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Female Genitourinary Tract Melanoma: Mutation Analysis with Clinicopathologic Correlation, a single-institution experience

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Abstract

Female genitourinary tract melanoma (FGTM) is a rare and often-fatal form of mucosal melanoma. We describe our institutional experience with 55 cases of FGTM, 16 of which were evaluated with next-generation sequencing targeting 151 cancer-associated genes. Tumors tended to be thicker than conventional melanoma at presentation (median 3.2 mm), were frequently ulcerated (50%), and characterized by incomplete initial resections. Regional lymph nodes showed tumor involvement at presentation in 28% of cases. With a median followup of 23.6 months, the median recurrence free survival was 14.5 months, and median overall survival at 29.6 months. Genomic analysis revealed mutually exclusive mutations in *TP53* and *KIT* in 25%, while 19% of cases demonstrated *BRAF* mutation. *NRAS* mutation was found in 13% of cases. Mutation in *ATRX*, previously undescribed in mucosal melanoma, was seen in 3/16 (10%) of patients. Only invasive melanoma cases were included in statistical analyses. Patients with three or more mutations had marginally worse **overall** survival rates than those with two or less ($p=0.007$). Further studies are required for potential adjuvant treatment modalities in order to improve survival outcomes of FGTM.

Keywords

Female genital tract melanoma; *ATRX*; *p53*

Introduction

Female genitourinary tract melanoma (FGTM) is rare, accounting for 3% of all melanoma [1] Survival Epidemiology and End Results (SEER) data shows 817 new genitourinary tract melanoma cases between 1992 and 2012 [2]. Among these cases, female genital tract is the

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most common site (89.4%) followed by male genital tract (6.6%) and urinary tract (4.3%) melanoma. The overall annual incidence of vulvar melanoma is around 0.2 in 100,000 women, making it the second most common malignancy of the site [3, 4]. Genitourinary melanomas are 10 times more common in women compared to men, and women have better overall survival rates [2]. Compared to conventional melanoma, genitourinary melanoma is thicker at presentation, with a median **tumor thickness** of 3.2 to 12.7 mm in recent series [5–7]. Prognosis is uniformly poor, with a median overall survival of 25 to 73.9 months [5–9]. Studies are limited to single institution series, and have pointed to a number of clinicopathologic factors in determining outcome. Of these, five of the six largest recent series all demonstrated that tumor **thickness** is an important predictor of disease free and overall survival [6–10]. Surgery remains the mainstay of treatment for genital tract melanoma [11], although it does not appear to influence overall survival [6]. Although there are numerous options for effective adjuvant therapy in cutaneous melanoma [12, 13], data on FGTM is scant and treatment modalities are relatively limited. The most widely studied adjuvant therapy is imatinib, targeting *KIT* mutations, has thus far shown modest response rates in mucosal melanoma trials [14]. Other adjuvant therapy modalities such as radiation treatment do not appear to add survival benefit for genital tract melanoma [5].

Recent studies of genital tract melanoma have revealed their unique clinicopathologic, molecular and biologic features [15–17]. Mutations and increased copy numbers encoding the receptor tyrosine kinase *KIT* have been described in up to 40% of mucosal melanomas [18–20]. Mutations involving *KIT* and *NRAS* genes along with wild-type *BRAF* are present in approximately 20% of patient cases with vulvo-vaginal melanomas in another study [21]. In contrast, *BRAF* mutation involving the V600E locus was reported in around 40% of atypical genital nevi; this interesting finding implies that ultraviolet exposure is not essential in generating this mutation [22]. Identifying site-specific mutations involved in the pathogenesis of genitourinary tract melanoma can be contributory for further treatment options. We sought to further investigate the mutational landscape of this rare melanoma in a series of 20 cases, culled from our institutional experience with 55 FGTM. We used a broad panel (Agilent ClearSeq Comprehensive Cancer Panel), which focuses on hot spots involving 151 common cancer genes, with comparison to normal tissue when available. We then attempted to identify clinicopathologic correlates with mutation status.

Methods

Clinicopathologic features

After institutional review board approval, a search of the pathology database revealed 55 cases of melanoma arising in the vulva, vagina, uterus, and/or urethra between 1995 and 2016. Clinical outcome data, including treatment, recurrence, and survival was obtained from chart review and the institutional cancer registry. Pathology reports and all available slides were reviewed. From these, twenty cases with archival tissue available for DNA retrieval were available for use; 16 of these had matching normal tissue available for use as a control.

DNA isolation

After pathologist review and identification of tumor from the H&E-slides, DNA was extracted from macrodissected 20-micron sections of the formalin fixed paraffin embedded (FFPE) tissue block, using the QIAamp DNA FFPE Tissue Kit, catalog #56404, and dissolved in water. DNA was quantified first using Nanodrop 1000 instrument and dsDNA quantity was assessed with Qubit instrument. Agilent's Tape station instrument with genomic screen tape, cat#5067-5365, was used to assess DNA quantity.

Agilent ClearSeq Comprehensive Cancer Panel and Illumina sequencing

Targeted DNA sequencing was performed to identify somatic mutations in the coding regions of 151 cancer-associated genes, using 200 ng of DNA and the Agilent SureSelect XT ClearSeq Comprehensive Cancer Panel kit. Briefly, for each tumor DNA sample, a genomic DNA library was constructed according to the manufacturer's protocol and the size and quality of the library was evaluated using the Agilent BioAnalyzer. Equimolar amounts of library DNA were used for targeted enrichment using the Agilent capture baits, and after quantitative PCR library quantitation and QC analysis on the BioAnalyzer, approximately 4.5 million 75-base paired-end sequence reads were generated using v2 chemistry on an Illumina NextSeq 500 sequencer in order to generate average target coverage greater than 150X. Sequence reads were aligned to the reference human genome (hs37d5) with the Burrows-Wheeler Aligner (BWA) [23] and insertion/deletion realignment and quality score recalibration were performed with the Genome Analysis ToolKit (GATK) [24]. Tumor specific mutations were identified with Strelka [25] and MuTect [26], and annotated to determine genic context (ie, non-synonymous, missense, splicing) using ANNOVAR [27] and summarized using spreadsheets and a genomic data visualization tool, VarSifter [28]. Additional contextual information was be incorporated, including allele frequency in other studies such as 1000 Genomes and the NHLBI Exome Sequence Project, in silico function impact predictions, and observed impacts from databases like ClinVar (<http://www.ncbi.nlm.nih.gov/clinvar/>), and the Collection Of Somatic Mutations In Cancer (COSMIC).

Statistical Analysis

Student's t-test and chi-square association test were performed to examine the relationship between mutation type(s) and **tumor thickness**, lymph node status, and ulceration. Based on the sum of all mutations for each patient, patients were then divided into two groups: low mutation group (0-2 mutations) and high mutation group (3 or more mutations). Kaplan-Meier survival analysis was conducted to examine patient survival time associated with mutation frequencies. Log-rank test was used to test the **overall** survival difference between low and high mutation groups. A two-sided p-value less than 0.05 was considered significant. All statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary, NC).

Results

Clinicopathologic features, treatment and clinical outcome

There were 55 cases identified, with an anatomic distribution as follows: 30 vulva, 20 vaginal, 2 cervix and uterus, 2 cervix, 1 uterus. The median patient age was 63.6 years. The median tumor **thickness** was 3.2 mm (range 0-20 mm). Ulceration was present in 16/33 (50%) of evaluable tumors. The tumor stage at diagnosis was as follows: Stage I-11 patients, Stage II-28 patients, Stage III-14 patients, and Stage IV-2 patients. 50 patients (90%) underwent surgery as the initial treatment, with 16 patients receiving wide excision alone, 16 patients undergoing wide excision and sentinel lymph node (SLN) biopsy, and 13 patients undergoing wide excision and complete groin lymph node dissection. The initial wide excision showed positive margins in 15/42 (40%) patients where known. Nodal involvement was present at diagnosis in 8/29 patients (28%): 5/16 patients undergoing SLN biopsy (31%) and 3/13 (23%) patients undergoing complete node dissection. The initial treatment for the remaining patients consisted of chemotherapy for locally advanced disease in 2 patients, local radiation for locally advanced disease in one patient, and unknown in 2 patients. At a median followup of 23.5 months, **there were 34 recurrences: 10 local, 12 regional, and 12 distant. The median time to recurrence was 14.5 months. There was no significant difference in recurrence rate with anatomic location: 62% of vulvar melanomas recurred, and 60% of vaginal melanomas recurred.** Within this followup interval, 23 patients (41%) died of disease, 5 patients were alive with disease, 5 were dead of other causes, and 19 had no evidence of disease, with 3 patients having unknown status. The median overall survival was 29.6 months.

Cases undergoing mutational evaluation

Twenty patients had available tissue for DNA analysis; sixteen of these had matching normal tissue to use as control. The distribution of cases by anatomic location was as follows: 11 vulvar, 6 vaginal and 3 periurethral melanomas. **All samples were obtained from primary anatomic sites.** Three of these were melanoma in-situ involving vulvar skin. One patient had separate vaginal and vulvar primary melanomas diagnosed 5 years apart. In this group, the median **tumor** thickness was 4.42 (range 0.42 mm-17.2 mm). Ulceration was identified in seven cases (35%). Five of nine (55%) of cases had regional lymph node involvement at presentation, where known. Two patients developed lung metastases and one patient developed brain metastasis during followup.

No mutations were detected in melanoma in situ cases (n=3). Only invasive melanoma samples with normal controls were included for statistical analysis (n=13). Mutations were identified in 12 cases (Table 1). Mutually exclusive *TP53* and *KIT* mutations were most frequent, and were identified in four cases each (25%), equally divided between vulvar and vaginal origin. *BRAF* mutations were noted in 3 vulvar melanomas (10%); 2 were p.V600E and 1 was p.G466'V'. *NRAS* mutations were found in 2 vulvar melanomas (13%), one p.G13D and one p.G12S. The *BRAF* and *NRAS* mutations were also mutually exclusive. Truncating mutations in *ATRX* were found in 3 patients (p.Q732X/p.Q770x in 2 and p.Q251X/p.Q289X in one); these accompanied a *TP53* mutation in all 3 cases. The supplemental file shows all detected mutations. The patient with separate primary vulvar and

vaginal melanomas showed distinct mutations in each: the vaginal melanoma showed *KIT* mutation and the vulvar melanoma *NRAS* mutation.

The relationship between mutation status and clinicopathologic variables was examined. **The summary of clinicopathologic features is presented in Table 2. *ZMYM3* mutational status was correlated with stromal invasion ($p=0.034$) in univariate analyses after Bonferroni correction. Only one sample out of 13 showed the mutation.** There was no correlation between mutation status with lymph node involvement and ulceration. The histomorphologic patterns of the tumors did not appear related to the mutation status (figure 1). Survival data was available for 15 of the 16 patients and varied from 11 to 91 months with median of 39 months. The cases were divided into two groups based on the total number of mutations in the individual tumors: two or less (5 patients) and three or more (7 patients). **There was a trend with worse overall survival outcome in patients with 3 or more mutations when these groups were compared by the log-rank test ($p=0.07$).** The patients who developed lung metastases each had one mutation: one had a *TP53* mutation and the other *KIT* mutation. The patient case with brain metastasis had both *KIT* and *NRAS* mutations.

Discussion

The prognosis of patients with FGTM remains very poor. There are diagnostic and treatment challenges due to difficulties in clinical and pathologic recognition of these lesions and lack of clearly effective ancillary treatment modalities [7, 17]. One of the largest series in literature ($n=98$) showed that stage of disease did not influence survival outcomes [5]. Other series have demonstrated that the choice of surgical treatment affected disease free survival but not overall survival [6, 29]. Although there is increasing data about genital melanoma, our knowledge concerning molecular targets for therapy lags far behind that of cutaneous and uveal melanoma.

In this small series, we confirm that the most common mutations in FGTM affect the *KIT* and *TP53* genes. *KIT* alterations are more common in vulvar than vaginal melanoma [30, 31], and our findings confirm this. Historically, the incidence of *c-KIT* mutation in cutaneous melanoma is between 2-8% and the rate is increased to 15-20% in mucosal melanomas [18, 20, 32]. Amino acid substitution at positions W555R (10%), V559A (20%), V559Q (5%), L576P (25%), K642E (20%) and D816H (5%) are the common mutational loci; specific mutations may affect response to targeted therapy with nilotinib and imatinib in clinical trials [14, 33, 34]. In our cases, the *KIT* mutations involved K642E, W557G and A529D loci.

TP53 mutation was as frequent as *KIT* mutation in our cases, affecting four patients, with equal distribution between vulvar and vaginal lesions. Aberrations of this gene in cutaneous melanoma are typically associated with chronic sun damage, and *TP53* mutation has been reported as rare to absent in acral and mucosal melanoma.(35) Its reactivation has been associated with acquired resistance to MAP-kinase inhibitors in *NRAS*-mutant melanoma (36). Aberrant *TP53* expression is also more commonly associated with undifferentiated tumor cells in primary mucosal melanomas of the head and neck [37]. The finding of this

mutation in two vulvar and two vaginal melanomas clearly indicates that sun exposure is not the only mechanism for mutation in this gene. One of the common mutations in cutaneous melanoma is the *BRAF* mutation; this confers sensitivity to *BRAF* therapy inhibitor, although patients typically ultimately develop drug resistance [38]. We identified this mutation in 3 cases; two of these involved the V600E locus. A *BRAF*^{G466V} mutation was found in the third case. Similar to prior studies, all *BRAF*-mutant lesions were located in the vulva; no vaginal lesions contained this mutation. This mutation has been reported in neurocutaneous melanosis and a case of primary mesenchymal brain neoplasm [39], non-small cell lung carcinoma [40] and colorectal carcinoma [41]. To the best of our knowledge, this is the first FGTM case with *BRAF*^{G466V} mutation in the literature. Finally, the finding of *NRAS* mutation in two vulvar melanomas is in keeping with the reported incidence of this mutation in mucosal melanoma [42].

Our initial hypothesis that differences exist in mutation rates between vulvar and vaginal primaries could not be proven statistically due to the small sample size. However, it is worth noting that *BRAF* and *NRAS* mutations were exclusive to the vulvar **melanoma** in our series, while *TP53*, *KIT* and *ATRX* were evenly distributed between the two sites. In cutaneous melanoma, associations between specific clinical parameters and mutational status are well described. For example, *BRAF* mutation is commonly associated with younger age in patients with metastatic melanoma [43]. *NRAS* mutation is associated with more aggressive disease and lower overall survival [44]. *NRAS*-mutated melanoma has higher lung metastasis rates and decreased overall survival compared to *BRAF*-mutated subtype in addition to decreased overall survival [45]. One of the *NRAS* mutant cases was associated with brain metastasis in our series; this case also had *KIT* mutation.

Male patients with high log-transformed missense mutation rates have better survival rates in cutaneous melanoma [46]. We found that a mutation burden of three or more was associated with marginally worse overall survival rates. Total mutation burden may be considered a significant predictor of survival and perhaps modify treatment choices in these patients. This observation deserves further investigation. In summary, patients with genitourinary tract melanoma have poor clinical outcomes compared to patients with cutaneous melanoma. Our series confirms the presence of thicker tumors at diagnosis, which are frequently ulcerated and often have positive margins at the time of initial resection. Almost one-third of patients present with regional nodal disease, and outcome is poor, with a median survival of less than two years. Mutations in cancer-associated genes are frequently found, and their role in choice of adjuvant treatment survival and determination of overall outcome certainly deserves larger-scale studies of this rare disease.

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Figure 1. Representative examples of female genital tract melanomas showing no distinct morphology based on mutation status. A: *ATRX*-mutated vulvar melanoma. B: *BRAF*-mutated vulvar melanoma. C: *KIT*-mutated vulvar melanoma. D: *NRAS*-mutated vaginal melanoma.

Table 1

Summary of clinically significant mutations detected in female genitourinary tract melanoma.

Sample No	Site	Mutation/loci	Type
1	Vulva	KIT (p.K638E/p.K642E)	nonsynonymous
2	Vulva	BRAF (p.G466V) NF1 (c.2990+1G>T,c.5749+1G>A/c.5812+1G>A)	Nonsynonymous Truncating
3	Vulva	ATR1 (p.Q732X/p.Q770X) P53 (p.R174X/p.R267X/p.R306X)	Truncating Truncating
4	Vagina	ATR1 (p.Q732X/p.Q770X) P53 (p.R148K/p.R241K/p.R280K)	Truncating nonsynonymous
5 *	Vagina	KIT (p.W553G/p.W557G)	nonsynonymous
6	Vagina	KIT (p.L572P/p.L576P)	nonsynonymous
7	Vulva	P53 (p.G113V/p.G206V/p.G245V)	nonsynonymous
8	Vagina	N/A	
9	Vagina	ATR1 (p.Q251X/p.Q289X) P53 (p.S176N/p.S215N/p.S83N)	Truncating nonsynonymous
10 *	Vulva	NRAS (p.G13D)	nonsynonymous
11	Vulva	N/A	
12	Vulva	N/A	
13	Vulva	BRAF (p.V600E) EZH2(p.Y590H/p.Y602H/p.Y632H/p.Y641H/p.Y646H)	Nonsynonymous nonsynonymous
14	Vulva	N/A	
15	Vulva	EZH2 (p.Y590N/p.Y602N/p.Y632N/p.Y641N/p.Y646N) KIT (p.A529D/p.A533D) NRAS (p.G12S)	Nonsynonymous Nonsynonymous nonsynonymous
16	Urethra	BRAF (p.V600E)	nonsynonymous

* Samples from same patient.

Table 2

Cases Undergoing Mutational Evaluation and primary tumor parameters.

Case #	Age at diagnosis	LVI	perineural invasion	Midline/bilateral involvement	regression	satellitosis	tumor thickness	ulceration	mitotic rate per sq mm	LN	Tumor
1	60	absent	absent	no	absent	absent	2.0 mm	no	1 per sq mm	2+	primary
2	65	absent	present	yes	absent	present	6.0 mm	yes	2	1+	primary
3 *	68	absent	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
4	72	present	present	n/a	absent	absent	13.0 mm	yes	8	n/a	primary
5	69	absent	absent	n/a	absent	absent	3.0 mm	yes	1	5-	primary
6 *	74	absent	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
7	59	absent	absent	n/a	present	absent	2.45 mm	yes	1	5-	primary
8	80	absent	absent	no	absent	present	2.4 mm	no	0	2+	primary
9	77	absent	absent	n/a	absent	absent	4.3 mm	yes	1	3-	primary
10 *	70	absent	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
11	54	absent	absent	yes	present	absent	1.4 mm	no	1	4-	primary
12	87	absent	absent	n/a	absent	absent	6.0 mm	yes	11	n/a	recurrent lesion
13	66	absent	absent	n/a	absent	absent	4.1 mm	yes	2	n/a	recurrent lesion
14	70	absent	absent	n/a	absent	absent	17.2 mm	yes	3	2+	primary
15	82	absent	absent	n/a	present	absent	0.45 mm	no	0	n/a	primary
16	62	absent	absent	n/a	n/a	absent	6.0 mm	no	2	2-	primary

LVI: Lymphovascular invasion. "Sq": Square. LN: Lymph node. n/a: not available or applicable.

* melanoma in situ cases.