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ORIGINAL ARTICLE

Mesothelin expression has prognostic value in stage II/III colorectal cancer

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Abstract

Mesothelin (MSLN) is a cell-surface glycoprotein present on mesothelial cells and in many cancers, where expression is generally associated with an unfavorable prognosis. The clinical significance and pathological characteristics of MSLN expression were evaluated by immunohistochemical staining of tissues from 530 stage II/III colorectal cancer (CRC) patients with R0 resection. Eighty-eight (16.6%) were MSLN-positive; 33 (37.5%) showed a luminal staining pattern whereas 55 (62.5%) showed a non-luminal staining pattern. MSLN expression, including the luminal and non-luminal staining patterns, was associated with shorter cancer-specific survival (CSS) period in stage II ($n = 314$, $P = 0.024$) and stage III ($n = 216$, $P = 0.0002$) CRC patients. The non-luminal staining pattern was correlated with poor prognosis in stage II ($P = 0.0006$) and III ($P < 0.0001$) CRC, but a luminal staining pattern was not significantly correlated with prognosis. Cox's multivariate analysis revealed that a non-luminal staining pattern was associated with CSS independently of other conventional parameters in stage II ($P = 0.040$, hazard ratio (HR) = 2.92) and III ($P = 0.020$, HR = 2.13) CRC patients. Immunohistochemical evaluation of MSLN expression was helpful in the prediction of patient prognosis in stage II/III CRC.

Keywords Colorectal cancer · Mesothelin · Glycoprotein · Immunohistochemistry · Prognosis

Abbreviations

CI	Confidence interval
CSS	Cancer-specific survival
EGFR	Epidermal growth factor receptor
HR	Hazard ratio
ROC	Receiver operating characteristic
SAGE	Serial analysis of gene expression

Introduction

Colorectal cancer (CRC) is a major cause of cancer-related death worldwide [1] and its prevalence in Japan is increasing [2]. Currently, the chemotherapy regimens include molecular targeted drugs are effective [3–6]; however, the accurate evaluation of the malignant potential is important for the choice of the most suitable treatment. The mesothelin (MSLN) gene, which is located on chromosome 16p13.3 and comprises 17 exons, encodes a 71-kDa precursor protein named mesothelin/megakaryocyte potentiating factor (MPF) family proteins. The 71-kDa precursor protein is cleaved with furin-like protease into two glycoproteins: a 30-kDa N-terminal fragment (MPF) and a 41-kDa C-terminal fragment (MSLN) [7]. The MSLN glycoprotein is linked to cell membrane with glycophosphatidylinositol (GPI) anchor and lines the pleura, peritoneum, and pericardium [7–9].

MSLN is also expressed in many cancers, such as malignant mesothelioma, pancreatic cancer, ovarian cancer, and lung adenocarcinoma [10–14]. With regard to gastrointestinal cancers, MSLN overexpression has been correlated with poor prognosis in esophageal and gastric cancer [15–19]. The published data available on its prognostic value in CRC are few. MSLN has been identified as a tumor antigen by the serial analysis of gene expression (SAGE), is thought to have high immunogenicity [13], and has been evaluated as a target for new therapies for pancreatic cancer and mesothelioma [20].

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Recombinant anti-MSLN immunotoxin SS1P (CAT-5001) and a high-affinity chimeric anti-MSLN monoclonal antibody (MORAb-009) have been evaluated in phase II clinical trials [21–23], and a vaccine including MSLN peptide epitope peptides has been clinically studied [24–26]. MSLN expression may be a candidate for novel targeted therapy and/or immunotherapy for CRC.

This study investigated the clinical and pathological significance of MSLN expression in 530 stage II/III CRC patients by immunohistochemical staining to determine the patterns and extent of expression. In addition, MSLN expression was assayed by western blotting and quantitative real-time polymerase chain reaction (RT-qPCR) to corroborate the immunohistochemistry results.

Materials and methods

Patients

The Ethics Committee of the National Defense Medical College Hospital, Tokorozawa, Japan, approved this study. Written informed consent for the experimental use of tissue samples was given by each patient following institutional regulations. The study patients had received curative resection for CRC between January 1997 and December 2005 at this center. The medical records of the 530 pathological stage II or III CRC patients were re-examined. Curatively resected and histologically proven stage II (T3–4, N0, M0) or III (any T, N1–2, M0) CRC was eligible [27]. Excluded from the analysis were patients who received preoperative chemotherapy or radiotherapy. Blood vessel invasion was recorded as negative or positive and tumor budding was evaluated as per the Japanese Society for Cancer of the Colon and Rectum Guidelines 2014 for treatment of colorectal cancer [28]. The mean postoperative follow-up was 64.9 months, during which time 101 patients (19.1%) experienced a recurrence. Twenty-nine of the 314 stage II CRC patients received adjuvant chemotherapy with 5-FU regimens; 139 patients of the 216 stage III CRC patients received the same type of adjuvant chemotherapy. Table 1 summarizes the clinicopathological characteristics of the study patients.

Immunohistochemical staining and evaluation

Immunohistochemical staining was performed as described previously [29]. In brief, sections from central portions of the tumor were selected for pathological evaluation. The 4-μm sections were mounted on silane-coated glass slides, deparaffinized, and rehydrated in a graded ethanol series. Antibody retrieval was heated for 15 min at 121 °C in an autoclave at pH 9.0 using a commercially available reagent kit (415211; Nichirei Bioscience, Tokyo, Japan). The sections were incubated with a mouse monoclonal antibody against MSLN (clone 5B2 diluted 1:30; Novocastra, Newcastle Upon Tyne, UK) at a 1:30 dilution and reacted with a dextran polymer reagent combined with secondary antibodies and peroxidase (Envision+System-HRP; Dako, Grostrup, Denmark). The anti-MSLN antibody used was raised against recombinant protein corresponding to the membrane-bound form of the MSLN molecule [9].

The slides were evaluated independently by two observers (T.S., E.S.) who did not know the clinical outcomes. The extent and pattern of MSLN immunostaining of tumor cells were evaluated. Positive (≥ 30%) and negative (< 30%) MSLN staining were scored as percentages of immunopositive cells in the tumor tissue sample (Fig. 1). The positive cutoff score was 30% positive cells based on receiver operating characteristic (ROC) curve analysis of death from CRC recurrence within 5 postoperative years. MSLN-positive staining was histologically described as a luminal or non-luminal pattern. A luminal staining pattern included positive staining of the apical/endoluminal surface in ≥ 50% MSLN-expressing cancer cells (Fig. 1c, d). A non-luminal staining pattern included dominant positive staining of the cell membrane and/or cytoplasm. In case of membrane staining, the surface facing the stroma or adjacent cancer cells were positive distinctly or relatively distinctly (Fig. 1e, f), and in case of cytoplasmic staining, the cytoplasm was stained either diffusely or in the form of cytoplasmic deposits or granules (Fig. 1g). Usually, the non-luminal staining pattern was distributed throughout the cancer tissue, but sometimes the staining pattern was limited to the basal portion of cancer cell clusters (Fig. 1h). The non-luminal staining pattern was frequently observed in the areas where cancer cell polarity had been lost. When the result was not concordant between the observers, a consensus was reached after reevaluation. The degree of interobserver agreement for the evaluation of immunoreactivity was measured with a generalized κ test for two observers following the criteria of Landis and Koch [30]. The κ values for strength of agreement were assigned to poor (< 0.00), slight (0.00–0.20), fair (0.21–0.40), moderate (0.41–0.60), substantial (0.61–0.80), and almost perfect (0.81–1.000).

Detection of mismatch repair deficiency

In this study, we retrospectively verified the microsatellite instability (MSI) status using immunohistochemical staining of MLH1 (Clone G168-15; BD Biosciences, San Jose, CA) and MSH2 (FE11; Invitrogen, Carlsbad, CA). We performed immunohistochemistry as described previously [29]. We adjudged tumor cells to be negative for the protein expression only if they lacked staining in a sample in which healthy colonocytes and stroma cells were stained. The normal colonic crypt epithelium adjoining the tumor served as the internal

Table 1 Correlation between clinicopathological characteristics and mesothelin expression

Parameters	Categories	Total ($n = 530$) n (%)	Mesothelin expression			Luminal pattern-positive ($n = 33$)		Non-luminal pattern-positive ($n = 55$)	
			Negative ($n = 442$) n (%)	Positive ($n = 88$) n (%)	P value	n (%)	P value*[4]	n (%)	P value*[5]
Sex	Male	305 (57.5)	254 (83.3)	51 (16.7)	0.93	20 (6.6)	0.72	31 (10.1)	0.88
	Female	225 (42.5)	188 (83.6)	37 (16.4)		13 (5.8)		24 (10.6)	
Age (years)	< 65	242 (45.7)	195 (80.6)	47 (19.4)	0.11	18 (7.4)	0.25	29 (12.0)	0.23
	≥ 65	288 (54.3)	247 (85.8)	41 (14.2)		15 (5.2)		26 (9.0)	
Location	Right side	155 (29.2)	125 (80.6)	30 (19.4)	0.28	12 (7.8)	0.32	18 (11.6)	0.49
	Left side	375 (70.8)	317 (84.5)	58 (15.5)		21 (5.6)		37 (9.9)	
Histopathological grading*[1]	G1	215 (40.6)	197 (91.6)	18 (8.4)	< 0.0001*[6]	15 (7.0)	0.90*[6]	3 (1.4)	< 0.0001*[6]
	G2	273 (51.5)	207 (75.8)	66 (24.2)		16 (5.9)		50 (18.3)	
	G3	15 (2.8)	14 (93.3)	1 (6.7)		1 (6.7)		0 (0)	
	G4	27 (5.1)	24 (88.9)	3 (11.1)		1 (3.7)		2 (7.4)	
Depth of tumor*[1]	≤ T3	421 (79.4)	361 (85.7)	60 (14.3)	0.0062	24 (5.7)	0.21	36 (8.6)	0.0047
	T4	109 (20.6)	81 (74.3)	28 (25.7)		9 (8.3)		19 (17.4)	
Lymph node metastasis*[1]	N0	314 (59.2)	263 (83.8)	51 (16.2)	0.48	24 (7.6)	0.44*[6]	27 (8.6)	0.073
	N1	152 (28.7)	129 (84.9)	23 (15.1)		7 (4.6)		16 (10.5)	
	N2	64 (12.1)	50 (78.1)	14 (21.9)		2 (3.1)		12 (18.8)	
Pathological stage*[1]	Stage II	314 (59.2)	263 (83.8)	51 (16.2)	0.79	24 (7.6)	0.13	27 (8.6)	0.14
	Stage III	216 (40.8)	179 (82.9)	37 (17.1)		9 (4.1)		28 (13.0)	
Venous invasion	Negative	82 (15.5)	73 (89.0)	9 (11.0)	0.12	5 (6.1)	0.84	4 (4.9)	0.078*[6]
	Positive	448 (84.5)	369 (82.4)	79 (17.6)		28 (6.2)		51 (11.4)	
Tumor budding*[2]	Grades 1, 2	380 (71.7)	338 (88.9)	42 (11.1)	< 0.0001	22 (5.8)	0.20	20 (5.3)	< 0.0001
	Grade 3	150 (28.3)	104 (69.4)	46 (30.6)		11 (7.3)		35 (23.3)	
MMR status*[3]	MMRp	494 (93.2)	408 (82.6)	86 (17.4)	0.065*[6]	33 (7.5)	0.098*[6]	53 (11.5)	0.27*[6]
	MMRd	36 (6.8)	34 (94.4)	2 (5.6)		0 (0)		2 (5.6)	
Adjuvant chemotherapy	Surgery alone	362 (68.3)	306 (84.5)	56 (15.5)	0.31	21 (5.8)	0.50	35 (9.7)	0.40
	Chemotherapy	168 (31.7)	136 (81.0)	32 (19.0)		12 (7.1)		20 (11.9)	

MMRp DNA mismatch repair proficiency, *MMRd* DNA mismatch repair deficiency

*[1] TNM Classification (8th Edition, 2017)

*[2] Japanese Classification of Colorectal Carcinoma (8th Edition, 2013)

*[3] DNA mismatch repair (MMR) status was verified using immunohistochemical staining of MLH1 and MSH2

*[4] P value was calculated between mesothelin expression negative and luminal pattern-positive

*[5] P value was calculated between mesothelin expression negative and non-luminal pattern-positive

*[6] Fisher's exact test

control. When expressed, both MLH1 and MSH2 proteins stained positively in nuclei [31]. Cancers whose MLH1 or MSH2 expression was negative were considered to be DNA mismatch repair deficiency (MMRd).

Total RNA extraction and first-strand cDNA synthesis

Frozen tissue samples from the 80 patients who underwent surgery in 2002–2003 were used in the RT-qPCR assays. At the time of surgery, a sample of resected CRC tissue was immediately embedded in the Tissue-Tek OCT medium (Sakura, Tokyo, Japan), frozen in liquid nitrogen, and stored at − 80 °C until assayed. The specimens were cut into serial sections and total cellular RNA was extracted by using the ISOGEN reagent (Nippon Gene, Tokyo, Japan) following the manufacturer's protocol. First-strand cDNA was synthesized using a PrimeScript RT reagent kit (Takara Bio Inc., Otsu, Japan) following the manufacturer's instructions.

RT-qPCR

RT-qPCR was performed as described elsewhere [32]. Briefly, *MSLN* gene and the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a reference gene were amplified using 5′-CTCAACCCAGATGCGTTCTCG-3′ (forward) and 5′-AGGTCCACATTGGCCTTCGT-3′ (reverse) primers for the *MSLN*-, and using 5′-GCAC CGTCAAGGCTGAGAAC-3′ (forward) and 5′-TGGT

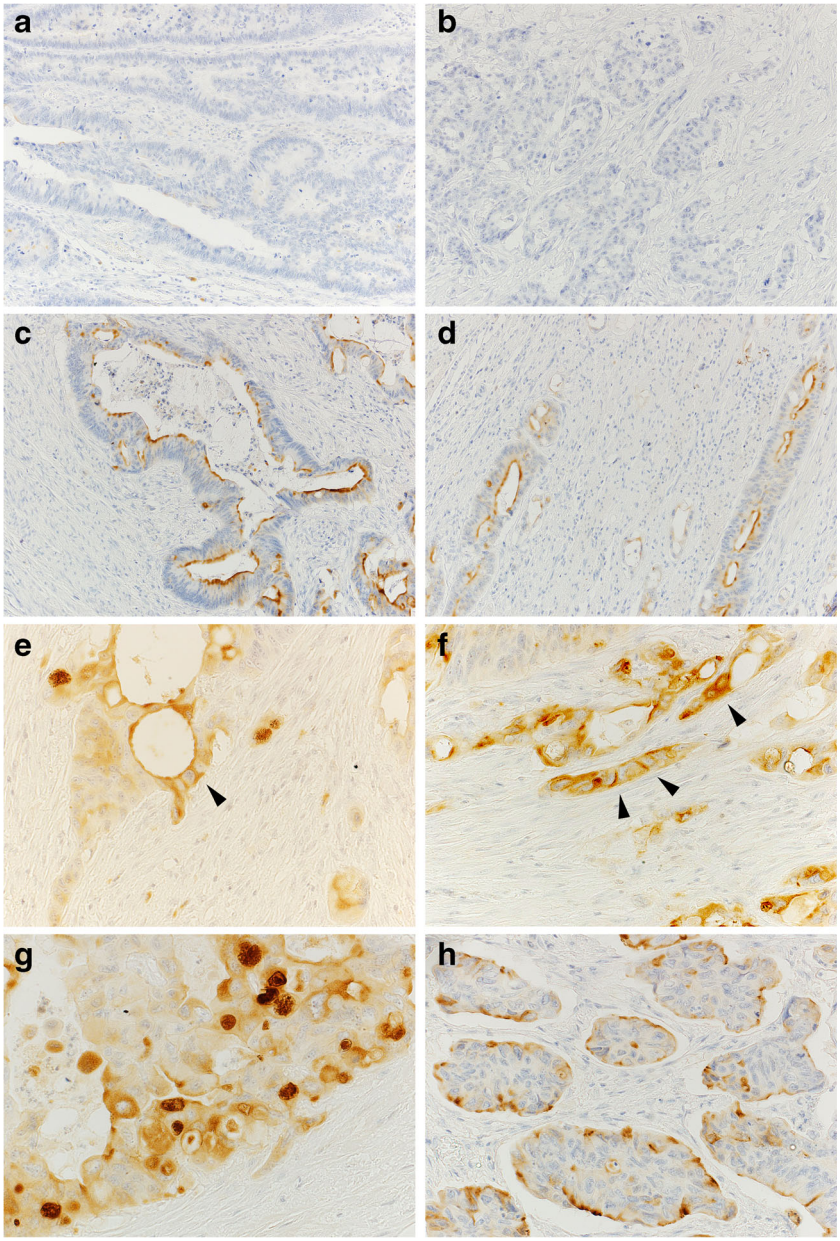

Fig. 1 Variation of MSLN expression and cellular localization in colorectal cancer tissues. **a**, **b** MSLN-negative tissue with no stained cells. **c**, **d** The luminal staining pattern with positive staining localized at the apical regions of cells at the endoluminal surface. **e**–**h** A case of the non-luminal staining pattern. **e**, **f** The non-luminal staining pattern with positive staining on the cell surfaces facing the stroma, or adjacent cancer cells (arrowheads). **g** The non-luminal staining pattern with positive staining in cytoplasmic deposit or granules. **h** The non-luminal staining pattern with positive staining on the periphery of cell clusters (so-called the inside-out pattern). Magnification: **a**–**d**, × 200; **e**–**h**, × 400. MSLN mesothelin

GAAGACGCCAGTGGA-3′ (reverse) primers for the *GAPDH* (Perfect Real-Time Support System; Takara Bio, Inc.). SYBR green RT-qPCR was performed using a Thermal Cycler Dice Real-Time System TP800 (Takara Bio, Inc.). The PCR reaction mixtures included distilled water, primers, and SYBR Premix Ex Taq II polymerase (Takara Bio, Inc.). PCR conditions included initial denaturation for 30 s at 95 °C, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. All the assays were performed in duplicate. A melting curve was constructed for each primer pair to confirm product specificity. RT-qPCR data were analyzed with Multiplate RQ software (Takara Bio, Inc.), which is able to analyze data from reactions with multiple reference genes. The comparative $2^{-\Delta\Delta Ct}$ method was used to determine relative gene expression. The mean of the two values was used in the data analysis.

Western blot analysis

Twenty-nine frozen tissue samples were used in the western blot assays. The specimens were cut into serial sections, and total protein was isolated after treatment with ISOGEN (Nippon gene, Tokyo, Japan) following the manufacturer's protocol. For sample preparation, 50 μl aliquots of each lysates was mixed with 50 μl of 2× sample buffer with reducing conditions, placed on a hotplate for 10 min at 100 °C and, then, separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The

separated proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Tokyo, Japan). The membranes were blocked for 2 h in block-Ace (DS pharma biomedical, Osaka, Japan) followed by incubation with primary MSLN antibodies (clone 5B2 diluted 1:1000; Novocastra, Newcastle Upon Tyne, UK) at 4 °C overnight with gentle shaking. The membranes were washed five times with PBS-T for 10 min, before incubation with horseradish peroxidase-conjugated secondary antibodies (Envision+System-HRP; Dako, Grostrup, Denmark) for 2 h at room temperature. Protein bands were visualized using enhanced chemiluminescence detection reagents (SuperSignal West Dura Extended Duration Substrate, Thermo Fisher Scientific Inc., Tokyo, Japan) and read with an ImageQuant LAS 4000 system (GE Healthcare, Tokyo, Japan). Anti-GAPDH antibodies (1:1000 dilution; catalog no. ab125247; Abcam, Cambridge, MA, USA) were used as a loading control. Image J software was used to perform the densitometric analysis of the protein bands.

Statistical analysis

Correlations of MSLN expression scores and clinicopathological variables were calculated and tested for significance with χ^2 tests or Fisher's exact method. Unpaired *t* tests were used to compare differences in continuous variables that had a normal distribution. A *P* value of < 0.05 was considered statistically significant. Cancer-specific survival (CSS) was the interval from surgery to death from CRC recurrence. Survival probabilities were calculated by the Kaplan–Meier method, and comparisons were made with the log-rank test. The significance of the association of clinical and pathological variables and postoperative survival was tested by Cox's proportional hazard regression to determine the hazard ratio (HR) and 95% confidence interval (CI). All the statistical procedures were performed with JMP Pro 13.1.0 software (SAS Institute, Cary, NC, USA).

Results

Interobserver agreement and correlations of MSLN immunohistochemical expression and clinicopathological characteristics

The tumor cells were MSLN-positive in 88 of the 530 patients (16.6%) and MSLN-negative in 442 (83.4). Of the 88 MSLN-positive tissues, a luminal staining pattern was seen in 33 (37.5%) and a non-luminal staining pattern was seen in 55 (62.5%). Interobserver agreement for evaluation of MSLN immunostaining was almost perfect, 96.4% ($\kappa = 0.87$) for percentage of tumor positive cells and 93.0% ($\kappa = 0.85$) for the staining pattern. Table 1 shows the correlations of MSLN immunoreactivity and clinicopathological characteristics. MSLN expression was correlated with depth of tumor ($P = 0.0062$) and tumor budding ($P < 0.0001$). Non-luminal staining was correlated with depth of tumor ($P = 0.0047$) and tumor budding ($P < 0.0001$), respectively.

Correlation of MSLN immunohistochemistry and mRNA expression

In the 80 evaluated patients, 12 were MSLN-positive (15.0%) and 68 were MSLN-negative (85.0%). The patients with MSLN-positive immunostaining had significantly higher MSLN mRNA expression (median MSLN/GAPDH ratio of 1.99, range 0.25–9.45) than those who were MSLN-negative (median MSLN/GAPDH ratio of 0.33, range 0.01–3.84, $P = 0.0014$; Fig. 2a). Immunohistochemistry in the 12 MSLN-positive patients revealed that mRNA expression did not differ significantly between those with luminal staining ($n = 2$, median MSLN/GAPDH ratio 2.23, range 0.60–3.87) and those with non-luminal staining ($n = 10$, median MSLN/GAPDH ratio 1.99, range 0.25–9.45).

Correlations of MSLN immunohistochemistry and western blot assays

The Western blot assays showed significantly higher MSLN protein expression in patients with immunohistochemically MSLN-positive immunostaining ($n = 8$, median MSLN/GAPDH ratio 7.34, range 1.24–14.68) compared with MSLN-negative patients ($n = 21$, median MSLN/GAPDH ratio 2.97, range 0.51–8.38; $P = 0.021$, Fig. 2b). The MSLN protein expression in tumor tissue with a luminal ($n = 2$, median MSLN/GAPDH ratio 7.38, range 1.24–13.52) did not differ significantly from that in tissue with a non-luminal ($n = 6$, median MSLN/GAPDH ratio 7.34, range 3.64–14.68) staining pattern.

Prognostic implications of MSLN status

The 5-year CSS rates in stage II/III CRC patients with MSLN-positive (73.4%) and MSLN-negative (89.3%) tumors were significantly different ($P < 0.0001$). When the MSLN-positive patients were stratified by the staining pattern, the 5-year CSS survival in those with luminal staining (87.1%) did not differ from that of patients who were MSLN-negative (89.3%, $P = 0.96$). The 5-year CSS was significantly worse in patients with non-luminal staining (65.2%) than in those who were MSLN-negative (89.3%, $P = 0.0001$).

In stage II CRC patients, the 5-year CSS was worse in the MSLN-positive (87.9%) than in the MSLN-negative patients (96.0%, $P = 0.024$). The difference in 5-year CSS in the patients with luminal staining (95.2%) and in those with MSLN-negative (96.0%) was not significant ($P = 0.81$). The 5-year survival rate of those with non-luminal staining (81.3%) was

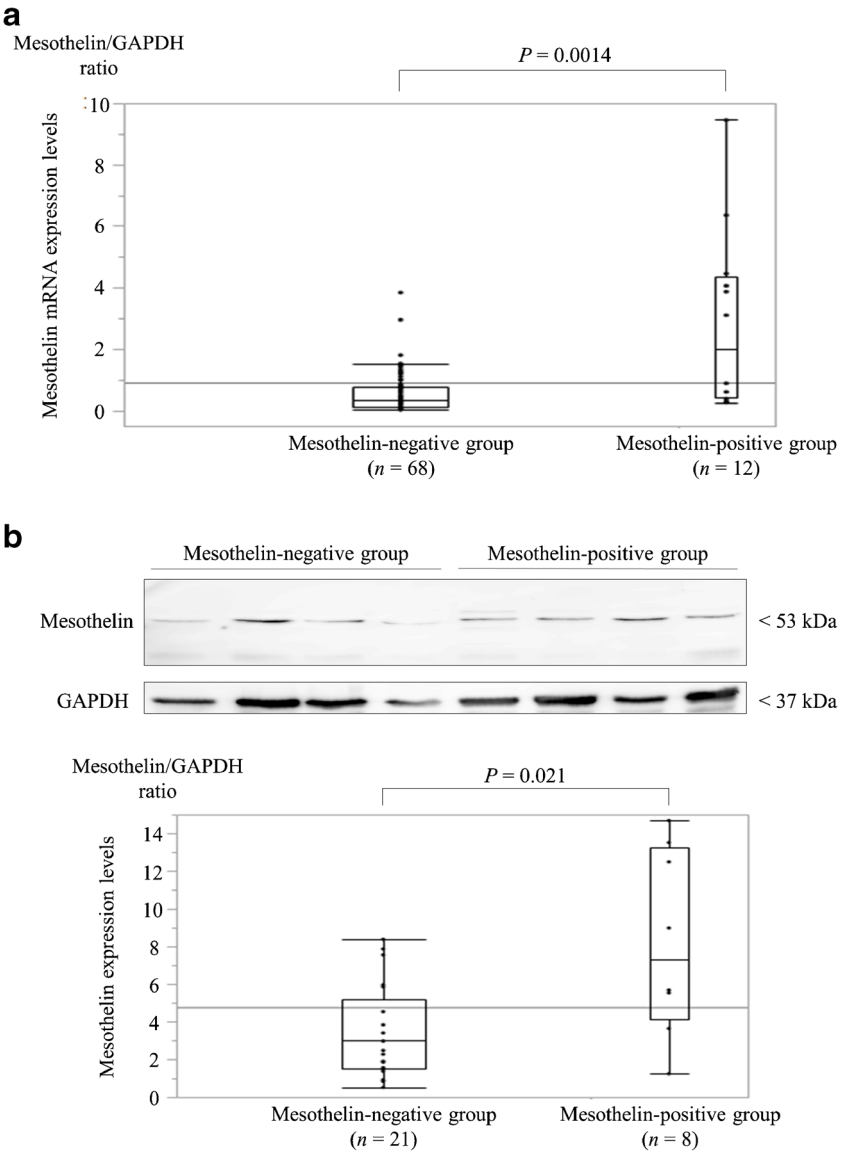

Fig. 2 **a** Correlation between immunohistochemistry and mRNA expression. Cancer tissue with MSLN-positive immunoreactivity has significantly higher MSLN mRNA expression (median MSLN/GAPDH ratio 1.99, range 0.25–9.45) than tissue with MSLN-negative immunoreactivity (median MSLN/GAPDH ratio 0.33, range 0.01–3.84, $P = 0.0014$). MSLN mesothelin. **b** Western blotting of tissues that were MSLN-negative and MSLN-positive by immunohistochemistry. Analysis of western blot assays showed significantly higher MSLN protein expression levels in immunohistochemically MSLN-positive tissues ($n = 8$, median MSLN/GAPDH ratio 7.34, range 1.24–14.68) than in MSLN-negative cases ($n = 21$, median MSLN/GAPDH ratio 2.97, range 0.51–8.38, $P = 0.021$). The molecular weight of MSLN is 41 kDa, but the band in the figure is approximately 53 kDa, which might be the result of glycosylation. MSLN mesothelin

significantly worse than that of those who were MSLN-negative (89.3%, $P = 0.0006$, Fig. 3a, b). Univariate analyses using the Cox proportional hazards model revealed that depth of tumor ($P = 0.012$), tumor budding ($P = 0.012$), and non-luminal staining ($P = 0.0061$) were significantly correlated with the risk of cancer death. Following the Cox multivariate proportional hazard model analysis, including variables with a P value of < 0.1, non-luminal staining remained as the only variable that was independently associated with poor CSS in stage II CRC ($P = 0.040$; HR = 2.92, Table 2).

In stage III CRC patients, the 5-year CSS rates in MSLN-positive (53.8%) patients were significantly worse than in those who were MSLN-negative (79.3%, $P = 0.0002$). The 5-year survival rates of patients with luminal staining (66.7%) and those who were MSLN-negative (79.3%) were not significantly different ($P = 0.36$). Tumors with a non-luminal staining pattern had a significantly poorer prognosis, a 5-year CSS of 50.0% compared with the patients who had MSLN-negative tumors (79.3%, $P < 0.0001$, Fig. 3c, d). Univariate analyses using the Cox proportional hazards model indicated that depth of tumor ($P = 0.029$), lymph node metastasis ($P = 0.058$), venous invasion ($P = 0.017$), tumor budding ($P = 0.0004$), adjuvant chemotherapy ($P = 0.043$), and non-luminal staining pattern ($P = 0.0006$) were significantly correlated with the risk of cancer death. Following the Cox multivariate analysis, including only variables with a P value of < 0.1, only non-luminal staining was independently associated with poor 5-year CSS in stage III CRC patients ($P = 0.020$; HR = 2.13, Table 3).

Discussion

In this study, the prognostic value of MSLN was investigated in an analysis of staining extent and expression pattern in stage

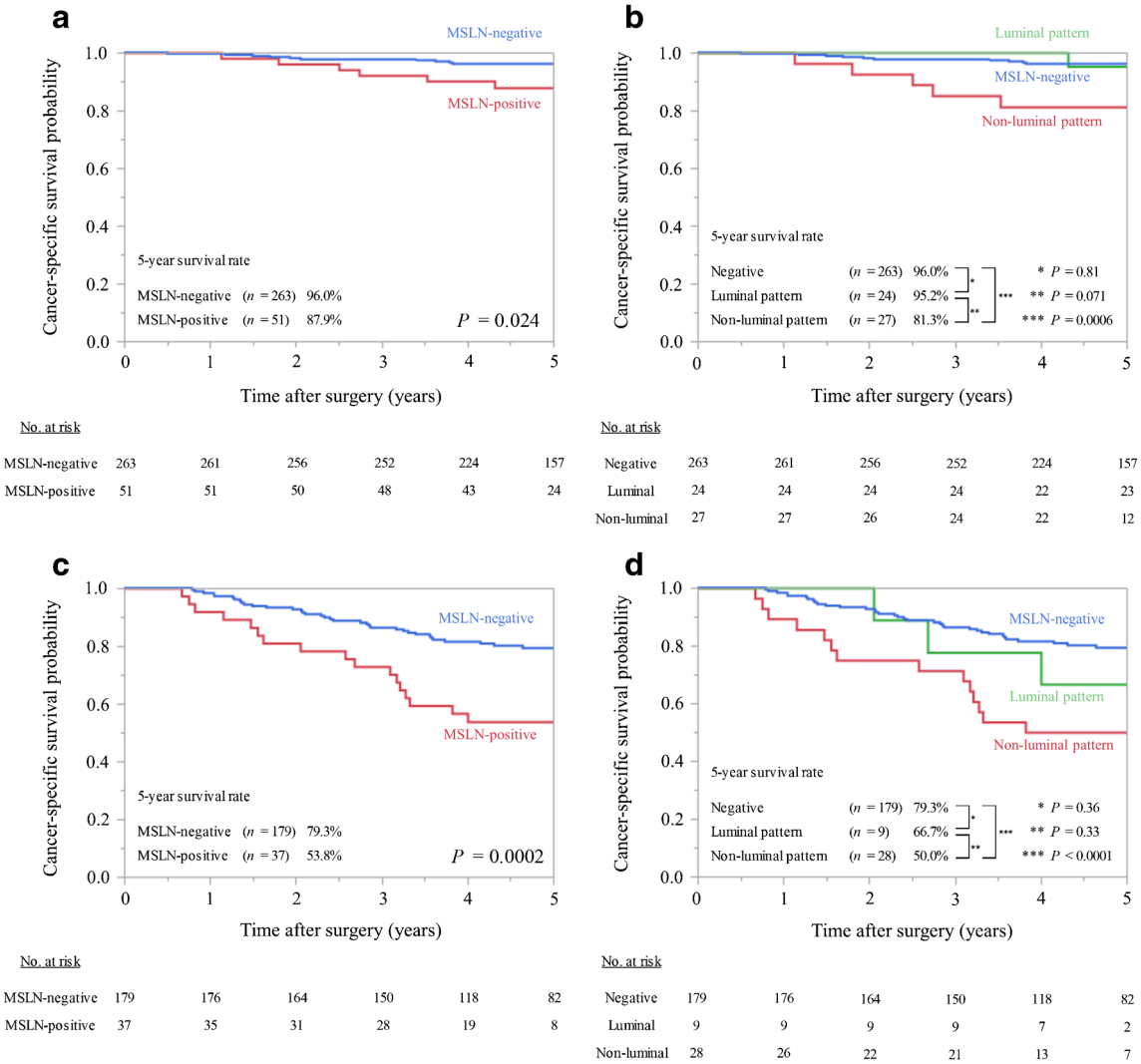

Fig. 3 Kaplan–Meier survival estimates of stage II/III colorectal cancer patients with differences in MSLN expression detected by immunohistochemistry. **a** MSLN-negative vs. MSLN-positive with stage II CRC. The difference in 5-year CSS (87.9% vs. 96.0%) was significant ($P = 0.024$). **b** The MSLN-negative vs. luminal staining pattern vs. non-luminal staining pattern in stage II CRC. There was no significant difference ($P = 0.81$) in the 5-year CSS of those with the luminal staining pattern (95.2%) and MSLN-negative staining (96.0%). Five-year CSS was significantly worse with non-luminal staining (81.3%) than with MSLN-negative staining ($P = 0.0006$). **c** MSLN-negative vs. MSLN-positive staining in stage III CRC. There was a significant difference in the 5-year CSS of the MSLN-positive (53.8%) and MSLN-negative (79.3%) patients ($P = 0.0002$). **d** The negative vs. luminal staining pattern vs. non-luminal staining pattern in stage III CRC. The 5-year CSS of patients with the luminal staining pattern (66.7%) and MSLN-negative staining (79.3%) were not significantly different ($P = 0.36$). The 5-year CSS of patients with the non-luminal staining pattern (50.0%) was significantly worse than that of the MSLN-negative patients ($P < 0.0001$). MSLN mesothelin

II/III CRC patients. MSLN expression was shown to be a prognostic factor in stage II/III CRC patients with the non-luminal staining pattern, including cell membrane and/or cytoplasmic staining, sometimes outer surfaces of cell clusters were stained (so-called the inside-out pattern), was found to be independently associated with worse survival.

High MSLN expression may indicate a poor prognosis in pancreatic, ovarian, and gastric cancer [15, 16, 26, 33, 34]; however, it seems that only one study of the significance of MSLN expression in CRC has been published [35]. In that study of patients with stages I–IV CRC, Kawamata et al. reported MSLN-positivity, a staining percentage ≥ 10%, in 45 of the 91 tumors (49.5%) but that the results of immunohistochemical staining were not correlated with survival [35]. In this study, based on the ROC curve analysis for death from CRC recurrence, the optimal cutoff score of immunoreactivity was 30%. Consequently, the MSLN-positive rate was 16.6% and clearly showed that MSLN expression indicated a poor prognosis. In the two studies, the MSLN expression pattern was also scored differently. Kawamata et al. classified MSLN

Table 2 Univariate and multivariate analyses based on Cox's proportional hazards model for cancer-specific survival according to the clinicopathological features in stage II colorectal cancer patients

Variables	No. of cases	Univariate analysis			Multivariate analysis		
		Hazard ratio	95% confidence interval	*P* value	Hazard ratio	95% confidence interval	*P* value
Sex							
Male/female	183/131	0.86	0.37–2.05	0.74			
Age							
< 65/≥ 65	133/181	0.75	0.30–1.74	0.50			
Location							
Right side/left side	106/208	0.54	0.18–1.37	0.20			
Histological grading							
G1, 2/G3, 4	296/18	1.08	0.22–19.34	0.94			
Depth of tumor							
T3/T4	254/60	0.31	0.14–0.76	0.012	0.38	0.16–0.90	0.028
Venous invasion							
Negative/positive	53/261	1.18	0.34–3.16	0.77			
Tumor budding							
Grades 1, 2/grade 3	242/72	0.33	0.14–0.78	0.012		Not selected	
MMR status							
MMRp/MMRd	300/14		Not available*				
Adjuvant chemotherapy							
Surgery alone/chemotherapy	285/29	0.83	0.24–5.26	0.81			
Type of mesothelin expression							
Luminal/negative	24/263	0.79	0.044–3.90	0.81	0.67	0.089–5.09	0.70
Non-luminal/negative	27/263	4.53	1.61–11.17	0.0061	2.92	1.05–8.13	0.040

*All cases showing cancer-specific death were categorized as DNA mismatch repair deficiency (MMRd) group

expression into luminal membrane-positive and luminal membrane-negative patterns; the tumor was classified as luminal membrane-positive when the luminal membrane was even partially or faintly stained or the entire circumference of the luminal membrane was clearly stained and the tumor was classified as luminal membrane-negative when the luminal membrane was not stained or when only the cytoplasm was stained. Based on these criteria, luminal membrane-positive MSLN staining was significantly correlated with unfavorable patient outcome in stage III/IV CRC. On the other hand, in this study, the relationship of the stained area and cancer cell polarity was taken into account. Tissues in which cell polarity was maintained and the lumen side of the tubules was stained were defined as a luminal staining pattern. Tissues with positive staining away from the lumen surface had a non-luminal staining pattern.

The luminal staining pattern was interpreted as indicating that the MSLN molecules were positioned facing the lumen and did not affect other tumor or stromal cells after shedding. The non-luminal staining pattern was believed to indicate MSLN secretion into the surrounding tissue where it interacted with other tumor and stromal cells, and may have promoted aggressiveness. A poor prognosis indicated by non-luminal staining in this population of CRC patients may have been related to MSLN activity in the tissue surrounding the lumen that affected cancer progression.

The activity of MSLN in cancer pathogenesis has not been explored in depth; however, there is evidence that MSLN-mediated activation of specific signaling pathways is important for cancer progression. Bharadwaj et al. found that MSLN overexpression in pancreatic cancer cells led to the constitutive activation of the transcription factor Stat3, increased expression of cyclin E and cyclin E/cyclin-dependent kinase 2 complex, and enhanced G1/S cell-cycle progression [36]. MSLN-activated nuclear factor-kappa B (NF-kB) induces increased expression of IL-6, which acts as a growth factor to support pancreatic cancer cell survival and proliferation by auto/paracrine IL-6/soluble IL-6R trans-signaling [37]. MSLN is also known to bind to the ovarian cancer antigen MUC16 (also known as CA125) [38] with which it is often coexpressed. Their binding has been reported to mediate cell adhesion, infiltration, and migration [38–41]. The present study clearly demonstrated that the expression of MSLN, particularly the non-luminal staining pattern expression, is closely linked to high grade of tumor budding, which is an

Table 3 Univariate and multivariate analyses based on Cox's proportional hazards model for cancer-specific survival according to the clinicopathological features in stage III colorectal cancer patients

Variables	No. of cases	Univariate analysis			Multivariate analysis		
		Hazard ratio	95% confidence interval	*P* value	Hazard ratio	95% confidence interval	*P* value
Sex							
Male/female	122/94	1.10	0.66–1.89	0.71			
Age (years)							
< 65/≥ 65	109/107	0.86	0.51–1.47	0.59			
Location							
Right side/left side	49/167	0.83	0.42–1.52	0.56			
Histological grading							
G1, 2/G3, 4	192/24	0.61	0.31–1.32	0.20			
Depth of tumor							
< T3/T4	167/49	0.53	0.31–0.93	0.029		Not selected	
Lymph node metastasis							
N1/N2	152/64	0.59	0.35–1.02	0.058		Not selected	
Venous invasion							
Negative/positive	29/187	0.31	0.075–0.83	0.017		Not selected	
Tumor budding							
Grades 1, 2/grade 3	138/78	0.39	0.23–0.65	0.0004	0.45	0.26–0.79	0.0056
MMR status							
MMRp/MMRd	194/22	1.29	0.57–3.71	0.58			
Adjuvant chemotherapy							
Surgery alone/chemotherapy	77/139	1.72	1.02–2.90	0.043	2.38	1.35–4.21	0.0029
Type of mesothelin expression							
Luminal/negative	9/179	1.72	0.41–4.76	0.40	1.91	0.57–6.42	0.30
Non-luminal/negative	28/179	3.17	1.69–5.65	0.0006	2.13	1.13–4.01	0.020

important histological feature of the loss of cell-cell adhesion as well as increased ability of migration of cancer cells. The evidence of basic and clinical studies is in line with MSLN involvement in cancer progression and metastasis and consistent with the study data.

Currently, molecular targeted therapy is indicated for treatment of diverse cancers. Recent approvals include antibodies against epidermal growth factor receptor (EGFR), such as cetuximab and panitumumab for CRC, and against c-erbB-2 (HER2), such as trastuzumab for breast cancer and gastric cancer. MSLN, a cell membrane binding protein similar to EGFR or HER2, may become a candidate target for antibody therapy, because MSLN expression has a poor prognosis. Currently, MSLN is being tested as a target of antibody-mediated pancreatic cancer therapy [20]. Investigation of anti-MSLN molecular targeting in the treatment of MSLN-expressing CRC is warranted.

The study limitations include potential changes in tissue MSLN antigenicity associated with tissue processing from fixation to section preparation. The changes could result in insufficient detection sensitivity. However, the evaluation of MSLN expression by immunohistochemical staining was validated by RT-qPCR and western blot assays, showing that the immunohistochemical procedures were reliable. Second, the retrospective study design, postoperative adjuvant chemotherapy, surgical procedures, and/or treatments for recurrent cases that were influenced by age or performance status may have been sources of bias.

In conclusion, a non-luminal MSLN staining pattern was independently associated with reduced 5-year survival and indicated a poor prognosis in stage II/III CRC patients. Evaluation of MSLN immunoexpression in surgical specimens may be useful in accurately predicting prognosis. Future studies will investigate the activity and prognostic value of MSLN expression in metastatic lesions. These and future study results will ultimately allow a determination to conduct clinical trials on the administration of MSLN antibody as adjuvant chemotherapy.

Authors' contributions TS and ES conceived and designed the experiments. TS and SM performed the experiments. TS, ES, and HT analyzed histopathological data. TS and ES drafted the manuscript. HT, YK, KO, KH, and HU revised the manuscript. TS finalized the manuscript. All authors reviewed and approved the manuscript.

Compliance with ethical standards The experiments reported here were carried out in agreements with the Declaration of Helsinki principles and in agreement with the Ethics Committee of the National Defense Medical College Hospital, Tokorozawa, Japan.

Conflict of interest The authors declare that they have no conflicts of interest.

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