Published OnlineFirst August 12, 2010; DOI:10.1158/1078-0432.CCR-10-0349

Table 2. Clinicopathologic significance of the CD47/GAPDH mRNA expression ratio in bone marrow and in peripheral blood

CD47/GAPDH clinicopathologic factors	BM			PB		
	Low ratio (%)	High ratio (%)	*P*	Low ratio (%)	High ratio (%)	*P*
Age, y (mean ± SD)	55.7 ± 11.2	55.1 ± 11.5	n.s.	55.8 ± 10.9	54.9 ± 11.8	n.s.
Menopause status			0.01*			n.s.
Pre	71 (31.4)	96 (42.5)		76 (33.6)	91 (40.3)	
Post	155 (68.6)	130 (57.5)		150 (66.4)	135 (59.7)	
Tumor stage			n.s.			0.0011*
T_1	103 (45.6)	123 (54.4)		96 (42.8)	130 (57.2)	
T_{2-4}	123 (54.4)	103 (45.6)		130 (57.2)	96 (42.8)	
Lymph node metastasis			n.s.			n.s.
Absent	138 (61.1)	137 (60.6)		135 (59.7)	140 (61.9)	
Present	88 (38.9)	89 (39.4)		91 (40.3)	86 (38.1)	
Lymphatic invasion			n.s.			n.s.
Absent	127 (56.2)	146 (64.6)		130 (58.0)	143 (62.3)	
Present	99 (43.8)	80 (35.4)		96 (42.0)	83 (36.7)	
Venous invasion			n.s.			n.s.
Absent	215 (95.1)	208 (92.0)		215 (95.1)	208 (92.0)	
Present	11 (4.9)	18 (8.0)		11 (4.9)	18 (8.0)	
Distant metastasis			n.s.			n.s.
Absent	221 (97.8)	222 (98.2)		221 (98.2)	222 (97.8)	
Present	5 (2.2)	4 (1.8)		5 (1.8)	4 (2.2)	
Stage			n.s.			n.s.
Stage I	78 (34.5)	86 (38.1)		73 (32.3)	91 (40.7)	
Stage II-IV	148 (65.5)	140 (62)		153 (67.7)	135 (59.3)	
ER			n.s.			n.s.
Absent	56 (24.8)	74 (32.7)		68 (30.1)	62 (27.0)	
Present	170 (75.2)	152 (67.3)		158 (69.9)	164 (73.0)	
PgR			n.s.			n.s.
Absent	102 (45.1)	101 (44.7)		95 (42.0)	108 (47.8)	
Present	124 (54.9)	125 (55.3)		131 (58.0)	118 (52.2)	
Her2 score			0.03*			n.s.
0-1	157 (69.5)	135 (59.7)		152 (67.3)	140 (61.9)	
2-3	69 (30.5)	91 (40.3)		74 (32.7)	86 (38.1)	
ER, PgR, Her2 status			0.0097*			n.s.
Triple negative	26 (11.5)	46 (20.4)		26 (11.5)	46 (20.4)	
Either one positive	200 (88.5)	180 (79.7)		200 (88.5)	180 (79.7)	
Recurrence			0.04*			<0.0001*
Absent	189 (83.6)	204 (90.3)		181 (80.1)	212 (93.8)	
Present	37 (16.4)	22 (9.7)		45 (19.9)	14 (6.2)	

Abbreviation: n.s., not significant.
*$P < 0.05$, statistical significance.

venous invasion, estrogen receptor, progesterone receptor, Her2 score, and *CD47* expression. Multivariate analysis indicated that the high expression ratio of *CD47* was found to be an independent and significant prognostic factor for survival ($P = 0.024$). Univariate and multivariate analyses of clinicopathologic factors affecting OS rate in bone marrow are shown in Table 3. Univariate analysis revealed a significant relationship between OS and the following factors: menopause, lymph node metastasis, estrogen receptor, progesterone receptor, recurrence, and *CD47* expression. Multivariate analysis indicated that the high expression ratio of *CD47* was not an independent and significant prognostic factor for survival ($P = 0.41$).

The clinicopathologic factors analyzed in relation to *CD47* mRNA expression in peripheral blood are shown in Table 2. The incidence of recurrence was significantly

Published OnlineFirst August 12, 2010; DOI:10.1158/1078-0432.CCR-10-0349

Table 3. Univariate and multivariate analyses of clinicopathologic factors affecting disease-free survival and overall survival rate in bone marrow

Clinicopathologic factors	Disease-free survival				Overall survival			
	No. of patients	Univariate analysis HR (CI)	Multivariate analysis		No. of patients	Univariate analysis HR (CI)	Multivariate analysis	
			Relative risk (CI)	*P*			Relative risk (CI)	*P*
Age (years)								
55	233	0.98 (0.58-1.67)	—	—	237	0.55 (0.22-1.32)	—	—
>55	210				215			
Menopause								
Pre	165	0.86 (0.47-1.49)	—	—	167	0.21 (0.03-0.72)	0.25 (0.04-0.92)	0.035*
Post	278				285			
Tumor stage								
T_1	226	0.60 (0.33-1.04)	—	—	226	0.61 (0.22-1.52)	—	—
T_{2-4}	217				226			
Lymphatic invasion								
Present	172	2.08 (1.22-3.70)	1.22 (0.66-2.32)	0.54	179	1.85 (0.78-4.55)	—	—
Absent	271				273			
Lymph node metastasis								
Present	170	3.60 (2.04-6.67)	2.63 (1.31-5.56)	0.0057*	177	3.97 (1.61-11.17)	1.72 (0.07-5.00)	0.27
Absent	273				275			
Venous invasion								
Present	29	3.64 (1.23-8.33)	2.50 (0.84-6.25)	0.1	29	4.00 (0.62-15.02)	—	—
Absent	414				423			
Distant metastasis								
Present	0	—	—	—	9	5.32 (0.83-19.23)	—	—
Absent	443				443			
ER								
Present	318	0.49 (0.29-0.84)	0.80 (0.38-1.69)	0.56	322	0.27 (0.11-0.64)	1.67 (0.48-5.39)	0.40
Absent	125				130			
PgR								
Present	245	0.55 (0.32-0.93)	0.71 (0.35-1.49)	0.36	249	0.21 (0.07-0.54)	0.19 (0.04-0.79)	0.022*
Absent	198				203			
Her2 score								
0-1	285	0.42 (0.25-0.72)	0.69 (0.38-1.26)	0.22	292	0.47 (0.19-1.14)	—	—
2-3	158				160			
Recurrence								
Present	56	—	—	—	59	19.08 (5.26-33.33)	1955	<0.0001*
Absent	387				393			
CD47 expression								
High	221	2.32 (1.28-4.17)	2.00 (1.10-3.61)	0.024*	226	2.99 (1.18-7.61)	1.54 (0.55-4.30)	0.41
Low	222				226			

Abbreviation: HR, hazard ratio.
*$P < 0.05$.

Published OnlineFirst August 12, 2010; DOI:10.1158/1078-0432.CCR-10-0349

Table 4. Univariate and multivariate analyses of clinicopathologic factors affecting disease-free survival and overall survival rate in peripheral blood

Clinicopathologic variable	Disease-free survival				Overall survival			
	No. of patients	Univariate analysis HR (CI)	Multivariate analysis		No. of patients	Univariate analysis HR (CI)	Multivariate analysis	
			Relative risk (CI)	*P*			Relative risk (CI)	*P*
Age (years)								
55	233	0.98 (0.58-1.67)	—	—	237	0.55 (0.22-1.32)	—	—
>55	210				215			
Menopause								
Pre	165	0.86 (0.47-1.49)	—	—	167	0.21 (0.03-0.72)	0.26 (0.04-0.94)	0.039*
Post	278				285			
Tumor stage								
T_{is-1}	226	0.60 (0.33-1.04)	—	—	226	0.61 (0.22-1.52)	—	—
T_{2-4}	217				226			
Lymphatic invasion								
Present	172	2.08 (1.22-3.70)	1.20 (0.65-2.27)	0.56	179	1.85 (0.78-4.55)	—	—
Absent	271				273			
Lymph node metastasis								
Present	170	3.60 (2.04-6.67)	2.63 (1.32-5.43)	0.0055*	177	3.97 (1.61-11.17)	1.58 (0.63-4.51)	0.34
Absent	273				275			
Venous invasion								
Present	29	3.64 (1.23-8.33)	2.76 (0.92-6.67)	0.07	29	4.00 (0.62-15.02)	—	—
Absent	414				423			
Distant metastasis								
Present	0	—	—	—	9	5.32 (0.83-19.23)	—	—
Absent	443				443			
ER								
Present	318	0.49 (0.29-0.84)	0.69 (0.34-1.41)	0.3	322	0.27 (0.11-0.64)	1.50 (0.45-4.47)	0.49
Absent	125				130			
PgR								
Present	245	0.55 (0.32-0.93)	0.71 (0.36-1.43)	0.35	249	0.21 (0.07-0.54)	0.18 (0.04-0.68)	0.011*
Absent	198				203			
Her2 score								
0-1	285	0.42 (0.25-0.72)	0.69 (0.38-1.24)	0.21	292	0.47 (0.19-1.14)	—	—
2-3	158				160			
Recurrence								
Present	56	—	—	—	59	19.08 (5.26-33.33)	2075	<0.0001*
Absent	387				393			
CD47 expression								
High	221	1.57 (0.79-3.03)	—	—	226	1.10 (0.30-3.22)	—	—
Low	222				226			

*$P < 0.05$.

lower ($P < 0.0001$) in the high-expression group than in the low-expression group. The incidence of tumor stage was significantly lower ($P = 0.0011$) in the high-expression group than in the low-expression group. Conversely, no significant differences were observed regarding age, menopause, lymph node metastasis, lymphatic invasion, venous invasion, distant metastasis, clinical stage, estrogen receptor, progesterone receptor, Her2 score, and ER, PgR, Her2 status. The 5-year DFS and OS rates in patients with high *CD47* mRNA and patients with low *CD47* mRNA are shown in Fig. 1D. The survival difference between these two groups was not statistically significant for DFS ($P = 0.18$, log-rank test) and OS ($P = 0.87$, log-rank test). Univariate and multivariate analyses of clinicopathologic factors affecting DFS rate in peripheral blood are shown in Table 4. Univariate analysis revealed a significant relationship between OS and the following factors: tumor stage, lymphatic invasion, lymph node metastasis, venous invasion, estrogen receptor, progesterone receptor, and Her2 score, but *CD47* expression was not included. Multivariate analysis indicated that the presence of lymph node metastasis was found to be an independent and significant prognostic factor for survival ($P = 0.0055$). Univariate and multivariate analyses of clinicopathologic factors affecting OS rate in peripheral blood are shown in Table 4. Univariate analysis revealed a significant relationship between OS and the following factors: menopause, lymph node metastasis, estrogen receptor, progesterone receptor, and recurrence. Univariate analysis indicated that the high expression ratio of *CD47* was

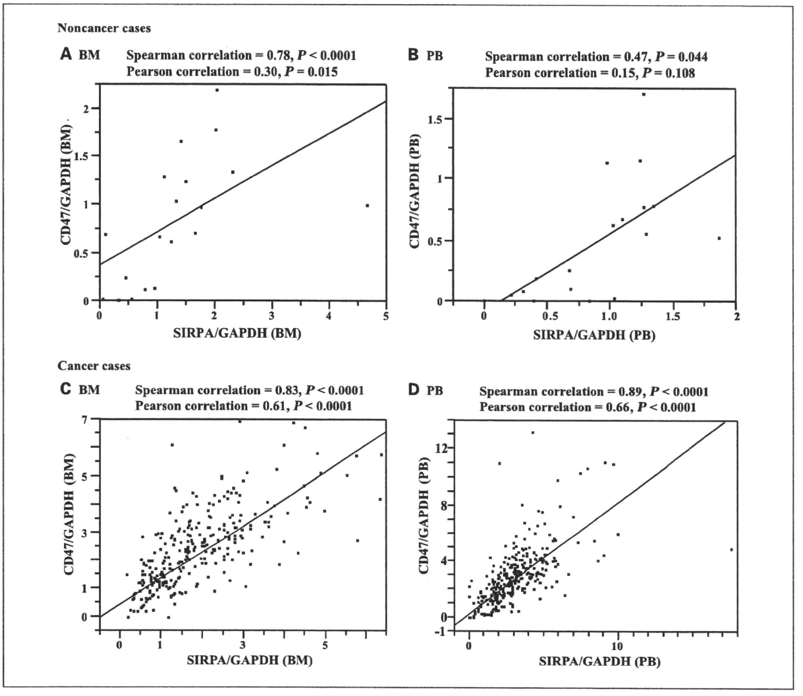

Fig. 2. The correlation between *CD47* expression and *SIRPA* expression. In control patients, *CD47* expression was correlated with *SIRPA* in both bone marrow (A) and peripheral blood (B). In breast cancer cases, *CD47* expression was even more strongly correlated with *SIRPA* in both bone marrow (C) and peripheral blood (D).

not an independent and significant prognostic factor for survival.

Correlation with SIRPA

We investigated *SIRPA* expression in the same breast cancer patients and controls. Figure 2 shows the correlation between *CD47* expression and *SIRPA* expression. In cancer cell lines, *CD47* expression did not correlate with *SIRPA* expression (Spearman correlation = 0.0319; P = 0.95; data not shown). In control patients, *CD47* expression was correlated with *SIRPA* in both bone marrow ($P < 0.0001$) and peripheral blood ($P = 0.0044$; Fig. 2A and B). In 32 noncancer cases, *CD47* expression was correlated with *SIRPA* in both bone marrow ($P = 0.004$) and peripheral blood ($P < 0.0001$; Supplementary Fig. S2-b).

In breast cancer cases, *CD47* expression is more strongly correlated with *SIRPA* in both bone marrow ($P < 0.0001$) and peripheral blood ($P < 0.0001$; Fig. 2C and D).

Discussion

CD47 is expressed on the surface of a wide variety of cells such as hematopoietic cells, keratinocytes, and cells of the brain (17). CD47 is associated with $\alpha_v\beta_3$ integrin and is implicated in the modulation of integrin functions, such as cell adhesion, phagocytosis, and cellular migration (18–20). It is known that CD47 is a marker of self on RBC. CD47 could work as a marker of self on cancer cells, and breast cancer cells may express high levels of CD47 by themselves. Our results showed that the *CD47/GAPDH* expression ratio in breast cancer cell lines was significantly lower than those found in the bone marrow and peripheral blood samples of breast cancer cases. This may indicate that *CD47* has various functions, and that the level of *CD47* expression was affected by the cell environment rather than by the number of cancer cells. Therefore, the high expression of *CD47* in the bone marrow and peripheral blood of breast cancer patients may represent the characteristic appearance of breast cancer and some evidence of a cancer-specific mechanism in the bone marrow and peripheral blood of breast cancer.

Recent reports have shown that CD47 plays a role in inhibiting macrophage phagocytosis of cancer stem cells and tumor-initiating cells (5–7). In the same manner as a cancer stem cell, the cancer cell itself may circumvent immune system surveillance by expressing CD47 as a marker of self, thereby evading natural killer cells (21, 22). In our study, we found that high *CD47* expression had a correlation with high *CK19* expression in the bone marrow and peripheral blood of breast cancer. This result strongly suggests that ITC of breast cancer patients may utilize the function of *CD47* in circulating circumstances such as bone marrow and peripheral blood. Moreover, expression of *CD47* in the bone marrow and peripheral blood of breast cancer patients was significantly higher than in control patients. Because expression of *CD47* in circulating tumor cells increases exponentially with the progress of the cancer stage, *CD47* derived from ITC may be an upregulating factor of breast cancer.

CD47 also promotes apoptosis, and the CD47 ligand thrombospondin (TSP) has been implicated as an antitumor and antimetastatic factor in breast cancer (23–33). Both TSP1 and a CD47 agonist peptide (4N1K, derived from TSP1) can induce a novel form of apoptosis in transformed and activated normal T cells (34, 35), chronic lymphocytic leukemia cells (36), erythroleukemia cells, and primary arterial smooth muscle cells (35). We supposed that CD47 may have a role not only as a marker of self but also as an inducer of apoptosis to inhibit phagocytosis.

In the present study, the mean ratio of *CD47/GAPDH* mRNAs in the high-expression group of cancer cases was three to five times higher than in noncancer cases. We suggest that *CD47* may be specifically expressed in the bone marrow and peripheral blood of breast cancer patients and that *CD47* expression may represent an important biomarker in breast cancer patients. As a result of the identification of the clinical significance of *CD47* expression in bone marrow and peripheral blood, we found that overexpression of *CD47* in bone marrow and peripheral blood correlated with the aggressiveness of breast cancer. This result might suggest that the more there are circulating tumor cells expressing increased *CD47* in bone marrow and peripheral blood, the more active the primary immune system is in inducing apoptosis in tumor cells in the circulating systems. Therefore, it is important to clarify the level of *CD47* expression in bone marrow and peripheral blood to indicate whether micrometastasis exists in the breast cancer cases. Thus, *CD47* may be a novel biological marker that predicts the number of highly malignant circulating tumor cells that escape from the immune systems in breast cancer.

To further characterize the function of *CD47* in bone marrow and peripheral blood, we examined *SIRPA* expression in the same breast cancer samples. In doing so, we obtained the novel finding that the expressions of *CD47* and *SIRPA* are markedly associated. The correlation between *CD47* and *SIRPA* was significantly stronger in breast cancer patients than in control cases. In control cases, the CD47-SIRPA signaling system is activated in bone marrow and in peripheral blood, reflecting homeostatic regulation in the hematopoietic system. In the breast cancer cases, carcinogenicity may promote the CD47-SIRPA cell signaling system in bone marrow and in peripheral blood, thereby possibly promoting micrometastases. We suggest that expression of the CD47/SIRPA signal system indicates the presence of cancer-specific microenvironmental areas that support micrometastasis.

In conclusion, our data indicate that *CD47* is a significant prognostic indicator for DFS, and our study is one of the first to report a host factor in bone marrow with prognostic significance. With regard to patient care, many cases require postoperative adjuvant chemotherapy. Due to the associated adverse effects of such treatment, reliable prognostic markers for recurrence and metastasis would greatly

Published OnlineFirst August 12, 2010; DOI:10.1158/1078-0432.CCR-10-0349

improve patient management. We suggest that this biomarker may fill that need for enhanced patient care.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank T. Shimooka, K. Ogata, M. Kasagi, Y. Nakagawa, and T. Kawano for their technical assistance, and Kelly K. Chong for her editorial assistance.

Grant Support

CREST, Japan Science and Technology Agency (JST); Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research, grant numbers 19591509, 19390336, 20390360, 20591547, 20659209, 20790960, 20790961 and 21791295.

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Received 02/08/2010; revised 07/28/2010; accepted 07/29/2010; published OnlineFirst 08/12/2010.

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Ann Surg Oncol (2010) 17:2601–2607
DOI 10.1245/s10434-010-1086-0

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Clinical Significance of *Stanniocalcin 2* as a Prognostic Marker in Gastric Cancer

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ABSTRACT

Background. Stanniocalcins are glycoprotein hormones that were originally found in the endocrine gland of bony fish. Microarray expression data from 32 paired samples of gastric cancer and normal mucosa in a public microarray database showed that the expression level of *Stanniocalcin 2* was higher in gastric cancer than in normal gastric mucosa. The clinical significance of *Stanniocalcin 2* expression has been observed for several cancers. However, the relationship between *Stanniocalcin 2* and clinicopathological factors in gastric cancer has not yet been investigated.

Materials and Methods. We examined the clinical significance of *Stanniocalcin 2* in gastric cancer in 108 gastric cancer samples using real-time reverse transcription-polymerase chain reaction (RT–PCR). Immunohistochemical studies were conducted with paraffin sections. The suppression analysis of *Stanniocalcin 2* using siRNA was done to determine *Stanniocalcin 2*'s biological roles.

Results. The level of *Stanniocalcin 2* in cancer tissues was higher than in normal tissues ($P = .0002$). The high *Stanniocalcin 2* expression group ($n = 54$) had more progressive lymph node metastasis ($P = .07$) and venous invasion ($P = .028$) than the low-expression group ($n = 54$). High *Stanniocalcin 2* expression was an independent prognostic factor in gastric cancer patients ($P = .02$). Moreover, siRNA suppression of *Stanniocalcin 2* in a gastric cancer cell line inhibited cellular proliferation ($P < .05$).

Conclusions. The high expression level of *Stanniocalcin 2* in gastric cancer tissues could be a very powerful marker of poor prognosis. Therefore, *Stanniocalcin 2* is a promising candidate for a molecular target for the treatment of gastric cancer.

Electronic supplementary material The online version of this article (doi:10.1245/s10434-010-1086-0) contains supplementary material, which is available to authorized users.

First Received: 31 January 2010;
Published Online: 27 April 2010

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The incidence of gastric cancer has been decreasing recently, and many cancer patients remain free of the disease following curative resection and postoperative adjuvant chemotherapy.[1,2] Postoperative adjuvant chemotherapy significantly improves the prognosis of gastric cancer patients compared with untreated patients.[3] However, if we could identify patients with a low risk of metastasis/recurrence just after surgery, those patients could avoid expensive postoperative adjuvant chemotherapy with adverse events. To provide optimal treatments for individual patients, identification of the true predictors of poor prognosis/recurrence is extremely important in gastric cancer patients. Moreover, it is expected that such a marker might also be a promising treatment target.

We first focused on genes that are specifically expressed in gastric cancer tissues to detect candidate markers for cancer progression and poor prognosis. We analyzed the expression microarray data of gastric cancer and normal gastric mucosa registered with the Gene Expression Omnibus public database by D'Errico et al.(GSE13911) and found high expression of the *stanniocalcin 2* (*STC2*) gene in gastric cancer (Supplementary Fig. A, Supplementary Table A).[4] *STC2* was of particular interest since we had previously reported that its expression level is higher in colorectal cancers and that it is an independent prognostic factor in that disease.[5]

Stanniocalcins are glycoprotein hormones that are involved in calcium and phosphate homeostasis.[6–9] *STC2* expression is clinically significant in several cancers, including colorectal cancer, neuroblastoma, prostate cancer, and renal cell carcinoma.[5,10–12] It is clear that *STC2* overexpression in cancer tissues is associated with carcinoma development and poor prognosis. On the other hand, *STC2* expression is also upregulated in estrogen receptor (ER)-positive breast cancer patients; however, the prognosis for these cases is good. The clinical significance of *STC2* in cancer is controversial and depends on cancer type.[13–15] The relationship between *STC2* expression and clinicopathological factors in gastric cancer has not yet been investigated.

In this study, we examined the effect of *STC2* by reanalysis of a GEO database to assess if this gene might be a good prognostic indicator and candidate molecular target in gastric cancer as in colorectal cancer.[5] We examined the expression levels of *STC2* in gastric cancer samples using real-time reverse transcription-polymerase chain reaction (RT–PCR) to evaluate whether the expression level of *STC2* in cancer tissues can be used as a prognostic tool in gastric cancer patients. Moreover, we performed *STC2* suppression analysis to determine if *STC2* might play a role in the proliferation of gastric cancer cells.

MATERIALS AND METHODS

Clinical Samples and Cell Lines

A total of 108 gastric cancer samples and paired noncancerous samples were obtained during surgery. These samples were used in accordance with the institutional ethical guidelines of Kyushu University after obtaining written informed consent. All patients underwent resection of the primary tumor at Kyushu University Hospital and affiliated hospitals between 1990 and 2001. All patients were clearly identified as having gastric cancer based on the clinicopathological findings: age 65.7 ± 11.3; male:female = 76:32; histological type well, mod:poor, sig = 54:53; tumor depth m:sm:mp:ss:se:si = 16:13:16:27:30:6; lymph node metastasis (+):(−) = 69:39; lymphatic permeation (+):(−) = 69:39; venous permeation (+):(−) = 28:80; and stage I:II:II:III:IV = 44:19:23:22. Resected (T) and paired (N) tissues were immediately cut and embedded in Tissue Tek OCT medium (Sakura, Tokyo, Japan), frozen in liquid nitrogen, and kept at −80°C until RNA extraction. Complementary DNA (cDNA) was synthesized from 8.0 μg total RNA as previously described.[16] None of the patients had received preoperative chemotherapy and/or radiotherapy. Among 108 cases of gastric cancer entered in this study, 31 cases had 5-FU based postoperative chemotherapy.

Human gastric cancer cell lines NUGC4 were provided by the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). NUGC4 was maintained in RPMI1640 containing 10% fetal bovine serum (FBS) and supplemented with 100 units/mL penicillin and 100 units/mL streptomycin sulfates, and cultured in a humidified 5% CO_2 incubator at 37°C.

Real Time Quantitative RT–PCR

Gene-specific oligonucleotide primers were designed for PCR. The following primers were used: *STC2*: 5′-GGA GATGATCCATTTCAAGGAC-3′ (sense) and 5′-AGAGC TTGGTTCTGTCCACCT-3′ (antisense); glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): 5′-GTCAACG GATTTGGTCTGTATT-3′ (sense) and 5′-AGTCTTCTGG GTGGCAGTGAT-3′ (antisense). These primers spanned more than 2 exons to avoid amplification of contaminating genomic DNA. PCR amplification was performed in the LightCycler System (Roche Applied Science, Indianapolis, IN), using the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Applied Science) as described previously.[17] *STC2* mRNA amplification conditions consisted of initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and elongation at 65°C for 10 s. Melting curve analysis was performed to distinguish specific products from nonspecific products and primer dimers. The relative expression levels of these genes were obtained by normalizing the amount of mRNA to that of *GAPDH* mRNA as an endogenous control in each sample.

Immunohistochemistry

Immunohistochemical studies of STC2 were performed on 15 surgical specimens from gastric cancer patients. Formalin-fixed, paraffin-embedded tissues were deparaffinized, blocked, incubated with specific antibodies for 1 h at room temperature, and detected using ENVISION reagents (ENVISION1 Dual Link/HRP, Dako Cytomation, Glostrup, Denmark). All sections were counterstained with hematoxylin. Primary mouse monoclonal anti-STC2 antibody (Abnova, Taipei City, Taiwan) was used at a dilution of 1:50.

STC2 RNA Interference

STC2-specific siRNA (Invitrogen Corp., Carlsbad, CA) was used in this assay. Silencer Negative Control No. 1 siRNA (Applied Biosystems/Ambion, Austin, TX) was applied as a negative control siRNA. Lipofectamine RNAi MAX (Invitrogen Corp.) and *STC2*-specific siRNA were mixed in 6-well flat-bottom microtiter plates. After incubation, the gastric cancer cell line NUGC4 was seeded in a volume of 2 mL in the microtiter plates and incubated in a humidified atmosphere (37°C and 5% CO_2). Reduced *STC2* expression was confirmed by quantitative RT–PCR and Western blot analysis.

Immunoblot Analysis

Total protein was extracted from NUGC4 after *STC2* RNA interference. Aliquots of total protein were electrophoresed in NuPAGE 4–12% Bis–Tris Gels (Invitrogen Corp.). STC2 proteins were detected using anti-STC2 antibody (Abnova) diluted 1:250. These proteins were normalized to the level of β-actin protein (Cytoskeleton, Denver, CO) diluted 1:500. Western blot analysis was performed using the iBlot Western Detection Chemiluminescent Kit (Anti-Mouse) (Invitrogen Corp.). Band intensity was calculated using ImageJ software.

In Vitro Proliferation Assay

Proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Roche Diagnostics Corp.). After the addition of siRNA, the cells were further cultured for 0–72 h and the absorbance of the samples was measured as previously described.[18]

Statistical Analysis

For continuous variables, the data were expressed as the means ± standard deviation (s.d.). The relationship between *STC2* expression and clinicopathological factors and the in vitro assay data were analyzed using *t* test, chi-square test, and ANOVA. Overall survival curves were plotted according to the Kaplan–Meier method measured from the day of surgery, and the log-rank test was applied for comparison. All differences were statistically significant at the level of $P < .05$. Relative multivariate significance of potential prognostic variables was examined. Cox proportional hazard regression was used to test the independent prognostic contribution of *STC2*. Statistical analysis was performed with the JMP software package (SAS Institute Inc., Cary, NC).

RESULTS

Expression of STC2 in Clinical Gastric Cancer Tissues

We examined the clinical significance of *STC2* in 108 primary gastric cancer tissues. Real-time RT–PCR was

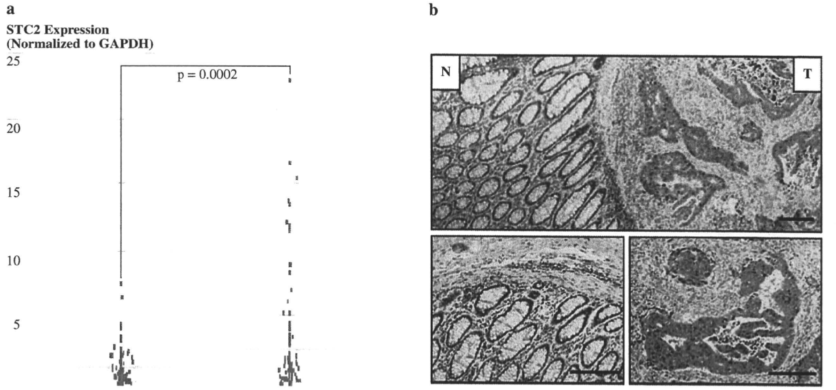

FIG. 1 *STC2* expression in normal gastric mucosa and gastric cancer tissues. **a** *STC2* mRNA expression in cancerous (T) and noncancerous (N) tissues from gastric cancer patients as determined by real-time RT-PCR. T group (n = 108), *STC2* mRNA (T)/*GAPDH* mRNA (T); N group (n = 108), *STC2* mRNA (N)/*GAPDH* mRNA (N) (P = .0002). Horizontal lines indicate the mean value of each group. **b** Representative immunohistochemical staining of STC2 was stronger in gastric cancer tissues (T) than in the corresponding noncancerous tissues (N). Scale bar; 200 μm (original magnification 200×)

TABLE 1 *STC2* gene expression and clinicopathological factors for 108 gastric cancer patients

Factors	*STC2/GAPDH*		*P* value
	High expression $n = 54$	Low expression $n = 54$	
Age	64.9 ± 11.3	66.5 ± 11.4	.48
Gender			
Male	37	39	.67
Female	17	15	
Histology			
Well, moderate	30	24	.21
Poor, signet	23	30	
Depth			
m, sm, mp	13	16	.51
ss, se, si	41	38	
Lymph node metastasis			
Absent	15	24	.07
Present	39	30	
Lymphatic invasion			
Absent	18	21	.55
Present	36	33	
Venous invasion			
Absent	35	45	.028*
Present	19	9	
Liver metastasis			
Absent	51	51	1
Present	3	3	
Peritoneal dissemination			
Absent	46	48	.57
Present	8	6	
Stage			
1, 2	30	33	.55
3, 4	24	21	

Well well differentiated, *Moderate* moderately differentiated, *Poor* poorly differentiated, *Signet* signet ring cell

* $P < .05$

used to define the expression levels of *STC2* mRNA in gastric cancer tissues (T) (2.74 ± 4.3; mean ± S.D.) ($n = 108$) and the corresponding normal tissues (N) (1.07 ± 1.21) ($n = 108$) in 84 of the 108 cases (77.7%). *STC2* mRNA expression levels in (T) were significantly higher than in (N) ($P = .0002$) (Fig. 1a).

To investigate protein expression of STC2, immunohistochemical analysis was performed on 15 gastric cancer samples. Expression of STC2 was localized to the cytoplasm in the cancer and the normal tissues. Consistent with RT–PCR data, STC2 staining was stronger in gastric cancer tissues than in the corresponding normal gastric epithelial tissues (Fig. 1b).

Clinicopathological Significance of STC2 mRNA Expression

Clinicopathological factors differed significantly in the high *STC2* expression group ($n = 54$). There was more venous invasion ($P = .028$) than in the low *STC2* expression group ($n = 54$) (Table 1). With regard to overall survival (Fig. 2), patients in the high *STC2* expression group ($n = 54$) had a significantly poorer prognosis than those in the low *STC2* expression group ($n = 54$) ($P = .0081$). Univariate analysis showed that depth of tumor invasion ($P < .0001$), lymph node metastasis ($P < .0001$), lymphatic invasion ($P < .0001$), venous invasion ($P = .0001$), peritoneal dissemination ($P < .0001$), and *STC2* expression ($P = .0072$) were significantly correlated with overall survival (Table 2). The multivariate regression analysis revealed that inclusion in the *STC2* high-expression group [relative risk (RR), 1.53; 95% confidence interval (CI), 1.06–2.29; $P = .02$] was an independent predictor of overall survival.

STC2 RNAi Inhibits Proliferation in Vitro

Because *STC2* mRNA upregulation in cancer tissues is associated with poor prognosis, *STC2* suppression analysis was performed with 2 different *STC2* siRNA (siRNA1 or siRNA2) using the gastric cancer cell line NUGC4. *STC2* suppression by siRNA was confirmed with quantitative RT–PCR and Western blots of the control siRNA and *STC2* siRNA groups (Fig. 3a, b). Evaluation of proliferation potency in the *STC2* siRNA groups using the MTT assay showed that proliferation rates were significantly inhibited in both *STC2* siRNA groups compared with the control siRNA group and the parent cell line NUGC4 (Fig. 3c).

DISCUSSION

In this study we found that the expression level of *STC2* in primary gastric cancer is higher than in corresponding normal gastric tissues, which was consistent with the results of a previous expression microarray study. We also showed that the high expression level of *STC2* is an independent prognostic factor in gastric cancers.

One of the important characteristics of *STC2* is that its contribution to carcinoma development depends on cancer type. *STC2* overexpression contributes to poor prognosis or cancer recurrence in colorectal cancer, neuroblastoma, prostate cancer, and renal cell carcinoma.[5,10–12] However, *STC2* overexpression in breast cancer is associated with good prognoses, particularly ER-positive breast cancers.[13–15] ER-positive breast cancers are usually low-grade malignancies and are effectively treated with hormonal

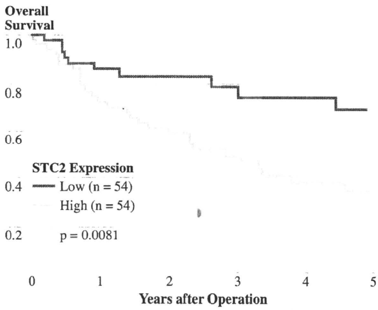

FIG. 2 High expression level of *STC2* in gastric cancer is associated with poor prognosis. Kaplan–Meier overall survival curves of gastric cancer patients according to the level of *STC2* mRNA expression. High *STC2* expression group ($n = 54$), Low *STC2* expression group ($n = 54$) (* $P = .0081$)

therapies, unlike HER2 and triple-negative types, which may be the basis of the good prognosis.[19,20]

STC2 (−/−) mice are 10%–15% larger and grow at a faster rate than wild-type mice; however, the growth of transgenic mice overexpressing human *STC2* is inhibited.[21,22] Therefore, it has been suggested that the function of *STC2* is related to the inhibition of cellular proliferation. However, the in vitro data collected in this study show that *STC2* siRNA treatment inhibits the proliferative capacity of a gastric cancer cell line. As for gastric cancers, *STC2* may contribute to cancer development and poor prognosis by controlling proliferative capacity.

Some reports have suggested that cells expressing *STC2* show resistance to apoptosis. Ito et al. reported that *STC2* expression is induced by oxidative stress and hypoxia and that this expression contributes to antiapoptotic activity and survival of ischemia nerve cells.[23] Moreover, *STC2* functions to protect cells from apoptosis in hypoxic ovarian cancer cell lines.[24] On the other hand, late relapse cases in breast cancer had overexpressed *STC2* in both the primary and recurrence sites.[25] From these data, breast cancer cells expressing *STC2* appear to have antiapoptotic activity and act to maintain cancer dormancy for late relapse, as well as in carcinoma development.

We examined the relationships between *STC2* expression and clinicopathological factors in gastric cancer. As a result, the high expression levels of *STC2* were related to a positive rate of venous invasion in clinical gastric cancer patients, suggesting that cancer cells that overexpress *STC2* confer aggressive invasive ability. Some reports have shown a connection between the high expression levels of *STC2* in cancer cells and the invasive ability by activation of HIF-1 alpha and MMP.[10,24] Moreover, an additional interesting characteristic of *STC2* expression can be found in ovarian cancer. Buckanovich et al. compared the expression microarray profiles between tumor vessels and normal ovary vessels from ovarian cancer cases using endothelial cells obtained by laser microdissection. They found that *STC2* is highly expressed in tumor vascular endothelial cells and that the overexpression was associated with postoperative recurrences.[26] These observations suggest that *STC2* expression in cancer samples contributes to carcinoma development through vascular endothelial cells of the host, as well as cancer cells.

Because *STC2* has been associated with cancer progression and prognosis in several cancers, it has been speculated that this gene may be important for progression and survival of several cancers including gastric cancer. To use *STC2* inhibition as a molecular therapy, the issue of side effects is very important. STC2 (−/−) mice experience growth retardation; however, lethality is rare.[21] When

TABLE 2 Results of univariate and multivariate analyses of clinicopathological factors affecting overall survival rate following surgery

Clinicopathological variable	Univariate analysis			Multivariate analysis		
	RR	95% CI	*P* value	RR	95% CI	*P* value
Gender (male/female)	1.01	0.69–1.44	.94	–	–	–
Histology grade (well, moderate/poor, signet)	1.32	0.96–1.84	.09	–	–	–
Depth (m, sm, mp/ss, se, si)	2.96	1.63–7.34	<.0001*	1.00	0.5–2.63	.9
Lymph node metastasis (negative/positive)	12.5	3.83–77.2	<.0001*	5.75	1.64–36.7	.003*
Lymphatic invasion (negative/positive)	12.6	3.84–77.5	<.0001*	5.57	1.46–37.3	.009*
Venous invasion (negative/positive)	3.76	1.96–7.16	.0001*	1.34	0.65–2.75	.43
Peritoneal dissemination (negative/positive)	6.54	3.09–13.1	<.0001*	5.41	2.37–12.1	.0001*
STC2 mRNA expression (low/high)	1.58	1.13–2.29	.0072*	1.53	1.06–2.29	.02*

RR relative risk, *CI* confidence interval

* $P < .05$

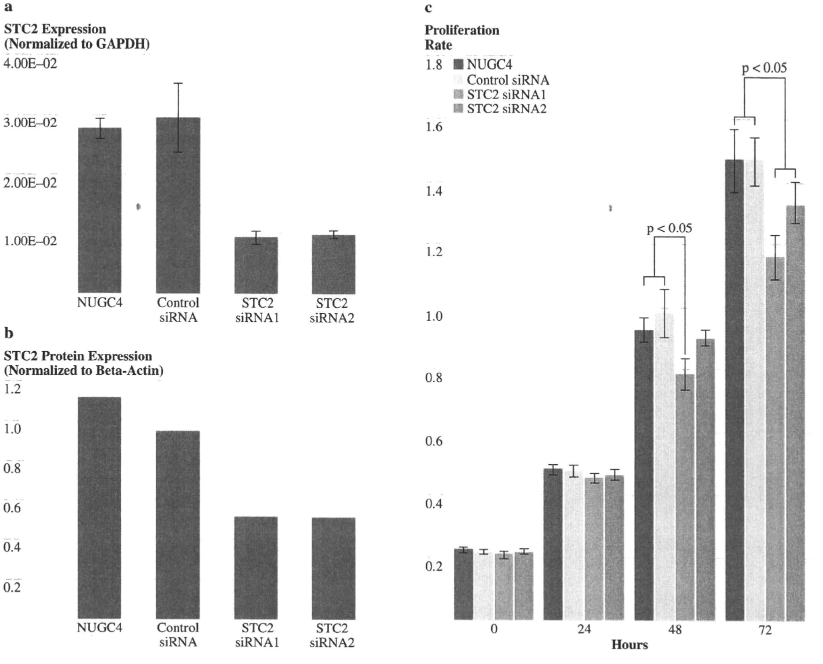

FIG. 3 Proliferation assay with RNA interference of *STC2* in the NUGC4 gastric cancer cell line. **a** Reduced *STC2* expression was confirmed by quantitative RT-PCR analyses in *STC2* siRNA cells, compared with parent NUGC4 cells and control siRNA cells. *STC2* expression was normalized by *GAPDH* expression. The data represent the mean ± S.D. **b** Western blot analysis of STC2 in *STC2* siRNA cells, control siRNA cells, and parent NUGC4 cells. These proteins were normalized to the level of β-actin expression. Intensity was measured using ImageJ software. **c** MTT assay. The proliferation rate of *STC2* siRNA (1 and 2) cells was suppressed compared with that of control siRNA and parent NUGC4 cells. The data represent the mean ± SD (* $P < .05$)

we think about clinical application of *STC2* inhibition, this report will become very important evidence. Further study is needed to determine if serious side effects would result from strong inhibition of *STC2* as a treatment target.

In this study, it was clear that the expression levels of *STC2* in gastric cancer were higher than those in normal gastric mucosa and that they are associated with cancer progression and poor prognosis. The high expression level of *STC2* could be a powerful marker of poor prognosis. Moreover, *STC2* may be a promising candidate for targeted treatment of gastric cancer.

ACKNOWLEDGMENT We thank T. Shimooka, K. Ogata, M. Kasagi, Y. Nakagawa, and T. Kawano for their technical assistance. This work was supported in part by the following grants and foundations: CREST, Japan Science and Technology Agency (JST); Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research, grant numbers 21679006, 20390360, 20590313, 20591547, 21591644, 21592014, 20790960, 21791297, 21229015, 20659209, and 20012039; NEDO (New Energy and Industrial Technology Development Organization) Technological Development for Chromosome Analysis; Grant of Clinical Research Foundation (2008–2010).

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Biochemical and Biophysical Research Communications 395 (2010) 258–263

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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Long-term culture following ES-like gene-induced reprogramming elicits an aggressive phenotype in mutated cholangiocellular carcinoma cells

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ARTICLE INFO

Article history:
Received 17 March 2010
Available online 7 April 2010

Keywords:
Cholangiocelluar carcinoma
Malignancy
Reprogramming
Induced pluripotent cancer
Cancer stem cells

ABSTRACT

Background: We recently reported that gastrointestinal (GI) cancer cells can be reprogrammed to a pluripotent state by the ectopic expression of defined embryonic stem (ES)-like transcriptional factors. The induced pluripotent cancer (iPC) cells from GI cancer were sensitized to chemotherapeutic agents and differentiation-inducing treatment during a short-term culture, although a phenotype induced by long-term culture needs to be studied.
Methods: A long-term cultured (Lc)-iPC cells were produced in GI cancer cell lines by virus-mediated introduction of four ES-like genes-*c-MYC*, *SOX2*, *OCT3/4*, and *KLF4*-followed by a culture more than three months after iPC cells induction. An acquired state was studied by expression of immature-related surface antigens, Tra-1-60, Tra-1-81, Tra-2-49, and Ssea-4; and epigenetic trimethyl modification at lysine 4 of histone H3. Sensitivity to chemotherapeutic agents and tumorigenicity were studied in Lc-iPC cells.
Results: Whereas the introduction of defined factors of iPC cells once induced an immature state and sensitized cells to therapeutic reagents, the endogenous expression of the ES-like genes except for activated endogenous *c-MYC* was down-regulated in a long-term culture, suggesting a high magnitude of the reprogramming induction by defined factors and the requirement of therapeutic maintenance in Lc-iPC cells from cholangiocellular carcinoma HuCC-T1 cells, which harbor $TP53^{R175H}$ and $KRAS^{G12D}$. The Lc-iPC cells showed resistance to 5-fluorouracil in culture, and high tumorigenic ability with activated endogenous *c-MYC* in immunodeficient mice.
Conclusion: The Lc-iPC cells from HuCC-T1 might be prone to an undesirable therapeutic response because of an association with the activated endogenous *c-MYC*. To consider the possible therapeutic approach in GI cancer, it would be necessary to develop a predictive method for evaluating the improper reprogramming-associated aggressive phenotype of iPC cells.

1. Introduction

Cancer is a genetic and epigenetic disorder [1] characterized by abnormal differentiation of cells [2]. Although genetic alterations, including activation of tumor-promoting oncogenes and inactivation of growth constraint tumor suppressor genes, are involved in stepwise carcinogenesis, abnormal epigenetic modifications, which are irrelevant to genetic codes, are undoubtedly important for generating malignant cancer cell phenotypes. Nevertheless, the magnitude of effect of epigenetic corrections remains to be understood.

Non-cancerous somatic cells have recently been reprogrammed to a pluripotent state (induced pluripotent stem [iPS] cells) by the ectopic expression of defined embryonic stem (ES)-like transcriptional factors, *c-MYC*, *SOX2*, *OCT3/4*, and *KLF4* [3]. We have shown that introducing defined factors induced pluripotent cancer (iPC) cells from human gastrointestinal (GI) cancer [4]. The iPC cells were sensitized to chemotherapeutic agents and differentiation-inducing treatment, and tumorigenicity was reduced after a short-term culture. While the defined factor-induced reprogramming occurs fundamentally at the epigenetic level [5], the study

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doi:10.1016/j.bbrc.2010.03.176

indicates the possibility that epigenetic modifications could generate a significant magnitude to induce the sensitivity to differentiation induction and chemotherapy reagents in GI cancer. Nevertheless, long-term behavior of iPC cells with deleterious mutations remains to be studied. Furthermore, safety issues involved in synthesizing tumor-producing iPS cells from non-cancerous somatic cells should be addressed to avoid an unexpected malignant transformation in the course of medical innervations in human diseases.

In the present study, we introduced defined factors in three GI cancer cell lines to establish short-term cultured (Sc)-iPC cells, similar to iPS/iPC cells production [3,4], which were cultured to assess long-term cultured (Lc)-iPC cells. The aggressive phenotype was observed in a cell line with genetic mutations, which were associated with endogenous *c-MYC* activation.

2. Materials and methods

For detailed information see Supplementary Information.

2.1. Cell culture

Three GI cell lines were maintained in DMEM (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS) at 37 °C in a 5% humidified CO_2 atmosphere, and used for reprogramming. Colorectal cancer DLD-1 and hepatocellular carcinoma PLC/PRF/5 (PLC) cells were purchased from the Japanese Cancer Research Resources Bank (Tokyo, Japan), and cholangiocellular carcinoma HuCC-T1 cells were a gift from Dr. Gregory J. Gores. PLAT-E cells (Cell Biolabs, San Diego, CA) were maintained in DMEM containing 10% FBS, 1 μg/ml puromycin (Sigma), and 10 μg/ml blasticidin (Sigma). 293FT (Invitrogen, Carlsbad, CA) cells were maintained in DMEM containing 10% FBS and 500 μg/ml geneticin (Invitrogen). The medium was replaced every 2 or 3 days.

2.2. Transfection and Sc-iPC cells production

293FT cells were used to produce the lentiviral vector harboring the mouse retroviral receptor by introducing the pLenti6/UbC/mSlc7a1 plasmid (Addgene Cambridge, MA). After transfection, the lentivirus was purified by filtration. Cancer cells transfected using the lentivirus mSlc7a1, as described above, were infected with retroviruses in the medium. Retroviral vectors were produced by transfecting constructed plasmids into PLAT-E cells, and the culture medium was purified by filtration, concentrated, and used for infection.

Eight days after transduction, the transfected cells in 10% FBS–DMEM were harvested and re-plated on culture plates coated with Matrigel hESC-qualified Matrix (BD Biosciences, Bedford, MA). The medium was replaced the next day with mTeSR1 medium (StemCell Technologies, Vancouver, BC, Canada). Post-Sc-iPC production was induced as described previously [4]. Post-Sc-iPC cells were cultured for an additional 10 weeks in 10% FBS–DMEM primary culture medium until day 90 to induce Lc-iPC cells.

2.3. Quantitative reverse transcription (RT)-PCR

Total RNA was extracted, reverse transcribed, and subjected to quantitative real-time RT-PCR using the LightCycler TaqMan Master kit (Roche Diagnostics GmbH, Mannheim, Germany) and the LightCycler FastStart DNA Master SYBR Green I kit (Roche).

2.4. Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde, immunostained with specific antibodies, and visualized by fluorescence microscopy (BZ-8000; Keyence, Osaka, Japan). For adipogenic or osteogenic differentiation, iPC cells were treated with the supplements (R&D Systems, Minneapolis, MN) in culture medium for 2 weeks.

2.5. Chromatin immunoprecipitation assay

Cells were cross-linked with 1% formaldehyde, immunoprecipitated using the anti-trimethyl lysine 4 histone H3 antibody (Nippon Gene, Toyama, Japan), and used for semi-quantitative PCR.

2.6. Invasion and chemosensitivity assay

Cell invasion was assessed using a CytoSelect Cell Invasion Assay kit (Cell Biolabs) with or without 100 nM retinol (RA; Sigma) or 10 nM 1,25-dihydroxy-vitamin D3 (VD3; Sigma) treatment. The *in vitro* chemotherapeutic sensitivity to 5-fluorouracil (5-FU; Kyowa Hakkou, Tokyo, Japan) was assessed by the 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay.

2.7. In vivo tumorigenicity assay

In vivo tumorigenicity was evaluated by transplanting cells into immunodeficient NOD.CB17-Prkdcscid/J (NOD-scid) mice (Charles Liver, Yokohama, Japan). Eight weeks after injection, tumors were dissected, measured, and fixed with 4% paraformaldehyde. Paraffin-embedded tissue was sliced, stained with hematoxylin–eosin (HE), and subjected to immunohistochemistry using the anti-c-Myc antibody.

3. Results

3.1. Introduction of defined ES-like transcriptional factors elicited an immature state in cancer cells

The experimental schedule is shown in Fig. 1A. Four defined factors, *c-MYC, SOX2, OCT3/4*, and *KLF4*, were transfected into three cancer cell lines, HuCC-T1, PLC, and DLD-1, using the retroviral packaging cell line PLAT-E at day 0. Cells were trypsinized, harvested, and re-plated onto Matrigel-coated plates at day 8 after transfection. The next day, culture dishes were replaced with human ES cell culture medium (mTeSR1). Three weeks after transduction, round-shaped, colonies, which were distinct from background cells, started appearing. The colonies were picked up at day 30 (Supplementary Fig. S1) and *NANOG* expression was confirmed using fluorescence microscopic observation for green fluorescent protein (*Gfp*) expression after transiently transfecting the *NANOG* promoter-*Gfp* vector (data not shown). We obtained approximately ~10 *Gfp*-positive Sc-iPC colonies from 1×10^4 cancer cells.

Quantitative RT-PCR analysis with specific primers showed that a temporal increase in the expression of four transfected genes (Tgs), *c-MYC, SOX2, OCT3/4*, and *KLF4*, was observed at day 5, although they were absent at day 0. The expression decreased to low or undetectable levels on day 30, suggesting gene silencing (Fig. 1B). In contrast, the transfected and endogenous expression levels of ES-like genes, *c-MYC, SOX2, OCT3/4, KLF4, NANOG*, and *REX1*, apparently increased at day 30, compared with those at day 0 (Fig. 1C). The Sc-iPC cells data were consistent with previous findings on non-cancerous somatic cells (iPS cells; [3]) and other cancer cells (iPC cells; [4]).

3.2. Sc-iPC cells expressed immature-related surface antigens and associated epigenetic modifications

To assess the acquisition of an immature state, Sc-iPC cells at day 30 were stained with immature-related surface antigens.

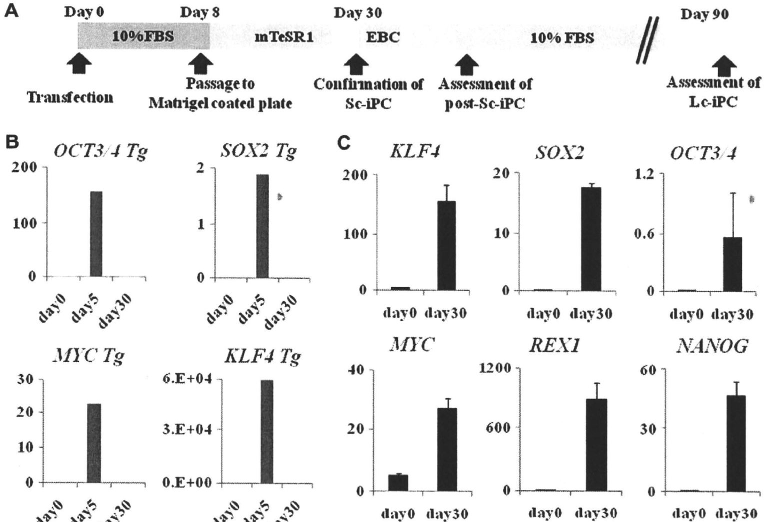

Fig. 1. Induction of ES-like genes in human GI cancer cells. Lentiviral and retroviral-mediated ES-like gene transfer induced a pluripotent state in three GI cancer cell lines to form iPC cells from three GI cancer cell lines. (A) Time course schedule of Sc-iPC, post-Sc-iPC, and Lc-iPC cells production. (B,C) Quantitative RT-PCR of Tg (B) and total mRNA (C) demonstrated temporal transgene expression after transfection and expressed undifferentiated ES-like genes *c-MYC*, *SOX2*, *OCT3/4*, *KLF4*, *NANOG*, and *REX1* in Sc-iPC cells from HuCC-T1 cells. The expression of mRNA copies was normalized against *GAPDH* mRNA expression.

Immunocytochemistry revealed that induced cells were positively stained with tumor-related antigens (Tra)-1-60, Tra-1-81, Tra-2-49, and stage-specific embryonic antigen (Ssea)-4 (Fig. 2A), indicating the maintenance of an immature state in Sc-iPC cells. Similar data were obtained for all three cell lines, and a representative result from HuCC-T1 and PLC cells are shown.

Epigenetic modifications were confirmed by assessing histone methylation. Chromatin immunoprecipitation using the trimethyl histone H3 protein at lysine 4 (H3K4) antibody indicated that H3K4 of *NANOG* and *OCT3/4* promoters were trimethylated in Sc-iPC cells, but not in parent nor post-Sc-iPC cells; post-Sc-iPC cells were prepared from Sc-iPC cells by culturing cells in embryonic body culture conditions (EBC) for 1 week and then in primary culture medium for another 1 week (Fig. 2B). Trimethylation of the *SOX2* promoter was detected in Sc-iPC cells and to a lesser extent in parental cells. The results indicated that the promoters of ES-like genes were activated in Sc-iPC cells.

3.3. Sc-iPC cells showed differentiation in vitro

To assess their ability to differentiate, we placed Sc-iPC cells in differentiation culture medium with an osteogenic or adipogenic supplement for 2 weeks (Fig. 2C and E). The data indicated that Sc-iPC cells were susceptible to differentiation, which was studied by positive staining for Osteocalcin (specific for osteocytes) or Fabp4 (specific for adipose cells), but not for parental cells (Fig. 2D and F; data not shown). Quantitative RT-PCR analysis with specific primers showed that post-Sc-iPC cells from HuCC-T1 were expressing paired box 6 (*PAX6*, representing ectoderm), microtubule-associated protein 2 (*MAP2*, representing ectoderm) and E-cadherin (*CDH1*, representing endoderm). Taken together, it is suggested that Sc-iPC cells have ability to express differentiation markers into three germ layers.

3.4. Sc-iPC cells were sensitized to differentiation-inducing reagents

The proliferation of Sc-iPC (Supplementary Fig. S2A) after 48 h incubation was not significantly different from that of parental HuCC-T1 cells. In contrast, treatment with differentiation inducers of RA or VD3 for 48 h resulted in a significant decrease in the invasiveness ratio of post-Sc-iPC cells compared to HuCC-T1 parental cells (Supplementary Fig. S2B and C). RA is commonly used for the treatment of acute promyelocytic leukemia, which involves differentiation of immature leukemic promyelocytes into mature granulocytes [6]. It has been suggested that RA or VD3 treatment is effective for inducing differentiation of post-Sc-iPC cells.

3.5. Cultured Lc-iPC cells showed increased proliferation and 5-FU resistance

In proliferation assay, Lc-iPC cells from HuCC-T1 proliferated in a high magnitude, compared with parental cells significantly (Fig. 3A). To compare the sensitivity of Lc-iPC cells to anti-cancer drugs with that of parental cells, we performed the MTT assay for 5-FU. The IC_{50} value of Lc-iPC cells from HuCC-T1 cells was significantly higher than that of parental cells (Fig. 3B), indicating that a long-term culture may elicit malignant transformation of iPC cells compared to clones immediately after inducing reprogramming [4].

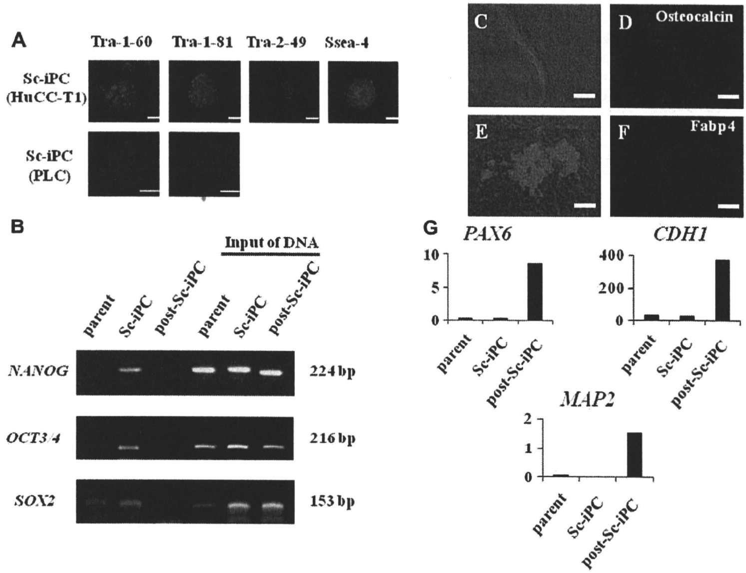

Fig. 2. An immature state and multi-differentiation potential of Sc-iPC cells. (A) Immature-related surface antigens in Sc-iPC cells from HuCC-T1 and PLC were analyzed; Tra-1-60, Tra-1-80, Tra-2-49, and Ssea-4. Bar, 200 μm; Original magnification, 200×. (B) Histone modification status in parental, Sc-iPC, and post-Sc-iPC cells from HuCC-T1 was analyzed using chromatin immunoprecipitation with the trimethyl-K4 H3 antibody. H3 lysine 4 was methylated in the promoter regions for *NANOG* and *OCT3/4* in Sc-iPC cells, but not in parental and post-Sc-iPC cells. Respective sheared chromatin samples were used as control for semi-quantitative PCR. Lineage-directed differentiation of Sc-iPC cells to osteocytes or adipocytes demonstrated that osteocyte-differentiated Sc-iPC cells (C) were positive for Osteocalcin (D) and adipocyte-differentiated Sc-iPC cells (E) were positive for Fabp4 (F). Bar, 200 μm; Original magnification, 100×. (G) *PAX6*, *CDH1*, and *MAP2* expressions were evaluated by quantitative RT-PCR in parental HuCC-T1, Sc-iPC, and post-Sc-iPC cells. The expression of mRNA copies was normalized against *GAPDH* mRNA expression.

3.6. Lc-iPC cells formed c-Myc-positive tumors in immunodeficient mice

Since the preceding proliferation assay indicated high activity, we inoculated HuCC-T1-derived Lc-iPC cells into NOD-scid mice to assess the *in vivo* tumorigenicity. Tumors were formed 4 weeks after inoculation (Fig. 3C and D). Interestingly, 8 weeks after injection the size of these tumors was significantly larger in Lc-iPC cells as compared to that in parental cells (Fig. 3E). The H-E staining indicated that tumors of Lc-iPC cells showed no apparent teratomas, but indicated the presence of a proliferating phenotype as compared to those of parental cells (Fig. 3F and G). Immunohistochemical staining with anti-c-Myc antibody indicated that Lc-iPC-formed tumors were positive for c-Myc, compared to parental cell-derived tumors (Fig. 3H and I), suggesting that c-Myc activation may play a role, at least partially, in the development of a malignant Lc-iPC cell phenotype.

3.7. Lc-iPC cells expressed the activated endogenous c-MYC gene but not other ES-like transcriptional factors

To elucidate the mechanism of activated *c-MYC* expression in Ls-iPC cell-derived tumor in NOD-scid mice, the expressions of endogenous and transgenic ES-like transcriptional factors were investigated in Lc-iPC cells from HuCC-T1. As shown in Fig. 4A, the expression of the ES-like transcriptional factor mRNAs including *SOX2, OCT3/4, KLF4, and NANOG* was decreased drastically, whereas total amount of *c-MYC* expression was detectable in an appreciable level. The origin of *c-MYC* was found to be endogenous, since transgenic *c-MYC* expression was undetected (Fig. 4B), indicating endogenous *c-MYC* activation of Lc-iPC cells increased tumorigenisity in NOD-scid mice.

3.8. Lc-iPC cells lost the expression of immature-related surface antigens and associated epigenetic modifications

To elucidate the immature status of Lc-iPC cells from HuCC-T1, we investigated immature-related surface antigens and histone modification status. Lc-iPC cells from HuCC-T1 lost their expression of immature-related surface antigens including Tra-1-60, Tra-1-81, Tra-2-49, and Ssea-4 (Supplementary Fig. S3); *NANOG, OCT3/4*, and *SOX2* promoters of Lc-iPC cells were slightly trimethylated, indicating that these three ES-like genes were inactivated (Fig. 4C), and suggesting that *c-MYC* is prone to activation in the Lc-iPC cells.

4. Discussion

GI cancer is a major cause of death in several developed countries, including Japan. After therapeutic interventions, such

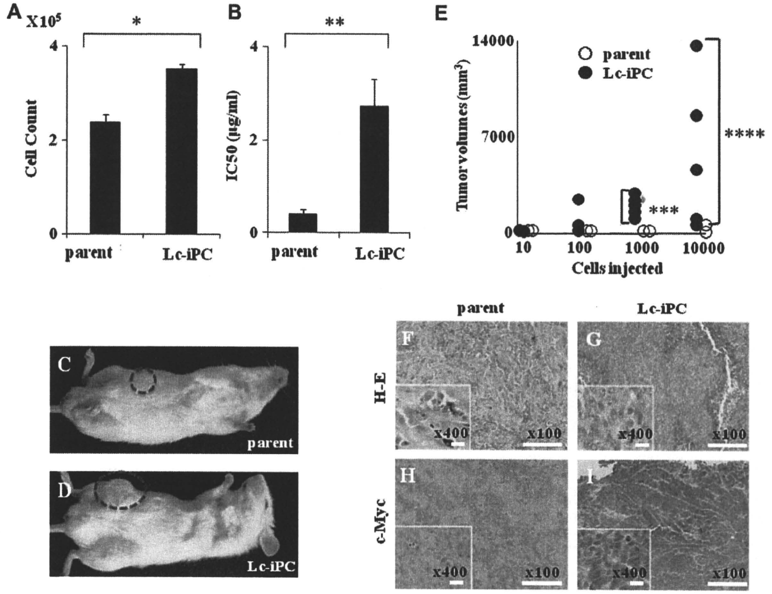

Fig. 3. MTT assay, proliferation *in vitro*, tumor formation *in vivo*, and c-Myc immunohistochemistry by induced Lc-iPC cells from HuCC-T1. Proliferation assay showed an increased proliferation (A; $n = 5$, $^{*}p = 0.012$) and MTT assay showed an increased IC_{50} for 5-FU (B; $n = 7$, $^{**}p = 0.001$) in Lc-iPC cells from HuCC-T1 as compared with parental cells. Lc-iPC and parental cells from HuCC-T1 were subcutaneously transplanted into three parts of NOD-scid mice. Four weeks after injection (C and D), tumors were palpable subcutaneously. Tumors were dissected and measured 8 weeks after injection. Tumors from Lc-iPC cells were larger than those from parental cells when 1000 and 10,000 cells were injected ($n = 6$, $^{***}p = 0.005$, $^{****}p = 0.005$, respectively). H–E staining of dissected tumor, (F) parental cells from HuCC-T1; (G) Lc-iPC cells from HuCC-T1. Tumors from Lc-iPC cells were less differentiated than parental cells. c-Myc immunohistochemistry showed that tumors of Lc-iPC cells from HuCC-T1 (I) expressed c-Myc protein more than those from parental cells (H). Bar, 100 μm; Original magnification, 100×, 400×.

as surgery and conventional chemoradiation therapy, tumor reduction and remission occur in more than half of the cases, although tumors can relapse and spread to other organs, i.e., metastasis. To overcome resistance to therapy, we recently showed that GI cancer reprogramming can sensitize cancer cells to differentiation and chemotherapeutic agents [4], indicating that further investigation is required on reprogramming of cancer cells to discover novel therapeutic approaches.

Several studies have reported reprogramming of cancer cells, including skin cancer or melanoma cells, using vectors harboring micro RNA-302 [7], *OCT3/4*, *c-MYC*, and *KLF4* [8], by nuclear transplantation [9]; but also GI cancer by introducing ES-like genes, which have been mentioned as iPS genes [4]. Introducing defined factors could be the advancement for science and technology as compared with reprogramming by nuclear transplantation, which might be necessary for determining safety issues. iPS cells, similar to ES cells, have the potential to form teratomas following inoculation in immunodeficient mice, presumably through the involvement of retroviral integration, retaining immature clones, and oncogenic *c-MYC* activation, which is consistent with the findings of the present study.

However, the involvement of *c-MYC* should be further investigated. A previous report indicated that tumors formed in iPS cell-derived chimeric mice could be attributed to the reactivation of the *c-MYC* retroviral transgene [10], whereas another report stated that the propensity for teratoma formation from a secondary neurosphere, derived from mice iPS cells, may depend on the tissue of origin but not on *c-MYC* transgene reactivation [11]. In our study, induced Lc-iPC cells showed increased proliferation and chemoresistance to 5-FU and stained strongly positive for c-Myc, which may be relevant to endogenous c-Myc activation in tumors, as detected by quantitative RT-PCR (Fig. 4A and B).

Nevertheless, we must consider other factors involved in iPC cells induction, such as genomic abnormalities, which are the usual characteristics of cancer cells. A possibility is that $TP53^{R175H}$ and $KRAS^{G12D}$ genomic mutations of HuCC-T1 may be relevant to the present observation [13] (data not shown). $KRAS^{G12D}$, a common mutation in solid cancers, is an active form of the *KRAS* gene. Mice expressing oncogenic $Kras^{G12D}$ and mutant *Tp53* accelerated the onset of cancer [12]. Taken together, data suggest that HuCC-T1 reprogramming may affect the pathways of these two mutated proteins.

Based on our results, we are confident of developing more effective differentiation therapies to conquer cancer if we find more appropriate differentiation pathways; however, further analysis of Sc-iPC and Lc-iPC cell properties is needed. Our data suggest that this new reprogramming technology will be a key to conquer bile duct carcinoma through its high magnitude of effect on sensitiza-

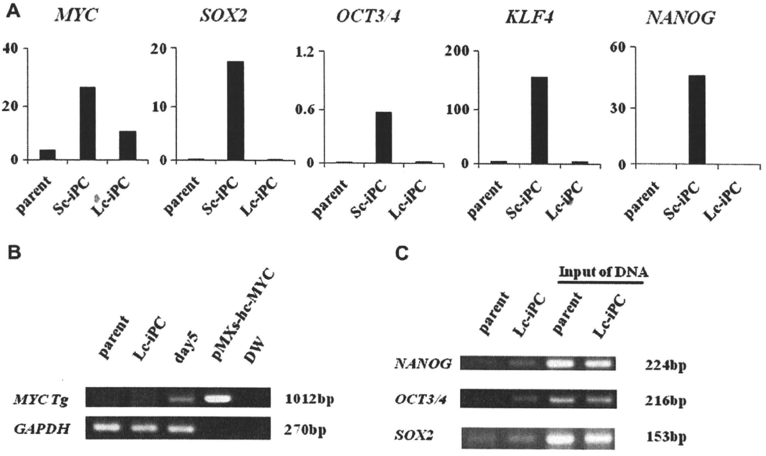

Fig. 4. Immature state of induced Lc-iPC cells from HuCC-T1. (A) Quantitative RT-PCR of total mRNAs of ES-like genes demonstrated genes expression, including *c-MYC, SOX2, OCT3/4, KLF4*, and *NANOG*, of Lc-iPC cells from HuCC-T1, decreased drastically compared to that of Sc-iPC cells except *c-MYC*. The expression of mRNA copies was normalized against *GAPDH* mRNA expression. (B) The RT-PCR of Lc-iPC from HuCC-T1 did not detect transgene (*Tg*) *c-MYC*. Day5, HuCC-T1 cells five days after transfection (*c-MYC, SOX2, OCT3/4*, and *KLF4*); pMXs-hc-MYC, positive control reaction of vector; DW, negative control with water. (C) Histone modification status in parental and Lc-iPC cells from HuCC-T1 was analyzed using chromatin immunoprecipitation with anti-trimethyl-K4 H3 antibody. The methylation signal at H3 lysine 4 was detected slightly in *NANOG, OCT3/4* and *SOX2* promoters in Lc-iPC cells, and *SOX2* in parental cells.

tion to a series of reprogramming-mediated, anti-cancer therapies, and that a predictive method will be necessary for evaluating the improper reprogramming-associated aggressive phenotype of iPC cells. In future, a day will come when cancer will be cured more effectively by newly discovered pharmacogenomic medicine based on reprogramming technology.

5. Conclusion

Although defined factor-induced reprogramming of gastrointestinal cancer cells is a promising approach for the treatment of cancer, we noted that Lc-iPC cells may be prone to genomic instability presumably due to genetic and epigenetic alterations including endogenous *c-MYC* activation, which is characteristic of cancer cells and is associated with reprogramming technology. To exclude therapy-resistant clones in GI cancer, it is necessary to develop a predictive method for evaluating improper reprogramming-associated aggressive phenotype of reprogrammed cells.

Acknowledgments

We thank Dr. Gregory J. Gores, Mayo Clinic College of Medicine, Rochester, MN, for providing cholangiocellular carcinoma HuCC-T1 cells and Kimie Kitagawa for excellent technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (20012039), a Grant-in-Aid for Scientific Research (S, 21229015; C, 20590313), and a Grant-in-Aid for Young Scientists (B, 21791287) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan; the Kobayashi Foundation for Cancer Research and the Uehara Memorial Foundation, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.03.176.

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Loss of FBXW7, a cell cycle regulating gene, in colorectal cancer: clinical significance

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This study focused on a cell cycle regulatory gene, *FBXW7*, which ubiquitinates c-Myc and cyclin E and promotes exit from the cell cycle. We determined the expression level of *FBXW7* in colorectal cancer (CRC) cases, correlated those values with clinicopathologic features, and characterized the molecular mechanism of reduced expression of *FBXW7* in CRC cells *in vitro*. *FBXW7* mRNA and protein expression were evaluated in 93 CRC cases. Using CGH array, the copy number aberrations of the flanking region of *FBXW7* were evaluated in another 130 CRC specimens. *In vitro* analysis of *FBXW7* gene silencing in CRC cells was conducted. *FBXW7* mRNA expression was significantly lower in tumor tissues than the corresponding normal tissues. The low *FBXW7* expression group showed a significantly poorer prognosis than patients in the high expression group. A concordant relationship was observed between the incidence of *FBXW7* repression and the genetic alteration. The incidence of genetic alteration was associated with the stage of disease progression. *In vitro*, *FBXW7*-specific siRNA enhanced expression of c-MYC and cyclin E proteins and up-regulated cell proliferation. Genetic alterations in tumors led to the loss of *FBXW7* expression and increased cell proliferation. *FBXW7* expression provides a prognostic factor for patients with CRC.

Normal cell growth and differentiation require appropriate regulation of the cell cycle. Deregulated cell cycle control is a fundamental aspect of cancer, resulting from mutation, deletion and transcriptional repression of genes such as pRB, and p53. Ubiquitin-mediated proteolysis is known to regulate the degradation of many proteins involved in the control of cell differentiation and growth.[1] Skp2 and FBXW7, F-box proteins containing components of the Skip1-Cull-F-box (SCF) ubiquitin ligase complexes, have been well characterized and shown to play important roles in degradation of proteins regulating cell cycle progression. Therefore, the altered expression of *FBXW7* is recognized to be one of the major causes of carcinogenesis or cancer development.[2–4] We have been focusing on expression of these cell cycle regulating genes in breast and gastric cancer.[5] In the current study, we examined FBXW7 which promotes the degradation of cyclin E, c-Myc, c-Jun and Notch and thereby negatively regulates these key oncoproteins.[6]

In an animal model, Onoyama *et al.* demonstrated conditional inactivation of Fbw7 in the T cell lineage of mice which later manifested thymic hyperplasia and eventually developed thymic lymphoma.[7] These results showed that FBXW7 plays an important role in malignant alterations of solid tumors. Thus far, there have been few studies regarding the clinicopathologic significance of *FBXW7* expression in human colorectal cancer (CRC).

In the present study, we examined copy number aberrations of *FBXW7* in a series of 130 CRC specimens using laser microdissection and a comprehensive genome hybridization (CGH) array. Then, we investigated *FBXW7* gene expression in another subset of 93 CRC samples with well-known clinicopathologic characteristics, including prognosis. The clinicopathologic significance of FBXW7 loss was validated biologically by CRC cell lines using siRNA interference.

Key words: FBXW7, colorectal cancer, c-Myc, cyclin E, CGH array
Additional Supporting Information may be found in the online version of this article.
Grant sponsor: CREST, Japan Science and Technology Agency (JST) and Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research; **Grant numbers:** 17109013, 18659384, 18390367, 18590333, 18015039, 19591509, 19390336, 20390360, 20591547, 20790961, 20790960; **Grant sponsor:** The Ministry of Education, Culture, Sports, Science and Technology (MEXT) Grant-in-Aid for Scientific Research on Priority Areas; **Grant number:** 18015039; **Grant sponsor:** Third Term Comprehensive Ten-year Strategy for Cancer Control; **Grant number:** 16271201; **Grant sponsor:** New Energy and Industrial Technology Development Organization (NEDO) Technological Development for Chromosome Analysis
DOI: 10.1002/ijc.24879
History: Received 5 May 2009; Accepted 18 Aug 2009; Online 8 Sep 2009
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