Original Article

Significance of a calcium-binding protein S100A14 expression in colon cancer progression

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Background: The S100 proteins are characterized by two distinct EF-hand calcium-binding motifs. Several members of the S100 family are involved in cancer progression. S100A14, a member of the S100 family, has been cloned and reported in colon-specific microarray databases. In this study, we assessed the biological and functional significance of S100A14 in normal colon and in colon cancer.

Methods: The expression of S100A14 in normal colon and colon cancer was assessed. We used *in situ* hybridization, reverse transcription polymerase chain reaction (RT-PCR), cell migration assay, cell growth assay, western blotting, and immunohistochemical analysis. Clinicopathological examinations were also performed.

Results: *S100A14* is expressed in normal colon epithelium and several colon cancer cell lines. However, it is not expressed in SW480 cells. S100A14-overexpressing SW480 cells were established by stable transfection. Interestingly, a decrease in cell motility was observed, as determined using two independent migration assays. S100A14-overexpressing SW480 cells showed increased E-cadherin levels but decreased ability to form colonies in soft agar. Moreover, S100A14 expression was decreased in 95 of 154 human colon cancers, and lower S100A14 expression led to worse prognoses.

Conclusions: Our results suggest that S100A14 is involved in cell motility, adhesion, and growth changes in colon cancer and that its expression is inversely correlated with colon cancer progression. S100A14 expression in colon cancer is a useful prognostic marker.

Keywords: S100A14; calcium-binding protein; S100 family; colon cancer; cell motility

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Introduction

The S100 proteins are distinguished by two different EF-hand calcium-binding motifs with different affinities. There are approximately 20 human S100 protein family members, which display unique cell- and tissue-specific expression patterns and different functions (1). In addition, genes encoding more than 15 of these S100 proteins are located in a cluster on human chromosome 1q21 (2). Several S100 proteins are involved in cellular activities, such as cell differentiation, cell cycle progression, signal

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Table 1 Relation of S100A14 expression and various prognostic factors in 154 patients with colon cancer

S100A14	Total no.	Positive	Negative	P value
Age (years)				0.808
≤60	79	31	48	
>60	75	28	47	
Gender				0.372
Female	67	23	44	
Male	87	36	51	
Tumour status				0.456
T1	19	9	10	
T2	33	14	19	
T3	74	25	49	
T4	28	10	18	
Nodal status				0.032
N0	75	36	39	
N1	37	13	24	
N2	42	10	32	
Pathological stage				0.052
I	36	18	18	
II	39	18	21	
III	79	23	56	
Differentiation				0.890
Well	36	15	21	
Moderately	105	39	66	
Poorly	13	5	8	
Total	154	59	95	

transduction, transcription, and cell migration (1), and have garnered attention owing to their involvement in several human diseases such as acute inflammatory lesions, cardiomyopathy, Alzheimer's disease, rheumatoid arthritis, and cancer (3,4).

S100A4 has been shown to be upregulated in several malignant tumors (5), and it is involved in cell migration (6,7). The overexpression of S100A4 leads to poor prognoses of colon and breast cancers (8,9). In addition, S100A6 is related to several kinds of malignant neoplasm (10,11). In contrast, *S100A2* has been described as a potential tumor suppressor gene that is downregulated in several kinds of tumor (12), and S100A2 reduces the migration of cancer cells (13).

S100A14, located on human chromosome 1q21 and consists of 4 exons and 3 introns, spanning 2,165 bp, has been cloned by analyzing a human lung cancer cell line (14). The complementary DNA (cDNA) of the open reading frame is 315-bp long and is predicted to encode 104 amino acids. The S100A14 sequence contains two Ca^{+}-binding EF-hand motifs, a myristoylation motif, a glycosylation site, and potential protein kinase phosphorylation sites (14).

A gene expression database for the gastrointestinal tract was created using a microarray technique (15), and using this archived database, it was found that S100A14 is more highly expressed in the colon than in the other parts of the gastrointestinal tract. Moreover, using suppression subtractive hybridization, S100A14 was found to be expressed in normal bronchial epithelium and suppressed in lung carcinoma cell lines (16). Recently, accumulating evidence has indicated that S100A14 dysregulation is correlated with tumor progression in various types of cancer (17-22). S100A14 is expressed with an epithelial phenotype; we selected this gene since it is highly expressed in the colon. Analyses of colon-specific genes may provide new insights regarding therapeutic perspectives. In this study, we assessed the biological and functional significance of S100A14 in normal colon and in colon cancer. We present the following article in accordance with the ARRIVE reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-21-528/rc).

Methods

Clinical characteristics of the patients

The study involved 154 patients with stage I–III colorectal cancer who underwent curative surgical resection in Kitano Hospital (Osaka, Japan). The postsurgical staging of each tumor was classified according to the tumor-node-metastasis (TNM) staging system. The clinical characteristics of the patients are presented in *Table 1*. Eighty-seven of the patients were men and 67 were women. The median age of the patients was 61.2 (range, 29–90) years. There were 36 patients with pathological stage I, 39 with stage II, and 79 with stage III disease. The mean follow-up period was 46.5 (range, 12–60) months. The present study was approved by the Ethics Committee of the Kitano Hospital (No. 2005-11). All procedures performed in studies involving human participants were in accordance with the

ethical standards of the institutional research committee and the Declaration of Helsinki (as revised in 2013). We obtained consent for publication from the patients.

Tissue samples

Tissue samples were obtained from patients with colon cancer who underwent colon resection in Kitano Hospital. The tumor tissues and adjacent normal tissues were kept in liquid nitrogen after surgical resection and stored at –80 ℃ until use.

Cell lines

DKO-1, HCT-8, SW480, SW620, HCT116, HT-29 (colorectal cancer cell line), HCT-15, DLD-1, Caco-2, and HCA-7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) (23). All cell lines were authenticated by short tandem repeat (STR) profiling and all experiments were performed with mycoplasma-free cells. The cells were cultured in an appropriate culture medium in an incubator with a humidified atmosphere containing 95% air and 5% CO_2.

Mouse samples

A total of 2 Balb/c mice (1 male and 1 female), weighing 20 g and aged 5 weeks, were used. Various organs, including the brain, thymus, lung, heart, kidney, placenta, testis, spleen, liver, pancreas, esophagus, stomach, duodenum, jejunum, ileum, cecum, and colon, were collected from the mice. Mice were housed (n=5 per cage) with 12 h light/dark cycle. CO_2 gas was used for mice euthanasia. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC), in compliance with the Kitano Hospital institutional guidelines for the care and use of animals. A protocol was prepared before the study without registration.

In situ hybridization

In situ hybridization was performed following the manufacturer's instructions (Roche, Indianapolis, IN, USA). The mouse colons were harvested, fixed in 4% paraformaldehyde, embedded in optimal cutting temperature (OCT) compound, and cut into 10 μm sections. The sections were placed in methanol for 2 h at –20 ℃, acetylated in acetic anhydride/0.1 M triethanolamine (pH 8.0), equilibrated for 10 min in 5 × SSC [3 M sodium chloride and 0.3 M sodium citrate (pH 7.0)], and prehybridized for 2 h at 60 ℃ in mRNA hybridization buffer (50% formamide, 5 × SSC, 0.5 mg/mL salmon sperm DNA, 0.25 mg/mL yeast RNA, and 1 × Denhardt's reagent). The sections were then hybridized with digoxigenin riboprobes (200 ng/mL) in mRNA hybridization buffer overnight at 60 ℃. After removing the unbound riboprobes by washing, the sections were blocked with 2% blocking reagent (Roche). Antidigoxigenin antibody (Roche) was used to detect the riboprobes according to the manufacturer's instructions. Final detection was accomplished using BM purple AP substrate (Roche/Merck, Darmstadt, Germany), and the sections were counterstained with nuclear fast red. As the negative control, sense riboprobes were used to detect any nonspecific sequences.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

The total RNA was isolated from the cell lines, and the frozen normal and cancer tissues, using TRIzol reagent (Invitrogen, San Diego, CA, USA), according to the manufacturer's protocol. First-strand cDNA was synthesized with 5 mg of total RNA using the SuperScript II RT cDNA synthesis kit (Invitrogen), following the manufacturer's protocol. For PCR amplification, 1 mL of reaction mixture was prepared. We titrated the amount of starting cDNA and determined the number of amplification cycles necessary to obtain reproducible quantitative performance in RT-PCR assays for mouse and human *S100A14*. The generated cDNAs were amplified using primers for mouse *S100A14* (5'-AAACACTGACCCCTGCTGAG-3' and 5'-ACTCTTGGCTGCTTCTCCAA-3') and human *S100A14* (5'-CTGACCCCTTCTGAGCTACG-3' and 5'-GCCTCTCCAGCTTCACACTC-3'), using mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5'-CTTCACCACCATGGAGAAGGC-3' and 5'-GGCATGGACTGTGGTCATGAG-3') and human GAPDH (5'-GACAACAGCCTCAAGATCATCA-3' and 5'-GGTCCACCACTGACACGTTG-3') as the internal controls. All subsequent assays were performed under conditions used to amplify *S100A14* and GAPDH within a linear range. Twenty-eight cycles of mouse and human *S100A14* PCR amplification were performed as follows: denaturation at 94 ℃ for 30 s, annealing at 60 ℃ for 30 s, and extension at 72 ℃ for 90 s. Twenty-four cycles of mouse and human GAPDH PCR amplification were performed as

follows: denaturation at 94 ℃ for 40 s, annealing at 60 ℃ for 40 s, and extension at 72 ℃ for 90 s. Ten-milliliter aliquots of the PCR products were electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining under a ultraviolet (UV) transilluminator. The negative control, *S100A14* without reverse transcriptase, was not amplified. The densitometric value for *S100A14* in a given tumor or normal tissue sample was normalized by dividing by the corresponding GAPDH value. The expression ratio of the tumor was divided by that of the corresponding normal sample.

S100A14 construct and stable transfectants

According to the nucleotide sequence of human *S100A14* cDNA, specific PCR primers were designed to recognize the full coding region: forward 5'-HindIII-ATGGGACAGTGTCGGTCAG-3' primer 1, reverse 5'-NotI-TCAGTGCCCCCGGACAGGCC-3' primer 2. To clone the full-length cDNA encoding human *S100A14* into the expression vector pcDNA3.1 (Invitrogen), the entire coding region of *S100A14* cDNA was amplified from the total RNA derived from normal colon mucosa by RT-PCR, using primers 1 and 2, as described above. The resulting 315-bp PCR product was designed with HindIII and NotI and subcloned between the HindIII and NotI sites of pcDNA3.1. The construct was confirmed by sequencing. *S100A14*-pcDNA3.1 and empty pcDNA3.1 were transfected into separate SW480 cells. The transfections were initiated following the FuGENE 6 (Roche Diagnostics Corporation, IN, USA) technique. Two micrograms of plasmid were mixed with 6 mL of FuGENE 6 reagent and incubated in a six-well tissue culture plate containing 70% confluent cells for 24 h at 37 ℃ in a CO_2 incubator. After incubation, the cells were detached using trypsin-ethylene diamine tetraacetic acid solution and passaged, at a ratio of 1:10, into selective medium containing 800 μg/mL G418 (Invitrogen). The surviving clones were identified and characterized by RT-PCR using their *S100A14* mRNA expression.

Cell motility

The motility of transfected cell clones was evaluated in 24-well Transwell chambers of pore size 8 μm (Coster, Corning Incorporated, NY, USA). In the motility assay, 1×10^5 cells/well were seeded on the filters in an appropriate culture medium and incubated for 24 h. The cells on the upper surface were then removed by wiping with cotton swabs. The cells that had migrated through the membrane to the lower surface were fixed with ethanol and stained with Giemsa's staining solution. To quantify cells on the lower surface, cells were counted in four fields under a microscope (magnification of ×200).

Wound healing assay

Transfectants (1×10^5) were seeded in 0.5 mL of culture medium with G418 in a 24-well culture plate. After 36 h, a wound was made in the confluent monolayer with a pipette tip. The medium and debris were aspirated and replaced with 0.5 mL of new medium. Photographs of the wound area were captured, and again after 24 h of incubation. Migration was calculated as follows: filling rate (%) = (A – B)/A ×100; where, A is the initial width of the wound area and B is the width of the wound area after 24 h. Each sample was tested in triplicate.

Soft agar growth assay

Cells (1×10^5) of each cell line were suspended in 3 mL of Dulbecco's modified Eagle's medium with 10% fetal calf serum and warmed to 37 ℃, before mixing with 300 mL of prewarmed (52 ℃) 3% agarose/phosphate-buffered saline solution. Each cell suspension was then layered into three wells (pre-coated with 1 mL of Dulbecco's modified Eagle's medium containing 0.6% agarose) of a six-well plate (1 mL/well). The number of colonies in each well was counted under a microscope after the cells had been cultured for 10 days.

Western blotting

Cells were lysed at 4 ℃ for 30 min in buffer containing 50 mM Tris-HCl, (pH 7.6), 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 0.5% NP-40, and 1% protease inhibitor cocktail (Sigma). Samples containing equal protein concentrations were mixed with an equivalent volume of the sample buffer [50 mM Tris-HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.5% 2-mercaptoethanol, and 0.5% bromophenol blue] and boiled for 5 min. Next, they were electrophoresed on a 10% SDS-polyacrylamide gel and transferred on to Immobilon-P membranes (Millipore, MA, USA). After washing, the nonspecific binding sites of the membrane were blocked in poly(butylene succinate) (PBS) containing 50 mg/mL nonfat

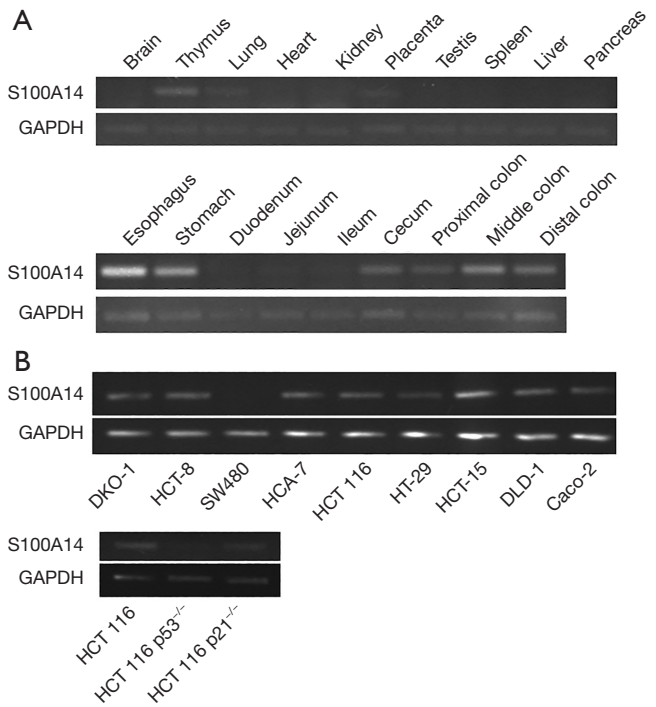

Figure 1 *S100A14* expression in mouse tissues and human colon cancer cell lines. (A) Expression of *S100A14* in mouse normal tissues. RT-PCR analysis showed 167 bp band following the designed primers. The same column was GAPDH as an internal control. *S100A14* was expressed in thymus, lung, esophagus, stomach and colon; (B) expression of *S100A14* in human colon cancer cell lines. RT-PCR analysis showed 178 bp band following the designed primers. The same column was GAPDH as an internal control. *S100A14* was not expressed in SW480 cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription polymerase chain reaction.

skimmed milk for 1 h. The membranes were then probed with an anti-E-cadherin monoclonal antibody (mAb) (BD Transduction Laboratories, CA, USA) diluted to 1:2,500 or anti-β-actin mAb (clone AC-74, Sigma, MO, USA) diluted to 1:10,000 for 16 h at 4 ℃. Following 1 h of incubation with peroxidase-conjugated donkey anti-mouse IgG (H + L) (Jackson ImmunoResearch Laboratories, PA, USA) diluted to 1:2,500, immunoreactive bands were visualized using Renaissance Chemiluminescent Reagent (Perkin Elmer, MA, USA).

Immunohistochemical analysis

Rabbit polyclonal antibodies against S100A14 anti-S100A14 (Proteintech, Chicago, IL, USA) were used in this study. Immunochemical staining was performed using an indirect streptavidin-biotin immunoperoxidase method [SAB-PO(M) kit, Nichirei Corporation, Japan]. After antigen retrieval, performed for 15 min in a microwave oven using 10 mM citrate buffer, endogenous peroxidase activity was blocked with 3% H_2O_2-methanol solution. The slides were incubated with primary antibodies (diluted 1/100) overnight at 4 ℃, washed with phosphate-buffered saline, and incubated with secondary biotin-labeled antibodies for 30 min at room temperature. Antibody localization was detected by treating the slides with peroxidase-conjugated streptavidin for 30 min at room temperature, followed by the diaminobenzidine reaction. The slides were counterstained with hematoxylin.

Statistical analysis

Differences between the control and experimental groups were evaluated using Analysis of Variance (ANOVA) and a post hoc test for multiple comparisons (Tukey's honestly significant difference test). The statistical significance of the differences between the incidence of S100A14 expression and clinical and pathologic parameters was assessed using the χ^2 test. A receiver operating characteristics curve was designed to determine specificity and sensitivity. The disease-free survival rate was calculated using the Kaplan-Meier method. Statistical significance was examined using the log-rank test. The prognostic significance of clinicopathological factors was evaluated using univariate and multivariate regression techniques (Cox's proportional hazards model). Statistical significance was considered as $P<0.05$. JMP statistical software (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses.

Results

S100A14 expression in normal mouse tissues and human colon cancer cell lines

We examined the expression of *S100A14* in normal mouse tissues using RT-PCR (*Figure 1A*). *S100A14* RNA was detected in the thymus, lungs, esophagus, stomach, and colon, but not in the small intestine. *S100A14* expression was determined in 10 colon cancer cell lines (*Figure 1B*). *S100A14* was not expressed in SW480 cells, but all the other colon cancer cell lines expressed *S100A14*. In addition, *S100A14* expression was lower in $p53^{-/-}$ HCT116

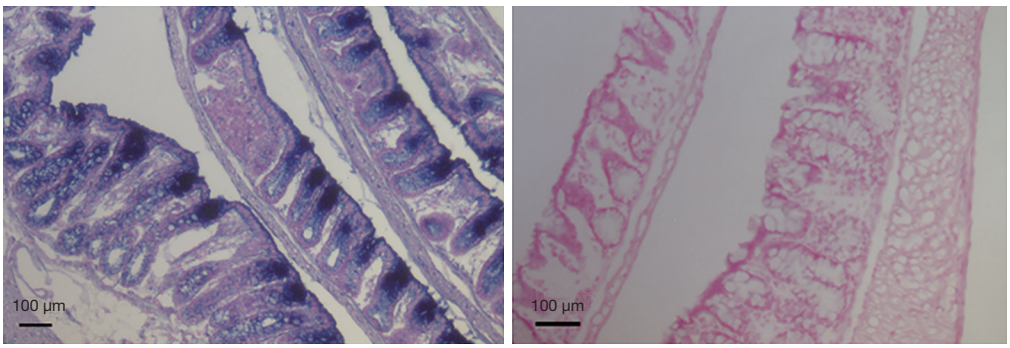

Figure 2 Expression of *S100A14* in mouse normal colon. The *S100A14* RNA was showed using antisense probe, and the negative control staining was showed using sense probe in *in situ* hybridization (BM purple staining).

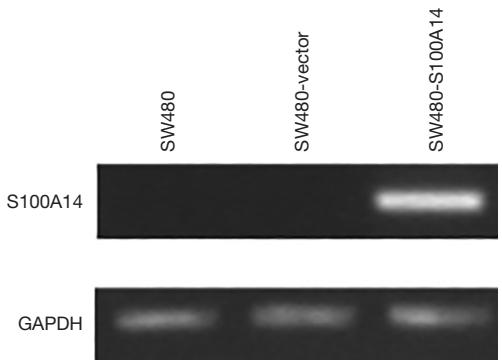

Figure 3 Expression of *S100A14* in transfectant clones was analyzed by RT-PCR, showing 315 bp band. *S100A14* RNA level in transfectants were higher than both parental SW480 cell and the vector-transfected cells. RT-PCR, reverse transcription polymerase chain reaction.

cells than in parental HCT116 cells.

The results of *in situ* hybridization showed that *S100A14* was distributed in the epithelial cells of normal mouse colon (*Figure 2*) and strongly expressed at the top of the crypt.

Preparation of S100A14 stable transfectants

To analyze the role of *S100A14* in colon cancer cells, stable transfectants were established. SW480 cells with the lowest *S100A14* expression were transfected with *S100A14* cDNA. Some cells were transfected with the empty vector, pcDNA3.1, as a control. The expression of *S100A14*, showing a 315-bp band, in the transfectants was analyzed by RT-PCR. As shown in *Figure 3*, the mRNA expression of *S100A14* in the cells transfected with *S100A14* was higher than that in parental SW480 cells and vector-transfected cells.

Effect of S100A14 on cell motility

Although several members of the S100 family have been reported to have effects on cell motility, the effect of S100A14 overexpression on cell motility has not been established. To establish this effect, we first analyzed the number of S100A14 transfectant cells that had penetrated an 8 μm pore filter after 24 h of incubation. This number was significantly lower than that for the parental and vector-transfected cells (P<0.001, *Figure 4*). Next, cell motility was assessed using the scratch wound assay. As shown in *Figure 5*, there was a four-fold decrease in the wound area after 24 h of incubation in the case of S100A14 transfectants (P<0.001). These findings show that the overexpression of S100A14 reduces cell motility.

Colony formation of SW480 cells in soft agar

The interactions between extracellular substrates and the cytoskeleton play a crucial role in cell proliferation. To examine this effect of S100A14, the transfected cells were grown in soft agar. As shown in *Figure 6*, the colony numbers of the S100A14 transfectants were three-fold lower than those of the parental and vector-transfected SW480 cells.

Effect of S100A14 on E-cadherin expression in SW480 cells

To examine the effect of S100A14 on E-cadherin expression, western blotting using an anti-E-cadherin mAb

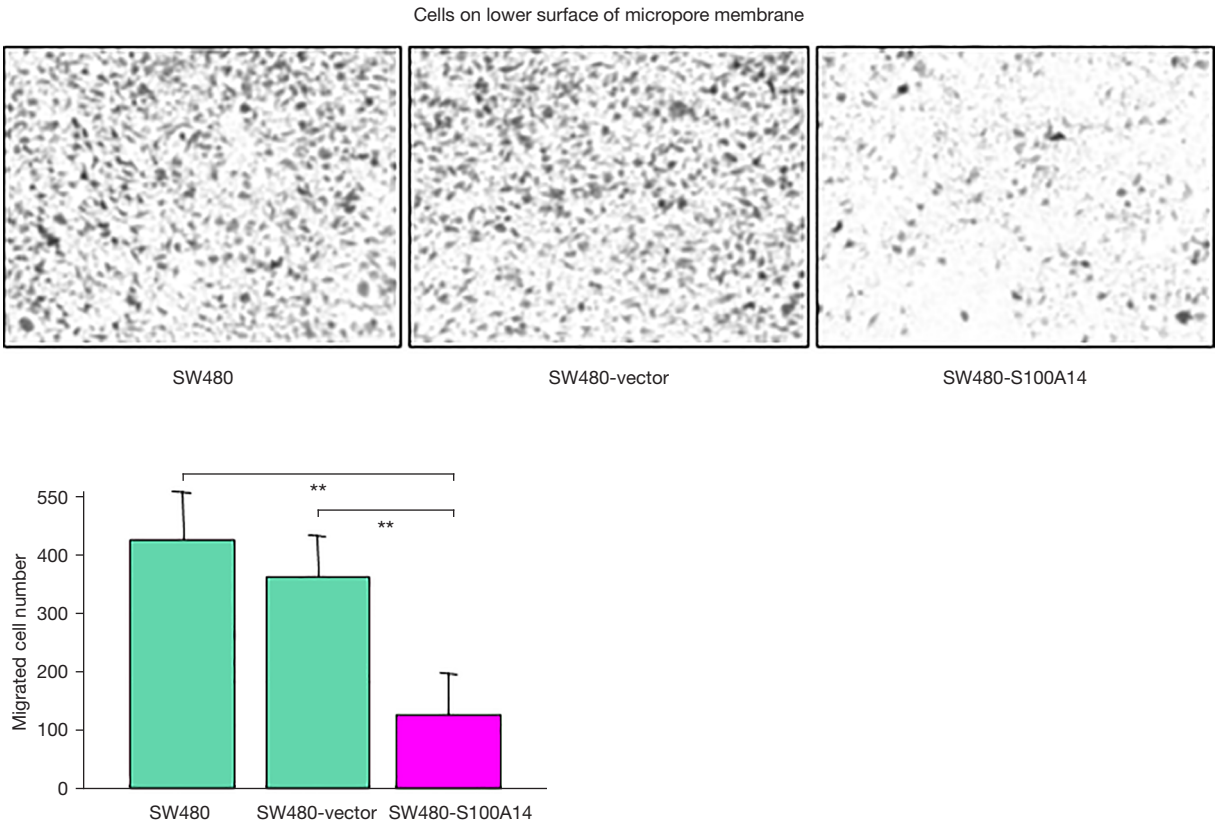

Figure 4 Effect of S100A14 on migration of SW480 cells. The number of migrated cells through the 8 μL pore filter was counted. To quantify cells that had migrated to the lower surface, cells were counted in four fields, under a microscope, at a magnification of ×200. Each sample was tested in triplicate and data represent the mean plus or minus the standard error. Cell migration was inhibited significantly by S100A14 over expression. **, P<0.01.

was performed. As shown in *Figure* 7, E-cadherin expression in the S100A14 transfectants was lower than that in the parental SW480 and vector-transfected cells. S100A14 overexpression increased E-cadherin expression.

S100A14 expression in human colon tumor tissues

S100A14 expression was immunohistochemically screened. We performed immunochemical staining for S100A14 expression in tissue samples from 154 patients who underwent curative resection for colon cancer. The S100A14 proteins were found to be expressed mainly on the membranes of colon cancer cells, whereas no or faint staining was observed in cancer cells of the samples (*Figure 8*). In colon cancer, although there are various staining patterns, the high parts of the well-differentiated areas show stronger expression.

Immunoreactivity was evaluated using a semiquantitative scoring method that was based on the proportion of positively stained tumor cells and intensity of staining. Tumor samples that showed staining of the entire membrane, as strong as that of the positive control, were assigned an intensity score of 2; tumor samples with weaker staining than the control were assigned an intensity score of 1; and tumor samples with negative staining were assigned an intensity score of 0. The expression level of S100A14 in the tumor samples was scored by multiplying the percentage of positively stained cells by the intensity score. Of the 154 colon cancers studied, 68 (44.2%) were classified as S100A14 positive. Immunostaining was intense and uniform on the cell membranes and 86 (55.8%) were determined as S100A14 negative.

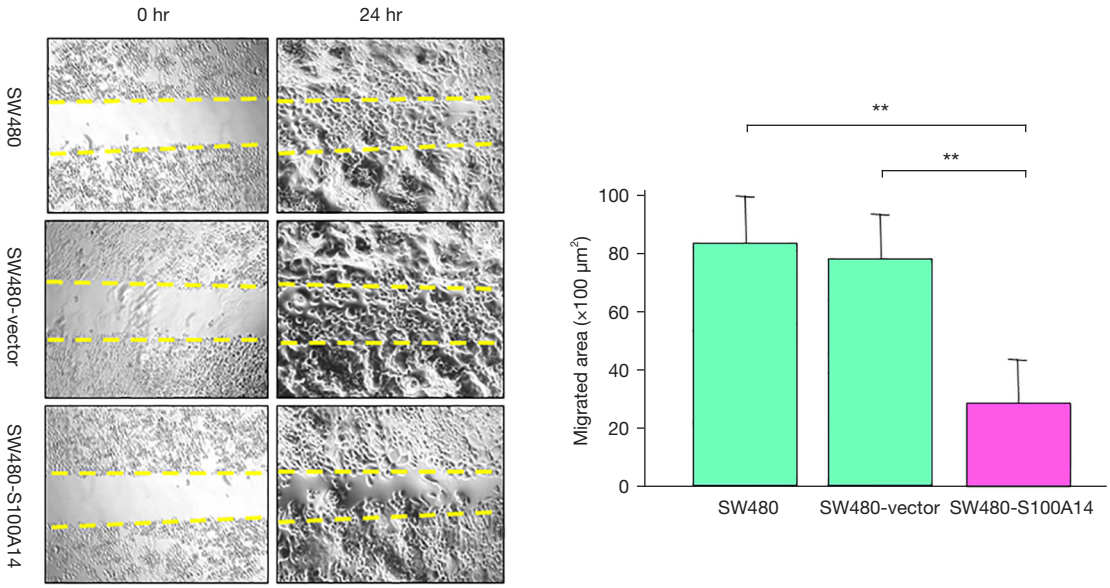

Figure 5 Scratch wound closure assay was conducted by measuring the filling rate of the wounded area. Each sample was tested in triplicate and data represent the mean plus or minus the standard error. Cell migration was inhibited significantly by S100A14 overexpression. **, P<0.01.

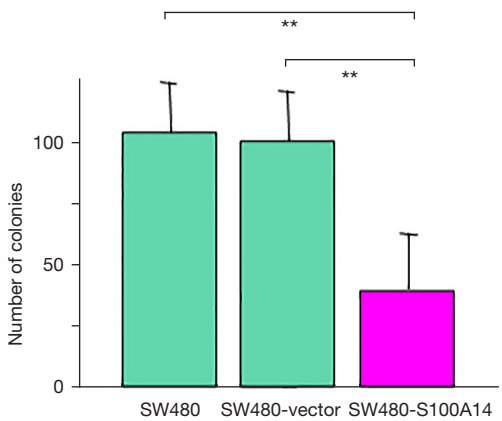

Figure 6 Effect of S100A14 in anchorage-independent growth of SW480 cells. The number of colonies in each well was counted. Colony number of the S100A14 transfectants was lower than those of parental and vector-transfected SW480 cells. **, P<0.05.

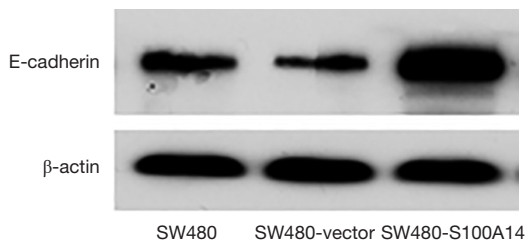

Figure 7 Effect of S100A14 in E-cadherin expression in SW480 cells. Western blot analysis was done using anti-E-cadherin mAb and anti-β-actin mAb. E-cadherin expression in S100A14 transfectant was lower than that in parental SW480 and vector-transfected cells. mAb, monoclonal antibody.

S100A14 gene expression in human colon cancer tissues

A receiver operating characteristics curve was plotted to determine the cut-off value for *S100A14* scores. *S100A14* showed a significant area under the curve (AUC) of 0.835 (P<0.001), and 0.651 was selected as the cut-off score (*Figure 9*). The relative expression rates (tumor/normal ratio) for *S100A14* ranged from 0 to 1.2. The mean relative expression rate was 0.422. Of the 154 colon cancer samples, 95 showed decreased *S100A14* expression (*Figure 10*). The immunohistochemistry results agreed well with the RT-PCR results, with the same results for 95.5% of the samples.

Correlation between S100A14 expression and clinicopathological factors in colon cancer

We assessed the correlation between the expression of S100A14 and clinicopathological factors (*Table 1*). Lower

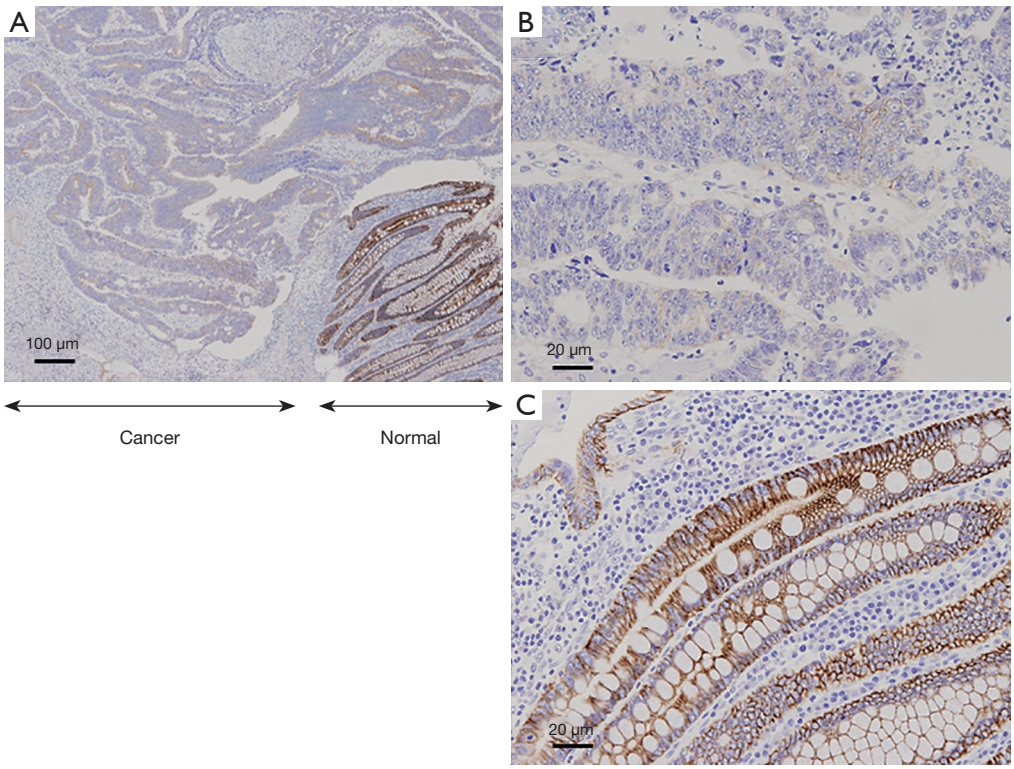

Figure 8 S100A14 expression in human colon tumor tissues. Immunostaining was performed using an indirect streptavidin-biotin immunoperoxidase method (diaminobenzidine staining). S100A14 proteins were found to be expressed mainly on the membranes of colon cancer cells. (A) ×40 magnification; (B,C) ×200 magnification.

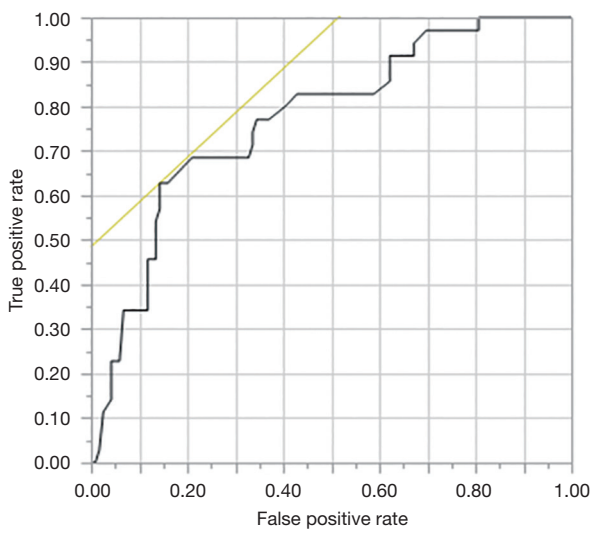

Figure 9 A receiver operating characteristics curve was plotted and analyzed to determine the optimal cut-off value for *S100A14* scores.

S100A14 expression was significantly associated with nodal status (P=0.032).

Expression of S100A14 predicts the prognosis of colon cancer

To analyze the correlation between S100A14 expression and the prognosis of colon cancer, Kaplan-Meier survival curves were generated. Low S100A14 expression correlated with poor outcomes (P=0.029, *Figure 11*). In the multivariate analysis, S100A14 expression (P=0.004) had a significant prognostic value for overall survival (*Table 2*).

Discussion

The S100 protein family is involved in various intracellular and extracellular activities, such as the modulation of cell differentiation and proliferation, cell-cell communication, intracellular signaling, cell motility, and cell structure.

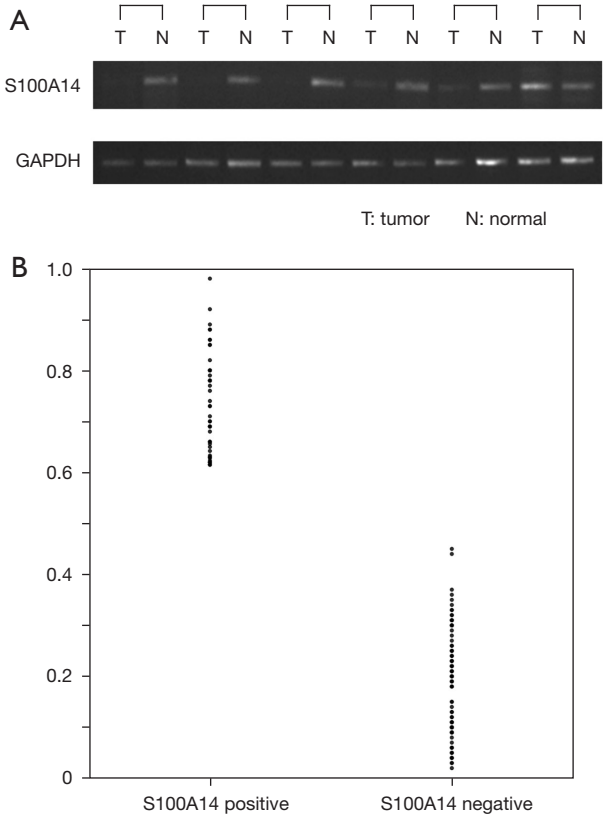

Figure 10 *S100A14* expression values in human colon tissues. (A) *S100A14* expression in human colon tumor tissues and normal colon tissues. The values were obtained from the expression ratio of a given tumor sample divided by that of the corresponding normal sample; (B) the scatter plot of relative expression ration of *S100A14*. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

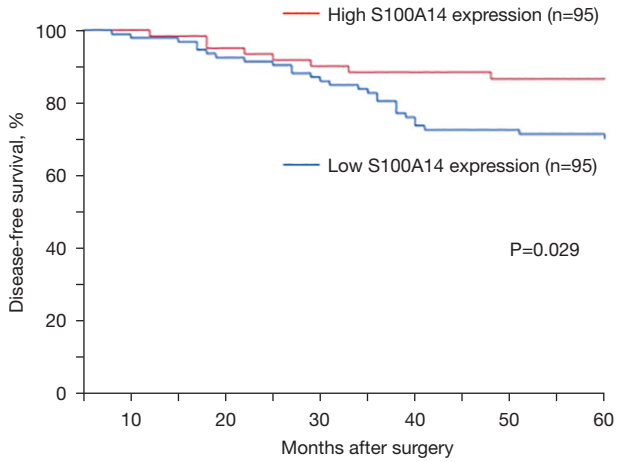

Figure 11 Expression of *S100A14* predicts the prognosis of colon cancer patients. Low *S100A14* expression was correlated with poor outcomes (P=0.029). Red line: high *S100A14* expression. Blue line: low *S100A14* expression.

The members of this family show cell- and tissue-specific expression (1), and some members are secreted from cells exerting extracellular, cytokine-like activities, through the surface receptor for glycation end-products, and have paracrine effects (2).

The malignancy-promoting effects of several S100 members have been proposed. The altered expression of *S100* in malignant tumors plays important roles as a prognostic and diagnostic factor (9). In addition, the S100A4 protein acts as an angiogenic factor (24), and S100P has been identified as a hypomethylation target in pancreatic cancer (25). In contrast, *S100A2* has been described as a potential tumor suppressor gene and is downregulated in several tumors (26). Briefly, *S100A2* is inversely correlated with tumor progression. Moreover, the S100 proteins are regulated by various kinds of molecules. For example, erbB2 upregulates S100A4 expression in medulloblastomas (27) and S100A4 regulates the membrane-induced activation of matrix metalloproteinase-2 in osteosarcoma cells (28).

S100A14, a new member of the S100 protein family, is differentially regulated in tumors. S100A14 is encoded in several epithelial tissues, and it is highly expressed in the colon. Different S100A14 expression patterns have been reported in different tumors. Genomic instability of the human chromosomal region 1q21 is often observed and is involved in various tumors. *S100A14* is closely related to *S100A16/S100A13/S100A1*, which has previously been physically mapped to the 1q21 chromosomal region (14).

Many molecular markers of colon cancer have been examined to date, and an enhanced understanding of the genetic events that occur during neoplastic progression in the colon could lead to improvements in survival. Therefore, analyses of colon-specific genes may be useful for new therapeutic perspectives. Our data show that *S100A14* is highly expressed in the epithelial cells of the mouse colon. *S100A14* was expected to be expressed in the colon based on a microarray database of the gastrointestinal tract (15). We found that the potentially tissue-specific expression of S100A14 might be important for regulating cell proliferation and differentiation. In addition, we found that S100A14 was downregulated in human colon tumors. Moreover, using the suppression subtractive hybridization technique, it was previously found that *S100A14* is also downregulated in lung cancer cell lines (16). These data

Table 2 Multivariate regression analysis in predicting the disease-free and overall survival of 154 patients

Variable	Assigned score	Hazard ratio (95% CI)	P value
S100A14			0.004
Positive	0	0.309 (0.114–0.708)	
Negative	1		
Tumor status			0.841
T1	1	0.862 (0.171–3.440)	
T2	2		
T3	3		
T4	4		
Nodal status			0.365
N0	0	0.667 (0.273–1.611)	
N1	1		
N2	2		
Differentiation			0.127
Well	0	0.476 (0.143–1.299)	
Moderately	1		
Poorly	2		

suggest the distinct regulation and potential roles of S100A14 in malignant tumor progression.

Cell motility is an essential function in tumor progression and metastasis. Several members of the S100 family regulate cell motility and metastasis in various kinds of malignant cells. For example, a positive correlation was observed between the metastatic potential of murine mammary adenocarcinoma cells and the level of expression of *S100A4* (29). A similar correlation has been found in B16 melanoma cells (30). S100A4 expression in mouse mammary tumour virus-neu (MMTV-neu) transgenic mice leads to the metastasis of mammary tumors (31), and the antisense RNA to *S100A4* in highly metastatic Lewis lung carcinoma cells suppresses cell motility and invasiveness (32). In addition, S100A6 is related to cell migration in glioblastoma cells (33) and S100A2 reduces cell migration (13). In this study, we addressed these points using SW480 colon carcinoma cells overexpressing S100A14 and elucidated the mechanisms leading to these changes. Briefly, to test the effect of S100A14 on the invasive abilities of colon cancer cells, we performed two *in vitro* motility assays. They revealed an inverse correlation between the expression level of S100A14 and cell motility. This study suggests that S100A14, like other parts of the S100 family, is involved in cell motility and cancer progression.

p53 is a tumor suppressor gene and the loss of normal function of the p53 protein is associated with carcinogenesis. *p21* is the downstream target gene of *p53*, and it is responsible for *p53*-induced G_1 arrest and inhibits the protein kinase activities of G_1 cyclin/cyclin-dependent protein kinase (CDK) complexes, thereby preventing the phosphorylation of retinoblastoma (RB). This in turn inhibits cell growth. S100A4 interacts with wild-type p53 to stimulate apoptosis, and the interaction with S100A4 may modulate the functions of p53 (34,35). Interestingly, we found that *S100A14* expression was lower in $p53^{-/-}$ HCT116 cells than in parental HCT116 cells. These findings suggest that the expression of S100A14 may be regulated by p53. In addition, it was reported that S100A14 is involved in cell invasion by affecting the expression and function of matrix metalloproteinase (MMP)-2 via p53-dependent transcription (17).

The attachment of cancer cells to extracellular matrices and cell motility are essential steps for invasion. E-Cadherin plays an important role in this process, as it is a powerful molecule involved in epithelial cell adhesion and epithelial organization. Although β-catenin binds directly to the cytoplasmic tail of E-cadherin, α-catenin links the bound β-catenin to the actin cytoskeleton (36). Calcium ions are known to play an important role in cell motility via the regulation of E-cadherin proteolysis (37). In the S100 family, S100A4 is involved in E-cadherin expression in gallbladder carcinomas (38) and gastric cancer (39). To analyze the mechanism underlying the decreased invasiveness of SW480 cells overexpressing S100A14, we examined the effects of E-cadherin on the adhesive abilities of SW480 cells. We found that the expression of E-cadherin in the S100A14 transfectants was higher than that in the parental and vector-transfected SW480 cells. Furthermore, growth in soft agar, which affects the loss of cell-substrate adhesion, cell growth, and survival, is often used as an indicator of enhanced metastatic ability of cancer cells. The effect of S100A14 overexpression on the anchorage independence of tumor cells is still unknown. In this study, we demonstrated that S100A14 might be involved in these functions.

The expression of S100A14 was not correlated with any prognostic parameters, such as tumor status, nodal status, pathologic stage, or tumor differentiation, of colon cancer but was correlated with disease-free survival of patients

with colon cancer. In multivarious analysis, S100A14 was an independent prognostic factor, and its expression was inversely correlated with the prognosis of colorectal cancer. By regulating the expression of S100A14, the progression of colorectal cancer can be controlled.

This study has some limitations. The mechanism of S100A14 in promoting cell migration and invasion requires further elucidation to provide insights into colon cancer metastasis. Analysis of intracellular signal transduction may help clarify the mechanism. In addition, animal model assays are required to confirm S100A14 function in metastasis *in vivo*. The underlying potential mechanism between S100A14 and epithelial-mesenchymal transition warrants further investigations.

Our current data from S100A14 transfectants show the suppressive effect of S100A14 on cell adhesion, cell motility, and tumor progression. S100A14 could be viewed as a new tool to gain insights into the epithelial organization of normal colon and colon cancer cell lines. Although further detailed studies on S100A14 in different organ tumors are needed, this molecule may be a promising target for new therapeutic strategies.

Conclusions

S100A14 is highly expressed in the gastrointestinal tract, especially in the colon. Using a colorectal cancer cell line, we investigated the expression mechanism of S100A14 and analyzed its expression in actual human colorectal cancer. The expression of S100A14 is inversely related to prognosis. It means that by regulating the expression of S100A14, the progression of colorectal cancer can be controlled.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The present study was approved by the Ethics Committee of the Kitano Hospital (No. 2005-11). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and the Declaration of Helsinki (as revised in 2013). We obtained consent for publication from the patients. All animal experiments were approved by the IACUC, in compliance with Kitano Hospital institutional guidelines for the care and use of animals. A protocol was prepared before the study without registration.

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