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The Role of Mutation Rates of *GNAQ* or *GNA11* in Cases of Uveal Melanoma in Japan

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Abstract: *GNAQ* and *GNA11* mutations are thought to be important for the tumorigenesis of uveal melanoma. Although previous studies have reported on mutation rates in cases of uveal melanoma, presently, no such report for the Japanese population exists. In this study, we examined the frequency of *GNAQ* and *GNA11* somatic mutations in cases of uveal melanoma in Japan and their relationship with clinicopathologic features or Ki-67-positive cell rates (Ki-67 labeling index: Ki-67 LI) using immunofluorescence methods. The study involved 19 cases of uveal melanoma. We extracted the template DNA from formalin-fixed, paraffin-embedded specimens using a DNA extraction kit. We amplified the DNA sequences of *GNAQ* and *GNA11* using polymerase chain reaction and analyzed mutations by direct sequencing. We evaluated Ki-67 LI using immunofluorescence methods. The frequencies of *GNAQ* and *GNA11* somatic mutations were 26.3% (5/19) and 31.6% (6/19), respectively. The *GNAQ* and *GNA11* mutations were mutually exclusive, as indicated in previous reports. The frequency of *GNA11* mutations was significantly higher in epithelioid cells; however, no significant association between *GNAQ* mutations and cell type was evident, and there was no significant difference in Ki-67 LI between the mutation-positive and mutation-negative tumors. *GNAQ* and *GNA11* mutations were identified in cases of uveal melanoma in Japan, although at lower frequencies than in white counterparts. The mutation frequency of *GNA11* was significantly higher in epithelioid cells.

Key Words: uveal melanoma, *GNAQ/11* mutation, cell types, Ki-67

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Uveal melanoma is the most common primary malignant intraocular tumor in adults. Many cases arise in the choroid; however, a small number arises in the ciliary body and iris.[1] The incidences of uveal melanoma differ between ethnic groups.[2] Among whites in the United States, uveal melanoma occurs in ~6 cases per million people annually; however, the incidence of uveal melanoma is extremely low in Japan.[3] Factors that determine survival are thought to include the site of tumor onset, histopathologic cell type, mitotic rate, diameter of the largest tumor, and scleral invasion.[4] Uveal melanomas have a strong metastatic affinity to the liver, and there is currently no effective treatment for cases, once metastasis has occurred. Once metastasis has been detected, the survival duration is ~8 months.[5,6]

The mitogen-activated protein kinase (MAPK) signaling pathway is known to be important for the proliferation of malignant melanoma cells. In cutaneous malignant melanomas, mutations occurring in *BRAF* and *NRAS*, which encode MAPK pathway proteins, are thought to promote the activation of the MAPK pathway and cell proliferation.[7–9] However, in uveal melanomas, the MAPK pathway is known to be activated, regardless of the absence of mutations in *BRAF* and *NRAS*.[10,11] Although the reason for this was previously unknown, it has recently been considered that mutations in *GNAQ* and *GNA11* detected in uveal melanomas may be responsible.[12–14]

GNAQ and *GNA11* encode G-protein alpha subunit q and alpha subunit 11, respectively, and are paralogs. Alpha subunits mediate between 7 transmembrane domain receptors and intracellular signaling machinery.[15,16] Recently, oncogenic mutations have been identified in these alpha subunits in uveal malignant melanomas.[12,13] Alpha subunits act as a molecular switch for G-proteins and play an important role in the hydrolysis of guanosine triphosphate (GTP). However, when mutations arise in *GNAQ* or *GNA11*, GTP hydrolysis by alpha subunits is blocked, leaving the alpha subunits locked in a state of activity with GTP, thereby causing signal transmission to become permanent.[17] Thus, the proliferation of uveal melanoma cells is promoted, thereby contributing to its development.[12,13]

Somatic mutations in *GNAQ* and *GNA11* are 2 mutations commonly found in uveal melanoma cases.[12,13]

Currently, there are no effective drugs for uveal melanoma. *GNAQ* and *GNA11* present possible targets for therapeutic intervention in uveal melanoma; therefore, it is important to determine the frequency of *GNAQ* and *GNA11* mutations. Although there have been reports showing a positive correlation of *GNAQ* and *GNA11* mutations with uveal melanomas in whites and Chinese people, the strength of the relationship in Japanese cases remains unknown. Therefore, the present study aimed to investigate the link between *GNAQ* and *GNA11* mutations and uveal melanoma in Japan and to evaluate the relationship with clinicopathologic features. In addition, we investigated the relationships of *GNAQ* and *GNA11* mutations with the proportion of Ki-67-positive cells (Ki-67 labeling index: Ki-67 LI). Ki-67 LI is used as an index for tumor cell proliferation ability.[18,19] We considered that immunofluorescence (IF) identifies Ki-67-positive cells in melanin-rich uveal melanomas more clearly than immunohistochemistry, and therefore, we used IF to evaluate Ki-67 LI. We also examined the relationship between *GNAQ* and *GNA11* mutations and uveal melanoma, with mitotic rate as another indicator of proliferative activity.

MATERIALS AND METHODS

Patients and Clinicopathologic Features

In the present study, we examined 19 cases of enucleated uveal melanomas. The study was approved by the medical ethics committee of Niigata University School of Medicine. The diagnosis of uveal melanoma was confirmed by hematoxylin and eosin-stained slides as well as by immunohistochemistry for HMB-45 and Melan-A. Clinicopathologic data included age, sex, histopathologic cell types (epithelioid, spindle, mixed with epithelioid predominant, and mixed with spindle predominant) of the tumors, pT (according to *AJCC*),[20] diameter of the largest tumor, and scleral invasion (Table 1). None of the cases showed evidence of lymph node involvement and/or distant metastasis at diagnosis.

TABLE 1. Clinicopathologic Features of 19 Cases of Uveal Melanomas

Clinicopathologic Features	Mean (± SD) or n (%)
Age (y)	64.9 (± 11.5)
Men/women	10 (52.6)/9 (47.4)
pT	
pT1	7 (36.8)
pT2	4 (21.1)
pT3	6 (31.6)
pT4	2 (10.5)
Largest tumor diameter (mm)	11.5 (± 7.5)
Scleral invasion	
Present	6 (31.6)
Absent	13 (68.4)
Histopathologic cell types	
Epithelioid	3 (15.8)
Spindle	3 (15.8)
Mixed with epithelioid predominant	7 (36.8)
Mixed with spindle predominant	6 (31.6)

Analysis of Mutations

The method of analyses of DNA extraction and mutation is described below. For each case, a 10-μm-thick, formalin-fixed, paraffin-embedded uveal melanoma specimen was prepared for microdissection. Tumor DNA was extracted from dissected tissues using a DNA isolator PS kit (Wako Pure Chemical Industries, Osaka, Japan). Fragments including *GNAQ* exons 5 and 4 and *GNA11* exons 5 and 4 were amplified by polymerase chain reaction (PCR) of the tumor template DNA. The primer sequences used are shown in Table 2. Genomic DNA was amplified by PCR using the following cycles: 1 minutes at 95°C, 1 minutes at 58°C, and 1 minutes at 72°C for 40 cycles. PCR products were purified with ExoSAP-IT (Affymetrix, Cleveland, OH). The products were directly sequenced on an Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA) using the BigDye Terminator version 1.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing reaction was performed in the forward and reverse directions to detect mutations.

IF and Mitotic Rate

Formalin-fixed, paraffin-embedded specimens of uveal melanoma were sliced in 5-μm-thick sections for processing by IF. Deparaffinized sections were incubated in hot distilled water, with ImmunoSaver (Wako Pure Chemical Industries), which is an antigen-retrieval drug used to retrieve antigen from the sections. The sections were then incubated in a moist chamber with Ki-67 antibody (monoclonal, mouse, 1:100; Dako, Glostrup, Denmark) overnight at 4°C. Following this, the sections were incubated with Alexa Fluor 568 (polyclonal, goat, 1:200; Thermo Fisher Scientific, MA) for 1 hour at room temperature. After nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) (1.5 μg/mL; Santa Cruz Biochemistry, California), the slides were mounted using a mounting medium. The samples were protected from direct exposure to light throughout processing.

Positive and negative controls were concurrently stained to ensure the reliability of the process. A Ki-67-positive case of amelanotic melanoma was used as a positive control. Omission of Ki-67 antigen or Alexa Fluor 568 was used as a negative control.

The slides were observed under a fluorescence microscope (BZ-9000; KEYENCE, Osaka, Japan), and Ki-67-positive cells were verified by fluorescence emission of a clear red color (Fig. 1). The percentage of Ki-67-positive cells (Ki-67 LI) was evaluated by counting at the

TABLE 2. Primer Sequences Used

GNAQ exon5-F	5′-AACCTTGCAGAATGGTCGAT-3′
GNAQ exon5-R	5′-TCTGACTCCACGAGAACTTGA-3′
GNA11 exon5-F	5′-GCTGTGTCCTTTCAGGATGG-3′
GNA11 exon5-R	5′-TTGGTCGTATTCGCTGAGG-3′
GNAQ exon4-F	5′-TACCTGCCTACGCAAGA-3′
GNAQ exon4-R	5′-GCTGGGAAATAGGTTTCATGG-3′
GNA11 exon4-F	5′-CTACCTGACCGACGTTGACC-3′
GNA11 exon4-R	5′-GGTCGAAAGGGTACTCGATG-3′

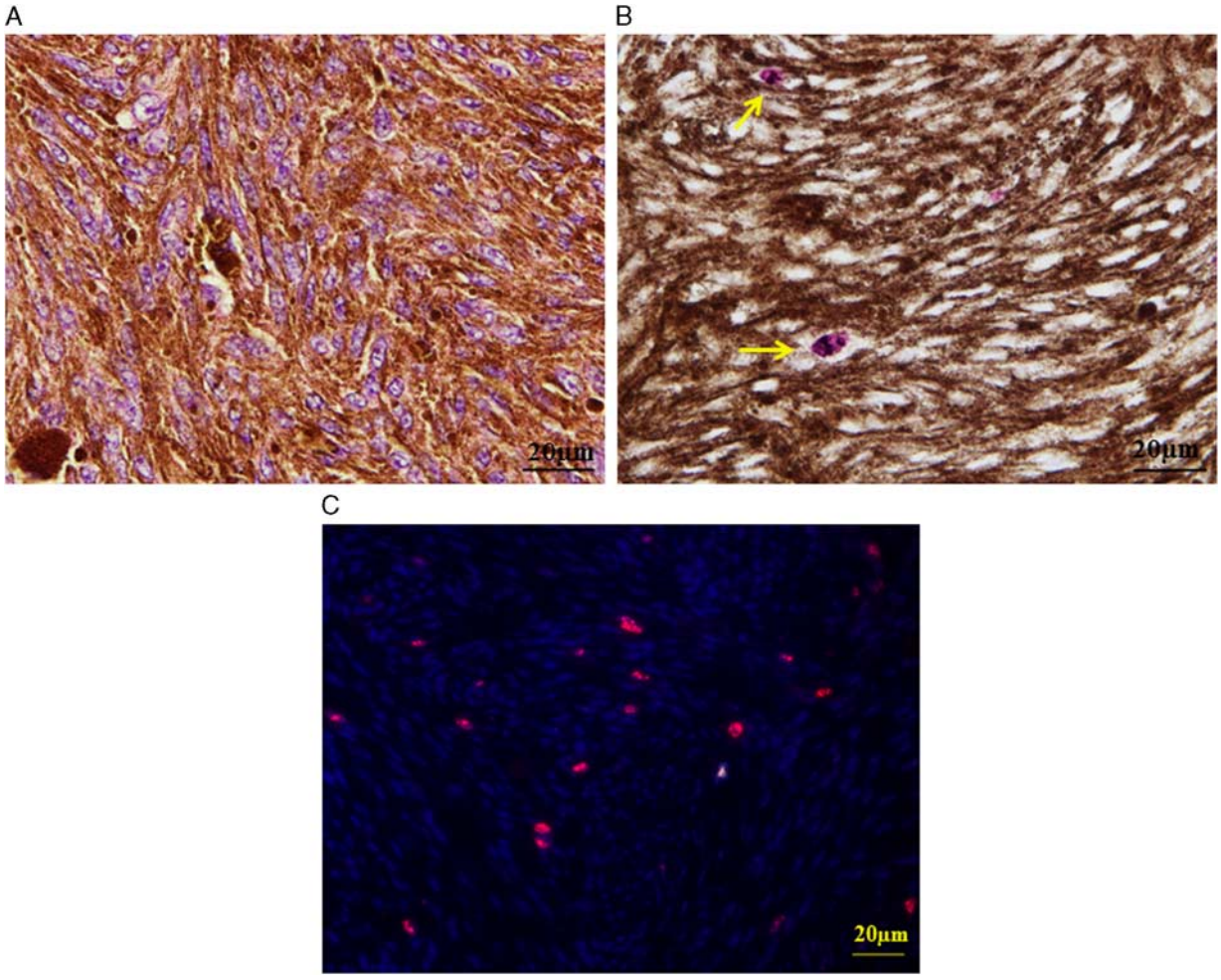

FIGURE 1. Comparison of immunohistochemistry and immunofluorescence methods. A, Uveal melanoma section with hematoxylin and eosin staining (×400). B, Ki-67 immunohistochemistry (positive cells stained violet, shown by arrow, ×400). C, Ki-67 IF method (positive cells stained red, ×400).

hot spot with ×400 magnification using the analysis software BZ-H3C (KEYENCE).

Mitotic cells were counted in 15 consecutive high-power fields with a total magnification of ×400.

Statistical Analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 23.0 (IBM Japan Inc., Tokyo, Japan). The association between the mutation status of *GNAQ* or *GNA11* and clinicopathologic features was evaluated by the Student *t* test and the Fisher exact test. The association between Ki-67 LI, mitotic rate, and the mutation status of *GNAQ* or *GNA11* was evaluated by the Mann-Whitney *U* test. A $P < 0.05$ was regarded as statistically significant.

RESULTS

Among the 19 cases of uveal melanoma screened for *GNAQ* and *GNA11* mutations, 5/19 (26.3%) and 6/19 (31.6%) of the cases were associated with *GNAQ* and *GNA11* mutations, respectively. The codons in *GNAQ* and *GNA11* affected by mutations were Q209L and Q209P, and Q209L and R183C, respectively. In all the samples, *GNAQ* or *GNA11* mutations were mutually exclusive (Table 3). All the cases were simultaneously sequenced for *BRAF* and *NRAS* mutations; however, no mutations were found.

The association between the presence or absence of *GNAQ* or *GNA11* mutations and clinicopathologic features of the cases was analyzed. Features included age, sex, pT, diameter of largest tumor, scleral invasion, and histopathologic cell types. There was a statistically significant association between *GNA11* mutations and histopathologic cell types ($P = 0.009$ by the Fisher exact test). *GNA11* mutations were found more commonly in cases related to epithelioid cells and rarely in cases related to spindle cells. Conversely, there was no statistical significance between *GNAQ* mutations and histopathologic cell types. No further associations between the mutations and other clinicopathologic features were observed (Table 4).

TABLE 3. Results of *GNAQ* and *GNA11* Mutations in 19 Cases of Uveal Melanomas and Their Mutated Forms

Number	Sex	Age (y)	*GNAQ*	*GNA11*	Histopathologic Cell Types
1	F	68	Q209L	wt	Spindle
2	F	74	Q209L	wt	Mixed with spindle predominant
3	M	74	Q209L	wt	Mixed with epithelioid predominant
4	F	57	Q209P	wt	Mixed with spindle predominant
5	F	59	Q209P	wt	Mixed with epithelioid predominant
6	M	58	wt	Q209L	Epithelioid
7	F	59	wt	Q209L	Epithelioid
8	M	62	wt	Q209L	Epithelioid
9	M	76	wt	Q209L	Mixed with epithelioid predominant
10	M	72	wt	Q209L	Mixed with epithelioid predominant
11	M	47	wt	R183C	Mixed with epithelioid predominant
12	M	51	wt	wt	Spindle
13	F	66	wt	wt	Spindle
14	F	92	wt	wt	Mixed with spindle predominant
15	M	52	wt	wt	Mixed with spindle predominant
16	M	55	wt	wt	Mixed with spindle predominant
17	F	83	wt	wt	Mixed with spindle predominant
18	F	62	wt	wt	Mixed with epithelioid predominant
19	F	66	wt	wt	Mixed with epithelioid predominant

F indicates female; M, male; wt, wild-type.

To evaluate the associations between *GNAQ* or *GNA11* mutation and the proliferation of uveal melanoma, Ki-67 LI was evaluated by IF. The average Ki-67 LI values in cases of *GNAQ* and *GNA11* mutations were 5.5% and 6.5%, respectively. There were no statistical differences between the 2 groups of mutant and wild-type cases for either *GNAQ* or *GNA11* (Fig. 2). Similarly, in the evaluation using the mitotic rate, there were no statistical differences depending on the presence or absence of the mutations (Fig. 3).

DISCUSSION

Uveal melanoma is the most common primary malignant intraocular tumor in adults.[1] *GNAQ* and *GNA11* mutations can act as molecular targets for the treatment of uveal melanoma. It is, therefore, important to determine the frequency of *GNAQ* and *GNA11* mutations. No previous study has investigated the frequency of somatic gene mutations in Japanese cases of uveal melanoma. In the present study, we were able to demonstrate this frequency. We found that somatic gene mutation rates

TABLE 4. Associations of *GNAQ* and *GNA11* Mutations With Clinicopathologic Features of Uveal Melanomas

Clinicopathologic Features	*GNAQ* mut	*GNAQ* wt	*P*	*GNA11* mut	*GNA11* wt	*P*
Cases [n (%)]	5 (26.3)	14 (73.7)		6 (31.6)	13 (68.4)	
Age (y)						
Mean (± SD)	66.4 (± 7.2)	64.4 (± 12.3)	0.74*	62.3 (± 9.5)	66.1 (± 11.7)	0.53*
Sex [n (%)]						
Men	2 (10.5)	8 (42.1)	0.44†	5 (26.3)	5 (26.3)	0.091†
Women	3 (15.8)	6 (31.6)		1 (5.3)	8 (42.1)	
pT staging [n (%)]						
pT1	2 (10.5)	5 (26.3)	0.89†	1 (5.3)	6 (31.6)	0.41†
pT2	1 (5.3)	3 (15.8)		2 (10.5)	2 (10.5)	
pT3	1 (5.3)	5 (26.3)		3 (15.8)	3 (15.8)	
pT4	1 (5.3)	1 (5.3)		0 (0)	2 (10.5)	
Largest tumor diameter (mm)						
Mean (± SD)	10.8 (± 5.6)	11.8 (± 8.3)	0.81*	11.8 (± 2.0)	11.4 (± 9.1)	0.91*
Scleral invasion [n (%)]						
Present	1 (5.3)	5 (26.3)	0.48†	1 (5.3)	5 (26.3)	0.35†
Absent	4 (21.1)	9 (47.4)		5 (26.3)	8 (42.1)	
Histopathologic cell types [n (%)]						
Spindle	1 (5.3)	2 (10.5)	0.9†	0 (0)	3 (15.8)	**0.009**†
Mixed with spindle predominant	2 (10.5)	4 (21.1)		0 (0)	6 (31.6)	
Mixed with epithelioid predominant	2 (10.5)	5 (26.3)		3 (15.8)	4 (21.1)	
Epithelioid	0 (0)	3 (15.8)		3 (15.8)	0 (0)	

*Calculated with the Student *t* test.
†Calculated with the Fisher exact test.
Bold value are statistically significant.
mut indicates mutant cases; wt, wild-type cases.

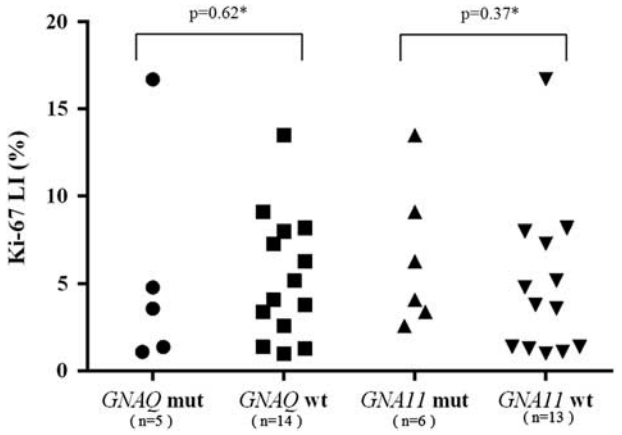

FIGURE 2. Association between the *GNAQ* or *GNA11* mutation status and Ki-67 LI. LI indicates labeling index; mut, mutant cases; wt, wild-type cases. *Calculated by Mann-Whitney *U* test.

were 26.3% and 31.6% for *GNAQ* and *GNA11* mutations, respectively. A *GNAQ* mutation rate of 40% to 50% has previously been reported in white cases of uveal melanoma,[12,14,21,22] whereas a *GNA11* mutation rate of 30% to 40% has been reported.[13,22] Compared with the *GNAQ/11* mutation rates reported in white cases of uveal melanoma by Koopmans et al,[22] our results showed that the *GNAQ/11* mutation rate in Japanese cases tended to be lower than that in whites. Xu et al[23] investigated the *GNAQ/11* mutation rates in Chinese cases and reported that both rates were lower than those in white cases. A comparison of the report by Xu and colleagues with our results indicated no differences in mutation rates. It seems that the *GNAQ/11* mutation rates in association with uveal melanoma are roughly the same in Japanese and Chinese cases, suggesting that the mutation rates differ between white and Asian races.

Most *GNAQ* and *GNA11* mutations arise in codon 209, which is located in exon 5. In some cases, mutations

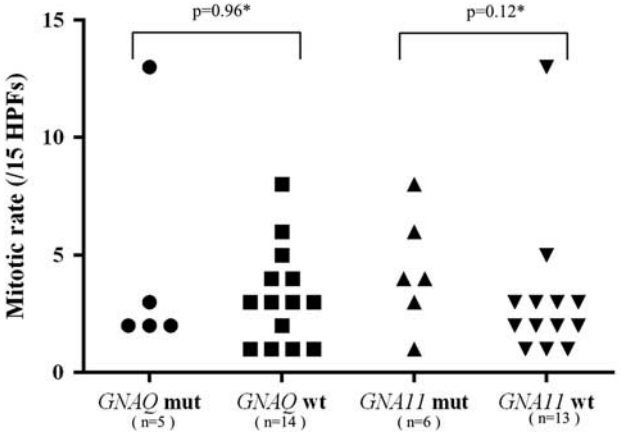

FIGURE 3. Association between the *GNAQ* or *GNA11* mutation status and mitotic rate. HPFs indicates high-power fields; mut, mutated cases; wt, wild-type cases. *Calculated by Mann-Whitney *U* test.

may arise in codon 183, which is located in exon 4.[12,13] In previous studies, these *GNAQ* and *GNA11* mutations were mutually exclusive, and this was also observed in our results. *GNAQ* mutations identified in the present study were Q209L or Q209P mutations, and *GNA11* mutations identified were Q209L or R183C mutations. These mutant forms were consistent with those reported previously.[12,13] It was confirmed that these genetic mutations were the same between Japanese and white/Chinese cases. The present study demonstrated that codon 183 mutations, in which infrequent occurrence has previously been reported, can also occur in Japanese patients.

Earlier studies have also investigated the associations of *GNAQ* and *GNA11* mutations with the clinicopathologic features of uveal melanomas. It was reported that age, sex, pT, diameter of the largest tumor, scleral invasion, and histopathologic cell type are not significantly associated with the presence or absence of genetic mutations.[20,23] Our results also showed no significant association of genetic mutations with age, sex, pT, diameter of the largest tumor, or scleral invasion. However, notably, our results showed that the rate of *GNA11* mutations varies according to histopathologic cell type of the tumor. The *GNA11* mutation rate was significantly higher in epithelioid cells, whereas it tended to be lower in spindle cells. No such trends were noted for positive cases of *GNAQ* mutations. Van Raamsdonk et al[13] reported that the number of cases of *GNAQ* and *GNA11* mutations tended to be higher in patients with predominant epithelioid cells, which was confirmed by our results for *GNA11* mutations. In the study by Xu et al,[23] no significant association between genetic mutations and histopathologic cell type was found in Chinese patients, and spindle-type cases accounted for >80% of the total number. In our study, spindle-type cases accounted for ~16% of the total number. A previous study that compiled data on Japanese cases of uveal melanoma indicated that spindle-type cases accounted for 38% of the total cases.[3] Thus, the proportions of different genetic backgrounds and histopathologic cell types may vary by region among Asian cases of uveal melanoma.

In the present study, we investigated the association between uveal melanoma genetic mutations and Ki-67 LI results. We believe that IF facilitates easier recognition of Ki-67-positive cells in melanin-rich uveal melanomas than immunohistochemistry. Hence, IF was used for Ki-67 LI evaluation. Mooy et al,[24] who used immunohistochemistry for evaluation, reported that the mean Ki-67 LI score for uveal melanomas was 1.23%. In our results, the mean Ki-67 LI score was 5.41%, which was higher than that reported previously. This may have been because of the easier recognition of Ki-67-positive cells due to the use of IF. However, no significant difference in Ki-67 LI scores was noted between the *GNAQ* mutation-positive and mutation-negative groups. This is in agreement with the results of a previous study that used immunohistochemistry.[25] In addition, no significant difference was noted in Ki-67 LI scores between the *GNA11* mutation-positive and mutation-negative groups. Indeed, IF enabled easier recognition of

Ki-67-positive cells, and no significant associations with genetic mutations and Ki-67 LI were found. We also evaluated the proliferative activity using mitotic rate, but no significant difference was observed between *GNAQ/11* mutation-positive and mutation-negative groups and the mitotic rate. *GNAQ* and *GNA11* may be involved in the proliferation activity not expressed by Ki-67 LI or mitotic rate.

Although the present study has provided reasonable data on Japanese cases of uveal melanoma, it has few limitations. First, the sample size, despite being the largest that we were able to gather, was smaller than that of previous studies.[22,23] The incidence of uveal melanoma within Japan is extremely low[3]; therefore, it is unlikely that a large number of cases can be investigated at a single hospital. This study is a pilot study, and it is necessary to increase the number of cases by multicenter collaborative research in the future, to further confirm the results. Furthermore, because this was a single-center, hospital-based study, patient selection bias may have occurred.

The aim of the present study was to investigate *GNAQ* and *GNA11* mutation rates in cases of uveal melanoma in Japan. In conclusion, *GNAQ* and *GNA11* mutations were found in cases of uveal melanomas in Japan, as observed in previous reports on cases of uveal melanomas among white and Chinese cases, and the phenotypes of these mutations were also identical. Although *GNAQ/11* mutation rates tended to be lower in Japanese cases than in white cases, it was demonstrated that the development of treatment for uveal melanoma targeting the *GNAQ/11* mutation can still prove beneficial for certain Japanese patients in future. Our investigation of a possible association between genetic mutations and tumor proliferative activity using IF revealed no significant association. As mentioned previously, the present study had a smaller sample size than previous studies. In the future, the findings of the present study need to be further verified via investigations using a larger sample size.

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