Diffuse mesothelin expression leads to worse prognosis through enhanced cellular proliferation in colorectal cancer

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Abstract. Mesothelin (MSLN) is a glycophosphatidylinositol (GPI)-linked cell surface protein that is highly expressed in several types of malignant tumor, including malignant pleural mesothelioma, ovarian cancer and pancreatic adenocarcinoma. Recently, a comprehensive immunohistochemical study using MN-1 monoclonal antibody identified a significant number of colorectal tumors in which MSLN was expressed. However, the clinicopathological profiles and survival of patients with MSLN-positive colorectal cancer have not been fully analyzed. In the current study, the expression of MSLN in 270 primary and 44 metastatic colorectal tumors was immunohistochemically analyzed to determine the clinical usefulness of MSLN immunohistochemistry and to identify potential candidates for future anti-MSLN therapy. *In vitro* experiments using colon cancer cell lines were performed to investigate the biological significance of MSLN expression in tumors. The results of univariate analyses identified a significant correlation between MSLN expression and females (P=0.0042). Furthermore, an inverse correlation between MSLN expression and solid/sheet-like proliferation (P=0.014) was also revealed. Additionally, overall survival was significantly shorter in patients with diffuse luminal/membranous expression of MSLN (P=0.018). Multivariable Cox hazards regression analysis revealed diffuse MSLN expression (hazard ratio, 2.26; 95% confidence interval, 1.04-4.91; P=0.039) as a potential risk factor. When comparing primary CRCs and the metastasis of each, a weakly positive correlation was identified for MSLN positivity (% positive cells; R=0.484; P<0.0001). The *in vitro* experiments revealed a positive role for MSLN in colon cancer cell proliferation. Thus, MSLN immunohistochemistry may be useful in the prognostication of patients with CRC. The results demonstrated that significant numbers of patients with MSLN-positive CRC exhibiting metastasis could be targeted by anti-MSLN therapies.

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Abbreviations: CRC, colorectal cancer; HR, hazard ratio; CI, confidence interval; GPI, glycophosphatidylinositol; MPF, megakaryocyte potentiating factor; CAR, chimeric antigen receptors; DAB, 3,3-diaminobenzidine; SDS, sodium sodecyl sulfate

Key words: mesothelin, colorectal cancer, immunohistochemistry, prognosis, cellular proliferation

Introduction

Mesothelin (MSLN) is a glycophosphatidylinositol (GPI)-linked cell surface protein that is usually expressed in mesothelial cells. *MSLN* encodes a precursor protein of 71 kDa that is processed to a shed 31 kDa protein, megakaryocyte potentiating factor (MPF), and 40-kDa membrane-bound MSLN protein (1). Based on normal growth and reproduction in a *Msln*-deficient mouse model, the biological function of MSLN is not fully understood (2).

In neoplastic conditions, MSLN was reported to be highly expressed in several types of malignant tumors, including malignant pleural mesothelioma, ovarian cancer, pancreatic adenocarcinoma, and gastric cancer (3-10). Recently, our group observed significant expression of MSLN in colorectal tumors, with up to 60% of cases demonstrating positivity (11).

Prognostication using MSLN immunohistochemistry has been reported in several types of tumors. In breast and lung adenocarcinoma, high-level MSLN expression was reported to be associated with poor prognosis (8,9,12). In contrast, prolonged survival in patients with MSLN-expressing tumors was noted in ovarian serous and thymic carcinomas as well as malignant pleural mesothelioma (11,13,14). In colorectal carcinomas, the clinicopathological profiles and prognosis of MSLN-positive CRC patients have not been fully studied. Furthermore, the biological significance of MSLN expression in CRC development remains to be elucidated (15-17).

Anti-MSLN immunotherapies using anti-MSLN monoclonal antibodies, antibody drug conjugates, and chimeric

antigen receptors (CAR) T cells have been developed and are believed to be promising therapeutics for patients with MSLN-expressing tumors (18). MSLN expression in immunohistochemistry is envisaged to be a suitable biomarker for predicting clinical response to these therapeutics, but its efficacy has never been fully evaluated.

The aim of the present study was to evaluate the clinicopathological, prognostic, and biological significance of MSLN expression in colorectal cancer (CRC) to elucidate the usefulness of MSLN immunohistochemistry for determining the prognosis of CRC patients and to identify potential candidates for MSLN immunotherapy.

Materials and methods

Tissue samples. The Institutional Ethical Review Board of Aichi Medical University Hospital approved this project to perform without collecting patient consent by giving them the opportunity for opt out. Two hundred and seventy primary colorectal tumors, resected at Aichi Medical University Hospital during 2009-2012, were collected according to the availability of tissue samples and clinical information. Fifty-two normal colonic mucosae samples adjacent to tumors as well as 44 metastases from 27 patients were also collected. After surgery, patients were followed up for up to 90 months. All the tumors were diagnosed to be invasive and naïve to chemotherapy or radiotherapy. Tumors with glandular formation (>50%) were defined as differentiated histology. Single 4.5-mm core tumor tissue samples derived from surgical specimens were assembled into tissue arrays containing up to 30 samples. The size of tumor tissue samples was estimated to exceed the size of a single 0.6 mm^2 core by a factor of 8-9.

Antibodies. The antibodies used in this study are as follows: MSLN (Rockland Immunochemicals Inc.), MLH1 (Clone G168-728; BD Biosciences), MSH2 (Clone G219-1129; BD Biosciences) MSH6 (Clone 44; BD Biosciences) PMS2 (Clone A16-4; BD Biosciences) CCNA (sc-751; Santa Cruz Biothechnology, Inc.), GAPDH (Clone 6C5; Santa Cruz Biothechnology), ERK (Clone 137F5; Cell Signaling Technology, Inc.), P-ERK (Clone 20G11; Cell Signaling Technology, Inc.).

Immunohistochemistry. Immunohistochemistry was performed using the Ventana BenchMark XT automated immunostainer (Roche Diagnostics). The conditions for immunohistochemistry are summarized in Table I. Signals were visualized by 3,3-diaminobenzidine (DAB) staining. MSLN immunoreactivity (luminal/membranous) was evaluated with a detection cut-off of 5% for any signal intensity, as described in our previous report (11). Cyclin A (CCNA) labeling indices were determined by counting more than 500 tumor cells per case.

Statistical analyses. All statistical analyses were performed with EZR version 1.32. software (19). Chi-squared or Student's t-test were performed to investigate the statistical association. The Bonferroni-corrected P-value for significance was P=0.0042 (0.05/12). The Spearman's rank correlation coefficient was used to analyze positivity (% positive cells) between primary tumors and their metastases. According to previous reports (11,14), the impact of diffuse (100% positive cells on the lumen/cell membrane) MSLN expression on overall survival was analyzed using Kaplan-Meier survival estimates with log-rank tests. Cox proportional hazards regression analysis was used to analyze the association of MSLN with survival and other factors. The initial model included age (<70 years vs. ≥70 years), sex (male vs. female), primary tumor location (right-sided colon vs. left-sided colon vs. rectum), tumor size (<5 cm vs. ≥5 cm), T stage (2 vs. 3 vs. 4), surgical status (complete resection vs. residual tumor), tumor histology (well to moderately vs. poorly differentiated), lymph node metastasis (positive vs. negative), distant organ metastasis (positive vs. negative), peritoneal metastasis (positive vs. negative), mismatch repair (MMR) system status (deficient vs. preserved), and data from MSLN immunohistochemistry (diffuse MSLN expression: 100% positive cells on the lumen/cell membrane vs. negative or partial expression in any location). Backward elimination with a threshold of P=0.05 was used to select variables in the final model. The Mann-Whitney U or Kruskal-Wallis with post-hoc test (Dunnett's test) was used for the statistical analyses in molecular experiments.

In vitro molecular experiments. The origins of other colon cancer cell lines were described previously (20). The human colon cancer cell lines, COLO205, CW-2, HCT116 and LoVo were obtained from the RIKEN BioResource Center. SW480 and Caco2 were from American Type Culture Collection (ATCC). The human colon cancer cell line SW48 was kindly provided by Dr. Yutaka Kondo (Nagoya University, Aichi, Japan). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). For β-estradiol stimulation experiments, activated charcoal-treated FBS was used. β-estradiol stimulation was performed for 24 h at a concentration of 10 μM, as defined in our previous studies (21-23).

CW-2 and HCT-116 cell lines expressing exogenous MSLN or its control LacZ were established by stable transfection with pcDNA 3.1 vectors (Invitrogen; Thermo Fisher Scientific, Inc.) containing *MSLN* or *LacZ* followed by IRES2 and puromycin resistance genes. The 21-nucleotide duplex siRNAs were synthesized as follows: si*MSLN*-1, 5'-CCCGUUUCUUCUCCCGCAUTT-3' and 5'-AUGCGGGAGAAGAAACGGGTT-3'; si*MSLN*-2, 5'-GCCUCAUCUUCUACAAGAATT-3' and 5'-UUCUUGUAGAAGAUGAGGCTT-3'; siControl, 5'-GACGUAUGACUAACUAACATT-3' and 5'-UGUUAGUUAGUCAUACGUCTT-3' (Nippon Gene Material Co., Ltd.). Transient transfection of siRNAs was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Inc.).

Immunoblot analyses were performed as previously reported (21-23). In short, whole cell lysates were prepared using 1x Sodium Dodecyl Sulfate (SDS) sample buffer, containing 50 mM Tris-HCl and 2% SDS. The SDS polyacrylamide gel electrophoresis was performed using 12% polyacrylamide gel and separated proteins were transferred to a PVDF membrane. Antibody dilutions are summarized in Table I. Each immunoblot panel was made from one membrane. For sequential detection, antibody stripping buffer (0.1 M Glycine-HCl pH 2.5) was used. Signal intensity was measured by ImageJ software (National Institutes of Health).

Cellular proliferation activity was measured using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega

Table I. Antibodies and conditions for immunohistochemistry and immunoblot analysis.

	Immunohistochemistry		Immunoblot	
Genes	Reagent	Dilution	Dilution	Antibodies
MSLN	OV	2,000	20,000	Rockland Immunochemicals Inc.
MLH1	OV	200	-	Clone G168-728, BD Biosciences
MSH2	OV	200	-	Clone G219-1129, BD Biosciences
MSH6	OV	400	-	Clone 44/MSH6, BD Biosciences
PMS2	OV+Linker	50	-	Clone A16-4, BD Biosciences
CCNA	IV	100	500	sc-751, Santa Cruz Biothechnology, Inc.
ERK	-	-	1,000	Clone 137F5, Cell Signaling Technology, Inc.
P-ERK	-	-	500	Clone 20G11, Cell Signaling Technology, Inc.
GAPDH	-	-	3,000	Clone 6C5, Santa Cruz Biothechnology, Inc.

Antigen retrieval was performed with heat activation in high pH buffer. MSLN, mesothelin; OV, OptiView reagent; IV, iVIEW reagent; MLH1, mutL homolog 1; MSH, mutS homolog; PMS2, PMS1 homolog 2; CCNA, cyclin A.

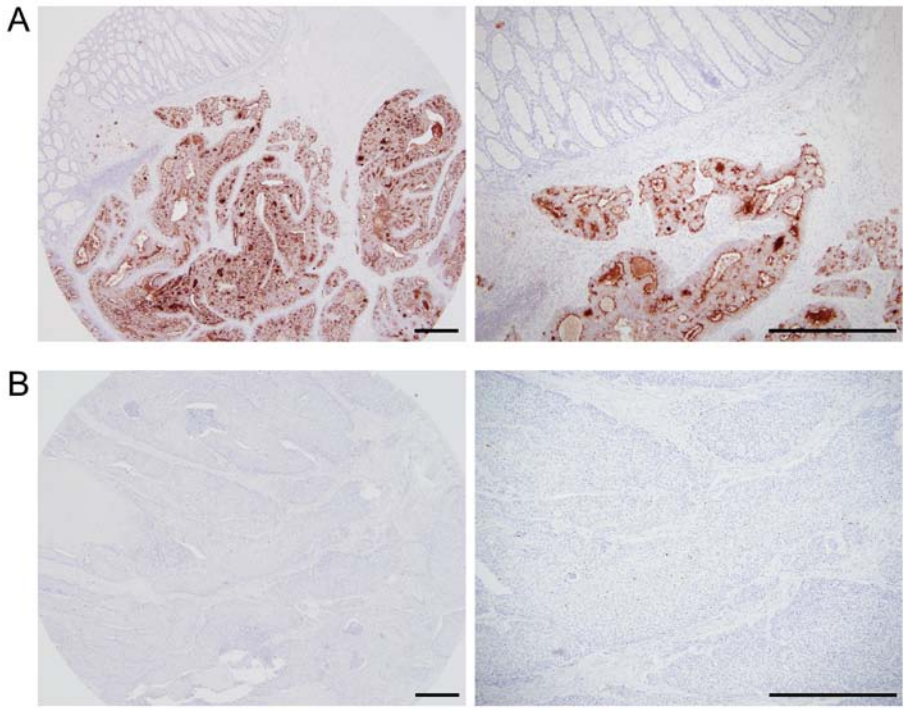

Figure 1. MSLN immunohistochemistry in colon cancer tissue. (A) Case of tubular adenocarcinoma. Diffuse and luminal MSLN expression was detected. (B) Case of poorly differentiated adenocarcinoma exhibiting solid/sheet-like proliferation with undetectable MSLN expression. Left, low-power magnification; right, high-power magnification. Scale bar, 500 μm. MSLN, mesothelin.

Corporation) in accordance with the manufacturer's instructions. Anchorage-independent cell proliferation in soft agar was measured using a Cytoselect 96-well cell transformation assay kit (Cell Biolabs Inc.) according to the manufacturer's protocol.

Results

MSLN expression in 52 normal colonic mucosae and 270 primary colorectal tumors. None of the normal colonic mucosae evaluated in the present study expressed MSLN. Representative images for MSLN immunohistochemistry are shown in Fig. 1. The results of MSLN immunohistochemistry in primary colorectal tumors are summarized in Table II. MSLN expression was detected in 53% (142/270) of CRC cases. Significantly higher MSLN-positivity was observed in tumors from female patients (P=0.0042).

Tumors presenting solid/sheet-like proliferation tended to show a lower rate of MSLN expression than those with tubular structures (P=0.014).

In the present study, 11% (31/270) showed MMR-deficient phenotypes. Similar to our previous report (11), however, no

Table II. Characteristics of colorectal carcinomas with or without MSLN expression.

Variables	Total, n (%) (n=270)	MSLN expression, n (%)		P-value
		Positive (n=142; 53%)	Negative (n=128; 47%)	
Sex				0.0042[a]
Male	143 (53)	63 (45)	80 (63)	
Female	127 (47)	79 (56)	48 (38)	
Age, years (mean ± SD)	68.6±12.6	67.4±14.3	69.9±10.3	0.093[b]
Size, cm (mean ± SD)	4.99±2.6	4.9±2.3	5.1±2.8	0.52[b]
Tumor location				0.77[a]
Right-sided colon	125 (46)	64 (45)	61 (48)	
Left-sided colon	86 (32)	45 (32)	41 (32)	
Rectum	58 (22)	33 (23)	25 (20)	
T stage				0.14[a]
T2	37 (14)	14 (10)	23 (18)	
T3	189 (70)	105 (74)	84 (66)	
T4	44 (17)	23 (16)	21 (16)	
Histological differentiation				0.94[a]
Well to moderate	242 (90)	128 (90)	114 (89)	
Poor	28 (10)	14 (10)	14 (11)	
Solid/sheet-like proliferation				0.014[a]
Positive	13 (5)	2 (1)	11 (9)	
Negative	257 (95)	140 (99)	117 (91)	
Lymph node metastasis				0.64[a]
Positive	124 (49)	70 (52)	54 (45)	
Negative	130 (51)	64 (48)	66 (55)	
Omental metastasis				0.49[a]
Positive	50 (19)	29 (20)	21 (16)	
Negative	220 (81)	113 (80)	107 (84)	
Distant organ metastasis				0.65[a]
Positive	44	25	19	
Negative	226	117	109	
Operation status				0.80[a]
Complete resection	238 (88)	124 (87)	114 (89)	
Residual disease	32 (12)	18 (13)	14 (11)	
MMR system status				0.066[a]
Deficient	31 (11)	11 (8)	20 (16)	
Preserved	239 (89)	131 (92)	108 (84)	

[a]P-values were calculated by the Chi-square test for mesothelin expression. [b]A t-test was used to compare the means of age. The Bonferroni-corrected P-value for significance was P=0.0042 (0.05/12). MSLN, mesothelin; SD, standard deviation.

significant correlation was detected between MSLN expression and MMR system status.

MSLN expression in 44 CRC metastases. The results of MSLN immunohistochemistry in metastatic tumors are summarized in Table III. Among the metastases analyzed, lymph node (39%) and liver (30%) metastases were dominant. Metastases in the liver and peritoneum tended to exhibit higher levels of MSLN expression than those in lymph nodes and other organs.

A weakly positive correlation was detected in MSLN positivity (% positive cells) between primary sites and their metastases (R=0.484, P<0.0001; Fig. S1).

Survival analysis of CRC patients. Survival was significantly shorter for patients with diffuse expression of MSLN (100% positive cells) on the lumen/cell membrane (47.8% vs. 75.6% in 5-year survival; P=0.018; Fig. 2A). Male CRC patients with diffuse MSLN expression (P=0.010) but not female patients (P=0.312) showed a significantly worse clinical outcome

Table III. MSLN expression in metastatic lesions.

Metastatic site	Total, n (%) (n=44;)	MSLN expression		Percentage of positive cells, median (range)	P-value
		Positive, n (%) (n=30; 68%)	Negative, n (%) (n=14; 32%)		
Liver	13 (30)	8 (27)	5 (36)	75 (20-80)	0.15
Peritoneum	7 (16)	7 (53)	0 (0)	60 (5-100)	
Lymph nodes	17 (39)	12 (40)	5 (36)	25 (5-100)	
Others[a]	7 (16)	3 (10)	4 (29)	10 (5-20)	

P-value was calculated by the Mann-Whitney U test for mesothelin expression. [a]Others include 1 brain, 1 lung, 2 ovary, 2 skin and 1 small intestine metastases. MSLN, mesothelin.

(Fig. 2B and C). Multivariate Cox hazards regression analysis revealed younger age (<74 years) to be a favorable prognostic factor (hazard ratio (HR), 0.44; 95% confidence interval (CI), 0.25-0.76; P=0.0033). Poorly differentiated histology (HR, 4.27; 95% CI, 2.30-7.92; P<0.0001), peritoneal metastasis (HR, 2.34; 95% CI, 1.26-4.33; P<0.0001), diffuse MSLN expression (HR, 2.26; 95% CI, 1.04-4.91; P=0.039), and lymph node metastasis (HR, 2.21; 95% CI, 1.28-3.38; P=0.0046) were identified as potential independent risk factors (Table IV).

MSLN expression in colon cancer cell lines. In cultured colon cancer cell lines, four out of seven cell lines (57%) expressed MSLN with no association with sex (Fig. 3). Further *in vitro* studies showed no effect of β-estradiol, a major female sex hormone, on MSLN expression in CW-2 and SW48 cells, both of which were established from female patients (Fig. S2).

MSLN regulates colon cancer cell proliferation but not migration and invasion. Additional experiments were performed to examine the effects of MSLN expression in colon cancer cells. Forced expression of MSLN in CW-2 and HCT-116 cells enhanced their proliferation or survival significantly with phospho-ERK accumulation under serum-reduced conditions (Fig. 4A-C). MSLN also enhanced anchorage-independent cell proliferation of colon cancer cells (Fig. 4D). CCNA, one of markers for S phase, were upregulated in MSLN-transfected cells (Fig. 4E). Furthermore, transient knock down of *MSLN* significantly suppressed the proliferation of COLO205 and SW48 cells in both adherent and anchorage-independent conditions with downregulation of CCNA (Fig. 5). In contrast, MSLN did not alter the migration and invasion of colon cancer cells under our experimental conditions (Fig. S3). Based on these observations, the proliferative activity of 21 diffusely MSLN-expressing CRC cases and 30 arbitrary selected control cases with negative or partial MSLN expression were compared by analyzing CCNA labeling indices. Significantly higher rates of CCNA labeling indices were observed in CRC with diffuse MSLN expression (P=0.011; Fig. 6).

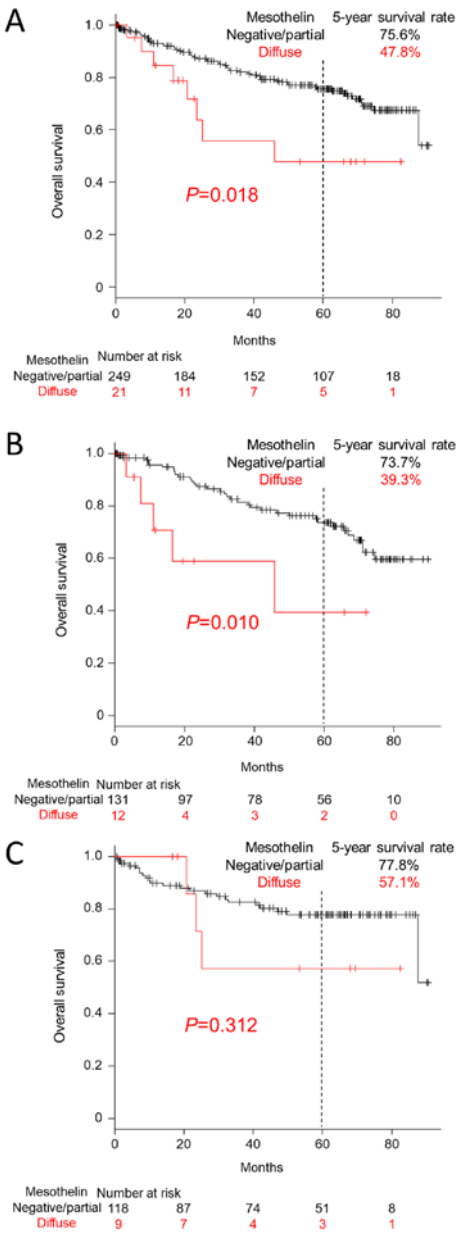

Figure 2. Overall survival of patients with CRC classified according to MSLN expression. (A) Kaplan-Meier curves for patients with colorectal tumors grouped by diffuse luminal/membranous or partial/negative MSLN expression. Kaplan-Meier curves for (B) male and (C) female patients with colorectal tumors grouped by diffuse luminal/membranous or partial/negative MSLN expression. CRC, colorectal cancer; MSLN, mesothelin.

Discussion

MSLN is a cell surface protein that is highly expressed in several types of malignant tumors and is associated with clinical outcome. Recently, our research group identified a

Table IV. Multivariate Cox hazards analysis of patients with CRC.

Variables	Hazard ratio	95% CI		P-value
		Min	Max	
Age (<74)	0.44	0.25	0.76	0.0033
Poorly differentiated histology	4.27	2.30	7.92	<0.0001
Peritoneal metastasis	2.34	1.26	4.33	<0.0001
Diffuse MSLN expression	2.26	1.04	4.91	0.039
Lymph node metastasis	2.21	1.28	3.38	0.0046

The multivariable Cox hazards analysis model initially included age, sex, primary tumor location, tumor size, T stage, operation status, tumor histology, mucus production, solid/sheet-like proliferation, lymph node metastasis, distant organ metastasis, omental metastasis, mismatch repair system status, diffuse mesothelin expression. A backward elimination with a threshold of P=0.05 was used to select variables in the final model. CRC, colorectal cancer; MSLN, mesothelin.

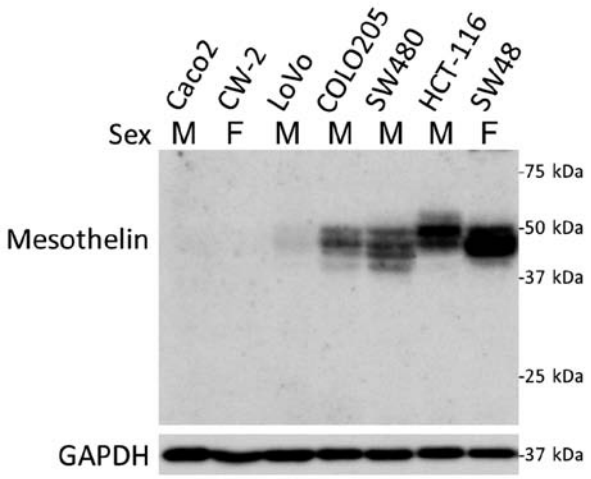

Figure 3. Expression of MSLN in cultured colon cancer cells. MSLN was expressed in four of seven colon cancer cell lines with no association with sex. MSLN, mesothelin; F, female; M, male.

significant number of MSLN-positive CRC cases in a comprehensive immunohistochemical study using MN-1 monoclonal antibody (11). In the present study, the clinicopathological profile and survival impact of MSLN were analyzed by immunohistochemistry in 270 CRC cases. Furthermore, molecular studies were performed to reveal the tumor biological significance of MSLN in colon cancer cell lines.

In the present study, 53% (142/270 cases) of CRCs were positive for luminal/membranous MSLN expression. This positivity was slightly lower than that identified in our previous study (61%, 115/188 cases) (11). This might be due to the different autoimmunostaining systems used (Ventana BenchMark XT and OptiView DAB universal kit vs. Leica Bond-Max automation and Leica Refine detection kit). It is unclear whether ethnicity (Japanese vs. Western populations) has an impact on MSLN expression. From univariate analyses, a significant correlation between MSLN expression and female sex (P=0.0042) was identified (Table II). Overall survival was significantly decreased in the cohort of patients with diffuse MSLN expression (Fig. 2). Furthermore, the multivariate Cox hazards regression analysis identified diffuse MSLN expression (P=0.039) as a potential independent risk factor (Table IV).

Previous studies analyzed the impact of diffuse (100% positive cells on the lumen/cell membrane) MSLN expression on overall survival (11,14). The present study analyzed the impact of diffuse MSLN expression on the survival of stage II to IV CRC patients and determined its expression as a potential independent risk factor (HR, 2.26; 95% CI, 1.04-4.91; P=0.039). Several studies have analyzed the impact of tumor MSLN expression on the survival of CRC patients (15-17). Shiraishi *et al* (15) found an adverse impact of MSLN expression on the survival of stage II/III CRC patients with statistical significance; however, Kawamata *et al* (16), found no significant difference in stage I to IV patients. Both studies performed MSLN immunohistochemistry using 5B2 anti-MSLN antibody. In contrast, the present study used MN-1 antibody, which has a higher affinity and positivity (% positive cells in immunohistochemistry) than 5B2 (11,24). Kim *et al* reported the prognostic role of MSLN in microsatellite unstable CRCs using SP74 antibody (17); however, this type of association was not confirmed in our cohort (data not shown). The discrepancies in these studies might result from the patient cohort (patient number and pathological stage) as well as the anti-MSLN antibody used.

Prognosis prediction using MSLN immunohistochemistry has also been reported in other tumor types. In cases of breast and lung adenocarcinoma, aberrant high MSLN expression is reported to be associated with poor prognosis (8,9,12). In contrast, prolonged survival in patients with MSLN-expressing tumors was shown in ovarian serous and thymic carcinomas (13,14). Our group also reported diffuse (100% positive cells) MSLN expression as a favorable prognostic factor in malignant pleural mesothelioma patients (11). Recently, prognostication by comprehensive molecular profiling of malignant pleural mesothelioma identified a poor prognosis cluster with an epithelial-mesenchymal transition phenotype distinguished by high mRNA expression of *VIM*, *PECAM1*, and *TGFB1*, and low *miR-200* family expression. Interestingly, these tumors also showed low *MSLN* mRNA expression with *MSLN* promoter methylation (25). These results indicate that high MSLN expression might be a favorable prognostic marker without tumor biological significance in some tumor

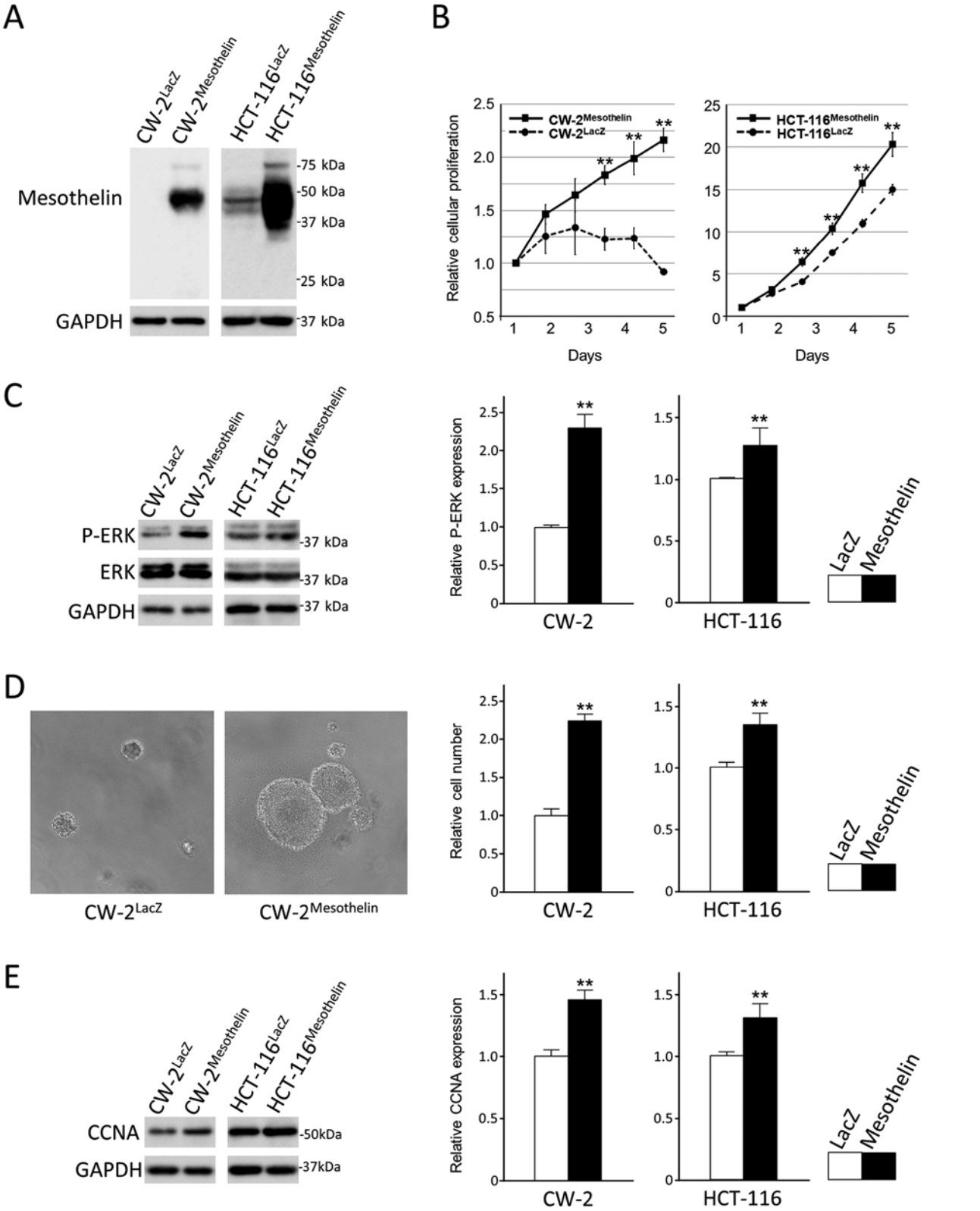

Figure 4. Forced-expression of MSLN upregulates colon cancer cell proliferation. (A) Immunoblot analyses of CW-2 and HCT-116 cells with stable expression of MSLN or its control, LacZ. (B) Colon cancer cells with forced MSLN expression exhibited significantly higher rates of cellular proliferation compared with their controls in serum-reduced conditions (CW-2, 1% serum; HCT-116, 5% serum). The expression of (C) p-ERK, (D) anchorage-independent cell proliferation and (E) CCNA expression in CW-2 and HCT-116 cells with or without MSLN is presented. Experiments were performed in triplicate. Data are presented as the mean ± standard deviation. **P<0.01 vs. LacZ group. MSLN, mesothelin; CCNA, cyclin A.

types including malignant pleural mesothelioma. In the present study, we performed further experiments to reveal the malignant potential of MSLN using colon cancer cells and found that enhanced cellular proliferation was a potential mechanism for the worse prognosis of MSLN-positive CRC patients.

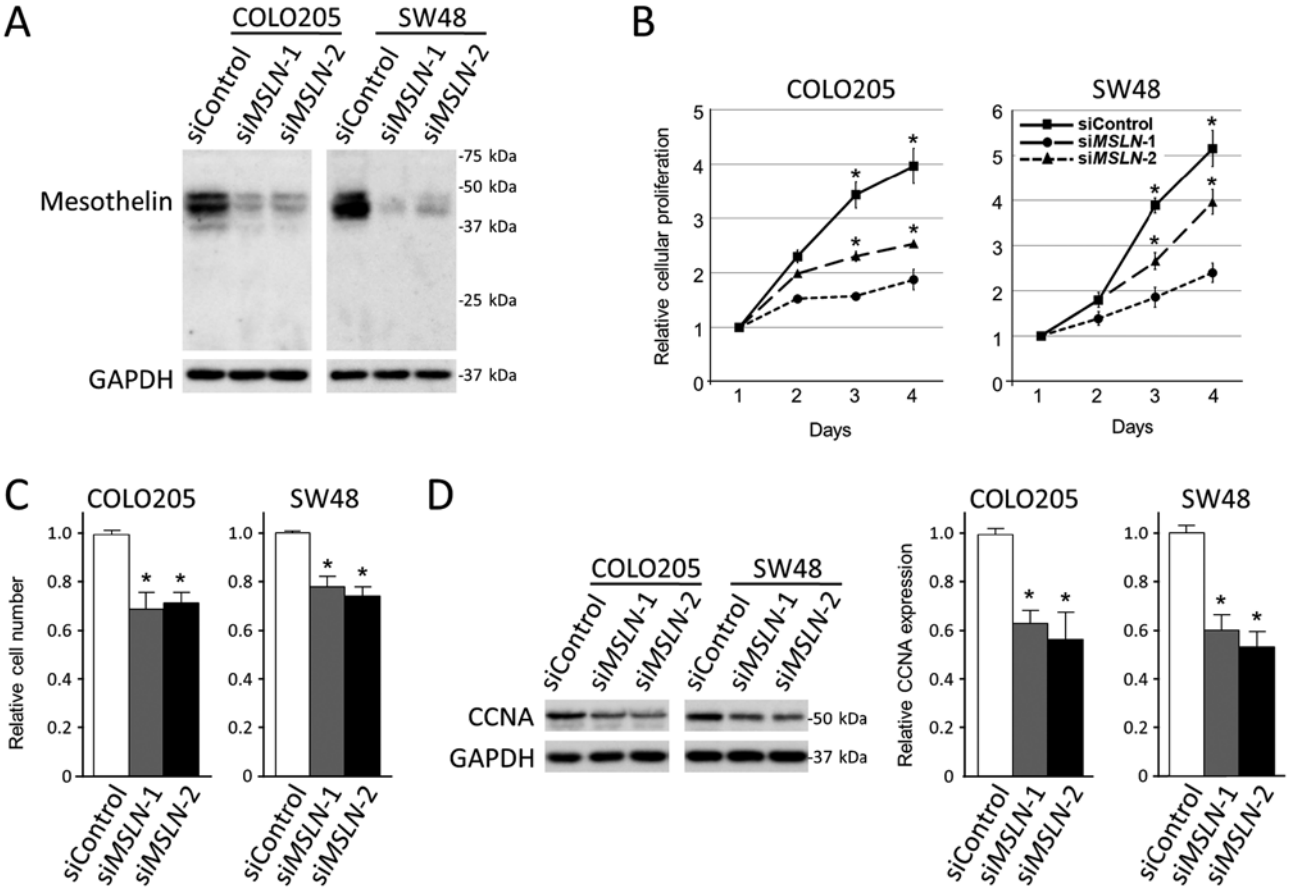

Figure 5. MSLN knockdown downregulates colon cancer cell proliferation. (A) Immunoblot analyses of COLO205 and SW48 cells transfected with siRNAs. Significantly decreased (B) cellular proliferation, (C) anchorage-independent cell proliferation and (D) CCNA expression were detected in MSLN-downregualted CRC cells. Experiments were performed in triplicate. Data are presented as the mean ± standard deviation; *P<0.05 vs. siControl group. MSLN, mesothelin; siRNA, small interfering RNA; CCNA, cyclin A; CRC, colorectal cancer.

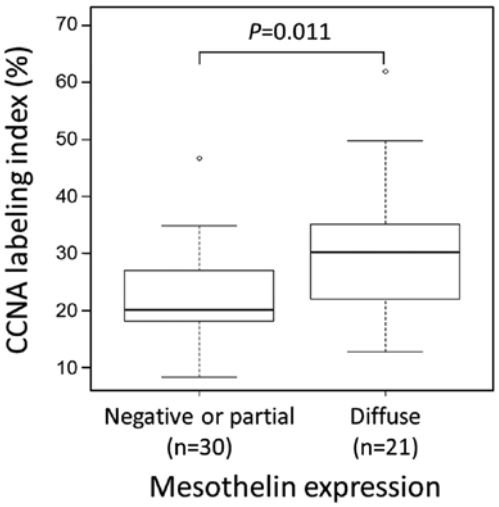

Figure 6. CRC cases with diffuse MSLN expression exhibited a significantly higher rate of CCNA labeling. A total of 21 CRC patients with diffuse MSLN expression and 30 arbitrarily selected cases with negative or partial MSLN expression were compared according to CCNA labeling indices. MSLN, mesothelin; CRC, colorectal cancer; CCNA, cyclin A.

It has been reported that MSLN has pivotal roles in tumor cell proliferation, invasion, and chemotherapy resistance through the activation of oncogenic signaling such as PI3K/AKT and ERK (26-28). However, the details of these signaling events have not been fully identified. Our study demonstrated the MSLN-dependent cellular proliferation of colon cancer cells (Figs. 4 and 5). In our experimental conditions, ectopically expressed MSLN enhanced the proliferation or survival of colon cancer cells with an accumulation of phosphorylated-ERK alone in serum-reduced conditions (Fig. 4B and C). This might be due to the enhanced basal activation of ERK signaling by the serum components.

The regulatory mechanisms of MSLN are not fully understood. Like CD274 (PD-L1) and PDCD1LG2 (PD-L2) (20,29), female-dominant tumor MSLN expression was identified. In cultured colon cancer cells, however, no clear association was identified between sex and MSLN expression (Fig. 3). This might be due to the small number of colon cancer cell lines analyzed in this study. Further analysis using β-estradiol, a major female sex hormone, failed to modulate MSLN levels even in MCF-7 cells with ESR1 expression (Fig. S2). In the present study, the weakly positive correlation between primary tumors and metastases for MSLN expression was found. This may indicate, in part, some preserved characteristics of CRC cells before and after metastasis. Thus, the regulatory mechanisms of MSLN should be elucidated in the future.

Many anti-MSLN therapies such as a high-affinity chimeric monoclonal antibody (MORAb-009), recombinant

immunotoxins (SS1P, RG7787/LMB-100), anti-MSLN antibody drug conjugates (anetumab ravtansine, DMOT4039A, BMS-986148), or adoptive T-cell immunotherapy using MSLN-specific CARs in autologous T lymphocytes are currently being investigated in phase I and II studies targeting advanced solid tumors including pancreatic cancer and malignant mesothelioma with high MSLN expression (18). In the present study, over half of the CRC patients showed tumor-specific MSLN expression. This observation suggests that MSLN might be a good diagnostic and therapeutic target in CRC patients. Furthermore, based on the weakly positive correlation between MSLN expression in primary tumors and their metastases (Fig. S1), not only primary CRC tumors but also metastases might be targeted by anti-MSLN therapeutics.

In the present study, a significant impact of MSLN immunohistochemistry on the prognostication of CRC patients was demonstrated. Additional molecular studies indicated the importance of enhanced cellular proliferation induced by MSLN for worse patient prognosis. Thus, MSLN-positive CRC patients with metastatic lesions might be good candidates for MSLN-targeting therapeutics.

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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

ShI and IP conceived the current study. ShI designed and supervised the study. SaI and ShI performed molecular experiments, histological and statistical analyses, constructed the figures and tables, and wrote the manuscript. MR, HidI, TT, AI, HM, KeK and HirI performed the immunohistochemical staining. SaI, ME, NO and KuK collected and analyzed the clinical data. IP, KuK, KeK and HirI critically reviewed the manuscript. All authors have read and gave final approval to the submitted version.

Ethics approval and consent to participate

The Institutional Ethical Review Board of Aichi Medical University Hospital approved that the present study could be performed without collecting patient consent by giving patients the opportunity to opt-out.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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