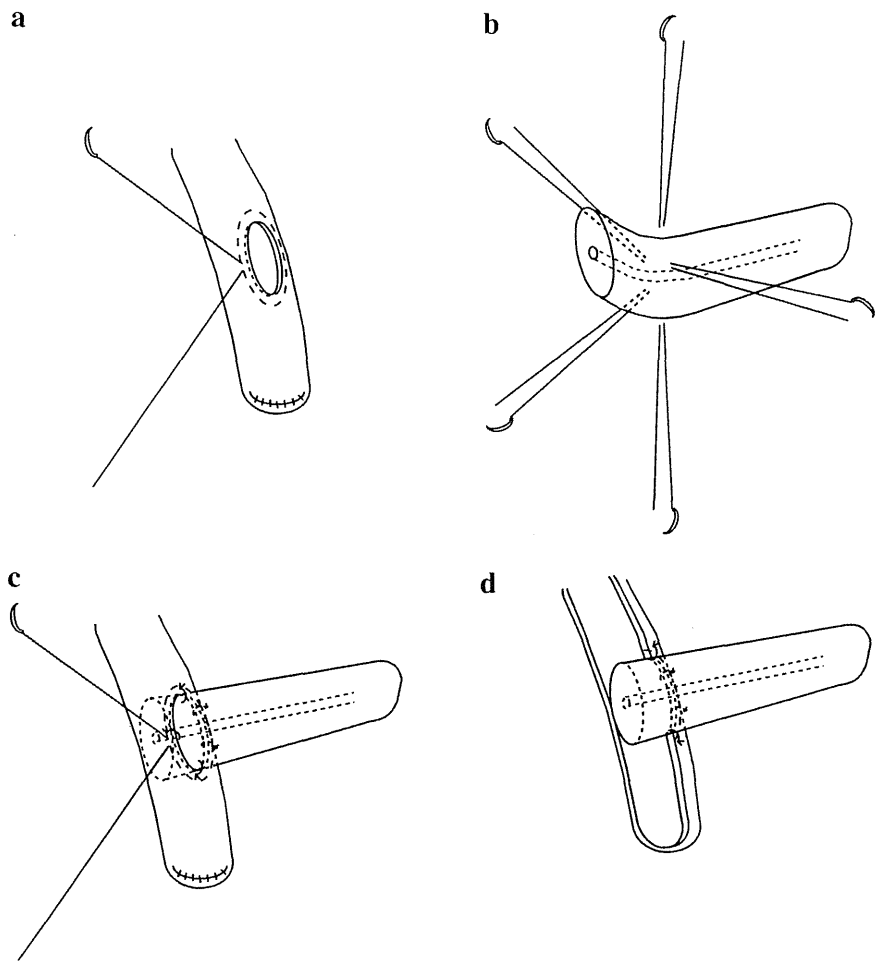

Fig. 1 A schematic drawing of end-to-side pancreaticojejunostomy without stitches in the pancreatic stump. An incision that matches the diameter of the pancreatic stump was made on the opposite side of the mesenterium of the jejunum, and a purse string suture of absorbable 3-0 monofilament thread was applied around the incision of the jejunum (**a**). The pancreatic stump was first isolated from the surrounding tissue with up to a 30-mm margin from the cut surface (**b**). Four to five stay sutures of absorbable 4-0 monofilament thread were also applied around the pancreas (**b**). The pancreatic stump was inserted into the jejunal incision in an end-to-side fashion applying the stay sutures on the incision to secure the depth of insertion (**c**, **d**). Finally, the purse string suture was tied to seal the anastomosis. The pancreatic remnant was sunk into the jejunum without any stitches on the cut end of the pancreas (**d**)

Table 1 Patient characteristics

	New method group	Traditional method group	*P* value
n	4	4	
Age	67 (63–79)	63 (58–68)	0.48
Male:female	3:1	4:0	0.28
Final diagnosis			
Pancreatic cancer	1	2	
Bile duct cancer	1	2	
Vater's papilla cancer	1	0	
Duodenal cancer	1	0	

Table 2 Operative parameters and outcomes after surgery

	New method group	Traditional method group	*P* value
Operating time (min)	420 (293–522)	422 (361–475)	0.48
Operating bleeding (g)	513 (170–890)	745 (210–1040)	0.36
Postoperative complication	1 (DGE)	0	0.28
Hospital stay after surgery (days)	33 (28–37)	32 (29–34)	0.48

DGE delayed gastric empting

developed a new end-to-end pancreaticojejunal anastomosis technique without the use of stitches going through the pancreatic stump. In their end-to-end anastomosis technique, there is a possible problem for a mismatch in the size of the jejunum and the pancreatic remnant. To avoid this problem, we modified Nordback et al.'s anastomosis technique in an end-to-side manner. An incision in the jejunum is made that is the same size as the diameter of the pancreatic remnant. We are not aware of any similar end-to-side pancreaticojejunal anastomosis techniques published in the English literature.

This new end-to-side pancreaticojejunostomy technique did not increase operative times or amounts of bleeding in the four pilot patients. In addition, the patients recovered without developing grade B or C POPF. These results prove that the four patients underwent successful operations,

even those with soft pancreatic textures. It is preferable for surgeons to master conventional techniques before performing this novel technique. This anastomosis technique can be performed safely, independent of the pancreatic texture or the size of the pancreatic duct (and can be used even in patients with a soft pancreas and small pancreatic duct). Additionally, pancreatic juice secreted from the transected pancreatic duct branches faced on the pancreatic stump is drained into the jejunal lumen [10]. Because this is a pilot study to determine whether this new end-to-side anastomosis technique can be performed as designed, further studies including more patients should be conducted to confirm the safety of the technique and whether it significantly decreases the incidence of pancreatic fistulas.

In conclusion, this pilot study showed that it is feasible to create a pancreaticojejunostomy anastomosis without applying any stitches at the cut end of the pancreas or pancreatic duct, independent of the size of the pancreas and the jejunum.

Conflict to interest No authors have any conflicts to interest.

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LINE-1 Hypomethylation Is Associated With a Poor Prognosis Among Patients With Curatively Resected Esophageal Squamous Cell Carcinoma

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Objective: To investigate the relationship between the long interspersed nucleotide element-1 (L1/LINE-1) methylation level and the disease-free survival and cancer-specific survival in patients with esophageal squamous cell carcinoma (ESCC).

Background: Cancer cells exhibit 2 types of deoxyribonucleic acid (DNA) methylation alterations: global DNA hypomethylation and site-specific CpG island promoter hypermethylation. Global DNA hypomethylation plays a role in genomic instability and carcinogenesis. DNA methylation in the LINE-1 repetitive element is a good indicator of the global DNA methylation level. Although the LINE-1 methylation level is attracting interest as a useful marker for predicting cancer prognosis, the prognostic significance of LINE-1 hypomethylaiton in ESCC remains unclear.

Methods: Using 217 curatively resected ESCC specimens, we quantified the LINE-1 methylation by utilizing the bisulfite pyrosequencing technology. Promoter methylation levels of *MGMT* and *MLH1* were also evaluated by pyrosequencing.

Results: ESCC showed significantly lower LINE-1 methylation levels in comparison with matched normal esophageal mucosa ($P < 0.0001$; N = 50). LINE-1 hypomethylation was significantly associated with disease-free survival [log-rank $P = 0.0008$; univariate hazard ratio (HR): 2.32, 95% confidence interval (CI): 1.38–3.84, $P = 0.0017$; multivariate HR: 1.81, 95% CI: 1.06–3.05, $P = 0.031$] and cancer-specific survival (log-rank $P = 0.0020$; univariate HR: 2.21, 95% CI: 1.33–3.60, $P = 0.0026$; multivariate HR: 1.87, 95% CI: 1.12–3.08, $P = 0.018$]. *MGMT* and *MLH1* hypermethylation were not associated with patient prognosis.

Conclusions: LINE-1 hypomethylation in ESCC is associated with a shorter survival, thus suggesting that it has potential for use as a prognostic biomarker.

Keywords: epigenetics, esophageal cancer, LINE-1, methylation, prognosis, pyrosequencing

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Esophageal squamous cell carcinoma (ESCC), the major histological type of esophageal cancer in east Asian countries, is one of the most aggressive malignant tumors.[1] Despite the development of multimodality therapies, the prognosis of patients remains poor, even for those who undergo complete resection of their carcinomas.[2–4] The limited improvements in treatment outcomes provided by conventional therapies have prompted us to seek innovative strategies for treating ESCC, especially those that are molecularly targeted. Importantly, epigenetic changes, including alterations in deoxyribonucleic acid (DNA) methylation, are reversible, and can thus be targets for therapy or chemoprevention.[5–7] In addition, the identification of new prognostic or predictive molecular markers for ESCC could improve the risk-adapted treatment strategies and help stratify patients in future clinical trials for drugs targeting these molecular changes.[8,9]

DNA methylation is a major epigenetic mechanism involved in X-chromosome inactivation, imprinting, and the repression of endogenous retroviruses.[5–7] DNA methylation alterations associated with human cancers include global DNA hypomethylation and site-specific CpG island promoter hypermethylation. Promoter hypermethylation can silence tumor suppressor genes, DNA mismatch repair genes (eg, *MLH1*), or DNA repair genes (eg, *MGMT*), thereby contributing to esophageal carcinogenesis.[10] Global DNA hypomethylation appears to play an important role in genomic instability, leading to cancer development.[11–13] As long interspersed nucleotide element-1 (LINE-1 or L1) retrotransposon constitutes a substantial portion (approximately 17%) of the human genome, the level of LINE-1 methylation is regarded to be a surrogate marker of global DNA methylation.[14] Measurement of LINE-1 methylation by pyrosequencing technology has emerged as a cost-effective and high-throughput method to assess the global DNA methylation status.[15–17] In several types of human neoplasms, LINE-1 methylation has been shown to be highly variable, and LINE-1 hypomethylation is strongly associated with a poor prognosis.[18–20] However, the influence of LINE-1 hypomethylation on the prognosis of ESCC patients remains unclear.

In this study, we quantified the LINE-1 methylation in 217 samples of curatively resected ESCCs by utilizing a bisulfite–polymerase chain reaction (PCR)–pyrosequencing assay, and examined prognostic significance of LINE-1 hypomethylation in ESCC. In addition, the promoter hypermethylation of *MGMT* and *MLH1* was also evaluated in association with patient survival. Our data suggest that LINE-1 hypomethylation can have a potential role as a prognostic biomarker.

METHODS

Study Subjects

A total of 231 consecutive patients with ESCC who were undergoing curative resection at Kumamoto University Hospital (Kumamoto, Japan) between April 2005 and December 2010 were enrolled in this study. Nine patients were excluded due to the unavailability of adequate tissue samples. We initially quantified LINE-1 methylation in 222 cancer specimens, and obtained valid results in 217 (98%) of the cases. Thus, a total of 217 ESCCs were finally included in this study. Patients were observed at 1 to 3-month

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intervals until death or June 30, 2011, whichever came first. Tumor staging was done by the American Joint Committee on Cancer's *Cancer Staging Manual* (7th edition).[21] Sixty-nine patients received preoperative treatment {41 patients: chemotherapy [cisplatin, 5-fluorouracil either with or without docetaxel], 4 patients: radiation therapy, 24 patients: chemoradiotherapy}. Disease-free survival was defined as the length of time after surgical treatment for the cancer during which the patient survived with no sign of cancer recurrence. Overall survival was defined as the time between the date of the operation and the date of death. Cancer-specific survival was defined as the time between the date of operation and the date of death that was confirmed to be attributable to ESCC. Written informed consent was obtained from each subject, and the study procedures were approved by the Institutional Review Board. The term "prognostic marker" is used throughout this paper according to the REMARK Guidelines.[22]

DNA Extraction and Sodium Bisulfite Treatment

Hematoxylin and eosin-stained slides of the tumors were reviewed by 1 pathologist, who marked the areas of the tumor and normal mucosa. Hematoxylin and eosin-stained tissue sections (depending on tissue and tumor size: on average, large tumor tissue 10 μm × 1 section) from each case were scraped off slides for DNA extraction. Genomic DNA extraction from the tumor and normal mucosa, and sodium bisulfite treatment on genomic DNA were performed as previously described by Ogino et al.[18,23–25]

Pyrosequencing to Measure LINE-1 Methylation

Polymerase chain reaction and subsequent pyrosequencing for LINE-1 were performed as previously described by Ogino et al, using the PyroMark kit (Qiagen).[18,23,24] This assay amplifies a region of LINE-1 element (position 305–331 in accession no. X58075), which includes 4 CpG cites. The PCR conditions were 45 cycles of 95°C for 20 seconds, 50°C for 20 seconds, and 72°C for 20 seconds, followed by 72°C for 5 minutes. The biotinylated PCR product was purified and made single-stranded to act as a template in a pyrosequencing reaction, using the Pyrosequencing Vacuum Prep Tool (Qiagen). Pyrosequencing reactions were performed in the PyroMark Q24 System (Qiagen). The nucleotide dispensation order was: ACT CAG TGT GTC AGT CAG TTA GTC TG. The non-CpG cytosine in LINE-1 repetitive sequences has been documented to be rarely methylated. Thus, complete conversion of cytosine at a non-CpG site ensured successful bisulfite conversion. The amount of C relative to the sum of the amounts of C and T at each CpG site was calculated as the percentage (ie, 0 to 100). The average of the relative amounts of C in the 4 CpG sites was used as the overall LINE-1 methylation level in a given tumor (Fig. 1).

Pyrosequencing to Measure Promoter Methylation of *MGMT* and *MLH1*

Pyrosequencing for *MGMT* and *MLH1* was performed using the PyroMark kit (Qiagen). We used the previously defined cutoff of ≥8% methylated alleles for *MGMT* and *MLH1* hypermethylated tumors.[26]

Statistical Methods

For the statistical analyses, we used the JMP (Version 9, SAS Institute, Cary, NC) and the SAS software programs (Version 9.1, SAS Institute, Cary, NC). All *P*-values were 2-sided. To compare the means, we performed the *t* test assuming unequal variances. For the survival analysis, the Kaplan-Meier method was used to assess the survival time distribution, and the log-rank test was used. To assess the independent effect of the LINE-1 methylation level on mortality, the tumor stage (IA + IB, IIA + IIB, IIIA + IIIB + IIIC) was used as a stratifying (matching) variable in Cox models using the "strata" option in the SAS "proc phreg" command to avoid residual confounding and overfitting. We constructed a multivariate, stage-stratified Cox proportional hazard model to compute a hazard ratio (HR) according to LINE-1 methylation status, containing sex (male vs female), age at surgery (continuous variable), tobacco use (yes vs no), alcohol use (yes vs no), year of diagnosis (2005–2007 vs 2008–2010), preoperative treatment (present vs absent), and histological grade (G1 vs G2–4). A backward stepwise elimination with a threshold of $P = 0.20$ was used to select variables in the final model. For cases with missing information in any of the categorical variables [tobacco use (5.1%) and alcohol use (6.9%)], we used a complete-subject method for model selection and final model. After the selection was done, we assigned separate missing indicator variables to those cases with missing information in the final model. An interaction was assessed by including the cross product of the LINE-1 variable and another variable of interest (without data-missing cases) in a multivariate Cox model, and thereafter the Wald test was performed. To validate the cutoff value, the *C* statistics for survival data established by Uno et al was estimated, which is similar to time-dependent receiver operating characteristic curve analysis.[27] The "survivalROC" package in

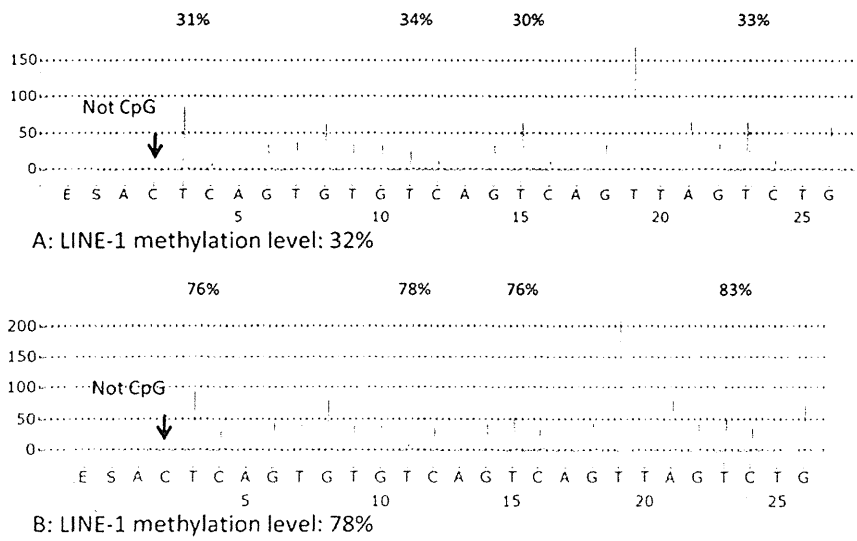

FIGURE 1. The pyrosequencing assay used to measure the LINE-1 methylation level. A, A LINE-1 hypomethylated tumor (methylation level, 32). B, A LINE-1 hypermethylated tumor (methylation level, 78). The percentages (in blue) are the proportion of C at each CpG site after bisulfite conversion, and the methylation level of each CpG site was estimated by the proportion of C (%). The overall LINE-1 methylation level was calculated as the average of the proportions of C (%) at the 4 CpG sites. The first, third, and fourth CpG sites follow mononucleotide T repeats, resulting in higher T peaks than in the case of the second CpG site, and the proportion of C (%) has been adjusted accordingly. The arrows indicate no residual C at the non-CpG site, ensuring complete bisulfite conversion.

the open-source statistical software R (http://www.R-project.org) was used for the analysis.

RESULTS

LINE-1 Methylation in Esophageal Squamous Cell Carcinoma and Matched Normal Mucosa

We first examined the LINE-1 methylation level in 50 ESCC tissues and matched normal esophageal mucosa samples. The cancer tissues exhibited significantly lower levels of LINE-1 methylation [median, 65.1; mean, 63.3; standard deviation, 12.7 (all in 0–100 scale)] than the matched normal mucosa (median, 79.3; mean, 78.8; standard deviation, 6.2) ($P < 0.0001$ by the paired t test) (Fig. 2A).

Evaluation of Association of LINE-1 Methylation Level and Clinical, Epidemiological, and Pathological Variables

Next, we quantified the LINE-1 methylation in 222 cancer specimens, and obtained valid results in 217 (98%) of cases. The distribution of the LINE-1 methylation level in the 217 cancers (Fig. 2B) was as follows: mean, 64.5; median, 65.0; standard deviation, 12.8; range, 24.8 to 91.8; interquartile range, 55.5 to 74.5 (all in 0–100 scale). The LINE-1 methylation level was then divided into quartiles [Q1 (≥74.6, n = 54), Q2 (65.0–74.5, n = 55), Q3 (55.5–64.9, n = 54), and Q4 (<55.5, n = 54)] for further analyses. However, we found that the LINE-1 methylation level was not associated with any of the clinical, epidemiological, or pathological characteristics (all $P > 0.18$, Table 1).

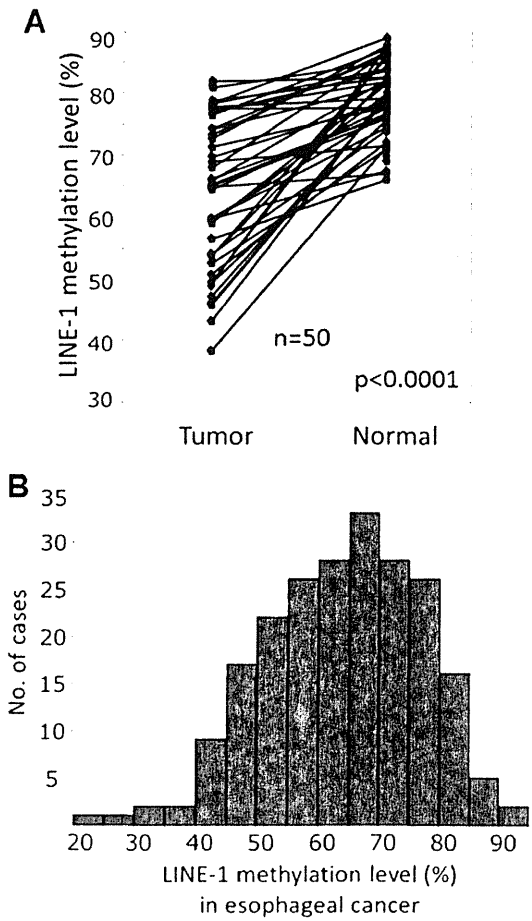

FIGURE 2. The LINE-1 methylation levels in esophageal cancer. A, The LINE-1 methylation levels in 50 esophageal cancer and matched normal mucosa specimens. The cancer tissues showed significantly lower levels of methylation than the matched normal mucosa ($P < 0.0001$ by the paired t test). B, The distribution of the LINE-1 methylation levels in 217 esophageal cancers.

LINE-1 Hypomethylation and Patient Survival

During the follow-up of the 217 patients, there were a total of 63 esophageal cancer recurrences and 51 deaths that were confirmed to be attributable to esophageal cancer. The median follow-up time for censored patients was 2.6 years.

We performed the Cox regression analysis with LINE-1 methylation as a continuous variable. LINE-1 hypomethylation was associated with a statistically significant increase in disease recurrence (univariate analysis $P < 0.001$) and cancer-specific mortality (univariate analysis $P < 0.001$). The univariate HR for disease recurrence rate associated with a 20% decrease in LINE-1 methylation was 2.23 (95% confidence interval [CI]: 1.50–3.32), whereas that of the cancer-specific mortality was 2.39 (95% CI: 1.54–3.70).

We performed the Cox regression analysis using a categorical variable [ie, the first quartile cases (Q1), the second quartile cases (Q2), the third quartile cases (Q3), and the fourth quartile cases (Q4)]. In a univariate Cox regression analysis, compared with Q1 cases, Q4 cases experienced a significantly higher disease recurrence rate [$P = 0.0020$, HR: 3.39; 95% CI: 1.64–7.70], whereas Q2 and Q3 cases experienced a slightly, but not significantly, higher disease recurrence rate compared with Q1 cases ($P = 0.12$ for Q2 and $P = 0.46$ for Q3) (Table 2). Similar results were observed in the multivariate analysis for disease-free survival (Table 2), in the univariate and multivariate analyses for cancer-specific survival (Table 2), and in the univariate and multivariate analyses for overall survival (data not shown). Thus, we made a dichotomous LINE-1 methylation variable, defining Q4 as the "hypomethylated group" and combining Q1, Q2, and Q3 into the "hypermethylated group."

In the Kaplan-Meier analysis, LINE-1 hypomethylators (ie, Q4 cases) experienced significantly shorter disease-free survival (log-rank $P = 0.0008$) and cancer-specific survival (log-rank $P = 0.0020$) than those with hypermethylation (Fig. 3). In the univariate Cox regression analysis, compared with LINE-1 hypermethylated cases, LINE-1 hypomethylated cases experienced a significantly higher disease recurrence rate (HR: 2.32; 95% CI: 1.38–3.84; $P = 0.0017$] (Table 2). In the multivariate Cox model adjusted for the clinical, pathological, and epidemiological features, LINE-1 hypomethylation was found to be associated with a significantly higher disease recurrence rate (multivariate HR: 1.81; 95% CI: 1.06–3.05; $P = 0.031$). Similar results were observed in an analysis of the cancer-specific mortality (Table 2) and overall mortality (data not shown).

The C statistics for survival data were also estimated to validate the cutoff value of LINE-1 hypomethylation. We generated a Cox regression model with LINE-1 methylation level and obtained the estimated C statistics, as the cutoff value of LINE-1 "hypomethylation," "<30," "<40," "<50," "<60," "<70," or "<80," has been tested. The estimated risk score was highest (0.71) in the case where LINE-1 hypomethylation was defined as "<50." This result may support the validity of our cutoff value (ie, <55.5).

Interaction Between LINE-1 Hypomethylation and Other Variables in Survival Analyses

We also examined whether the influence of LINE-1 hypomethylation on the disease-free survival was modified by any of

TABLE 1. LINE-1 Methylation in Esophageal Cancer Specimens and Association with Clinical and Tumor Features

Clinical, Epidemiological, or Pathological Feature	Total N	LINE-1 Methylation (Quartile)				P
		Q1 (≥74.6)	Q2 (65.0–74.5)	Q3 (55.5–64.9)	Q4 (<55.5)	
All cases	217	54	55	54	54	
Mean age ± standard deviation	65.9 ± 9.1	66.3 ± 8.6	66.8 ± 9.1	65.1 ± 8.4	65.6 ± 10.5	0.78
Sex						0.44
Male	192 (88%)	45 (83%)	50 (91%)	47 (87%)	50 (93%)	
Female	25 (12%)	9 (17%)	5 (9.1%)	7 (13%)	4 (7.4%)	
Tobacco use						0.81
Yes	164 (80%)	34 (76%)	45 (83%)	42 (79%)	43 (81%)	
No	41 (20%)	11 (24%)	9 (17%)	11 (21%)	10 (19%)	
Alcohol use						0.60
Yes	171 (87%)	34 (81%)	46 (90%)	44 (88%)	47 (88%)	
No	25 (13%)	8 (19%)	5 (9.8%)	6 (12%)	6 (12%)	
Year of diagnosis						0.33
2005–2007	86 (40%)	19 (35%)	19 (35%)	27 (50%)	21 (39%)	
2008–2010	131 (60%)	35 (65%)	36 (65%)	27 (50%)	33 (41%)	
Preoperative treatment						0.33
Present	69 (32%)	18 (33%)	14 (25%)	15 (28%)	22 (41%)	
Absent	148 (68%)	36 (67%)	41 (75%)	39 (72%)	32 (59%)	
Stage						0.51
I (IA, IB)	99 (46%)	27 (50%)	29 (53%)	20 (37%)	23 (43%)	
II (IIA, IIB)	47 (22%)	11 (20%)	13 (24%)	11 (20%)	12 (22%)	
III (IIIA, IIIB, IIIC)	71 (33%)	16 (30%)	13 (24%)	23 (43%)	19 (35%)	
Lymph node metastasis						0.18
Positive	96 (44%)	21 (39%)	20 (36%)	30 (56%)	25 (46%)	
Negative	121 (56%)	33 (61%)	35 (64%)	24 (44%)	29 (54%)	
Histological grade						0.55
G1	90 (41%)	26 (48%)	22 (40%)	25 (46%)	17 (31%)	
G2	92 (42%)	22 (41%)	22 (40%)	21 (39%)	27 (50%)	
G3–4	35 (16%)	6 (11%)	11 (20%)	8 (15%)	10 (19%)	

Percentage (%) indicates the proportion of cases with a specific clinical, pathological, or epidemiological feature among each quartile group (Q1, Q2, Q3, or Q4).

TABLE 2. Association of LINE-1 Methylation Status in Esophageal Cancer With Patient Mortality

LINE-1 Methylation Level (Quartile)	Total N	Disease-Free Survival			Cancer-Specific Survival		
		Univariate HR (95% CI)	Stage-matched HR (95% CI)	Multivariate stage-matched HR (95% CI)	Univariate HR (95% CI)	Stage-matched HR (95% CI)	Multivariate stage-matched HR (95% CI)
Q1 (≥74.6)	54	1 (referent)	1 (referent)	1 (referent)	1 (referent)	1 (referent)	1 (referent)
Q2 (65.0–74.5)	55	1.92 (0.87–4.54)	1.98 (0.89–4.68)	1.83 (0.80–4.46)	1.77 (0.82–4.05)	1.73 (0.79–3.94)	1.76 (0.80–4.09)
Q3 (55.5–64.9)	54	1.45 (0.64–3.60)	1.30 (0.56–3.15)	1.25 (0.52–3.13)	1.39 (0.62–3.23)	1.26 (0.56–2.94)	1.24 (0.54–2.95)
Q4 (<55.5)	54	3.39 (1.64–7.70)	2.86 (1.38–6.52)	2.50 (1.16–5.86)	3.04 (1.51–6.62)	2.72 (1.35–5.93)	2.47 (1.20–5.48)
P for trend		0.0062	0.019	0.072	0.011	0.024	0.055
Q1–3 (≥55.5)	163	1 (referent)	1 (referent)	1 (referent)	1 (referent)	1 (referent)	1 (referent)
Q4 (<55.5)	54	2.32 (1.38–3.84)	2.04 (1.21–3.38)	1.81 (1.06–3.05)	2.21 (1.33–3.60)	2.06 (1.24–3.37)	1.87 (1.12–3.08)
P		0.0017	0.0079	0.031	0.0026	0.0060	0.018

the clinical, pathological, and epidemiological variables. We found a significant modifying effect of the tumor stage on the relationship between LINE-1 methylation and the recurrence rate (*P* for interaction = 0.031; Fig. 4), although this could be a chance event considering multiple hypothesis testing. Among the patients with stage I tumors, LINE-1 hypomethylated cases experienced a significantly shorter disease-free survival (log-rank $P = 0.0006$; Fig. 5). In contrast, among patients with stage II and III tumors, the effect of LINE-1 hypomethylation on disease-free survival was slightly attenuated (log-rank, $P = 0.11$; Fig. 5). Similar results were obtained when cancer-specific mortality was used as the endpoint (data not shown). We did not observe a significant interaction with LINE-1 hypomethylation in the survival analysis for any other variables (all *P* for interactions > 0.12; Fig. 4).

Analyses Excluding Patients With Preoperative Therapy

The effect of preoperative chemotherapy and/or radiotherapy on LINE-1 methylation level is not known. Therefore, we also performed additional analyses excluding ESCC patients who had received preoperative therapy. The distribution of LINE-1 methylation level in 148 patients without preoperative therapy was very similar to that in 217 patients with or without preoperative therapy: mean,

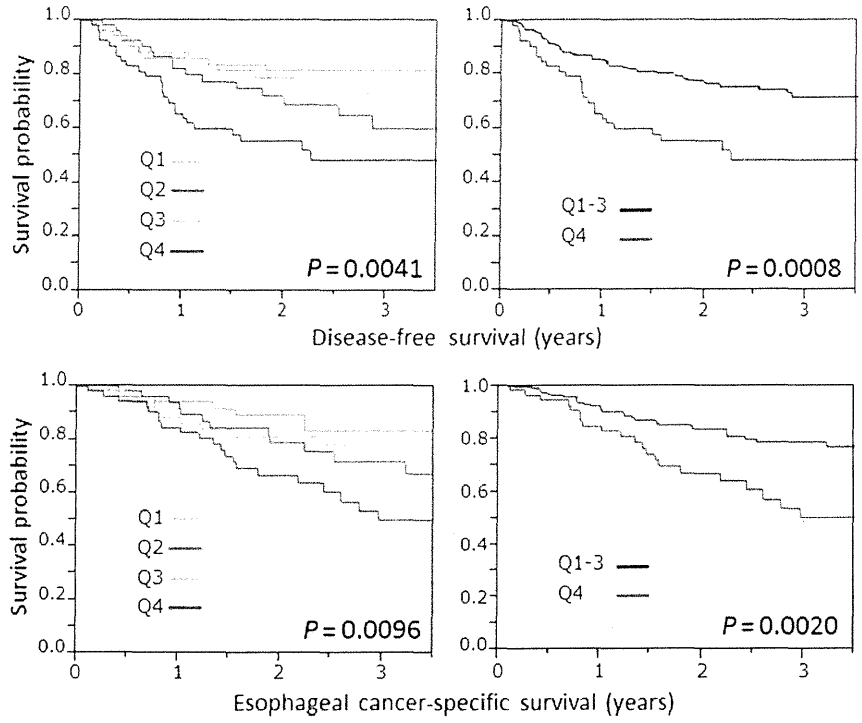

FIGURE 3. The Kaplan-Meier curves for disease-free survival (top panels) and cancer-specific survival (bottom panels) according to quartiles (Q1–4) of LINE-1 methylation in esophageal cancer. In the right panels, Q4 represents the "hypomethylated group" and Q1, Q2, and Q3 represent the "hypermethylated group".

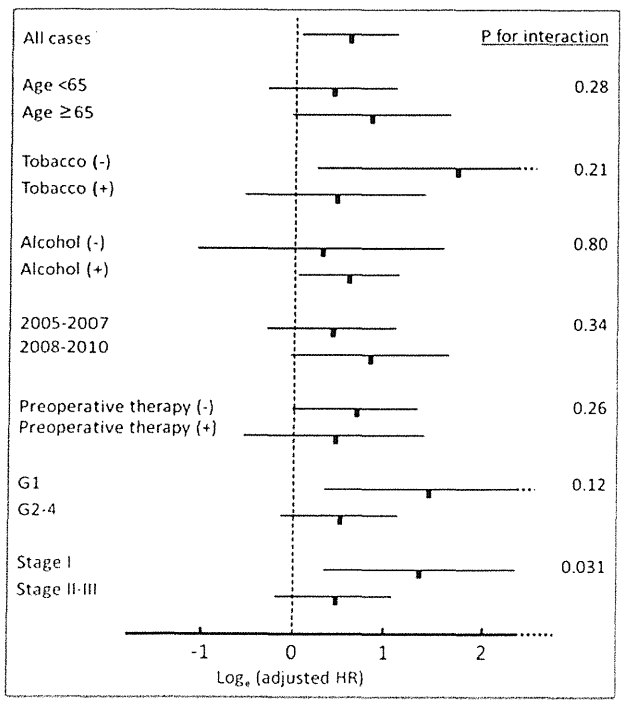

FIGURE 4. The LINE-1 methylation status and disease-free survival in various strata. The $\log_e$ (adjusted HRs) plot with the 95% CI for the disease recurrence rate in LINE-1 hypomethylated tumors (vs LINE-1 hypermethylated tumors) is shown.

64.7; median, 65.4; standard deviation, 12.6; range, 24.8 to 89.6; interquartile range, 55.7 to 74.2. We divided LINE-1 methylation level into quartiles [q1 (≥74.3, n = 37), q2 (65.4–74.2, n = 37), q3 (55.7–65.3, n = 37), and q4 (<55.7, n = 37)]. LINE-1 methylation level was not associated with any clinical, epidemiological, or pathological characteristics (all $P > 0.07$). The univariate and multivariate Cox regression analyses that utilize a tetrachotomous variable (ie, q1–4) also exhibited similar results with original analyses (see Table 1, Supplemental Digital Content 1, available at: http://links.lww.com/SLA/A298). We therefore made a dichotomous LINE-1 methylation variable, defining q4 as the "hypomethylated group" and combining q1, q2, and q3 into the "hypermethylated" group. Importantly, the cutoff value of "LINE-1 hypomethylated group" in this analysis (ie, <55.7) was quite close to that in the original analyses for patients with or without preoperative therapy (ie, <55.5). LINE-1 hypomethylators experienced a significantly higher disease recurrence rate compared with LINE-1 hypermethylated cases (log-rank $P = 0.0024$, univariate HR: 2.59, 95% CI: 1.34–4.83; $P = 0.0057$, multivariate HR: 2.08, 95% CI: 1.01–4.14; $P = 0.032$; see Figure 1A, Supplemental Digital Content 2, available at: http://links.lww.com/SLA/A299, which shows the Kaplan-Meier curve for disease-free survival). Similar results were seen regarding the interaction between LINE-1 hypomethylation and tumor stage [see Figure 1B, Supplemental Digital Content 2, available at: http://links.lww.com/SLA/A299, which shows Kaplan-Meier curves for disease-free survival among patients with stage I tumors (left panel) and those with stage II and III tumors (right panel)]. We did not observe a significant interaction in the survival analysis for any other variables (all P for interactions > 0.23).

Promoter Hypermethylation of *MGMT* and *MLH1* in Association With Patient Survival

We obtained valid results for *MGMT* methylation in 202 cases and for *MLH1* methylation in 173 cases. Sixty-nine cases (34%) exhibited *MGMT* hypermethylation, and 110 cases (64%) exhibited *MLH1* hypermethylation. There was no significant relationship between promoter hypermethylation and disease-free survival (log-rank $P = 0.41$ for *MGMT* and $P = 0.12$ for *MLH1*) [see Figure 2, Supplemental Digital Content 3, available at: http://links.lww.com/SLA/A300, which demonstrates the Kaplan-Meier Curves for disease-free

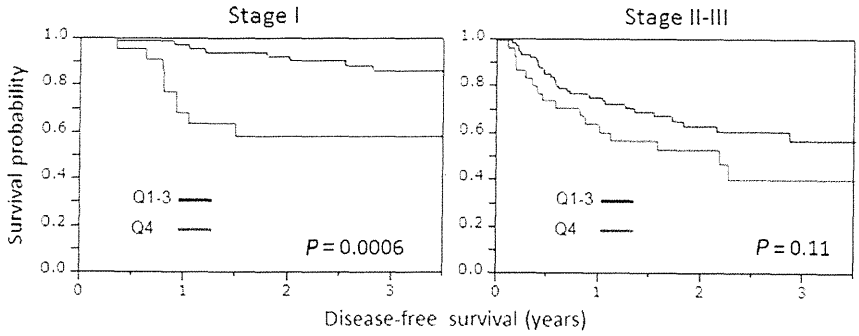

FIGURE 5. Kaplan-Meier curves for disease-free survival among patients with stage I tumors (left panel) and those with stage II and III tumors (right panel). Q4 represents the "hypomethylated group" and Q1, Q2, and Q3 represent the "hypermethylated group".

survival according to *MGMT* methylation status (left panel) and according to *MLH1* methylation status (right panel)]. Similar results were observed in an analysis of the cancer-specific mortality and overall mortality (data not shown).

DISCUSSION

We conducted this study to examine the prognostic impact of LINE-1 hypomethylation among 217 patients with curatively resected ESCCs. As LINE-1 constitutes a substantial portion of the human genome, the methylation status of LINE-1 reflects the global DNA methylation level.[14] We have found that LINE-1 hypomethylation (ie, global DNA hypomethylation) in esophageal cancer is associated with a poor prognosis, suggesting that LINE-1 hypomethylation may be a biomarker that can be used to identify patients who will experience an inferior outcome.

Although the prognostic factors in ESCC have been extensively studied,[28–30] little is known regarding the prognostic value of global DNA hypomethylation. However, the relationship between genome-wide DNA hypomethylation and the clinical outcome has been examined in several types of human neoplasms (eg, glioma, cutaneous melanoma, chronic myeloid leukemia, ovarian cancer, non-small-cell lung cancer, and colon cancer).[17–20,31–33] Studies of glioma, ovarian cancer, and colon cancer have shown a statistically significant association between global DNA hypomethylation and poor survival.[17–20] Our current findings in ESCC are in agreement with these results. In addition, global DNA hypomethylation was associated with clinically aggressive disease in patients with prostate cancer and gastrointestinal stromal tumors.[34,35] On the other hand, a study of cutaneous melanoma demonstrated that LINE-1 hypomethylation was associated with a favorable prognosis,[31] which did not agree with our current findings. This discrepancy might be due to differences in the tumor histological type. Nonetheless, our data support a potential role for LINE-1 hypomethylation as a prognostic biomarker for ESCC. This study also demonstrated that promoter hypermethylation of *MGMT* and *MLH1* was not associated with patient survival; the results of *MGMT* hypermethylation were consistent with the previous study.[36] This is the first study evaluating the prognostic value of *MLH1* hypermethylation.

The mechanism by which global DNA hypomethylation may confer a poor prognosis remains to be fully explored. Genome-wide DNA hypomethylation has been shown to be associated with genomic instability,[11–13,37] which may confer a poor prognosis. Transcriptional dysregulation might be another possible mechanism, and activation of proto-oncogenes, endogenous retroviruses, or transposable elements might affect the tumor's aggressiveness. A third possible mechanism involves inflammatory mediators and oxidative stress; the latter has been associated with genomic DNA hypomethylation.[38] The activation of the inflammatory pathway is also associated with a poor prognosis in esophageal cancer.[39] Furthermore, in addition to its role as a surrogate marker for global DNA methylation, the LINE-1 methylation status by itself likely has biological effects, as retrotransposons, such as LINE-1 elements, can provide alternative promoters,[40] and contribute to noncoding ribonucleic acid expression, which regulates the functions of a number of genes.[41,42] Further studies are necessary to validate our findings, as well as to elucidate the mechanism(s) by which LINE-1 hypomethylation affects tumor behavior.

Interestingly, the effect of LINE-1 hypomethylation on the prognosis appears to differ according to tumor stage; the effect is particularly prominent among stage I ESCCs. This finding is in agreement with the results of non-small–cell lung cancer, showing that LINE-1 hypomethylation is a marker of a poor prognosis in patients with early-stage (ie, stage IA) tumors, but not in those with advanced-stage tumors.[33] Even for patients with stage I disease, the prognosis of ESCC is relatively poor. Thus, accurate prediction of the likely outcome of stage I ESCC patients is important for selecting their postoperative management (eg, the use of adjuvant chemotherapy or the frequency of follow-up examination). Given that LINE-1 hypomethylation was found to be independently associated with the patient prognosis, even in the analysis including all tumor stages, our data support a potential role for LINE-1 hypomethylation as a prognostic biomarker for ESCC.

Given that the effect of preoperative chemotherapy and/or radiotherapy on LINE-1 methylation level is not known, we performed the analysis excluding ESCC patients who had received preoperative therapy. In this analysis, the distribution of LINE-1 methylation levels and the cutoff value of the LINE-1 "hypomethylated group" were quite close to those of 217 ESCCs with or without preoperative therapy. In addition, these analyses also demonstrated that patients with LINE-1 hypomethylation experienced significantly worse outcomes compared with those with LINE-1 hypermethylation, thus indicating that LINE-1 hypomethylation has potential for use as a prognostic biomarker, both for the cohort of ESCC patients without preoperative therapy and for the cohort of ESCC patients with preoperative therapy. In this respect, our finding may have clinical implications. The relationship between LINE-1 hypomethylation, preoperative therapy, and patient outcome needs to be confirmed in independent cohorts in the future.

In summary, this study suggests that genome-wide DNA hypomethylation, as measured in LINE-1, is independently associated with poor survival among patients with ESCC. In addition, the effect of LINE-1 hypomethylation on the prognosis is especially prominent in patients with stage I ESCCs. Future studies are needed to confirm this association and to examine the potential mechanism(s) by which genome-wide DNA hypomethylation affects tumor behavior.

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K. Sakamaki, and Y. Ohashi; manuscript writing: S. Iwagami, Y. Baba, M. Watanabe, K. Sakamaki, and H. Baba; and final approval of manuscript: all authors.

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Clinical Cancer Research

Enhancement of human cancer cell motility and invasiveness by anaphylatoxin C5a via aberrantly expressed C5a-receptor (CD88).

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Enhancement of human cancer cell motility and invasiveness by anaphylatoxin C5a via aberrantly expressed C5a-receptor (CD88).

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Running title: C5a-C5aR axis enhances cancer cell motility and invasion

Key words: C5a, C5a receptor, migration, invasion, MMP

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Translational Relevance

The anaphylatoxin C5a is a product of complement system activation that occurs in the cancer microenvironment, however, the role of C5a generated in cancer tissues is largely unknown. We found C5a-receptor (C5aR) expression in cancer cells of human cancer tissues samples. Moreover, by Matrigel chamber assay and nude mouse skin implantation we showed C5a-elicited enhancement of C5aR-expressing cell invasion through motility activation and matrix metalloproteases (MMP) release. Indeed, anti-C5aR antibody and MMP inhibitor GM6001 suppressed C5aR-expressing cancer cell invasion enhanced by C5a. These results illustrate a novel activity of the C5a-C5aR axis in cancer and suggest that it might be beneficial to target this signaling pathway for cancer therapy. Furthermore, since C5aR expression was seen in adenocarcinoma, squamous cell carcinoma and transitional cell carcinoma, C5a-C5aR-targeting therapy may work for those different types of cancer.

Abstract

Purpose: The anaphylatoxin C5a is a chemoattractant that induces leukocyte migration via C5a receptor (C5aR). There is emerging evidence that C5a is generated in the cancer microenvironment. We therefore sought C5aR expression and a direct influence of the C5a-C5aR axis on cancer cells.

Experimental Design: C5aR expression was investigated in human cancer tissues and cell lines. Effects of C5a stimulation on cancer cells were studied by cytoskeletal rearrangement, time-lapse analysis, Matrigel chamber assay and invasion in nude mouse in a comparison of C5aR-expressing cancer cells with control cells.

Results: C5aR was aberrantly expressed in various human cancers. Several cancer cell lines also expressed C5aR. C5a triggered cytoskeletal rearrangement and enhanced cell motility 3-fold and invasiveness 13-fold of C5aR-expressing cancer cells. Such enhancement by C5a was not observed in control cells. Cancer cell invasion was still enhanced in the absence of C5a concentration gradient and even after the removal of C5a stimulation, suggesting that random cell locomotion plays an important role in C5a-triggered cancer cell invasion. C5a increased the release of matrix

metalloproteinases (MMPs) from cancer cells by 2 to 11-fold, and inhibition of MMP activity abolished the C5a enhancing effect on cancer cell invasion. Compared with control cells, C5aR-expressing cells spread 1.8-fold more broadly at implanted nude mouse skin sites only when stimulated with C5a.

Conclusions: These results illustrate a novel activity of the C5a-C5aR axis that promotes cancer cell invasion through motility activation and MMP release. Targeting this signaling pathway may provide a useful therapeutic option for cancer treatment.

Introduction

The complement system is a biochemical cascade involved in immune responses (1). Previous reports showed that the complement system is activated on cancer cells in both an animal model (2) and in tissue specimens (3). It was initially suggested that the complement system might be involved in cancer immune surveillance by its direct cytolytic effect (3) and the sensitization of cancer cells to the immune effector cells via release of chemoattractants (4). However, cancer cells seem to evade the complement attack by expressing either soluble or membrane-associated regulators of complement e.g. CD55, which protects cancer cells from complement-dependent cytolysis (5, 6) and anti-cancer immune responses (7, 8). Thus, the complement system in cancer tissues does not seem to lead to cancer cell eradication.

Anaphylatoxin C5a is an N-terminal 74 amino acid fragment of the α-chain of the complement fifth component (C5), and is well known to act as a leukocyte chemoattractant and inflammatory mediator (9, 10). C5a is released by C5-convertase formed during the process of complement system activation (11), possibly triggered in response to cancer cells (3). Other C5a producing pathways include C5 cleavage by

thrombin (12), the ultimate product of the coagulation reaction. This cascade reaction can be triggered by tissue factor, which is expressed in a wide range of cell types including cancer cells (13). C5a is also generated by serine proteases from activated phagocytes (14), which frequently accumulate in cancer tissues. These findings lead us to the idea that C5a is also likely to be generated in the cancer microenvironment.

C5a activities are mediated by its binding to the membrane-associated C5a receptor (C5aR; CD88) which was originally identified in leukocyte cell lines (15). C5aR has since been reported to be expressed in other types of cells such as vascular endothelial cells, mesangial cells and renal proximal tubular cells. Further studies have revealed that C5aR expression is also inducible in epithelial cells by inflammatory and infection stimuli (16). Regarding cancer cells, functional C5aR expression was shown in a human hepatoma cell line HepG2 (17), whereas normal hepatocytes lack in its expression (16). These suggest that expression of C5aR is induced in cancer cells as a consequence of malignant transformation.

C5a and chemokines are chemoattractants, and a body of evidence indicates that a network of chemokines and their receptors influences the development of primary

cancers (18-22). Recently, C5a was reported to recruit myeloid-derived suppressor cells for suppressing the antitumor $CD8^+$ T cell response (2, 23), suggesting its indirect role in fostering cancer cells by protecting them from the antitumor $CD8^+$ T cells. However, the direct biological role of C5a-C5aR system in cancer cells is largely unknown. In this study, we investigated the expression of C5aR in cancer cells of various origins and analyzed its impact on cancer cell motility and invasiveness upon C5a stimulation.

Materials and Methods

Cell lines.

The human bile duct cancer cell lines MEC and HuCCT1 and the human colon cancer cell lines HCT15, COLO205, and DLD1 were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan). Human bile duct cancer cell lines SSp-25 and RBE were obtained from the Riken Cell Bank (Tsukuba, Japan). Human colon cancer cell lines HCT116 and SW620 were gifts from Dr. B. Vogelstein, Johns Hopkins University, and Dr. Kyogo Ito, Kurume University respectively. Cells were cultured in RPMI 1640 or DMEM

supplemented with 10% fetal bovine serum (FBS), penicillin (40 U/mL), and streptomycin (40 μg/mL) and were maintained at 37°C in 5% CO_2.

Tissue samples, immunohistochemistry and retrospective analysis.

Cancer tissue samples were obtained by surgical resection or core needle biopsy in Kumamoto University Hospital, and usage of those samples for this study was approved by the internal ethics committee. Deparaffinized 2-μm-thick sections were pretreated with 0.3% H_2O_2 in methanol for 20 min, followed by Protein Block, Serum-Free (Dako Cytomation, Glostrup, Denmark) treatment for 20 min. Sections were incubated with the primary antibody against C5aR (2 μg/mL; Hycult Biotechnology, Uden, the Netherlands) at 4°C overnight, and subsequently stained using EnVision+ solution (Dako Cytomation) and 3,3'-diaminobenzidine tetrahydrochloride solution containing 0.006% H_2O_2, according to the manufacturer's instructions. Nuclei were counterstained with hematoxylin. Retrospective analysis was performed on 42 intrahepatic cholangiocarcinoma patients who had undergone liver resection from May 2000 to November 2009. The relationship between cancer cell C5aR expression and vascular

invasiveness was investigated and analyzed by Fisher's exact test.

RT-PCR.

RNA was isolated from cancer cells using the Qiagen RNAeasy kit (Qiagen, Valencia, CA). cDNA was synthesized from extracted RNA using the RNA PCR kit AMV (Takara, Shiga, Japan), according to the manufacturer's instructions. PCR was performed using TaKaRa Ex Taq HS and primers (sense 5'-CGGGAGGAGTACTTTCCACC-3' and anti-sense 5'-CTACACTGCCTGGGTCTTCTG-3' for human C5aR, and sense 5'-CATCCACGAAACTACCTTCAACT-3' and anti-sense 5'-TCTCCTTAGAGAGAAGTGGGGTG-3' for β-actin) under the following conditions: 36 cycles for C5aR and 32 cycles for β-actin, 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C. PCR products were resolved by electrophoresis using 1% agarose gels and were visualized by ethidium bromide staining.

Immunoblotting.

To detect C5aR, cell lysates obtained from bile duct or colon cancer cells were analyzed