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Increased expression of Slug and Vimentin as novel predictive biomarkers for lymph node metastasis and poor prognosis in colorectal cancer

Yuji Toiyama[1,2,*], Hiromi Yasuda[1], Susumu Saigusa[1], Koji Tanaka[1], Yasuhiro Inoue[1], Ajay Goel[2] and Masato Kusunoki[1]

[1]Department of Gastrointestinal and Pediatric Surgery, Division of Reparative Medicine, Institute of Life Sciences, Mie University Graduate School of Medicine, Mie 514-8507, Japan and [2]Gastrointestinal Cancer Research Laboratory, Division of Gastroenterology, Department of Internal Medicine, Charles A. Sammons Cancer Center and Baylor Research Institute, Baylor University Medical Center, Dallas, TX 75246-2017, USA

*To whom correspondence should be addressed. Department of Gastrointestinal and Pediatric Surgery, Division of Reparative Medicine, Institute of Life Sciences, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan. Tel: +81 59 231 5294; Fax: +81 59 232 6968;
Email: ytoi0725@clin.medic.mie-u.ac.jp
Correspondence may also be addressed to Ajay Goel. Gastrointestinal Cancer Research Laboratory, Baylor University Medical Centre, 3500 Gaston Avenue, Suite H-250, Dallas, TX 75246, USA. Tel: +1 214 820 2692; Fax: +1 214 818 9292;
Email: ajay.goel@baylorhealth.edu

Slug and Vimentin genes play a critical role in regulating epithelial–mesenchymal transition (EMT) via downregulation of epithelial markers and upregulation of mesenchymal markers. The present study evaluated the clinical significance of Slug and Vimentin expression as potential disease biomarkers in colorectal cancer (CRC). At first, the biological role of Slug in CRC was assessed by RNA interference in CRC cell lines to assess tumor progression, invasion and migration. Next, we analyzed Slug and Vimentin expression in surgical tissue specimens from 181 CRC patients (Cohort 1) by quantitative real-time reverse transcription–PCR and 208 patients (Cohort 2) by immunohistochemistry. Knockdown of Slug using small interfering RNA in CRC cell lines resulted in inhibition of EMT, reduced cell proliferation, invasion and migration in CRC cells. Interestingly, Slug and Vimentin expression in cancer tissues was significantly higher in patients with higher T stage, lymph node involvement, liver metastasis and advanced tumor node metastasis stages. A significant correlation was observed between Slug and Vimentin expression in CRC (messenger RNA: ρ = 0.546, protein: ρ = 0.405), and increased expression of Slug and Vimentin was significantly associated with poor prognosis. Furthermore, increased expression of Slug emerged as an independent prognostic factor and a predictive marker of lymph node metastasis in CRC patients. Our data provide novel evidence for the biological and clinical significance of Slug and Vimentin expression as potential predictive biomarkers for identifying patients with lymph node metastasis or poor prognosis in CRC.

Introduction

Colorectal cancer (CRC) is one of the most common malignant diseases in the developed countries, and it remains a major public health problem (1). Survival rates of patients with CRC have improved in past few years, probably due to earlier diagnosis and improved treatment of this disease. In spite of this, following potentially curative surgery, ~30% of patients eventually develop local tumor recurrence or distant metastasis, resulting in an overall poor prognosis (2,3). Patients with local recurrence or distant metastases usually receive chemotherapy in combination with targeted monoclonal antibody therapy yielding response rates of at best 50% (4). This limitation is further compounded by the fact that even within this population, almost half of the patients frequently experience treatment-related side effects with no therapeutic benefit. Therefore, identification of CRC patients who are at higher risk for developing metastatic disease would aid in better stratification and selection of candidate patients for standard or intensive adjuvant chemotherapy.

The metastatic process consists of four distinct steps: local invasion, intravasation, extravasation and growth at metastatic sites. Recent research has focused on the involvement of epithelial–mesenchymal transition (EMT) in cancer invasion and metastasis. In CRC, tumor cells undergoing EMT are histologically represented by the presence of tumor buds defined as single cells or small clusters of dedifferentiated tumor cells at the invasive front (5). Tumor budding is a predictor of lymph node metastasis, vascular and lymphatic invasion, distant metastasis, local recurrence and poor disease-specific survival duration (6–8). Moreover, the Union for International Cancer Control (UICC) recognizes tumor budding as a highly relevant, additional prognostic factor (9). Therefore, it is very likely that further research into the molecular genomic targets and biomarkers underpinning EMT regulation holds tremendous potential in identifying the subset of patients who are at highest risk of developing metastasis in CRC.

One of the distinguishing features for the establishment of an EMT phenotype is upregulated expression of mesenchymal markers, such as Vimentin and fibronectin, and reduced expression of structural adhesion proteins such as E-cadherin (10). In a clinical context, increased Vimentin and reduced E-cadherin expression in various cancers is associated with tumor progression and metastasis (11,12). Several transcription factors, including Snail (13), SIP1 (14), Twist (15) and ZEB1 (16), have been shown to repress E-cadherin expression by targeting E-boxes proximal to the E-cadherin promoter. Slug is a member of the Snail family of zinc finger transcription factors that have been shown to participate in mesoderm formation, neural crest cell formation and migration, cellular differentiation, adhesion, invasion, and cell cycle and apoptosis regulation (17–21). The importance of Slug to allow cancer cells to downregulate epithelial markers and upregulate mesenchymal markers in order to become motile and invasive has also been reported (22,23). Moreover, recent studies showed that overexpression of Slug is associated with enhanced metastatic capacity and reduced postoperative survival from several human cancers such as breast, endometrial, ovarian, renal cell, esophageal, gastric and CRCs (24–30). However, to the best of our knowledge, there are no data describing direct associations between Slug and Vimentin expression in primary CRC, which will help establish not only their functional role, but possibly reveal their clinical usefulness as potential cancer metastasis biomarkers.

Accordingly, the present study aimed to evaluate the clinical significance of Slug and Vimentin expression and to determine association between the expression of these proteins in different stages of primary CRC. In addition, we also investigated the functional role of Slug in CRC by RNA interference analysis in cultured CRC cells. Using a multitude of approaches, we, for the first time, demonstrate that high expression of Slug and Vimentin promotes proliferation, invasion and migratory ability in colonic cells. In addition, our data provide novel evidence that Slug and Vimentin expression can also serve as predictive biomarkers of lymph node metastasis, and poor prognosis in CRC patients.

Abbreviations: CI, confidence interval; CRC, colorectal cancer; EMT, epithelial–mesenchymal transition; HR, hazard ratio; mRNA, messenger RNA; OS, overall survival; siRNA, small interfering RNA; ROC, receiver operating characteristic; TNM, tumor node metastasis.

Materials and methods

Cell lines

The human CRC cell lines SW480, SW620, Lovo, Caco2, HT29 and RKO were provided by the Cell Resource Center of Biomedical Research, Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan) and maintained in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics at 37°C in a 5% humidified CO_2 atmosphere. The authenticity of various cell lines was routinely monitored by analyzing a series of genetic and epigenetic markers specific for each cell line.

Slug RNA interference

Slug-specific small interfering RNA (siRNA) (Silencer® Select Validated siRNA, standard purity) and negative control siRNA (Silencer™ Negative Control siRNA) were purchased from Ambion (Austin, TX). Transfections were performed by mixing cell suspensions with siRNA oligonucleotides (20 nM), Opti-MEM I (Invitrogen, Carlsbad, CA) and Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) before cell plating. Cells were maintained in a humidified atmosphere, and assays were performed after 24 h incubation.

MTT assay

Twenty-four hours after transfection, Slug siRNA and control siRNA-transfected cells were seeded at 5×10^3 cells per well in 96-well flat-bottomed microtiter plates, in a final volume of 100 µl culture medium per well, and incubated in a humidified atmosphere. After 0–72 h culture, MTT assays were used to assess cell viability. Briefly, 200 µl sterile MTT dye (5 mg/ml; Sigma, St Louis, MO) was added. After incubating for 4 h at 37°C in 5% CO_2, MTT medium mixture was removed, and 200 µl of dimethyl sulfoxide was added to each well. Absorbance was measured by SoftMax Pro (Molecular Devices Corp., Sunnyvale, CA) at a wavelength of 450 nm. Each experiment was performed independently three times in triplicates.

Bromodeoxyuridine proliferation assay

The proliferation index was measured by bromodeoxyuridine incorporation in colon cancer cells 72 h after transfection of either control siRNA or Slug siRNA following manufacturer's instructions (Cell Proliferation ELISA, bromodeoxyuridine; Roche). Experiments were performed in three independent experiments.

Invasion assay

Transfected cells (2.5×10^5 cells per well) were seeded in serum-free media (in triplicate) in 24-well (8 µm pore size) Matrigel™ Invasion Chambers (BD Biosciences, Franklin Lakes, NJ). Inserts were placed into Falcon companion plates containing 10% fetal bovine serum and incubated for 48 h. The incubation media and cells were then removed from the top chamber using cotton swabs and phosphate-buffered saline, and the number of cells invading the membrane underside was determined. Membranes were fixed and stained with Diff-Quik stain™ (Sysmex, Kobe, Japan) and mounted on glass slides. The numbers of migrating or invading cells in 10 microscopic fields were subsequently counted with a light microscope at ×10 magnification.

Wound healing assay

Transfected CRC cells were incubated until confluent in 6-well plates, and wounds were generated using a sterile 200 µl pipette tip. Cells were then grown for an additional 48 h. Wound closure was assessed using an Olympus IX71 microscope (Olympus, Center valley, PA) at ×40 magnification. Cell migration distance was measured using Adobe Photoshop 9.0.2 software and compared with baseline measurements.

Tissue samples and patient characteristics

One hundred eighty-one colorectal tumor samples were frozen in liquid nitrogen immediately after surgical resection and kept at −80°C until RNA extraction for investigating the messenger RNA (mRNA) expression of Slug and Vimentin in CRC tissues by real-time PCR (Cohort 1). The surgical samples were obtained from the Department of Gastrointestinal and Pediatric Surgery, Division of Reparative Medicine, Institute of Life Sciences, Mie University Graduate School of Medicine, Japan, from 2000 to 2005. The patients included 117 men and 64 women with a mean age of 67 years (standard deviation = 11.6). The primary lesion was located in the rectum in 79 patients, sigmoid colon in 48, ascending colon in 27, transverse colon in 20 and descending colon in 7. Thirty-three patients were diagnosed with synchronous liver

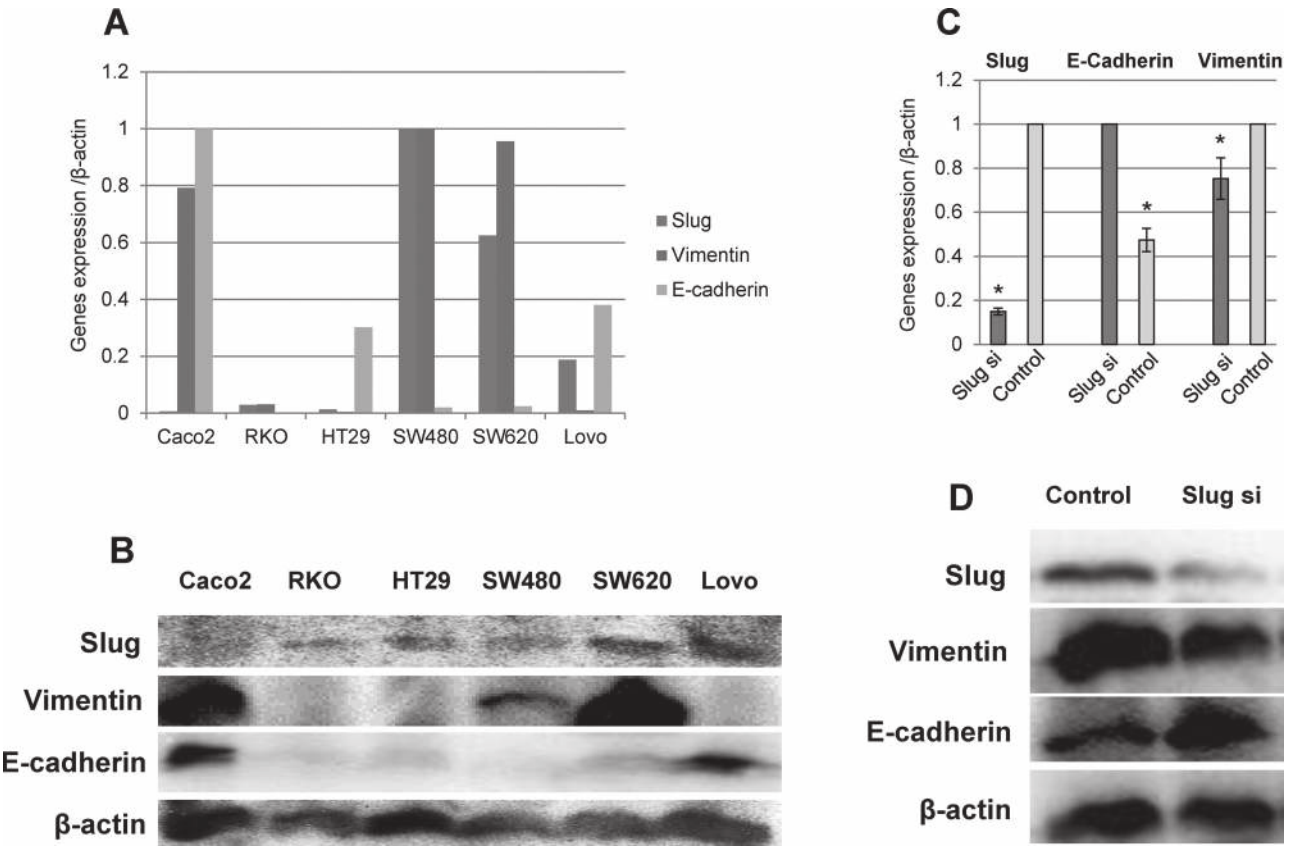

Fig. 1. Expression of Slug, Vimentin and E-cadherin in six colorectal carcinoma cell lines. (**A**) The histograms illustrate comparisons between relative gene expression of Slug, Vimentin and E-cadherin as determined by quantitative real-time reverse transcription—PCR (qRT–PCR). (**B**) Western blotting to determine protein expression of Slug, Vimentin and E-cadherin in six CRC cell lines. (**C**) Expression of Slug and different EMT markers (Vimentin and E-cadherin) after knockdown of Slug expression. Relative expression of Slug, E-cadherin and Vimentin genes was determined by qRT–PCR. *$P < 0.05$. (**D**) Western blotting to demonstrate protein expression of Slug, Vimentin and E-cadherin after transfection of Slug siRNA and control siRNA.

metastasis. Clinicopathological findings were based on the criteria of the UICC's tumor node metastasis (TNM) classification. There were 26 patients with stage I (T1–2 N0M0), 40 with stage II (T3–4 N0M0) and 69 with stage III (TXN1–2M0) disease. Forty-six patients with distant metastases were classified as having stage IV (TX NXM1) disease. Seventeen patients had poorly differentiated or mucinous adenocarcinomas, whereas 165 patients had well or moderately differentiated colorectal tumors. Postoperative follow-up data were obtained from all patients, and the median follow-up duration was 40 months (range: 1–140).

On the other hand, formalin-fixed and paraffin-embedded samples from primary CRC (n = 208) for protein expression of Slug and Vimentin by immunohistochemistry (Cohort 2) were obtained from the patients enrolled between 2005–2008 at the Department of Gastrointestinal and Pediatric Surgery, Division of Reparative Medicine, Institute of Life Sciences, Mie University Graduate School of Medicine, Japan. The patient age ranged from 12 to 91 years. Patients who underwent neoadjuvant therapy, treated by endoscopic mucosal resection and had non-colonic carcinomas were excluded. The patients included 121 men and 87 women with a mean age of 67 years (standard deviation = 11.7). Thirty-one patients were diagnosed with synchronous liver metastasis. Clinicopathological findings were based on TNM classification. There were 51 patients with stage I, 67 with stage II and 47 with stage III cancers. Forty-three patients with distant metastases were classified as having a stage IV disease. Eighteen patients had poorly differentiated or mucinous adenocarcinomas, whereas 190 patients had well or moderately differentiated colorectal tumors. Postoperative follow-up data were obtained from all patients, and the median follow-up duration was 50 months (range: 1–118).

All patients were followed up after the initial hospital discharge, with physical examination and tumor marker assays (CEA, CA19-9) performed every 1–3 months and computed tomography performed every 6 months. Endoscopic examinations were performed when necessary. None of the patients received preoperative treatment such as radiation or chemotherapy. A written informed consent was obtained from all patients according to guidelines approved by the institutional research board.

Total RNA extraction and complementary DNA synthesis

The surgical specimens were homogenized using a Mixer Mill MM 300 homogenizer (Qiagen, Chatsworth, CA). Total RNA from tissues and cell lines were isolated using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Complementary DNA was synthesized by random hexamers using Superscript III reverse transcriptase (Invitrogen).

Quantitative real-time reverse transcription–PCR

We performed quantitative real-time reverse transcription–PCR analysis using the StepOne™ Real Time PCR System (Applied Biosystems, Foster City, CA). Primers for Slug, E-cadherin, Vimentin and β-actin were designed by Primer 3 software (Biology Workbench version 3.2, San Diego Supercomputer Center, University of California, San Diego, CA). The sequences used were: Slug: forward, 5′-ATATTCGGACCCACACATTACC-3′; reverse, 5′-ACATTCTGGAGAA GGTTTTGGA-3′; Vimentin: forward, 5′-GTTTCCAAGCCTGACCTCAC-3′; reverse, 5′-GCTTCAACGGCAAAGTTCTC-3′; E-cadherin: forward, 5′-GCCGAGAGCTACACGTTCAC-3′; reverse, 5′-ACTTTGAATCGG GTGTCGAG-3′; β-actin: forward, 5′-ACAGAGCCTCGCCTTTGC-3′; reverse, 5′-GCGGCGATATCATCATCC-3′. PCR was performed with Power SYBR Green PCR Master Mix (×2) (Applied Biosystems, Carlsbad, CA). The following cycling conditions were used: 95°C, 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min.

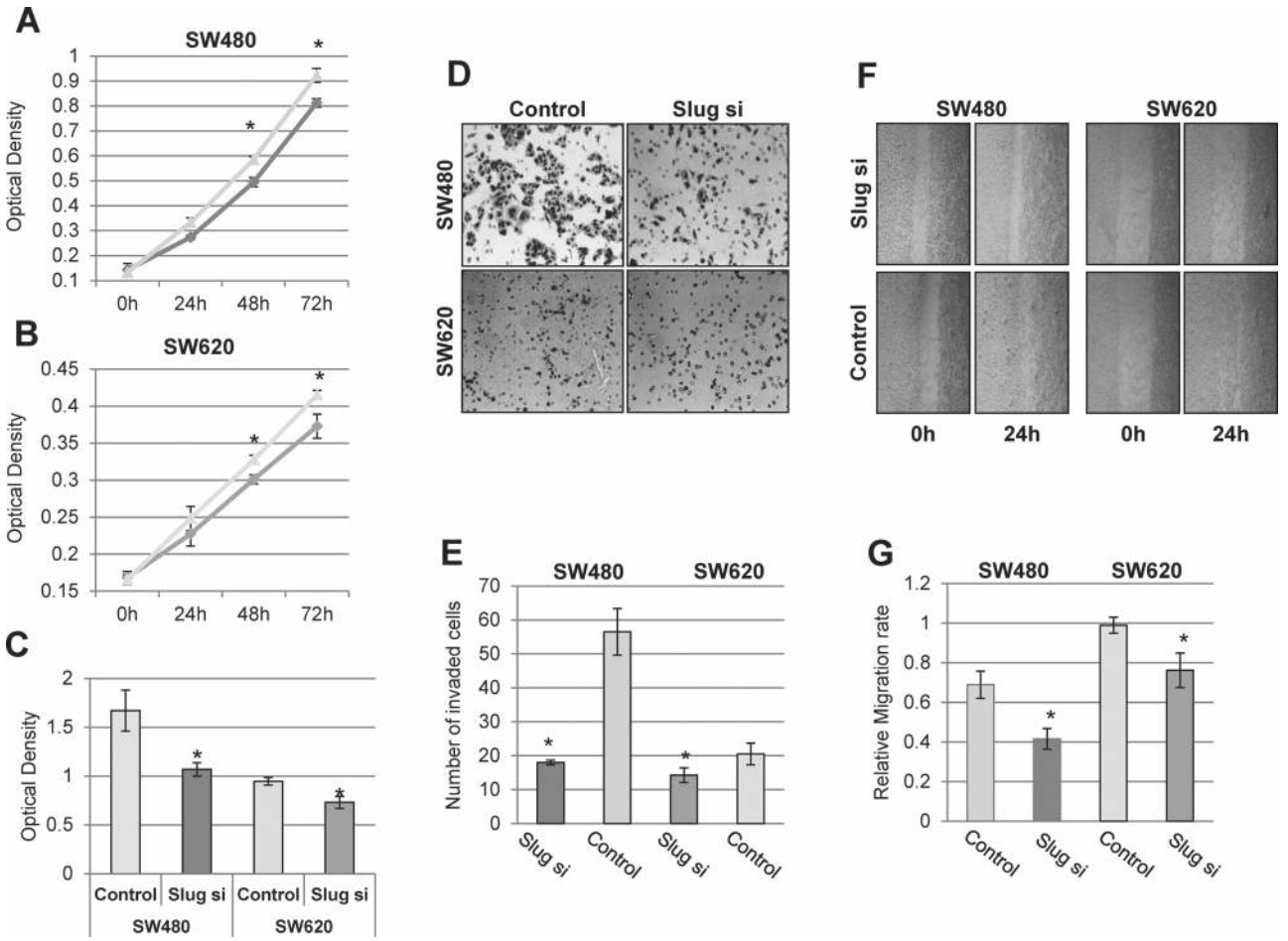

Fig. 2. Reduction of Slug expression enhances cancer cell proliferation, invasion and migration. MTT assays in SW480 (**A**) and SW620 (**B**) cell lines at 0–72 h after Slug siRNA transfection. Cell growth of SW480 and SW620 treated with Slug siRNA (diamond shape) is significantly inhibited compared with those with control (triangle shape) at 48 and 72 h. (**C**) Effect of Slug gene on cell proliferation (bromodeoxyuridine assay). (**D**) The transwell invasion system demonstrates enhanced invasive capacity after Slug knockdown. Images of invading cells were taken by phase contrast microscopy at ×100 magnification. (**E**) Quantitative transwell invasion assays, in which y-axis represents the number of invading cells. (**F**) Wound healing assays were performed to investigate the migratory potential after Slug knockdown. (**G**) Quantitative migration assay results, in which y-axis represents migration rates relative to control cells. Error bars represent standard deviation. $^*P < 0.05$.

Table I. Association between Slug and Vimentin mRNA and protein expression in colorectal cancer and clinicopathological characteristics

Factors	Cohort 1 (mRNA analysis)						Cohort 2 (protein analysis)					
	Slug mRNA			Vimentin mRNA			Slug protein			Vimentin protein		
	High (92)	Low (89)	*P*	High (90)	Low (91)	*P*	High (110)	Low (98)	*P*	High (140)	Low (68)	*P*
Age												
≤68[a]	46	52	0.32	46	52	0.5	55	50	0.98	67	38	0.34
>68[a]	46	37		44	39		55	48		73	30	
Gender												
Male	61	56	0.75	60	57	0.68	64	57	0.99	84	37	0.42
Female	31	33		30	34		46	41		56	31	
Histological grade												
Well and moderate	81	84	0.33	82	82	0.98	102	88	0.6	129	61	0.67
Poor and mucinous	11	6		9	8		8	10		11	7	
Tumor size												
≤40 (small)[a]	44	49	0.41	47	46	0.93	53	63	0.028*	72	47	0.034
>40 (large)[a]	48	40		43	45		57	35		68	21	
Serosal invasion												
Absent	18	40	0.0005*	19	39	0.003*	10	51	<0.0001*	25	36	< 0.0001
Present	74	49		71	52		100	47		115	32	
Lymph node metastasis												
Absent	24	43	0.003*	25	42	0.016*	45	83	<0.0001*	73	55	0.0005
Present	68	46		65	49		65	15		67	13	
Venous invasion												
Absent	27	34	0.27	28	33	0.56	25	44	0.0012*	35	34	0.0024
Present	65	55		62	58		85	54		105	34	
Lymphatic invasion												
Absent	7	18	0.024*	13	12	0.97	7	34	<0.0001*	20	21	0.0053
Present	85	71		77	79		103	64		120	47	
Liver metastasis												
Absent	69	79	0.027*	66	82	0.0063*	82	95	<0.0001*	116	61	0.3
Present	23	10		24	9		28	3		24	7	
Peritoneal dissemination												
Absent	81	86	0.06	82	85	0.76	98	97	0.007*	130	85	0.68
Present	11	3		8	6		12	1		10	3	
Distant metastasis												
Absent	61	73	0.025*	61	73	0.08	92	91	0.067	120	63	0.25
Present	31	16		29	18		18	7		20	5	

[a]The median age and tumor size are 68 years and 40 mm, respectively.
*$P < 0.05$.

Relative Slug and Vimentin expression levels

The relative gene expression levels for Slug and Vimentin were determined by the standard curve method. Standard curves and linear equations were generated using 5-fold serial dilutions of human reference complementary DNA. Within the range analyzed, all standard curves were linear with an acceptable correlation coefficient (R^2). The extent of target gene expression was calculated from the standard curve, and the complementary DNA in each sample was quantitatively normalized with respect to the β-actin gene, which served as an internal control. Finally, the target gene mRNA levels were expressed as respective gene ratios relative to β-actin mRNA levels. Real-time PCR assays were performed in duplicate for each sample, and the mean values were used to calculate mRNA expression levels.

Western blot analysis

Briefly, 30 µg of cell lysates was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, the proteins were transferred electrophoretically to an Immobilon membrane (Millipore). After blocking, the membrane was incubated for 1 h with the respective antihuman primary antibody at the recommended dilution (anti-Slug, E-cadherin, Vimentin, β-actin; Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was washed three times with Tween/phosphate-buffered saline for 15 min each time and then incubated with the appropriate secondary antibody for 1 h. After washing with Tween/phosphate-buffered saline, the membrane was treated with ECL-western blotting detecting reagent (Amersham Biosciences, Piscataway, NJ).

Immunohistochemistry

Formalin-fixed and paraffin-embedded sections (2–3 µm thickness) from 208 CRC patients were used for immunohistochemical analysis of Slug and Vimentin expression. Following deparaffinization and dehydration, specimens were boiled in 10 mM sodium citrate buffer to unmask antigens. Specimens were then blocked and incubated with primary antibody overnight at 4°C. Antibody binding was detected by Dako's horseradish peroxidase Envision kit (Dako Cytomation, Glostrup, Denmark). All sections were counterstained with hematoxylin. Primary antibody against Slug (Cell Signaling Technology, Boston, MA) and Vimentin (BD Biosciences, San Jose, CA) were diluted 1:50 and 1:100, respectively. Positive and negative controls were also run simultaneously.

Evaluation of immunohistochemistry

The Slug and Vimentin expression in formalin-fixed and paraffin-embedded stained sections from CRCs were analyzed separately by two expert pathologists without knowledge of the clinicopathological or survival data of any of the patients. Expression of Slug and Vimentin was evaluated by scanning the entire tissue specimen under low-power magnification (×40) and then confirmed under high-power magnification (×200 and ×400). An immunoreactivity scoring system was applied using the following criteria: (i) fraction of positive stained cells ≤5% scored 0, 6–25% scored 1, 26–50% scored 2, 51–75% scored 3 and >75% scored 4; (ii) intensity of stain: colorless scored 0, pallide-flavens scored 1, yellow scored 2 and brown scored 3 (Supplementary Figure S1A–H, available at *Carcinogenesis* Online). Scores obtained from A and B were multiplied together to make the staining score according to the proportion

and intensity of positively stained cancer cells. Specimens were rescored if the difference between the scores by the two pathologists was more than 3.

Statistical methods

The significance of mRNA levels was determined by the Mann–Whitney test, Kruskal–Wallis test or the χ^2 test where appropriate. Association between Slug and Vimentin was analyzed by Spearman correlation. Receiver operating characteristic (ROC) curves were established for determining cutoff values for analyzing prediction of lymph node metastasis and prognosis by Youden's index. Logistic regression analysis was used to predict the factors influencing lymph node metastasis. Overall survival (OS) curves were analyzed using the Kaplan–Meier method, and differences were examined using log-rank tests. Cox's proportional hazard regression test was used to estimate univariate and multivariate hazard ratios for prognosis. All *P* values were two sided, and those <0.05 were considered statistically significant. All statistical analyses were carried out using Medcalc 12.3 for Windows (MedCalc Software bvba, Mariakerke, Belgium).

Results

Functional analyses of Slug in CRC cells

Slug is expressed in a subset of CRC cell lines. We investigated Slug, E-cadherin and Vimentin expression by quantitative real-time reverse transcription–PCR in established CRC cell lines (Figure 1A). Of all CRC cell lines analyzed, SW620 cell line with lymph node metastasis and the paired SW480 cell line derived from the primary CRC from the same patient showed highest Slug and Vimentin expression. In all other cell lines, Slug expression was distinctly lower. In addition, E-cadherin expression in SW480 and SW620 was lower compared to the other cell lines. To confirm the protein expression of Slug, Vimentin and E-cadherin using CRC cell lines, western blotting was performed, and the result showed consistent pattern with the quantitative real-time reverse transcription–PCR (Figure 1B) data. Based on these results, we selected SW480 and SW620 cell lines for further knockdown experiments.

Slug inhibition leads to altered expression of EMT-related genes in CRC cells. Transfection of CRC cell lines with Slug siRNA resulted in dramatic reduction in Slug mRNA expression (up to 90%) compared with negative control siRNA-treated cells 48 h posttransfection. Next, we investigated the effect of Slug knockdown on the expression of key EMT-related markers such as E-cadherin and Vimentin (Figure 1C). We noted that E-cadherin expression was increased following Slug siRNA treatment in SW480 cells, whereas Vimentin expression showed a decrease in SW480 cell lines. Western blotting results were consistent with the real-time PCR data (Figure 1D), suggesting that Slug mediates EMT process in CRC.

Slug promotes proliferation, invasion and migration ability in CRC cells. We assessed various cellular functions such as proliferation, migration and invasion after treatments of non-silencing siRNA and

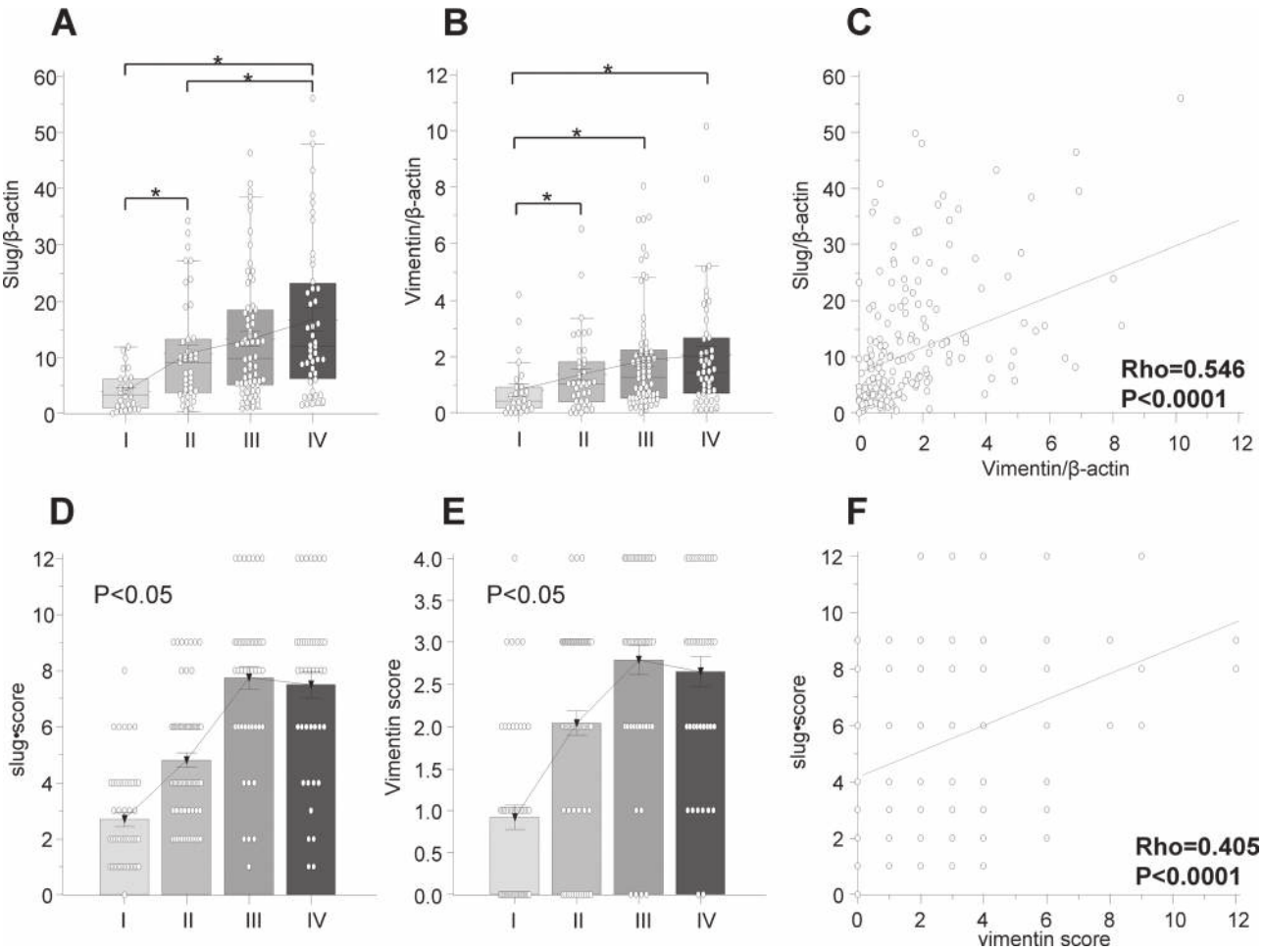

Fig. 3. Slug and Vimentin expression in 181 CRC patients (Cohort 1) subdivided by TNM staging. (**A**) Box plots of Slug mRNA expression and (**B**) Vimentin mRNA expression. Box represents interquartile ranges; line across the box indicates median value. Data were normalized to β-actin. Statistical analysis was performed using Kruskal–Wallis tests. **P* < 0.05. (**C**) Scatter plot of gene expression levels between Slug and Vimentin in CRC. Significant positive correlation was found by Spearman correlation; ρ = 0.546 (95% CI: 0.434–0.640, *P* < 0.0001). Slug and Vimentin immunostaining scores in 208 CRC patients (Cohort 2) subdivided by TNM staging. (**D**) Box plots of Slug and (**E**) Vimentin immunostaining scores. Box represents interquartile ranges; line across the box indicates median value. Statistical analysis was performed using Kruskal–Wallis tests. (**F**) Scatter plot of immunostaining scores between Slug and Vimentin in CRC. Significant positive correlation was found by Spearman correlation; ρ = 0.405 (95% CI: 0.284–0.513, *P* < 0.0001).

Slug siRNA. MTT assays did not reveal any significant differences between non-silencing siRNA and mock-transfected cells. In contrast, downregulation of Slug resulted in significant inhibition of tumor cell growth at 48 and 72 h after Slug siRNA transfection (Figure 2A and B). In addition, we performed bromodeoxyuridine incorporation assays after transfection of either control siRNA or Slug siRNA in SW480 and SW620 cell lines. Inhibition of Slug significantly enhanced cell proliferation in both cell lines (Figure 2C). We next performed invasion assays to determine whether attenuated Slug levels might affect cellular invasion. Slug siRNA transfection of SW480 and SW620 colon cancer cells showed weakened invasive capacity compared with cells transfected with non-silencing siRNA (Figure 2D and E). In addition, wound healing assays were performed to compare the migratory potential of CRC cells transfected with Slug siRNA. The number of migratory cells treated with Slug siRNA was markedly decreased compared with non-silencing siRNA-treated cells (Figure 2F and G). Taken together, these results demonstrate that Slug expression induces concurrent cell proliferation, invasion and migration in CRC cells.

Slug and Vimentin expression are associated with tumor malignancy in CRC. For clinicopathological evaluation, the experimental samples were divided into two groups according to the expression status of each gene from Cohort 1. Colorectal tumors with higher than median expression of both Slug and Vimentin were assigned to the high-expression group (Slug: $n = 92$, Vimentin: $n = 90$), whereas the remaining tumors were assigned to the low-expression group (Slug: $n = 89$, Vimentin: $n = 91$). The relationship between Slug and Vimentin mRNA in CRC and clinicopathologic factors is summarized in Table I. The tumors with high Slug expression invaded the serosal layer ($P = 0.0005$) and had more lymphatic duct invasion ($P = 0.024$), lymph node metastasis ($P = 0.003$), liver metastasis ($P = 0.027$) and distant metastasis ($P = 0.025$) than those with low Slug expression. Figure 3A illustrates the distribution of Slug mRNA expression relative to TNM stage. The expression of Slug increased in accordance with tumor stage progression. Likewise, Vimentin expression increased in accordance with tumor progression (Figure 3B), and the tumors with high expression of Vimentin had a greater extent of tumor

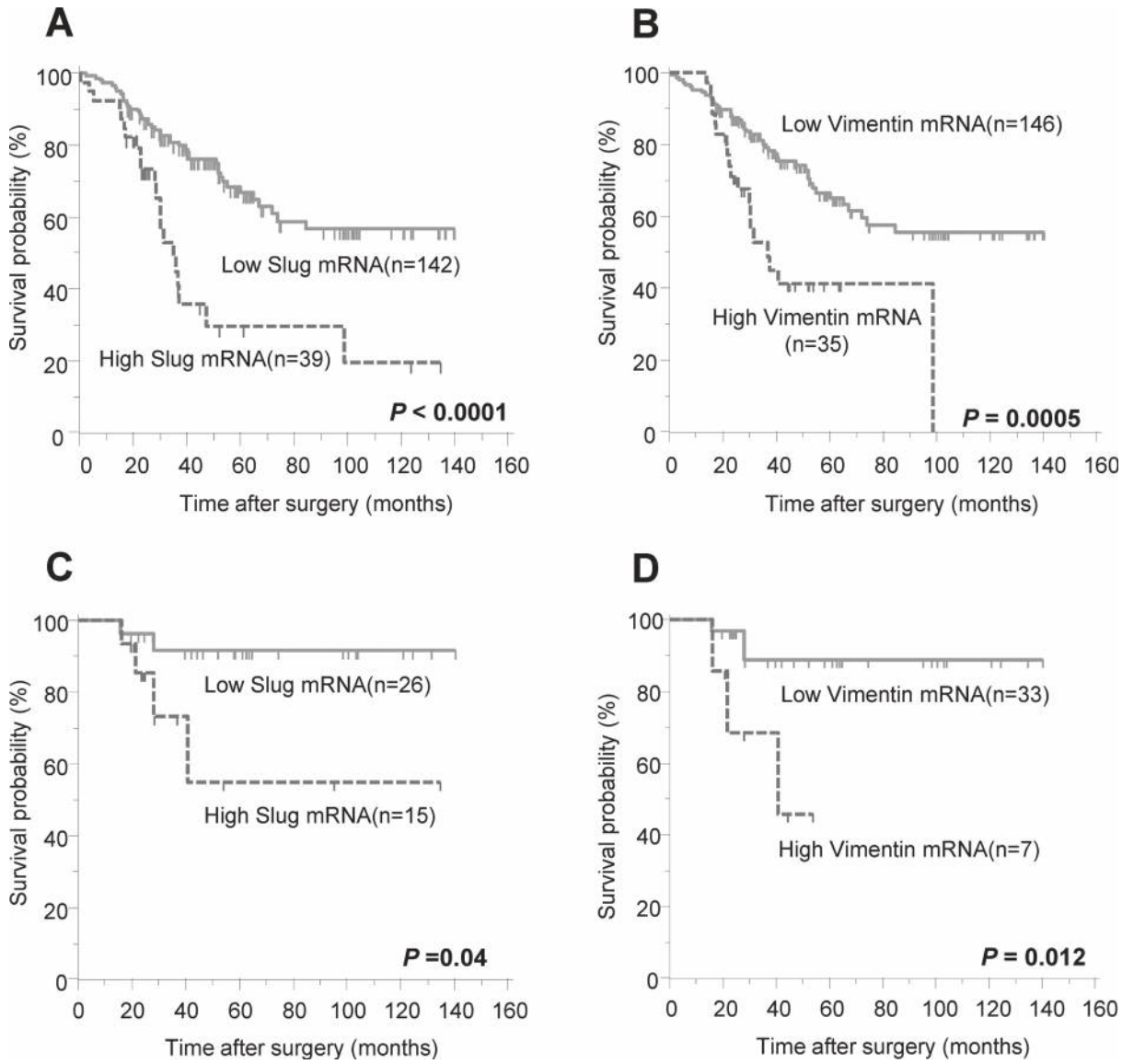

Fig. 4. Survival curves of patients with stages I–IV CRC according to the status of Slug or Vimentin mRNA expression. (**A**) Patients with higher Slug expression showed a significantly poor prognosis than those with lower Slug expression ($P < 0.0001$, log-rank test; cutoff value = 19.4). (**B**) Patients with higher Vimentin expression showed a significantly poor prognosis than those with lower Vimentin expression ($P = 0.0005$, log-rank test; cutoff value = 2.61). Survival curves of patients with stage II CRC according to the status of Slug or Vimentin mRNA expression. (**C**) Patients with higher Slug expression also showed a significantly poor prognosis than those with lower Slug expression ($P = 0.04$, log-rank test; cutoff value = 10.3). (**D**) Patients with higher Vimentin expression showed a significantly poorer prognosis than those with lower Vimentin expression ($P = 0.012$, log-rank test; cutoff value = 2.82).

invasion to the serosa ($P = 0.003$), lymph node metastasis ($P = 0.016$) and liver metastasis ($P = 0.0063$) than those with low expression of Vimentin (Table I). Interestingly, Slug mRNA expression was significantly positively correlated with Vimentin expression in CRC specimens [$\rho = 0.546$, 95% confidence interval (CI): 0.434–0.640, $P < 0.0001$; Figure 3C]. However, Slug and Vimentin expression was not associated with any specific histopathological subtypes (Table I).

On the other hand, immunohistochemical analysis was also performed to investigate the cellular distribution of Slug and Vimentin protein expression and evaluate associations between protein expression results and clinicopathological data. Slug protein expression was mainly observed in the cytoplasm and nucleus of tumor cells (Supplementary Figure S1A and B, available at *Carcinogenesis* Online). In contrast, Vimentin expression was primarily detectable in cancer stroma and diffusely expressed in the cytoplasm of primary CRC cells (Supplementary Figure S1E and F, available at *Carcinogenesis* Online). According to the ROC analyses with Youden's index correction for Slug expression analysis, we defined a cutoff value of >6 as high-staining group ($n = 110$) and <6 as low-staining group ($n = 98$). In contrast, the cutoff value of Vimentin was >2 as high-staining group ($n = 140$) and <2 as low-staining group ($n = 68$). High-staining group of Slug was significantly associated with large tumor size ($P = 0.028$), serosal invasion ($P < 0.0001$), metastasis of lymph node ($P < 0.0001$), liver ($P < 0.0001$) and peritoneum ($P = 0.007$) (Table I). In addition, high Vimentin staining was also associated with large tumor size ($P = 0.034$), serosal invasion ($P < 0.0001$) and lymph node metastasis ($P = 0.0005$) (Table I). Furthermore, Slug and Vimentin staining scores were significantly increased according to TNM stage (Figure 3D and E), and Figure 3F shows that staining score of Slug was significantly correlated with that of Vimentin ($\rho = 0.405$, 95% CI: 0.284–0.513, $P < 0.0001$). These results demonstrated that protein expression of Slug and Vimentin were consistent with their gene expression in CRC.

High Slug expression is an independent prognostic factor. To examine the predictive potential of Slug and Vimentin mRNA expression based on data from Cohort 1 for determining CRC prognosis, we determined cutoff values for the expression of both genes according to the ROC analyses with Youden's index. Figure 4A and 4B illustrates survival curves from CRC patients subdivided according to Slug mRNA expression (cutoff value = 19.4) and

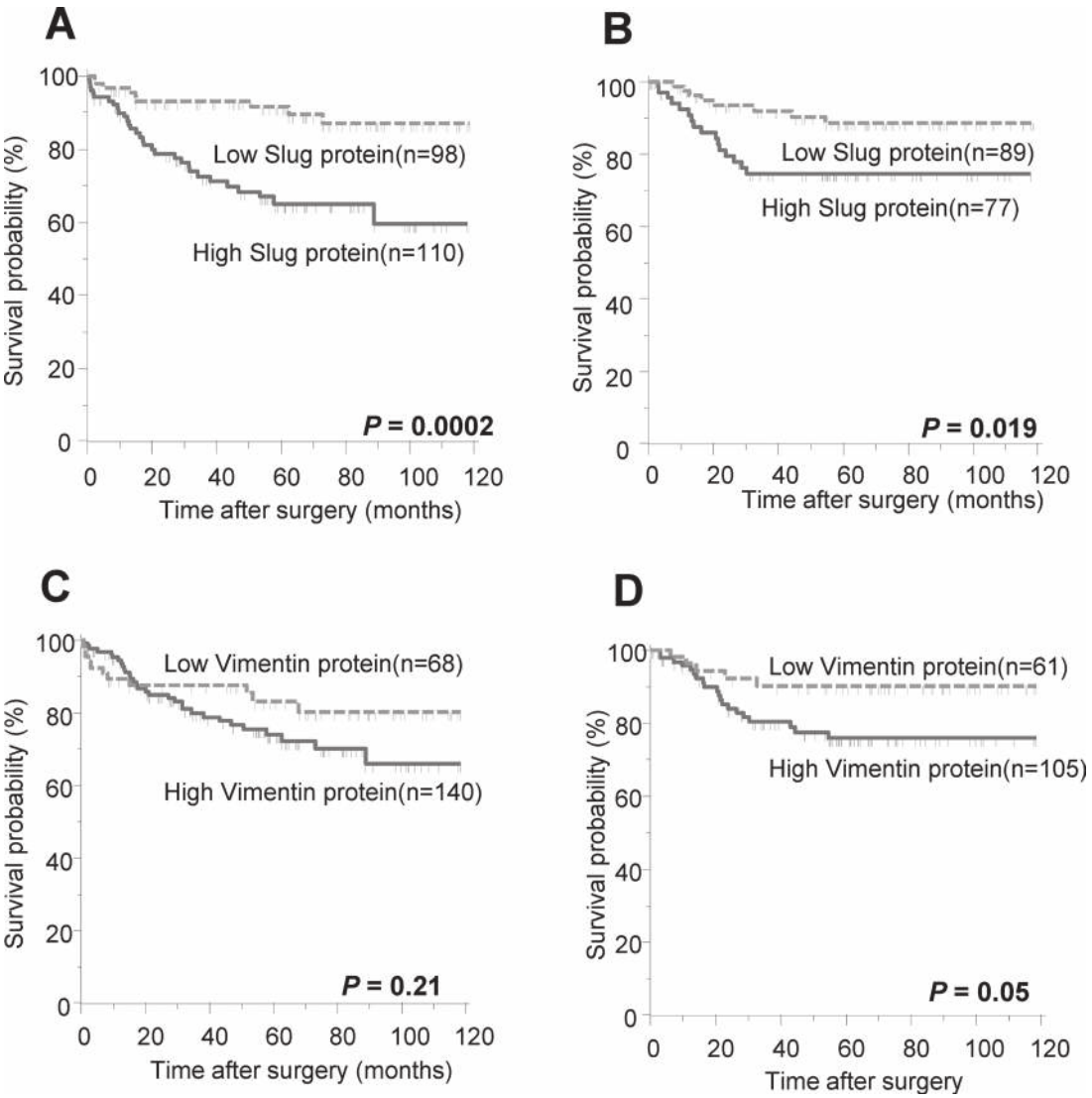

Fig. 5. Survival curves of patients with stages I–IV CRC according to the status of Slug or Vimentin immunostaining scores. (**A**) Patients with high Slug immunostaining showed a significantly poorer OS than those with low immunostaining ($P = 0.0002$, log-rank test; cutoff value = 6). (**B**) Patients with high Slug immunostaining also showed a significantly poorer disease-free survival (DFS) than those with low immunostaining ($P = 0.019$, log-rank test; cutoff value = 6). Survival curves of patients with stages II and III CRC according to the status of Slug or Vimentin immunostaining scores. (**C**) Patients with high Vimentin immunostaining showed a poorer OS than those with low immunostaining ($P = 0.21$, log-rank test; cutoff value = 2). (**D**) Patients with high Vimentin immunostaining showed a significantly poorer DFS than those with low immunostaining ($P = 0.05$, log-rank test; cutoff value = 2).

Table II. Univariate and multivariate analysis for overall survival (Cox proportional hazards regression model)

Factors	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*	HR	95% CI	*P*
Cohort 1 (mRNA analysis)						
Age (>68/68)[a]	1.38	0.85–2.25	0.19	—	—	—
Gender (male/female)	1.06	0.63–1.79	0.81	—	—	—
Histology (well/ moderately, poorly and mucinous)	1.96	0.93–4.14	0.1	—	—	—
Tumor size (>40/40 mm)[a]	1.15	0.70–1.88	0.57	—	—	—
Serosal invasion (absent/present)	2.7	1.45–4.95	0.0016	1.48	1.52–3.12	0.25
Lymph node metastasis (absent/present)	4.52	2.24–9.14	<0.0001*	1.82	1.26–2.76	0.0012*
Lymphatic invasion (absent/present)	3.57	1.12–11.33	0.03*	1.58	0.46–5.42	0.47
Venous invasion (absent/present)	2.17	1.22–3.87	0.009*	1.75	0.97–3.17	0.06
Vimentin mRNA expression (low/high)	2.54	1.47–4.38	0.0008*	1.47	0.79–2.75	0.22
Slug mRNA expression (low/high)	2.96	1.74–5.03	0.0001*	1.97	1.09–3.55	0.025*
Cohort 2 (protein analysis)						
Age (>68/68)[a]	0.74	0.39–1.39	0.35	—	—	—
Gender (male/female)	1.53	0.79–2.96	0.2	—	—	—
Histology (well/moderately, poorly and mucinous)	0.9	0.28–2.92	0.86	—	—	—
Tumor size (>40/40 mm)[a]	1.77	0.95–3.30	0.07	1.11	0.55–2.22	0.76
Lymph node metastasis (absent/present)	4.62	2.38–8.96	<0.0001*	1.95	0.92–4.13	0.08
Distant metastasis (absent/present)	9.26	4.78–17.94	<0.0001*	6.4	3.12–13.15	<0.0001*
Lymphatic invasion (absent/present)	2.69	0.96–7.54	0.06	0.82	0.24–2.78	0.75
Venous invasion (absent/present)	3.18	1.33–7.57	0.009*	1.53	0.54–4.34	0.42
Vimentin protein expression (low/high)	1.77	0.84–3.71	0.13	—	—	—
Slug protein expression (low/high)	3.81	1.81–7.99	0.0004*	2.42	1.10–5.31	0.02*

[a]The median age and tumor size are 68 years and 40 mm, respectively.
*$P < 0.05$.

Vimentin mRNA expression (cutoff value = 2.61). These data clearly suggest that high Slug and Vimentin mRNA expression was associated with poor OS in CRC patients ($P < 0.0001$, $P = 0.0005$, respectively; log-rank test). In addition, Figure 4C and D illustrates survival curves from stage II CRC patients subdivided by Slug (cutoff value = 10.3) and Vimentin mRNA expression (cutoff value = 2.82). These results indicate that high Slug and Vimentin expressions was associated with poor prognosis in stage II CRC patients ($P = 0.04$, $P = 0.012$, respectively; log-rank test).

Next, we evaluated whether protein expression of Slug and Vimentin could predict prognosis in CRC patients. The patients with high Slug expression had significantly poorer OS than those with low expression ($P = 0.0002$; Figure 5A). In addition, high Vimentin expression was also associated with poor OS in CRC ($P = 0.21$; Figure 5C). High expression of Slug and Vimentin was significantly associated with poor disease-free survival (Slug: $P = 0.019$, Vimentin: $P = 0.05$; Figure 5B and D).

Table II presents the results of Cox univariate and multivariate proportional hazards analysis of various factors that influence patient prognosis using the data from Cohorts 1 and 2. Univariate analysis using Cohort 1 data showed that the following factors were significantly related to OS: lymph node metastasis ($P < 0.0001$), Vimentin mRNA expression ($P = 0.0008$) and Slug mRNA expression ($P = 0.0001$). Multivariate analysis indicated that increased Slug expression was an independent prognostic factor (hazard ratio [HR]: 1.97, 95% CI: 1.09–3.55, $P = 0.025$). Univariate analysis based on data from Cohort 2 were consistent with the results of Cohort 1, showing that high Slug expression in CRC was significantly associated with poor OS ($P < 0.0001$, Table II), and high Slug expression was an independent factor for poor OS in CRC (HR: 2.42, 95% CI: 1.10–5.31, $P = 0.02$; Table II).

High Slug expression levels predict patients with lymph node metastasis in CRC. We defined elevated Slug and Vimentin mRNA levels from Cohort 1 according to the cutoff values determined from the ROC analysis with Youden's index for lymph node metastasis (Slug = 11.89, Vimentin = 1.18). Univariate logistic analysis showed that clinical parameters for significantly predicting lymph node involvement were large tumor size ($P = 0.019$), serosal invasion ($P < 0.0001$), lymphatic invasion ($P = 0.0031$), vessel invasion ($P = 0.037$), elevated Vimentin ($P = 0.0005$) and Slug expression levels ($P < 0.0001$). Furthermore, multivariate logistic analysis showed that elevated Slug level was an independent predictive factor for lymph node involvement (HR: 1.97, 95% CI: 1.09–3.55, $P = 0.012$; Table III). To confirm its predictive ability, univariate logistic analysis based on data from Cohort 2 also revealed that high Slug and Vimentin expression in CRC was significantly associated with lymph node metastasis (Slug: $P < 0.0001$, Vimentin: $P = 0.0002$, respectively). In addition, Slug expression was an independent predictor for lymph node metastasis (HR: 4.62, 95% CI: 2.13–10.01, $P = 0.0001$; Table III), suggesting that Slug is a promising factor for selecting the patients with lymph node metastasis.

Discussion

In the present study, we, for the first time, demonstrate a strong correlation between Slug and Vimentin expression in different stages of primary CRCs. Interestingly, we observed that high levels of Slug and Vimentin expression in CRC were significantly associated with disease progression, including tumor invasion to serosa, lymph node metastasis and liver metastasis. In addition, we found that elevated expression of both Slug and Vimentin expression has the potential to serve as a novel biomarker for worse prognosis and poor OS in CRC. Taken together, our data not only provide previously unrecognized mechanistic role for Slug and Vimentin overexpression in imparting tumor aggressiveness and induction of an epithelial-to-mesenchymal phenotype in CRC cells but also highlight their potential utility to serve as metastasis-predictive biomarkers in CRC.

In this context, we demonstrated that high intensity of Slug and Vimentin protein expression was significantly associated with poor disease-free survival in stages II and III CRC, and high expression of both genes predicted poor prognosis in stage II CRC. These data are in line with a previous report by Shioiri *et al.* (30), who reported that expression of Slug was the only independent prognostic factor in stages II and III CRC. However, this previous study investigated a very small group

Table III. Univariate and multivariate analysis for lymph node metastasis (logistic regression model)

Factors	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*	HR	95% CI	*P*
Cohort 1 (mRNA analysis)						
Age (>68/68)[a]	0.97	0.53–1.78	0.93	—	—	—
Gender (male/female)	1.26	0.68–2.37	0.46	—	—	—
Histology (well/ moderately, poorly and mucinous)	2.03	0.63–6.49	0.23	—	—	—
Tumor size (>40/40 mm)[a]	2.07	1.12–3.85	0.019*	1.61	0.80–3.24	0.18
Serosal invasion (absent/present)	4.86	2.49–9.48	<0.0001*	1.48	1.52–3.12	0.006*
Lymphatic invasion (absent/present)	3.66	1.51–8.85	0.0031*	1.58	0.46–5.42	0.35
Venous invasion (absent/present)	1.95	1.04–3.68	0.037*	1.75	0.97–3.17	0.52
Vimentin mRNA expression (low/high)	3.01	1.58–5.71	0.0005*	1.88	0.86–4.11	0.12
Slug mRNA expression (low/high)	4.58	2.22–9.46	<0.0001*	1.97	1.09–3.55	0.012*
Cohort 2 (protein analysis)						
Age (>68/68)[a]	0.58	0.33–1.02	0.055	—	—	—
Gender (male/female)	1.17	0.66–2.06	0.57	—	—	—
Histology (well/moderately, poorly and mucinous)	1.65	0.62–4.35	0.31	—	—	—
Tumor size (>40/40 mm)[a]	2.18	1.23–3.84	0.007*	1.13	0.56–2.31	0.71
Serosal invasion (absent/present)	9.58	3.89–23.62	<0.0001*	1.86	0.57–6.04	0.3
Lymphatic invasion (absent/present)	35.9	4.82–267.32	<0.0001*	6.99	0.77–63.03	0.08
Venous invasion (absent/present)	8.19	3.65–18.39	<0.0001*	3.2	1.18–8.70	0.02*
Vimentin protein expression (low/high)	3.4	1.73–6.70	0.0002*	1.84	0.84–4.19	0.14
Slug protein expression (low/high)	8.21	4.21–16.0	<0.0001*	4.62	2.13–10.01	0.0001*

[a]The median age and tumor size are 68 years and 40 mm, respectively.
*$P < 0.05$.

of CRCs and was unable to determine such associations within different tumor stages, particularly stage II tumors or node-negative groups. Clinically, the majority of patients with stage II CRC do not receive adjuvant treatment since at present there is no evidence for a beneficial effect of adjuvant treatment for this patient group. However, 20–25% of all stage II CRC patients will experience relapse and subsequently die from the disease (31). In this scenario, quantification of Slug and Vimentin expression in primary tumor might aid to select high-risk patients with stage II CRC that might experience tumor recurrence.

Vimentin overexpression in CRCs is mainly associated with the stromal component and is restricted to stromal fibroblasts, endothelial cells lining the microvessels and tumor-infiltrating lymphocytes (32,33). To date, fibroblasts, which are activated by cytokines from tumors and produce cytokines or other soluble factors at the same time, were proposed to modulate different aspects of tumor progression including proliferation or invasion (34,35), angiogenesis (36) or inhibition of cell death (37). However, Ngan *et al*. (33) showed that there was no association between Vimentin expression in stromal tissue and tumor progression in CRC. On the other hand, tumor-infiltrating lymphocytes, commonly identified by the appearance of unique subset of lymphocytes often observed in the tumor stroma, also express Vimentin. Thus, increased Vimentin expression could indicate increased number of tumor-infiltrating lymphocytes. However, this group of cells protects the host against the tumor cells and prevents a tumor-specific immune response. In fact, increased immune cells in CRC are reportedly associated with better survival (38). Therefore, the significance of Vimentin expression in the tumor stroma to tumor progression remains controversial.

In contrast, in our study we demonstrated that a significant correlation existed between Slug and Vimentin expression in CRC tissues, and our immunostaining results revealed that both Slug and Vimentin were primarily expressed in CRC cells from the patients with advanced CRC. In addition, our *in vitro* experiments revealed that suppression of Slug expression induced a dramatic reduction of Vimentin expression in CRC cell lines, which in turn leads to decreased rates of cell proliferation, invasion and migration. These results suggest that overexpression of Slug accompanied by simultaneous increase in Vimentin helps CRC cells to acquire mesenchymal phenotype and may help promote cell invasiveness and metastasis, which is responsible for clinical aggressiveness and poor prognosis in CRC patients.

Another interesting point learnt from this study is that we demonstrated Slug mRNA and protein expression in tumors served as an independent marker for prediction of lymph node metastasis. Currently, there are several options for curative treatment of early CRC, such as endoscopic mucosal resection, endoscopic submucosal dissection and laparoscopy-assisted colectomy with regional lymphadenectomy. Patients suitable for endoscopic treatments are selected by preoperative diagnosis of lymph node metastasis, such as macroscopic type, tumor size, presence of an ulcer and the histology of biopsy specimens. Since some proportion of patients are erroneously misdiagnosed and present with lymph node metastasis before surgery, incorporating the quantification of Slug gene expression or intensity score using biopsied specimens and polypectomy samples followed by preoperative selection of patients without lymph node metastasis may be possible and could promote minimally invasive treatments during early CRC.

In conclusion, we demonstrate several novel evidences for the clinical significance of Slug and Vimentin expression in CRC. First, we observed a significant correlation between Slug and Vimentin expression in CRC, and the patients with increased Slug and Vimentin expression had an overall worse prognosis in CRC, particularly in stage II or stages II and III CRC patients. Our data highlight that Slug and Vimentin expression may serve as potentially important disease biomarkers for the identification of patients who are at high risk for tumor recurrence and require adjuvant chemotherapy and strict surveillance in curative CRC patients. Finally, we provide encouraging evidence that indicates the clinical significance of Slug as a valuable predictive marker of lymph node metastasis, which can help reduce CRC-associated mortality and morbidity by stratifying patients for minimally invasive and curative treatments during earlier stages of CRC, bringing us a step closer towards personalizing therapeutic intervention for this malignancy.

Supplementary material

Supplementary Figure S1 can be found at http://carcin.oxfordjournals.org/

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