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Identification of vital genes and pathways associated with mucosal melanoma in Chinese

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ABSTRACT

Mucosal melanoma is a rare malignant melanoma with more aggressive and poorer outcomes. The incidence of mucosal melanoma varies greatly among different ethnic groups. We herein sought to characterize the vital genes and pathways of Chinese mucosal melanoma patients. By whole-exome sequencing in six patients with mucosal melanoma, we detected a total of 21,733 CNVs and 2372 SNPs. The CNV/SNP burden varies greatly between individuals, including recurrent CNV targeting PIK3 family, *KRAS, APC* and *BRCA1*. Significantly mutated genes were *NUDT5, ZBTB18, NEURL4, ZNF430, RBM44, GAK, PCDHA13, STK38* and *UBR5*. Besides, *FAT1* gene was identified frequently mutated in anorectal melanoma patients (3/3, 100%). Moreover, our result showed that HPV infection may be associated with mucosal melanoma. In conclusion, this study indicated that mucosal melanomas have a low SNPs burden and a high number of CNVs and expand the spectrum of mucosal melanoma variants, also provided an insight for the pathological mechanism of mucosal melanoma.

1. Introduction

Mucosal melanoma (MCM) is a common subtype of malignant melanoma, which is a relatively rare malignant tumor in Asia [1], with a median survival of 3.58 years and the overall 5-year survival rates of only 26.8%, lower than non-MCM and ALM, respectively [1]. The incidence of mucosal melanoma increased with age and was significantly higher in people of color than in Caucasians (Asia versus Western: 22.6% versus 1.3%), and higher in women than in men [1,2].

Clinically, mucosal melanoma can be divided into head and neck melanoma, anorectal melanoma, female genital tract melanoma, and urinary tract melanoma, and the incidence of them is 55.4%, 23.8%, 18.0%, and 2.8%, respectively [2]. Patients affected with mucosal melanoma may manifest greatly varieties in the disease course, and mucosal melanoma presentations can overlay with other cancer, such as rectal cancer and esophagus cancer.

A large number of mutations, such as *BRAF, TERT, BAP1, SF3B1, TSC1,* and *TP53* are frequently observed in sporadic melanoma [3-8]. Although melanoma genomes have the highest mutation load of any cancer [9,10], since to the frequency of the disease, the genetic mechanisms involved in the pathogenesis and progression of mucosal melanoma remain unclear.

Herein, we employed whole-exome sequencing (WES) to analyze melanoma and paired paracancerous tissues derived from six pathologically confirmed mucosal melanoma patients to characterize the vital driver genes and pathways of Chinese patients. We detected a large amount of single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) in tumor tissues. One most prominent mutated gene *FAT1* may be related to the pathogenesis of anorectal melanoma, as well as HPV infection.

2. Materials and methods

2.1. Tumor specimens

We collected seven patients affected mucosal melanoma, of whom one patient was excluded for DNA quality did not meet the sequencing criteria. Specimens included three anorectal melanoma, two vaginal melanoma and one esophageal melanoma that were obtained at the first affiliated hospital of Soochow University from June 2016 to October 2017 (Table 1). Following resection, the tissues were paraffin-embedded according to routine protocol. The diagnosis of mucosal melanomas was made by histological analysis. All the patients were sporadic.

The study was reviewed and approved by the Institutional Review

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Table 1
Patient sample and clinical information.

No.	Sex	Date	Onset age	Tumor position	Tumor size (cm)	Lymph node	Metastasis time	Metastasis site	Relapse time
1	F	2016-08	50	Vagina	2.5*2.5*1.5	0/29	14 months	Multiple lung metastases	–
2	M	2017-01	54	Esophagus	4.0*2.2*1.8	0/26	4 months	Multiple intrahepatic metastases	–
3	F	2017-10	50	Vagina	3.5*2.0*1.2	\	–	–	2 years
4	F	2016-05	50	Anorectum	7.5*5.5*4.0	5/10	2 months	Lung and mediastinal metastases	\
5	F	2016-06	59	Anorectum	12.0*7.0*4.0	5/7		\	\
7	M	2012-11	60	Anorectum	5.0*4.0*3.0	0/5	5 years	Lung and mediastinal metastases	–

Date represents operation date. "\" represents no information, "–" represents negative.

Board of the authors' affiliated institution and patient consent for the study was waived due to the retrospective nature of this study.

2.2. DNA extraction

Genomic DNA was extracted using Tissue Kit (Qiagen) according to the manufacturer's protocol. Purified DNA was quantitated with a Nanodrop ND 1000 spectrophotometer (Fisher Scientific), and stored at 4 °C. Molecular weight and the quality of DNA were tested by gel electrophoresis, and the sample was excluded if their concentration of DNA was less than 50 ng/μl, or absence of a high molecular mass band in electrophoresis gels.

2.3. Whole-exome sequencing

Genomic DNA was sheared to an average size of ~300 bp with the Covaris S220 system (Covaris). Next, sequencing libraries were prepared using NanoPrep™ DNA kit (Nanodigmbio) for Illumina sequencing according to the manufacture's instructions. Finally, libraries were sequenced on Illumina NextSeq500 System (Illumina).

2.4. Quality control and bioinformatics analysis

DNA sequencing quality was analyzed by FastQC. BWA/SAMtools were used for alignment of raw reads and SNP detection, and VarDict was used for variant calling. Mutations were identified by SNPeff. Pathway and network analyses were performed using Ingenuity (IPA). Gene set enrichment analysis was performed based on the Kyoto encyclopedia of genes and genomes (KEGG) using the clusterProfiler package.

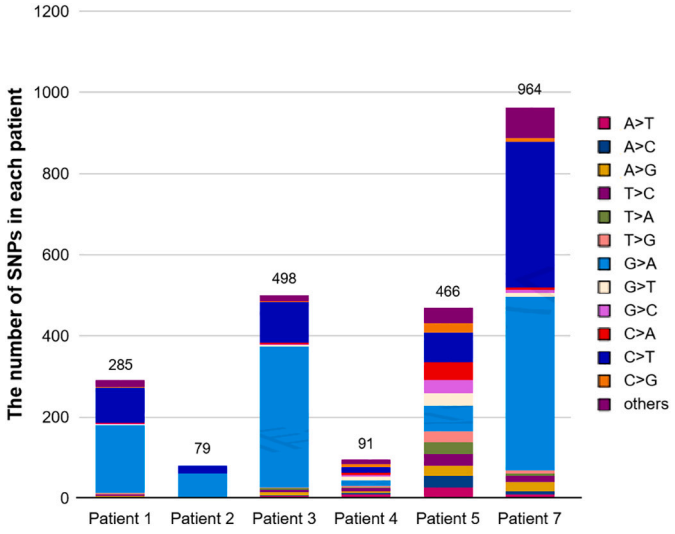

Fig. 1. Summary of CNVs in each patient.

3. Results

3.1. Patient sample and clinical information

The six mucosal melanomas comprised four primary tumors, one relapse tumor and one lymph node from metastases; three anorectal melanoma (AM), two vaginal melanoma and one esophageal melanoma (Table 1). All patients aged 50–60 years had undergone complete resection of their disease as soon as they were diagnosed, but all had relapsed or metastasized within five years except for patient 5, for we only have a record of her death within 1 year after surgery. Anorectal melanoma patients' primary tumor size is generally larger, and the incidence of nodal involvement was also higher at presentation (Table 1). Those characteristics may, at least in part, explain the poor prognosis of this disease. Just like patient 4, at the time of surgery, the tumor was 7.5*5.5*4.0 cm^3 in size with nodal involvement (5/10), and multiple metastases (lung and mediastinum) occurred only 2 months after surgery (Table 1).

3.2. Single nucleotide polymorphisms (SNPs) identified in tumor tissues

We identified 2374 SNPs (range 79-964 per tumor) (variant reads >10), and the number and richness of SNPs differs greatly between six patients (Fig. 1). While we can see that there is no relativity between the number and richness of SNPs. By overlapping SNPs between patients, we obtained nine significant genes, *NUDT5, ZBTB18, NEURL4, ZNF430, RBM44, GAK, PCDHA13, STK38* and *UBR5* (Fig. 2). Of which, many are known to be involved in some cancers, such as glioblastoma, renal cell carcinoma, Gastric cancer and colorectal cancer [11-15]. However, no study to date suggested a link between these genes and mucosal melanomas. Our study is the first to observe that these genes may be associated with mucosal melanoma.

3.3. Copy number variations (CNVs) identified in tumor tissues

WES identified 21,733 CNVs (range 1047-7699 per tumor) comprising 8029 gain of function (range 273-3340 per tumor) and

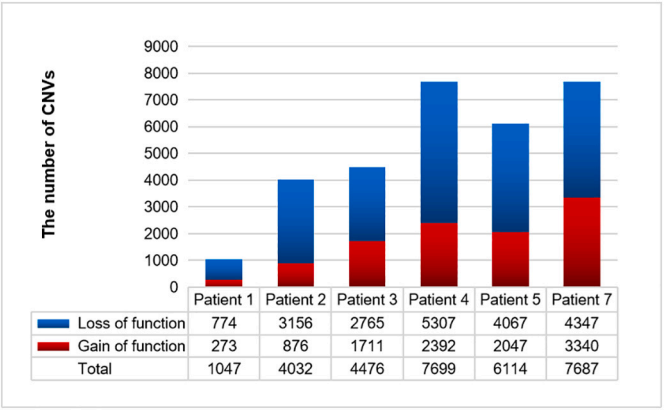

Fig. 2. Summary of SNPs in each patient.

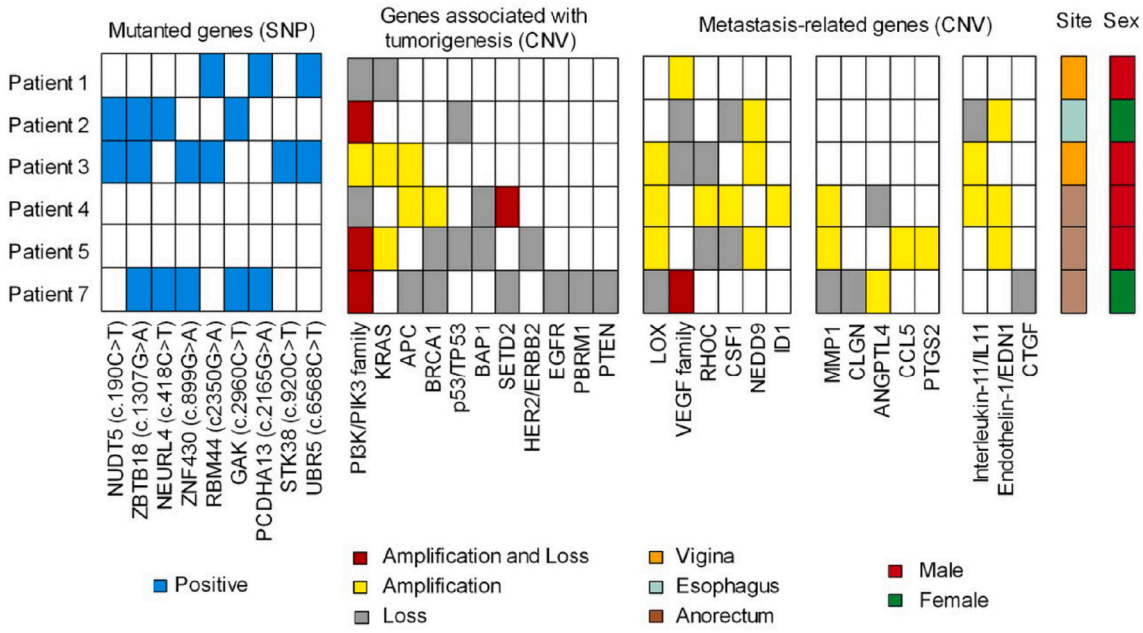

Fig. 3. Summary of significantly mutated genes affected by SNPs and CNVs.

13,704 loss of function (range 774-5307 per tumor) excluding those recurrent CNVs between patients (Fig. 3). In particular, we detected some CNVs mutation in some known melanoma-related genes, including *PI3K*, *KRAS, PTEN, EGFR, BAP1* and *BRCA1* genes CNVs (Fig. 2), consistent with the Cancer Genome Atlas (TCGA) study of 333 melanomas [2], suggesting that those genes may have closely relationship with Chinese melanoma patients.

The CNV load varies greatly between patients, and all three patients with anorectal melanomas exhibited substantially more CNVs than the other patients, consistent with their bigger primary tumors (Fig. 3). Interestingly, patient 4 had the largest CNV load and her disease progress was also the fastest, suggesting that CNV load maybe has something with the prognosis of melanomas patients.

3.4. CNV associated with cancer and metastasis

We then analyzed the genes associated with tumorigenesis and metastasis in raw data. This analysis revealed that each patient carried several CNVs (range 2–7) in genes associated with tumorigenesis: *PI3K/PIK3* family, *KRAS, APC, BRCA1, p53, BAP1* and *SETD2* (Fig. 2). In addition to *p53* and *BAP1* gene are copy loss, other genes in patients have copy amplified and loss. *PI3K/PIK3* family genes CNVs were observed in all patients (Fig. 2). Moreover, we also detected CNVs in multiple genes (range 1–8 per tumor) related to tumor metastasis, such as *LOX, VEGF family, RHOC, CSF1, MEDD9, MMP1, ANGPTL4, IL-11,* and *EDN11*. As we can see, patient 4 and patient 5 carried the greatest number of CNV in genes associated with metastasis, and most of them were gain of function, consistent with their rapid clinical metastasis and progress.

Table 2
The information of *FAT1* mutations.

Sample	Mutation	Exome	Annotation	Transcript ID
Patient 4	c.3713A > G (p. Glu1238Gly)	5	Missense variant	ENST00000441802.2
Patient 5	c.11429G > A (p. Trp3810*)	20	Stop gain	ENST00000441802.2
Patient 7	c.13717G > A (p. Glu4573Lys)	27	Missense variant	ENST00000441802.2
Patient 7	c.10955C > T (p. Pro3652Leu)	19	Missense variant	ENST00000441802.2
Patient 7	c.8176C > T (p. Arg2726*)	10	Stop gain	ENST00000441802.2

3.5. FAT1 gene mutations detected in three anorectal melanoma patents

FAT1 was identified as the most significantly mutated gene, for we identified five variations in all three anorectal melanoma patents, including three missense mutations and two stop gains; especially patient 7, who has three non-synonymous variants in *FAT1*, including one stop gain (Table 2 and Fig. 4A). Those variants have not been reported previously and were not found in the 1000 genomes. Four of the five mutations were highly evolutionarily conserved (Fig. 4B), suggesting that a critical functional role for those residues and non-synonymous substitution at those sites are not likely to be tolerated.

3.6. Gene-set pathway enrichment analysis

We undertook gene-set enrichment analyses based upon SPNs identified in all patients using clusterProfiler [16]. The most significant SNPs enrichments were observed in human papillomavirus (HPV) infection (Fig. 5). While no studies to date have reported the association between HPV infection and mucosal melanoma, HPV infection is known to be associated with cervical cancer, and also has an association with vaginal cancers and colorectal cancer [17,18]. Therefore, HPV infection may be also contribute to mucosal melanoma.

4. Discussion

Due to the low incidence of malignant melanoma in Asian population [1], the pathogenesis of melanoma in Asian population is still unclear. The incidence of mucosal melanoma varies greatly between Asian and western countries [1,2]. The mean age for diagnosis of mucosal melanoma is 63 years in Western population [2], while that is 53 years in China [19]. To identify key driver variants specific to mucosal melanomas patients in China, we performed whole-exome sequencing of six pairs of tumor tissue and pericarcinomatous tissue derived from individuals affected with mucosal melanomas, including three anorectal melanomas, two vaginal melanomas and one esophageal melanoma.

We finally filtrated 21,733 CNVs and 2372 SNPs in six patients consistent with previous report that mucosal melanoma carries a large

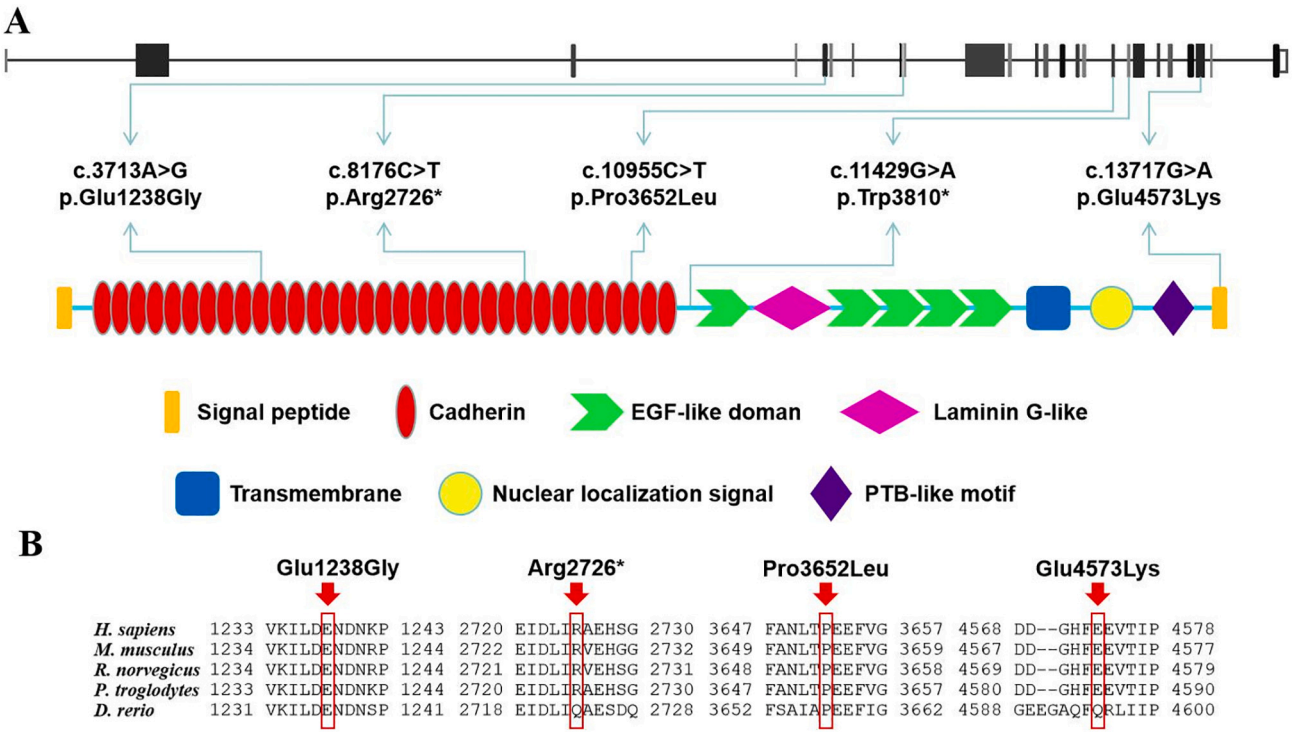

Fig. 4. Mutations identified in *FAT1* gene. (A) The genome and protein structures of *FAT1* are presented. Schematic representation of the relative linear location of all five *FAT1* mutations identified in this study in context of the genome structure (upper) and protein structure (below) Domains of the *FAT1* protein are indicated. (B) Evolutionary conservation of the G1238, A2726, P3652 and G4573 residues in the *FAT1* protein of five species.

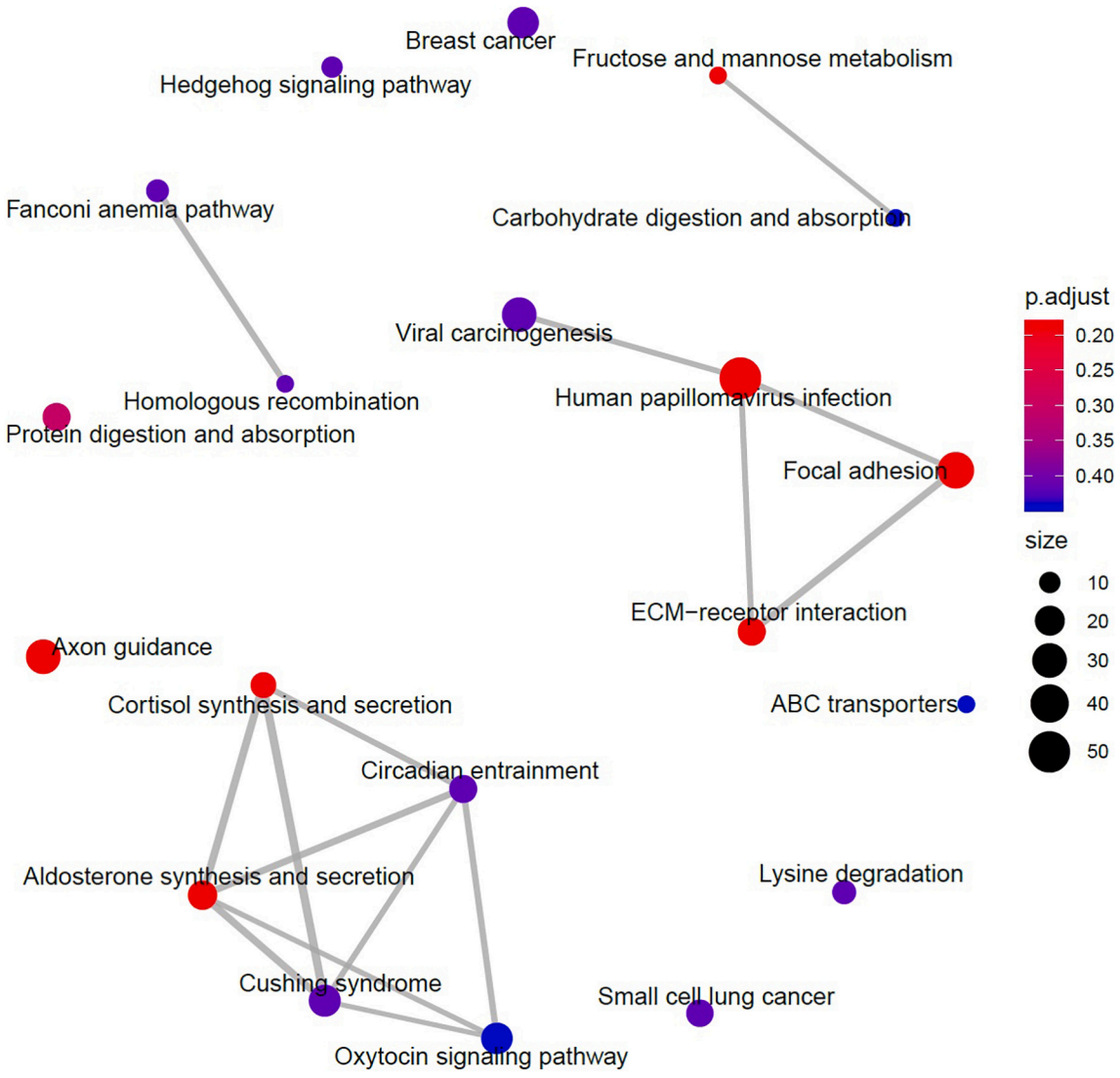

Fig. 5. Signaling pathways enriched with SNP genes.

amount of CNVs and low SNPs burden [19]. Nine SNPs were present in both patients: *NUDT5, ZBTB18, NEURL4, ZNF430, RBM44, GAK, PCDHA13, STK38* and *UBR5*, though the relationship between the above genes and melanoma remains unknown. Therefore, further work is required to investigate the role of these genes in mucosal melanoma.

All patients carried several CNVs in genes associated with tumorigenesis and metastasis, including *PI3K/PIK3* family, *KRAS, APC, BRCA1, LOX, VEGF* family, *RHOC, CSF1, MEDD9, MMP1, IL-11,* and *EDN11*. *PI3K/PIK3* family genes were the most significantly one presented in all tumors. All together, these results indicated that multiple molecular mechanisms involved in mucosal melanoma.

FAT1 is a candidate tumor suppressor gene on chromosome 14 identified by linkage studies [20]. In line with previous studies, recurrent somatic mutation of *FAT1* is associated with a variety of tumors, including colorectal cancer (7.7%) [21,22]. *FAT1* is broadly expressed in colorectal cancer cells and is expected to be as a new potential target for the treatment of colon cancer [23]. In this study, *FAT1* mutations were observed in three anorectal melanoma patients (100%), including two stop-gain variants (66.7%). Most of them (4/5) are located in highly conserved regions, which may affect protein function. Therefore, we speculated that *FAT1* gene mutations may play a pivotal role in anorectal melanoma and provide the therapeutic target, which requires and deserves further study.

It is generally known that HPV infection is very closely related to cervical cancer, as well as vaginal cancer and colorectal cancer risk [17,18]. Since the incidence of mucosal melanoma in women is much higher than that in men, and the incidence of female genital tract melanoma (18.0%) is only second to that of head and neck melanoma (55.4%) and anorectal melanoma (23.8%) [1,2]. In consequence, we conjectured that HPV infection may be associated with mucosal melanoma.

We have described mutation analysis of six China patients with mucosal melanoma. The study has discovered some gene mutations providing important clues for further research on the pathogenesis of mucosal melanoma. The association between HPV infection and mucosal melanoma needs to be further verified with more sample.

Declaration of competing interest

The authors declare no competing financial interests.

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