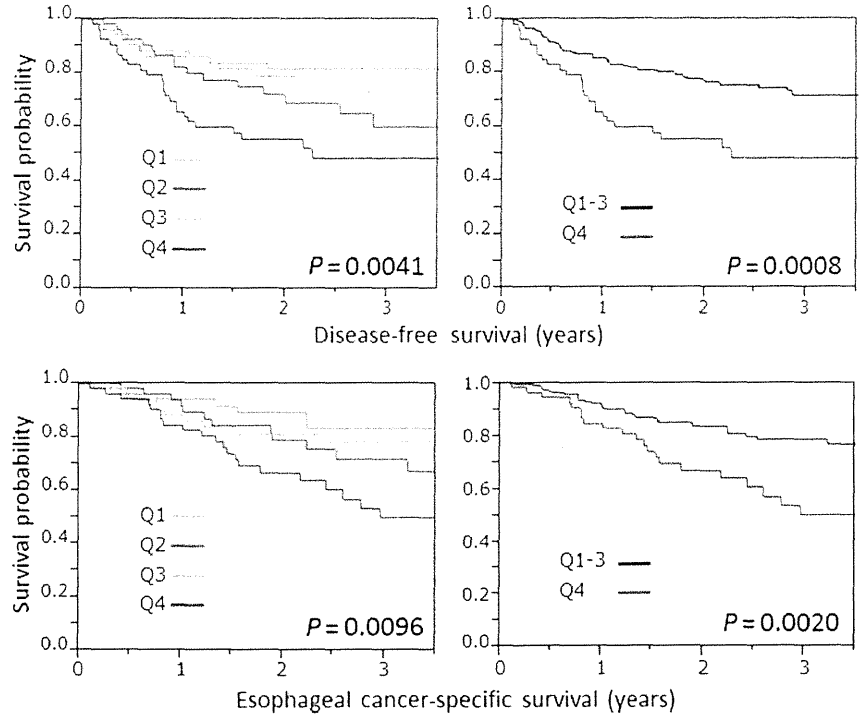

FIGURE 3. The Kaplan-Meier curves for disease-free survival (top panels) and cancer-specific survival (bottom panels) according to quartiles (Q1–4) of LINE-1 methylation in esophageal cancer. In the right panels, Q4 represents the "hypomethylated group" and Q1, Q2, and Q3 represent the "hypermethylated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

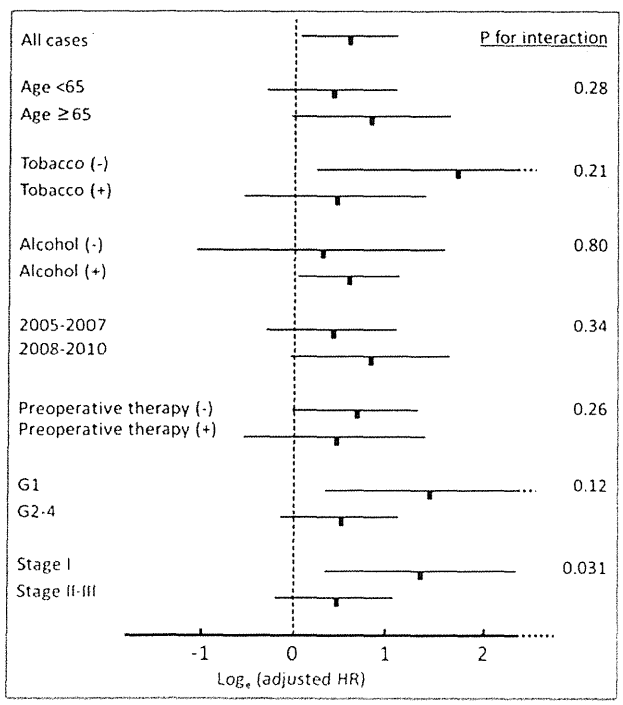

FIGURE 4. The LINE-1 methylation status and disease-free survival in various strata. The $\log_e$ (adjusted HRs) plot with the 95% CI for the disease recurrence rate in LINE-1 hypomethylated tumors (vs LINE-1 hypermethylated tumors) is shown.

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

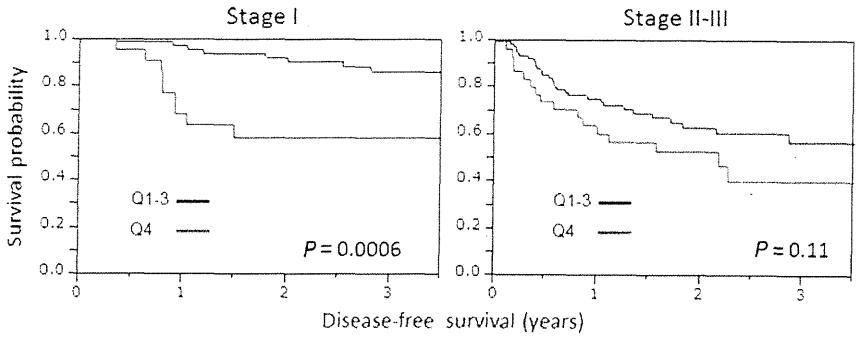

FIGURE 5. Kaplan-Meier curves for disease-free survival among patients with stage I tumors (left panel) and those with stage II and III tumors (right panel). Q4 represents the "hypomethylated group" and Q1, Q2, and Q3 represent the "hypermethylated group".

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

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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

by SDS-PAGE under reducing conditions using 10% polyacrylamide gels and were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon Transfer Membranes; Millipore). After blocking with 5% fat-free milk, the membranes were incubated with anti-human C5aR rabbit IgG (1000-fold dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or polyclonal anti-actin rabbit IgG (500-fold dilution; Santa Cruz Biotechnology). This was followed by incubation with HRP-conjugated anti-rabbit IgG goat antibody (1000-fold dilution; Amersham Biosciences, Blauvelt, NY) and bands were visualized via enhanced chemiluminescence (ECL), according to the manufacturer's instructions.

Establishment of C5aR stably expressing HuCCT1 cells.

Full-length human C5aR cDNA of 1053 bp was amplified by PCR using human macrophage cDNA library and subsequently subcloned into the pENTR/D-TOPO-vector (Invitrogen, Carlsbad, CA). After confirming the sequence, the cDNA was inserted into pCAG-IRES-puro vector using the Gateway system (Invitrogen). The purified plasmid was transfected into HuCCT1 cells using

Lipofectamine 2000 (Invitrogen). After 48 h, medium was replaced with selection medium supplemented with puromycin (1 μg/mL) to be cultured for 2 weeks. Puromycin resistant cells were collected and were subjected to cell sorting by FACS Vantage to isolate those cells highly expressing C5aR (designated HuCCT1/C5aR). HuCCT1 cells transfected with empty-pCAG-IRES-puro vectors were used as the control (HuCCT1/mock).

Flow cytometric analysis.

MEC, HuCCT1/mock or HuCCT1/C5aR cells were treated for 30 min with a murine monoclonal FITC-conjugated anti-C5aR antibody (Serotec Ltd., Oxford, UK), or a FITC-conjugated isotype matched control antibody (Serotec Ltd.), followed by washing with PBS twice. C5aR antigen was quantified by FACScan (BD Biosciences).

Immunofluorescence analysis.

Filamentous actin (F-actin) formation was visualized as previously described (24). Cells were seeded at a low density on glass coverslips and were incubated for 24 h. After 2 h

serum starvation, cells were stimulated with 100 nM human C5a (Sigma, St Louis, MO) for the stated time periods. Cells were then fixed in 4% paraformaldehyde, permeabilized in 0.2 % Triton X-100 for 5 min, and were incubated with 5 U/mL Alexa 488-phalloidin (Molecular Probes, Eugene, OR) for 40 min, followed by washing with PBS. Images were obtained and processed by FluoView 300 Laser Scanning Confocal Microscope (Olympus, Melville, NY).

Time-lapse video analysis.

Cells (1 x 10^4/well in RPMI1640) were cultured in a 24 well-glass bottom plate (Iwaki, Funabashi, Japan) for 24 h. After addition of C5a (final concentration: 100 nM), cells were maintained at 37°C in 5% CO_2 within the chamber set under the camera during the observation. Images were obtained using 20X UPlan SApo objective (Olympus IX81, Tokyo, Japan). The camera, shutters, and filter wheel were controlled by MetaMorph imaging software (Molecular Devices, Sunnyvale, CA), and images were collected every 10 min with an exposure time of 50 ms. Cell migration distance was measured by tracing individual cells using MetaMorph imaging software according to the

manufacturer's instructions.

Invasion assay *in vitro*.

To assess invasion of cancer cell lines *in vitro,* BioCoat Matrigel invasion chambers were utilized (24-well plate, 8-μm pore; BD Biosciences) (25). HuCCT1-derived (3.75×10^4 cells), or MEC (7.5×10^4 cells) cells were suspended in serum-free RPMI 1640 then seeded into the upper chamber. RPMI 1640 supplemented with either C5a or carrier solution (PBS) was placed in the lower chamber. To block C5aR-mediated signaling, anti-human C5aR rabbit IgG (10 μg/mL) or nonspecific control IgG (10 μg/mL) was added to the cell suspension before seeding. For analyzing the effect of discontinuous stimulation with C5a, cells were cultured at 37°C in serum-free RPMI 1640 supplemented with C5a for 12 h (HuCCT1-derived cells) or 24 h (MEC) at indicated concentrations. Cells were then washed with serum-free RPMI 1640 and were seeded into the upper chamber. RPMI 1640 containing 10% FBS was set in the lower chamber. Chambers were incubated for 24 h (HuCCT1-derived cells) or 36 h (MEC) at 37°C. Cells on the upper surface of the filter were removed with a cotton wool swab,

and cells that migrated to the lower surface were fixed in 100% methanol and were stained with 1% toluidine blue. Invaded cells were counted in five power fields (×20). The invasion-enhancing effect was shown as the ratio of cell invasion by C5a stimulation versus PBS controls. To determine whether MMPs were involved in C5a-elicited cancer cell invasion, GM6001 (5 μM; Merck, Darmstadt, Germany) was added to the cell suspension when cell invasion activity of 100 nM C5a was measured. For checkerboard analysis for C5a cancer cell invasion activity, various concentrations of C5a were added to the HuCCT1/C5aR cell suspension in the upper chamber together with the lower chamber, and cell invasion was assessed as described above.

Invasion assay *in vivo*.

HuCCT1/mock and HuCCT1/C5aR were incubated in serum-free medium in the presence or absence of C5a (10^{-7} M) at 37°C for 12 h. This was followed by labeling with CellTracker™ Orange CMTMR (20 μM) or CellTracker™ Green BODIFY (25 μM) (Molecular Probes) for HuCCT1/C5aR and HuCCT1/mock, respectively, at 37°C for 45 min. After washing with serum-free medium, HuCCT1/mock cells and

HuCCT1/C5aR cells were equally mixed to create a concentration of 3×10^7 cells/mL each. The cell mixture (50 μL) was injected intradermally into 7-week-old BALB/cA Jcl-nu/nu mice (CLEA Japan, Tokyo). After 1, 2, or 3 days, the nude mice were sacrificed by cervical dislocation, and the cell injection sites including surrounding tissues were excised to prepare frozen sections in liquid nitrogen. Labeled cells in 4-μm-thick sections were observed with a fluorescence microscope (BIOREVO; KEYENCE, Osaka, Japan). To quantify the distribution of HuCCT1-derived cells, regions of fluorescent dots of labeled cells were encircled (Fig. 6*A*) then the area of each region was measured using an imaging processor (VH-Analyzer; KEYENCE). The ratio of the distribution area of HuCCT1/C5aR versus HuCCT1/mock was calculated. Some endogenous green fluorescence background was observed in mice skin, therefore these spots were avoided and cancer cells were specifically encircled, which was confirmed by observation of the adjacent section HE-stained. This experiment was performed according to the criteria of animal experiments of the Kumamoto University Animal Experiment Committee.