Decreased expression of SCUBE2 is associated with progression and prognosis in colorectal cancer

QI SONG[1], CHUANWEI LI[1], XIAODONG FENG[1], AILIAN YU[2], HUAMEI TANG[3], ZHIHAI PENG[4] and XUECHUN WANG[1]

Departments of [1]Pathology and [2]Etiology, Taishan Medical University, Taian, Shandong; Departments of [3]Pathology and [4]General Surgery, Shanghai Jiaotong University Affiliated First People's Hospital, Shanghai, P.R. China

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Abstract. Signal peptide-CUB-epidermal growth factor-like domain-containing protein 2 (SCUBE2), a member of the SCUBE protein family, is a secreted and membrane-associated multi-domain glycoprotein. SCUBE2 is known as a novel tumor suppressor and a useful prognostic marker in breast cancer. In the present study, we investigated the expression (including mRNA and protein levels) of SCUBE2 in colorectal cancer (CRC) and adjacent normal tissues, using quantitative real-time PCR, western blot analysis and immunohistochemistry on a tissue microarray. Upregulation of SCUBE2 was achieved by transient transfection in RKO cell lines, and the effects of SCUBE2 on tumor proliferation, invasion, migration and apoptosis were evaluated by a series of functional experiments. The results indicated that SCUBE2 expression was decreased at the transcriptional and translational levels in CRC tissues and significantly associated with clinical stage, the depth of tumor invasion, lymph-node metastasis, distant metastasis and histological grade. Patients with SCUBE2-positive tumors had a lower recurrence rate and better survival than patients with SCUBE2-negative tumors. Moreover, upregulation of SCUBE2 had a limited effect on cell apoptosis but significantly inhibited tumor cell proliferation, migration and invasion *in vitro*. In conclusion, SCUBE2 plays an important role in suppressing CRC progression and prognosis. Our findings suggested that SCUBE2 may serve as a novel tumor suppressor and a potential therapeutic target for CRC patients.

Introduction

Colorectal cancer (CRC) is a major cause of cancer incidence and mortality worldwide (1) and its incidence has increased particularly among adults <50 years (2). Due to the high recurrence and distant metastases, the long-term outcome of CRC patients remains unsatisfactory. Although by improving access to and use of screening and standard treatment have led to significant advances in early detection and reduction of the death rates of CRC, these methods are not cost-effective. Molecular genetics have provided evidence that the occurrence of CRC is a series of molecular events, including the accumulation of genetic and epigenetic changes (3). Sensitive biomarkers can contribute to early diagnosis and prognosis prediction, therefore, novel factors for predicting tumor recurrence following surgery remain to be defined and novel therapeutic strategies should be identified.

Signal peptide-CUB-epidermal growth factor-like domain-containing protein 2 (SCUBE2) belongs to a novel, small and evolutionarily conserved family that comprises three different members have been designated as SCUBE1 to SCUBE3 in the sequence of identification (4,5). These SCUBE proteins contain ~1,000 amino acids and share an organized protein domain structure with five motifs: an NH2 terminal signal peptide sequence, nine copies of EGF-like repeats, a spacer region followed by three repeated stretches of 6-cysteine residues and one CUB domain at the COOH terminus (6-9). SCUBE2 is predominantly expressed in vascular endothelial cells (4) and is a secreted cell-surface glycoprotein which is a novel positive component of Sonic hedgehog (SHH) signaling and can specifically interact with SHH and its receptor PTCH1 to enhance the SHH signaling activity acting upstream of ligand binding at the plasma membrane (7). Increased angiogenesis (10,11) and dysregulation of the SHH pathway (12,13) have been shown to contribute to colorectal carcinogenesis.

Mounting evidence suggested that SCUBE2 act as a novel breast tumor-suppressor gene that serves as a useful prognostic marker (8,9). Furthermore, SCUBE2 expression is associated with better prognosis and longer disease-free survival in breast cancer (8) and is part of the 8-gene expression score, which has a prognostic value for early breast cancer (14). Similarly, ectopic overexpression of the full-length SCUBE2 protein resulted in the suppression of breast cancer cell proliferation *in vitro* and *in vivo* through co-ordinated regulation of the suppression of the bone morphogenetic protein and β-catenin signaling pathways (8,9). Furthermore, SCUBE2 plays a key role in the suppression of breast cancer cell migra-

Correspondence to: Professor Xuechun Wang, Department of Pathology, Taishan Medical University, No. 2 Yingsheng East Road, Taian, Shandong 271000, P.R. China
E-mail: alyu@tsmc.edu.cn

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tion and invasion by increasing the formation of epithelial E-cadherin-containing adherens junctions and driving the reversal of epithelial-mesenchymal transition (EMT) (15). In prostate cancer and endometrial cancer, SCUBE2 expression is reduced in high-grade tumors (16,17).

In the present study, we investigated SCUBE2 expression (mRNA and protein level) in CRC and then analyzed the correlations between SCUBE2 expression and its clinicopathological parameters. The effect of SCUBE2 expression on RKO CRC cell growth, migration, invasion and apoptosis was also analyzed.

Materials and methods

Clinical samples and cell lines. The present study was approved by the Ethics Committee of Shanghai Jiaotong University Affiliated First People's Hospital. A total of 120 patient-derived specimens were collected between January, 2001 and December, 2003 and archived under protocols approved by our Institutional Review Board. None of the patients underwent therapy prior to surgery. The diagnoses were confirmed by two pathologists, and the tumor stage was determined on the basis of pathological findings in accordance with the American Joint Committee on Cancer (AJCC). Disease-free survival (DFS) and overall survival (OS) durations were defined as the interval from initial surgery to clinically or radiologically proven metastasis or recurrence and death, respectively. There were 48 male and 72 female patients, with a mean age of 64±14 years (range, 22-85 years) at the time of surgery. The median patient follow-up time was 61 months after surgery (range, 10-81 months). Each patient provided informed consent for the use of their tissue samples in the present study.

The LoVo, RKO, HCT8, HT29, HCT116, SW480 and SW620 CRC cell lines were obtained from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured according to the manufacturer's instructions.

RNA extraction, reverse transcription PCR and quantitative PCR. Total RNA in 40 pairs of frozen primary tumor and adjacent normal mucosa of CRC specimens were extracted according to the manufacturer's instructions (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 1 μg of RNA using the A3500 RT-PCR System (Promega, Madison, WI, USA). The primers used for quantitative PCR were: SCUBE2, forward: 5'-CCCCCAAGCGCCGCATCCTGA-3' and reverse: 5'-TATTGAGTGGCACGTGGGCTGAGT-3'; GAPDH, forward: 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'. Quantitative SCUBE2 mRNA levels were assessed using Mastercycler ep realplex® (Eppendorf, Hamburg, Germany) with a SYBR-Green RNA PCR kit (Fermentas, Waltham, MA, USA) according to the manufacturer's instructions. The cycling conditions used were: initial denaturation (10 min at 95˚C), 40 cycles of denaturation (10 sec at 95˚C) followed by annealing (30 sec at 60˚C), and a final elongation (30 sec at 72˚C). Each reaction was repeated three times and the average SCUBE2 mRNA level for each tumor was compared with the level of its matched normal mucosa. The fold-change ($2^{-\Delta\Delta Ct}$) of SCUBE2 expression was calculated for each group (18).

Western blot analysis. Total proteins of four randomly selected, paired, frozen CRC tissues and adjacent normal mucosa specimens were extracted and measured using the BCA protein assay kit (Beyotime Biotechnology Co., Jiangsu, China). Equivalent amounts of protein were separated on a 10% polyacrylamide gel and transferred onto polyvinylidene difluoride membranes, which were blocked in 5% non-fat milk for 1 h at room temperature and incubated overnight with the appropriate primary antibodies: SCUBE2 (1:300 dilution) and TUBB2C (1:3,000 dilution) (both from Abgent, San Diego, CA, USA). After washing with TBST buffer, the membranes were incubated with a HRP-conjugated goat anti-rabbit (1:5,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) secondary antibody. The membranes were visualized using the ECL Plus enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL, USA) and exposed to X-ray film. TUBB2C expression was used to confirm and normalize equal loading of the samples.

Tissue microarray construction and immunohistochemistry. Tissue microarray (TMA) was made from paired tumor and adjacent normal mucosa from the 120 patients in our archive (in collaboration with Shanghai Biochip, Shanghai, China). Two cores, which were validated as having high accordance with the whole archived section, were obtained from each specimen of formalin-fixed, paraffin-embedded CRC tissue and normal mucosa using punch cores measuring 2.0 mm in greatest dimension from the non-necrotic area of the tissue (19).

The primary antibody against SCUBE2 (1:50; Sigma-Aldrich, St. Louis, MO, USA), which was produced primarily by the Human Protein Atlas (HPA), as HPA performs antigen microarray, was chosen for use in the present study. The sections were incubated with primary antibody at 4˚C overnight and then incubated with the secondary antibody (Gene Tech, Shanghai, China) for 30 min at 37˚C. After rinsing in PBS, the sections were incubated with 3,3'-diaminobenzidine (DAB) liquid, counterstained with Mayer's hematoxylin, dehydrated and then mounted.

Immunoreactivity was evaluated independently by two investigators who were blinded to patient outcome according to the intensity and extent of staining. Staining intensity for SCUBE2 was scored as: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong), and the staining extent was scored as: 0 (0%), 1 (<10%), 2 (10-50%) and 3 (>50%), on the basis of the percentage of positively stained cells. The sum of the intensity and extent scores was used as the final staining score, which was defined as: 0-2, negative; 3-4, weakly positive; and 5-6, strongly positive.

Transfection of SCUBE2 in CRC RKO cell line. RKO cells ($5x10^5$/well) were seeded in 6-well plates and cultured to 70-80% confluence. The cells were then transiently transfected with GV144-SCUBE2 (Jikai Gene Chemical Co., Ltd., Shanghai, China) or empty vector controls using Lipofectamine 2000 (Invitrogen). SCUBE2 expression was confirmed using quantitative PCR and western blot analysis.

Cell proliferation assay and plate colony formation. Exponentially growing RKO, RKO-vector and RKO-SCUBE2 cells were trypsinized and re-suspended in DMEM supplemented with 10% fetal bovine serum (FBS), and then seeded in 96-well plates ($2x10^3$ cells/well). The cells were incubated for 7 days, and the number of living cells in each well was determined daily using a Cell Counting Kit (Rainbio, Shanghai, China). The experiments were independently repeated three times.

Colony formation was determined by preparing single-cell suspension solutions and seeding in 6-well plates with $1x10^3$ cells. Following incubation for 14 days, the colonies were washed three times with phosphate-buffered saline (PBS), fixed with paraformaldehyde for 30 min, and stained with crystal violet for 10 min. The stained colonies were counted, and the plates were photographed. The experiments were performed in triplicate.

Apoptosis assay. Apoptosis was analyzed using the Annexin V-FITC Apoptosis kit (Rainbio). After a 48-h transfection, the cells were collected, rinsed with PBS, and stained with Annexin V-FITC and PI. Apoptotic rates were determined using a Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA, USA). At least $1x10^4$ cells were captured in each sample.

Cell migration assay and invasion assay. The migration and invasion assays were performed using 24-well Transwell chambers with polycarbonate membranes of 8-μm pore size (Corning Inc., New York, NY, USA) that were uncoated or coated with Matrigel (BD Biosciences). After a 24-h transfection, cells were cultured for 12 h in serum-free medium. A cell suspension containing $5x10^5$ cells/ml in serum-free medium was prepared, and 200 μl were added to the upper chamber and 500 μl medium with 20% FBS was added to the lower chamber. After 48 h for the migration assay and 72 h for the invasion assay, the incubation was terminated, and the cells were fixed with 95% ethanol and stained with crystal violet. The images were captured using a microscope (Nikon, Tokyo, Japan) at a magnification of x200. The experiments were carried out in triplicate.

Statistical analysis. SPSS 19.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used to perform the statistical analyses. Data were presented as means ± standard deviations for continuous variables or frequencies and percentages for categorical data. The Student's t-test was used for comparisons of means between two groups and the three-group comparisons were conducted using one-way analysis of variance (ANOVA). The χ^2 or Fisher's exact tests were used to determine the significance of differences between SCUBE2 and clinicopathological variables. Kaplan-Meier curves with log-rank tests were used to calculate the cumulative survival proportion for OS and DFS by SCUBE2 expression level. A Cox proportional hazards model was applied to investigate the univariate and multivariate hazard ratios for the study variables. P<0.05 was considered to indicate a statistically significant result.

Results

Expression of SCUBE2 mRNA and protein in CRC tissues. SCUBE2 gene expression in mRNA level was confirmed by the quantitative PCR analysis of 40 pairs of CRC tissue and matched adjacent normal mucosa. As shown in Fig. 1A, the relative level of SCUBE2 mRNA in 31/40 (77.5%) tumor tissues showed a ≥2-fold decrease in the SCUBE2 mRNA level, compared with that of the adjacent normal mucosa (P<0.001). Subsequent western blot analysis also confirmed that SCUBE2 protein levels were decreased in tumor tissue as compared with the paired normal mucosa (Fig. 1B), consistent with the results of quantitative PCR.

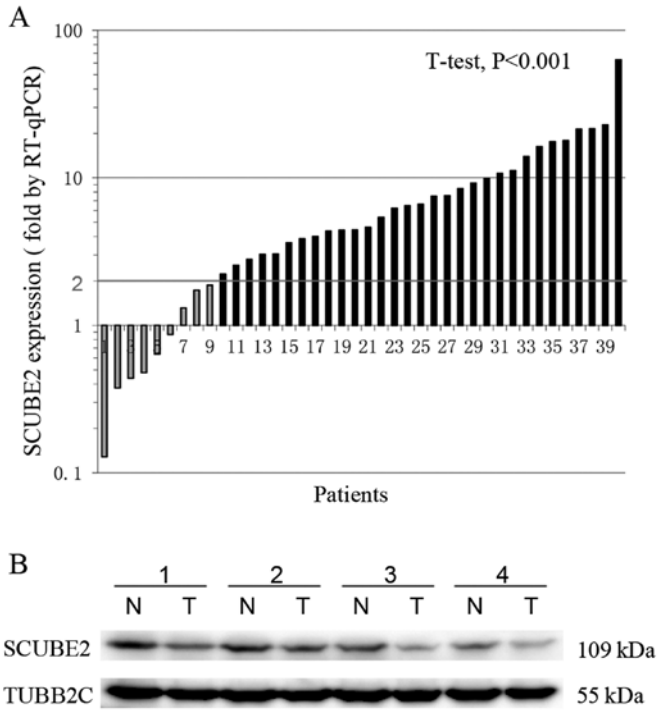

Figure 1. Expression of SCUBE2 in CRC tissues and adjacent normal mucosa. (A) Quantitative PCR analysis of SCUBE2 mRNA expression in 40 paired CRC samples and adjacent normal mucosa. A logarithmic scale of $2^{-\Delta\Delta Ct}$ was used to represent the fold-change. (B) Western blot analysis of SCUBE2 protein expression in four paired CRC tissues, with TUBB2C being the loading control. P<0.001. SCUBE2, signal peptide-CUB-epidermal growth factor-like domain-containing protein 2; CRC, colorectal cancer.

Association of SCUBE2 immunohistochemical staining with clinicopathological characteristics of CRC. To analyze the clinicopathological characteristics of SCUBE2 expression, immunohistochemistry was used to detect the SCUBE2 protein expression in TMA containing 120 cases of primary CRC and paired adjacent normal mucosa.

Consistent with its endothelial origin, SCUBE2 protein was detected in the endothelial cells of small vasculars (Fig. 2A and E). As shown in Fig. 2, SCUBE2 was mainly expressed in the membrane of colorectal epithelium, with cytoplasmic staining only observed with strong positive staining. Notably, in some well-differentiated CRC tissues, positive staining was detected in the membrane of colorectal mucosa (Fig. 2D) and was expressed in the cytoplasm of goblet cells (Fig. 2E).

Of the 120 normal mucosa specimens in the TMA 16/120 (13.33%) showed a negative SCUBE2 expression, 56/120 (46.67%) specimens had weak staining, and 48/120 (40%) specimens exhibited strong staining. By contrast, the

Table I. Expression of SCUBE2 in normal colonic mucosa and cancerous tissues.

Tissue sample	n	Expression of SCUBE2			P-value
		Negative (n, %)	Weak (n, %)	Strong (n, %)	
Normal mucosa	120	16 (13.33)	56 (46.67)	48 (40)	<0.001[a]
Cancer tissue	120	57 (47.50)	39 (32.50)	24 (20)	

[a]P-value derived from the χ^2 test. SCUBE2, signal peptide-CUB-epidermal growth factor-like domain-containing protein 2.

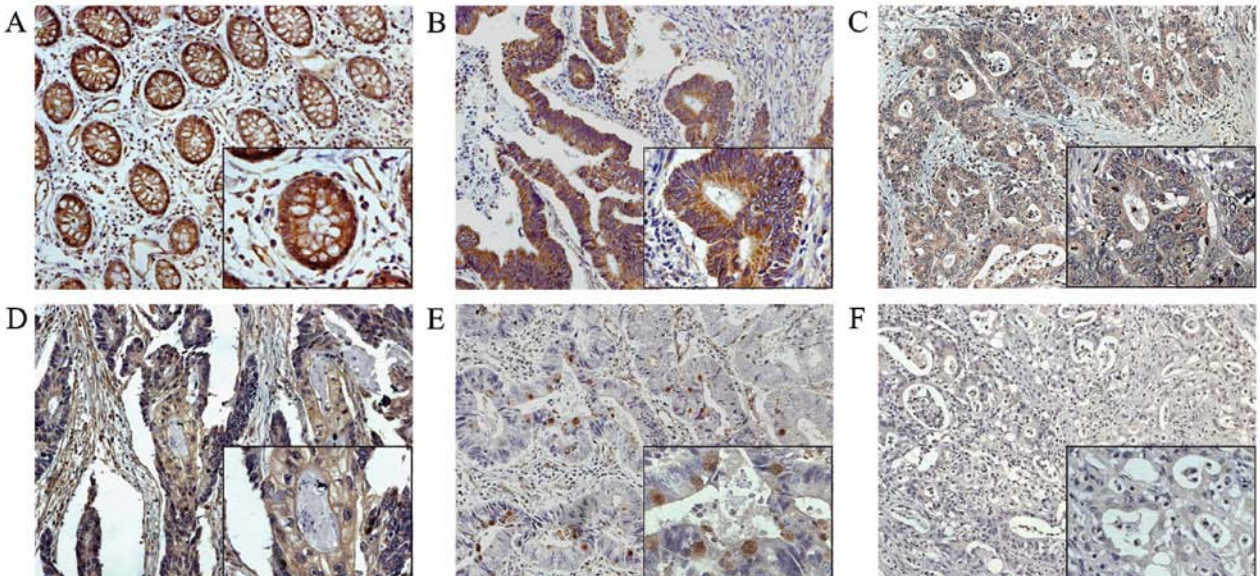

Figure 2. Immunohistochemical staining of SCUBE2 expression in normal and CRC tissue. (A) Positive SCUBE2 expression in normal colonic epithelium and (B) well-differentiated tumor. (C-E) Weak SCUBE2 staining in moderately differentiated colon tumor. (F) Negative SCUBE2 staining in poorly-differentiated colon tumors. Original magnification, x200 (x400 for insets). SCUBE2, signal peptide-CUB-epidermal growth factor-like domain-containing protein 2; CRC, colorectal cancer.

immunoreactive patterns of SCUBE2 were not predominantly positively identified in the majority of CRC specimens. Of these CRC tissues, 57/120 (47.50%) showed negative SCUBE2 expression, 39 (32.50%) cases showed weak staining and 24 (20%) exhibited strong staining (Table I). The distribution of SCUBE2 expression was significantly different between normal mucosa and tumor tissues and SCUBE2 was significantly downregulated in the cancer tissues compared with the corresponding non-cancer mucosas (P<0.001).

Associations between SCUBE2 expression and clinicopathological characteristics for the 120 subjects are shown in Table II. The downregulation of SCUBE2 was significantly correlated with that of the American Joint Committee on cancer (AJCC) stage (P<0.001), depth of tumor invasion (P<0.001), nodal involvement (P=0.016), distant metastasis (P=0.044) and histological differentiation (P=0.031). No correlations were identified between SCUBE2 expression and age, gender, tumor location and vascular invasion.

Survival analysis and prognostic significance of SCUBE2 expression. To assess the possible association between CRC SCUBE2 expression and patient survival, Kaplan-Meier curves with a log-rank test for OS and DFS were undertaken. As shown in Fig. 3, the estimated mean OS time was significantly different between patients with SCUBE2-positive and -negative tumors (63.38±1.86 and 53.98±2.09 months, respectively; P=0.001). The estimated mean DFS time was 61.05±2.11 and 45.32±2.66 months for subjects with SCUBE2-positive and SCUBE2-negative tumors (P<0.001). Kaplan-Meier curves showed that the rate of recurrence was significantly elevated with the negative SCUBE2 expression. In the 37 recurrence cases, patients with a negative SCUBE2 expression had a higher recurrence rate than patients with a positive expression (negative, 75.68% and positive, 24.32%; P<0.001).

The results of the univariate analysis revealed that patients with positive tumor SCUBE2 expression had a significantly higher overall survival (OS) and disease-free survival (DFS) rate than patients with a negative SCUBE2 expression (HR 0.356, 95% CI 0.178-0.714, P=0.004; HR 0.226, 95% CI 0.107-0.481, P<0.001, respectively; Tables III and IV). In addition, both OS and DFS were significantly associated with AJCC stage, LNM (P<0.001), distant metastasis (P<0.001), histological differentiation, and vascular invasion (Tables III and IV). However, the multivariate analysis revealed that a positive SCUBE2 expression could not be a significant independent

Table II. Association between SCUBE2 expression and the clinicopathological characteristics in CRC tissues.

Characteristics	SCUBE2 expression			P-value
	Negative (n=57)	Weak (n=39)	Strong (n=24)	
Age (years)				0.211
<65	27	14	14	
≥65	30	25	10	
Gender				0.708
Male	25	14	9	
Female	32	25	15	
Location				0.560
Right	22	9	8	
Transverse	9	10	3	
Left	12	7	4	
Sigmoid	14	13	9	
AJCC stage				<0.001[a]
I	12	10	15	
II	13	21	7	
III	18	5	1	
IV	14	3	1	
T stage				<0.001[a]
T1	2	5	9	
T2	4	6	7	
T3	22	16	5	
T4	29	12	3	
N stage				0.016[a]
N0	25	28	19	
N1	19	7	3	
N2	13	4	2	
M stage				0.044[a]
M0	48	37	23	
M1	9	2	1	
Differentiation				0.031[a]
Well	19	17	17	
Moderate	23	14	6	
Poor	15	8	1	
Vascular invasion				0.079
Yes	8	2	1	
No	49	37	23	

[a]P-values based on the χ^2 test. SCUBE2, signal peptide-CUB-epidermal growth factor-like domain-containing protein 2; CRC, colorectal cancer; AJCC, American Joint Committee on Cancer.

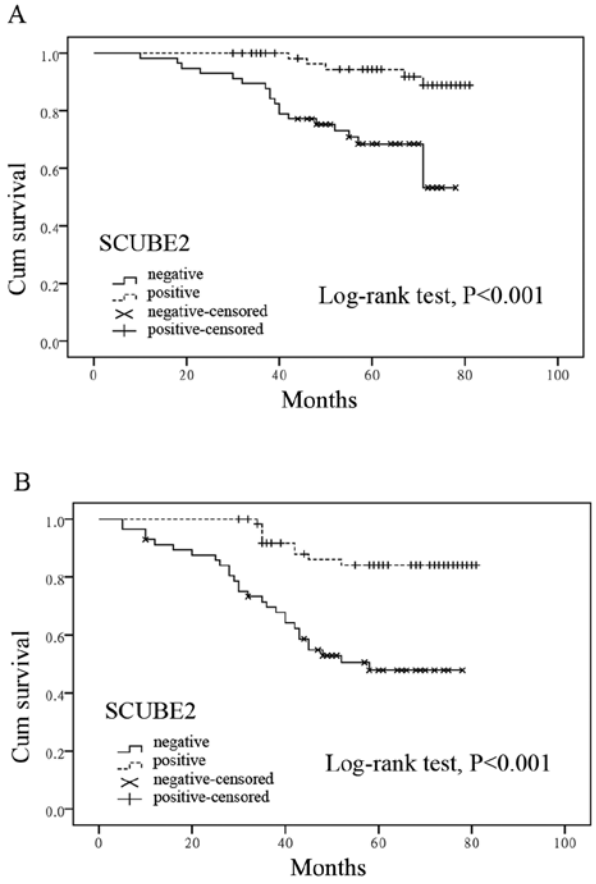

Figure 3. Kaplan-Meier analyses with a log-rank test of survival. Kaplan-Meier plots of (A) overall survival and (B) disease-free survival of patients with CRC who underwent curative resections on the basis of the immunohistochemical UHRF2 expression. SCUBE2, signal peptide-CUB-epidermal growth factor-like domain-containing protein 2; CRC, colorectal cancer.

prognostic factor for decreased disease recurrence and increased survival (Tables III and IV).

Overexpression of SCUBE2 protein suppresses proliferation and inhibits cell migration and invasion of RKO CRC cell line, but does not increase apoptosis. To examine the effect of SCUBE2 on CRC, its mRNA and protein expression was evaluated in the CRC cell lines (data not shown). The expression level of the RKO CRC cell line was decreased as compared to the remaining cell lines and was more easily transfected. Thus, RKO cell line was selected for the subsequent *in vitro* experiments.

RKO was transfected with a SCUBE2 expression plasmid and a series of functional experiments concerning cell proliferation, apoptosis and metastasis was performed. Upregulation of SCUBE2 expression was confirmed in transfected RKO cells by quantitative PCR and western blot analysis (Fig. 4).

Significantly decreased proliferation of SCUBE2-transfected RKO cells was observed after 3-7 days as compared to the two control groups (Fig. 5A). In addition, RKO colony formation was significantly decreased upon SCUBE2 expression (P<0.05, Fig. 5B). Cell migration and invasion were significantly reduced in SCUBE2-transfected cells as compared to parental and empty vector-transfected cells (P<0.05, Fig. 6A and B). Furthermore, the RKO cell apoptotic rate was not significantly increased from 0.2 and 0.1% among parental and empty vector-transfected cells, respectively, to 0.5% in SCUBE2-transfected cells (data not shown).

Table III. Association between clinicopathological characteristics and OS by COX regression model analysis.

	Univariate			Multivariate		
Overall survival	HR	CI (95%)	P-value	HR	CI (95%)	P-value
Age (years)						
<65	1					
≥65	1.988	0.850-4.649	0.113			
Gender						
Male	1					
Female	1.399	0.598-3.270	0.439			
Vascular invasion						
No	1			1		
Yes	4.417	1.632-11.953	0.003[a]	3.645	1.165-11.401	0.026[a]
AJCC stage						
I-II	1			1		
III-IV	5.042	2.130-11.939	<0.001[a]	1.948	0.776-4.893	0.156
T stage						
T1-T2	1			1		
T3-T4	2.468	0.840-7.249	0.100	1.669	0.421-6.612	0.466
N stage						
N0	1			1		
N1-N2	12.465	3.715-41.818	<0.001[a]	13.661	1.463-127.602	0.022[a]
M stage						
M0	1			1		
M1	6.684	2.844-15.705	<0.001[a]	3.140	1.223-8.062	0.017[a]
Differentiation						
Well	1			1		
Moderate/poor	5.875	1.752-19.703	0.004[a]	0.416	0.042-4.103	0.453
SCUBE2 expression						
Negative	1			1		
Positive	0.356	0.178-0.714	0.004[a]	0.465	0.128-1.682	0.243

OS, overall survival; AJCC, American Joint Committee on Cancer; CI, confidence interval; HR, hazard ratio; SCUBE2, signal peptide-CUB-epidermal growth factor-like domain-containing protein 2. [a]P<0.05 indicated that the 95% CI of HR was not including 1.

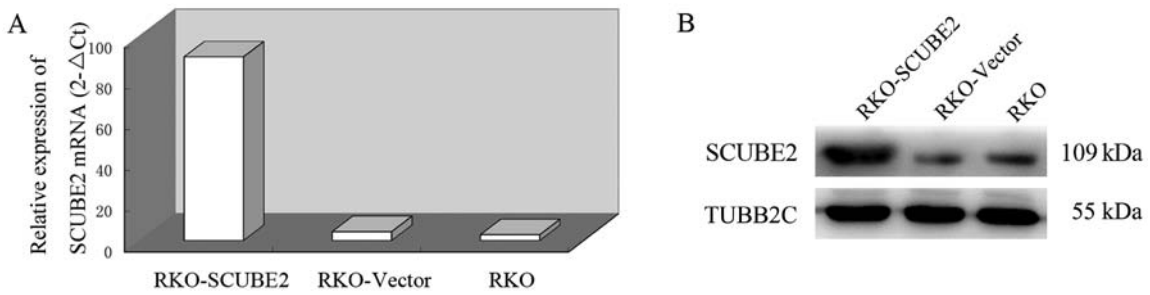

Figure 4. SCUBE2 expression in transfected RKO cells was confirmed through (A) quantitative PCR and (B) western blot analysis. Parental or empty vector-transfected cells were used as controls. SCUBE2, signal peptide-CUB-epidermal growth factor-like domain-containing protein 2.

Discussion

In the present study, we used a variety of experimental approaches to examine protein localization, function and clinical implications of a newly described human gene, *SCUBE2*, and its a role in human CRC. Our findings have demonstrated the importance of SCUBE2 as a potential tumor suppressor in CRC and correlations were observed between

Table IV. Association between clinicopathological characteristics and DFS by COX regression model analysis.

	Univariate			Multivariate		
Disease-free survival	HR	CI (95%)	P-value	HR	CI (95%)	P-value
Age (years)						
<65	1					
≥65	1.274	0.665-2.444	0.465			
Gender						
Male	1					
Female	1.115	0.574-2.168	0.747			
Vascular invasion						
No	1			1		
Yes	4.753	2.162-10.452	<0.001[a]	4.327	1.781-10.510	0.001[a]
AJCC stage						
I-II	1			1		
III-IV	2.506	1.314-4.781	0.005[a]	1.410	0.683-2.915	0.353
T stage						
T1-T2	1			1		
T3-T4	2.454	1.023-5.887	0.044[a]	2.246	0.813-6.208	0.119
N stage						
N0	1			1		
N1-N2	3.673	1.868-7.224	<0.001[a]	1.896	0.637-5.645	0.250
M stage						
M0	1			1		
M1	6.136	2.856-13.181	<0.001[a]	4.207	1.729-10.239	0.002[a]
Differentiation						
Well	1			1		
Moderate/poor	3.008	1.419-6.377	0.004[a]	1.460	0.452-4.711	0.527
SCUBE2 expression						
Negative				1		
Positive	0.226	0.107-0.481	<0.001[a]	0.438	0.183-1.049	0.064

DFS, disease-free survival; AJCC, American Joint Committee on Cancer; CI, confidence interval; HR, hazard ratio; SCUBE2, signal peptide-CUB-epidermal growth factor-like domain-containing protein 2. [a]P<0.05 indicated that the 95% CI of HR was not including 1.

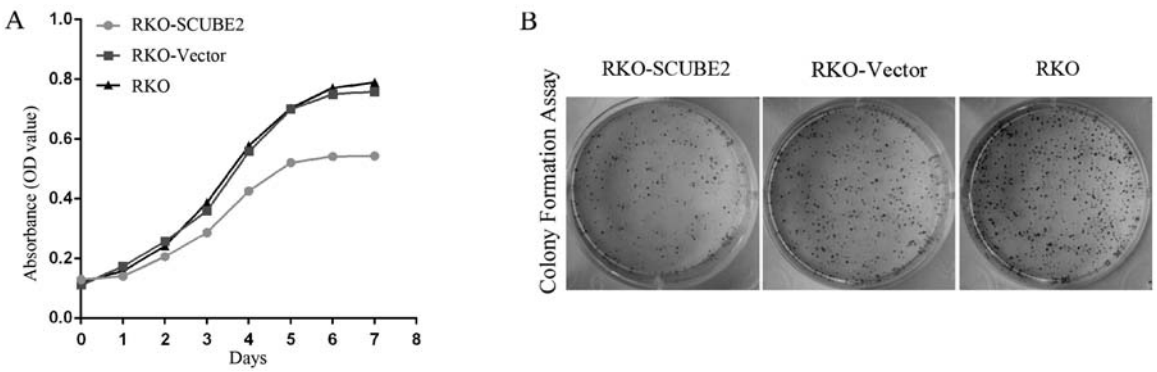

Figure 5. (A) Cell proliferation and (B) colony formation of RKO cells *in vitro*. SCUBE2, signal peptide-CUB-epidermal growth factor-like domain-containing protein 2.

the downregulated SCUBE2 expression and advanced cancer biology, OS and DFS.

SCUBE2 belongs to a small, evolutionarily conserved SCUBE protein family comprising the members SCUBE1 to

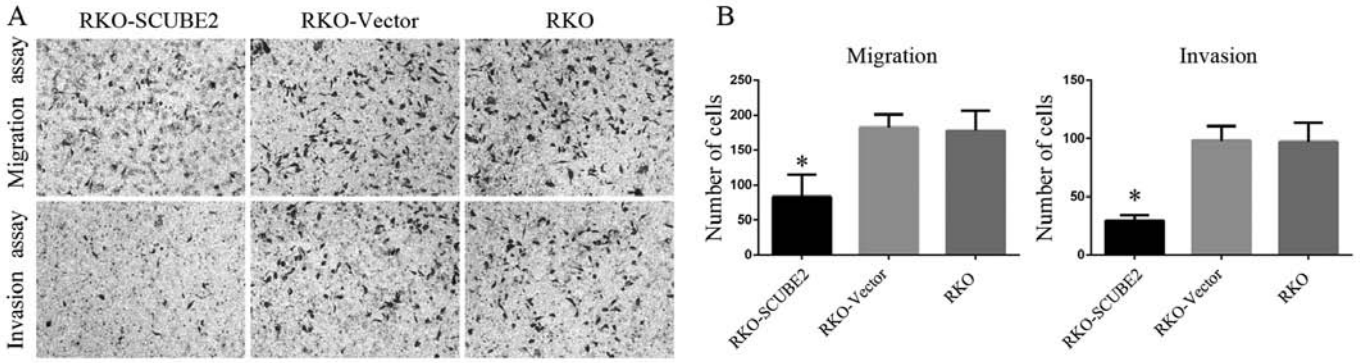

Figure 6. Cell migration and invasion assays of RKO cells *in vitro*. (A) Image shows RKO cell migration and invasion following transfection as described in Materials and methods. (B) Histogram shows the number of migrated and invaded cells. Error bars represent standard deviation (n=5). *P<0.001 in a comparison of the RKO-SCUBE2 with the RKO-vector and RKO cells. SCUBE2, signal peptide-CUB-epidermal growth factor-like domain-containing protein 2.

SCUBE3 by sequence of identification (4,5). These proteins, containing ~1,000 amino acids, are characterized by nine tandem copies of EGF-like repeats, a spacer region, three repeated stretches of 6-cysteine residues and one CUB domain at the COOH terminus. The EGF motif is identified in many types of proteins functioning as adhesion molecules, secreted growth factors, signaling molecules, transmembrane receptors, and important components of the extracellular matrix. The CUB domain regulates cell-cell communication and has been found in several proteins involved in developmental processes (4). SCUBE2 is known to enhance the HH signaling pathway (6,20,21), the dysregulation of which is known to contribute to carcinogenesis (12,13). SCUBE2 expression has been observed in vascular endothelium primarily and thus may affect CRC angiogenesis (10,11,22). SCUBE2 has been reported to act as a tumor suppressor in human breast cancer (8,9,14,15). To the best of our knowledg, the present study is the first to examine SCUBE2 expression in CRC, suggesting that SCUBE2 has a similar role in this tumor type.

Consistent with its endothelial origin, SCUBE2 immunoreactive staining was localized in vascular endothelial cells, and can be detected in colorectal mucosa epithelium. Notably, we found that the SCUBE2 transcript and translational levels were significantly decreased in CRC tissues compared with adjacent normal tissues. The relative level of SCUBE2 mRNA in 34/40 (85%) tumor tissues showed a ≥2-fold decrease in the corresponding non-cancer mucosa. These results suggest that similar to breast cancer (14,23) and endometrial cancer (16), the transcript levels of SCUBE2 are an essential component of predictive gene signatures. Moreover, immunohistochemistry results revealed that only 20% (24/120) of primary CRC had strong positive-SCUBE2 protein staining and 40% (48/120) of normal colorectal epithelium. These data indicate that SCUBE2 is invovled in the progression of colorectal carcinogenesis. Negative correlations were observed between SCUBE2 expression and clinicopathological characteristics of the patients, including AJCC stage (P<0.001), tumor invasion depth (P<0.001), lymph-node metastasis (P=0.016), distant metastasis (P=0.044) and tumor differentiation (P=0.031). The data suggest that downregulated SCUBE2 may contribute to tumor invasion and metastasis. Therefore, SCUBE2 is a potential biomarker for the identification of subsets of CRC with a less aggressive phenotype. In breast cancer, SCUBE2 is reduced in high-grade tumors and is a biomarker for a good prognosis (24-27). Parris *et al* (28) identified that SCUBE2 is a putative target for clinical management and drug development of squamous cell carcinoma of the oral cavity.

In addition, we found that individuals with SCUBE2-positive tumors have higher OS and DFS as compared to those with SCUBE2-negative tumors according to the Kaplan-Meier curves and univariate analysis. These results are partly consistent with those of a previous report (8) whereby SCUBE2 acted as an independent prognostic factor for DFS in breast cancer. The multivariate analyses revealed that SCUBE2 expression in CRC was not an independent prognostic factor for survival. This may be due to the relatively small number of CRC patients and requires further confirmation in a larger group of CRC patients from multicenter trials.

In the present study, restoration of SCUBE2 gene expression in RKO cells was reduced in *in vitro* proliferation and *in vitro* colony formation. The mechanism by which SCUBE2 suppresses CRC progression remains to be determined. According to previous reports, SCUBE2 suppresses cancer cell growth through, at least in part, a coordinated regulation of two distinct mechanisms: antagonizing bone morphogenetic protein activity by releasing an active COOH terminal fragment and suppressing the β-catenin pathway by the NH2-terminal EGF-like repeats (8,9). However, the molecular mechanism of SCUBE2 governing the effects on CRC cell growth needs further exploration. SCUBE2 expression was significantly correlated with N stage and distant metastasis, suggesting that SCUBE2 may modulate the metastatic process. In concordance with findings of those reports, restoration of SCUBE2 inhibited RKO cell migration and invasion *in vitro*. According to previous reports, SCUBE2 increased the formation of epithelial E-cadherin-containing adherens junctions to promote epithelial differentiation and drive the reversal of EMT (15). The molecular mechanism of SCUBE2 governing the effects on CRC cell migration and invasion needs further exploration.

In conclusion, a specific downregulated expression of SCUBE2 and its association with multiple clinicopathological factors in CRC patients has been identified in the present study. This downregulation may possess an important role in

promoting CRC. Moreover, it is closely correlated with aggressive malignant behavior and predicted poor survival in CRC patients. In future studies, large clinical sample validation and mechanism investigations are required to fully elucidate the molecular mechanisms and the role of SCUBE2 in CRC progression.

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