ORIGINAL ARTICLE

C-ERC/mesothelin provokes lymphatic invasion of colorectal adenocarcinoma

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ABSTRACT

Background: Lymph node metastasis is a key event of colorectal cancer (CRC) progression. Mesothelin is expressed in various types of malignant tumor, and associated with the unfavorable prognosis. The full-length of mesothelin (Full-ERC) is cleaved by protease into membrane-bound C-ERC/mesothelin and N-ERC/mesothelin which is secreted into the blood. The aim of this study was to examine the biological role of mesothelin in CRC by clinicopathological analysis and *in vitro* lymphatic invasion assay.

Methods: Ninety-one cases of CRC specimens were immunohistochemically examined and the localization of mesothelin in luminal membrane and/or cytoplasm was also evaluated. Lymphatic invasion assay using the human CRC cell line, WiDr, which was transfected with Full-, N- and C-ERC/mesothelin expression plasmids (Full-WiDr, N-WiDr and C-WiDr) was also performed.

Results: Immunohistochemically, "luminal membrane positive" of mesothelin was identified in 37.4 %, and correlated with lymphatic permeation and lymph node metastasis, but not with patients' prognosis. Interestingly, among the patients with lymph node metastasis (N = 38), "luminal membrane positive" of mesothelin significantly correlated with unfavorable patients' outcome. In addition, lymphatic invasion assay revealed that Full-WiDr and C-WiDr more significantly invaded through human lymphatic endothelial cells (hLEC) than the Mock-WiDr ($P < 0.01$).

Conclusion: The luminal membrane expression of mesothelin was associated with unfavorable prognosis of CRC patients with lymph node metastasis. Moreover, this is the first report to prove the biological function of C-ERC/mesothelin associated with lymphatic invasion of cancer *in vitro*. (234 words)

Key words

C-ERC/mesothelin; colorectal cancer, lymphatic invasion

INTRODUCTION

Colorectal cancer (CRC) is one of the most common types of cancer in the world [1] and its prevalence is also increasing rapidly in Japan [2]. Although surgery is a common treatment option for CRC, a previous study reported the recurrence rate after curative surgery to be 17.3 % [3] and an overall 5-year survival rate of surgically treated CRC patients to be 65 % [3, 4]. In about 40% of patients, the cancer has already spread to local lymph nodes at the time of diagnosis, and in about 20% of patients, distant metastases are present at that time [4]. Chemotherapy has improved the clinical outcome and survival rate in cases of non-resectable and recurrent CRC [5, 6], however, there is still a need to identify patient subgroups with high or low risk of tumor recurrence and to tailor individual therapeutic interventions. In particular, lymph node metastasis is widely accepted as one of the most important prognostic factors in CRC patients [7, 8], and there is an urgent need to identify molecular markers that can be used as predictors of lymph node metastasis.

Mesothelin is expressed on normal mesothelial cells lining the pleura, pericardium and peritoneum [9, 10]. In addition, the overexpression of mesothelin has been found in several cancer types, including malignant mesothelioma, ovarian cancer, and pancreatic cancer [11-15]. The full length of human *mesothelin* gene (Full-ERC/mesothelin) codes the primary product, which is a 71-kDa precursor protein. This protein can be physiologically cleaved by some furin-like proteases into a 40-kDa *C*-terminal fragment (C-ERC/mesothelin) that remains membrane-bound, and a 31-kDa *N*-terminal fragment (N-ERC/mesothelin), which is secreted into the blood [9]. The *C*-terminal 40-kDa fragment (C-ERC/mesothelin) is attached to the cell membrane through a glycosyl-phosphatidylinositol (GPI) anchor [16]. Many researchers have investigated the role of the mesothelin expression in tumor biology, and demonstrated the importance of mesothelin expression for tumor progression *in vitro* [17-20] and *in vivo* [20, 21], and we recently explored that the luminal membrane expression of mesothelin was related to an unfavorable patient outcome in gastric carcinoma by immunohistochemistry [22]. In CRC, Liebig, who first studied mesothelin expression in CRC, reported that mesothelin was expressed in 28 of 46 adenocarcinomas (58 %), and mesothelin positive tumor cells were restricted to the invasive front [23]. However, the critical biological role of mesothelin in cancer progression resulting in patients' poor prognosis has not been clarified yet.

In this study, we evaluated the clinicopathological significance of mesothelin expression in 91 CRCs,

especially in terms of its association with the staining pattern, luminal membrane and/or cytoplasm. Furthermore, we performed enforced expression of Full-, C- and N-ERC/mesothelin in human CRC cell lines, and examined the role of mesothelin associated with lymphatic invasion.

Materials and Methods

Patients' Demography and Tumor Specimens

This retrospective study was performed with the approval of the Internal Review Board on ethical issues of Hokkaido University Hospital, Sapporo, Japan. The samples and the patients' information were obtained under a blanket written informed consent. The subjects of this study were 91 patients who underwent radical surgery for colorectal carcinoma between 2002 and 2004 at the Department of General Surgery, Hokkaido University, Graduate School of Medicine, Sapporo, Japan. Patient status was checked in October, 2011. The median follow-up was 62 months. The clinicopathological characteristics of these cases are summarized in Table 1. The location of the tumor was the colon in 68 cases (74.7 %) and the rectum in 23 cases (25.2 %). The median survival time of the patients was 62.2 months (± 5.2 standard deviation: S.D.) T-factor, N-factor, M-factor, and clinical stage were assigned according to the TNM classification of the Union Internationale Contre le Cancer (UICC) [24].

Pathological and immunohistochemical evaluation

Formalin-fixed paraffin-embedded tissue blocks were prepared from surgical specimens, and sections were sliced and stained with hematoxylin and eosin (HE) for routine histopathological examination. All specimens were diagnosed as colorectal cancers, and lymphatic permeation and blood vessel invasion were evaluated using elastica-Masson staining and anti-podoplanin (D2-40) immunostaining, respectively, in addition to the routine HE staining. Immunohistochemical staining against mesothelin was performed as described previously [15]. In brief, the tissue sections were incubated with a mouse monoclonal antibody against mesothelin (clone 5B2 diluted 1:50; Novocastra, Newcastle Upon Tyne, United Kingdom) at a 1:50 dilution, and reacted with a dextran polymer reagent combined with secondary antibodies and peroxidase (Envision/HRP; Dako, Tokyo, Japan). All assessments were made on the tumor region of the specimen (×400). Each slide was evaluated independently by three pathologists (F. K., Y. K., and H. N.) who did not know the clinical outcomes. Among

the 91 cases of CRC, the expression level of mesothelin in tumor cells was classified into high and low according to the staining intensity and proportion as described previously [22]. Furthermore, among the 91 cases of CRC, the staining localization of mesothelin was evaluated in the luminal membrane or cytoplasm (Fig. 1). Cases in which the luminal membrane was stained even partially or faintly, or the entire circumference of the luminal membrane was explicitly stained, were judged as "luminal membrane positive (Fig. 1-d, f)." In cases with no membrane staining (Fig. 1-b) and those in which only cytoplasmic staining was observed in any intensity level, the term "luminal membrane negative" was given (Fig. 1-b, h, j). Meanwhile, the mesothelin cytoplasmic expression was evaluated as follows: in a case in which the cytoplasmic staining was even barely perceptible or clearly observed in the constituent cancer cells, including the cytoplasmic granular staining, we judged it to be "cytoplasmic positive (Fig. 1-h, j)". In cases with no cytoplasmic staining (Fig. 1-b) and those in which only luminal membrane staining was observed in any intensity level, the term "cytoplasmic negative" was given (Fig. 1-b,d,f)(Table 2).

Cell culture

Human CRC cell lines, CaCo-2, LoVo, and HCA7 were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin (Life Technologies, Rockville, MD). Human CRC cell line, WiDr cells were cultured in RPMI 1640 medium with the same concentrations of FBS and antibiotics. Human CRC cell line, T84 cells were cultured in DMEM mixed 1:1 with Ham's F-12 nutrient mixture and 6 % newborn calf serum with the same concentrations of antibiotics. For lymphatic invasion and adhesion assay, we used primary human lung lymphatic endothelial cells (hLEC; Lonza Japan, Tokyo), which were cultured in fully supplemented EBM2 endothelial basal medium (with EGM2 bullet kit; Lonza). All cells were maintaind at 37°C in 5 % CO2.

Full-, N- and C-ERC/mesothelin expression plasmids.

The pcDNA3.1(+) vector containing the full coding region of human ERC/mesothelin cDNA (pcDNA3.1(+) Full-ERC), and similarly, a C-ERC expressing vector (pcDNA3.1(+) C-ERC) and a N-ERC expressing vector (pcDNA3.1(+) N-ERC) were reported previously [25]. To generate a stable cell line of WiDr which permanently expressed exogenous GFP (GFP-WiDr), cells were transduced with a GFP-expression construct by using pCX4 retrovirus vectors [26] and selected by puromycin (100mg/mL, Sigma, St. Louis, MO) for 7 days.

GFP-WiDr cells were transiently transfected with Full-, N- and C-ERC expression plasmids (Full-WiDr, N-WiDr and C-WiDr) using FuGENE 6 (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. pcDNA3.1(+) without an insert was used as the mock vector.

Western blotting

Anti-Full-ERC/mesothelin antibody (Abcam, ab-3362) [27], mouse monoclonal anti-human N-ERC (7E7) [28] , and anti-human C-ERC (22A31) [29, 30] antibodies were prepared as described previously. Forty-eight hours after transfection, cells and culture medium were harvested. The lysate (20 μg) and culture medium (20 μl) were adjusted to be in a solution containing 2 % SDS, 10 % glycerol, 50 mM Tris-HCl (pH 6.8) and 100 mM dithiothreitol (DTT). 20 μg of cytoplasmic protein from cells and culture medium (20 μl) were diluted in SDS-PAGE loading buffer, boiled for 5 min and loaded onto 10 % or 12 % SDS-PAGE gel. Proteins on the membranes were blocked in 1% skim milk in phosphate-buffered saline with 0.1 % Tween-20 (PBS-T) for 1 h at RT. The membranes were then incubated with either 1 μg/ml anti-Full-ERC/mesothelin antibody (Abcam, ab-3362), 1 μg/ml anti N-ERC/mesothelin (7E7), or 2 μg/ml anti C-ERC/mesothelin (22A31) at RT for 1 h, at 4˚C for overnight in PBS-T with 1 % skim milk. Bound antibodies were detected with peroxidase-labeled goat antibody to mouse IgG or goat antibody to rabbit IgG, and visualized by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Immunofluorescence of mesothelin

Full-WiDr, N-WiDr and C-WiDr were fixed in 3 % paraformaldehyde for 15 minutes at room temperature (RT) permeabilized with 0.1% Triton X-100 in PBS for 4 minutes at RT, and then incubated with 1% bovine serum albumin to block non-specific binding of antibodies. The cells were incubated with anti N-ERC/mesothelin (7E7) and anti C-ERC/mesothelin (22A31) antibodies at 4°C overnight, followed by AlexaFluor488-conjugated secondary antibodies (1:200 dilution, Molecular Probes, Invitrogen) for 1 hour at RT in the dark. The cells were further incubated with Alexa 594-conjugated phalloidin (1:50 dilution, Molecular Probes) at 37°C for 30 min. Images were acquired using an FV-1000 confocal microscope (Olympus, Tokyo, Japan)

Lymphatic invasion assay

Primary hLEC were used in passage 4 to 6. 5 x 10^4 hLEC were seeded on the surface of BD Matrigel Matrix (BD BioCoat™ 24-Multiwell Tumor Cell Invasion System) in EBM2 endothelial basal medium to cover the

upper side of transwell inserts (8 μm pore size), and incubated at 37˚C, 5 % CO_2 for 2 days. After the confirmation of hLEC in confluence and monolayer by microscope, the rehydration solution was carefully removed. 5 x 10^4 stable GFP-expressing WiDr cells transfected with Full-, N-, C-ERC or Mock expression plasmids (Full-WiDr, N-WiDr, C-WiDr and Mock-WiDr, respectively) were resuspended in 0.5 ml of RPMI without FBS and loaded on top of the upper side of transwell inserts and 0.75 ml RPMI containing 10 % FBS to induce chemotaxis was added to the lower side of transwell inserts. After the incubation for 24 hours at 37 °C, 5 % CO2, the remaining cells were removed by cell scraping of the upper side of transwell inserts. Invaded cells through the hLEC and transwell insert were counted by a fluorescence microscope (BZ-9000, KEYENCE, Tokyo). The cells in full visual field (FF) (x200) per filter were counted; results are reported as the means of triplicate assays (Supplemental Fig. 1).

Adhesion assay to lymphatic endothelial cells

1 x 10^5 hLEC were plated on a 12-well dish and incubated at 37 ˚C, 5 % CO_2 for 3 days. After confirmation of hLEC in confluence and monolayer by microscope, the rehydration solution was carefully removed. 1 x 10^5 stable GFP-expressing Full-WiDr, N-WiDr, C-WiDr and Mock-WiDr were resuspended in 1.0 ml of RPMI with FBS and plated on a 12-well dish at 37˚C for 30 min. Non-adherent or loosely attached cells were removed by gently washing the wells twice with PBS. The attached GFP-expressing cells (Full-WiDr, N-WiDr, C-WiDr and Mock-WiDr) on hLEC were counted by a fluorescence microscope (BZ-9000). The cells in 3 microscopic fields (x400) per filter were counted; results are reported as the means of triplicate assays (Supplemental Fig. 1).

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Statistical analysis

We used $\chi 2$ test or Fisher's exact test to determine the correlation between mesothelin and clinicopathological data. Survival curves for patients were drawn by the Kaplan-Meier method. For the i*n vitro* assay, we used the Student's t-test (two sided). All differences were considered significant at a p-value of less than 0.05. All statistical analyses were performed using the Ekuseru-Toukei 2010 software for Windows (Social Survey Research Information Co., Ltd., Tokyo, Japan).

RESULTS

High-level expression of mesothelin was observed in about 50 % of CRC samples.

The overexpression of mesothelin has been found in several cancer types, including malignant mesothelioma, ovarian cancer, and pancreatic cancer. Thus, we first evaluated the comprehensive expression of mesothelin in CRC patients. As summarized in Table 3, "high-level expression" was detected in 45 cases (49.5 %), whereas "low-level expression" was detected in 46 cases (50.5 %), although "high-level expression" of mesothelin was not correlated with clinicopathological parameters such as histological grade, T-factor and metastasis. Recent studies reported that higher mesothelin expression was found to be associated with shorter patients' survival in ovarian and pancreatic cancer [31-33]; therefore, we also examined the correlation of mesothelin overexpression with overall survival (OS) in the CRC patients. Contrary to our expectation, the group of "high-level expression" of mesothelin was not correlated with OS compared to the group of "low-level expression" of mesothelin (P = 0.26, Supplemental Fig. 2). While mesothelin-positive tumor cells in CRC specimen were localized in the invasive front in previous report [23], the mesothelin-positive cells were found throughout the tumor area, not restricted in the invasive front in our study.

Luminal membrane expression of mesothelin was associated with lymphatic invasion, and correlated with unfavorable prognosis in CRC patients with lymph node metastasis.

We recently explored that the luminal membrane expression of mesothelin was related to an unfavorable patient outcome in gastric carcinoma [22]. Thus we next analyzed the intracellular localization of mesothelin by immunostaining to explore the clinicopathological significance of its translocation. As shown in Table 2, the group "luminal membrane positive" (Fig. 1-d, f) was identified in as many as 34 (37.4 %) cases, while the group "luminal membrane negative (Fig. 1-b, h, j) was comprised of 57 cases (62.6 %) including 29 cases that were completely mesothelin negative (Fig. 1-b). Interestingly, the statistical analysis revealed that the incidence of luminal membrane positivity was significantly correlated with lymphatic permeation (P ＝0.009), lymph node metastasis (P ＝0.048) and p-Stage (P ＝0.048) (Table 3). In addition, although the luminal membrane expression of mesothelin was not correlated with total CRC patients' prognosis (N = 91, P = 0.13) (Fig. 2-a), "luminal membrane positive" of mesothelin (N =19) revealed significant unfavorable patients' outcome

compared to "luminal membrane negative" of mesothelin (N =19) among the patients with lymph node metastasis (N = 38, *P* = 0.033) (Fig 2-b). The detailed clinicopathological information of 19 patients with lymph node metastasis and luminal membrane expression of mesothelin was summarized in Supplemental Table 1. Moreover, we performed $\chi 2$ test to clarify whether the clinical parameters can affect the prognosis in these groups (luminal membrane positive (N=19) vs. negative patients (N=19)). As a result, we did not find a significant clinicopathological difference between these groups except for luminal membrane expression of mesothelin (Supplemental Table 2).

Meanwhile, "cytoplasmic positive" (Fig. 1-h, j) was detected in 38 cases (41.8 %), which contained 10 cases that were both luminal membrane and cytoplasmic positive (Table 2), while the group "cytoplasmic negative" (Fig. 1-b, d, f), was detected in 53 cases (58.2 %). The statistical analysis revealed that the incidence of cytoplasmic expression revealed no correlation with clinicopathological factor (Table 3), or even patients' prognosis (P = 0.73) (Supplemental Fig. 2). These results enforced us to speculate that membrane localized mesothelin plays a pivotal role in the lymphatic invasion of CRC.

ERC/mesothelin expression in CRC cell lines

First we employed 5 types of CRC cell lines (WiDr, LoVo, CaCo2, T84 and HCA7) to examine the endogenous expression of ERC/mesothelin by Western blotting. The full length of human *mesothelin* gene (ERC/mesothelin) codes the primary product, which is a 71-kDa precursor protein. This protein can be physiologically cleaved by some furin-like proteases into a 40-kDa *C*-terminal fragment (C-ERC/mesothelin) that remains membrane-bound, and a 30-kDa *N*-terminal fragment (N-ERC/mesothelin), which is secreted into the blood. As shown in (Fig. 3-a), Full-ERC/mesothelin was not detected in these CRC cell lines except in the CaCo2 cell line, and C-ERC/mesothelin was not identified in any of the 5 types of CRC cell lines. Interestingly, (Fig. 3-a), right panel showed the enhanced expression of the 71 kDa protein in the C-WiDr lysate, compared with that in the WiDr lysate. This result suggested us the possibility of positive feedback mechanism in which the over-expressed C-ERC could enhance the endogenous gene expression. To analyze the biological function of each of the types of ERC/mesothelin, we transfected with Full-, N-, C-ERC or Mock expression plasmids (Full-WiDr,

N-WiDr, C-WiDr and Mock-WiDr respectively) in WiDr cells, and confirmed the expression of 71-kDa, 40-kDa and 30-kDa forms of ERC in the cell lysate and/or culture medium by Western blotting (Fig. 3-b). The N-ERC in the medium, derived from the Full-ERC expression vector, migrated to a position identical to that derived from the N-ERC expression vector (Fig. 3-b, left panel, lanes Full-WiDr and N-WiDr in medium), while the C-ERC in the lysate, derived from the Full-ERC expression vector, was similar to that derived from the N-ERC expressing vector (Fig. 3-b, right panel, lanes Full-WiDr and C-WiDr in lysate). These results were consistent with those of Wang, T. et al [25], and also meant that the proper cleavage of Full-ERC/mesothelin by furin-like protease was processed.

Immunofluorescence of mesothelin

We next examined the intracellular localization of Full-, N- and C-ERC-transfected cell expression by fluorescence microscopic analysis. As indicated in (Fig. 3-c, d), 7E7 antibody which recognizes the N-terminus of ERC/mesothelin revealed the diffuse cytoplasm expression of Full- and N-ERC mesothelin in Full-WiDr and N-WiDr. By contrast, 22A31 antibody which recognizes the C-terminus of ERC/mesothelin demonstrated dot-like expression of Full- and C-ERC in Full-WiDr and C-WiDr (Fig. 3-e, f). Because the diffuse cytoplasmic expression of Full- and N-ERC was confirmed by 7E7 antibody (Fig. 3-c, d), the dot-like pattern of ERC by 22A31 antibody should be derived from C-ERC (Fig. 3-e, f). Moreover, some of the dot-like spots along with the cellular membrane were merged with actin showing yellow signals. Thus, we finally confirmed the membranous expression of C-ERC/mesothelin in Full-WiDr and also C-WiDr.

Enforced expression of Full, C-ERC/mesothelin promotes lymphatic invasion of CRC.

To explore the possible biological role of mesothelin in lymphatic invasion of CRC, we conducted an *in vitro* lymphatic invasion assay using hLEC as described in (Supplemental Fig. 1). In our transient expression system, the transfection efficiency was up to 60 %, evaluated by the immunofluorescent staining (data not shown). GFP-expressing Full-WiDr, N-WiDr, C-WiDr and Mock-WiDr were loaded on the top of the upper side of transwell inserts which were covered with a confluent monolayer of hLEC, and invaded cells which were found on the bottom side of the inserts were counted. As shown in (Fig. 4-a, b), Full-WiDr (526 ± 68 cells /FF) significantly invaded toward hLEC compared to the Mock-WiDr (412 ± 50 cells /FF) ($P < 0.01$). In particular,

C-WiDr (614 ± 74 cells /FF) significantly invaded toward hLEC compared to the N-WiDr (430 ± 31 cells /FF) and Mock-WiDr (412 ± 50 cells /FF) ($P < 0.001$). Furthermore, to elucidate the mechanism by which mesothelin enhances lymphatic invasion of CRC, we performed a cell adhesion assay using hLEC as indicated in (Supplemental Fig 1). GFP-expressing Full-WiDr, N-WiDr, C-WiDr and Mock-WiDr were seeded and incubated on 12-well dishes which were confluenced by an hLEC monolayer at 37˚C for 30 min. As indicated in (Fig. 4-c, d), Full-WiDr (60.6 ± 4.9 cells /HPF) and C-WiDr (60.0 ± 4.9 cells /HPF) more significantly attached on hLEC than did the Mock-WiDr (51.1 ± 3.4 cells /HPF) ($P < 0.01$). These results suggested that membrane-localized C-ERC/mesothelin provoked lymphatic invasion of CRC through the increase of cell adhesion to lymphatic endothelial cells.

DISCUSSION

Here we explored that membrane-localized C-ERC/mesothelin promoted lymphatic invasion of CRC *in vitro*, verifying the result of clinicopathological analysis for CRC in which "luminal membrane positive" of mesothelin was statistically correlated with lymphatic permeation and lymph node metastasis.

To our knowledge, this is the first study to demonstrate the association of luminal membrane-localized C-ERC/mesothelin and lymphatic permeation. The mechanism for the membranous localization of mesothelin should be explained as follows: the full length of the human *mesothelin* gene encodes a 71-kDa precursor protein (Full-ERC) that is proteolytically cleaved by some furin-like proteases into an *N*-terminal secreted form (N-ERC) and a *C*-terminal fragment (C-ERC), 40-kDa mesothelin, which is a glycosyl-phosphatidylinositol (GPI)-linked glycoprotein [16]. Interestingly, by western blot analysis, we found the enhanced endogenous expression of the 71 kDa protein in the C-WiDr lysate, compared to that in the WiDr lysate (Fig. 3-a). This result might be explained by the possible positive feedback mechanism in which the over-expressed C-ERC could enhance the endogenous gene expression, and could be another aspect for biological malignancy of mesothelin. GPI-anchored proteins such as T-cadherin can transduce molecular signals that modulate cell migration and invasion with integrin-linked kinase [34] and integrin β3 [35]; in addition, an association between the expression of integrin and mesothelin in malignant pleural mesothelioma was reported using gene array [21]. Moreover, a recent study has shown that integrin α4β1 is expressed on lymphatic endothelium and its signaling

is required for lymphangiogenesis and tumor metastasis [36]. Further studies to better define the association between mesothelin and integrin may ultimately help to elucidate the relationship of this pathway of lymphatic adhesion and invasion in CRC.

In our previous report in gastric cancer, luminal membrane expression of mesothelin was correlated with histological grade, T-factor, p-Stage, lymph node metastasis, blood vessel invasion, lymphatic invasion, recurrence and poor patient outcome [22]. Meanwhile the present study in CRC revealed that luminal membrane expression of mesothelin was correlated with lymph node metastasis, p-Stage and lymphatic invasion, but not with the generalized patients' prognosis (N =91, P = 0.13), although among the patients with lymph node metastasis (N =38), "luminal membrane positive" of mesothelin (N =19) significantly indicated an unfavorable patients' outcome compared to "luminal membrane negative" of mesothelin (N =19) (P = 0.033, Fig. 2-b, Supplemental Table 1, 2). Such discrepancy between gastric cancer and CRC, even though both types of cancer are derived from gastrointestinal tract, might be explained by their distinct genomic backgrounds. In fact, the recent report revealed that gastrointestinal adenocarcinoma of stomach and colon exhibited distinct patterns of genome instability and oncogenesis using genomic profiling arrays, in particular, focal amplification of receptor tyrosine kinases were substantially more prevalent in gastric carcinoma (28 %) than in CRC (14 %) [37]. These results may suggest that mesothelin expression might be more amplified and associated with tumor development in gastric carcinoma rather than in CRC.

Although, this is significant information for the clinicians who treat CRC patients, especially those with lymph node metastasis. If the pathological evaluation of the surgical specimen of patients reveals the luminal membrane expression of mesothelin in tumor cells in addition to lymph node metastasis, such patients should be treated with a more powerful first line adjuvant therapy to avoid the risk of further progression and metastasis of CRC [38, 39]. Moreover, in case that an endoscopically resected specimen was evaluated as having "luminal membrane positive" of mesothelin, we may consider a much higher risk of lymph node metastasis; this might warrant our offering additional surgery and/or adjuvant chemotherapy to such patients, even if the endoscopic resection without adjuvant chemotherapy is a standard treatment for early stage CRC patients.

Currently, C-ERC is being tested as a possible target for antibody-mediated cancer therapy. Recombinant anti-mesothelin immunotoxin SS1P (CAT-5001) and a high affinity chimeric anti-mesothelin monoclonal antibody MORAb-009 recently entered phase II clinical trials [40, 41]. Our results might offer such possible

anti-mesothelin molecular targeting therapy for the high-risk CRC patients who have advanced lymph node metastasis. To evaluate the therapeutic effect of such antibody-based medicine, pathological verification of membranous expression of the target molecule must be performed, because antibody-based drugs can usually access the molecules located on the cell membrane. Herein, we believe that luminal membrane positivity for mesothelin in CRC would be of clinical benefit not only as a prognostic factor but also as a predictive factor for the eligibility to mesothelin-targeting therapies in the future [16, 20, 42-44].

In conclusion, luminal membrane expression of mesothelin elucidated unfavorable prognosis of CRC patients with lymph node metastasis. Moreover, our studies demonstrate the important role of the C-ERC/mesothelin in lymphatic invasion and suggest that C-ERC/mesothelin targeted drugs could be novel therapeutics to suppress the spread of tumors through the lymphatic system in not only CRC, but also in various types of tumors with "luminal membrane positive" of mesothelin.

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Figure 1.

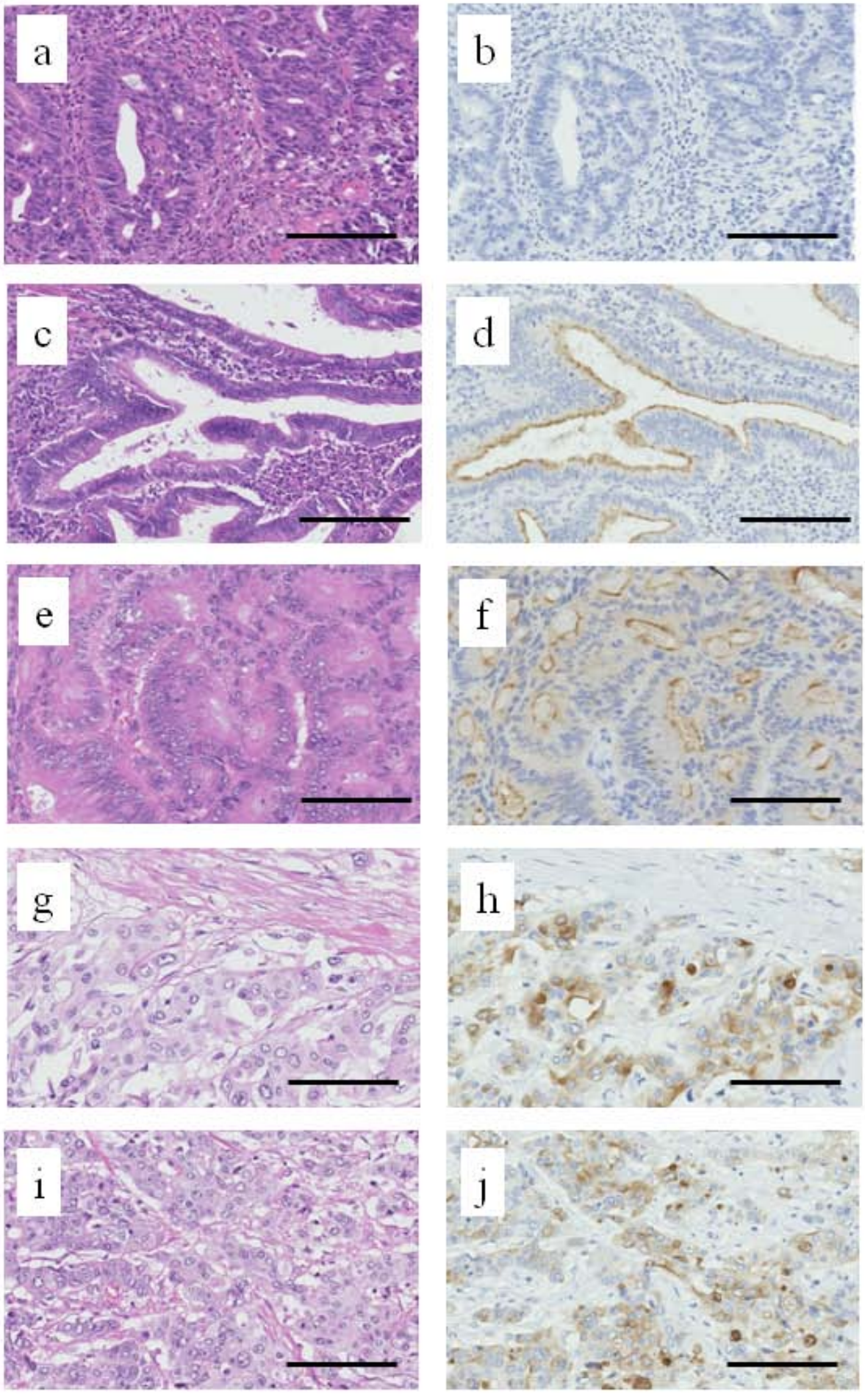


Figure 2.

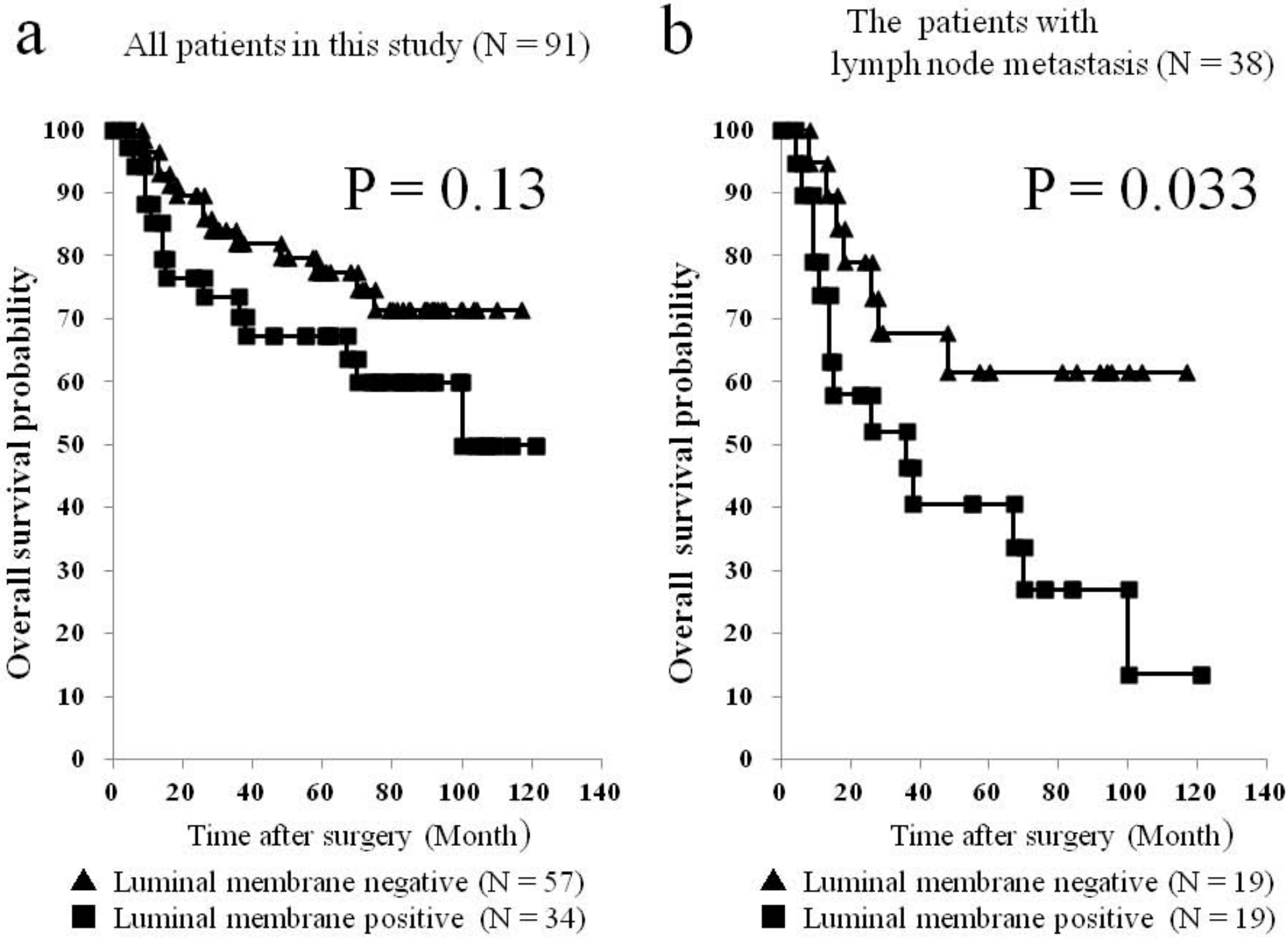
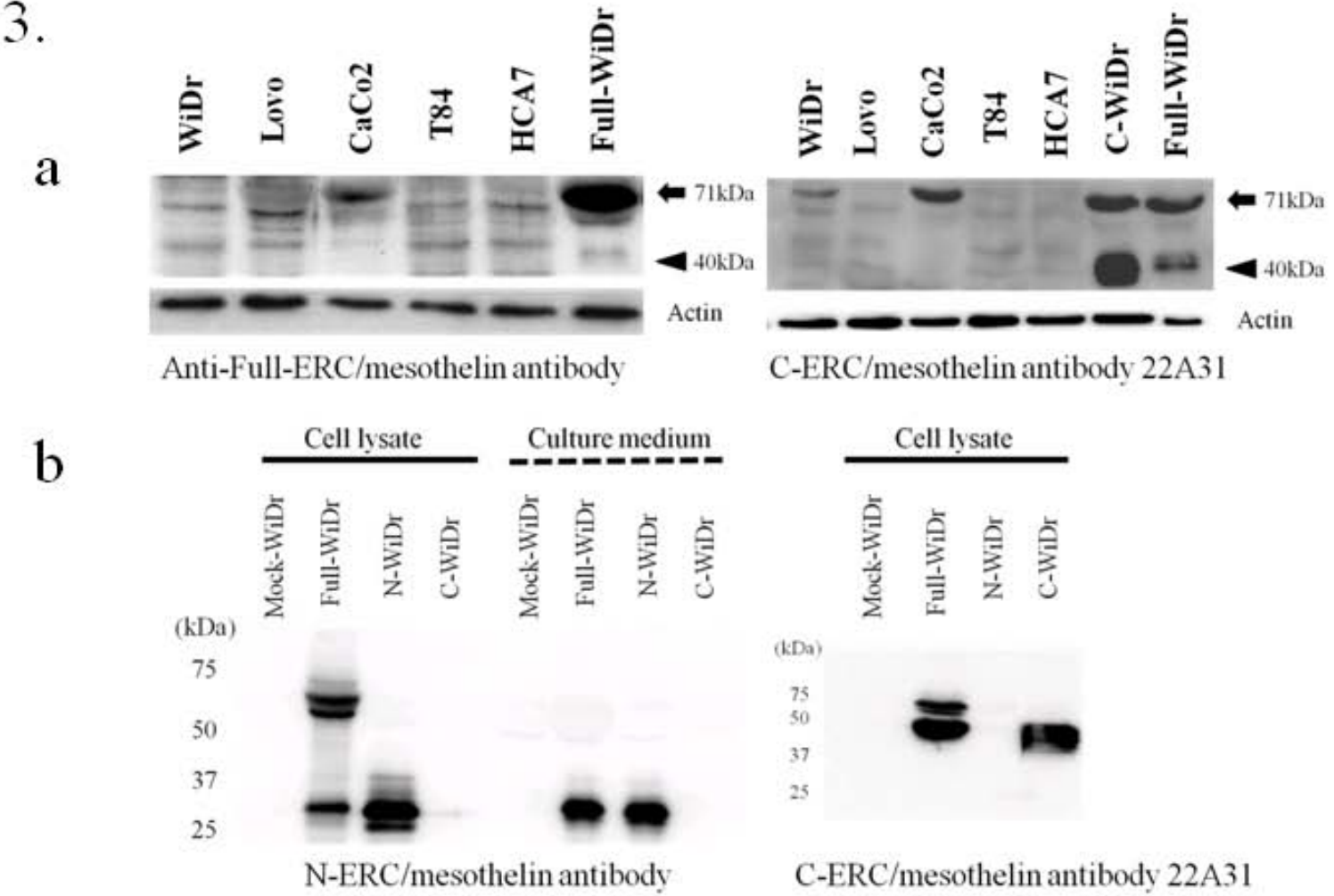

a
All patients in this study (N = 91)
P = 0.13
Overall survival probability
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
Time after surgery (Month)
Luminal membrane negative (N = 57)
Luminal membrane positive (N = 34)
b
The patients with lymph node metastasis (N = 38)
P = 0.033
Overall survival probability
Time after surgery (Month)
Luminal membrane negative (N = 19)
Luminal membrane positive (N = 19)

Figure 3.


a
WiDr
Lovo
CaCo2
T84
HCA7
Full-WiDr
71kDa
40kDa
Actin
Anti-Full-ERC/mesothelin antibody
WiDr
Lovo
CaCo2
T84
HCA7
C-WiDr
Full-WiDr
71kDa
40kDa
Actin
C-ERC/mesothelin antibody 22A31
b
Cell lysate
Culture medium
Mock-WiDr
Full-WiDr
N-WiDr
C-WiDr
(kDa)
75
50
37
25
N-ERC/mesothelin antibody
Cell lysate
Mock-WiDr
Full-WiDr
N-WiDr
C-WiDr
(kDa)
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C-ERC/mesothelin antibody 22A31

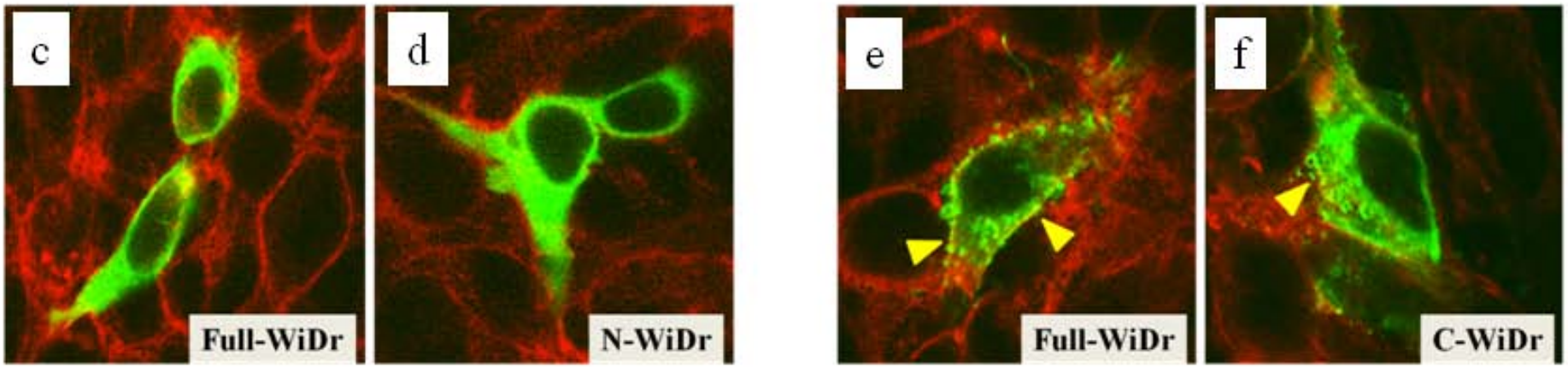
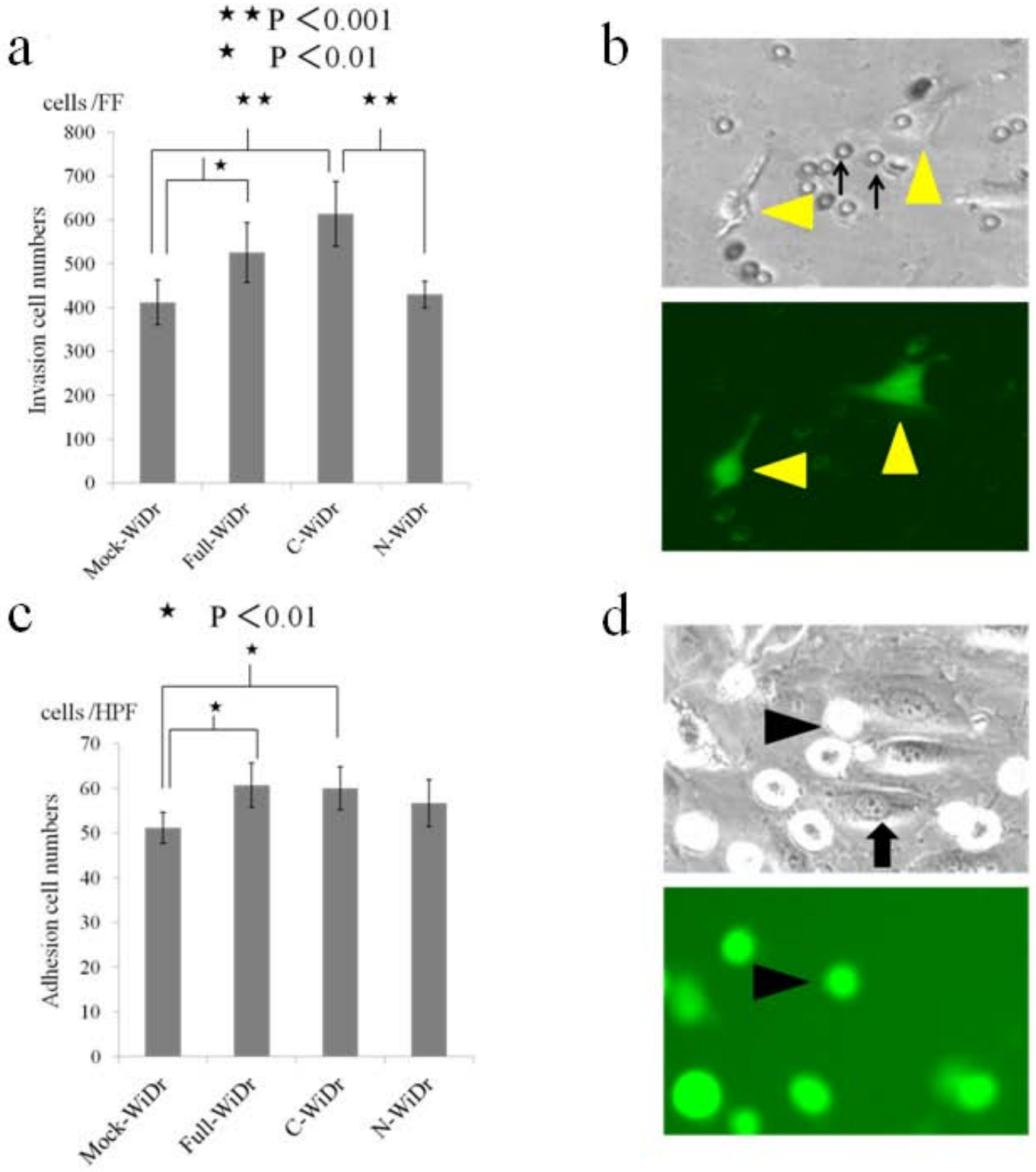

c
Full-WiDr
d
N-WiDr
Anti-N-ERC Antibody 7E7
e
Full-WiDr
f
C-WiDr
Anti-C-ERC Antibody 22A31

Figure 4.


a
★★ P <0.001
★ P <0.01
cells /FF
Invasion cell numbers
800
700
600
500
400
300
200
100
0
Mock-WiDr
Full-WiDr
C-WiDr
N-WiDr
b
c
★ P <0.01
cells /HPF
Adhesion cell numbers
70
60
50
40
30
20
10
0
Mock-WiDr
Full-WiDr
C-WiDr
N-WiDr
d

Supplemental Table 2. Correlation between luminal membrane expression and clinicopathological features among the patients with lymph node metastasis

Parameter	Total	Luminal membrane expression		P-value
		Positive (n=19)	Negative (n=19)	
1.Histopahologic grade				
well	10	4	6	0.714
mod por	28	15	13	
2.pT-factor				
pT1-2	6	2	4	0.660
pT3-4	32	17	15	
3.pStage				
III	33	15	18	0.340
IV	5	4	1	
4.Lymphatic permeation				
Negative	4	0	4	0.105
Positive	34	19	15	
5.Blood vessel permeation				
Negative	5	3	2	1.000
Positive	33	16	17	
6.Resection margin				
pR0	30	15	15	1.000
pR1	8	4	4	
7.Recurrence				
No	19	9	10	1.000
Yes	19	10	9	
8.Liver Metastasis				
No	26	12	14	0.728
Yes	12	7	5	
9.Local Recurrence				
No	37	18	19	1.000
Yes	1	1	0	
10.Lung Metastasis				
No	29	14	15	1.000
Yes	9	5	4	

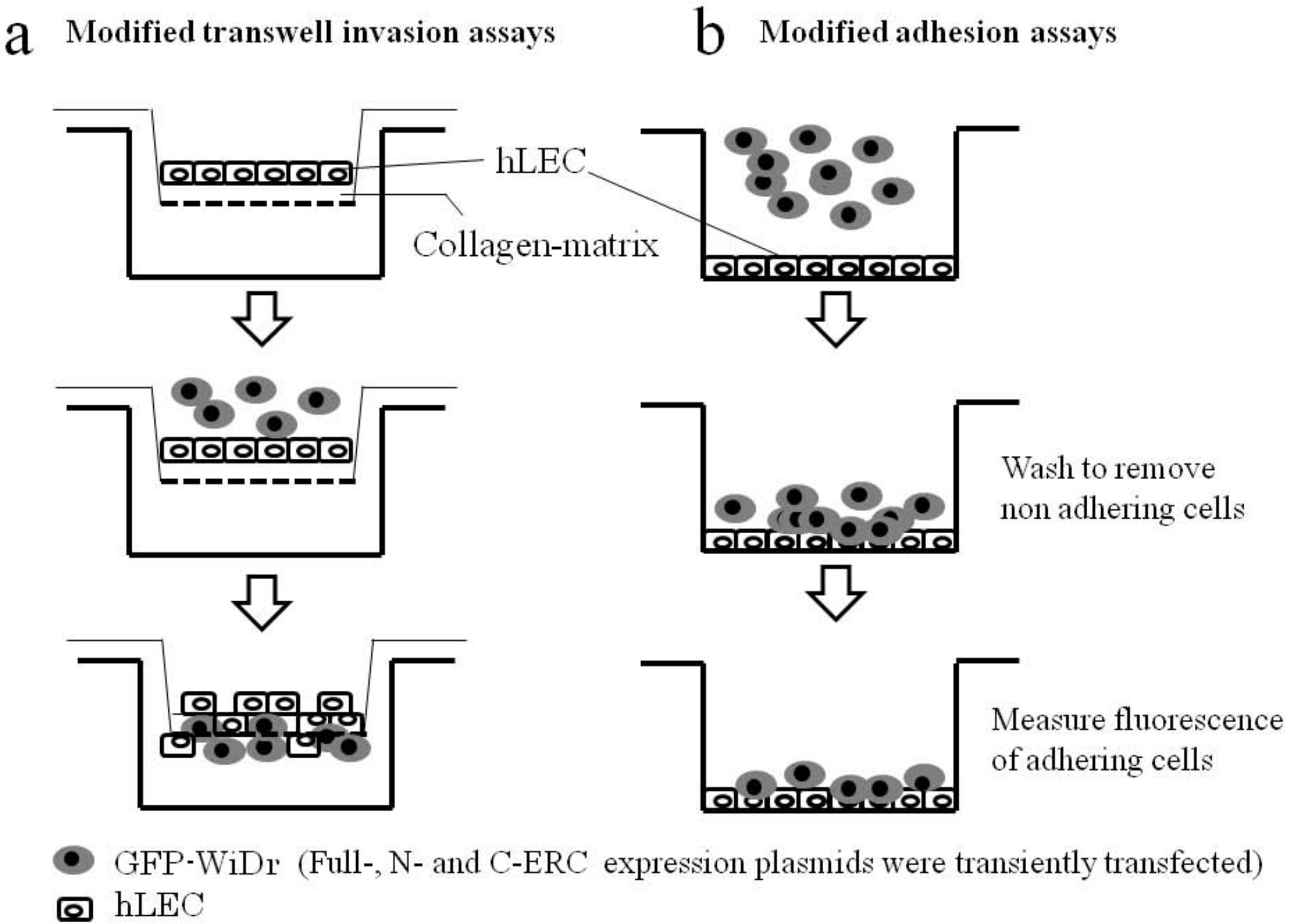
*$\chi 2$ test

Supplemental Table 1. Summary of 19 patients with lymph node metastasis and luminal membrane expression of mesothelin

Case No.	Age	Sex	Histological classification	pT-factor	pN-factor	pStage	Lymphatic permeation	Blood vessel permeation	Curability	Site of reccurence	Outcome (month)
1	55	F	mod	3	1b	IIIB	positive (1)	positive (1)	B	lung, peritoneum	dead (100)
2	71	M	mod	3	1b	IV	positive (1)	positive (1)	C	liver	dead (14)
3	53	F	por	4a	2a	IV	positive (3)	positive (3)	C	liver	dead (9)
4	56	M	well	4a	2b	IV	positive (2)	positive (2)	B	liver	dead (36)
5	56	F	mod	4a	1b	IIIB	positive (1)	positive (1)	A	-	alive (84)
6	62	M	well	3	2a	IIIB	positive (1)	positive (2)	A	lung	dead (67)
7	64	F	mod	3	1a	IIIB	positive (1)	positive (1)	A	-	alive (121)
8	68	M	mod	4a	2a	IIIC	positive (3)	positive (3)	B	-	dead (15)
9	83	F	mod	3	1b	IIIB	positive (1)	positive (1)	A	-	dead (38)
10	58	F	well	3	2b	IV	positive (2)	positive (2)	C	liver	dead (4)
11	68	F	well	3	1a	IIIB	positive (1)	negative	A	-	alive (23)
12	56	F	mod	4a	2b	IIIC	positive (3)	positive (3)	C	liver, lung, local	dead (9)
13	66	F	mod	4a	2a	IIIC	positive (1)	positive (2)	A	liver, lung, bone	dead (11)
14	75	M	mod	3	1b	IIIB	positive (2)	positive (1)	A	-	alive (76)
15	71	M	mod	3	2a	IIIB	positive (3)	positive (2)	A	-	dead (70)
16	67	F	por	2	1b	IIIA	positive (1)	negative	A	liver	dead (14)
17	79	M	mod	3	2b	IIIC	positive (2)	positive (1)	B	lung	dead (6)
18	79	M	mod	2	2a	IIIB	positive (2)	positive (1)	A	-	alive (55)
19	81	M	mod	4a	1b	IIIB	positive (1)	negative	A	-	dead (26)

Lymphatic and blood vessel permeation: (1): mild, (2): moderate, (3) severe Case No.8, 15: unrelated death

Supplemental Figure 1.



Supplemental Figure 2.

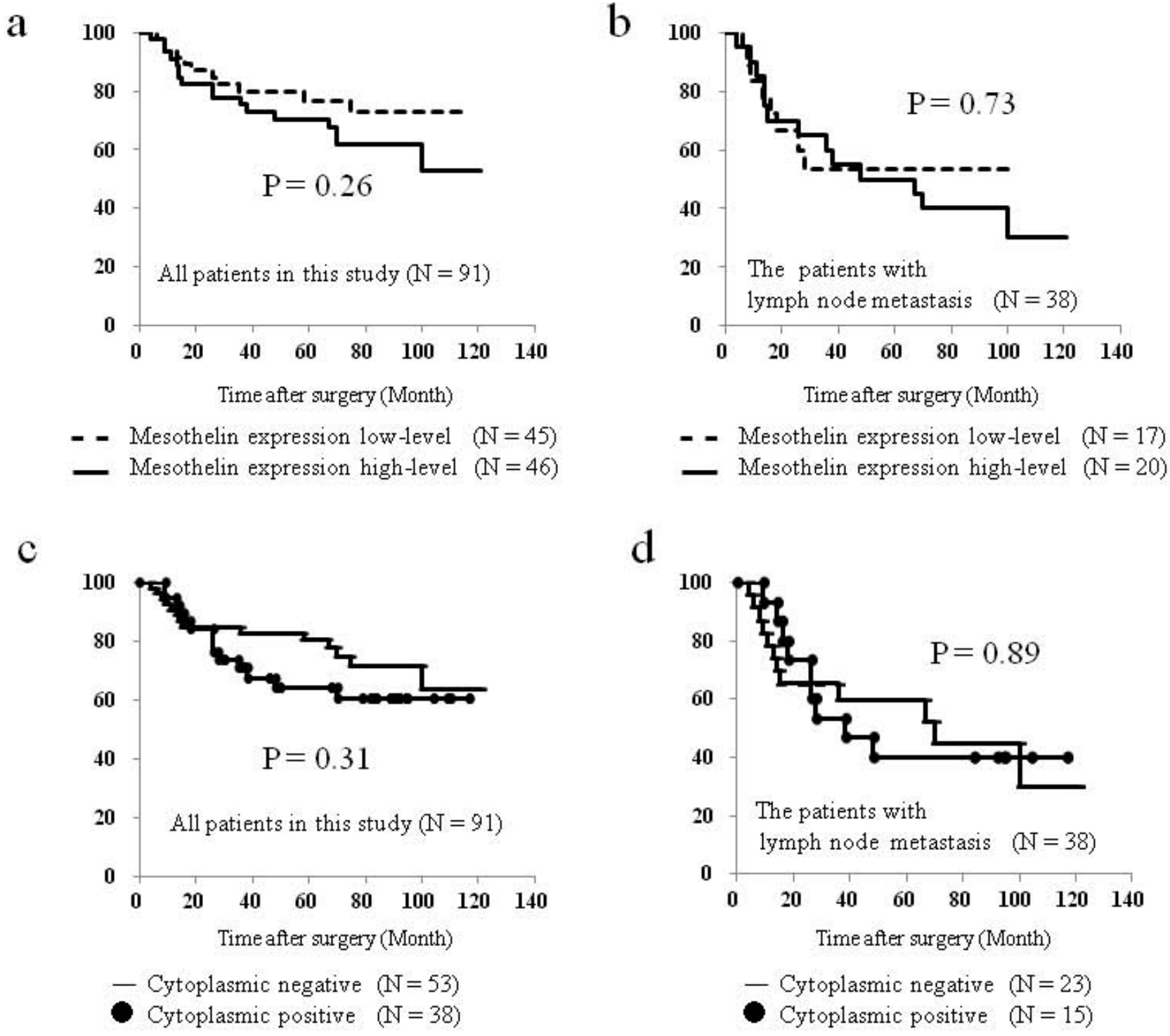


Table 1. Clinicopathological characteristics of 91 patients with CRC in this study

Parameter	No.Case (%)
1.Age(y)	
< 60	32 (35.2)
≧60	59 (64.8)
Mean±SD	65.2 ± 12.2
2.Sex	
Male	39 (42.9)
Female	52 (57.1)
3.Location	
Colon	68 (74.7)
Rectum	23 (25.3)
5.Resection status	
R0	83 (91.2)
R1	8 (8.8)
6.T-factor	
T1	16 (17.6)
T2	19 (20.9)
T3	42 (46.2)
T4	14 (15.4)
7.N-factor	
N0	53 (58.2)
N1	22 (24.2)
N2	16 (17.6)
8.M-factor	
M0	86 (94.5)
M1	5 (5.5)
9.Stage	
I	29 (31.9)
II	24 (26.4)
III	33 (36.3)
IV	5 (5.5)
10. Median Survival (month)	62.2

Table 2. Immunohistochemical findings of mesothelin expression.

Luminal membrane expression	Cytoplasmic expression	
	Positive (N = 38)	Negative (N = 53)
Positive (N = 34)	10	24
Negative (N = 57)	28	29

Table 3. Correlation between mesothelin expression pattern and clinicopathological features

Parameter	Total	Mesothelin			Luminal membrane expression			Cytoplasmic expression		
		High-level (n=45)	Low-level (n=46)	P-value *	Positive (n=34)	Negative (n=57)	P-value *	Positive (n=38)	Negative (n=53)	P-value *
1.Histopahologic grade										
well	36	17	19	0.831	11	25	0.376	13	23	0.395
mod por	55	28	27		23	32		25	30	
2.pT-factor										
pT1-2	35	14	21	0.197	10	25	0.189	14	21	0.830
pT3-4	56	31	25		24	32		24	32	
3.pN-factor										
Negative	53	25	28	0.673	15	38	**0.048**	23	30	0.830
Positive	38	20	18		19	19		15	23	
4.pStage										
I - II	53	25	28	0.673	15	38	**0.048**	23	30	0.830
III - IV	38	20	18		19	19		15	23	
5.Lymphatic permeation										
Negative	41	20	21	1.000	9	32	**0.009**	17	24	1.000
Positive	50	25	25		25	25		21	29	
6.Blood vessel permeation										
Negative	32	15	17	0.827	10	22	0.497	16	16	0.271
Positive	59	30	29		24	35		22	37	

7.Resection margin										
pR0	83	42	41	0.713	30	53	0.466	36	47	0.461
pR1	8	3	5		4	4		2	6	
8.Recurrence										
No	62	29	33	0.505	23	39	1.000	23	39	0.254
Yes	29	16	13		11	18		15	14	
9.Liver Metastasis										
No	74	35	39	0.431	26	48	0.411	29	45	0.414
Yes	17	10	7		8	9		9	8	
10.Local Recurrence										
No	87	44	43	0.617	33	54	1.000	35	52	0.305
Yes	4	1	3		1	3		3	1	
11.Lung Metastasis										
No	79	39	40	1.000	29	50	0.757	32	47	0.547
Yes	12	6	6		5	7		6	6	

*$\chi 2$ test