

## INVESTIGATIVE REPORT

**Kinesin Family Member 20A is a Novel Melanoma-associated Antigen**

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**It has been shown recently that immunotherapy for advanced melanoma is effective. However, in order to improve the efficacy of immunotherapy, the identification of more specific melanoma-associated antigens is urgently needed. Kinesin family member 20A (KIF20A) has been reported to be a promising immunotherapeutic target for pancreatic cancer. To investigate the expression of KIF20A in melanoma, we performed quantitative reverse transcript (RT)-PCR and western blotting analyses of melanoma cell lines. We also investigated primary melanomas and naevus tissues with immunohistochemistry and real-time RT-PCR. KIF20A expression was detected in 59% of melanomas and 12% of naevi by immunohistochemistry, and 64% of melanomas and 60% of naevi by real-time RT-PCR. The primary melanomas that were positive for KIF20A showed a significantly greater thickness than those that were negative, and patients with KIF20A<sup>+</sup> melanoma tended to develop recurrence earlier. These results suggest that immunotherapy with KIF20A may be a novel treatment option for advanced melanoma. *Key words: KIF20A; melanoma; antigen.***

(Accepted May 8, 2012.)

Acta Derm Venereol 2012; 92: 593–597.

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The worldwide incidence of melanoma has gradually increased (1), and the mortality rate is high because malignant melanoma is often associated with early metastatic spread and a high level of resistance to existing treatment strategies. Median survival is 6 months, and the 5-year survival rate for metastatic melanoma is less than 5% (2–4). Previously, surgical resection was the only curative treatment. However, ipilimumab, a fully human monoclonal antibody against cytotoxic T-lymphocyte antigen 4, has been shown recently to have consistent activity against malignant melanoma in a randomized phase III trial (5, 6). Immunotherapy is currently thought to be a candidate treatment modality for advanced melanoma. However, in previous studies, ipilimumab, with or without gp100 peptide vaccine, improved overall patient survival (5). In other words, the efficacy of ipilimumab

was not improved by the addition of gp100 vaccine. Therefore, more specific and powerful peptide vaccines that can improve the overall survival rate for patients with metastatic melanoma are urgently required.

Imai et al. (7) reported recently that kinesin family member 20A (KIF20A) is overexpressed in pancreatic cancer, based on cDNA microarray analyses, and this may be a potential immunotherapeutic target for pancreatic cancer (8). They also analysed KIF20A gene expression in normal tissues, and revealed its exclusive expression in the testes and thymus. KIF20A, also known as RAB6KIFL, was first identified to localize in the Golgi apparatus, where it plays an important role in the dynamics in the organelle via interaction with the GTP-bound form of Rab6 (9). KIF20A belongs to a large family of proteins that share a conserved motor domain that binds to microtubules and couples ATP hydrolysis to generate mechanical force (10). KIF20A has previously been reported to accumulate in mitotic cells (11). Knockdown of the KIF20A gene in pancreatic cancer cell lines by small interfering RNA drastically inhibited the growth of these cell lines (12). Therefore, KIF20A is suspected to be involved in carcinogenesis. KIF20A is also reported to be highly detected among other types of cancer, such as small-cell lung cancer, bladder cancer, non-small-cell lung cancer, cholangiocellular carcinoma, and breast cancer (7). To the best of our knowledge, there has been no previous study examining KIF20A expression in melanocytic lesions. We report here an analysis of the relationship between KIF20A expression and the characteristics of melanoma patients.

## MATERIALS AND METHODS

*Clinical assessment and patient material*

Tissue samples were obtained from melanomas and naevi during routine diagnostic procedures. Institutional review board approval and written informed consent were obtained before patients were entered into this study, according to the Declaration of Helsinki. The tissue samples were stored at –80°C until use. The 26 benign naevi were from 26 subjects (11 males and 15 females). Their ages ranged from 1 to 86 years (mean 39 years). Histologically, they included junctional, compound and intradermal variants. The 51 primary cutaneous melanomas were obtained from 51 subjects (20 males and 31 females) whose ages ranged from 35 to 97 years (mean 68 years). The primary cutaneous melanomas had Clark's levels ranging from I to V and the Breslow depths ranged

from *in situ* to 53 mm. The 10 metastatic melanomas were from 10 subjects (4 males and 6 females) whose ages ranged from 52 to 85 years (mean 68 years). Three metastatic melanomas were localized to regional lymph nodes, while the others were from skin metastases. Patient information was gathered from medical records to determine the clinical stages according to the American Joint Committee on Cancer (AJCC) Cancer Staging Manual, 6<sup>th</sup> edition, staging system for melanoma of the skin.

#### Immunohistochemical analysis

Immunohistochemical studies were performed on 4- $\mu$ m sections of formalin-fixed, paraffin-embedded tissue, using a rabbit polyclonal antibody against KIF20A (A300-879A, Bethyl Laboratories, Montgomery, TX, USA). Melanoma and naevus sections were cut from paraffin-embedded archival blocks, dried at 37°C, deparaffinized in xylene and rehydrated in a series of alcohols. Antigen retrieval was performed using a microwave in citrate buffer at pH 6.0 for 20 min and cooling for 60 min at room temperature. Non-specific staining was blocked with 5% normal horse serum. A KIF20A antibody was used at a concentration of 1:250 and incubated with the specimens overnight in a humidified chamber. Endogenous peroxidase activity was blocked by immersing the sections in 0.3% hydrogen peroxidase diluted with methanol for 15 min. Using the EnVision method, an anti-rabbit polyclonal antibody (EnVision+; DakoCytomation, Carpinteria, CA, USA) was used neat and incubated for 45 min at room temperature. Staining was developed with a 3,3'-diaminobenzidine tetrahydrochloride- (Dojindo, Kumamoto, Japan) based detection method. Slides were rinsed in running distilled water and counterstained with Giemsa stain. The slides were mounted using aqueous medium and viewed under a microscope. The intensity of staining was classified as (-): the same or weaker than the adjacent epidermis or (+): stronger than the adjacent epidermis. The samples were estimated independently by 2 observers in a blinded manner (J.Y. and S.F.).

#### Cell lines

The human melanoma cell lines and pancreatic cancer cell line, PANC1, were maintained *in vitro* in RPMI 1640 or DMEM medium supplemented with 20% foetal calf serum (FCS) in a 5% CO<sub>2</sub> atmosphere at 37°C. Primary normal human epidermal melanocytes (NHEM) in the CSF-4HM-500D culture medium supplemented with human melanocyte growth supplements, was maintained in a 5% CO<sub>2</sub> atmosphere at 37°C. The human melanoma cell lines were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and Dr Y. Kawakami (Keio University; Tokyo, Japan). NHEM was purchased from DS Pharma Biomedical (Osaka, Japan).

#### Reverse transcription PCR

The real-time PCR primer was purchased from Takara Bio Inc. (Shiga, Japan). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer was purchased from SABioscience (Frederick, USA).

#### Western blotting analysis

The cultured cells were homogenized, and the cells lysates were electrophoresed on 10% sodium dodecyl sulphate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes. Blocking was achieved by incubating the membrane in 1% skimmed milk/Tris-buffered saline 0.2% Tween-20 (TBST) for 1 h. Anti-KIF20A rabbit polyclonal IgG (A300-879A, Bethyl Laboratories, Inc., USA) was applied at a dilution of 1:2000 and incubated at 4°C,

after which the membrane was incubated with anti-rabbit IgG-HRP (Biorad, Hercules, CA, USA) for an hour. Membranes were washed thoroughly with TBST, and the signals were detected using the Enhanced Chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA).  $\beta$ -actin was used as a loading control.

## RESULTS

### High expression of KIF20A mRNA and protein in cancer cell lines

We first performed a quantitative RT-PCR analysis of KIF20A expression in 10 malignant melanoma cell lines and a human pancreatic cell line, PANC1, as a positive control. PANC1 has been reported to have a high level of expression of KIF20A (7). As shown in Fig. 1a, all of the malignant melanoma cell lines showed comparable levels of KIF20A expression to the PANC1 cells. The results of a Western blot analysis of the melanoma cell lines and a normal human epidermal melanocyte cell line (NHEM) to detect the KIF20A protein expression are shown in Fig. 1b. All of the melanoma cell lines showed positivity for KIF20A, while the NHEM cells showed low levels of KIF20A.

### Immunohistochemical analysis revealed high level of expression of KIF20A in malignant melanoma

Immunohistochemical analysis of KIF20A expression was performed in 51 primary cutaneous melanoma, 9 metastatic melanoma and 26 naevus tissue specimens. Representative examples are shown in Fig. 2, and the

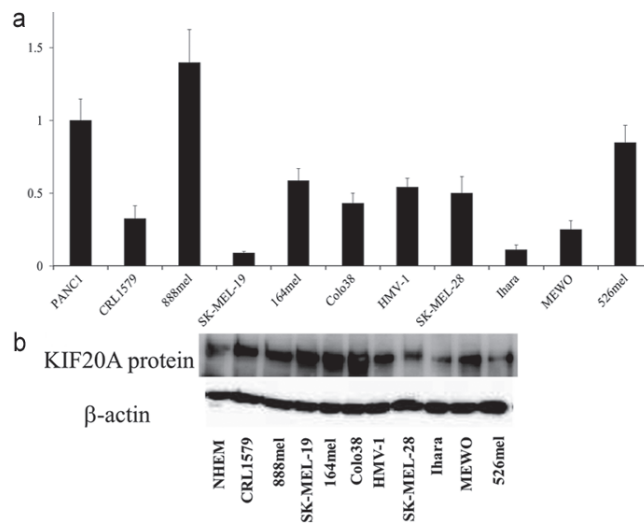


Fig. 1. Expression of KIF20A mRNA and protein in melanoma cell lines. Analysis of KIF20A mRNA and protein expression in malignant melanoma cell lines, normal human epidermal melanocyte cell line (NHEM) cells, and the PANC1 pancreatic cancer cell line. PANC1 cells were used as a positive control. (a) Results of a quantitative reverse transcript (RT)-PCR analysis of the KIF20A mRNA expression in malignant melanoma cell lines and the pancreatic cancer cell line. We compared the relative transcript levels of KIF20A normalized with that of PANC1. (b) Expression of KIF20A protein in the NHEM and malignant melanoma cell lines.

results are summarized in Table I. The melanoma cells positive for KIF20A showed homogenous cytoplasmic staining. KIF20A was highly expressed in melanoma samples, but showed minimal expression in the melanocytic naevus samples. Thirty of the 51 primary melanomas (59%) and 3 of the 26 melanocytic naevi (12%) showed overexpression of KIF20A. There was no correlation between the positivity for KIF20A and AJCC staging or histological types of melanoma (Table I). Metastatic melanoma cells are known to escape from the immune response through various mechanisms, including the loss of tumour-associated antigens. However, KIF20A was highly expressed not only at the primary tumour sites of melanoma, but also in the metastatic lesions (Table I).

#### *mRNA detection of KIF20A in tissue samples*

Quantitative RT-PCR analysis was performed for primary melanoma, metastatic melanoma and naevus samples. Gene-specific primers for KIF20A and GAPDH amplified each mRNA specifically (Fig. 3). We found that there were differences in the Ct values of KIF20A among the primary melanoma, metastatic melanoma and naevus samples. Eighteen of the 28 primary melanoma samples (64%), 8 of 10 metastatic melanoma samples (80%) and 6 of 10 melanocytic naevi (60%) showed positive expression of KIF20A. When normalized to GAPDH, the primary and metastatic melanoma samples showed significantly higher levels of expression of KIF20A than did the naevi (Fig. 3).

The results of the RT-PCR analysis of KIF20A expression in the tissue samples of 28 primary cutaneous

Table I. Results of the immunohistochemical analysis of KIF20A and KIF20A, reverse transcript (RT)-PCR

	KIF20A		KIF20A, RT-PCR	
	Total <i>n</i>	Positive <i>n</i> (%)	Total <i>n</i>	Positive <i>n</i> (%)
Naevi	26	3 (12)	10	6 (60)
Melanomas	51	30 (59)	28	18 (64)
Stage 0	3	3 (100)	5	2 (40)
Stage I	15	6 (40)	4	3 (75)
Stage II	19	12 (63)	11	5 (45)
Stage III	12	7 (58)	6	6 (100)
Stage IV	2	2 (100)	2	2 (100)
Metastatic melanomas	9	6 (67)	10	8 (80)
ALM	22	13 (59)	12	6 (50)
LMM	13	7 (54)	7	4 (57)
SSM	7	5 (71)	4	3 (75)
NM	9	5 (56)	5	5 (100)

ALM: acral lentiginous melanoma; LMM: lentigo maligna melanoma; SSM: superficial spreading melanoma; NM: nodular melanoma.

melanomas, 10 metastatic melanomas and 10 naevi are summarized in Table I. There was no correlation between the positivity of KIF20A and the AJCC staging or histological types of melanoma. However, KIF20A expression was highly conserved in both advanced malignant melanoma samples and samples of metastatic lesions.

#### *Correlation between KIF20A expression and tumour thickness*

The tumour thickness of primary malignant melanomas was divided into 2 groups according to KIF20A expression detected by an immunohistochemical analysis (the results are shown in Fig. 4). Patients with stage 0 disease

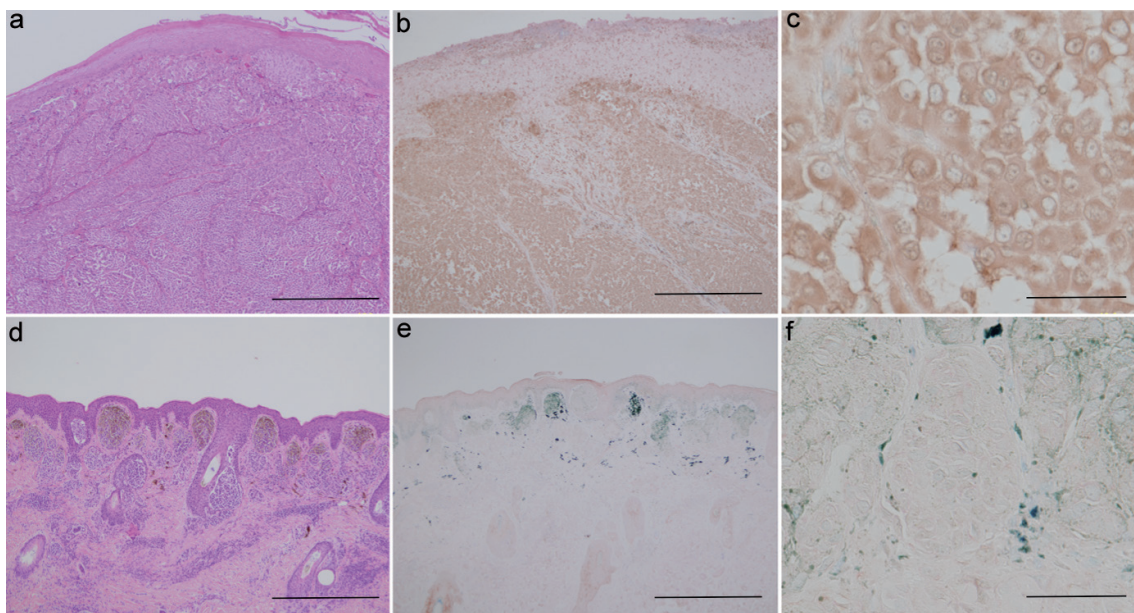


Fig. 2. Immunohistochemical staining for KIF20A. Representative immunohistochemical staining for KIF20A in primary malignant melanoma (a, b, c) and naevi (e, f, g). Haematoxylin and eosin staining showed atypical, mitotically-active malignant melanoma (nodular melanoma) (a:  $\times 40$ ). KIF20A immunohistochemistry (b:  $\times 40$ , c:  $\times 400$ ) showed homogenous cytoplasmic staining. Haematoxylin and eosin staining of a naevus (c:  $\times 40$ ) and KIF20A immunohistochemical staining in a corresponding normal section (e:  $\times 40$ , f:  $\times 400$ ). Melanin granules are indicated by blue staining, but these did not show KIF20A expression. Bars: 500  $\mu\text{m}$  (a, b, d, e), 50  $\mu\text{m}$  (c, f).



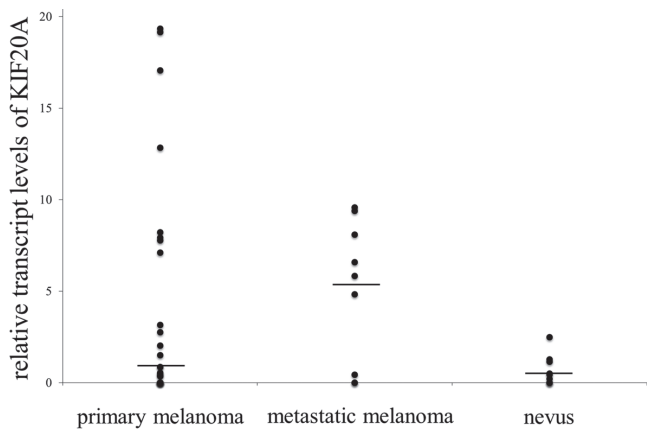


Fig. 3. Results of the quantitative reverse transcript (RT)-PCR analysis of primary melanoma, metastatic melanoma and naevus tissue samples. The results of the quantitative RT-PCR analysis of KIF20A expression (normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) in patients with primary melanomas ( $n=28$ ), metastatic melanomas ( $n=10$ ) and naevi ( $n=10$ ). Bars show median values.

were excluded from the study. There was a significant difference in the values between the 2 groups. The tumour thickness in the KIF20A-positive patients was greater than that in the patients who were negative for KIF20A. In addition, the median thickness of KIF20A-positive primary malignant melanoma was 3.2 mm, in contrast with 1.9 mm for KIF20A-negative patients.

Relapse-free survival

Fig. 5 shows the relapse-free survival, estimated by the Kaplan–Meier method, for the malignant melanoma

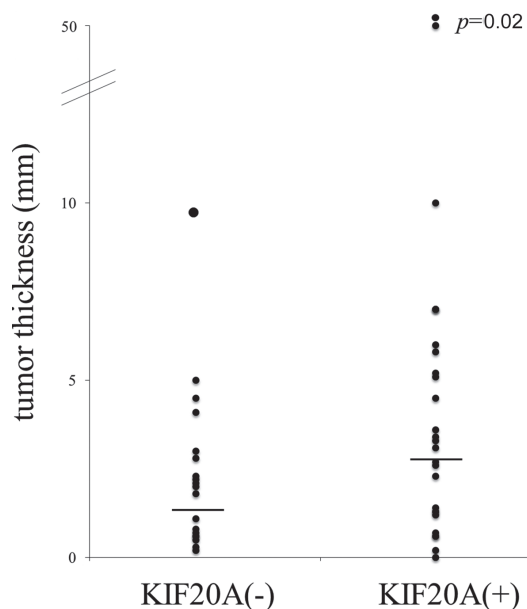


Fig. 4. Relationship between KIF20A expression and tumour thickness. Comparison of the tumour thickness between patients positive and negative for KIF20A by immunohistochemical staining. Bars show median values, which were 1.9 mm in KIF20A<sup>-</sup> and 3.2 mm in KIF20A<sup>+</sup> patients.  $p$ -values were determined by a Mann–Whitney  $U$  test.

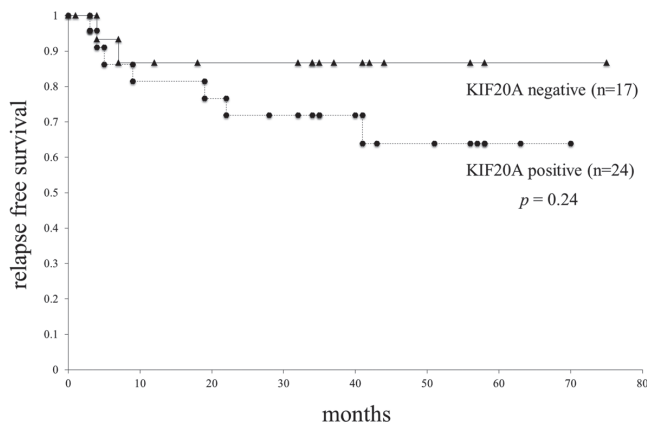


Fig. 5. Relationship between KIF20A expression and relapse-free survival. A comparison of the relapse-free survival between patients who were negative and positive for KIF20A expression. The curves are shown for patients who were positive and negative for KIF20A expression as determined by immunohistochemical staining.  $p$ -values were determined by log-rank test.

patients. The patients were divided into 2 groups based on the KIF20A expression of their primary melanoma, as determined by immunohistochemical staining. Probably because of the small number of samples, there were no significant differences between the 2 groups in their relapse-free survival ( $p=0.24$ ). However, patients with KIF20A positive melanoma tended to develop earlier recurrence.

DISCUSSION

This study is the first to demonstrate KIF20A expression in melanocytic lesions. We have shown that KIF20A was overexpressed in approximately 60% of primary malignant melanomas and metastatic melanomas in the RT-PCR and immunohistochemical analyses, but that there is a markedly lower rate of expression in benign melanocytic naevi. Naevus tissue expresses some KIF20A mRNA, but it leads to only low levels of KIF20A protein expression. It was revealed that the KIF20A expression levels are not absolutely specific for malignant melanoma, and can also be detected in naevus tissues, but that the positive rates are much lower than those in melanoma tissues. Previous studies have shown that HLA Class I molecules are downregulated in naevus tissue samples (13); therefore, naevi are isolated from adoptive immunity. As 60% of malignant melanomas expressed KIF20A, it would not be sufficient for all patients with melanoma to be treated with a KIF20A-targeted vaccine. In fact, we have demonstrated that immunotherapy employing multiple tumour-associated antigens (TAA) is more effective than that employing a single TAA (14). In future clinical trials, the use of multiple TAA-targeted immunotherapy should be considered.

As noted in the Introduction, KIF20A is thought to be involved in carcinogenesis. Therefore, KIF20A-targeted immunotherapy might inhibit cancer progres-

sion. KIF20A showed a lower expression rate than other melanocyte differentiation antigens, such as gp100 or tyrosinase (15, 16). However, it is likely that it would be more effective if we were to use immunotherapy against antigens related to carcinogenesis, such as KIF20A. Metastatic melanoma cells are known to escape from the immune response through various mechanisms, including the loss of the tumour-associated antigens. However, KIF20A was highly conserved in metastatic lesions (Table I). Furthermore, KIF20A was overexpressed in all of the histological types of melanoma (Table I).

In a previous study, it was reported that KIF20A was overexpressed in various cancers, including pancreatic cancer, bladder cancer, non-small-cell lung cancer and cholangiocellular carcinoma (7). The testes and thymus are the only normal tissues that overexpress KIF20A (7). Therefore, KIF20A could be considered as an ideal cancer-testis antigen, and the KIF20A peptide might be a "commodity cancer vaccine". In Japan, a clinical trial utilizing peptide vaccines including KIF20A has been conducted against pancreatic cancer. Our investigations may lead to a new therapeutic option for malignant melanoma targeting this antigen.

The progression of melanoma is defined according to its tumour-node-metastasis (TNM) classification. It has been shown that as the tumour thickness increases, there is a highly significant decrease in the 5- and 10-year survival rates ( $p < 0.0001$ ) (17). The 10-year survival was 92% among 11,841 patients with T1 melanomas (<1.00 mm thickness), while it was 80% in 8,046 patients with T2 melanomas (1.01–2.00 mm), 63% in 5,291 patients with T3 melanomas (2.01–4.00 mm), and 50% in 2,461 patients with T4 melanomas (>4.00 mm) (17). Since we found that there was a correlation between KIF20A expression and tumour thickness (Fig. 4), it can be assumed that KIF20A expression might be correlated directly or indirectly with the prognosis of melanoma patients. We also found that the patients with KIF20A expression tended to have a shorter relapse-free survival (Fig. 5). An analysis of a larger number of samples will be needed to clarify whether KIF20A can be a prognostic marker. Therefore, studies using a larger population are needed to elucidate whether a direct relationship exists between KIF20A and the prognosis of melanoma.

In summary, our present results revealed that KIF20A is a novel melanoma-associated antigen, and suggested the possibility that KIF20A could be a diagnostic and prognostic marker of melanoma. A KIF20A peptide vaccine may represent a potential novel treatment option for advanced malignant melanoma.

#### ACKNOWLEDGEMENTS

This work was supported by a JSPS Grant-in-Aid for Young Scientists (B) 23791280.

*The authors declare no conflicts of interest.*

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