Characterization of Single Nucleotide Variants of OPN3 Gene in Melanocytic Nevi and Melanoma

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In this study, we examined single nucleotide variants (SNVs) of the OPN3 gene in malignant melanoma and melanocytic nevi. A total of 20 variants of SNVs were detected. Of these variants, five nonsynonymous mutations of OPN3 were identified, including c.T152C, c.T401C, c.G547A, c.G768A, and c.G992A. Three prediction tools, MutationTaster2, Polymorphism Phenotyping version 2, and PROVEAN (Protein Variation Effect Analyzer), which predict possible impact of an amino acid substitution, suggested that the mutations could be deleterious. Nine SNVs occurred in 3’ untranslated regions, whereas two were observed in 5’ untranslated regions. In all cases, four intronic variants were identified. In addition, we identified nine 3’ untranslated region SNVs in OPN3; one of them (OPN3[NM_014322.c.*83C>T]) is predicted to disrupt a conserved microRNA (has-miR-376c-3p) target site, located in position 86–93 of OPN3 3’ untranslated region. Our findings suggest that there is a strong possibility that OPN3 SNVs play a role in the pathogenesis of melanocytic tumors via prediction of functional phenotype.

INTRODUCTION
First described by Blackshaw and Snyder (1999), OPN3 was identified as a mammalian extraocular opsin. It is highly conserved throughout vertebrates as an ancestral type of opsin (Fischer et al., 2013). On the genomic level, OPN3 is composed of four exons on human chromosome 1q43, encompassing two flanking genes, CHML and KMO. CHML, a single exon gene, resides in intron 1 of OPN3, whereas KMO and OPN3 overlap with their 3’-untranslated regions (UTRs), which are transcribed in opposite directions (Halford et al., 2001). Recently, we and others have found that OPN3 is highly expressed in human epidermal melanocytes compared with other opsins (Ozdelslik et al., 2019; Regazzetti et al., 2018; Wang et al., 2020). These studies further demonstrated that OPN3 mediates nonvisual functions such as melanogenesis and apoptosis in melanocytes. In addition, previous studies of the relationship between OPN3 and human diseases showed that OPN3 is identified as an asthma susceptibility gene (White et al., 2008). Miyanaga et al. (2020) recently found that OPN3 is upregulated in pulmonary carcinoid tumors that developed postsurgical metastasis. With The Cancer Genome Atlas data analysis, we also observed that OPN3 expression is upregulated in human melanoma compared with normal skin (NS) (Figure 1). Although its role in benign and malignant melanocytic lesions remains uncharacterized, based on these studies, it is likely that OPN3 modulates proliferation, pigmentation, and apoptosis of malignant melanocytes in tumor initiation and progression.

RESULTS
Here, we performed single nucleotide variant (SNV) analysis of OPN3 in 68 malignant melanoma (MM), 166 melanocytic nevi (MNs), and 42 NS tissues from a Chinese population (Table 1). This study of formalin-fixed, paraffin-embedded tissues was approved by the ethics committee of Affiliated Hospital of Guizhou Medical University (Guiyang, China). Five nonsynonymous mutations were identified in the melanocytic lesions, including c.T152C, c.T401C, c.G547A, c.G768A, and c.G992A (Table 2). The c.T152C mutation was detected in one case of acral lentiginous melanoma. For the c.T401C mutation, MM mutations were much more common than MN and NS mutations, which were not associated with sex, age, localization, or histological subtype of samples. In addition, no significant differences were seen for SNVs in OPN3 in MM, MN, and NS (Table 2). In the c.G768A mutation of MM and MN, all five MM lesions displayed acral lentiginous melanoma. Of them, three MNs were found to be junctional nevi, and two were compound nevi. Overall, 3 of 68 MMNs (4.41%) and 14 of 166 MNs (8.43%) revealed simultaneous OPN3 and BRAF V600E mutations. In 2 of 40 acral lentiginous melanoma cases (5%) with hotspot mutant BRAF V600E, the c.G768A mutation in OPN3 was also identified. In MM, the remaining one case with two mutations was metastatic melanoma (Table 3).

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Abbreviations: miRNA, microRNA; MM, malignant melanoma; MN, melanocytic nevus; NS, normal skin; SNV, single nucleotide variant; UTR, untranslated region

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We predicted the functional consequences of amino acid substitutions via three prediction tools (Polymorphism Phenotyping version 2, PROVEAN [Protein Variation Effect Analyzer], MutationTaster2) to evaluate the pathogenic potential of these nonsynonymous mutations (Table 4) (Schwarz et al., 2014). The prediction results of the c.G768A variant by the three tools was the most consistent, suggesting that it has deleterious effects. The results of two prediction tools showed that the c.T152C variant was also probably damaging. Other mutations were predicted to be deleterious by one of the three prediction tools. Moreover, mutant protein three-dimensional models were generated via the remote homology detection method of Phyre2 (Kelley et al., 2015) (Figure 2).

In addition, nine SNVs in the 3′-UTR and two SNVs in the 5′-UTR of OPN3 were found in all MN and MM samples, including four novel SNVs of 3′-UTR: KMO(c.*1932G>C), OPN3(c.*343C>G); KMO(c.*1955_*1956insA), OPN3(c.*319_*320insT); KMO(c.*2146C>T), OPN3(c.*129G>A); and KMO(c.*2192G>A), OPN3(c.*83C>T) (Table 2). Two SNVs (OPN3[c.-80A>G] and OPN3[c.-102T>C]) in the 5′-UTR occurred in the same cases. Only two SNVs in the 3′-UTR (OPN3[c.*319_*320insT], rs3765809) were detected in the NS control group. The homozygote (TT) variant of rs3765809 had significant differences between the MN and NS groups (P < 0.05). Furthermore, we predicted the effects of SNVs in the 3′-UTR via miRDB and TargetscanHuman7.2 (Peng et al., 2020), which predicted microRNA (miRNA) targets in mammals. In 3′-UTR variants, as biological targets of miRNAs, seven miRNAs (has-miR-1272, has-miR-1267, has-miR-376c-3p, has-miR-6507-3p, has-miR-10399-5p, has-miR-137-5p, and has-miR-376c-3p) were predicted, one (has-miR-376c-3p) of them with a conserved 8mer (monomorphic unit) site. This 3′-UTR SNV was detected in one intradermal nevus. In addition, four intronic SNVs were detected in all samples, including rs45572340, OPN3[241767624_53 T>G, rs140858921, and rs632966 (Table 2).

![Figure 1. OPN3 expression profile between SKCM (n = 461) and paired normal tissues (n = 558) based on TCGA data analysis. The height of each bar represents the median expression. The expression of OPN3 in SKCM is higher than that in normal tissues. SKCM, skin cutaneous melanoma; TCGA, The Cancer Genome Atlas.](image)

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**Table 1. Participants’ Characteristics of the Study from Chinese Patients with MN, MM, and NS**

| Subject                  | Total (n) | BRAFT600E (n) | Total (n) | BRAFT600E (n) | Total (n) |
|--------------------------|-----------|--------------|-----------|--------------|-----------|
| Number                   | 166       | 81           | 68        | 7            | 42        |
| Sex                      |           |              |           |              |           |
| Male                     | 67        | 27           | 29        | 4            | 18        |
| Female                   | 99        | 54           | 39        | 3            | 26        |
| Age, median (IQR)        | 30.0 (18.0–47.0) | 33.00 (23.00–47.00) | 65.0 (53.0–72.0) | 42.0 (39.0–57.0) | 44.0 (35.0–60.0) |
| Anatomical site          |           |              |           |              |           |
| Head                     | 16        | 10           | 1         | 0            | 3         |
| Face                     | 78        | 54           | 4         | 0            | 13        |
| Neck                     | 4         | 3            | 0         | 0            | 4         |
| Trunk                    | 15        | 9            | 3         | 2            | 11        |
| Limbs                    | 24        | 1            | 6         | 2            | 6         |
| Hand                     | 9         | 0            | 10        | 1            | 3         |
| Foot                     | 20        | 4            | 39        | 2            | 2         |
| Brain                    | –         | –            | 2         | 0            | z         |
| Lymph node               | –         | –            | 3         | 0            | z         |
| Subtype                  |           |              |           |              |           |
| Junctional nevus         | 28        | 3            | –         | –            | –         |
| Compound nevus           | 52        | 29           | –         | –            | –         |
| Intradermal nevus        | 53        | 49           | –         | –            | –         |
| Blue nevus               | 33        | 0            | –         | –            | –         |
| Superficial spreading melanoma | –     | –            | 10        | 1            | –         |
| Nodular melanoma         | –         | –            | 7         | 2            | –         |
| Lentigo maligna melanoma | –         | –            | 4         | 0            | –         |
| Acral lentiginous melanoma | –     | –            | 40        | 2            | –         |

Abbreviations: IQR, interquartile range; MM, malignant melanoma; MN, melanocytic nevus; NS, normal skin. BRAFT600E status was previously assessed by direct sequencing (Sanger) in all cases.
| Exon | SNP | MM (n = 68), % | MN (n = 166), % | NS (n = 42), % | P-Value |
|------|------|----------------|----------------|---------------|----------|
| Exon 1 c.T152C A>G | AG | 1.47 (1/68) | 0 | 0 | 0.2106 |
| | AA | 97.06 (66/68) | 100 (166/166) | 100 (42/42) | |
| | NA | 1.47 (1/68) | 0 | 0 | |
| Exon 2 c.T401C A>G | AG | 4.41 (3/68) | 1.20 (2/166) | 2.38 (1/42) | 0.1122 |
| | AA | 92.65 (63/68) | 98.80 (164/166) | 97.6 (41/42) | |
| | GG | 1.47 (1/68) | 0 | 0 | |
| | NA | 1.47 (1/68) | 0 | 0 | |
| Exon 2 c.G547A C>T | CT | 10.29 (7/68) | 9.04 (15/166) | 14.28 (6/42) | 0.6810 |
| | CC | 93.93 (60/66) | 90.36 (150/166) | 85.71 (36/42) | |
| | TT | 0 | 0.60 (1/166) | 0 | |
| | NA | 1.47 (1/68) | 0 | 0 | |
| Exon 3 c.G768A C>T | CT | 7.35 (5/68) | 3.01 (5/166) | 9.52 (4/42) | 0.1440 |
| | CC | 92.66 (63/68) | 95.78 (159/166) | 90.48 (38/42) | |
| | NA | 0 | 1.20 (2/166) | 0 | |
| Exon 4 c.G992A C>T | CT | 0 | 1.81 (3/166) | 2.38 (1/42) | 0.4948 |
| | CC | 98.53 (67/68) | 97.60 (162/166) | 97.6 (41/42) | |
| | NA | 1.47 (1/68) | 0.60 (1/166) | 0 | |
| 3'-UTR | | | | | |
| KMO(c.*1253G>A), OPN3(c.*1022C>T) rs371271799 | GA | 1.47 (1/68) | 0 | 0 | 0.2663 |
| | GG | 66.18 (45/68) | 47.59 (79/166) | 100 (42/42) | |
| | NA | 32.35 (22/68) | 52.41 (87/166) | 0 | |
| KMO(c.*1498A>G), OPN3(c.*777T>C) rs144936606 | AG | 1.47 (1/68) | 0.60 (1/166) | 0 | 0.6418 |
| | AA | 97.06 (66/68) | 99.40 (165/166) | 100 (42/42) | |
| | NA | 1.47 (1/68) | 0 | 0 | |
| KMO(c.*1932G>C), OPN3(c.*343C>G) | GC | 1.47 (1/68) | 0 | 0 | 0.2086 |
| | GG | 94.12 (64/68) | 96.99 (161/166) | 100 (42/42) | |
| | NA | 4.41 (3/68) | 3.01 (5/166) | 0 | |
| KMO(c.*1955_*1956insA),OPN3(c.*319_*320insT) | A/ins_A | 38.24 (26/68) | 37.35 (62/166) | 28.57 (12/42) | 0.5028 |
| | AA | 60.29 (41/68) | 62.05 (103/166) | 71.43 (30/42) | |
| | NA | 1.47 (1/68) | 0.60 (1/166) | 0 | |
| KMO(c.*2094G>T), OPN3(c.*181C>A) rs3765809 | GT | 13.24 (9/68) | 15.06 (25/166) | 19.05 (8/42) | 0.7272 |
| | TT | 0 | 0 | 4.76 (2/42) | 0.0404 |
| | GG | 85.29 (58/68) | 84.34 (140/166) | 76.19 (32/42) | |
| | NA | 1.47 (1/68) | 0.60 (1/166) | 0 | |
| KMO(c.*2146C>T), OPN3(c.*129G>A) | CT | 0 | 1.20 (2/166) | 0 | 0.5140 |
| | CC | 98.53 (67/68) | 98.19 (163/166) | 100 (42/42) | |
| | NA | 1.47 (1/68) | 0.60 (1/166) | 0 | |
| KMO(c.*2192G>A), OPN3(c.*83C>T) | GA | 0 | 0.60 (1/166) | 0 | 0.7171 |
| | GG | 100 (68/68) | 99.40 (165/166) | 100 (42/42) | |
| | NA | 0 | 0 | 0 | |
| KMO(c.*2258G>A), OPN3(c.*17C>T) rs199779503 | GA | 1.47 (1/68) | 0 | 0 | 0.2122 |
| | GG | 97.06 (66/68) | 99.40 (165/166) | 100 (42/42) | |
| | NA | 1.47 (1/68) | 0.60 (1/166) | 0 | |

(continued)
DISCUSSION
In this study, we comprehensively identified OPN3 genetic variants in patients with MM or MN. Five nonsynonymous SNVs of OPN3 were detected in our results, and predictions of functional phenotypes provided evidence that these SNVs altered OPN3 conservation, especially for c.G768A, suggesting that this missense variant is deleterious. Crystal structure of OPN3 has been not resolved; we constructed the

| Table 2. Continued |
|---------------------|
| **OPN3 SNVs** | **MM (n = 68), %** | **MN (n = 166), %** | **NS (n = 42), %** | **P-Value** |
| KMO(c.*2267C>G), OPN3(c.*8G>C) rs201495076 | | | | |
| GG | 1.47 (1/68) | 1.20 (2/166) | 0 | 0.7469 |
| CC | 97.06 (66/68) | 98.19 (163/166) | 100 (42/42) | 0 |
| NA | 1.47 (1/68) | 0.60 (1/166) | 0 | 0 |
| 5'UTR | | | | |
| OPN3(c.-80A>G) rs7513575 | | | | |
| TC | 1.47 (1/68) | 0.60 (1/166) | 0 | 0.2244 |
| CC | 1.47 (1/68) | 0 | 100 (42/42) | 0.2244 |
| NA | 97.06 (66/68) | 99.40 (165/166) | 0 | 0.2244 |
| OPN3(c.-102T>C) rs7513451 | | | | |
| AG | 1.47 (1/68) | 0.60 (1/166) | 0 | 0.2244 |
| GG | 1.47 (1/68) | 0 | 100 (42/42) | 0.2244 |
| NA | 97.06 (66/68) | 99.40 (165/166) | 0 | 0.2244 |
| Introns | | | | |
| rs45572340 T>C | | | | |
| TC | 8.82 (6/68) | 9.04 (15/166) | 11.90 (5/42) | 0.8494 |
| CC | 2.94 (2/68) | 0.60 (1/166) | 0 | 0.2244 |
| TT | 85.29 (58/68) | 89.76 (149/166) | 88.10 (37/42) | 0.2244 |
| NA | 2.94 (2/68) | 0.60 (1/166) | 0 | 0.2244 |
| OPN3 241767624_53 T>G | | | | |
| TG | 1.47 (1/68) | 0 | 0 | 0.2051 |
| TT | 92.65 (63/68) | 96.39 (160/166) | 0 | 0.2051 |
| NA | 5.8 (4/68) | 3.61 (6/166) | 100 (42/42) | 0.2051 |
| rs140858921 A>G | | | | |
| AG | 4.41 (3/68) | 4.22 (7/166) | 0 | 0.3931 |
| GG | 0 | 0.60 (1/166) | 0 | 0.7185 |
| AA | 89.71 (61/68) | 91.57 (152/166) | 100 (42/42) | 0.7185 |
| NA | 5.8 (4/68) | 3.61 (6/166) | 0 | 0.7185 |
| rs632966 G>A | | | | |
| GA | 30.88 (21/68) | 33.73 (56/166) | 21.43 (9/42) | 0.3058 |
| AA | 64.71 (44/68) | 62.05 (103/166) | 76.19 (32/42) | 0.3058 |
| GG | 4.41 (3/68) | 4.22 (7/166) | 2.38 (1/42) | 0.3058 |
| NA | 0 | 0 | 0 | 0.3058 |

Abbreviations: MM, malignant melanoma; MN, melanocytic nevus; NA, not applicable; NS, normal skin; SNV, single nucleotide variant; UTR, untranslated region.

1Two groups were compared by Fisher's exact test.

DISCUSSION
In this study, we comprehensively identified OPN3 genetic variants in patients with MM or MN. Five nonsynonymous SNVs of OPN3 were detected in our results, and predictions of functional phenotypes provided evidence that these SNVs altered OPN3 conservation, especially for c.G768A, suggesting that this missense variant is deleterious. Crystal structure of OPN3 has been not resolved; we constructed the

| Table 3. **OPN3 Nonsynonymous SNVs in MM, MN, and NS** |
|---------------------|
| **OPN3 Mutation** | **Base Substitution** | **Amino Acid Substitution** | **MM (n = 68), %** | **MN (n = 166), %** | **NS (n = 42), %** | **Concomitant BRAF V600E Mutation** |
| Exon 1 | c.T152C A>G | Ile>Thr | 1 | 0 | 0 | — |
| Exon 2 | c.T401C A>G | Val>Ala | 4 | 2 | 1 | 0 | 2 |
| Exon 2 | c.G547A C>T | Val>Ile | 7 | 15 | 6 | 1 | 9 |
| Exon 3 | c.G768A C>T | Met>Ile | 5 | 5 | 4 | 2 | 1 |
| Exon 2 | c.G992A C>T | Cys>Tyr | 0 | 3 | 1 | — | 2 |

Abbreviations: Ala, alanine; Cys, cysteine; Ile, isoleucine; Met, methionine; MM, malignant melanoma; MN, melanocytic nevus; NS, normal skin; SNV, single nucleotide variant; Thr, threonine; Val, valine.
mutant three-dimensional structures of OPN3 by homology modeling based on OPN2. Although we could not demonstrate a significant statistical difference between MM and MN in OPN3 SNVs, which may be due to the small sample size and therefore lack of statistical power, some implications can be suggested. OPN3 is a cell surface receptor of the G-protein coupled receptor family that plays a vital role in the posttranscriptional regulation of proliferation, migration, and survival (Bar-Shavit et al., 2016). In addition, OPN3 expression influences melanocyte apoptosis (Wang et al., 2020). Therefore, c.G768A or other nonsynonymous SNVs could alter OPN3 protein structure and function and have an impact on melanocytic proliferation and apoptosis in MM and MN. BRAF mutations are low frequency in nature (about 15%) (Nakamura and Fujisawa, 2018). In all 40 samples of acral lentiginous melanoma, only two cases showed the BRAF V600E mutation. Both of them also contained the c.G768A variant in OPN3. This suggested that the products of multiple gene mutations may affect melanocytic proliferation and tumor formation. In addition, the 3′-UTR SNV (OPN3 NM_014322.c.*83C>T) is predicted to disrupt a conserved miRNA (has-miR-376c-3p) target site, located in position 86–93 of OPN3 3′-UTR. It is possible that this UTR SNV might influence RNA stability or posttranscriptional regulation of OPN3 (Mayr, 2017), which may restrain melanocytic proliferation and malignant transformation by downregulation of OPN3 expression.

In this study, because we focused on overall OPN3 SNVs, additional investigation will be necessary to further elucidate whether or not these variants affect the formation and growth of melanocytic lesions.

### MATERIALS AND METHODS

#### Study population and data collection

All subjects with MMs and MNs were collected at Affiliated Hospital of Guizhou Medical University from January 2015 to December 2019 (Table 1). The control skin samples were obtained from normal adjacent nevi tissues. H&E-stained sections were reviewed by an experienced pathologist, and cases fulfilling criteria for the appropriate diagnoses (MM and MN) were selected for study. The study was approved by the Ethics Committees of our institution (Affiliated Hospital of Guizhou Medical University) and was performed according to the Declaration of Helsinki.

#### DNA extraction

DNA extraction from formalin-fixed, paraffin-embedded tissue was performed using an FFPE DNA Extraction Kit (AmoyDx, Xiamen, China), following the manufacturer’s instructions. We measured the concentration of DNA using a Qubit 2.0 (Thermo Fisher Scientific, Waltham, MA) to ensure that adequate amounts of high-quality genomic DNA had been extracted.

#### Multiplex PCR and sequencing as described by Wei et al. (2020)

Library preparation was performed by two-step PCR. The first round PCR reaction was set up as follows: DNA (10 ng/µl) 2 µl; ampiclon PCR forward primer mix (10 µM) 1 µl; ampiclon PCR reverse primer mix (10 µM) 1 µl; and 2 × PCR Ready Mix 15 µl (total 25 µl) (Kapa HiFi Ready Mix). The plate was sealed and PCR performed in a thermal instrument (T100TM, Bio-Rad, Hercules, CA) using the following program: one cycle of denaturing at 98 °C for 5 minutes, eight cycles of denaturing at 98 °C for 30 seconds, annealing at 50 °C for 30 seconds, elongation at 72 °C for 30 seconds, 25 cycles of denaturing at 98 °C for 30 seconds, annealing at 66 °C for 30 seconds, elongation at 72 °C for 30 seconds, and a final extension at 72 °C for 5 minutes with a final hold at 4 °C. The PCR products were checked using electrophoresis in 1 % (w/v) agarose gels in Tris, boric acid, and EDTA buffer stained with ethidium bromide and visualized under UV light. Then we used AMPure XP beads to purify the amplicon product. After that, the second round of PCR was performed. PCR reaction was set up as follows: DNA (10 ng/µl) 2 µl; universal P7 primer with barcode (10 µM) 1 µl; universal P5 primer (10 µM) 1 µl; and ×2 PCR Ready Mix 15 µl (total 30 µl) (Kapa HiFi Ready Mix). The plate was sealed, and PCR performed in a thermal instrument (T100TM, Bio-Rad) using the following program: one cycle of denaturing at 98 °C for 3 minutes, five cycles of denaturing at 94 °C for 30 seconds, annealing at 55 °C for 20 seconds, elongation at 72 °C for 30 seconds, and a final extension at 72 °C for 5 minutes. Then we used AMPure XP beads to purify the amplicon product. The libraries were then quantified and pooled. Paired-end sequencing of the library was performed on the HiSeq XTen sequencers (illumina, San Diego, CA).

### Table 4. Prediction Evaluation for OPN3 Nonsynonymous SNVs with Three Kinds of Bioinformatics Software (PolyPhen-2, PROVEAN, MutationTaster2)

| OPN3 Mutation | Base Substitution | NCBI dbSNP Reference ID | PolyPhen-2 | PROVEAN | MutationTaster2 |
|---------------|-------------------|-------------------------|------------|---------|-----------------|
| Exon 1        | c.T152C A>G       | rs201734451             | 0.180      | 0.025   | Benign          |
|               |                   |                         |            | −7.63   | Deleterious     |
| Exon 2        | c.T401C A>G       | rs117720055             | 0.116      | 0.057   | Benign          |
|               |                   |                         |            | −9.15   | Deleterious     |
| Exon 2        | c.G547A C>T       | rs2273712               | 0.001      | 0.002   | Benign          |
|               |                   |                         |            | −8.83   | Deleterious     |
| Exon 3        | c.G768A C>T       | rs78202695              | 0.985      | 0.977   | Probably        |
|               |                   |                         |            | −4.76   | Deleterious     |
| Exon 4        | c.G992A C>T       | rs180909883             | 0.146      | 0.024   | Benign          |
|               |                   |                         |            | −2.10   | Neutral         |

Abbreviations: dbSNP, SNP database; Div, division; Hum, human; ID, identification; NCBI, National Center for Biotechnology Information; PolyPhen-2, Polymorphism Phenotyping version 2; PROVEAN, Protein Variation Effect Analyzer; SNV, single nucleotide variant; Var, variant.
Figure 2. Mutant 3D models for all five nsSNVs in *OPN3* generated by Phyre2 server. 3D, three-dimensional; Ala, alanine; Cys, cysteine; Ile, isoleucine; Met, methionine; nsSNV, nonsynonymous single nucleotide variant; Thr, threonine; Tyr, tyrosine; Val, valine.
Data quality control and SNV calling as described by Wei et al. (2020)

Raw reads were filtered according to two steps: (i) removing adaptor sequence if reads contain by cutadapt (version 1.2.1) and (ii) removing low quality bases from reads 3′–5′ (Q < 20) by PRINSEQ-lite (version 0.20.3). The remaining clean data were mapped to the reference genome by BWA (version 0.7.13-r1126) with default parameters. SAMtools (version: 0.1.18) was used to calculate each genotype of target site. ANNOVAR (16 April 2018) was used to detect genetic variants.

Prediction of amino acid substitution

We evaluated the functional consequences of amino acid substitutions via three prediction tools, Polymorphism Phenotyping version 2 (http://genetics.bwh.harvard.edu/pph2/), PROVEAN (http://provean.jcvi.org/index.php), and MutationTaster2 (http://www.mutationtaster.org/), and compared predictions of the three tools on OPN3 nonsynonymous SNVs. The web versions of the three prediction tools are used to predict the pathogenic potential of DNA sequence alterations. In addition, we built three-dimensional models of OPN3 to analyze the effect of SNVs on the protein sequence using SWISS-MODEL (https://swissmodel.expasy.org/). We also predicted the effects of SNVs in the 3′-UTR via miRDB (http://mirdb.org/) and TargetscanHUMAN7.2 (http://www.targetscan.org/vert_72/), which predicted miRNA targets in mammals. Details about the methods and further statistics followed their websites and previous reports.

Statistical analyses

All data were entered into GraphPad Prism (version 8.0) for statistical analysis. Categorical data were analyzed using Fisher’s exact test. A two-tailed P < 0.05 was considered statistically significant.

Data availability statement

No large datasets were generated or analyzed during this study. Minimal datasets necessary to interpret and/or replicate data in this paper are available on request to the corresponding author.

Ethics Statement

This study of formalin-fixed, paraffin-embedded tissues was approved by the ethics committee of Affiliated Hospital of Guizhou Medical University (Guiyang, China). Under Chinese law, written consent from the patients was not required because the material used had been collected for diagnostic and therapeutic purposes in the archives of the Institute for Pathology, Affiliated Hospital of Guizhou Medical University (Guiyang, China) and used for this study in pseudonymized form. All authors contributed to results interpretations.

CONFLICT OF INTEREST

The authors state no conflict of interest.

REFERENCES

Bar-Shavit R, Maoz M, Kancharla A, Nag JK, Agranovich D, Grisaru-Granovsky S, et al. G protein-coupled receptors in cancer. Int J Mol Sci 2016;17:1320.

Blackshaw S, Snyder SH. Encephalopsin: a novel mammalian extraretinal opsin discretely localized in the brain. J Neurosci 1999;19:3681–90.

Fischer RM, Fontinha BM, Kirchmaier S, Steger J, Bloch S, Inoue D, et al. Co-expression of VAL- and TMT-opsins uncovers ancient photosensory interneurons and motoneurons in the vertebrate brain. PLoS Biol 2013;11:e1001585.

Halford S, Bellingham J, Oalca L, Fox M, Johnson S, Foster RG, et al. Assignment of panopsin (OPN3) to human chromosome band 1p43 by in situ hybridization and somatic cell hybrids. Cytogeten Cell Genet 2001;95:234–5.

Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE. The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc 2015;10:845–58.

Mayr C. Regulation by 3′-untranslated regions. Annu Rev Genet 2017;51:171–94.

Miyanaga A, Masuda M, Motoi T, Tsuta K, Nakamura Y, Nishijima N, et al. Whole-exome and RNA sequencing of pulmonary carcinoid reveals chromosomal rearrangements associated with recurrence. Lung Cancer 2020;145:85–94.

Nakamura Y, Fujisawa Y. Diagnosis and management of acral lentiginous melanoma. Curr Treat Options Oncol 2018;19:42.

Ozdeslik RN, Olinski LE, Trieu MM, Oprian DD, Oancea E. Human nonvisual opsin 3 regulates pigmentation of epidermal melanocytes through functional interaction with melanocortin 1 receptor. Proc Natl Acad Sci USA 2019;116:11508–17.

Peng J, Liu F, Zheng H, Wu Q, Liu S. Long noncoding RNA ZFAS1 promotes tumorigenesis and metastasis in nasopharyngeal carcinoma by sponging miR-892b to up-regulate LPAR1 expression. J Cell Mol Med 2020;24:1437–50.

Regazzetti C, Somrani L, Debayle D, Berendt F, Tulic MK, De Donatis GM, et al. Melanocytes sense blue light and regulate pigmentation through Opsin-3. J Invest Dermatol 2018;138:171–8.

Schwarz JM, Cooper DN, Schuelke M, Seelow D, Oancea E. Human nonvisual opsin 3 regulates pigmentary epithelial melanocytes through functional interactions with melanocortin 1 receptor. Proc Natl Acad Sci USA 2019;116:11508–17.

Wang Y, Lan Y, Lu H. Opsin3 downregulation induces apoptosis of human epidermal melanocytes via mitochondrial pathway. Photochem Photobiol 2016;93:83–93.

Wei BL, Yin RX, Liu CX, Deng GX, Guan YZ, Zheng PF. The MC4R SNPs, their haplotypes and gene–environment interactions on the risk of obesity. Mol Med 2020;26:77.

White JH, Chiano M, Wigglesworth M, Geske R, Riley J, White N, et al. Identification of a novel asthma susceptibility gene on chromosome 1qter and its functional evaluation. Hum Mol Genet 2008;17:1890–903.