SPANDIDOS PUBLICATIONS

Minichromosome maintenance 2 is an independent predictor of survival in patients with lung adenocarcinoma

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Abstract. Minichromosome maintenance (MCM) protein deregulation is associated with tumor formation, progression and malignant transformation. MCM2 is frequently expressed during premalignant lung cell proliferation and is a sensitive marker for the early detection of pulmonary malignant lesions. The present study was undertaken to investigate whether MCM2 expression is of clinical and prognostic value in patients who have undergone lung adenocarcinoma resection. Between January 2009 and December 2010, 102 consecutive patients underwent complete pulmonary resection (involving lobectomy or more extensive resection) for lung adenocarcinoma at St. Marianna Medical University Hospital (Kanagawa, Japan). Among those, 73 patients, who had a final pathological diagnosis of lung adenocarcinoma measuring ≥10 mm, were enrolled in the present study. High MCM2 expression was found in 35 patients (48.0%). Univariate analysis of the overall survival (OS) revealed that pathological stage and MCM2 expression were significant prognostic factors in lung adenocarcinoma (P<0.001 and P<0.002, respectively). Univariate analysis of the recurrence-free survival (RFS), the significant prognostic factors included pathological stage, EGFR mutation status and MCM2 expression (P<0.001, P<0.034 and P<0.003, respectively). On multivariate survival analysis, high MCM2 expression and pathological stage II-III were identified as independent strong prognostic factors (OS: HR=5.084, 95% CI: 1.715-15.080, P=0.003; RFS: HR=2.761, 95% CI: 1.090-6.998, P=0.032). Therefore, the findings of the present study demonstrated that MCM2 may serve as a potential biomarker and therapeutic target for lung adenocarcinoma.

Introduction

Minichromosome maintenance (MCM) proteins are DNA helicases essential for DNA replication and participate in eukaryotic DNA replication by binding to chromatin before the initiation of DNA replication from the early G1 phase to the S phase of the cell cycle (1). Hence, MCM protein deregulation has been linked to tumor formation, progression and malignant transformation. MCM2, a member of the MCM family (MCM2-MCM7) that comprises 904 amino acids and has a molecular mass of 102 kDa, has been shown to be a cell proliferation marker (2).

MCM proteins have been shown to promote cell proliferation *in vitro* and *in vivo* in several types of cancer, and MCM expression is implicated in cancer cell proliferation mainly because these proteins can enhance DNA replication. MCM2 and MCM7, in particular, are involved in various cellular functions in humans and other eukaryotes (3). MCM7 expression in the bronchial brushing of patients with non-small cell lung cancer (NSCLC) has been found to be associated with prognosis (4), whereas MCM2 is frequently expressed during premalignant lung cell proliferation and is reportedly a sensitive marker for the early detection of pulmonary malignant lesions. MCM expression has also been associated with higher cell proliferation in non-dysplastic squamous epithelium, malignant fibrous histiocytomas and endometrial carcinoma. Furthermore, MCM2 expression has been found to be associated with a higher mitotic index and worse survival in patients with breast cancer, colon cancer, adrenocortical carcinoma and gliomas (5-7).

We previously identified the molecular signatures for malignancy in small cell lung cancer (SCLC), which is characterized by highly aggressive clinical features and poor prognosis, and detected the following five tumor proliferation proteins involved in SCLC malignancy: MCM2, MCM4,

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MCM6, MCM7 and MutS homolog 2 (MSH2) (1). Hence, the aim of the present study was to investigate whether MCM2 expression is of clinical and prognostic value in patients who have undergone resection of lung adenocarcinoma.

Materials and methods

Patients. Between January 2009 and December 2010, 102 patients with lung adenocarcinoma underwent complete pulmonary resection at St. Marianna Medical University Hospital (Kanagawa, Japan). Complete pulmonary resection was defined as lobectomy or a more extensive lung resection with ipsilateral hilar and mediastinal lymph node dissection and no evidence of residual cancer, either macroscopically or microscopically. Patients who had received preoperative chemotherapy, radiotherapy, or both, were excluded. Ultimately, 73 patients with a final pathological diagnosis of lung adeno-carcinoma measuring ≥10 mm were enrolled in the present study.

The following clinicopathological data were collected from the hospital charts of all patients: Age, sex, pathological T factor, histological type, surgical procedure(s), relapse type, EGFR mutation status, overall survival (OS) and recurrence-free survival (RFS). OS was defined as the time from the day of the surgery until the day of death from any cause; patients who remained alive at the end of the follow-up period were treated as censored cases. RFS was defined as the time from the day of surgery to the day of disease recurrence or death from any cause; patients who remained alive with no evidence of recurrence at the end of the follow-up period were treated as censored cases.

Preoperative evaluation included physical examina-tion, blood examination, chest radiography and chest and abdominal CT, as well as brain CT or MRI and positron emis-sion tomography (PET)-CT if clinically indicated. Staging and pathological findings for lung adenocarcinoma were based on the 7th TNM Classification for Lung and Pleural Tumors (8), the World Health Organization classification (9), and the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification of lung adenocarcinoma (10).

Following pulmonary resection, all patients were followed up at our institute every 3 months during the first year 1, every 6 months during years 2-5 and annually thereafter as outpatients; the follow-up continued for ≥5 years. The follow-up included physical examination, chest radiography and blood examination, including serum tumor markers such as carcinoembryonic antigen. Patients also routinely underwent chest and upper abdominal CT at every scheduled outpatient appointment. Brain MRI and/or PET-CT examina-tions were performed if any symptoms or signs of recurrence were detected. If metastasis was observed, the metastatic site was confirmed histologically or cytologically when clini-cally feasible. Additionally, EGFR-TKIs were administered to 11 patients with disease recurrence, including gefitinib in 10 cases, erlotinib in 4 cases, afatinib in 3 cases and osimer-tinib in 1 case.

Clinical samples were tested using cobas® EGFR Mutation Test version 2.0 (Roche Diagnostics) or the peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp method

(LSI Medience Corporation). Deletion mutations in exon 19 and point mutations in exon 18 or exon 21(L858R) were considered to be EGFR mutations in the present study.

MCM2 expression. All collected surgical specimens were fixed in 10% formalin for 24 h at room temperature and then embedded in paraffin. The representative samples were routinely stained with hematoxylin for 20 min at room temper-ature and eosin for 1 min at room temperature by experienced pathologists, followed by reviewing to confirm the inclusion of carcinoma cells. Next, the formalin-fixed, paraffin-embedded blocks were cut into serial 3-μm sections for immunohis-tochemistry (IHC). All assays were performed using the automated Histostainer 36 A immunostainer (Nichirei Biosciences Inc.) according to the manufacturer's instructions. In addition, the sections were incubated for 60 min at room temperature with anti-MCM2 monoclonal antibody (1:2,000; cat. no. ab4461; Abcam) and examined by IHC; specificity was confirmed by using large-cell neuroendocrine carcinoma as a positive control according to the expression data in the Human Protein Atlas database (https://www.proteinatlas.org/).

To evaluate MCM2 antibody immunoreactivity, five microscopic, high-power fields (magnification, x400) were randomly selected using a BX51 polarizing microscope (Olympus Corporation); each field was scored a positive rate calculated as the percentage of positive cells per total number of cells. Considering that MCM2 is expressed in the nucleus, the number of nucleus-positive cells was counted. Three inde-pendent evaluators, including a pathologist certified by the Japanese Society of Pathology, assessed the cells for immu-noreactivity and calculated the average value. The expression levels of MCM2 were assessed using the labeling index, which was determined by counting the number of distinctly stained malignant cells, regardless of the staining intensity, divided by the total number of tumor cells.

Statistical analysis. The OS and PRS were estimated using the Kaplan-Meier method, and differences in survival rates were determined by log-rank analysis. Univariate analysis was performed among different groups. The χ^2 test and Mann-Whitney U test were used for analyzing the categorical variables and identifying differences between two groups, respectively. For multivariate analysis, the Cox proportional hazards model with significant factors identified by the univar-iate analysis was used to determine the association between survival and potential prognostic factors. All P-values were two-sided, and P<0.05 was considered to indicate statistically significant differences.

All statistical data were analyzed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R, and a modified version of R commander that adds statistical functions frequently used in biostatistics (R Foundation for Statistical Computing, Vienna, Austria).

Results

Association between patient characteristics and MCM2 expression. The baseline characteristics of the 73 patients and the association between MCM2 expression and

Table I. Baseline characteristics of patients (n=73) and association between MCM2 expression and clinicopathological factors.

Variables	No. of patients	MCM2 expression[b]		P-value
		High (n)	Low (n)	
Sex				0.557
Male	36	20	16	
Female	37	17	20	
Age, years (mean, 70; range, 33-84)				0.727
≥70	37	20	17	
<70	36	17	19	
Smoking status				0.564
Current or former	40	22	18	
Never	33	15	18	
Adenocarcinoma subtypes				0.017[a]
In situ	2	0	2	
Minimally invasive	2	0	2	
Lepidic-predominant	8	0	8	
Papillary-predominant	19	10	9	
Acinar-predominant	29	17	12	
Solid-predominant	9	7	2	
Micropapillary-predominant	3	2	1	
Pathological stage				0.078
pIAB	53	23	30	
pII-III	20	14	6	
T factor (mean diameter, 23 mm; range, 10-74 mm)				0.362
T1	45	21	24	
T2	23	12	11	
T3-4	5	4	1	
N factor				0.020[a]
N0	56	25	31	
N1	4	1	3	
N2	13	11	2	
EGFR status				0.397
Positive	33	16	17	
Negative	35	17	18	
Unknown	5	4	1	
Distant recurrence				0.024[a]
Yes	15	12	3	
No	58	25	33	

[a]Statistically significant difference by Yates' correction; [b]30% cut-off was used to assign patients to the high- or low-expression groups, as ≥30% MCM2 expression in patients with resected lung adenocarcinoma served as the full cut-off value in the survival analysis. MCM2, minichromosome maintenance 2.

clinicopathological factors are presented in Table I. According to the IHC assessment of MCM2 expression in lung adenocarcinoma tissues (Fig. 1), 35 (48.0%) of the 73 patients exhibited high MCM2 expression levels. High MCM2 expression was statistically significantly associated with lung adenocarcinoma subtypes (P=0.0051), N factor (P=0.0117) and distant recurrence (P=0.0186). MCM2 is differentially expressed among different subtypes (lepidic-, acinar- and papillary-predominant)

of lung adenocarcinoma, with the lowest expression levels observed in lepidic-predominant lung adenocarcinoma (Fig. 1D).

Survival analysis and prognostic factors. The median follow-up time was 69 months (range, 6-120 months). To investigate the prognostic impact of MCM2 expression in patients with resected lung adenocarcinoma, patients were

Table II. Association between OS or RFS and MCM2 expression.

MCM2 expression			OS			RFS		
Positive (%)	Positive (n)	Negative (n)	P-value	HR	95% CI	P-value	HR	95% CI
15	60	13	0.237	2.399	0.562-10.240	0.677	1.254	0.431-3.644
20	50	23	0.040[a]	3.563	1.059-11.990	0.035[a]	3.143	1.082-9.130
25	46	27	0.012[a]	4.702	1.397-15.830	0.008[a]	4.204	1.447-12.220
30	37	36	0.001[a]	5.697	1.936-16.760	0.003[a]	3.745	1.568-8.944
35	31	42	0.001[a]	4.672	1.840-11.870	0.009[a]	2.873	1.299-6.356
40	26	47	0.002[a]	4.008	1.697-9.465	0.010[a]	2.784	1.283-6.043

[a]Statistically significant difference. OS, overall survival; RFS, recurrence-free survival; MCM2, minichromosome maintenance 2.
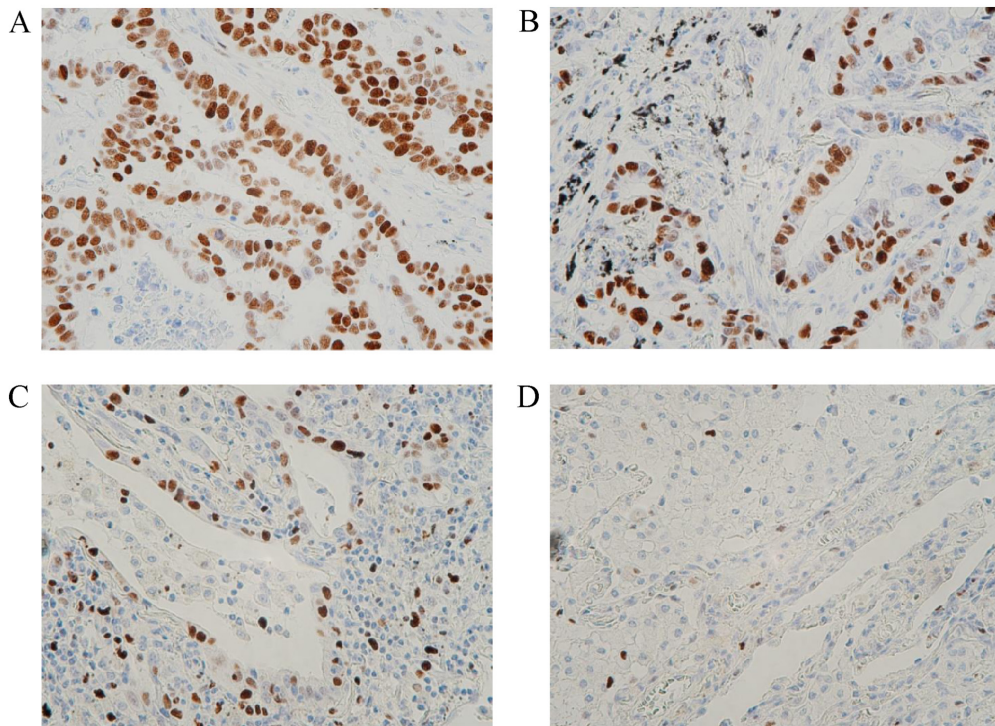

Figure 1. Expression status of MCM2 in lung adenocarcinoma tissue samples. (A-D) Representative immunohistochemistry microphotographs of MCM2 with (A) 90%, (B) 54%, (C) 33% and (D) 9% expression in lung adenocarcinoma tissues. MCM2, minichromosome maintenance 2. Magnification, x400.

grouped according to MCM2 expression (%) as follows: Groups 1 (<15 vs. ≥15), 2 (<20 vs. ≥20), 3 (<25 vs. ≥25), 4 (<30 vs. ≥30), 5 (<35 vs. ≥35) and 6 (<40 vs. ≥40). Group 4 exhibited the most significant difference in MCM2 expression in terms of the OS. Therefore, ≥30% MCM2 expression in patients with resected lung adenocarcinoma served as the cutoff value in survival analysis. The results of the comparisons among groups are summarized in Table II.

Patients with high MCM2 expression had significantly worse 5-year OS and 5-year RFS compared with those with low MCM2 expression (OS: 54.1 vs. 86.3%, respectively, P<0.001, Fig. 2A; RFS: 53.9 vs. 81.7%, respectively, P=0.001, Fig. 2B). Of the 11 patients with stage IB lung adenocarcinoma, 2 (18.2%) received postoperative adjuvant therapy with oral uracil-tegafur. Furthermore, 10 (50.0%) of 20 patients with stage II-III lung adenocarcinoma received intravenous

platinum doublet-based chemotherapy. However, 27 patients relapsed, of whom 17, 7 and 1 patients received standard anti-cancer therapy (according to the guidelines published by the Japan Lung Cancer Society in 2016; https://www.haigan.gr.jp/), EGFR-tyrosine kinase inhibitors and immune checkpoint inhibitors, respectively. In addition, 16 (59.3%), 8 (29.6%) and 4 (14.8%) patients received first-, second- and third-line therapies, respectively, but 6 (22.2%) did not receive treatment upon disease recurrence.

As mentioned above, univariate and multivariate survival analyses were performed to identify the potential prognostic factors for OS and RFS (Table III). Univariate analysis revealed that the significant prognostic factors for the OS of lung adenocarcinoma included pathological stage and MCM2 expression (P<0.001 and P<0.002, respectively), whereas those for the RFS included pathological stage, EGFR mutation

Table III. Univariate and multivariate survival analyses in patients with lung adenocarcinoma.

Variables	Univariate analysis			Multivariate analysis		
	P-value	HR	95% CI	P-value	Risk ratio	95% CI
OS						
Age ≥70 years (vs. <70)	0.089	2.114	0.893-5.002	-	-	-
Male sex (vs. female)	0.247	1.641	0.710-3.793	-	-	-
Smoking (vs. no)	0.909	0.960	0.480-1.923			
pStage I (vs. II-III)	<0.001[a]	7.249	3.054-17.210	<0.001[a]	6.688	2.783-16.070
EGFR mutation-positive (vs. negative)	0.8791	1.07	0.445-2.574	-	-	-
MCM2 high (vs. low)[b]	0.002[a]	5.697	1.936-16.760	0.003[a]	5.084	1.715-15.080
RFS						
Age ≥70 years (vs. <70)	0.300	1.514	0.691-3.318	-	-	-
Male sex (vs. female)	0.992	1.004	0.472-2.137	-	-	-
Smoking (vs. no)	0.550	0.824	0.436-1.556			
pStage I (vs. II-III)	<0.001[a]	6.728	3.017-15.000	0.001[a]	4.115	1.731-9.778
EGFR mutation-positive (vs. negative)	0.034[a]	2.491	1.074-5.778	0.055	2.304	0.981-5.409
MCM2 high (vs. low)[b]	0.003[a]	3.745	1.568-8.944	0.032[a]	2.761	1.090-6.998

[a]Statistically significant difference; [b]30% cut-off was used to assign patients to the high- or low-expression groups, as ≥30% MCM2 expression in patients with resected lung adenocarcinoma served as the full cut-off value in the survival analysis. OS, overall survival; MCM2, minichromosome maintenance 2; RFS, recurrence-free survival.
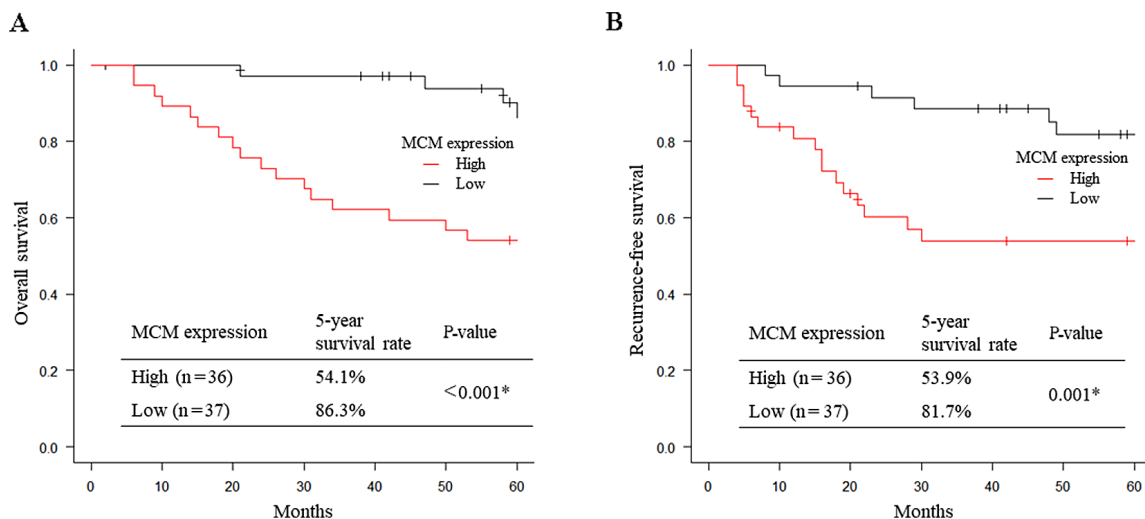

Figure 2. Association of MCM2 with OS and RFS in patients with adenocarcinoma. Kaplan-Meier curves showing the association between the MCM2 expression status in (A) OS and (B) RFS. All P-values were determined by the log-rank test and are shown in the graph. *Statistically significant difference OS, overall survival; RFS recurrence-free survival; MCM2, minichromosome maintenance 2.

status and MCM2 expression (P<0.001, P<0.034 and P<0.003, respectively). On multivariate survival analysis, high MCM2 expression (pathological stage II-III) was found to be a strong prognostic factor and was independent of and superior to the EGFR mutation status (OS: HR=5.084, 95% CI: 1.715-15.080, P=0.003; RFS: HR=2.761, 95% CI: 1.090-6.998, P=0.032).

Discussion

MCM proteins serve as biological markers of dysplasia and malignancy (11). Although MCM proteins promote cancer cell proliferation, MCM expression is a poor prognostic marker in several types of cancer, such as breast cancer, renal cell carcinoma, prostate cancer, colorectal cancer, lung cancer, glioma and cervical cancer (4-6,12-15). According to the protein-protein interaction network analysis of 201 proteins as previously reported (1), SCLC is functionally characterized by molecular pathway activation for spliceosome, RNA transport, and DNA replication and the cell cycle. In particular, the following 11 proteins have been identified as SCLC-specific proteins: MCM2, MCM4, MCM6, MCM7 and MSH2 for tumor proliferation; regulator of chromosome condensation 2,

coronin-1C, chromodomain helicase DNA-binding protein 4 and importin 9 for metastasis; and phosphoglycerate dehydrogenase and thymidine phosphorylase for cancer metabolism. For both SCLC and NSCLC, MCM2 is a potentially poor prognostic factor according to an online Kaplan-Meier survival analysis (1). Therefore, the present study was focused on MCM2 expression in resected NSCLC tissues and performed further survival analysis.

In our analysis of the correlation between MCM2 expression and clinicopathological characteristics of patients with resected lung adenocarcinoma, those with a history of smoking, male sex, or late-stage cancer exhibited significantly high MCM2 expression levels. Similarly, MCM2 has been found to be significantly associated with tumor stage in patients with lung cancer (16), and high MCM2 expression in NSCLC is more frequently observed among elderly patients and patients with late-stage disease (17). Overall, high MCM2 expression is linked to aggressive progression in the majority of cancer types.

Moreover, multivariate analysis of the OS showed that MCM2 expression may serve a biomarker and an independent factor of unfavorable prognosis, consistent with previous studies (17,18). As regards RSF, high MCM2 expression in lung adenocarcinoma was shown to be associated with early metastasis recurrence (Fig. 2B), particularly distant metastasis (Table I). These results provide useful information for thoracic surgeons and medical oncologists and for postoperative follow-up management.

MCM proteins, which are highly conserved helicases and key regulatory components of eukaryotic DNA replication, are overexpressed in several cancer types, and are therefore considered as important targets of chemotherapy. The potential of MCMs as a therapeutic target has been previously reported. Suppression of MCM complexes was found to sensitize pancreatic cancer cells to cytotoxic chemotherapy agents (gemcitabine and 5-FU) and enhance treatment efficacy (19). MCM7 was found to play an important role in cell proliferation by promoting quiescence-reactivated proliferation and paclitaxel resistance (20). In addition, MCM2 may serve as a functional target for long non-coding RNAs and microRNAs in regulating tumor cell proliferation, migration and apoptosis (21,22). Moreover, the anti-hyperlipidemic agent lovastatin, as an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A has demonstrated antitumor activity by silencing MCM2, triggering G1/S arrest in NSCLC cells (23). However, further research into targeting MCM is required.

There were certain limitations to the present study. First, this study was retrospective, rather than prospective or multicenter; therefore, bias might exist. Second, the sample size was small and possibly inadequate for obtaining reliable results. Third, the postoperative observation period was relatively brief. Further studies with a larger cohort are necessary to confirm our results. Finally, no data on CD56 positivity in the tumor cells were assessed in this study to evaluate neuroendocrine tumor characteristics in association with high MCM2 expression.

In conclusion, MCM2 overexpression in patients with lung adenocarcinoma appears to be an independent factor for an unfavorable prognosis in terms of OS and RFS. Therefore, MCM2 may represent a potential biomarker and therapeutic target for such patients.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HSakai, first author, and HSaji, corresponding author, served as the principal authors, performed data acquisition and had full access to all the data in the study, and are responsible for confirming the integrity and authenticity of the data and the accuracy of the data analysis. HSakai and HSaji contributed to study conception and design, coordination of the study and revised the article for important intellectual content. HK, TM, HM, NF, KK and KO enrolled patients and contributed to preparing the manuscript. MC, JK, KF, TN and HN assessed the cells for immunoreactivity and contributed to preparing the manuscript. All the authors have read and approved the final version of the manuscript to be published.

Ethics approval and consent to participate

The Institutional Review Board of the St. Marianna Medical University in Kanagawa, Japan approved this study (accession no. 1461). Prior to the study, written informed consent was obtained from all participants. All patient data remained confidential throughout this research.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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