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EZH2 and MMSET Were Identified as Potentially Useful Therapeutic Targets in Metaplastic Breast Carcinoma

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Abstract. *Background/Aim: Metaplastic breast carcinoma (MBC) is a rare malignancy, which is often triple-negative for the hormone receptors and human epidermal growth factor receptor 2, and thus, does not benefit from targeted therapy. In this study, we examined the expression of methylation and demethylation enzymes by immunostaining MBC and the adjacent normal tissues or triple-negative ductal carcinoma (TNDC), and identified alterations that may be used as therapeutic targets. Materials and Methods: We retrospectively studied surgical specimens from 15 patients who underwent surgery for MBC at Kanagawa Cancer Center between 2005 and 2016, and similarly from 14 patients with TNDC. The frequencies of high methylation/demethylation enzyme expression were compared among them. Results: The frequencies of high enhancer of zeste homolog 2 (EZH2) and multiple myeloma SET domain (MMSET) expression were significantly higher in both MBC and TNDC than in normal tissue. Conclusion: EZH2 and MMSET may be useful therapeutic targets in MBC.*

Metaplastic breast carcinoma (MBC) is a rare malignancy, accounting for 0.25% to 1% of all primary breast carcinomas (1). It is characterized by various combinations of adenocarcinoma with mesenchymal and epithelial components, such as squamous, chondroid, osseous, spindle-shaped, and polymorphic components (2-5). MBC is often triple-negative for the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (6), and thus, does not benefit from targeted antihormonal or anti-HER2 therapy. Compared with conventional triple-negative carcinomas, MBC is more often resistant to neoadjuvant chemotherapy (7), more likely to present with metastatic disease (8-10), and has a worse overall prognosis (7, 8, 11).

DNA is packaged as chromatin, which is composed of nucleosomes, in cells. Histones are the central component of the nucleosomal subunit, and form octamers containing the four core histone proteins (H3, H4, H2A, and H2B). A 147-base-pair segment of DNA is wrapped around each octamer. Each of the histone proteins has a characteristic side chain, a histone tail, which is densely populated with basic lysine and arginine residues. The histone tail undergoes cooperative post-translational modification to regulate the state of chromatin. Some post-translational modifications can alter the charge density between histones and DNA, which affects the organization of chromatin and the underlying transcription process, and are involved in regulating cell differentiation and proliferation. Furthermore, alterations in the patterns of post-translational histone modifications seem to play an important role in cancer development and proliferation. Post-translational histone modifications include acetylation, methylation, phosphorylation, and ubiquitination. Histone methylation has different effects on transcriptional control, depending on the position of the modified amino acid, and most methylation and demethylation enzymes modify amino acid residues at specific positions. Recently, the development of specific inhibitors of these modifying enzymes has advanced. Therefore, if characteristic methylation or demethylation enzyme expression patterns are identified in MBC, appropriate inhibitors may be used as selective therapeutic agents for the disease.

In this study, we examined the expression of methylation and demethylation enzymes by immunostaining MBC and the adjacent normal tissues or triple-negative ductal

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carcinoma (TNDC), and identified alterations that may be used as therapeutic targets.

Materials and Methods

Surgical specimens from 15 patients who underwent surgery for MBC at Kanagawa Cancer Center between 2005 and 2016 were studied retrospectively. We reviewed the archived hematoxylin and eosin (HE)-stained slides of the samples, selected suitable tissue blocks for immunohistochemical (IHC) analysis and then constructed tissue microarrays (TMA) using the MBC and the adjacent normal tissues. When various histological subtypes coexisted in an MBC, microarrays were created from each subtype. Similarly, TMA were created from 14 patients with TNDC, who were age- and cancer stage-matched with the MBC patients. All tissues were collected prior to the introduction of anticancer drug therapy.

IHC analysis. The TMA were cut into 4-μm-thick sections and mounted on pre-coated glass slides. IHC staining of the ER, PR, HER2, MLL2, MLL3, G9a, EZH2, MMSET, KMT4, JRID1A, LSD1, KDM6A, and KDM6B was performed in all cases, using an autostainer (Histostainer; Nichirei Biosciences Inc., Tokyo, Japan) and primary antibodies against ER (clone 1D5, dilution: 1:80; Nichirei Biosciences Inc., Tokyo, Japan), PR (clone A9621A, dilution: 1:100; Nichirei Biosciences Inc., Tokyo, Japan), HER2 (clone D8F12, dilution: 1:800; Cell Signaling Technology Inc., Danvers, MA, USA), MLL2 (dilution: 1:40; BETHYL Laboratories Inc., Montgomery, USA), MLL3 (dilution: 1:100; Biorbyt Ltd., Cambridge, UK), G9a (dilution: 1:100; Atlas Antibodies AB., Stockholm, Sweden), EZH2 (clone D2C9, dilution: 1:50; Cell Signaling Technology Inc., Danvers, MA, USA), MMSET (clone CL1063, dilution: 1:200; Atlas Antibodies AB., Stockholm, Sweden), KMT4 (dilution: 1:250; Abcam, Cambridge, UK), JRID1A (dilution: 1:200; Abcam, Cambridge, UK), LSD1 (clone C69G12, dilution: 1:500; Cell Signaling Technology Inc., Danvers, MA, USA), KDM6A (dilution: 1:500; Atlas Antibodies AB., Stockholm, Sweden), and KDM6B (dilution: 1:500; Gene Tex Inc., Los Angeles, CA, USA).

The results of the IHC analysis were evaluated by two researchers, a breast surgeon (the author) and a pathologist (K.K.). We examined each slide independently, discussed any unclear cases, and reached agreement. ER and PR staining were defined as positive if at least 1% of cell nuclei were stained. HER2 expression was defined as negative if HER2 score was 0 or 1+ in accordance with the guidelines of the American Society of Clinical Oncology/College of the American Pathologists (12). The expression of methylation and demethylation enzymes (MLL2, MLL3, G9a, EZH2, MMSET, KMT4, JRID1A, LSD1, KDM6A, and KDM6B) was evaluated by scoring the proportions of positively stained nuclei in cancer cells and normal cells, regardless of the staining intensity. Then, we classified them as follows: 1: <1/100 cells stained; 2: <1/10 cells stained; 3: <1/3 cells stained; 4: <2/3 cells stained; and 5: >2/3 cells stained. Scores of 4 or 5 were considered to indicate high expression. Representative MBC, adjacent normal and TNDC tissue sections stained with HE, EZH2 antibody and MMSET antibody are shown in Figure 1.

Statistical analysis. The frequencies of high MLL2, MLL3, G9a, EZH2, MMSET, KMT4, JRID1A, LSD1, KDM6A, and KDM6B expression in the normal and tumor tissues were compared using Fisher's exact test.

Table I. *Patient characteristics*.

Characteristics	MBC (15 cases)	TNDC (14 cases)
Age (years)		
Median age (range)	60 (43-85)	65 (35-78)
Estrogen receptor status		
Positive	0	0
Progesterone receptor status		
Positive	0	0
HER2 status		
Positive	0	0
Stage at presentation		
I	3 (20%)	0
II	9 (60%)	9 (64.3%)
III	3 (20%)	5 (35.7%)
IV	0	0

MBC: Metaplastic breast carcinoma; TNDC: triple-negative ductal carcinoma.

Results

The patients' characteristics are summarized in Table I. The median ages of the MBC patients and TNDC patients were 60 years (range=43-85 years) and 65 years (range=35–78 years), respectively (no significant difference). All cases belonged to the triple-negative type. Twenty percent of MBC cases and 35.7% of TNDC cases involved locally advanced cancer (Union for International Cancer Control stage III) at presentation (no significant difference).

In 15 cases of MBC, tissue samples were obtained from 22 MBC sites and 13 adjacent normal tissue sites. Different histological types were found in the same tumor in six cases (Table II). Even within the same tumor, the expression status of the modifying enzymes differed according to the histological type. Three histological types were present in one case (case 4). We constructed a TMA from each histological type. The most common histological type was squamous cell carcinoma (SCC) (50.0%), followed by matrix-producing carcinoma (13.6%) (Table III). Samples were not obtained from the adjacent normal tissues in two cases because there was not enough tissue to evaluate. Fourteen tissue samples were obtained from 14 TNDC patients.

The frequencies of the expression of high methylation/demethylation enzymes were compared between MBC and normal tissue, and between MBC and TNDC (Figure 2A). None of the examined tissues exhibited high MLL3 or JARID1A expression. In addition, none of the examined normal tissue samples displayed high EZH2, MMSET, KMT4, or KDM6A expression. The frequencies of high EZH2 and MMSET expression were significantly higher in both MBC and TNDC than in normal tissue, and were also higher in TNDC than in MBC. MLL2 and G9a were highly expressed more often in normal tissue than in MBC. The frequency of

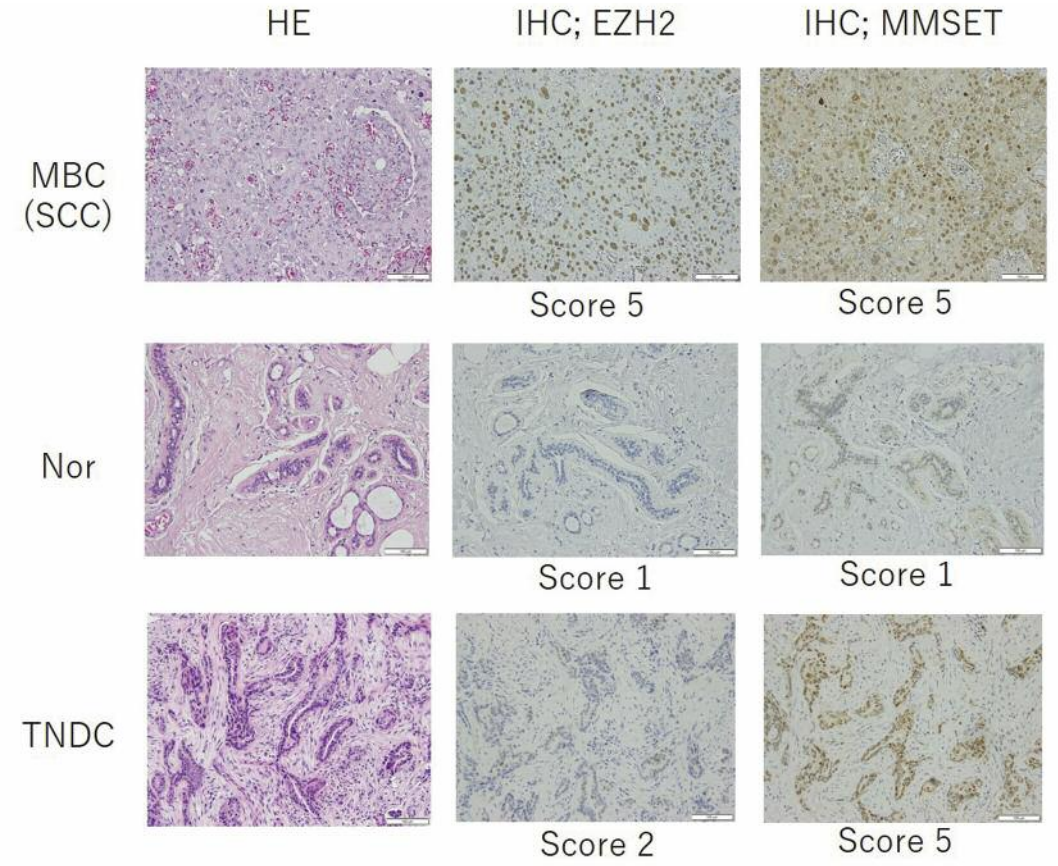

Figure 1. *Representative MBC, adjacent normal and TNDC tissue sections stained with HE, EZH2 antibody and MMSET antibody. (Original magnification, ×200). MBC: Metaplastic breast carcinoma; SCC: squamous cell carcinoma; Nor: adjacent normal tissue; TNDC: triple-negative ductal carcinoma; HE: hematoxylin and eosin stain; IHC: immunohistochemical stain.*

high KMT4 expression was significantly higher in TNDC than in MBC and normal tissue. KDM6A was not highly expressed in TNDC or normal tissue, whereas 22.7% of MBC exhibited high KDM6A expression, but there was no significant difference in the frequency of high KDM6A expression between the various tissue sample types.

The most common histological type of MBC was SCC (11 cases). The SCCs were compared with normal and TNDC tissue samples (Figure 2B). In addition to the above results, we found significant differences in the frequency of high KDM6A expression between SCC and normal tissue/TNDC.

Discussion

Currently, there is no standard treatment for MBC, and new molecular targeted therapies and novel treatment options are urgently needed. In this study, we found that the frequencies of high EZH2 and MMSET expression were significantly higher in MBC than in normal tissue.

EZH2 is a well-known histone modifier protein, which functions as a methyltransferase at H3K27 (lysine 27 of histone H3)(13). EZH2 is a member of the polycomb group of genes (14), which plays an important role in transcriptional regulation, involving chromatin remodeling, nucleosome modification, and interactions with other transcription factors. EZH2 has been demonstrated to be overexpressed in many types of malignancy, including breast, prostate, and endometrial cancer, and has been suggested to be a potential treatment target (15, 16). In primary breast cancer, Kleer *et al*. (15) showed that EZH2 overexpression was further associated with a larger tumor size, an ER- and PR-negative status, an advanced stage of disease, and significantly reduced disease-free survival and overall survival. Other investigators have reported that EZH2 promotes neoplastic progression in the breast, and that the downregulation of EZH2 expression reduces the *in vivo* growth of breast cancer (17, 18). In the current study, the frequency of high EZH2 expression was significantly higher not only in MBC, but also in TNDC; therefore, EZH2 may be a useful therapeutic target for both MBC and TNDC. Several inhibitors of EZH2, such as GSK126 (19), EPZ005687 (20), and EPZ6438 (21), have been developed.

Table II. *MBC cases in which different histological types were seen in the same case.*

	Histology	MLL2	MLL3	G9a	EZH2	MMSET	KMT4	JARID1A	LSD1	KDM6A	KDM6B
Case 1	Squamous	Low	Low	High	High	Low	Low	Low	High	Low	High
	Chondroid	Low	Low	Low	Low	Low	Low	Low	High	Low	Low
Case 2	Squamous	Low	Low	High	High	Low	Low	Low	High	High	High
	Adenosquamous	Low	Low	High	Low	Low	Low	Low	Low	Low	Low
Case 3	Spindle-shaped	Low	Low	Low	Low	Low	Low	Low	High	Low	High
	Squamous	Low	Low	Low	Low	Low	Low	Low	High	Low	High
Case 4	Pleomorphic	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low
	MPC	Low	Low	Low	Low	High	Low	Low	High	High	High
	Squamous	Low	Low	High	Low	High	Low	Low	High	High	High
Case 5	Adenosquamous	Low	Low	High	Low	Low	Low	Low	High	Low	High
	Squamous	Low	Low	High	High	Low	Low	Low	High	High	High
Case 6	Spindle-shaped	Low	Low	High	High	High	Low	Low	Low	Low	High
	Pleomorphic	Low	Low	High	High	High	Low	Low	High	Low	High

MBC: Metaplastic breast carcinoma; MPC: matrix-producing carcinoma.

The frequency of high MMSET expression was also higher in MBC and TNDC than in normal tissue. Asangani *et al*. reported that MMSET and EZH2 are coexpressed at high levels in cancers in which the oncogenic functions of EZH2 require MMSET activity (22). MMSET, which is also known as Wolf-Hirschhorn syndrome candidate 1 (WHSC1) or nuclear receptor-binding SET domain 2 (NSD2), is a member of the NSD histone methyltransferase family, which also includes NSD1 and NSD3 (23-25). NSD2 usually functions as a transcriptional repressor that mediates the dimethylation and trimethylation of H3K36 and the demethylation of H4K20 (26, 27). Several studies have reported that NSD2 is overexpressed in some types of human cancer, such as neuroblastoma, stomach cancer, colon cancer, small-cell lung cancer, and osteosarcoma (28). MMSET overexpression is associated with tumor aggressiveness (29). We consider that MMSET may also be a useful therapeutic target in MBC and TNDC. A number of MMSET inhibitors have been developed (30, 31).

In the current study, there was no significant difference in KDM6A expression between MBC and normal tissue or TNDC, but when we focused solely on MBC with an SCC histology, we found that high KDM6A expression was significantly more common in such tumors than in normal tissue and TNDC.

KDM6A may be a characteristic marker of SCC. KDM6A is a histone demethylase, which targets di- and trimethylated histone H3 lysine 27 (H3K27)(32). KDM6A was first reported as a highly mutated histone H3K27 demethylase in a survey of different human cancers and cancer cell lines, including acute myeloid leukemia, bladder carcinoma, breast cancer, chronic myeloid leukemia, colorectal adenocarcinoma, endometrial adenocarcinoma, and glioblastoma (33). EZH2 inhibitors are more effective in cell lines and mouse models exhibiting loss of KDM6A function (34). In the present study, many cases of TNDC displayed high EZH2 expression and low KDM6A expression, and EZH2 inhibitors may be effective in such cases. However, EZH2 inhibitors may be ineffective in cases of SCC, in which KDM6A is highly expressed simultaneously with EZH2.

Table III. *Metaplastic carcinoma components.*

Histology	Number of cases
Squomous	11 (50.0%)
Matrix-producing	3 (13.6%)
Adenosquamous	2 (9.1%)
Spindle-shaped	2 (9.1%)
Pleomorphic	2 (9.1%)
Chondroid	1 (4.5%)
Myoepithelial	1 (4.5%)

MLL2, like MLL3, is considered to be involved in the development of cancer (through the loss of its function) (35). It has also been reported that higher MLL2 and MLL3 expression is associated with a better prognosis in lung cancer (36). MLL2, which is also known as KMT2D (histone-lysine N-methyltransferase 2D), belongs to a family of mammalian histone H3 lysine 4 (H3K4) methyltransferases and represses genes in certain cell types, leading to the inhibition of cell growth (37). In the present study, there were few cases of MBC that exhibited high MLL2 expression. Although the low MLL2 expression seen in MBC is not observed in normal tissues or TNDC, MLL2 cannot be a therapeutic target because it is difficult to therapeutically target loss-of-function tumor suppressors directly.

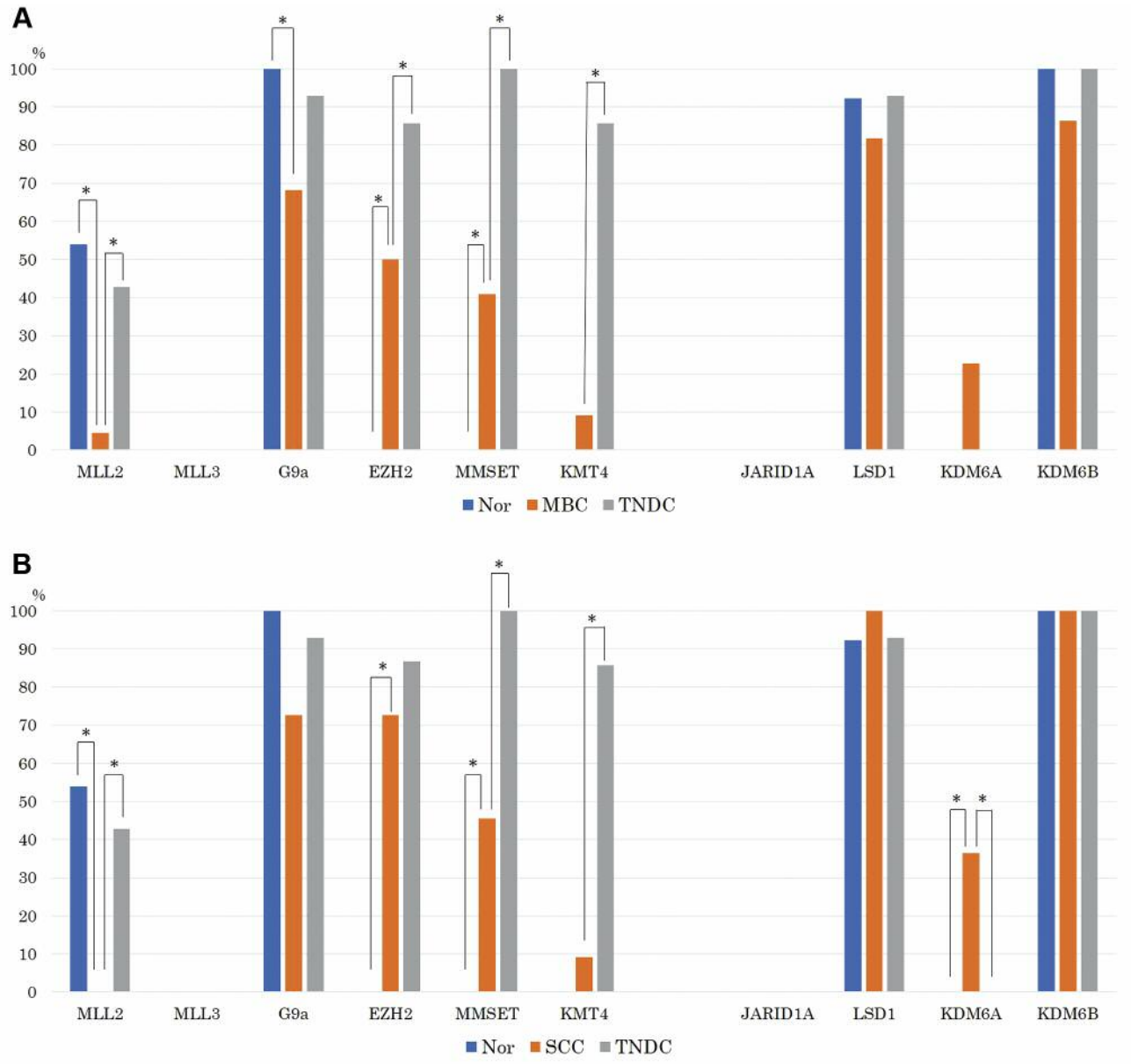

Figure 2. *Frequencies of high methylation/demethylation enzyme expression. A) MBC vs. normal or TNDC tissue. B) MBC with SCC histology vs. normal or TNDC tissue. Nor: Normal tissue; MBC: metaplastic breast carcinoma; TNDC: triple-negative ductal carcinoma; SCC: squamous cell carcinoma; *: statistically significant difference.*

Conclusion

In this study, the frequencies of high EZH2 and MMSET expression were significantly higher in MBC and TNDC than in normal tissue. These molecules may be useful therapeutic targets in MBC.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

Hirotaka Nakayama, Nobuyasu Suganuma, Yohei Miyagi, Yasushi Rino, and Munetaka Masuda designed the study. Hirotaka Nakayama performed clinical, pathological and statistical investigation, and drafted the manuscript. Kae Kawachi participated in the histological and immunohistochemical evaluation. Hirotaka Nakayama, Kae Kawachi, Yoshiyasu Nakamura, and Yohei Miyagi constructed TMAs. Yoshiyasu Nakamura performed the immunohistochemical staining. Nobuyasu Suganuma, Tatsuya Yoshida, Toshinari Yamashita, Takashi Yamanaka, Yuka Matsubara, Kaori Kohagura, and Soji Toda assisted the clinical investigation.

Nobuyasu Suganuma, Yohei Miyagi, Yasushi Rino, and Munetaka Masuda participated in preparing and drafting the manuscript. All Authors read and approved the final manuscript.

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