanomas, seven of 32 vaginal melanomas, and one of three cervical melanomas were PD-L1⁺/high tumor-infiltrating lymphocytes (Table 5). Immune checkpoint inhibitors may be more effective for these patients [25]. Moreover, these “hot” melanomas were more often located in the vulva than in the vagina or cervix. Given the complexity of the tumor microenvironment, future studies are needed to evaluate other lymphoid cell elements for more precise patient selection.

The reported frequency of *KIT* mutations in genital melanomas ranges from 7 to 45%; the frequency observed in our cases (14%) falls within this range [32]. Among the reported pathogenic *KIT* mutations, seven gain-of-function mutations (V599D, V560D, L576P, D820V, D816V, V654A, and N822K) and one loss-of-function mutation (V555A) have been characterized in detail [76–78]. Tumors with V559D, V560D, and L576P mutations were shown to be more sensitive to the C-KIT inhibitors imatinib and sunitinib, whereas those with D820V, D816V, V654A, N822K, and V555A mutations were associated with drug resistance [79–82]. Among our cases of genital melanoma, we discovered four pathogenic mutations: K642E, K642Q, E633G, and F681I. A previous report of K642E in a genital melanoma revealed that the mutated tyrosine kinase I domain resulted in a constitutively active conformation of the kinase [83]. In gastrointestinal stromal tumors, this

mutation was associated with imatinib treatment response in both in vitro drug testing and patient treatment. K642Q, E633G, and F681I were reported in a gastrointestinal stromal tumor, an acral melanoma (from a Chinese patient), and a desmoplastic melanoma, respectively [84–86]. Whether these mutations render the tumors sensitive to C-KIT inhibitors requires further evaluation.

The *BRAF* V600E mutation accounts for ~90% of *BRAF* mutations in cutaneous melanomas [87]. Targeting *BRAF* V600E, an activating mutation, with tyrosine kinase inhibitors, such as vemurafenib and dabrafenib has been shown to be effective with significant survival benefits. The V600E mutation has occasionally been identified in a subset of genital melanomas (frequency: 6–23%) [17, 88, 89]. The presence of this mutation may be reliably screened with immunohistochemistry using the specific antibody VE1, which targets the cytoplasmic protein [35, 90, 91]. However, melanin pigment present in tumors can make evaluation difficult, and an alkaline phosphatase-based red detection kit should be used. In our study, using this detection method, five vulvar and two vaginal melanomas showed diffuse (>66%) but weak cytoplasmic staining. None of these cases were subsequently found to harbor the V600E mutation when analyzed by Sanger sequencing. Based on the low frequency of *BRAF* V600E mutations in genital melanomas, difficulties in immunohistochemical analysis, and the high cost of the VE1 antibody, this approach may not be a practical or informative screening tool. Molecular analysis should be considered instead. Incidentally, a pathogenic mutation (COSM133631 c.1828 T>A) previously reported in basal cell carcinoma of the skin [40] was discovered in one of the vaginal melanomas in our study.

Tumors with either mutations or epigenetic inactivation of mismatch repair genes (i.e., *MLH1*, *MSH2*, *MSH6*, and *PMS2*) are considered microsatellite-high/mismatch repair deficient and cannot repair defects in DNA replication. These tumors are more prone to accumulate multiple genomic alterations, resulting in high mutational burden, increased neoantigen load, and increased tumor-infiltrating lymphocytes. These tumors have also been shown to be more responsive to immune checkpoint inhibitors [27, 92]. Screening for an underlying mismatch repair deficiency can be carried out using immunohistochemistry, and loss of staining for any of the mismatch repair proteins can be further verified with more detailed microsatellite instability tests. While the majority of analyses were performed with non-cutaneous and non-melanocytic tumors, a few cutaneous melanomas were reported to be microsatellite-high [22, 28–30, 93]. The status in genital melanomas is unclear. In our study, none of the cases showed loss of mismatch repair proteins, suggesting that microsatellite instability may be a rare event in genital melanomas and that

immunohistochemistry may not be a useful biomarker for screening. However, microsatellite-high is more frequently detected in metastatic than in primary tumors in cutaneous melanomas because of acquisition of new mutations as the tumor progresses and disseminates [28]. Whether this occurs in metastatic genital melanomas is unknown.

In summary, melanomas of the vulva, vagina, and cervix are aggressive, and affected patients have a poor prognosis. In our study, we found that tumor size greater than or equal to 33 mm, mitotic figures count of greater than or equal to 10 per mm², the presence of lymph node metastasis at diagnosis, and low CD8⁺ tumor-infiltrating lymphocytes density were adverse prognostic factors. Surgery may not always be feasible, and conventional adjuvant therapy is ineffective. Evaluation of multiple biomarkers should be considered to identify patients suitable for targeted therapy and immunotherapy. Our study was limited by the small sample size for cervical melanomas. For patients who had inoperable tumors, the assessment of biomarkers was only feasible in biopsy specimens, which may have affected the accuracy of the results in some cases. The retrospective nature of the study was also an inherent limitation because treatment modalities have changed and evolved over the study period of 22 years. In future studies, next-generation sequencing may enable more thorough elucidation of potential therapeutic targets. While a minority of the tumors had *KIT* mutations, none had *BRAF* V600E mutations, and loss of staining for any of the mismatch repair proteins was not observed. A subset of patients whose tumors express PD-L1 and have higher density of tumor-infiltrating lymphocytes may benefit from immune checkpoint inhibitors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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