

Research Paper

Potential Mutations in Uveal Melanoma Identified Using Targeted Next-Generation Sequencing

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Received: 2018.04.30; Accepted: 2018.11.18; Published: 2019.01.01

Abstract

Background/objective: Uveal melanoma (UM) is the most common intraocular malignancy and has a high tendency to metastasize to the liver. Although primary tumours can be successfully treated, there is currently no effective treatment for metastatic UM. To gain insight into the genetics of UM, we performed the targeted next-generation sequencing (NGS) of UM samples from a non-Caucasian population.

Methods: This study included tumour samples and blood samples from 107 UM patients at Peking University Cancer Hospital & Institute. Clinical data were collected. DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) specimens. Using the HaloPlex Target Enrichment System (Agilent Technologies), NGS was performed to investigate mutations in a 35-gene panel composed of cancer-related genes.

Results: Recurrent coding mutations were found in the known UM drivers *GNAQ* and *GNA11*. *FOXO1*, *PIK3R1* and *HIF1A* were also found to harbour somatic mutations in more than 20% of patients, a result that may indicate previously undescribed associations between these genes and UM pathogenesis. Patients with *HIF1A* and *FOXO1* mutations exhibited worse overall survival (OS). In multivariate analysis, *FOXO1* mutation was an independent prognostic factor for OS ($P < 0.05$) that was associated with an increase in the risk ratio by a factor of 1.35. Notably, we found that *HIF1A* and *FOXO1* mutations were associated with metastatic transformation of UM ($P < 0.05$ and $P < 0.001$, respectively).

Conclusion: Our findings from analyses of targeted NGS data shed new light on the molecular genetics of UM and facilitate the exploration of mutations associated with metastatic potential.

Key words: uveal melanoma, metastasis, *FOXO1*, *HIF1A*.

Introduction

Uveal melanoma (UM) is the most common intraocular malignancy and can arise at three sites: the iris, the choroid, and the ciliary body. [1]UM tumours are highly aggressive and exhibit a high tendency to metastasize to the liver. [2]Due to a lack of effective systemic therapy, the median survival time of patients with metastatic UM is 12.5 months. [3]

Dramatic breakthroughs in the treatment of cutaneous melanoma (CM) have been achieved over the past decade. Targeted therapy such as the use of BRAF and MEK mutation inhibitors can improve overall survival (OS) for CM patients who harbour specific mutations. [4] However, the development of

targeted therapy for UM is hampered by a lack of understanding of genetic changes associated with UM. Therefore, investigating potential high-frequency mutations in UM is crucial and could provide benefits for direct clinical applications.

Based on genomic, transcriptomic, and proteomic high-throughput screening of UM samples, several driver genes for UM have been detected. The molecular alterations associated with UM differ from those associated with CM. Driver mutations related to the pathogenesis of UM, such as mutations in *GNAQ*, *GNA11*, and *BAP1*, have also been revealed; these mutations are distinct from those related to CM. [5-7]

GNAQ or *GNA11* mutations lead to constitutive activation of the mitogen-activated protein kinase (MAPK) pathway and represent early events in tumorigenesis in UM. [8] *BAP1* mutations are strongly associated with the progression and metastasis of UM. [7] Most research on the molecular landscape of UM has focused on Caucasians, and little data are available for other races. Asian CM patients significantly vary from Caucasian CM patients with respect to genetic profiles and clinical characteristics; [9-11] however, the genetic mutations associated with UM in non-Caucasian populations remain unclear.

To gain insight into the genetics of UM, we performed the targeted NGS of UM samples from a non-Caucasian population. We evaluated 35 mutated genes in the MAPK and PI3K/AKT pathways. We then analysed correlations between mutation status and clinicopathological features, with a particular focus on metastatic transformation. Our findings can facilitate the selection of more appropriate therapeutic treatments for UM in clinical practice, especially for patients with a high risk of metastasis.

Patients and Methods

Patient samples

Matched Formalin-fixed, paraffin-embedded (FFPE) tumor tissues and blood samples were obtained from 107 Chinese UM patients who visited Peking University Cancer Hospital. All of these samples were collected between June 2009 and December 2017. Clinical data, including gender, age, tumour side, tumour thickness, metastasis status, and survival (with follow-up continuing until September 2018, loss to follow-up or patient death), were collected. Diagnoses of UM were confirmed histopathologically for all patients. This study was approved by the medical ethics committee of Peking University Cancer Hospital & Institute and was conducted according to the Declaration of Helsinki Principles.

DNA preparation and NGS

Genomic DNA was extracted from FFPE sections using a QIAamp DNA FFPE Tissue Kit (Qiagen) and from blood lymphocytes using a QIAamp DNA Mini kit (Qiagen). Human Genomic DNA (G3041, Promega) as normal control. Using the HaloPlex Target Enrichment System (Agilent Technologies), NGS was performed to investigate mutations in a panel of 35 cancer-related genes. The panel was designed contain common genes relevant to melanoma pathogenesis, include MAPK, PI3K/AKT and cell cycle pathway. In accordance with the manufacturer's instructions, we utilized a custom-designed HaloPlex Target Enrichment Kit

(Agilent) to capture target regions. In brief, genomic DNA was digested via eight different restriction reactions. The restricted segments were hybridized to a probe with ends complementary to those of the target fragment. During hybridization, the fragment was cyclized and integrated with the sequencing motif, which consisted of index sequences. The attachment of the cyclized target DNA fragment was captured using streptavidin magnetic beads. Finally, polymerase chain reaction (PCR) was performed to amplify the captured target libraries. Paired-end sequencing (with 100 bp reads) of all samples was performed on a HiSeq2500 instrument (Illumina). Clusters were generated using the TruSeq PE Cluster Kit V3 (Illumina), and the TruSeq SBS Kit V3 (Illumina) was used for sequencing. Image analysis and base calling were performed using Illumina RTA software. Sequence reads were trimmed to remove Illumina adapter sequences and aligned to the human reference genome (version hg19). Variants were invoked using Agilent SureCall software. Variants were further filtered using the dbSNP database and 1000 Genomes Project. The results were filtered by dbSNP database (<https://www.ncbi.nlm.nih.gov/projects/SNP/>) and 1000 Genomes projects (<https://ftp.ncbi.nih.gov/>). Shanghai Biotechnology Corporation (Shanghai, China) completed the targeted enrichment, sequencing, and data analysis for NGS.

Statistical analysis

IBM SPSS statistical software (version 20.0) was used for statistical assessments. Mean values were analysed using *t* tests for normally distributed continuous variables and estimated using the Mann-Whitney *U* test for abnormally distributed continuous variables. OS curves were evaluated using the Kaplan-Meier method. Cox univariate and multivariate analyses were performed to assess associations between prognostic indicators and OS. For all statistical analyses, $P < 0.05$ (for two-tailed tests) was considered to be statistically significant.

Results

Patient characteristics and gene mutation rates

The records of 107 patients with UM were reviewed in this study. Among them, 40 patients were classified as training cohort, 67 patients were classified as validation cohort. Clinicopathological characteristics of these patients are shown in Table 1.

In training cohort, the gene mutation rates of *GNAQ*, *GNA11*, *BRAF*, *NRAS*, *CCND1*, *HIF1A*, *AKT1*, *FOXO1*, *PIK3R1*, *PTEN*, *TSC1*, *TSC2*, *FGFR3*, *KIT* and *MET* were 45% (18/40), 20% (8/40), 10% (4/40), 10%

(4/40), 5% (2/40), 30% (12/40), 10% (4/40), 40% (16/40), 50% (20/40), 10% (4/40), 17.5% (7/40), 15% (6/40), 7.5% (3/40), 5% (2/40) and 5% (2/40), respectively.

Table 1. Baseline Characteristics of Uveal Melanoma Patients.

Characteristics	Training Cohort, n=40		Validation Cohort, n=67	
	N	%	N	%
Gender				
Male	24	60	36	53.7
Female	16	40	31	46.3
Age/year				
≥60	15	37.5	28	41.8
<60	25	62.5	39	58.2
Side				
Left eye	18	45	32	47.8
Right eye	22	55	35	52.2
Thickness				
≤2mm	8	20	16	23.9
2-4mm	12	30	27	40.3
>4 mm	20	50	24	35.8
Ciliary body involvement				
yes	22	55	31	46.3
no	18	45	36	53.7
Cell type				
Spindle	20	50	34	50.7
Mixed or epithelioid	20	50	33	49.3
Extraocular extension				
None or superficial	24	60	42	62.7
Deep, total, or episcleral	16	40	25	37.3
with metastasis				
without	24	60	38	56.7
with	16	40	29	43.3
GNAQ Mutation				
yes	18	45	32	47.8
no	22	55	35	52.2
GNA11 Mutation				
yes	8	20	15	22.4
no	32	80	52	77.6
BRAF Mutation				
yes	4	10	9	13.4
no	36	90	58	86.6
NRAS Mutation				
yes	4	10	6	9
no	36	90	61	91
HIF1A Mutation				
yes	12	30	18	26.9
no	28	70	49	73.1
FOXO1 Mutation				
yes	16	40	29	43.3
no	24	60	38	56.7
PIK3R1 Mutation				
yes	20	50	27	40.3
no	20	50	40	59.7
TSC1 Mutation				
yes	7	17.5	12	17.9
no	33	82.5	55	82.1
TSC2 Mutation				
yes	6	15	7	10.4
no	34	85	60	89.6

In validation cohort, the gene mutation rates of GNAQ, GNA11, BRAF, NRAS, CCND1, HIF1A, AKT1, FOXO1, PIK3R1, PTEN, TSC1, TSC2, FGFR3, KIT and MET were 47.8% (32/67), 22.4% (15/67), 13.4% (9/67), 9% (6/67), 6% (4/67), 26.9% (18/67), 7.5% (5/67), 43.3% (29/67), 40.3% (27/67), 7.5% (5/67), 17.9% (12/67), 10.4% (7/40), 7.5% (5/67), 9% (6/67) and 6% (4/67), respectively.

MAPK pathway gene mutations

Associations between mutations in MAPK pathway genes and patients' clinicopathological features were analysed. We found that *HIF1A* mutation was significantly correlated with tumour metastasis (Table 2, $P < 0.05$).

Table 2. Associations between mutation status of genes in the MAPK and PI3K/AKT pathways and OS for uveal melanoma patients in univariate Cox regression analyses.

Characteristics	HR	95%CI	P value
Gender (female/male)	0.85	0.47-1.27	0.245
Age(>60 years/ 60 years)	0.61	0.19-1.76	0.284
Side(left/right)	0.83	0.35-1.79	0.392
Thickness (2 mm/>2 mm)	0.77	0.14-1.84	0.289
Ciliary body involvement(with/without)	1.14	0.46-2.98	0.121
Cell type(spindle/ mixed or epithelioid)	0.91	0.07-1.48	0.348
Mixed or epithelioid(extraocular extension/none or superficial)	0.97	0.27-1.36	0.249
with metastasis(with/without)	2.34	1.13-4.14	0.008
GNA11 mutation(yes/no)	0.87	0.69-1.23	0.489
GNAQ mutation(yes/no)	1.13	0.31-3.02	0.941
BRAF mutation(yes/no)	1.07	0.09-2.38	0.125
NRAS mutation(yes/no)	1.32	0.37-3.12	0.637
HIF1A mutation(yes/no)	1.39	0.27-2.99	0.022
FOXO1 mutation(yes/no)	1.47	0.09-3.87	0.013
PIK3R1 mutation(yes/no)	0.52	0.03-1.69	0.524
TSC1 mutation(yes/no)	1.03	0.09-2.63	0.134
TSC2 mutation(yes/no)	0.39	0.11-2.98	0.537

We further analysed the types of gene mutations detected in *BRAF*, *NRAS*, *GNA11*, *GNAQ*, *HIF1A* and *PTEN* in training cohort, as shown in Figure 1. For the 4 patients harbouring *BRAF* mutations, the mutation, V600E, involved the well-known V600 amino acid residue. All 4 patients with *NRAS* mutations had hot-spot mutations, including mutations at Q61 (n=3, 75%) and G12 (n=1, 25%). The 8 patients carrying *GNA11* mutations shared a Q209 mutation. Among the 18 patients carrying *GNAQ* mutations, 11 (61%) patients shared the Q209P mutation, 5 (28%) patients shared the Q209L mutation, and 2 (11%) patients shared the R183Q mutation. Among the 12 patients with *HIF1A* mutations, 5 (42%) patients had the P606S mutation, 3 (25%) patients had the I136T mutation, and 4 (33%) patients had both the D373N and P606S mutations. All 4 patients harbouring *PTEN* mutations shared the M270I mutation.

We also investigated associations between MAPK pathway gene mutations and prognosis for UM patients and demonstrated that *HIF1A* mutation was associated with a worse prognosis in training cohort ($P < 0.05$), as shown in Figure 2A. The result were further verified in validation cohort ($P < 0.05$), as shown in Figure 2C.

In univariate analyses, *HIF1A* mutation status was significantly associated with OS for UM patients ($P < 0.05$), as shown in Table 2. However, in a multivariate Cox analysis, no gene mutation in the MAPK pathway was significantly correlated with OS.

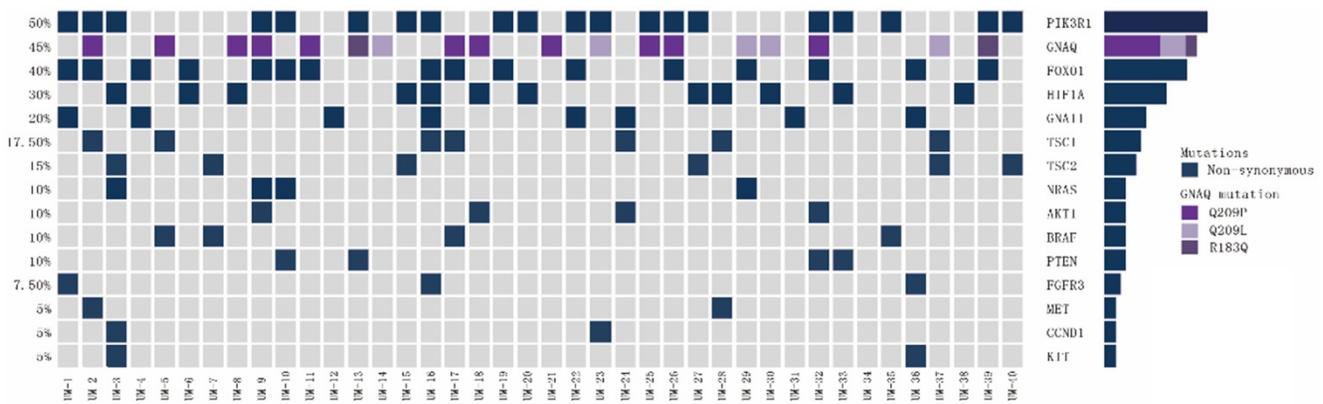


Figure 1. Mutation patterns for MAPK and PI3K/AKT pathway genes in uveal melanoma patients of training cohort (n=40).

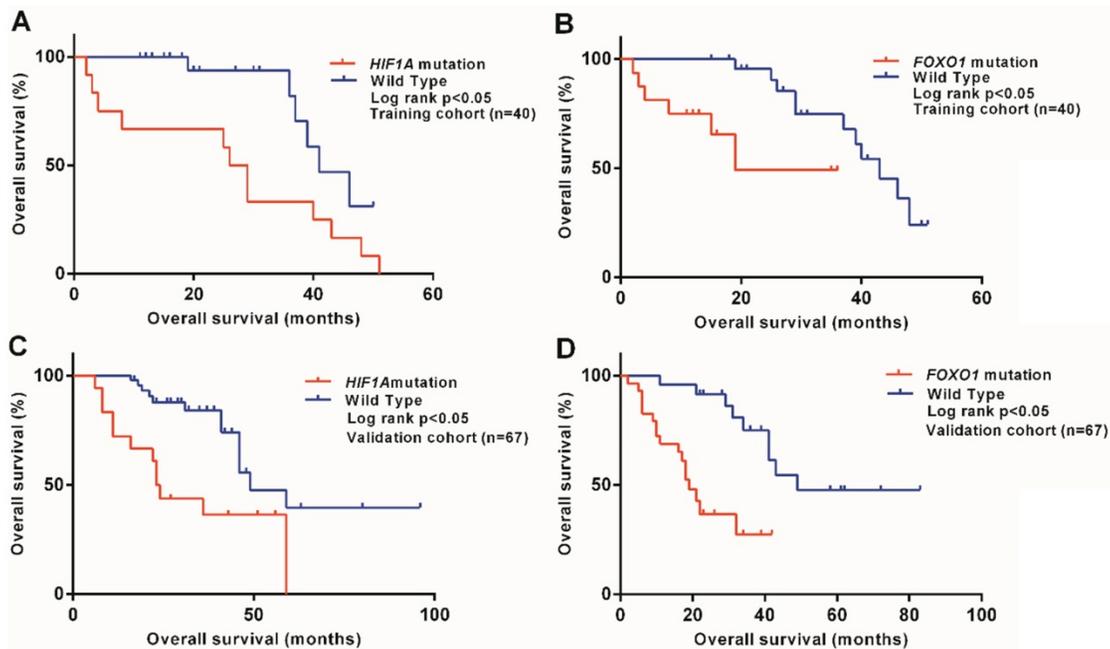


Figure 2. Kaplan-Meier survival curves for OS according to gene mutation status for patients with uveal melanoma. (A) In training cohort, Patients with HIF1A mutation had a shorter median OS than patients with wild-type HIF1A ($P<0.05$). (B) In training cohort, Patients with FOXO1 mutation had a shorter median OS than patients with wild-type FOXO1 ($P<0.05$). (C) In validation cohort, Patients with HIF1A mutation had a shorter median OS than patients with wild-type HIF1A ($P<0.05$). (D) In validation cohort, Patients with FOXO1 mutation had a shorter median OS than patients with wild-type FOXO1 ($P<0.05$).

PI3K/AKT pathway gene mutations

We evaluated associations between mutations in PI3K/AKT pathway genes and patients' clinicopathological features and found that FOXO1 mutation was associated with metastatic transformation ($P<0.001$).

Details regarding the types of gene mutations detected in FOXO1, PIK3R1, TSC1, TSC2 and AKT1 in training cohort are shown in Figure 1. Among the 16 patients carrying FOXO1 mutations, 10 (63%) patients had the D82N mutation, and 6 (37%) patients had the A511V mutation. The 20 patients harbouring PIK3R1 mutations had the M56I mutation. The 4 patients with NRAS mutations had hot-spot mutations, including mutations at Q61 (n=3, 75%) and G12 (n=1, 25%). Among the 7 patients with TSC1 mutations, 2 (29%) patients had the E894Q mutation, 2 (29%) patients had

the M271T mutation, 2 (29%) patients had the Q603E mutation, and 1 (13%) patient had the M374V mutation. Six patients harboured TSC2 mutations, including 3 (50%) patients with the R1115W mutation and 3 (50%) patients with the V591I mutation. The 4 patients carrying AKT mutations shared the V416A mutation.

We evaluated the effects of individual gene mutations in the PI3K/AKT pathway on UM patient survival and showed that FOXO1 mutation was correlated with a worse prognosis in training cohort ($P<0.05$), as shown in Figure 2B. The result were further verified in validation cohort ($P<0.05$), as shown in Figure 2D.

Cox analyses were performed to investigate the prognostic value of gene mutations in the PI3K/AKT pathway. In univariate analyses, FOXO1 mutation

status was significantly correlated with worse OS for UM patients ($P < 0.05$), as shown in Table 2. Parameters that were significant in univariate analyses were assessed in a multivariate Cox analysis. In this multivariate analysis, *FOXO1* mutation was an independent prognostic factor for OS ($P < 0.05$) that was associated with an increase in the risk ratio by a factor of 1.35, as shown in Table 3.

Table 3. Association between *FOXO1* gene mutation status and OS for uveal melanoma patients in multivariate Cox regression analyses.

Characteristics	HR	95%CI	P value
with metastasis(with/without)	2.83	1.03-4.98	0.009
<i>FOXO1</i> mutation(yes/no)	1.35	0.45-3.13	0.044

Discussion

Comprehensive genetic profiling of UM has been performed in Caucasian populations; [12] however, our research is the investigation involving genetic profiling of UM in a non-Caucasian population. Using NGS, we identified potential targeted therapy parameters and clinical biomarkers of disease metastasis and prognosis for UM.

With respect to genetic mutation profiles, UM is different from CM in that *BRAF* and *NRAS* mutations are not predominant. Instead, 83% of UM patients harbour mutations in *GNAQ* or *GNA11*. [6] The reported frequencies of *GNAQ* mutation range from 42-49% in Caucasian populations. [5 8 13] In the cohort examined in this investigation, the gene mutation rate for *GNAQ* was 46.7%, which was similar to previously reported rates in Caucasian populations. Although the *GNA11* mutation rate was reported to be 32.6% in a Caucasian population, [13] this rate was only 21.5% in the current study, a much lower frequency than that observed in the Caucasian cohort. It remains unclear whether this discrepancy in the frequency of *GNA11* mutation was attributable to ethnic differences.

UM is extremely deadly due to its high metastatic potential. Several biomarkers for predicting UM metastasis have been studied, such as *BAP1*, *EIF1AX* and *SF3B1*. [14-16] In our cohort, we found that *HIF1A* and *FOXO1* mutations were associated with metastatic transformation of UM ($P < 0.05$ and $P < 0.001$, respectively).

Tumour hypoxia is a prominent characteristic of the tumour microenvironment and could activate a series of genes that mediate metastasis. [17] Several studies have demonstrated the role of hypoxia in the metastatic behaviour of melanoma. [18] *HIF1A* (hypoxia-inducible factor 1 alpha) is an essential regulator of the hypoxic stress response. [19]

Furthermore, the association between *HIF1A* and melanoma metastasis has already been studied. [20] Hanna found that inactivation of *HIF1A* led to decreased melanoma metastasis. [20] Chang also demonstrated that *HIF1A* was an accurate predictor of UM metastasis in a Caucasian population. [21] Consistent with these previous results, we found that *HIF1A* mutation was significantly associated with tumour metastasis in UM. Cox univariate analyses also indicated that *HIF1A* mutation was significantly associated with outcome for UM.

There are four FOXO (Forkhead box class O) proteins: *FOXO1* (FKHR), *FOXO3a* (FKHRL1), *FOXO4* (AFX) and *FOXO6*. These proteins play crucial roles in regulating series of tumour-related processes. [21] Nakamura found that *FOXO1* is a critical effector of PTEN-mediated tumour suppression. [22] Zhang showed that in prostate cancer, inactivation of *FOXO1* could drive the promiscuous expression of *Runx2* target genes involved in cell migration and invasion and promote tumour progression. [23] Dong found that in hepatocellular carcinoma, *FOXO1* could suppress the ZEB2-induced epithelial-to-mesenchymal transition (EMT) to inhibit tumour metastasis. [24] In our cohort, the *FOXO1* mutation was significantly associated with metastatic transformation of UM. The *FOXO1* mutation was also found to be an independent prognostic factor for UM in both univariate and multivariate analyses.

The prognosis of patients with UM remains dismal due to a lack of effective treatment. Overall, 80% of UM patients have mutations in the *GNAQ* and *GNA11* genes, which constitutively activate the MAPK and PI3K/AKT pathways; therapies targeting downstream effectors of these pathways, such as MEK, AKT, and protein kinase C (PKC), are currently being investigated. Unfortunately, the results to date have been disappointing. Selumetinib, a highly selective inhibitor of MEK, improves progression-free survival (PFS) in UM, but no OS benefit has been observed. [25] Efforts to optimize the efficacy of targeted therapy remain ongoing.

In conclusion, in this study, we showed for the mutation patterns of UM in a non-Caucasian cohort, with a focus on gene mutations that affect key molecules in the MAPK and PI3K/AKT pathways. These findings illustrate the distinct biological mechanisms underlying UM pathogenesis and metastatic transformation and may provide evidence to improve targeted therapy for UM.

Abbreviations

UM: uveal melanoma; NGS: next-generation sequencing; FFPE: formalin-fixed, paraffin-

embedded; OS: overall survival; CM: cutaneous melanoma; MAPK: mitogen-activated protein kinase; PCR: polymerase chain reaction; FOXO: Forkhead box class O; EMT: epithelial-to-mesenchymal transition; PKC: protein kinase C; PFS: progression-free survival.

Acknowledgements

We would like to thank Shanghai Biotechnology Corporation (Shanghai, China) for targeted enrichment, sequencing, and data analysis of NGS. We would like to thank American Journal Experts (AJE) for English language editing.

Funding

This work was supported by grants from the National Natural Science Foundation of China (81672696; 81772912), Beijing Municipal Administration of Hospitals Clinical medicine Development of special funding support (ZYLX201603), Fostering Young Scholars of Peking University Health Science Center and Beijing Baiqianwan Talents Project.

Competing Interests

The authors have declared that no competing interest exists.

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