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Int. J. Cancer: **125**, 926–931 (2009)

Clinicopathological significance of *stanniocalcin 2* gene expression in colorectal cancer

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Laser microdissection (LMD) and microarray were used to identify genes associated with colorectal cancer. *Stanniocalcin 2* (*STC2*) expression and clinicopathological significance in 139 clinical colorectal cancer samples were specifically investigated using real-time quantitative reverse transcription-polymerase chain reaction. A number of genes upregulated in colorectal cancer cells compared to normal colorectal epithelial cells were identified including *STC2*. *STC2* gene expression in cancer tissue was higher than in corresponding normal colorectal epithelial tissue in 124 of 139 cases (89.2%, $p < 0.01$). Tumors with high *STC2* expression showed higher frequencies of lymph node metastasis, lymphatic invasion, tumor depth, tumor size and AJCC Stage classification ($p < 0.01$). Patients with high *STC2* expression also showed significantly worse overall survival rates than those with low *STC2* expression ($p < 0.01$). Furthermore, *STC2* gene appeared to be associated with colorectal cancer progression and may be a useful prognostic indicator for colorectal cancer.
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Key words: colorectal cancer; *Stanniocalcin 2 (STC2)*; microarray; laser microdissection (LMD)

Colorectal cancer is one of the most common cancers and is a significant contributor to cancer death.[1] Understanding how colorectal cancer arises, progresses and metastasizes will help the development of novel diagnostic methods and therapies. Therefore, it is important to examine differential gene expression between colorectal cancer and normal colorectal epithelium and elucidate gene function.

Laser microdissection (LMD) is helpful to isolate cells from tissues and microarray is useful for exhaustive analysis.[2] Many reports, including our own, have been published recently using these methods. We reported cancer-related genes relating to esophageal cancer,[3,4] gastric cancer[5,6] and hepatocellular carcinoma[7] using these methods. For colorectal cancer, we reported gene lists comparing 8 samples of colorectal cancer and normal colorectal epithelium using a cDNA microarray containing 12,814 genes.[8,9] In this study, *STC2* gene was identified as upregulated gene in colon cancer tissues.

The *stanniocalcin 2* (*STC2*) gene, located on chromosome 5q35.2, belongs to the *STC* family.[10–13] Stanniocalcins are glycoprotein hormones that are originally found in the corpuscle of Stannius, an endocrine gland of bony fish, involved in calcium and phosphate homeostasis.[14–16] The *STC* family includes 2 genes, *STC1* and *STC2*. It was reported that the *STC1* gene is associated with some cancers.[17–24] *STC2* was reported to be associated with breast cancer,[25–29] ovarian cancer[30] and renal cell carcinoma.[31] However, the relationship between *STC2* expression and clinicopathological factors in colorectal cancer has not yet been investigated.

In our study, we compared differentially expressed genes between colorectal cancer and normal colorectal epithelium and focused on *STC2* to clarify as a potential novel marker or therapeutic target. Additionally, we examined *STC2* gene expression in colorectal cancer tissues and paired normal colorectal tissues, and whether there is an association between *STC2* expression level and clinicopathological significance in colorectal cancer.

Material and methods

Clinical samples

One hundred and thirty-nine patients with colorectal cancer were enrolled into this study. All patients underwent resection of the primary tumor at the Kyushu University Hospital at Beppu and affiliated hospitals between 1993 and 2000. All patients were clearly identified as having colorectal cancer based on the clinicopathologic findings (age 66.1 ± 11.4, male/female = 79:60, tumor size (cm) 4.7 ± 2.0, histological type well/mod/poor = 52:77:8, tumor depth Tis/T1/T2/T3/T4 = 9:11:29:54:36, lymph node metastasis (+)/(−) = 56:83, lymphatic permeation (+)/(−) = 48:91, venous permeation (+)/(−) = 20:119, liver metastasis (+)/(−) = 19:120, Stage 0/I/IIA/IIB/III or IV = 9:31:26:11:62). No patients received chemotherapy or radiotherapy before surgery. Resected tumor and paired nontumor tissue specimens were immediately cut from the resected colorectum and placed in RNA Later (TaKaRa, Japan) or embedded in Tissue Tek OCT medium (Sakura, Tokyo, Japan), frozen in liquid nitrogen and kept at −80°C until RNA extraction. Written informed consent was obtained from all patients. The follow-up period ranged from 3 months to 11.3 years with a median of 2.8 years.

Laser microdissection and RNA extraction

Colon cancer tissues and colon normal epithelial tissues were microdissected using the LMD system (Leica Laser Microdissection System, Leica Microsystems, Wetzlar, Germany) as previously described.[3] Total RNA was extracted using an Rneasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purity and concentration of the RNA samples were determined with a Nano Drop (Nano Drop Technologies, Wilmington) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto) as previously described.[32]

DNA microarray

We used the commercially available Human 1 cDNA Microarray (Agilent Technologies) containing 12,814 genes. All microar-

Grant sponsor: 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, 20659209, 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 10-year Strategy for Cancer Control; Grant number: 16271201; Grant sponsors: CREST, Japan Science and Technology Agency (JST); NEDO (New Energy and Industrial Technology Development Organization) Technological Development for Chromosome Analysis; The Ministry of Education, Culture, Sports, Science and Technology of Japan for Scientific Research on Priority Areas, Cancer Translational Research Project, Japan; Grant of Clinical Research Foundation (2008-2010).

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Received 23 August 2008; Accepted after revision 2 March 2009
DOI 10.1002/ijc.24453
Published online 25 March 2009 in Wiley InterScience (www.interscience.wiley.com).

FIGURE 1 – Expression of *STC2* mRNA as assessed by RT-PCR in representative colorectal cancer cases (T, cancer tissue; N, noncancerous tissue; n, negative control; p, positive control; m, marker).

ray data including this study are available from Center for Information Biology Gene Expression database (http://cibex.nig.ac.jp/cibex2/index.jsp).[9]

Oligonucleotide primers for STC2 *gene amplification by RT-PCR*

Total RNA was extracted from each clinical sample and cDNA synthesized from 8.0 μg of total RNA as previously described.[32]

STC2-specific oligonucleotide primers were designed to give a 165 bp PCR product: sense primer 5′-CTTACATGGGATTTG CATGACTT-3′; antisense primer 5′-AATGGATCATCTCCAC TATCACC-3′.[13] Primers were also designed for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (270 bp): sense primer 5′-TTGGTATCGTGGAAGGACTCA-3′; antisense primer 5′-TGT CATCATATTTGGCAGGTT-3′. To avoid amplification of contaminating genomic DNA, the primers spanned more than 2 exons. Amplification was performed for 27 cycles (22 cycles for GAPDH) of 1 min at 95°C, 1 min at 60°C (56°C for GAPDH) and 1 min at 72°C. An 8.0 μl aliquot of each PCR-amplified DNA was electrophoresed on 2% agarose gels containing ethidium bromide.

Real-time quantitative RT-PCR

PCR amplification for quantification of *STC2* and *GAPDH* mRNA in 139 clinical samples was performed using the LightCycler system (Roche Applied Science, IN) and the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Applied Science, IN) as previously described.[33] Amplification conditions consisted of initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 64°C (60°C for *GAPDH*) for 10 sec and elongation at 72°C for 10 sec. Melting curve analysis and electrophoresis on 2% agarose gels were performed to ensure that the expected PCR products were generated. To quantitate specific mRNA in the samples, a standard curve was produced for each run based on 3 points from diluted human control cDNA. Relative *STC2* expression levels were then obtained by normalizing the amount of *STC2* mRNA divided by that of *GAPDH* mRNA as an endogenous control in each sample.

Immunohistochemistry

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

Statistical analysis

For continuous variables, data were expressed as the means ± SD. We classified the 139 colorectal cancer cases into 2 groups according to a cutoff value at median + SD of *STC2* mRNA expression level in nontumor tissues as determined by quantitative RT-PCR, to give high- (n = 84) and low-(n = 55) expression groups. Differences between groups were estimated using Student's t-test and χ^2 test. Overall survival curves and disease-free survival curves were plotted according to the Kaplan-Meier method, and measured from the day of surgery, with the log-rank test applied for comparisons. Variables with a value of $p < 0.05$ by univariate analysis were used in subsequent multivariate analyses based on Cox's proportional hazards model. All differences were deemed significant at the level of $p < 0.05$. Statistical analyses were performed using the JMP 5 for Windows software package (SAS Institute, Cary, NC).

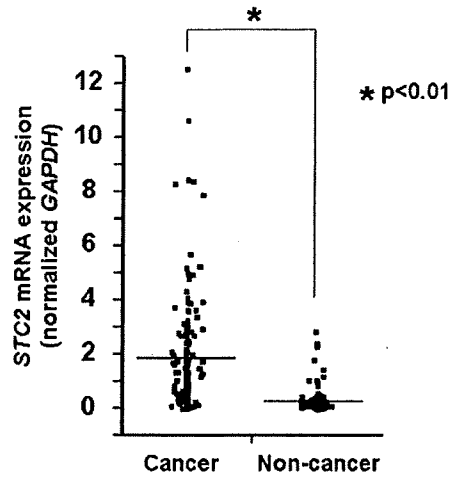

FIGURE 2 – *STC2* mRNA expression in cancer and noncancerous tissues from colorectal cancer patients as assessed by real-time quantitative PCR (n = 139). Horizontal lines indicate mean value of each group (T, cancer tissue; N, noncancerous tissue).

Results

Expression of STC2 *mRNA in clinical tissue specimens*

STC2 mRNA expression in cancerous and noncancerous tissues of colorectal cancer patients was examined by RT-PCR and real-time quantitative PCR and quantified using *STC2/GAPDH* expression ratios. Results indicated that *STC2* mRNA expression levels were higher in cancerous tissues (1.89 ± 2.13; mean ± SD) than in noncancerous tissues (0.29 ± 0.43) in 124 of the 139 cases (89.2%). This resulted in a significant difference in mRNA expression level between cancer and normal tissues ($p < 0.01$) (Figs. 1 and 2). To investigate protein expression of STC2, immunohistochemical staining was performed 5 each cases of high *STC2* mRNA expression group and low *STC2* mRNA expression group. STC2 staining was stronger in colorectal cancer tissues than in corresponding normal colorectal epithelial tissues. STC2 staining of cancer tissues in high mRNA expression group was stronger than in low mRNA expression group (Fig. 3).

The clinicopathological significance of STC2 *mRNA expression*

Clinicopathological features were analyzed in relation to *STC2* expression status (Table I). The incidence of deeply invading tumors was also significantly higher ($p < 0.01$) in the high-expression group (77.4%, 65/84) than in the low-expression group (45.4%, 25/55). Likewise, the incidence of lymph node metastasis and lymphatic permeation was higher ($p < 0.01$) in the high-expression group (51.2%, 43/84, 42.8%, 36/84) than in the low-expression group (23.6%, 13/55, 21.8%, 12/55). Furthermore, stage according to AJCC Stage classification correlated with higher *STC2* expression levels ($p < 0.01$). Incidence of larger tumors (>3 cm) was significantly higher ($p < 0.01$) in the high-expression group (89.3%, 75/84) than in the low-expression group (63.6%, 35/55). No other significant differences were observed with respect to age, gender, histology, venous permeation and liver metastasis.

The 5-year actuarial overall survival rates in patients with high *STC2* mRNA expression levels and those with low levels were 50.3 and 91.4%, respectively. Analysis of overall survival curves

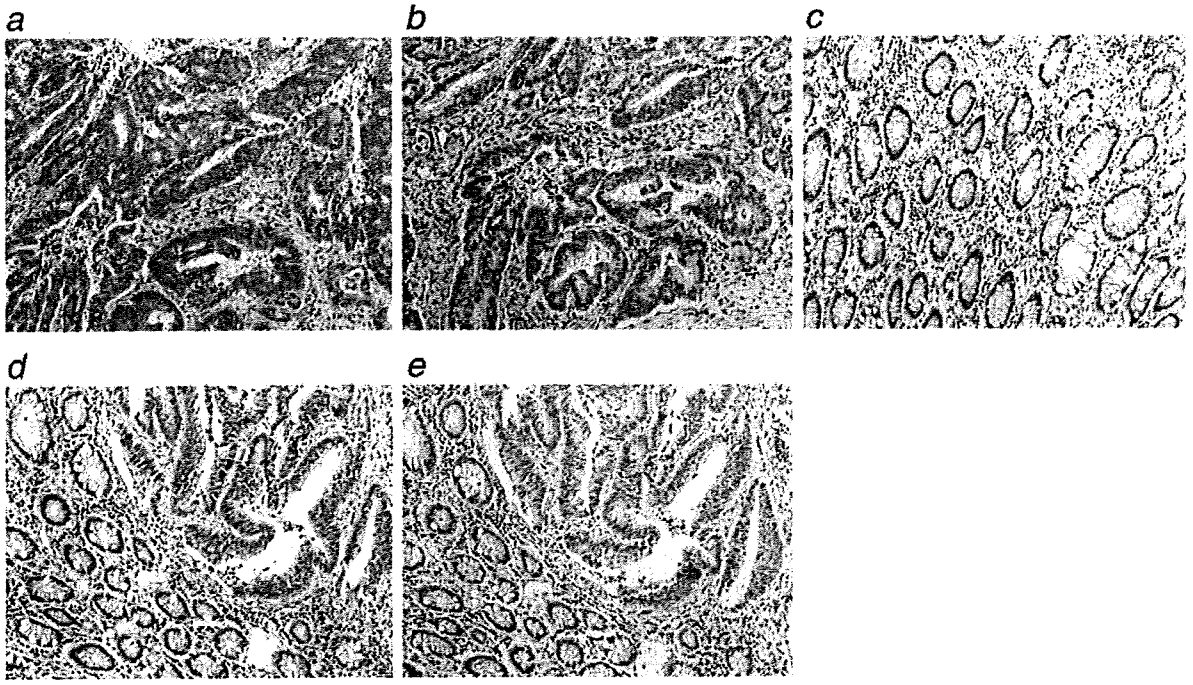

FIGURE 3 – Immunohistochemistry with STC2 antibody on colorectal cancer patient samples. The majority of staining occurred in cancer cells. (*a*) cancer tissue (mRNA high-expression group), STC2 stain; (*b*) cancer tissue (mRNA high-expression group), control stain (secondary antibody only); (*c*) noncancerous tissue (mRNA high-expression group), STC2 stain; (*d*) cancer and noncancerous tissue (mRNA low-expression group), STC2 stain; (*e*) cancer and noncancerous tissue (mRNA low-expression group), control stain (secondary antibody only), original magnification ×100. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I – *STC2* GENE EXPRESSION AND CLINICOPATHOLOGICAL FEATURES FOR 139 COLORECTAL CANCER PATIENTS

Clinicopathologic variable	High-expression group (n = 84)	Low-expression group (n = 55)	p-value
Age	65.7 ± 12.1	66.7 ± 10.2	0.63
Gender			
Male	44	35	0.19
Female	40	20	
Tumor size			
<3 cm	7	19	<0.01
>3 cm	75	35	
Histology			
Well	27	25	0.23
Moderate	50	27	
Poor	6	2	
Depth			
Tis, T1, T2	19	30	<0.01
T3, T4	65	25	
Lymph node metastasis			
Absent	41	42	<0.01
Present	43	13	
Lymphatic permeation			
Absent	48	43	<0.01
Present	36	12	
Venous permeation			
Absent	70	49	0.34
Present	14	6	
Liver Metastasis			
Absent	70	50	0.23
Present	14	5	
AJCC Stage classification			
0, I, II	37	40	<0.01
III, IV	47	15	

High-expression group (*STC2/GAPDH* ≥ cutoff value), low-expression group (*STC2/GAPDH* < cutoff value), well, well differentiated; poor, poorly differentiated; moderate, moderately differentiated.

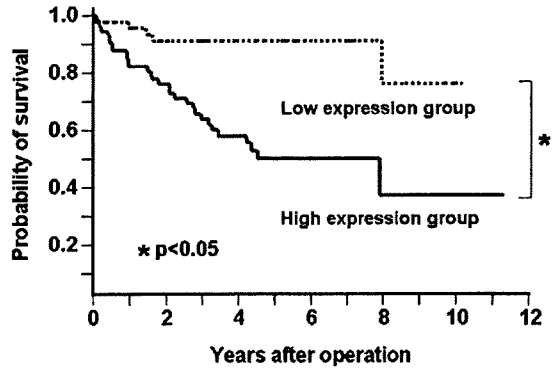

FIGURE 4 – Kaplan-Meier overall survival curves for colorectal cancer patients according to the level of *STC2* mRNA expression. The overall survival rate for patients in the high-expression group was significantly higher than that for patients in the low-expression group ($p < 0.01$). High-expression group (n = 84): *STC2/GAPDH* ≥ cutoff value, low-expression group: *STC2/GAPDH* < cutoff value (n = 55).

showed that patients in the high-expression group had a significantly poorer prognosis than those in the low-expression group ($p < 0.01$) (Fig. 4).

Univariate analysis identified *STC2* expression (low- or high-expression), tumor size, depth, lymph node metastasis, lymphatic permeation, venous permeation and liver metastasis as adverse prognostic factors for overall survival after colorectal resection. Variables with a p-value of less than 0.05 by univariate analysis were selected for multivariate analysis using Cox's proportional

TABLE II - RESULTS OF UNIVARIATE AND MULTIVARIATE ANALYSES OF CLINICOPATHOLOGICAL FACTORS AFFECTING OVERALL SURVIVAL RATE AFTER SURGERY

Clinicopathologic variable	*n*	5-year survival rate (%)	Univariate analysis *p*-value	Multivariate analysis	
				Relative risk (CI)	*p*-value
Gender					
Male	79	63.1	0.41		
Female	60	69.9			
Tumor size					
<3 cm	26	96.0	0.004	1.54	0.36
>3 cm	110	58.8		(0.65–6.66)	
Histology					
Well	52	69.5	0.61		
Mod + poor	85	32.6			
Depth					
Tis, T1, T2	49	90.7	<0.0001	1.78	0.04
T3, T4	90	51.9		(1.02–3.75)	
Lymph node metastasis					
Absent	83	82.9	<0.0001	1.97	0.0003
Present	56	43.1		(1.34–3.04)	
Lymphatic permeation					
Absent	91	78.7	<0.0001		
Present	48	43.9			
Venous permeation					
Absent	119	71.4	0.0053	1.29	0.21
Present	20	39.3		(0.86–1.87)	
Liver metastasis					
Absent	120	75.4	<0.0001		
Present	19	15.5			
STC2					
Low	55	91.3	0.0002	1.76	0.023
High	84	50.3		(1.07–3.31)	

n, number of patient; CI, confidence interval, *STC2*, *STC2* expression; high, high-expression group (*STC2/GAPDH* ≥ cutoff value); low, low-expression group (*STC2/GAPDH* < cutoff value).

hazards model. *STC2* expression (relative risk: 1.76, confidence interval: 1.07–3.31, $p = 0.023$) was found to be a factor affecting overall survival rate after lymph node metastasis (Table II).

Discussion

This study identified differentially expressed genes between colorectal cancer and normal colorectal epithelial tissues. We had previously reported that the expression of *FABP6* was higher in primary colorectal cancers and adenomas than in normal epithelium, and *FABP6* might play an important role in early carcinogenesis.[34] Here, we focused on *STC2* as it was one of the upregulated genes.

*STC*s represent a small family of secreted glycoprotein hormones, consisting of *STC1* and *STC2*, which are conserved from fish to mammals. The STC2 protein may play a role in the regulation of renal and intestinal calcium and phosphate transport, cell metabolism or cellular calcium/phosphate homeostasis, but the precise physiological function of *STC2* has not been clearly elucidated.[16]

Some studies on *STC2* gene expression relating to cancer have been reported. In breast cancer, *STC2* expression was found to be associated with tumor estrogen receptor (ER) status. High *STC2* expressions were associated with a good prognosis (disease-free survival) in ER-positive breast cancer patients.[22–24] It was reported that a significantly higher expression level of *STC2* in metastases after 5 and 10 years after surgery was shown when compared to the primary breast cancers displaying early metastatic lesions.[28] In ovarian cancer, *STC2* was one of the overexpressed genes as investigated by immunohistochemistry-guided laser capture microdissection and microarray, and overexpression of *STC2* was associated with a decreased disease-free interval.[30] Increased cytoplasmic STC2 expression correlated with aggressiveness of renal cell carcinoma and shorter overall patient survival times.[31] In colorectal cancer, it was reported that expression of *STC1* increased 10-fold in tumors compared to normal mucosa by microarray,[20] and *STC2* was one of the overexpression genes in colorectal cancer cells by microarray.[35] However, *STC2* expression status in clinical samples and clinicopathologic factors in colorectal cancer has not been investigated before this study.

Our study indicated that in colorectal cancer patients, *STC2* was more frequently overexpressed in cancerous tissues than in noncancerous tissues, and high mRNA expression of *STC2* was significantly associated with tumor size, depth, lymph node metastasis, lymphatic permeation, AJCC Stage classification and overall survival. Additionally, *STC2* expression was found to be a factor affecting overall survival rate by multivariate analysis. It was shown by means of immunohistochemical staining that STC2 protein expression was correlated with mRNA expression. Therefore, these results suggest that high *STC2* expression has an association with progression and malignancy of colorectal caner cells.

In human breast cancer cell lines, *STC2* represents a downstream target of estrogen and retinoic acid signaling pathways and constitutive *STC2* expression resulted in significant impairment of cell growth, migration and cell viability after serum withdrawal.[29] Additionally, it was reported that *STC2* expression was sporadically abrogated in human cancer cells by transcriptional silencing associated with CpG island promoter hypermethylation.[36] Constitutive expression of human *STC2* in transgenic mice acts as a potent growth inhibitor *in vivo* and results in a significant reduction of intramembranous and endochondral bone development, as well as high neonatal morbidity.[37] It seems that these findings correspond to clinical data of breast cancer. Meanwhile, in ovarian cancer, renal cell carcinoma and colorectal cancer, high *STC2* expression correlated with a poor prognosis. The reason why colorectal cancer with high *STC2* expression shows aggressive behavior remains unclear.

Endoplasmic reticulum (ER) stress results from physiological, pathological and experimental conditions that perturb ER function because of accumulation of misfolded proteins within the ER. Accumulation of misfolded protein in the ER induces a highly conserved homeostatic response in all eukaryotic cells, termed the unfolded protein response (UPR).[38,39] It was reported that *STC2* is a novel target of the UPR. *STC2* expression is upregulated after exposure to tunicamycin and thapsigargin and induced in cultured

cells downstream of *PERK-ATF4* activation by ER stress agents, oxidative stress and hypoxia.[40] Recent report revealed that endogenous *HIF-1α* plays an essential role in hypoxia-induced *STC2* expression and the direct binding of *HIF-1α* to *STC2* promoter with the chromatin immunoprecipitation assay.[36] These findings support the notion that *HIF-1α* is a potent stimulator of *STC2* expression. *STC2* may be a critical survival component of the UPR, and in colorectal cancer, cells may overexpress *STC2* for tolerance to the extreme microenvironment of tumor and ER stress including hypoxia. The contradictory findings in different types of cancer may be due to differences in variations of organs.

In conclusion, here, we identified several upregulated genes in colorectal cancer cells compared to normal colorectal cancer epithelial cells and focused on one of the most upregulated genes, *STC2*. *STC2* may play an important role in the progression of colorectal cancer and may prove useful as a novel prognostic marker and target for molecular treatment of patients with colorectal cancer. Furthermore, understanding the biological function of *STC2* in colorectal tissue may help to delineate its role in the pathophysiology of colorectal cancer.

Acknowledgements

The authors thank Ms. T. Shimooka, Ms. K. Ogata, Ms. M. Kasagi, Ms. Y. Nakagawa, Mr. M. Utou, Ms. Y. Miura, Mr. S. Matsuzaki, Ms. M. Kawamoto, Ms. Y. Fukuyoshi, Ms. A. Harada, Dr. M. Ohkuma, Dr. K. Sakashita, Dr. Y. Takatsuno, Dr. K. Motoyama, Dr. T. Yokoe, Dr. Z. Xiang, Dr. Y. Kosaka, Dr. S. Hirasaki, Dr. K. Ishikawa, Dr. Y. Nakamura and Dr. H. Sakashita for their technical assistance and advice.

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GASTROENTEROLOGY 2010;138:1312–1320

Effects of Ghrelin Administration After Total Gastrectomy: A Prospective, Randomized, Placebo-Controlled Phase II Study

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BACKGROUND & AIMS: Body weight (BW) loss and reduction of blood ghrelin level are commonly observed after total gastrectomy (TG). A prospective study was designed to elucidate whether exogenous ghrelin administration prevents postoperative BW loss by improving appetite and oral food intake in patients with gastric cancer after undergoing TG. **METHODS:** In this randomized phase II study, 21 patients undergoing TG were assigned to a ghrelin (11 patients) or placebo group (10 patients). They received intravenous infusion of synthetic human ghrelin (3 μg/kg) or saline twice daily for 10 days after starting oral food intake following surgery. Changes in BW, appetite visual analog scale score, food intake calories, body composition, basal metabolic rate, and various blood test results were evaluated. **RESULTS:** Excluding one patient who developed profound diaphoresis during ghrelin infusion, 20 patients completed the study. Food intake and appetite were significantly higher with ghrelin compared with placebo (average, 13.8 vs 10.4 kcal/kg/day [$P = .030$] and 5.7 vs 3.9 cm [$P = .032$], respectively). BW loss was significantly lower in the ghrelin than in the placebo group (−1.4% vs −3.7%; $P = .044$). Fat mass, lean body mass, and basal metabolic rate decreased significantly in the placebo group; however, the reductions in lean body mass and basal metabolic rate were not significant in the ghrelin group, although that of fat mass was significant. **CONCLUSIONS: Short-term administration of synthetic ghrelin was safe and successfully lessened postoperative BW loss and improved appetite and food intake after TG.**

Keywords: Ghrelin; Total Gastrectomy; Gastric Cancer; Body Weight Loss.

Body weight loss is common and a serious outcome in patients with gastric cancer who have undergone total gastrectomy. It correlates well with decline in postoperative quality of life and is the most reliable indicator of malnutrition, which impairs immune function, infection susceptibility, and survival.[1–3] Although various mechanisms have been considered, such as perturbation of absorption due to reduced pancreatic excretion,[4,5] decrease of gastric acid level,[6] reflux esophagitis,[7] intestinal floral alteration,[8] and increased peristalsis and diarrhea,[9] reduced food intake[10,11] is the most conceivable explanation for body weight loss after total gastrectomy. Therefore, surgeons dealing with gastric cancers have tried to increase food intake by producing a gastric substitute, such as a jejunal pouch, but such procedures have not always been successful.[12] Another study indicated that the majority of patients with total gastrectomy could eat food as much as healthy subjects under a regulated program.[13] Our own experience indicates that some patients do not show significant body weight loss after total gastrectomy by resorting to small but frequent meals. These changes suggest that reduced food intake after total gastrectomy could not be simply explained by loss of storage volume due to gastrectomy, but rather reflect a disturbance of eating activity through an unknown mechanism.

The 28-amino acid peptide ghrelin is the endogenous ligand for the growth hormone (GH) secretagogue receptor 1a, which stimulates GH release from the pituitary gland.[14] The majority of ghrelin is produced by X/A-like cells of the oxyntic glands in the stomach, and a smaller amount is secreted from other organs, such as the intestine, pancreas, kidney, and hypothalamus.[15,16] Ghrelin has various physiologic functions in addition to secretion of GH, such as promoting the appetite signal in the hypothalamus (in contrast to leptin),[17] stimulating gastrointestinal activity (such as peristalsis, gastric acid secretion, and pancreatic excretion through the vagal nerves),[18] and regulation of fat metabolism.[19] In addition, ghrelin mitigates proinflammatory cytokine production and attenuates the stress signal.[20] Among the pleiotropic functions of ghrelin, this peptide is the only gastrointestinal hormone known to stimulate appetite. A randomized double-blind study of healthy volunteers

Abbreviations used in this paper: ANOVA, analysis of variance; BMR, body metabolic rate; GH, growth hormone; IGF, insulin-like growth factor.

0016-5085/10/$36.00
doi:10.1053/j.gastro.2009.12.058

showed that ghrelin enhances appetite and increases food intake.[21,22] Thereafter, several clinical trials of patients with heart failure,[23] pulmonary disease,[24] and cancer cachexia[25] concluded that ghrelin successfully improved the diseases along with increased oral food intake and body weight. In the field of surgical treatment for obesity, reduction in ghrelin levels after sleeve gastrectomy is associated with successful body weight loss and appetite suppression.[26] Taken together, the discovery of ghrelin allows the proposal of a new concept, body weight regulation by the stomach, which could be applied to various diseases with malnutrition.

We reported previously that serum ghrelin levels decreased to 10% to 20% of the preoperative level immediately after total gastrectomy[27,28] and did not recover thereafter, accompanied by approximately 20% body weight loss.[10,12,27] These findings suggest that loss of ghrelin could be involved in body weight loss observed after total gastrectomy. The present prospective, randomized, placebo-controlled phase II study investigated the effects of exogenous ghrelin administration on postoperative body weight loss by improving appetite and oral food intake in patients with gastric cancer who had undergone total gastrectomy. We report here the successful results of the study, and further use of ghrelin for these patients is discussed.

Patients and Methods

Patients

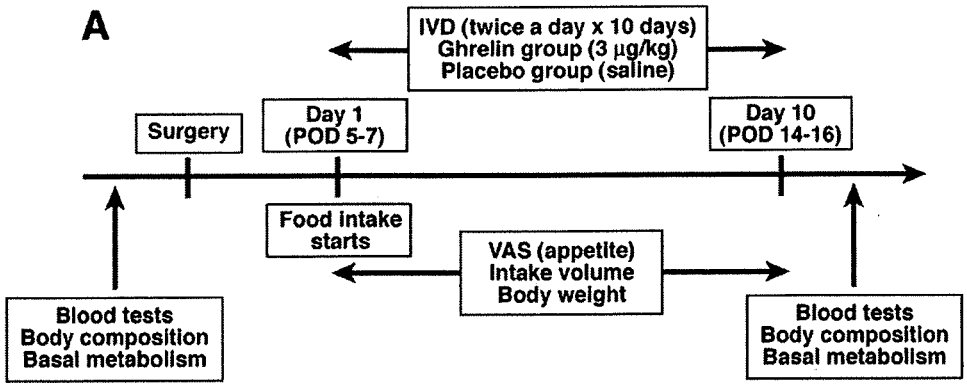
Twenty-one patients who underwent total gastrectomy at Osaka University Hospital between June 2006 and June 2008 were enrolled in the study. The inclusion criteria were as follows: (1) adenocarcinoma of the stomach confirmed by histopathologic examination, (2) preoperative clinical staging with less than stage II (International Union Against Cancer TNM stage classification), (3) curative surgical treatment (R0) (ie, total gastrectomy with D1 or D2 lymph node dissection), and (4) age between 20 and 80 years. The exclusion criteria were the presence of any of the following: (1) cardiopulmonary, liver, or renal dysfunction; (2) active dual malignancy; (3) pregnancy; (4) past history of gastrointestinal surgery; and (5) postoperative complications after total gastrectomy that could affect oral food intake, such as anastomotic leakage, pancreatitis, and mechanical ileus. Twenty-one patients were randomized by sealed envelope and divided into 2 study groups. The center office generated the allocation sequence and enrolled and assigned the patients to the 2 groups, and the random allocation sequence was concealed until interventions were assigned. Eleven patients received repeated administrations of ghrelin (ghrelin group), and 10 patients received repeated administrations of pure saline (placebo group). The study was approved by the Osaka University Ethics Committee, and all patients gave written informed consent before study entry in accordance with the Declaration of Helsinki. The study was registered at UMIN (http://www.umin.ac.jp; clinical trial no. UMIN000001925).

Preparation of Synthetic Human Ghrelin

Synthetic human ghrelin, which consists of 99.4% acyl ghrelin and 0.6% des-acyl ghrelin, based on analysis by high-performance liquid chromatography, was obtained from Peptide Institute Inc (Osaka, Japan). Endotoxin examinations and the pyrogen test for ghrelin solutions were conducted as described previously.[29] Synthetic human ghrelin was dissolved in distilled water with 3.75% D-mannitol and sterilized by passage through a filter. Ghrelin solution was stored in 2-mL volumes, each containing 210 μg. These solutions were stored at −20°C in sterile vials until preparation of ghrelin for administration.

End Points and Study Protocol

The primary end point of this study was an increase in orally ingested calories following ghrelin administration. The secondary end points included changes in body weight, appetite, body composition, basal metabolism, and blood tests. The study design is summarized in Figure 1*A*. The patient usually started oral food intake of rice porridge between postoperative day 5 and postoperative day 7. All patients were served standard postoperative meals, but they were always allowed to receive extra food when they desired. In the following 10 days after starting oral food intake, intravenous drip infusion of synthetic human ghrelin (3 μg/kg) or placebo was administered twice a day (before breakfast and before dinner). Ghrelin solution and placebo (pure saline) were added to a 50-mL saline bottle, which was intravenously infused over a 30-minute period. The same amount of ghrelin was administered through intravenous infusion during the 10-day treatment; the dose was calculated based on the body weight on the day before oral food intake. During the study period, the same protocol of intravenous infusion and the same menu of meals were provided for the 2 groups. The composition of the intravenous infusion fluid was 43.0 g glucose, 35 mEq Na, 20 mEq K, 35 mEq Cl, and 20 mEq lactate in 1000 mL. The protocol of intravenous infusion was 2000 mL/day from postoperative day 1 to postoperative day 7 and 1000 mL/day from postoperative day 8 to postoperative day 14. The study was performed in a single-blind manner; patients without knowledge of their treatment assessed the amount of food intake, appetite, and body weight every day during the treatment by themselves without any intervention by the hospital staff. Food intake calories based on the food weight measured by the patient, including standard meal and extra foods, were calculated by dietitians using a calorimeter. Preprandial appetite at every meal was scored by the visual analog scale (possible scales, 0–10 cm) recorded in the account sheet by each



POD; postoperative day, VAS; visual analogue scale; IVD; intravenous drip

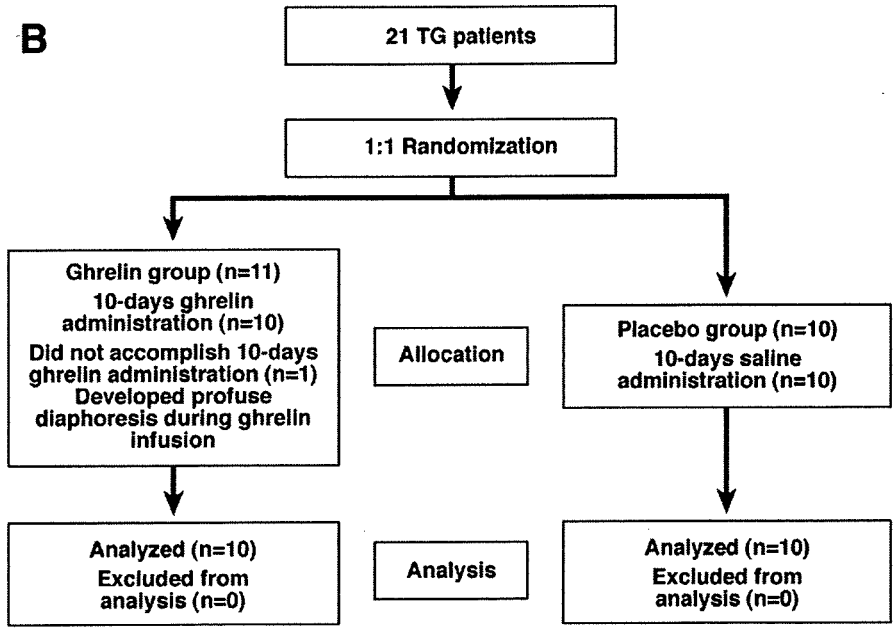


Figure 1. Study protocol and flow diagram.

patient. Body weight was measured with a beam scale to the nearest 0.1 kg, with patients standing barefoot and in light clothing.

Body composition was measured by using dual-energy x-ray absorptiometry (Hologic QDR-2000 instrument; Hologic Inc, Waltham, MA) to assess changes in lean body mass, fat mass, and bone mineral content before and after protocol treatment. The whole body was scanned in the single-beam mode, and the results were analyzed with body composition software. Basal metabolism was measured by using a metabolic analyzer (MedGem metabolic analyzer; HealtheTech, Golden, CO) to assess changes in basal metabolism before and after treatment. All subjects breathed through the MedGem using a disposable, scuba-type mouthpiece. During the measurement, oxygen consumption (VO_2) and body metabolic rate (BMR) were continuously and electronically recorded on a personal computer.

Blood Sampling and Assay

Blood samples were collected from patients before breakfast after an overnight fast, transferred into chilled tubes, stored on ice during collection, centrifuged, serum separated, and stored at −50°C until assay. Insulin-like growth factor (IGF)-I levels were measured by IGF-I IRMA "Daiichi" (TFB, Inc, Tokyo, Japan). Norepinephrine was measured using high-performance liquid chromatography (Tosoh Co, Tokyo, Japan). Cortisol and insulin were measured using the Cortisol Kit "TFB" (TFB, Inc, Tokyo, Japan) and chemiluminescent enzyme immunoassay (Fujirebio, Inc, Tokyo, Japan), respectively. Serum GH and leptin were

measured using GH Kit "Daiichi" (TFB, Inc, Tokyo, Japan) and Human Leptin RIA Kit (Linco Research Inc, St Charles, MO), respectively.

Sample Size Calculation and Statistical Analysis

We estimated that the difference in the effect of ghrelin or placebo on oral food intake calories should be at least 25% assuming 1200 and 1500 kcal/day in the placebo and ghrelin groups, respectively, with ±200 kcal for each SD. To analyze the difference in the effects in the ghrelin and placebo groups using Student *t* test, the study group should comprise at least 16 subjects, with a 5% α value and statistical power of 80%. Assuming that 20% of subjects in each group would not complete the study, the total number of subjects required in this study was estimated at 20.

Numerical values are expressed as mean ± SD unless otherwise indicated. Differences in parameters between the placebo and ghrelin groups were tested by Student *t* test or Mann–Whitney *U* test. Changes in parameters before and after total gastrectomy were tested statistically by the paired *t* test or Wilcoxon signed rank test. Changes in parameters between the 2 groups during the 10 days of follow-up were tested for significance by repeated-measures analysis of variance (ANOVA). A *P* value of $<.05$ was considered statistically significant. SAS for Windows software version 9 (SAS Institute, Inc, Cary, NC) was used to conduct repeated-measures ANOVA, whereas StatView version 5.0 (SAS Institute, Inc) was used for other tests.

Results

Patient Characteristics

The study flow diagram is summarized in Figure 1*B*. One of the 11 patients (9.1%) in the ghrelin group developed profuse diaphoresis during ghrelin infusion, equivalent to grade 1 by National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0. Accordingly, we decided to stop ghrelin administration and the patient was excluded from further analysis. The 10-day course of ghrelin administration was well tolerated by the remaining 10 patients without any adverse events, although some reported transient periods of feeling warm and/or peristalsis during ghrelin infusion. Table 1 summarizes the clinical background of the 20 patients who completed the study. There was no significant difference in age, sex, body weight, body mass index, and clinical stage of gastric cancer between the 2 groups.

Table 1. Patient Characteristics

Parameter	Ghrelin group	Placebo group	*P* value
n	10	10	
Age (*y*)	64.8 ± 10.4	61.6 ± 8.4	.46
Sex (male/female)	7/3	4/6	.19
Body weight (*kg*)	62.2 ± 13.6	62.9 ± 11.5	.89
Body mass index (kg/m^2)	23.1 ± 3.1	24.5 ± 3.8	.36
Procedure (LATG/COTG)	8/2	9/1	.54
Clinical TMN stage			
T (T1/T2/T3/T4)	7/1/2/0	8/2/0/0	.49
N (N0/N1/N2)	9/1/0	9/1/0	1.00
Stage (I/II/III/IV)	8/2/0/0	10/0/0/0	.15

LATG, laparoscopic assisted total gastrectomy; COTG, conventional open total gastrectomy.

Effects of Ghrelin on Appetite, Food Intake, and Body Weight Loss

Appetite, oral food intake, and body weight were recorded by the patients throughout 10 days of ghrelin/saline administration. During this period, the patients in the 2 groups received the same amount (ie, volume and calories) of intravenous infusion. The mean appetite visual analog scale score was significantly higher in the ghrelin group than the placebo group during the 10-day period (Figure 2*A*; repeated-measures ANOVA, $P = .032$).

Food intake calories (kcal/kg/day) during the 10-day period were significantly higher in the ghrelin group than in the placebo group (Figure 2*B*; repeated-measures ANOVA, $P = .030$). Food intake gradually increased at an earlier period of food intake and was then unchanged thereafter; both groups showed a similar difference throughout the 10-day period. The mean intake calorie over the 10-day period was 13.8 and 10.4 kcal/kg/day for the ghrelin and placebo groups, and ghrelin administration accounted for about 32.7% of the increase.

Body weight loss was calculated in reference to the first day of oral food intake. During this period, body weight gradually decreased in both groups, although the loss was more evident in the placebo group. At the end of the intravenous drip protocol (Day 10), body weight loss was −3.7% for the placebo group but only −1.4% for the ghrelin group. For the 10-day period, body weight loss of the ghrelin group was less than that of the placebo group (Figure 2*C*; repeated-measures ANOVA, $P = .044$).

Effects of Ghrelin on Body Composition and Basal Metabolism

Consistent with the body weight changes, both lean body mass and fat mass decreased gradually during the study period. The mean change in fat mass was −8.8% (14,100 ± 5400 to 12,900 ± 5200 g) and −7.6% (19,000 ± 8400 to 17,700 ± 8300 g) for the ghrelin and placebo groups, respectively. The reduction was statistically significant for each group (Figure 3*A*; $P < .001$). The mean change in lean body mass in the placebo group was −7.8% (41,800 ± 6500 to 38,500 ± 5700 g), which was also significant (Figure 3*B*; $P < .001$); however, the change in the ghrelin group was only −2.9% (44,600 ± 10,500 to 43,200 ± 9600 g, Figure 3*B*; $P = .076$). Figure 3*C* shows the BMR values before and after total gastrectomy. BMR decreased significantly after total gastrectomy in the placebo group (21.8 ± 4.0 to 19.4 ± 3.4

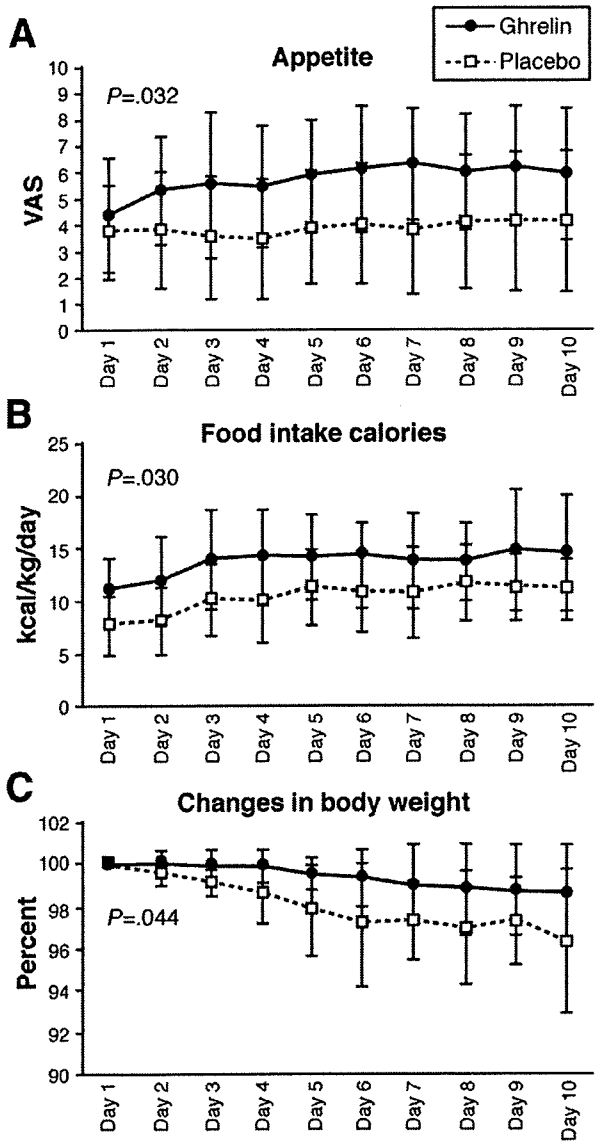


Figure 2. Serial changes in appetite, food intake, and body weight during the 10-day study in the ghrelin and placebo groups. Data are expressed as the mean ± SD of visual analog scale scores of (*A*) preprandial appetite at every meal, (*B*) daily total food intake calories per body weight (kcal/kg/day), and (*C*) percent body weight relative to the first day of oral intake in the ghrelin and placebo groups. The visual analog scale score throughout the study period, which was evaluated by repeated-measures ANOVA, was significantly higher in the ghrelin group than in the placebo group (5.7 vs 3.9 cm; $P = .032$). Likewise, food intake calories were significantly higher in the ghrelin group than in the placebo group (average, 13.8 vs 10.4 kcal/kg/day; repeated-measures ANOVA, $P = .030$). Body weight loss in the ghrelin group was significantly lower than in the placebo group (−1.4% vs −3.7%; repeated-measures ANOVA, $P = .044$).

kcal/kg; $P = .023$). In contrast, the reduction in BMR in the ghrelin group was smaller, and the difference between before and after treatment was not significant (22.6 ± 6.1 to 21.4 ± 6.0 kcal/kg; $P = .20$).

Blood Tests and Hormone Assays

Finally, we compared the results of certain blood tests that reflect the nutritional status and hormones (hemoglobin, total protein, albumin, total cholesterol, triglyceride, leptin, GH, cortisol, norepinephrine, insulin, and IGF-I) both before and after the 10-day period (Table 2). In the early recovery phase, the parameters associated with nutrition did not change in the placebo group but significantly improved in the ghrelin group. Leptin levels decreased significantly in both groups after total gastrectomy, consistent with the reduction in fat mass. On the other hand, there was no significant change in GH, cortisol, norepinephrine, insulin, and IGF-I levels after treatment in both groups.

Discussion

Body weight loss is a common finding in patients who undergo gastrectomy for gastric cancer, which not

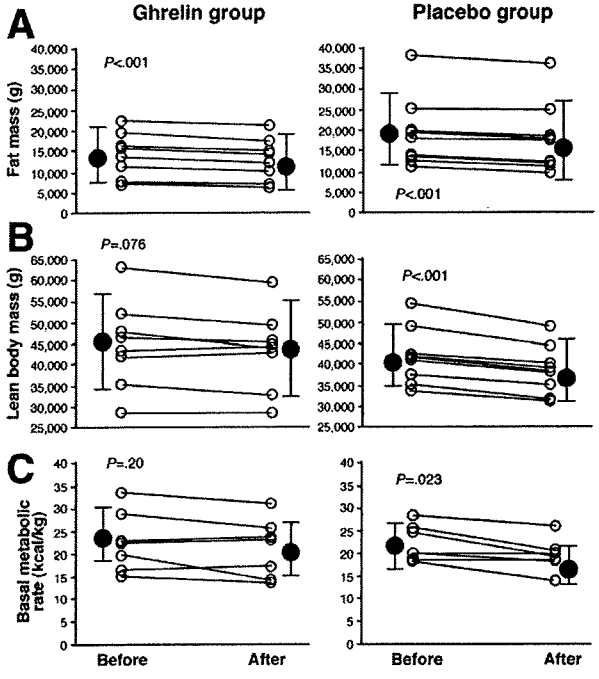


Figure 3. Body composition and basal metabolic rate before and after the study in the ghrelin and placebo groups. (*A*) Fat mass and (*B*) lean body mass measured by dual-energy x-ray absorptiometry and (*C*) basal metabolic rate were determined before and after the 10-day study. Changes for each patient (*open circles*) and the whole group (*closed circles*, ±SD) are shown for the ghrelin and placebo groups. The reductions in all 3 parameters were statistically significant in the placebo group by Student *t* test (fat mass, 19,000 ± 8400 to 17,700 ± 8300 g [$P < .001$]; lean body mass, 41,800 ± 6500 to 38,500 ± 5700 g [$P < .001$]; BMR, 21.4 ± 6.0 to 19.4 ± 3.4 kcal/kg [$P = .023$]). In the ghrelin group, the reduction of fat mass was significant while that of lean body mass and BMR was less than in the placebo group and not statistically significant (fat mass, 14,100 ± 5400 to 12,900 ± 5200 g [$P < .001$]; lean body mass, 44,600 ± 10,500 to 43,200 ± 9600 g [$P = .076$]; BMR, 22.6 ± 6.1 to 21.4 ± 6.0 kcal/kg [$P = .20$]).

Table 2. Results of Laboratory Tests

	Ghrelin group	Placebo group
Hemoglobin (*g/dL*)		
Before	12.2 ± 0.8	12.7 ± 2.4
After	11.8 ± 1.1	11.7 ± 1.4
Total protein (*g/dL*)		
Before	5.7 ± 0.3	6.0 ± 0.7
After	6.6 ± 0.4[a]	6.4 ± 0.3
Albumin (*g/dL*)		
Before	3.1 ± 0.2	3.4 ± 0.6
After	3.5 ± 0.4[a]	3.5 ± 0.2
Total cholesterol (*mg/dL*)		
Before	148 ± 43	176 ± 26
After	174 ± 37[a]	164 ± 32
Triglyceride (*mg/dL*)		
Before	82 ± 47	77 ± 29
After	113 ± 32[a]	99 ± 44
Leptin (*ng/mL*)		
Before	3.1 ± 1.4	7.9 ± 6.7
After	1.2 ± 0.4[b]	4.1 ± 4.0[b]
GH (*ng/mL*)		
Before	0.87 ± 1.5	0.62 ± 0.8
After	0.55 ± 0.7	1.65 ± 2.7
Cortisol (*μg/dL*)		
Before	18.6 ± 4.8	16.9 ± 4.6
After	17.0 ± 4.0	17.9 ± 6.7
Norepinephrine (*pg/mL*)		
Before	314 ± 132	294 ± 171
After	366 ± 122	269 ± 158
Insulin (*μIU/mL*)		
Before	6.1 ± 3.5	10.3 ± 5.5
After	5.1 ± 2.5	6.5 ± 6.0
IGF-I (*ng/mL*)		
Before	108 ± 33	92 ± 36
After	85 ± 53	76 ± 37

[a]$P < .05$, [b]$P < .01$ (paired *t* test; before vs after).

only associates with various pathologic conditions but also affects patients' social activity. Therefore, postoperative body weight loss needs to be investigated thoroughly, especially in Japan, where early gastric cancer accounts for more than 50% of the total incidence of gastric cancer,[30] and the 5-year survival rate of early gastric cancer is more than 90%.[31] Previous studies reported that body weight loss after total gastrectomy was approximately 15% to 20% of the preoperative weight.[10,12,27] Because the incidence of gastric cancer is associated with low body weight in not only Japan and Asian countries but also in the Western world, the estimated average body mass index after total gastrectomy is expected to be 18 to 20 kg/m^2, which is lower than the ideal body mass index. The correlation between low body weight and long-term survival rate has not been analyzed thoroughly even in healthy individuals. A large cohort study of healthy Japanese subjects surveyed over 10 years concluded that a body mass index less than 19 kg/m^2 was associated with high mortality risk at an odds ratio of 2.26 due to various diseases, including infectious, cardiovascular, and malignant diseases.[3] Although there are no concrete data for patients who undergo gastrectomy, these patients could be at risk for a higher mortality rate due to low body weight. Based on this background, the major purpose of this study was to minimize postoperative body weight loss by ghrelin administration through up-regulation of GH secretion and appetite.

At the end of the 10-day study period, ghrelin reduced more than half of postoperative body weight loss from −3.7% in the placebo group to −1.4% in the ghrelin group. Although this is a limited result in the early postoperative period, which is associated with the most profound body weight loss, to the best of our knowledge, ghrelin administration is the most effective procedure among various studies that were designed for the same purpose.[12,32]

After numerous experimental studies, clinical application of ghrelin commenced in healthy volunteers and then extended to patients with heart failure,[23] pulmonary disease,[24] and cancer cachexia.[25] The results of these studies confirmed the safety of ghrelin administration. In our study, the patients in the 2 groups showed no differences in postoperative complications (eg, infections, delayed wound healing, thromboembolism) and length of hospital stay. However, 1 of the 11 patients developed diaphoresis, corresponding to National Cancer Institute Common Terminology Criteria for Adverse Events grade 1. Although we stopped ghrelin administration following the study protocol, this symptom was consistently reported in previous trials although it was tolerated by patients.[23-25,29] The overall positive effects of ghrelin such as body weight gain and increase in food intake calories were observed consistently in all clinical trials, including the present study.[21-25] In addition, improvement of disease-specific status has been reported, including patients with chronic heart failure[23] and those with chronic obstructive pulmonary disease.[24]

To our knowledge, the present study is the first clinical trial in the field of gastroenterological surgery. Moreover, the present study differs in 2 aspects from previous studies.[23-25] The first difference related to the study subjects; the subjects enrolled in previous clinical studies were cachexic emaciated patients in whom the level of circulating ghrelin was predicted to rise. It has been considered that the efficacy of exogenous ghrelin is limited because of down-regulation by high endogenous ghrelin. In contrast, in the present study, in which circulating ghrelin levels were extremely low due to total gastrectomy, we replaced the low levels of endogenous ghrelin with an exogenous one; therefore, it seems more physiologically related to study the effect of ghrelin administration. Another point is that complete vagotomy at the esophagogastric junction was performed during total gastrectomy in our patients. Because the vagus nerve mediates both efferent and afferent ghrelin signals,[33-35] it was suspected that exogenous ghrelin would not adequately interact in the hypothalamus. In animal experiments, vagotomy or chemical blockade of the vagal

signal abolished the effects of intravenously administrated ghrelin.[36] In vagotomized patients, ghrelin administration did not increase food intake.[37] However, other studies reported that ghrelin administered intraperitoneally successfully stimulated food intake after vagotomy in rats[38] and that ghrelin administration in vagotomized patients enhanced GH secretion.[39] These animal experiments and clinical studies indicate that the effects of ghrelin administration are still controversial, at least in vagotomized patients. In the present study, intravenous administration of exogenous ghrelin successfully stimulated food intake and appetite immediately after total gastrectomy. Our results suggest that the administered ghrelin crossed the blood-brain barrier to the central nervous system, probably increasing the appetite signal through not only the vagal pathway but also the circulation. The relationship between ghrelin and vagotomy remains poorly defined, and further studies should be performed in the future.

BMR accounts for between 60% and 70% of the total energy expenditure in adults.[40] Furthermore, the fat-free mass is considered the best single predictor of energy expenditure, and 53% to 88% of the variation in BMR is accounted for by fat-free mass.[41] In the placebo group, the BMR decreased significantly after total gastrectomy, whereas it did not change in the ghrelin group. This result was consistent with the significant decrease in lean body mass, which was limited to the placebo group. In animal experiments, ghrelin enhances abdominal fat storage in white adipose tissue in rats,[19] whereas clinical studies, including the present study, have shown that ghrelin increases lean body mass relative to fat mass.[23,24] Differences in species and patient status may influence the effect of ghrelin administration on fat metabolism. Preservation of lean body mass against the postoperative catabolic metabolism might be caused by ghrelin-stimulated GH secretion from the pituitary gland. However, serum GH levels were stable in the 2 groups, probably due to the rapid turnover of GH. This phenomenon was already reported in a previous phase I study.[29] Baseline leptin levels tended to be lower in the ghrelin group, probably because this group included more men, who generally have lower leptin levels than women.[42,43] Leptin levels significantly decreased in parallel with the decrease in fat mass in both the ghrelin and placebo groups.

The influence of cancer proliferation is another issue of safety in ghrelin studies. Several in vitro studies reported the expression of ghrelin receptor in cancer cells and that ghrelin weakly enhanced their proliferation, for example, in prostate[44] and pancreatic[45] cancer cells. However, another study reported that ghrelin inhibited proliferation and increased apoptosis in a lung cancer cell line.[46] In a preliminary experiment in our laboratory using various gastric cancer cell lines, all cells examined were negative for ghrelin receptor and showed no growth response to exogenous ghrelin (unpublished observation, March 2005). In clinical studies of cancer cachexic patients, no adverse events concerning tumor growth stimulation have been reported.[25] With respect to the present clinical trial, this argument was partly evaded because patients who met the inclusion criteria accounted for more than 90% curability by surgery alone[31] and ghrelin was administered for only 10 days. However, care should be taken when administering ghrelin over longer periods.

Although we successfully demonstrated a short-term effect of ghrelin administration on food intake, appetite, body weight, and other parameters, its long-term effect and benefit still need to be evaluated before clinical application. Because ghrelin secretion does not recover even several years after total gastrectomy, long-term administration of ghrelin is probably required to maintain the short-term effects. For this purpose, ghrelin poses a practical problem in that it is an unstable short-acting peptide and needs to be administered intravenously. An easier administration route, such as subcutaneous injection and inhalation, should be investigated to allow outpatient and home use. GH secretagogues, which were discovered before ghrelin, are orally available and perhaps could be used as ghrelin substitutes. For example, RC-1291 is orally available, well tolerated, and effective in promoting body weight gain, as demonstrated in a phase I study in healthy volunteers.[47] Another issue worth further investigation is the clinical benefits of ghrelin therapy, because it is argued that increases in appetite and body weight are not sufficient reasons for medication. Thus, further studies are needed to evaluate other aspects of ghrelin administration, such as reduction of total medical cost and hospital admission, improvement of social activity and quality of life, and postoperative survival. For example, postoperative body weight loss is most progressive and rehabilitation is most important in the first 3 months after surgery. It is possible that ghrelin administration for at least 3 months would improve postoperative recovery.

In conclusion, this prospective randomized study in a limited number of patients provides convincing data for the beneficial effects of ghrelin on body weight and dietary activity after total gastrectomy. Although there are some issues to be resolved before clinical application, including drug delivery system, duration of administration, and adequate assessment of clinical benefits, surgeons dealing with gastric cancers and other gastroesophageal diseases should be encouraged by the availability of ghrelin. Because surgery is essentially not physiologic and highly invasive for the body but the most reliable therapeutic option to cure cancer, it is our obligation to invent novel procedures to minimize its side effects.

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Received July 20, 2009. Accepted December 17, 2009.

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Acknowledgments

Online registry: http://www.umin.ac.jp; clinical trial no. UMIN000001925.

The authors thank Tomoyuki Sugimoto from the Department of Biomedical Statistics, Osaka University, for the advice on statistical analysis as well as the nutritional management room staff of Osaka University Hospital for calculating food intake calories per day in this study.

Conflicts of interest

The authors disclose no conflicts.

ELSEVIER

Adenovirus-Mediated Gene Expression of the Human *c-FLIP$_L$* Gene Protects Pig Islets Against Human CD8$^+$ Cytotoxic T Lymphocyte-Mediated Cytotoxicity

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ABSTRACT

Cell-mediated immunity, especially of human CD8+ cytotoxic T lymphocytes (CTLs) is believed to have an important role in the long-term survival of pig islet xenografts. Protection against human CD8+ CTL cytotoxicity may reduce the direct damage to pig islets and enable long-term xenograft survival in pig-to-human islet xenotransplantation. We have previously reported that *c-FLIP$_{S/L}$* genes, which are potent inhibitors of death receptor–mediated proapoptotic signals through binding competition with caspase-8 for recruitment to the Fas-associated via death domain (FADD), markedly suppress human CD8+ CTL-mediated xenocytotoxicity. In addition, the cytoprotective effects of *c-FLIP$_L$* seem to be significantly stronger than those of *c-FLIP$_S$*. Accordingly, in the present study, expression of *c-FLIP$_L$* was induced in intact pig islets by adenoviral transduction. Consequently, the cytoprotective capacity of the transgene in pig islets was examined in in vitro and in vivo exposure to human CD8+ CTLs. Cells from untransduced islets or mock islets were sensitive to CD8+ CTL-mediated lysis (59.3% ± 15.9% and 64.0% ± 8.9% cytotoxicity, respectively). In contrast, cells from pig islets transduced with the *c-FLIP$_L$* gene were markedly protected from lysis (30.5% ± 3.5%). Furthermore, prolonged xenograft survival was elicited from pig islets transduced with this molecule as assessed using an islet transplant model using the rat kidney capsule. Thus, these data indicate that intact pig islets can be transduced to express *c-FLIP$_L$* with adenovirus. Pig islets expressing *c-FLIP$_L$* are significantly resistant to human CTL killing and further exhibit beneficial effects to prolong xenograft survival.

THE EDMONTON PROTOCOL for human allogenic islet transplantation can successfully restore enodogenous insulin production and glycemic stability in patients with type 1 diabetes mellitus. However, insulin independence is usually not sustained despite islet infusions from two or more donors.[1] The current supply of islets from deceased human donors will almost certainly never meet

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0041-1345/09/$–see front matter
doi:10.1016/j.transproceed.2008.10.030

the demand. Xenotransplantation using pig islets could potentially resolve the shortage of donor islets. Pigs are the favored donor species because of (1) their similar physiology to human beings; (2) unlimited availability owing to a short generation interval because of the high number of pregnancies; and (3) somatic cloning is possible, and, thus, production of transgenic animals can be substantially enhanced.[2] In particular, pig islets have precise glycobiologic advantages because they lack α-gal epitopes, which induce complement activation, hyperacute rejection, and acute vascular rejection of xenografts. Therefore, successful short-term xenograft survival of these islets is the result of prevention of hyperacute rejection. However, after initial survival of pig islets, infiltrating cells into pig islet xenografts, including natural killer cells, macrophages, and CD8+ CTLs, are directly cytotoxic to the islets.[3–6] In previous studies, we reported that direct cytotoxicity of human CD8+ CTLs to pig islets is mediated in major part by the Fas/FasL apoptotic pathway.[4–6] Cellular FLICE inhibitory protein (c-FLIP) was originally identified as an inhibitor of death-receptor signaling through binding competition with caspase-8 for recruitment to the Fas-associated via death domain.[7–9] Two major c-FLIP variants result from alternative messenger RNA splicing: a short 26-KDa protein (c-FLIP$_S$) and a long 55-KDa form (c-FLIP$_L$).[7–9] We have demonstrated that the overexpression of *c-FLIP$_{S/L}$* genes markedly suppresses human CD8+ CTL-mediated xenocytotoxicity. In addition, the cytoprotective effects of c-FLIP$_L$ seem to be significantly stronger than those of c-FLIP$_S$.[6] The cytoprotective effect of c-FLIP$_L$ in pig islet cells remains controversial. Accordingly, in the present study, we evaluated the cytoprotective activities of the c-FLIP$_L$ molecule by adenovirus-mediated gene expression in pig islets.

MATERIALS AND METHODS

Pig Islet Isolation

Adult pig pancreases were removed at a slaughterhouse that handles young market-weight pigs (Large White–Landrace X–Duroc; age, 2 years; weight, 200–300 kg). Pig islets isolated using the modified Ricordi method as previously described[10,11] were maintained in complete medium 199 containing 10% heat-inactivated pig serum. Pig islet purity was assessed using the percentage of dithizone-positive cells.

Construction of Adenovirus Vector

The pAdex1CAwt adenovirus vector, regulated by the CAG (chicken β-actin) promoter, containing the open reading frame of the human *c-FLIP$_L$* gene, was purchased from RIKEN BioResource Center, Wako, Japan. The adenovirus was propagated by infection of human embryonic kidney 293 cells. Subsequently, adenovirus was purified using a Cesium/Tris gradient, separated into aliquots, and stored at −80°C until use. The titer of recombinant adenoviruses (multiplicity of infection [MOI]) was measured using the 50% tissue culture infectious dose method.[12] The "empty" control adenovirus, which lacks the human c-FLIP$_L$ insert, was also amplified in the same manner.

Transduction of Pig Islets by Adenovirus Vector

Freshly obtained adult pig islets represented in 500 μL of serum-free medium 199 were exposed to adenovirus encoding human c-FLIP$_L$ at a MOI of either 10 or 30 for 1 hour at 37°C. The transfected pig islets were then rinsed with serum-free RPMI (Roswell Park Memorial Institute) medium and resuspended in complete medium 199. Pig islets transfected with empty control adenovirus, which lacks the complementary DNA fragments of human c-FLIP$_L$ (ie, mock islets), were used as the vehicle control. Western blot analysis was performed to identify the expression of this molecule in pig islets.

Generation of Human CD8+ CTLs

To generate human CD8+ CTLs, peripheral blood mononuclear cells, freshly obtained from the blood of healthy volunteers were separated. Then 10 to 15 × 10^6 cells of separated peripheral blood mononuclear cells were cocultured for 14 days with irradiated pig endothelial cell monolayers in the presence of 50 U/mL of recombinant human IL-2 as previously described.[5,6] Subsequently, human CD8+ CTLs were positively selected using magnetic beads (Dynal Biotech AS, Oslo, Norway) and subjected to an in vitro cytotoxicity assay.

In Vitro Cytotoxicity Assay

The cytotoxic activity of human CD8+ CTLs against pig islets was assessed using a chromium 51 (^{51}Cr) release assay.[13] Either parental pig islets, mock islets, or transfected pig islets were incubated with Na_2-$^{51}CrO_4$ for 24 hours (1 μCi of ^{51}Cr per 100 islets). Subsequently, ^{51}Cr-labeled pig islets were plated in 96-well plates as target cells for admixture with human CTLs isolated using magnetic beads at various effector-to-target ratios. The ^{51}Cr released from dead islet cells was measured in the supernatants.

Transplant Studies and Immunohistochemical Analysis

To prove the in vivo effectiveness of this molecule to prolong xenograft survival, parental, mock, or transfected pig islets were transplanted under the kidney capsule in 8- to 10-week-old Lewis rats (Oriental Yeast Co, Ltd, Tokyo, Japan). The animals were randomly distributed between the three experimental groups. Rats preimmunized intraperitoneally with 250 mg of pig kidney membranes three times at 1-week intervals were the recipients.[5,6] In each case, 3000 IEQ of either parental, mock, or transfected pig islets were transplanted under the kidney capsule in the absence of immunosuppression. Transplant recipient rats were monitored until the time of harvest at day 3 or day 5 posttransplantation. Each grafted kidney was analyzed at immunohistochemistry. Kidney specimens were cut into small blocks, fixed in formalin, and embedded in a single paraffin block. After quenching endogenous peroxidase activity by exposure to 3% hydrogen peroxide–methanol, paraffin sections were stained with anti-pig insulin antibody (DAKO, Glostrup, Denmark) to detect surviving pig islet xenografts. The sections were rinsed and incubated with link antibody, followed by incubation of with horseradish peroxidase–conjugated streptavidin. Immunostaining was visualized with 0.02% diaminobenzidine (Sigma-Aldrich Corp, St Louis, Missouri) as the chromogen.

Statistical Analysis

Data were evaluated using the t test, with $P < .05$ considered significant. Data are presented as means (SD).

RESULTS

Protein Expression of Human c-FLIP$_L$ in Pig Islets

No protein expression of c-FLIP$_L$ was observed in parental pig islets (Fig 1A). The MOCK pig islets transduced with