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ORIGINAL ARTICLE – COLORECTAL CANCER

TGM2 Is a Novel Marker for Prognosis and Therapeutic Target in Colorectal Cancer

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ABSTRACT

Background. Transglutaminase 2 (*TGM2*) plays a role in cell growth and survival through the antiapoptosis signaling pathway.

Methods. We analyzed *TGM2* gene expression in 91 paired cases of colorectal cancer (CRC) and noncancerous regions and seven CRC cell lines to demonstrate the importance of *TGM2* expression for the prediction of prognosis of CRC. *TGM2* expression was higher in CRC tissue than in corresponding normal tissue by real-time reverse transcriptase–polymerase chain reaction ($P = .015$).

Results. Patients in the high *TGM2* expression group showed a poorer overall survival rate than those in the low expression group ($P = .001$), indicating that the increase in *TGM2* expression was an independent prognostic factor. *TGM2* was also expressed in the seven CRC cell lines. The in vitro proliferation assay showed that *TGM2* expression is involved with tumor growth.

Conclusions. The present study suggests that *TGM2* is useful as a predictive marker for patient prognosis and may be a novel therapeutic target for CRC.

Cancer is a major public health problem in developed countries, while the incidence of colorectal cancer (CRC) has greatly increased in Japan in recent years as a result of lifestyle changes.[1] CRC is now one of the most important causes of death from neoplastic disease in Japan.[1] Therefore, identification of the genes responsible for the development and progression of CRC and understanding the clinical significance are critical for the diagnosis and adequate treatment of the disease.

Transglutaminase 2, *TGM2*, is a family of enzymes that catalyzes the formation of an amide bond between the γ-carboxamide groups of peptide-bound glutamine residues and the primary amino groups in various compounds.[2,3] Several studies have reported that that increased expression of *TGM2* indicates prolonged cell survival and the prevention of apoptosis.[4–9]

We analyzed *TGM2* in seven human gastrointestinal cancer cell lines and 91 paired cases of CRC and noncancerous regions to identify the importance of *TGM2* expression for prognosis and to suggest that it be a candidate novel marker for the prognosis with functional relevance in CRCs.

MATERIALS AND METHODS

Clinical Tissue Samples

From 1992 to 2002, 91 patients (62 men, 29 women) with CRC underwent surgery at the Medical Institute of Bioregulation at Kyusyu University. Primary CRC specimens and adjacent normal colorectal mucosa were obtained from patients after receiving informed consent in accordance with the institutional guidelines. Every patient was definitively identified with CRC on the basis of clinicopathological findings. Tissues were extracted immediately after surgical resections. The specimens were immediately fixed in formalin, processed through graded ethanol,

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embedded in paraffin, and sectioned with hematoxylin and eosin stain and elastica van Gieson stain, and the degree of the histological differentiation, lymphatic invasion, and venous invasion was examined. All specimens were frozen in liquid nitrogen immediately after resection and stored at −80°C until RNA extractions were performed.

None of the patients received chemotherapy or radiotherapy before surgery. After the surgery, the patients were followed up with a blood examination that included the tumor markers carcinoembryonic antigen and cancer antigen, and imaging modalities such as abdominal ultrasound, computed tomography, and chest x-ray every 3 to 6 months. Clinicopathological factors were assessed according to the criteria of the tumor node metastasis classification of the International Union Against Cancer.[10]

Cell Lines and Culture

Seven cell lines derived from human CRC (Caco2, DLD-1, HCT116, HT-29, KM12SM, LoVo, and SW480) were obtained and maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum and antibiotics at 37°C in a 5% humidified CO_2 atmosphere. For the siRNA knockdown experiment, double-stranded RNA duplexes targeting human *TGM2* (5′-UAGGAUCCCAUCUUCAA ACUGCCCA-3′/5′-UGGGCAGUUUGAAGAUGGGAUC CUA-3′, 5′-AUCCCAUUGUAGCUGACGGUGCGGG-3′/ 5′-CCCGCACCGUGAGCUACAAUGGGAU-3′, and 5′-UG UAGUUGGUCACGACGCGGGUAGG-3′/5′-CCUACCCG CGUCGUGACCAACUACA-3′) were purchased (Stealth RNAi) from Invitrogen (Carlsbad, CA). Negative control siRNA (NC) was also purchased from Invitrogen. CRC cell lines were transfected with siRNA at a concentration of 20 μmol/L with lipofectamine (RNAiMAX, Invitrogen), incubated in glucose-free Opti-MEM (Invitrogen) for the time indicated, and analyzed by the proliferation assay. All siRNA duplexes were used together as a triple transfection. siRNA knockdowns were performed in seven CRC cell lines to evaluate proliferation under *TGM2* suppression. Each cell line with siRNA was compared with the negative control. The values are presented as mean ± standard deviation (SD) from independent experiments conducted in triplicate.

RNA Preparation and Quantitative Real-Time Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was prepared by using a modified acid guanidium-phenol-chloroform procedure with DNase.[11] Reverse transcription was performed from 2.5 μg of total RNA as previously described.[12] A 143-bp *TGM2* fragment was amplified. Two human *TGM2* oligonucleotide primers for the polymerase chain reaction (PCR) reaction were designed as follows: 5′-ATAAGTTAGCGCCGCTCTCC-3′ (forward); 5′-CCAGCTCCAGATCACACCTC-3′ (reverse). The forward primer is located in exon 1 and the reverse primer in exon 2. The PCR assay with primers specific to the glyceraladehyde-3-phosphate dehydrogenase (*GAPDH*) gene was performed to evaluate expression. The *GAPDH* primers, 5′-TTGGTATCGTGGAAGGACTCA-3′ (forward) and 5′-TGTCATCATATTGGCAGGTT-3′ (reverse), produced a 270-bp amplicon. cDNA from the Human Reference Total RNA (Clontech, Palo Alto, CA) was studied concurrently as a positive control. Real-time monitoring of the PCRs was performed with the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Tokyo, Japan) for cDNA amplification of *TGM2* and *GAPDH*. The amplification protocol consisted of 35 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and elongation at 72°C for 10 seconds. The products were then subjected to a temperature gradient from 55°C to 95°C at 0.1°C per second with continuous fluorescence monitoring to produce product melting curves. The expression ratio of mRNA copies in tumor and normal tissues was calculated and normalized against *GAPDH* mRNA expression.

Proliferation Assays

In CRC cell lines transfected with siRNA, 1×10^5 cells were seeded in 12-well dishes and cultured for 96 hours to determine proliferation. The cell growth rate was measured by counting cells with a CellTac kit (Nihon Koden, Tokyo, Japan).

Statistical Analysis

Continuous variable data were expressed as mean ± SD. The relationship between mRNA expression and clinicopathological factors were analyzed by the χ^2 test and Student's *t*-test. Kaplan-Meier survival curves were plotted and compared with the generalized log rank test. Univariate and multivariate analyses to identify prognostic factors for overall survival were performed by the Cox proportional hazard regression model. All tests were analyzed by JMP software (SAS Institute, Cary, NC). *P* values of <.05 were considered statistically significant.

RESULTS

TGM2 mRNA Expression in Clinical Tissue Specimens

Reverse transcriptase–polymerase chain reaction (RT-PCR) of 91 paired clinical samples showed that 65 (71.4%) of the 91 cases exhibited higher levels of *TGM2* mRNA in tumors than paired normal tissues (Fig. 1). The mean *TGM2* mRNA expression value in tumor tissues was

significantly higher than that for corresponding normal tissues ($P = .015$; Student's *t*-test).

TGM2 Expression and Clinicopathological Characteristics

The experimental samples were divided into two groups according to expression status for the clinicopathological evaluation. Patients with tumors that had more than the median *TGM2/GAPDH* expression (median .329) were assigned to the high expression group ($n = 46$); the others were assigned to the low expression group ($n = 45$, Table 1). The number of cases that were based on histological grade was 37, 47, 4, and 3 in the well, moderate, poor, and mucinous adenocarcinoma categories, respectively. *TGM2* expression was correlated with tumor type ($P = .002$), tumor invasion ($P < .001$), lymph node metastasis ($P = .041$), lymphatic invasion ($P = .010$), metastasis ($P = .040$), and International Union Against Cancer stage ($P < .001$).

Relationship Between TGM2 Expression and Prognosis

Postoperative overall survival rate was statistically significantly lower in patients with increased *TGM2* expression (Fig. 2). The median follow-up was 4.12 years. Table 2 provides the univariate and multivariate analyses of factors related to patient prognosis. Univariate analysis showed that histological grade ($P = .040$), tumor type ($P = .003$), tumor size ($P = .004$), tumor invasion

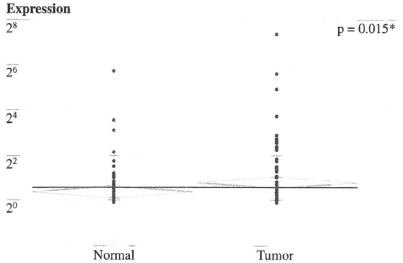

FIG. 1 Transglutaminase 2 (*TGM2*) mRNA expression in clinical tissue specimens. Quantitative real-time reverse transcriptase–polymerase chain reaction of 91 paired clinical samples showed that 65 (71.4%) of the 91 cases exhibited higher levels of *TGM2* mRNA in tumors than in paired normal tissues. The mean *TGM2* mRNA expression in tumor tissues (normalized by *GAPDH* gene expression) was significantly higher than that of the corresponding normal tissues ($P = .015$; Student's *t*-test)

TABLE 1 Clinicopathological factors and TGM2 mRNA expression in 91 colorectal cancers

Factor	High expression ($n = 46$)	Low expression ($n = 45$)	*P* value
Age (y)			
<68	25 (54.3%)	17 (37.8%)	.112
≥68	21 (45.7%)	28 (62.2%)	
Sex			
Male	31 (67.4%)	31 (68.9%)	.878
Female	15 (32.6%)	14 (31.1%)	
Histological grade			
Wel/Mod	41 (89.1%)	43 (95.6%)	.242
Others	5 (10.9%)	2 (4.4%)	
Tumor type			
0–2	0–2 3 (6.5%)	14 (31.1%)	.002*
3–4	3–4 43 (93.5%)	31 (68.9%)	
Tumor size			
<30 mm	39 (84.8%)	35 (77.8%)	.391
≥30 mm	7 (15.2%)	10 (22.2%)	
Tumor invasion			
Tis	0 (0%)	5 (11.1%)	≤.001*
T1	2 (4.3%)	6 (13.3%)	
T2	3 (6.5%)	12 (26.7%)	
T3	28 (60.9%)	18 (40.0%)	
T4	13 (28.3%)	4 (8.9%)	
Lymph node metastasis			
N0	22 (47.8%)	31 (68.9%)	.041*
N1–2	24 (52.2%)	14 (31.1%)	
Lymphatic invasion			
Absent	24 (52.2%)	35 (77.8%)	.010*
Present	22 (47.8%)	10 (22.2%)	
Venous invasion			
Absent	38 (82.6%)	40 (88.9%)	.392
Present	8 (17.4%)	5 (11.1%)	
Metastasis			
M0	29 (63.0%)	37 (82.2%)	.040*
M1	17 (37.0%)	8 (17.8%)	
UICC stage			
0	0 (0%)	5 (11.1%)	≤.001*
I	5 (10.9%)	12 (26.7%)	
IIA	11 (23.9%)	11 (24.4%)	
IIB	2 (4.3%)	1 (2.2%)	
IIIA	0 (0%)	5 (11.1%)	
IIIB	8 (17.4%)	3 (6.7%)	
IIIC	3 (6.5%)	0 (0%)	
IV	17 (37.0%)	8 (17.8%)	

Wel well differentiated adenocarcinoma, *mod* moderately differentiated adenocarcinoma, *others* poorly differentiated adenocarcinoma and mucinous carcinoma, *UICC* International Union Against Cancer

* Statistically significant

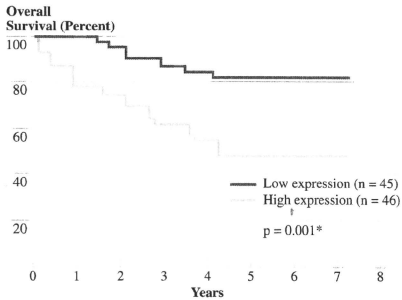

FIG. 2 Overall survival curves of colorectal cancer patients based on *TGM2* mRNA expression status. The postoperative overall survival rate was significantly lower among patients in the high *TGM2* expression group ($P = .001$, log rank test) than the low expression group. The median follow-up was 4.12 years

($P < .001$), lymph node metastasis ($P < .001$), lymphatic invasion ($P = .006$), venous invasion ($P = .001$), and *TGM2* mRNA expression ($P = .003$) were significantly related to overall survival. Multivariate analysis indicated that inclusion in the *TGM2* mRNA high expression group (relative risk, 2.40; 95% confidence interval, 1.03–6.11; $P = .041$) was an independent predictor of postoperative overall survival, as was metastasis (M1/M0, relative risk, 5.86; 95% confidence interval, 2.49–15.12; $P < .001$).

In Vitro Assessment of TGM2 Expression Knockdown

Seven CRC cell lines were used for the proliferation study because *TGM2* expression was higher than the median value of *GAPDH* in the primary CRC specimen by RT-PCR. A reduction in *TGM2* by siRNA was observed by quantitative real-time RT-PCR in all the cell lines examined (negative control [NC] and *TGM2* siRNAs; $P < .05$, Student's *t*-test). A reduction in *TGM2* expression was confirmed in the HT-29, HCT116, KM12SM, and LoVo cell lines (Suppl. Fig. S1). In proliferation assay, there were differences in cell numbers of HT-29 between NC and *TGM2* siRNA ($P < .05$) (Fig. 3). There was no statistically significant difference in the number between the NC and *TGM2* siRNA in the other cell lines.

DISCUSSION

Previous reports showed that *TGM2*, also known as *TG2*, is expressed in breast and pancreatic cancer cells and is associated with drug resistance and metastasis.[4–16] *TGM2* promotes a stable interaction with extracellular matrix protein components in association with some β members of the integrin family of proteins, which induce cell survival signaling pathways.[17] Other reports suggest that *TGM2* regulates activation of NF-κB by forming a ternary complex with NF-κB/IκBα, and inhibition of apoptosis through transamidation and GTP-binding activity.[4,9,18]

Seven distinct transglutaminases have been described.[19–22] *TGM2* is ubiquitously expressed as a single/polypeptide protein that exhibits Ca^{2+}-dependent protein cross-linking activity.[23]

We assessed *TGM2* gene expression and found that it was a statistically significant independent prognostic factor, similar to the well-known important predictive factor.[24] To our knowledge, the present study is the first report

TABLE 2 Univariate and multivariate analyses for overall survival (Cox proportional hazard regression model)

Factor	Univariate analysis			Multivariate analysis		
	RR	95% CI	*P* value	RR	95% CI	*P* value
Age (y), <68/≥68	1.47	0.70–3.11	.298			
Sex, male/female	1.40	0.64–3.38	.401			
Histological grade, por–others/well–mod	3.66	1.06–9.64	.040*	2.52	0.68–7.45	.148
Tumor type, 3–4/0–2	8.27	1.76–147.44	.003*	1.80	0.22–40.49	.615
Tumor size, ≥30 cm/<30 cm	2.82	1.30–11.91	.004*	1.26	0.45–6.02	.697
Tumor invasion, T3–4/Tis-2	7.60	2.27–47.16	≤.001*	1.13	0.36–2.68	.802
Lymph node metastasis, N1–2/N0	5.42	2.43–13.74	≤.001*	2.06	0.83–5.74	.119
Lymphatic invasion, present/absent	2.80	1.34–5.89	.006*	1.32	0.53–3.22	.532
Venous invasion, present/absent	4.20	1.81–9.03	.001*	2.24	0.85–5.80	.099
Metastasis, M1/M0	8.93	4.14–20.84	≤.001*	5.86	2.49–15.12	≤.001*
TGM2 mRNA expression, ≥median/median>	3.08	1.43–7.18	.003*	2.40	1.03–6.11	.041*

RR relative risk, *95% CI* 95% confidence interva, *wel* well-differentiated adenocarcinoma, *mod* moderately differentiated adenocarcinoma, *por* poorly differentiated adenocarcinoma, *others* poorly differentiated adenocarcinoma and mucinous carcinoma

* Statistically significant

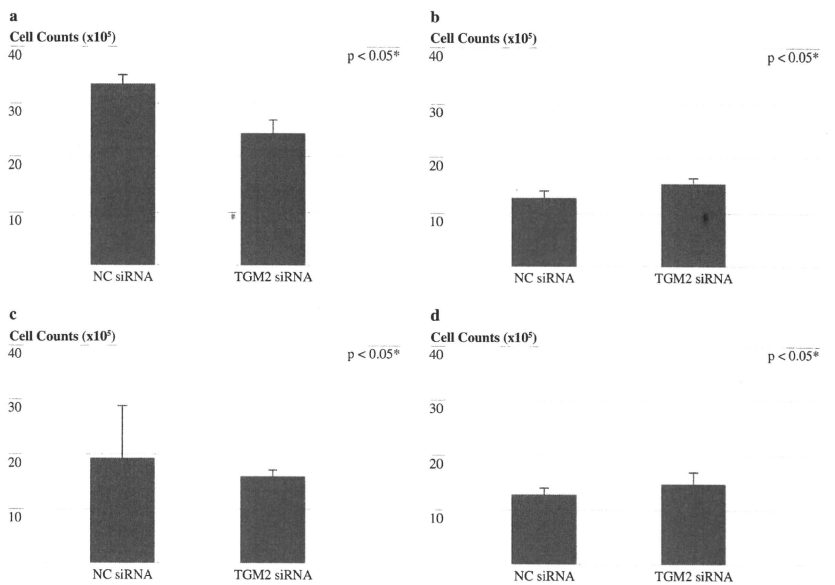

FIG. 3 Proliferation assay and siRNA inhibition in 4 colorectal cancer cell lines. The proliferation assay showed a difference in growth of colorectal cancer cell line HT-29. There were significant differences between NC and *TGM2* siRNA. In the other 3 cell lines, there was no significant difference between NC and *TGM2* siRNA (**a** HT-29; **b** HCT116; **c** KM12SM; **d** LoVo). Values are mean ± SD for three independent experiments. WT, wild type; NC, negative control

showing that *TGM2* is upregulated in CRCs, suggesting that it could be a novel predictive marker for the prognosis of CRCs that may contribute to further clinical cancer diagnosis.

Recently, the necessity of intensive follow-up and adjuvant therapy for CRC has been proposed to predict recurrence and metastasis in curative surgical resected cases.[25–27] In addition, there have been many recent reports on the use of less invasive surgery for CRC such as laparoscopic and endoscopic surgery.[28–31] For these cases, a predictive marker of tumor invasion, lymph node metastasis, and distant metastasis would play a very important role in cancer diagnoses and treatments, especially as a novel marker independent from the traditional tumor, node, metastasis factors. Thus, the *TGM2* expression profile could contribute to the predictive diagnosis of CRCs.

TGM2 plays an important role in antiapoptotic signaling pathways and several cancer cell lines that exhibit high *TGM2* expression levels and have been selected for resistance to chemotherapeutic drugs.[17,32,33] Downregulation of *TGM2* expression by siRNA rendered the cancer cells sensitive to chemotherapeutic drugs.[17]

The present in vitro study showed that *TGM2* expression is associated with tumor growth, and the inhibition of *TGM2* may lead to a reduction in CRC proliferation. *TGM2* is expressed in several cancers.[14–16] Our results suggest a rationale for further study of *TGM2* as a possible novel target for clinical cancer therapy such as anticancer agents and the sensitizer in addition to the novel marker of prognosis and prediction about the susceptibility of anticancer agents.

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ATP11A is a novel predictive marker for metachronous metastasis of colorectal cancer

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Abstract. The adenosine triphosphate-binding cassette transporter-homologous gene *ATP11A* belongs to an extended family of adenosine triphosphate-binding cassette transporters. We analysed the *ATP11A* gene in 7 colorectal cancer cell lines and 95 paired cases of colorectal cancer and non-cancerous regions to demonstrate the importance of *ATP11A* expression in colorectal cancer prognosis. *ATP11A* was expressed in the 7 colorectal cancer cell lines. *ATP11A* mRNA expression was higher in colorectal cancer tissue than in corresponding normal tissue ($P<0.001$). Patients with high *ATP11A* expression showed a poorer disease-free survival rate compared to those with low expression ($P<0.001$), thus indicating that increase in *ATP11A* expression was an independent predictor of metachronous metastasis of colorectal cancer. The present study suggests that *ATP11A* is a useful predictive marker of metastasis in colorectal cancer patients.

Introduction

Cancer is a major public health problem in developed countries. In Japan, the incidence of colorectal cancer (CRC) has significantly increased in recent years with changes in lifestyle (1). CRC is now one of the most important causes of death from neoplastic disease in Japan (1). The necessity of intensive follow-up and adjuvant therapy for CRC has been recently proposed in view of predicting recurrence and metastasis in curative surgical resection cases (2-4). Identification of genes responsible for development and progression of CRC and an understanding of their clinical significance are critical for diagnosis and adequate treatment of the disease.

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Key words: ATP11A, colorectal cancer, metastasis

The adenosine triphosphate-binding cassette (ABC) transporter-homologous gene *ATP11A* belongs to an extended family of ABC transporters that confer multi-drug resistance to cancer cells (5,6). In a previous study, *ATP11A* expression increased in leukaemia cells resistant to chemotherapy and the levels of *ATP11A* mRNA were 100-fold higher in cells that were intentionally made SCH66336-resistant (6).

We investigated the importance of the *ATP11A* gene by analysing it in 95 paired cases of CRC and non-cancerous regions as well as 7 CRC cell lines. We proposed the importance of *ATP11A* expression in prognosis evaluation, thus suggesting that *ATP11A* could be a novel predictive marker for prognosis of CRCs.

Materials and methods

Clinical tissue samples. Ninety-five patients (58 men and 37 women) with CRC underwent curative surgery at the Medical Institute of Bio-regulation at Kyusyu University from 1993 to 2001. We followed 61 of the 95 patients for over 5 years after the primary operation. Primary CRC specimens and adjacent normal colorectal mucosa were obtained from patients after receiving informed consent in accordance with the institutional guidelines. Each patient was definitively diagnosed with CRC based on clinicopathological findings. Resected surgical specimens were fixed in formalin, processed through graded ethanol, embedded in paraffin and sectioned with haematoxylin and eosin as well as elastica van Gieson stains. The degree of histological differentiation, lymphatic invasion and venous invasion was examined. Immediately after resection, all specimens were frozen in liquid nitrogen and kept at -80˚C until RNA extractions were performed.

None of the patients received chemotherapy or radiotherapy prior to surgery. After surgery, the patients were followed up with blood examinations, including serum carcinoembryonic antigen and cancer antigen (CA19-9) level and imaging modalities such as abdominal ultrasonography and/or computed tomography and chest X-ray every 3-6 months. Clinicopathological factors were assessed according to the tumour-node-metastasis (TNM) criteria of the International Union Against Cancer (7).

Cell lines and culture. Seven cell lines derived from human CRC (Caco2, DLD-1, HCT116, HT-29, KM12SM, LoVo and SW480) were obtained and maintained in Dulbecco's minimal essential medium containing 10% foetal bovine serum and antibiotics at 37˚C in a 5% humidified CO_2 atmosphere. For the siRNA knockdown experiment, double-stranded RNA duplexes targeting human *ATP11A* (5'-AGGAGCGUCCAG UAGAUGAACACGC-3'/5'-GCGUGUUCAUCUACUGGA CGCUCCU-3', 5'-UUCAAAUGCUUAAACUUUGGGA UUG-3'/5'-CAAUCCCAAAGUUUAAGCAUUUGAA-3' and 5'-UGGAAAGGAAGAUGAAGUCGCAGGG-3'/ 5'-CCCUGCGACUUGAUCUUCCUUUCCA-3') were purchased (Stealth RNAi; Invitrogen, Carlsbad, CA, USA). Negative control siRNA (NC) was also purchased from Invitrogen. CRC cell lines were transfected with siRNA at a concentration of 20 μmol/l using lipofectamine (RNAiMAX; Invitrogen), incubated in glucose-free Opti-MEM (Invitrogen) for the time indicated and analysed by proliferation assay. All siRNA duplexes were used together as triple transfection. The growth rate of cultured cells was measured by counting cells using a CellTac kit (Nihon Koden, Tokyo, Japan). siRNA knockdowns were performed in 7 CRC cell lines to evaluate proliferation under *ATP11A* suppression. Each cell line with siRNA was compared to the wild-type and a negative control. Values were expressed as the mean ± standard deviation (SD) from independent experiments performed in triplicate.

RNA preparation and quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was prepared using a modified acid guanidium-phenol-chloroform method with DNase (8). Reverse transcription was performed with 2.5 μg of total RNA as described previously (9) and a 143-bp *ATP11A* fragment was amplified. Two human *ATP11A* oligonucleotide primers for PCR were designed as follows: 5'-CACAGAGATACCCAGACAAC AGG-3' (forward) and 5'- CAACTGCACCAGAAATATGA TAAGG-3' (reverse). The forward primer was located in exon 2 and the reverse primer in exon 3. A PCR assay with primers specific to the glyceraladehyde-3-phosphate dehydrogenase (*GAPDH*) gene was performed. The *GAPDH* primers 5'-TTGGTATCGTGGAAGGACTCA-3' (forward) and 5'-TGTCATCATATTGGCAGGTT-3' (reverse) produced a 270-bp amplicon. cDNA from Human Reference total RNA (Clontech, Palo Alto, CA, USA) was used as a source for positive controls. Real-time monitoring of PCRs was performed using the LightCycler FastStart DNA Master SYBR-Green I kit (Roche Diagnostics, Tokyo, Japan) for cDNA amplification of *ATP11A* and *GAPDH*. The amplification protocol consisted of 35 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 10 sec and elongation at 72˚C for 10 sec. The products were then subjected to a temperature gradient from 55˚C to 95˚C at 0.1˚C s^{-1}, with continuous fluorescence monitoring to produce product melting curves. The expression ratio of mRNA copies in tumour and normal tissues was calculated and normalized against *GAPDH* mRNA expression.

Statistical analysis. Continuous variable data are expressed as the mean ± SD. The relationship between mRNA expression and clinico-pathological factors was analysed using the Chi-square test and Student's t-test. Kaplan-Meier survival curves were plotted and compared to the generalized log-rank test. Univariate and multivariate analyses to identify prognostic factors for overall survival were performed using the Cox proportional hazard regression model. All tests were analysed using JMP software (SAS Institute, Cary, NC, USA) and P-values <0.05 were considered statistically significant.

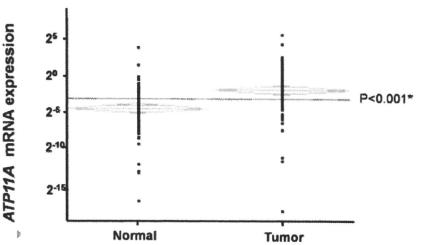

Figure 1. *ATP11A* mRNA expression in clinical tissue specimens. Quantitative real-time reverse transcriptase-polymerase chain reaction on 95 paired clinical samples showed that 80 of these cases (84.2%) exhibited higher levels of *ATP11A* mRNA in tumours than in paired normal tissues. The mean *ATP11A* mRNA expression level in tumour tissues (normalized by glyceraladehyde-3-phosphate dehydrogenase gene expression) was significantly higher compared to that of corresponding normal tissues (P<0.001; Student's t-test).

Results

ATP11A mRNA expression in clinical tissue specimens. We performed quantitative real-time RT-PCR analysis with paired primary and adjacent non-cancerous regions of CRCs. RT-PCR on 95 paired clinical samples showed that 80 of these cases (84.2%) exhibited higher levels of *ATP11A* mRNA in tumours than in paired normal tissues (Fig. 1). Mean *ATP11A* mRNA expression in tumour tissues was significantly higher than that for corresponding normal tissues (P<0.001; Student's t-test).

ATP11A expression and clinico-pathological characteristics. *ATP11A* expression was calculated by dividing the value of *ATP11A/GAPDH* expression of the tumour region by that of adjacent non-cancerous regions. For clinico-pathological evaluation, experimental samples were divided into 2 groups according to expression status. Patients with values more than the median *ATP11A* expression value (median, 5.800) were assigned to the high expression group and the others were assigned to the low expression group. Clinico-pathological factors related to *ATP11A* expression status of 95 patients are summarized in Table I and those of 61 patients that were followed over 5 years are summarized in Table II. Data indicate that *ATP11A* expression was correlated with tumour type, tumour invasion, lymph node metastasis, lymphatic invasion, venous invasion and metastasis.

Relationship between ATP11A expression and prognosis. Data show that the post-operative overall survival rate was

Table I. Clinico-pathological factors and *ATP11A* mRNA expression in 95 colorectal cancer patients.

Factors	High expression n=47 (%)	Low expression n=48 (%)	P-value
Age			
<68	26 (55.3%)	21 (43.8%)	0.259
≥68	21 (44.7%)	27 (56.2%)	
Gender			
Male	31 (66.0%)	27 (56.3%)	0.330
Female	16 (34.0%)	21 (43.7%)	
Histological grade			
Wel/Mod/	43 (91.5%)	47 (97.9%)	0.160
Por	4 (8.5%)	1 (2.1%)	
Tumour type			
Type 0-1	6 (12.8%)	13 (27.1%)	0.081
Type 2-4	41 (87.2%)	35 (72.9%)	
Tumour size			
<50 mm	23 (48.9%)	29 (60.4%)	0.260
≥50 mm	24 (51.1%)	19 (39.6%)	
Tumour invasion			
Tis	1 (2.1%)	4 (8.3%)	0.007
T1	3 (6.4%)	7 (14.6%)	
T2	3 (6.4%)	12 (25.0%)	
T3	33 (70.2%)	18 (37.5 %)	
T4	7 (14.9%)	7 (14.6%)	
Lymph node metastasis			
N0	21 (47.7%)	36 (75.0%)	0.002
N1-2	26 (55.3%)	12 (25.0%)	
Lymphatic invasion			
Absent	25 (53.2%)	38 (79.2%)	0.007
Present	22 (46.8%)	10 (20.8%)	
Venous invasion			
Absent	34 (72.3%)	45 (93.8%)	0.005
Present	13 (27.7%)	3 (6.2%)	
Metastasis			
M0	30 (63.8%)	46 (95.8%)	<0.001
M1	17 (36.2%)	2 (4.2%)	

Underlined values indicate statistical significance. Wel, well-differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma and Por, poorly differentiated adenocarcinoma.

Table II. Clinico-pathological factors and *ATP11A* mRNA expression in 61 patients followed over 5 years.

Factors	High expression n=31 (%)	Low expression n=30 (%)	P-value
Age			
<68	17 (54.8%)	14 (46.7%)	0.523
≥68	14 (45.2%)	16 (53.3%)	
Gender			
Male	22 (71.0%)	16 (53.3%)	0.154
Female	9 (29.0%)	14 (46.7%)	
Histological grade			
Wel-Mod	30 (96.8%)	29 (96.7%)	0.981
Por	1 (3.2%)	1 (3.3%)	
Tumour type			
Type 0-1	2 (6.5%)	11 (36.7%)	0.004
Type 2-4	29 (93.5%)	19 (63.3%)	
Tumour size			
<50 mm	14 (45.2%)	18 (60.0%)	0.245
≥50 mm	17 (54.8%)	12 (40.0%)	
Tumour invasion			
Tis	0 (0%)	3 (10.0%)	0.001
T1	0 (0%)	5 (16.7%)	
T2	3 (9.7%)	8 (26.7%)	
T3	22 (71.0%)	10 (33.3 %)	
T4	6 (19.3%)	4 (13.3%)	
Lymph node metastasis			
N0	12 (38.7%)	24 (80.0%)	0.001
N1-2	19 (61.3%)	6 (20.0%)	
Lymphatic invasion			
Absent	16 (51.6%)	23 (76.7%)	0.041
Present	15 (48.4%)	7 (23.3%)	
Venous invasion			
Absent	21 (67.7%)	28 (93.3%)	0.011
Present	10 (32.3%)	2 (6.7%)	
Metachronous Metastasis			
Absent	15 (48.4%)	29 (96.7%)	<0.001
Present	16 (51.6%)	1 (3.3%)	

Underlined values indicate statistical significance. Wel, well-differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma and Por, poorly differentiated adenocarcinoma.

lower in patients with elevated *ATP11A* expression (P=0.141). The median follow-up was 4.22 years (Fig. 2). We followed 61 of 95 patients over 5 years after the operation (median, 6.80 years). We also evaluated the metachronous metastasis-free over 5-year survival rate in these patients and found that the rate was significantly lower in patients with elevated *ATP11A* expression (P<0.001; Fig. 3). Table III shows univariate and multivariate analyses of factors related to patient prognosis. Univariate analysis showed that age (P=0.008), tumour type (P=0.042), tumour invasion (P=0.002), lymph node metastasis (P<0.001), lymphatic invasion (P<0.001), venous invasion (P=0.008) and *ATP11A* mRNA expression (P<0.001) were significantly correlated with post-operative metastasis. Multivariate regression analysis indicated that inclusion in the *ATP11A* mRNA high expression group [relative risk (RR), 8.79; 95% confidence interval (CI), 1.35-172.64; P=0.019] was an independent predictor of metastasis-free survival, as were age (RR, 5.57; 95% CI, 1.54-24.78; P=0.007) and venous invasion (RR, 4.56; 95% CI, 1.31-16.10; P=0.017).

Table III. Univariate and multivariate analyses for disease-free survival over 5 years (Cox proportional hazards regression model).

Factors	Univariate analysis			Multivariate analysis		
	RR	95% CI	P-value	RR	95% CI	P-value
Age (<68/≥68)	3.93	1.38-13.97	0.008	5.57	1.54-24.78	0.007
Gender (Male/Female)	1.56	0.57-4.91	0.389			
Histological grade (Por/Wel-Mod)	3.02	0.16-15.17	0.358			
Tumour type (Type 2-4/Type 0-1)	5.17	1.04-93.61	0.042	1.23	0.19-7.55	0.807
Tumour size (≥50/<50)	1.32	0.82-2.20	0.246			
Tumour invasion (T3-4/Tis-2)	9.31	1.89-168.23	0.002	1.06	0.12-4.97	0.948
Lymph node metastasis (N1-2/N0)	10.02	3.22-43.89	<0.001	3.22	0.94-15.54	0.063
Lymphatic invasion (Present/Absent)	5.51	2.04-17.35	<0.001	2.27	0.78-8.11	0.135
Venous invasion (Present/Absent)	4.05	1.46-10.63	0.008	4.56	1.31-16.10	0.017
ATP11A mRNA expression (≥5.8/<5.8)	20.07	4.08-362.59	<0.001	8.79	1.35-172.64	0.019

Underlined values indicate statistical significance. RR, relative risk; CI, confidence interval; Wel, well-differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma and Por, poorly differentiated adenocarcinoma and mucinous carcinoma.

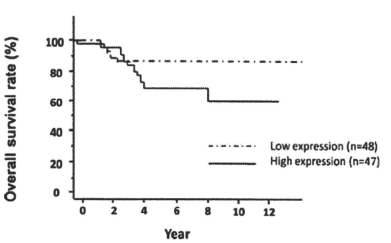

Figure 2. Overall survival curves based on *ATP11A* mRNA expression status of CRC patients. The post-operative overall survival rate was lower in patients in the high *ATP11A* expression group (P=0.141). The median follow-up was 4.22 years.

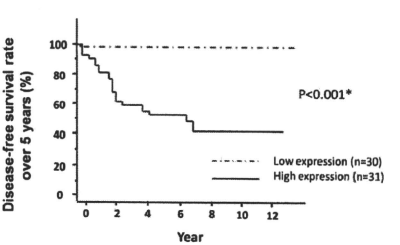

Figure 3. Disease-free survival curves based on *ATP11A* mRNA expression status of patients over 5 years after curative surgery for CRC. The post-operative disease-free survival rate was significantly lower in patients in the high *ATP11A* expression group (P<0.001). The median follow-up was 6.80 years.

In vitro assessment of ATP11A expression knockdown. Seven CRC cell lines were chosen for the proliferation study because their expression was higher than the median value of *ATP11A/GAPDH* in primary CRC specimens by RT-PCR. Significant reduction in *ATP11A* by siRNA was also confirmed by quantitative real-time RT-PCR in Caco2, HCT116, KM12SM and LoVo cell lines (NC and *ATP11A* siRNAs; P<0.05; Student's t-test; Fig. 4). Significant reduction in *ATP11A* expression was confirmed by seeding the cells ($1x10^5$) in 12-well dishes and culturing them for 96 h to determine proliferation. The results showed no significant difference in cell numbers between NC and *ATP11A* siRNA.

Discussion

ATP11A, also known as RP-11-120K24.1, ATPIH or ATPIS, is expressed in the heart, brain, muscle and liver of mice (10).

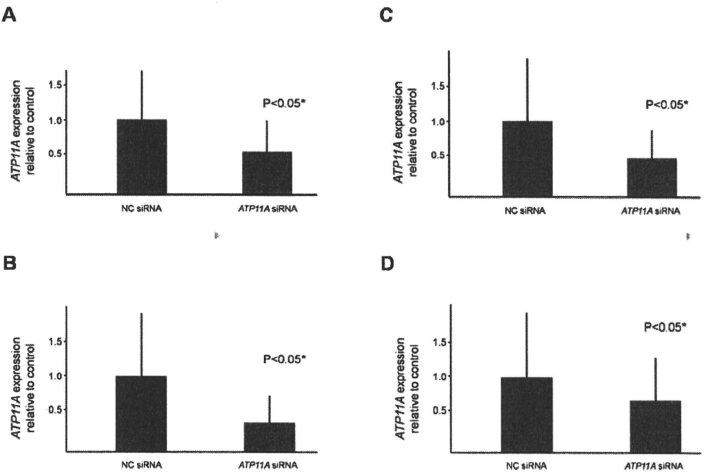

Figure 4. siRNA inhibition of *ATP11A* in 4 colorectal cancer cell lines. Suppression of *ATP11A* expression was confirmed by real-time reverse transcriptase-polymerase chain reaction. Reduction in the *ATP11A* siRNA experiment was significant compared to NC ($P<0.05$; Student's t-test) in 4 cell lines (A, Caco2; B, HCT116; C, KM12SM; D, LoVo). The results showed no significant difference in cell numbers between NC and *ATP11A* siRNA. NC, negative control.

It has also been reported to be expressed in human blood and colon (6).

The present study is the first to provide data indicating higher *ATP11A* expression in CRC and the possibility that *ATP11A* expression could be a useful predictive marker for metachronous metastasis of CRC.

Adjuvant chemotherapy for CRC after curative resection has been proposed; intensive follow-up is necessary (2-4). There are many recent studies related to less invasive surgery for CRC, such as laparoscopic and endoscopic surgery (11-14). For these cases, a predictive marker for tumour invasion as well as lymph node and distant metastasis would play a very important role in cancer diagnoses and treatments, especially as a novel marker independent of traditional TNM factors. Thus, the *ATP11A* expression profile may contribute to some kind of predictive diagnosis.

In a previous study, *ATP11A* was used as a drug transporter. Modulating *ATP11A* levels either through overexpression or siRNA knockdown caused decreased sensitivity of cells to SCH66336, thus indicating that *ATP11A* can directly transport SCH66336 out of cells (6).

The results of the present study showed that the metachronous metastasis-free survival rate was significantly lower in patients with elevated *ATP11A* expression; however, it was also shown that *ATP11A* expression was not related to tumour growth in CRC cell lines. Detailed investigation of *ATP11A* expression and adjuvant chemotherapy did not show any apparent correlation (data not shown). Furthermore, 33 of 61 (54.9%) cases in the present study had adjuvant chemotherapy, thus suggesting that *ATP11A* could be an independent predictive marker for metachronous metastasis in CRC patients; however, further investigation is necessary. Our results suggest a rationale for further study of *ATP11A* as a possible novel target for clinical cancer therapy.

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Epithelial–mesenchymal transition with expression of *SNAI1*-induced chemoresistance in colorectal cancer

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ABSTRACT

Background: Previous reports have demonstrated that *SNAI1* plays a role in epithelial–mesenchymal transition (EMT) through the suppression of *CDH1*. Its role in the pathology and regulation of EMT expression to chemoresistance in colorectal cancer (CRC) has not yet been fully elucidated.
Methods: Immunohistochemistry was performed to evaluate the expression of Snai1 protein in 30 primary CRC samples. The biological significance of Snai1 expression was studied by induction of the wild-type (*WT*) and mutant *SNAI1* gene in CRC SW480 cells.
Results: Examination of 20 surgical specimens of CRC indicated that Snai1 protein expression was localized outer regions of invasive tumors. Introduction of phosphorylation-defective active EMT forms, *SNAI1-6SA* and *SNAI1-8SA*, caused downregulation of *CDH1* and upregulation of *VIM* compared with *SNAI1-WT* and the negative control (NC). Chemoresistance to 5-fluorouracil (IC50) was higher in *SNAI1-6SA* and *SNAI1-8SA* transfectants compared with *SNAI1-WT* and NC. All the above results were significantly different.
Conclusion: The present study demonstrated that Snai1 plays a role in CRC invasion through phosphorylation, suggesting a plausible mechanism for overcoming chemoresistance that will lead to the development of effective treatments for CRC.

Introduction

Most cancer deaths happen due to local invasion and distant metastasis of tumor cells [1–3]. Thus, it is necessary to understand the mechanisms of invasion and metastasis to prevent and cure these malignancies. A recent report demonstrated that epithelial–mesenchymal transition (EMT) plays an important role in cancer invasiveness and metastasis, which might result in poor prognosis [4,5]. In EMT, epithelial cells acquire fibroblast-like properties and exhibit a reduction in intracellular adhesion. This process is associated with the functional loss of E-cadherin, which increases mortality in cancer, indicating that the downregulation of E-cadherin is a key step in cancer invasiveness [6–8]. Several transcriptional factors including zinc finger proteins of the Snail/Slug family [9,10], Eb1, Sip1, and the basic helix-loop-helix E12/E47 factor [11] are known to be responsible for the loss of E-cadherin expression.

Snai1 was first identified in *Drosophila* as the transcriptional regulator of gastrulation [12]. Its homologs have subsequently been found in many other species including humans, other vertebrates, non-vertebrate chordates (ascidians and *Amphioxus*), insects, nematodes, annelids, and molluscs [12]. Snai1 plays a fundamental role in EMT by suppressing E-cadherin expression [1,5]. Snai1 is highly unstable with a short half-life of about 25 min and has two consensus motifs for dual regulation of the function of this protein [13]. Phosphorylation of the first motif regulates its β-Trcp-mediated ubiquitination, whereas phosphorylation of the second motif controls its subcellular localization [13]. Previous reports have showed that Snai1 and Gsk-3β together function as a molecular switch for multiple signaling pathways that lead to EMT by *SNAI1* variants (*SNAI1-6SA* and *SNAI1-8SA*). These variants are much more stable than the parent and reside exclusively in the nucleus because of a loss of their phosphorylation ability [13–15]. Furthermore, Gsk-3β inhibition results in the upregulation of Snai1 and downregulation of E-cadherin *in vivo* [13].

Abbreviation: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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Recently, Snai1 was shown to repress E-cadherin expression and induced EMT in several cell lines [1,5,13]. Here, we report that EMT caused by Snai1-associated budding indicated local tumor invasion of colorectal cancer (CRC) and drug resistance that are usually very important clinical problems.

Materials and methods

Primary tumor samples. The study comprised 30 patients who underwent surgery for CRC at the Department of Gastroenterological Surgery at Osaka University from 2004 to 2005. Primary CRC specimens and adjacent normal colorectal mucosa were obtained from patients after acquiring their informed and written consent, in accordance with the Institutional Ethical Guidelines. The surgical specimens were fixed in formalin, processed with graded ethanol, embedded in paraffin, and sectioned and stained with hematoxylin and eosin (H&E) and Elastica van Gieson stains to analyze the degree of histological differentiation and lymphatic and venous invasion. None of the patients received chemotherapy or radiotherapy prior to surgery. Every patient was definitively identified as having CRC based on clinicopathological findings. Clinicopathological factors were assessed according to the tumor-node-metastasis (TNM) classification criteria of the International Union Against Cancer (UICC) [16].

Cell culture and transfection. The human CRC SW480 cells was maintained in DMEM containing 10% fetal bovine serum (FBS) and penicillin–streptomycin at 37 °C in 5% humidified CO_2 atmosphere. SW480 cells express very low levels of endogenous Snai1, which was evaluated using quantitative real-time reverse transcription (RT)-PCR as described below. Plasmids of green fluorescent protein (*GFP*)-*SNAI1-WT* (#16225; Addgene, Cambridge, MA), *GFP-SNAI1-6SA* (#16228; Addgene), and *GFP-SNAI1-8SA* (#16229; Addgene) were used; SW480 cells with *SNAI1-WT*, *SNAI1-6SA*, or *SNAI1-8SA* were established by transfecting the cells with plasmids, as described previously [13]. Products of *SNAI1-6SA* and *SNAI1-8SA* were phosphorylation-defective mutants of Gsk-3β kinase [13]. These transfectants were grown in DMEM supplemented with 10% FBS and G418 (600 μg/ml). CRC cells were transfected with or without pAcGFP1-C1 (Clontech, Palo Alto, CA) using lipofectamine (Lipofectamine 2000; Invitrogen, Carlsbad, CA) to be used as negative controls. In brief, CRC cells were transfected with adequate plasmid at a concentration of 5 μg/μl using lipofectamine and incubated in glucose-free Opti-MEM (Invitrogen). All experiments were performed at 50–70% cell confluence, and the results were confirmed by at least three independent experiments.

Morphological analysis. Gfp expression of transfectants, grown to 70% confluence in DMEM with 10% FBS and G418 (600 μg/ml), was visualized at several magnifications with a Nikon light microscope (Nikon Inc., Meville, NY) having digital photographic capability. The digital images of CRC and parental cells were compared with EMT for consistent morphological characteristics such as loss of spindle and cell polarity [13]. Four blinded observers classified the images for the presence or absence of morphological changes consistent with EMT.

RNA preparation and quantitative real-time RT-PCR. Total RNA was prepared using TRIzol reagent (Invitrogen). Reverse transcription was performed with SuperScript III (Invitrogen). For quantitative assessment, real-time quantitative RT-PCR was performed using a LightCycler TaqMan Master Kit (Roche Diagnostics, Tokyo, Japan) for cDNA amplification of *SNAI1* (forward: 5′-GCTGCAGGACTCTAATC CAGA-3′, reverse: 5′-ATCTCCGGAGGTGGGATG-3′, and Universal ProbeLibrary probe #11) and glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) (forward: 5′-AGCCACATCGCTCAGACAC-3′, reverse: 5′-GCCCAATACGACCAAATCC-3′, and Universal ProbeLibrary probe #60), *CDH1* (forward: 5′-CCCGGGACAACGTTTATTA-3′, reverse: 5′-GCTGGCTCAAGTCAAAGTCC-3′, and Universal ProbeLibrary probe #35), *VIM* (forward: 5′-AAAGTGTGGCTGCCAAGAAC-3′, reverse: 5′-AGCCTCAGAGAGGTCAGCAA-3′, and Universal ProbeLibrary probe #16). The amplification protocol consisted of 35 cycles of denaturation at 95 °C for 10 s and annealing and elongation at 60 °C for 30 s. The expression ratios of mRNA copies in tumor and normal tissues were calculated and normalized against *GAPDH* mRNA expression. Complementary DNA from human reference total RNA (Clontech, Palo Alto, CA) was studied concurrently as a source of positive controls.

Invasion assay. The invasion assay was performed using Transwell cell culture chambers (8 μm pore size polyethylene terephthalate membrane; BD Biosciences, Bedford, MA). The membrane was coated with Matrigel (BD Biosciences). Cells (5×10^4 cells/ml) were resuspended in DMEM. The upper chamber was loaded with 500 μl of cell suspension and the lower chamber was loaded with 750 μl of DMEM with 5% FBS. After incubation for 22 h at 37 °C in 5% CO_2, cells were fixed and stained with 100% methanol and 1% toluidine blue. Cells that had invaded the undersurface of the membrane were counted under a microscope. Four microscopic fields were randomly selected for cell counting. Each assay was performed in triplicate.

Drugs and antibodies. To assess the chemosensitivity for 5-fluorouracil (5-FU), CRC cells were evaluated with the MTT assay at different 5-FU concentrations. 5-FU was purchased from Kyowa Hakkou (Tokyo, Japan). Antibodies used for immunocytology were anti-E-cadherin (Zymed Laboratories, Carlsbad, CA), mouse anti-vimentin (Chemicon International, Temecula, CA), and anti-Snai1 (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunohistochemistry. Tissues from 30 CRC surgical specimens were formalin fixed, paraffin embedded, and assessed for Snai1 immunohistochemistry. The antigen–antibody reaction was incubated overnight at 4 °C after deparaffinization, and the blocking reagent Envision (Dako Cytomation, Glostrup, Denmark) was applied to detect the signal from the antigen–antibody reaction. All sections were counterstained with hematoxylin. A primary anti-Snai1 rabbit polyclonal antibody (Santa Cruz Biotechnology) was used at a dilution of 1:100.

Statistical analysis. For continuous variables, the data were expressed as means ± SD. The relationship between *SNAI1* mRNA expression and clinicopathological factors was analyzed with the chi-square and Student's *t*-tests. All tests were analyzed with the JMP software (SAS Institute, Cary, NC). Differences with p values < 0.05 were considered statistically significant.

Results

Immunohistochemistry of Snai1 and Cdh1

Snai1 staining was observed in the nucleus of cancer cells, but it was weak or undetectable in the cytoplasm of cancer cells as well as in the stromal cells of tumors (representative, positive immunohistochemical staining of the Snai1 protein in tissue from CRC is shown in Fig. 1). Twenty of 30 cases (63.3%) exhibited a positive expression level of Snai1 in the region of tumor invasion. In these cases, Snai1 expression was localized in the budding portion of the tumor (Fig. 1A). On the other hand, in 15 of 20 cases (75.0%), Cdh1 expression was lower in the region of tumor invasion (Fig. 1B) than in the superficial portion.

Significance of Snai1 phosphorylation sites in CRC cells

It has been shown previously that Snai1 contains two Gsk-3β phosphorylation motifs, which overlap with the β-Trcp destruction motif, and Gsk-3β physically interacts with Snai1 and induces its proteolysis [13]. To confirm that Snai1 in CRC cells can function under the control of phosphorylation-dependent proteolysis,

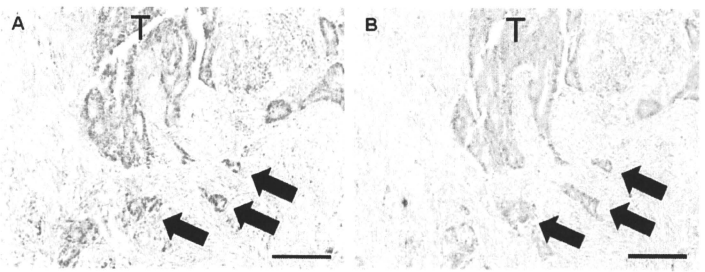

Fig. 1. Immunohistochemical staining for Snai1 and Cdh1. Positive expression level of Snai1 observed in the region of tumor invasion and in the budding portion (A; arrows), whereas the expression of Cdh1 was deeper in the body and budding portion of the tumor (B; arrows) than in the superficial portion. T, tumor; Bar, 200 μm. (Original magnification, 20×).

phosphorylation-defective mutants (*SNAI1-2SA*, *SNAI1-4SA*, *SNAI1-6SA*, and *SNAI1-8SA*) in which two serine residues corresponding to Gsk-3β and β-Trcp destruction motif are substituted by alanine were used. Gsk-3β and β-Trcp dependent degradation can be inhibited by the addition of MG132, a proteasome inhibitor [17]. The incubation of cells in a medium with MG132 for 5 h showed that *SNAI1-WT* protein accumulated in the cytoplasm, whereas in the absence of MG132, it localized predominantly in the nucleus, with a weak signal in the cytoplasm, but was not long before degradation. In contrast, *SNAI1-6SA* and *SNAI1-8SA* were localized in the nucleus with or without the addition of MG132 (Supplementary Fig. S1), suggesting that phosphorylation sites control the subcellular localization of Snai1 and are involved in the nuclear transcription of CRC. These data are in agreement with a previous report [13].

Effect of WT and mutant SNAI1 on EMT and chemoresistance

Transfection of SW480 with *SNAI1-WT*, *SNAI1-6SA*, and *SNAI1-8SA* was performed, and the expression of *SNAI1* mRNA was investigated (Supplementary Fig. S2). The mean expression value of *SNAI1*, normalized by *GAPDH* gene expression, was 1.03×10^{-2}, 6.37×10^{6}, 7.75×10^{8}, and 4.43×10^{8} in the mock control (NC), *SNAI1-WT*, *SNAI1-6SA*, and *SNAI1-8SA*, respectively. It was statistically significant among transfected cell lines and NC ($p = 0.014$; Student's *t*-test). No significant differences were noted among the transfectants.

Morphological characteristics of SW480 transfectants, with an overexpression of *SNAI1*, were compared (Supplementary Fig. S3 and Fig. 2). The transfected SW480 cell lines were visualized at 200-fold magnification with a Nikon light microscope (Nikon, Inc., Melville, NY) having digital photographic capability. Gfp was expressed in transfected cell lines (Fig. 2D–F). The digital images of cell lines were compared for morphological characteristics such as spindle and loss of cell polarity.

Quantitative real-time RT-PCR analysis of *CDH1* and *VIM* indicated that transfectants of *SNAI1-6SA* and *SNAI1-8SA* downregulated *CDH1* expression and upregulated *VIM* expression, compared with transfectants of *SNAI1-WT* and NC ($p < 0.001$;

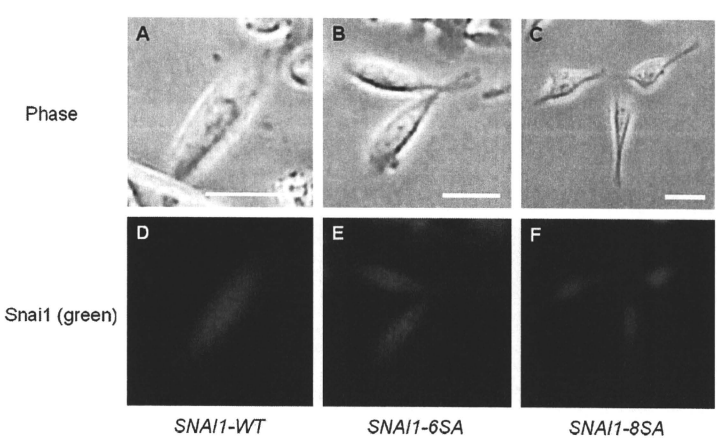

Fig. 2. Morphological analysis in transfected SW480 cell lines. Morphological characteristics of SW480 transfectants (A, *SNAI1-WT*; B, *SNAI1-6SA*; C, *SNAI1-8SA*), such as spindle and loss of cell polarity, were observed and Gfp was expressed in transfected cell lines (D, *SNAI1-WT*; E, *SNAI1-6SA*; F, *SNAI1-8SA*). Bar, 100 μm. (Original magnification, 200×).

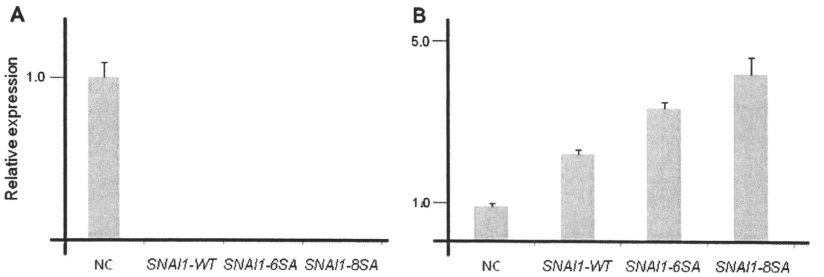

Fig. 3. Detection of mRNA expression of EMT-related genes by quantitative real-time RT-PCR analysis. *SNAI1-6SA* and *SNAI1-8SA* transfectants induced the downregulation of *CDH1* and the upregulation of *VIM*, compared with *SNAI1-WT* and NC. The expression values of *CDH1* and *VIM* are depicted with an error bar (mean ± SD, normalized by *GAPDH* gene expression).

Student's *t*-test; Fig. 3) from independent experiments performed in triplicate.

We studied the invasive ability of parental and transfected SW480 cells using the Transwell invasion assay to determine the effect of *WT* and mutant *SNAI1* on EMT (Fig. 4). Data showed that *SNAI1* enhanced the invasive ability of transfected SW480 cells. Among transfected cells, the invasive ability of SW480 cells after transfection with *SNAI1-6SA* and *SNAI1-8SA* was greater than that of NC ($p < 0.01$; Student's *t*-test). Furthermore, cells transfected with *SNAI1-6SA* and *SNAI1-8SA* were more invasive than cells transfected with *SNAI1-WT* ($p < 0.01$; Student's *t*-test).

To assess the relation of *SNAI1* expression with chemoresistance, the 50% inhibitory concentration (IC50) of cells was studied after incubation in a medium with 5-FU. Each assay was repeated at least three times. IC50 was calculated using the approximate curve. Data indicated that IC50 of transfectants, *SNAI1-WT*, *SNAI1-6SA*, and *SNAI1-8SA* were 5.6 ± 2.0, 23.6 ± 6.9, 33.9 ± 13.9, and 34.3 ± 2.5, respectively; IC50 of transfectants, *SNAI1-WT*, *SNAI1-6SA*, and *SNAI1-8SA* was higher than that of NC ($p < 0.05$; Student's *t*-test; Table 1). Furthermore, IC50 of *SNAI1-6SA* and *SNAI1-8SA* was higher than that of *SNAI1-WT* ($p < 0.05$; Student's *t*-test; Table 1).

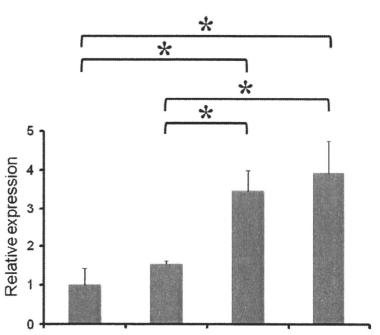

Fig. 4. Invasion assay of SW480 transfectants. The invasive ability of *SNAI1-6SA* and *SNAI1-8SA* transfectants was greater than the invasive ability of *SNAI1-WT* and NC (*$p < 0.01$; Student's *t*-test). The invasive values are indicated with error bars (mean ± SD normalized by invasiveness of NC) from independent experiments performed in triplicate.

Table 1
Analysis of chemoresistance to 5-FU in transfected cell lines by the MTT assay.

Cell line	IC50 (μg/ml)	SD
NC	5.6	2.0
SNAI1-WT	23.6	6.9
SNAI1-6SA	33.9	13.9
SNAI1-8SA	34.3	2.5

IC50, 50% inhibitory concentration; NC, negative control; SD, standard deviation.

Discussion

EMT during embryonic development plays a very important role in cancer invasiveness and metastasis resulting in poor prognosis [1,4,5,12]. In the EMT process, the downregulation of E-cadherin is very important with regard to outer regions of invasive tumors [6–8]. The previous report indicates that *SNAI1* expression induces transcription in the nucleus, and the effect would be apparent in cells with phosphorylation-defective mutants, *SNAI1-6SA* and *SNAI1-8SA* [13], which is consistent with the result of the present study. Our data indicates that *SNAI1-6SA* and *SNAI1-8SA* transfectants induce downregulation of *CDH1* and upregulation of *VIM* and elicit significant invasiveness and chemoresistance as compared with *SNAI1-WT* transfectants.

In the outer regions of invasive tumors, budding was reported as the predictive marker, whereas several other established markers were related to worse prognosis [18–23]. Budding refers to microtubular cancer nests and undifferentiated cancer cells surrounding tumor margins [18]. According to the present study, Snai1 protein expression spreads toward the outer regions of invasive tumors, and it may indicate not only tumor metastasis but also a refractory cell population resulting in poor prognosis.

Although advanced treatments, such as preoperative and postoperative chemotherapy and radiotherapy combined with surgery for CRC, have contributed to the reduction of recurrences and metastases, approximately 50% cases ultimately metastasize despite systemic chemotherapy followed by surgery [24]. Adjuvant chemotherapy for CRC is desirable in highly doubtful metastatic cases. Nowadays, it is useful to determine the necessity of intensive follow-up and adjuvant therapy for CRC in order to predict the development of recurrences and metastases in curative surgical resection cases [25–27]. Several adjuvant chemotherapies are helpful in certain disease stages, especially in CRC [27,28].

In previous reports, *SNAI1* was related to EMT, whereas the present study shows that *SNAI1* expression is related to chemoresistance in CRC cell lines during biological examinations [29,30],

and it becomes commonly a problem in the clinical course. The chemoresistance mechanisms in *SNAI1*-induced EMT have not been fully understood, though previous studies have suggested that the chemoresistance of CRC might be associated with cell cycle perturbation [31], expression of multidrug resistance genes [32], and other mechanisms [33,34]. In the present study, the expression of chemoresistance-related genes, *CCND1* and *ABCB1*, was studied by quantitative real-time RT-PCR using CRC SW480 transfectants, which would be useful in understanding chemoresistance mechanisms in *SNAI1*-induced EMT. The present data indicated that the mean value of *CCND1* expression was significantly higher in *SNAI1-8SA* cells, compared to those of *SNAI1-WT* and *SNAI1-6SA* ($p < 0.05$; Student's *t*-test; Supplementary Fig. S4). However, our study also shows that *ABCB1* expression increases in *SNAI1-8SA*, although not significantly (data not shown). The present study shows that *CCND1* is more significant. It was previously reported that the overexpression of *CCND1* confers resistance to anticancer drug-mediated apoptosis [35], which is consistent with the present observation that chemoresistance is associated with *CCND1* expression in phosphorylation-defective *SNAI1* mutants. Thus, our data suggests that *SNAI1*-induced EMT is involved in chemoresistance, at least via *CCND1* expression.

To our knowledge, this is the first study reporting that *SNAI1* enhances the effect of EMT and chemoresistance in CRC, using phosphorylation-defective *SNAI1* mutants. We hypothesize that overexpression of *CCND1* is involved in 5-FU resistance in *SNAI1*-induced EMT, a possible survival mechanism for chemoresistant CRC. The present study reinforces the theory that a specific stimulation or the enhancement of *SNAI1* phosphorylation may sensitize cancer cells to chemotherapy.

Conclusion

Snai1 not only plays a role in invasion but also in chemoresistance of CRC through phosphorylation-induced EMT. *SNAI1* can be a new molecular target for CRC treatment, and the stimulation of Snai1 phosphorylation might contribute to the control of invasion and metastasis of CRC.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.10.117.

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Clinical significance of ASB9 in human colorectal cancer

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Abstract. Ankyrin repeat and SOCS box-containing 9 (ASB9) is involved in the negative regulation of cytokine signaling. However, its biological function is largely undefined. The aim of this study was to assess the value and role of ASB9 as an indicator of prognosis in colorectal cancer (CRC). In order to demonstrate the importance of ASB9 expression for predicting the prognosis of CRC, we analyzed the ASB9 mRNA expression in 125 paired cases of CRC and non-cancerous regions and the protein expression by immunohistochemistry. To investigate the role of ASB9 *in vitro*, we performed proliferation and invasion assay with small interfering RNA against ASB9. ASB9 mRNA expression was higher in CRC tissue than corresponding normal tissue (P=0.0282). Patients expressing low levels of ASB9 had a poorer overall survival rate than those expressing high levels (P=0.0301), indicating that decreased ASB9 expression was an independent prognostic factor. The immunohistochemical study revealed that ASB9 was predominantly expressed in cancer cells. A multivariate analysis showed that ASB9 expression status was an independent prognostic factor of overall survival (relative risk, 4.09; 95% confidence interval, 1.47-11.88; P=0.007). In an invasion assay, ASB9 siRNA-transfected cells showed significantly high invasiveness. The results of the present study suggest that ASB9 is a useful prognostic marker for CRC.

Introduction

Cancer is a major public health problem in developed countries. In recent years, the incidence of colorectal cancer (CRC) has significantly increased with changes in lifestyle and CRC is now one of the most frequent causes of death from neoplastic disease in Japan (1). Identifying genes responsible for the development and progression of CRC and understanding their clinical significance is critical to the diagnosis and adequate treatment of the disease.

ASBs such as ASB5 and ASB8 have been reported to play a role in the initiation of arteriogenesis and cell growth (2,3) However, ASB9 is involved in the negative regulation of cytokine signaling (4), its functions in colorectal cancer is unknown. ASB9 was reported to have close relation to CKB. Creatine kinase B (CKB) is over-expressed in a wide range of solid tumors and has been used as a prognostic marker of cancer and metastasis (5). The CKB gene is positively regulated by the oncogene E1a and negatively regulated by the tumor suppressor gene p53 (6,7). Thus, many growth factors and hormones, such as estrogen, stimulate CKB activity and expression (8,9). Estrogen has been shown to induce the expression of CKB in the reproductive tract of female rats and human breast tumors and tissues (10). We hypothesized that ASB9 expression is a prognostic factor for CRC patients. We analyzed the ASB9 gene in 125 paired cases of CRC and non-cancerous regions, proposing an importance of ASB9 expression in evaluating prognosis and suggesting it as a novel candidate marker for CRC.

Materials and methods

Clinical tissue samples. A total of 125 patients (45 men, 80 women) with CRC underwent surgery at the Medical Institute of Bioregulation at Kyusyu University from 1992 to 2002. Primary CRC specimens and adjacent normal colorectal mucosa were obtained from patients after receiving informed consent in accordance with institutional guidelines. Every patient was definitively identified as having CRC based on clinicopathological findings. The resected surgical specimens were fixed in formalin, processed through graded ethanol, embedded in paraffin, sectioned with hematoxylin and eosin (H&E) stain and Elastica van Gieson stain, and the degree of the histological differentiation, lymphatic invasion and venous invasion examined. Immediately after resection, all specimens were frozen in liquid nitrogen and kept at -80°C until RNA extraction was performed.

None of the patients received chemotherapy or radiotherapy prior to surgery. After surgery, the patients were followed up with blood analysis, including serum tumor

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Key words: ASB9, gastrointestinal cancer, colorectal cancer

markers carcinoembryonic antigen (CEA) and cancer antigen (CA19-9), and imaging modalities, such as abdominal ultrasonography and/or computed tomography and chest X-ray, every 3-6 months. Clinicopathological factors were assessed according to the criteria of the Tumor-Node-Metastasis (TNM) classification of the International Union Against Cancer (UICC) (11).

RNA preparation and reverse transcription (RT)-PCR. Total RNA was prepared using a modified acid guanidium-phenol-chloroform procedure with DNAse (12). Reverse transcription was performed using 2.5 μg of total RNA as described previously (13). Two human ASB9 oligonucleotide primers were designed for the PCR reaction: 5'-CCTGGCATCAGGC TTCTTTC-3' (forward) and 5'-ACCCCTGGCTGATGAGG TTC-3' (reverse). To confirm adequate PCR products, 35 cycles of PCR were performed using a PCR kit (Takara, Kyoto, Japan) on a GeneAMP PCR system 9600 (PE Applied Biosystems, Foster City, CA) with the following parameters: 95°C for 40 sec, 45°C for 40 sec, and 72°C for 60 sec. An 8-μl aliquot of each reaction mixture was size-fractionated in a 1.5% agarose gel and visualized by ethidium bromide staining. To ensure that the RNA was not degraded, PCR specific to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was performed using 30 cycles of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C and the following primers: 5'-TTGG TATCGTGGAAGGACTCA-3' (forward) and 5'-TGTCAT CATATTGGCAGGTT-3' (reverse). A 270-bp amplicon was produced. cDNA from the human reference total RNA (Clontech, Palo Alto, CA, USA) was studied concurrently as a source of positive controls.

Quantitative real-time PCR. Real-time PCR monitoring was performed using the LightCycler FastStart DNA Master SYBR-Green I kit (Roche Diagnostics, Tokyo, Japan). The amplification protocol for ASB9 and GAPDH cDNA consisted of 35 cycles of denaturation at 95°C for 10 sec, annealing at 62°C for 10 sec, and elongation at 72°C for 10 sec. The products were then subjected to a temperature gradient from 55°C to 95°C at 0.1°C/sec with continuous fluorescence monitoring to produce a melting curve for the products. The ratio of ASB9 mRNA copies in tumor and normal tissues was calculated and normalized against GAPDH.

Immunohistochemistry. Surgical specimens from formalin-fixed, paraffin-embedded tissues were used for immunohistochemistry. After deparaffinization and blocking, the antigen-antibody reaction was incubated overnight at 4°C. Reagent (Envision, Dako Cytomation, Denmark) was applied to detect the signal from the antigen-antibody reaction. All sections were counterstained with hematoxylin. Primary anti-ASB9 mouse monoclonal antibody (WH0140462M1, Sigma Aldrich) was used at a dilution of 1:200. All sections were independently examined for protein expression and scored as positive when >10% of cancer tissues were stained in an examined area of a specimen and negative when <10% was stained.

Cell lines and culture. Three cell lines derived from human CRC (CaCo-2, HCT116 and SW480) were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum and antibiotics at 37°C in a 5% humidified CO_2 atmosphere. For siRNA inhibition, double-stranded RNA duplexes targeting human ASB9 and negative control siRNA (NC) were purchased in a Stealth RNAi kit (Invitrogen, Carlsbad, CA, USA): 5'-GCC UGUCUUGGAGGUCAUCUCUCUU-3' and 5'-UAUUAAA GCAUGGAGCUCAGGUGAA-3', 5'-CAGCCACCUGGGC ACUCCACUCUAU-3' and 5'-GGCUUCCAGAAGAUCUA CAAGCAAU-3', and 5'-ACGGGCGAAUUGAGUUGUCC GAGUU-3' and 5'-GACAUUGUGGAUGCCAUUUACC AGA-3'. The CRC cell lines were transfected with siRNA at a concentration of 20 μmol/l using lipofectamine RNAiMAX (Invitrogen), incubated in glucose-free Opti-MEM (Invitrogen), treated in accordance with the manufacturer's protocols, and analyzed by proliferation and invasion assay after 48 h. All siRNA duplexes were used together in a triple transfection. The culture growth rate was measured by counting cells using a CellTac kit (Nihon Koden, Tokyo, Japan). Each transfected cell line was compared to the wild-type and NC. Values were expressed as the standard error of the mean (SEM) of at least three independent experiments.

Proliferation assays. To assess proliferation, cells were grown an additional 24 h in DMEM containing 10% FBS. Cell viability was determined using the Cell Counting kit incorporating WST-8 (Dojindo Lab., Tokyo, Japan). WST-8 (10 μl) was added to 100 μl of medium containing each supplement above, and the absorbance was read at 450 nm using a microplate reader (Model 680XR; Bio-Rad Laboratories, Hercules, CA, USA). All experiments were performed at 30-80% cell confluence and the results confirmed in at least three independent experiments.

Invasion assays. Cell invasion was assessed using the Cyto-Select Cell Invasion Assay according to the manufacturer's protocol (Cell Biolabs, San Diego, CA) 48 h after transfection. Cells ($1.0x10^5$) in DMEM were placed on an 8.0-μm pore size membrane inserted in 96-well plates. DMEM with 10% FBS was placed in the bottom wells. After 24 h, non-invading cells were removed from the top side of the membrane chamber, and the cells from the underside of the membrane were completely dislodged by tilting the membrane chamber in Cell Detachment Solution (Cell Biolabs). Lysis buffer/CyQuannt GR dye solution (Cell Biolabs) was added to each well, the fluorescence of the mixture was read with a fluorescence plate reader at 480 nm/520 nm. The values are expressed as a ratio to control (each parental cell).

Statistical analysis. For continuous variables, the data are expressed as the mean ± SD. The relationship between mRNA expression and clinicopathological factors was analyzed by the Chi-square test and Student's t-test. Kaplan-Meier survival curves were plotted and compared with the generalized log-rank test. Univariate and multivariate analyses for the identification of prognostic factors for overall survival were performed using the Cox proportional hazard regression model. The tests were analyzed using JMP software (SAS Institute, Cary, NC, USA). Significance was set at $P<0.05$.