Table I. Summary of characteristics of 100 patients with endometrioid adenocarcinoma.

| Characteristics | No. of patients |
|---|---|
| Tumor | |
| T1 | 72 |
| T2 | 8 |
| T3 | 20 |
| Stage | |
| I | 63 |
| II | 4 |
| III | 27 |
| IV | 6 |
| Histological grade | |
| 1 | 40 |
| 2 | 39 |
| 3 | 21 |
| Lymph node metastasis | |
| Negative | 75 |
| Positive | 25 |
| MIB-1 labeling index (%) | |
| <20 | 16 |
| ≥20 | 84 |
| Response to chemotherapy | |
| Response | 21 |
| No response | 17 |
| Recurrence | |
| Negative | 80 |
| Positive | 20 |
| Survival | |
| Yes (with no recurrence) | 79 |
| Yes (with recurrence) | 6 |
| No | 15 |

12-month intervals. The follow-up period for survivors ranged from 7 to 122 months (median 82). The study was approved by the ethics review board of the Graduate School of Medicine, Osaka University.

*Immunohistochemistry for ELP3 and MIB-1.* ELP3 expression was immunohistochemically examined with anti-ELP3 antibody (Sigma, St. Louis, MO, USA). The proliferative activity of cancer cells was examined with monoclonal antibody MIB-1 (Immunotech, Marseilles, France), thereby identifying the proliferation-associated antigen Ki-67. Following antigen retrieval using a Pascal pressurized heating chamber (Dako A/S, Glostrup, Denmark), the sections were incubated with anti-ELP3 and MIB-1, diluted at x250 and x100 magnification, respectively, and then treated with a ChemMate EnVision kit (Dako). DAB (Dako) was used as a chromogen. As the negative control, staining was carried out in the absence of the primary antibody. Positive staining of endometrioid glandular epithelium was used as a positive control. Scoring for ELP3 staining was performed independently by two pathologists (J.I. and E.M.) who examined the samples in a blinded manner with respect to the clinical information of the subjects. Tumor cells expressing ELP3 revealed clear staining in the cytoplasm (Fig. 1), and the staining intensities were comparable to those of normal epithelia. Cases with <10% ELP3-positive cells among tumor cells were regarded as ELP3-low, those with 10-25% cells as ELP3-intermediate and those with >25% cells as ELP3-high. The MIB-1 labeling index was defined as the percentage of stained nuclei per 1,000 cells.

*Double staining of ELP3 with MIB-1.* Double staining of ELP3 with MIB-1 was carried out using the EnVision G/2 Doublestain system (Dako) according to the manufacturer's instructions. Sections were initially incubated with anti-ELP3 antibody (1:250), colored with DAB and, subsequently, Ki-67 expression was detected by MIB-1 (1:100), colored with Permanent Red (Dako).

*Statistical analysis.* Statistical analyses were performed using StatView software (SAS Institute Inc., Cary, NC, USA). The Chi-square and Fisher's exact probability tests were used to analyze the correlation between ELP3 expression and clinicopathological factors in endometrioid adenocarcinoma. Overall survival (OS) was measured from the time of diagnosis. Disease-free survival (DFS) was measured from the time of diagnosis until recurrence of the disease. The Kaplan-Meier method was used to calculate the OS and DFS rate, and differences in survival curves were evaluated with the log-rank test. Cox's proportional hazard regression model was used in a stepwise manner to analyze the independent prognostic factors. $P \leq 0.05$ was considered to be statistically significant.

## Results

*Immunohistochemical findings.* ELP3 expression was examined in normal endometrium. A strong ELP3 expression was detected in the endometrial glands of all the examined tissues (proliferative and secretory phases, Fig. 1A and B). The expression of ELP3 was then examined in 100 samples of endometrioid adenocarcinoma tissue. Tumor cells revealed variable ELP3 expression levels. Of the 100 cases, 13% were classified as ELP3-high, 56 (56%) as ELP3-intermediate and the remaining 31 cases (31%) as ELP3-low (Figs. 1C-E).

*Double staining of ELP3 with MIB-1.* To examine the proliferation status of ELP3-expressing cells, double staining of ELP3 was carried out with MIB-1. ELP3-expressing and MIB-1-stained cells were almost mutually exclusive; most of the ELP3-expressing cells were negative for MIB-1, whereas most MIB-1-positive cells revealed no ELP3 expression (Fig. 2).

*Correlation of ELP3 expression with clinical variables.* The correlation of ELP3 expression levels (ELP3-high, ELP3-intermediate and ELP3-low) with clinicopathological characteristics was evaluated (Table II). A low ELP3 expression was correlated with a high T-factor ($p=0.036$), stage

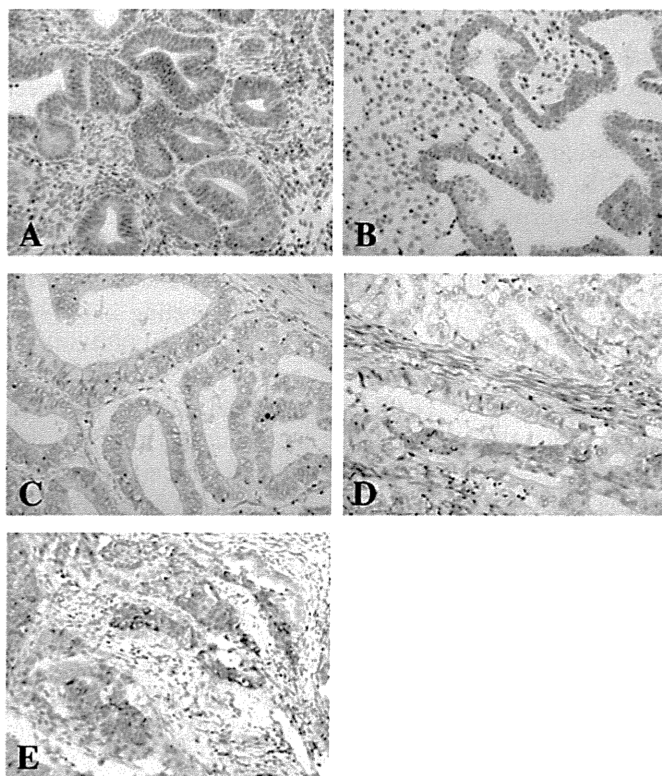


Figure 1. ELP3 expression in normal endometrium and endometrioid adenocarcinoma. Glandular epithelial cells in (A) normal proliferative and (B) secretory phase of endometrial epithelial cells expressed ELP3. Representative fields of (C) ELP3-low, (D) ELP3-intermediate and (E) ELP3-high expression in endometrioid adenocarcinoma. Magnification, x200.

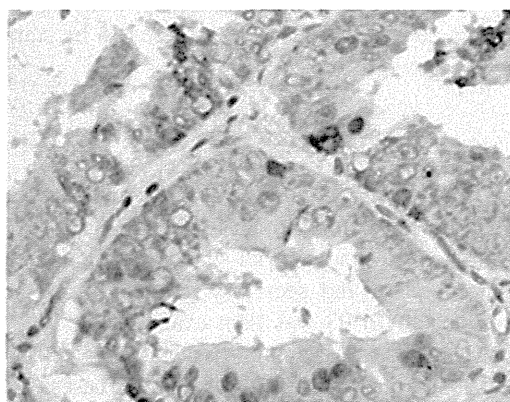

Figure 2. Double staining of ELP3 (brown color) and MIB-1 (red color) was performed. Magnification, x400.

Table II. Correlation between ELP3 expression and clinicopathological parameters.

| | ELP3 expression | | | p-value |
|---|---|---|---|---|
| | Low | Intermediate | High | |
| Tumor | | | | 0.036 |
| T1 | 17 | 42 | 13 | |
| T2 | 4 | 4 | 0 | |
| T3 | 11 | 9 | 0 | |
| Stage | | | | 0.001 |
| I | 11 | 39 | 13 | |
| II | 1 | 3 | 0 | |
| III | 17 | 10 | 0 | |
| IV | 2 | 4 | 0 | |
| Histological grade | | | | 0.379 |
| 1 | 10 | 24 | 6 | |
| 2 | 11 | 22 | 6 | |
| 3 | 10 | 10 | 1 | |
| Lymph node metastasis | | | | <0.001 |
| Negative | 15 | 47 | 13 | |
| Positive | 17 | 8 | 0 | |
| MIB-1 labeling index (%) | | | | 0.703 |
| <20 | 4 | 9 | 3 | |
| ≥20 | 28 | 46 | 10 | |
| Response to chemotherapy | | | | 0.045 |
| Response | 7 | 11 | 3 | |
| No response | 12 | 5 | 0 | |
| Recurrence | | | | 0.004 |
| Negative | 19 | 48 | 13 | |
| Positive | 12 | 8 | 0 | |
| Survival | | | | 0.003 |
| Yes (with no recurrence) | 19 | 48 | 12 | |
| Yes (with recurrence) | 1 | 5 | 0 | |
| No | 11 | 3 | 1 | |

(p=0.001), lymph node metastasis (p<0.001), resistance to chemotherapy (p=0.045), recurrence (p=0.004) and poor prognosis (p=0.003). Other parameters, including the histological grade of the tumor and the MIB-1 labeling index, did not correlate with ELP3 expression (Table II).

Five-year DFS and OS rates were 86.7 and 90.6%, respectively. Tumors recurred in 21 patients. Of these, 15 patients succumbed to the disease. A statistically significant difference was found in DFS (p<0.001) and OS rates (p=0.001) among patients with ELP3-high, ELP3-intermediate and ELP3-low tumors (Fig. 3A and B).

Univariate analysis showed that the T-factor, stage, histological grade, lymph node metastasis and ELP3 expression were significant factors for both OS and DFS (Table III). The multivariate analysis revealed that ELP3 expression and histological grade were independent factors for OS. None of the factors were significant for DFS (Table III).

## Discussion

In the present study, the characteristics of patients, such as age and tumor stage, were similar to those in a previous study by Steiner *et al*, indicating that our results are commonly applicable to endometrioid adenocarcinoma worldwide (16).

ELP3 expression was detected in non-cancerous endometrial glands of the proliferative and secretory phases. This is

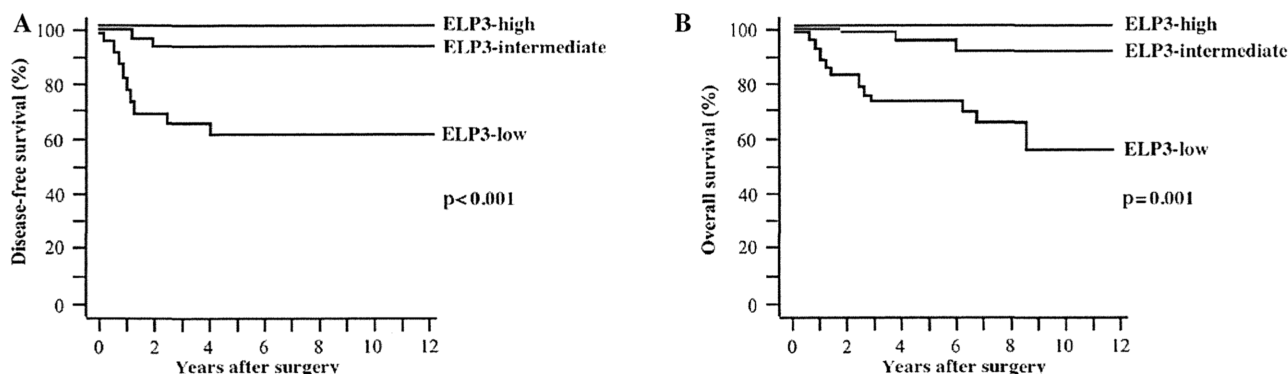


Figure 3. Kaplan-Meier plots. (A) Disease-free survival (DFS) and (B) overall survival (OS) curves are shown. ELP3-low cases showed less favorable DFS and OS.

Table III. Univariate and multivariate analyses of prognostic factors for overall survival (OS) and disease-free survival (DFS).

| | OS | | | | DFS | | | |
|---|---|---|---|---|---|---|---|---|
| | Univariate | | Multivariate | | Univariate | | Multivariate | |
| | HR (95% CI) | p-value | HR (95% CI) | p-value | HR (95% CI) | p-value | HR (95% CI) | p-value |
| Tumor | 1.54 (1.23-1.93) | 0.002 | 1.21 (0.82-1.80) | 0.337 | 1.59 (1.26-2.00) | <0.001 | 1.44 (0.94-2.22) | 0.094 |
| Stage | 2.60 (1.62-4.17) | <0.001 | 0.79 (0.27-2.30) | 0.660 | 2.63 (1.63-4.25) | <0.001 | 0.57 (0.18-1.77) | 0.329 |
| Histological grade | 3.32 (1.58-7.00) | 0.002 | 3.10 (1.20-8.01) | 0.020 | 3.29 (1.58-6.83) | 0.002 | 2.38 (0.89-6.37) | 0.084 |
| Lymph node metastasis | 8.34 (2.75-25.3) | 0.002 | 2.47 (0.60-10.1) | 0.210 | 7.83 (2.61-23.5) | <0.001 | 2.45 (0.55-10.9) | 0.240 |
| MIB-1 labeling index | 2.56 (0.34-19.5) | 0.364 | | | 2.85 (0.38-21.7) | 0.311 | | |
| ELP3 expression | 0.19 (0.07-0.52) | 0.001 | 0.24 (0.07-0.79) | 0.019 | 0.21 (0.08-0.57) | 0.002 | 0.33 (0.10-1.11) | 0.073 |

HR, hazard ratio; CI, confidence interval.

the first study showing ELP3 expression in the endometrium. Although the role of ELP3 in normal endometrium remains to be elucidated, ELP3 may function as a tumor-suppressor in endometrioid adenocarcinoma, since a reduced expression of ELP3 correlated with a poor prognosis for patients. Low ELP3 expression was correlated with a high T-factor, an advanced stage, the occurrence of lymph node metastasis, resistance to chemotherapy and a high recurrence rate. Recently, Gu *et al* reported that ELP3 overexpression inhibits cell growth and causes cell cycle arrest in a human embryonic kidney cell line (5). In the present study, ELP3-expressing cells stained negative with MIB-1, which is consistent with the findings of Gu *et al.*

Li *et al* reported that ELP3 is required for S-phase progression in yeast in the presence of DNA-damaging agents, such as hydroxyurea (4). By contrast, a high expression of ELP3 in humans was reported to correlate with vulnerability to anticancer drugs. This may be attributable to the difference in species. ELP3 is known to regulate the structure of chromatin and the methylation of genomes (2,17). Although target genes epigenetically regulated by ELP3 have not yet been reported, the identification of target genes of ELP3 may aid in understanding the various effects ELP3 has on the human and yeast cell cycle.

In conclusion, a low ELP3 expression is a poor prognostic factor in endometrioid adenocarcinoma. Further studies are required to clarify whether ELP3 expression would be a useful prognostic marker in other types of cancer.

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ORIGINAL ARTICLE

# Serum Leucine-rich Alpha-2 Glycoprotein Is a Disease Activity Biomarker in Ulcerative Colitis

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**Background:** Reliable biomarkers for monitoring disease activity have not been clinically established in ulcerative colitis (UC). This study aimed to investigate whether levels of serum leucine-rich alpha-2 glycoprotein (LRG), identified recently as a potential disease activity marker in Crohn's disease and rheumatoid arthritis, correlate with disease activity in UC.

**Methods:** Serum LRG concentrations were determined by enzyme-linked immunosorbent assay (ELISA) in patients with UC and healthy controls (HC) and were evaluated for correlation with disease activity. Expression of LRG in inflamed colonic tissues from patients with UC was analyzed by western blotting and immunohistochemistry. Interleukin (IL)-6-independent induction of LRG was investigated using IL-6-deficient mice by lipopolysaccharide (LPS)-mediated acute inflammation and dextran sodium sulfate (DSS)-induced colitis.

**Results:** Serum LRG concentrations were significantly elevated in active UC patients compared with patients in remission ($P < 0.0001$) and HC ($P < 0.0001$) and were correlated with disease activity in UC better than C-reactive protein (CRP). Expression of LRG was increased in inflamed colonic tissues in UC. Tumor necrosis factor alpha (TNF-$\alpha$), IL-6, and IL-22, serum levels of which were elevated in patients with active UC, could induce LRG expression in COLO205 cells. Serum LRG levels were increased in IL-6-deficient mice with LPS-mediated acute inflammation and DSS-induced colitis.

**Conclusions:** Serum LRG concentrations correlate well with disease activity in UC. LRG induction is robust in inflamed colons and is likely to involve an IL-6-independent pathway. Serum LRG is thus a novel serum biomarker for monitoring disease activity in UC and is a promising surrogate for CRP.

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**Key Words:** IBD, ulcerative colitis, biomarker, leucine-rich alpha-2 glycoprotein, DSS

The chronic inflammatory bowel diseases (IBDs), Crohn's disease (CD) and ulcerative colitis (UC), are typically characterized by episodes of acute flares and remission.[1,2] Depending on disease location and extent, exacerbation leads to diarrhea, abdominal pain, and systemic symptoms such as fatigue and weight loss.[3–5] Disease activity indices have been developed as outcome measures in clinical trials.[6,7] They may help to reproducibly and validly assess the patients' status and to support therapeutic decision-making.[6] Variables of disease activity indices comprise frequency of bowel movements, severity of abdominal pain, general well-being, occurrence of extraintestinal manifestations, and laboratory parameters.[8]

One of the most important protein biomarkers increased during the inflammatory state is C-reactive protein (CRP). However, elevation of serum CRP levels is not observed in certain inflammatory diseases. While serum CRP levels are highly increased in CD and rheumatoid arthritis (RA) patients and widely used for monitoring

Additional Supporting Information may be found in the online version of this article.

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**TABLE 1.** Characteristics of Patients with Ulcerative Colitis (UC)

| Characteristics | Patients with UC | Patients with Appendicitis and Diverticulitis |
|---|---|---|
| Number (male:female) | 82 (41:41) | 17 (8:9) |
| Age, yr, mean (SD) | 40.1 (15.7) | 33.1 (13.7) |
| Age at diagnosis, yr, mean (SD) | 34.7 (15.6) | 33.1 (13.7) |
| Bowel surgery (including appendectomy), *N* (%) | 7 (8.54) | |
| Treatment | | |
| Salazosulfapyridine or mesalazine, N (%) | 66 (80.5) | |
| Steroids, *N* (%) | 16 (19.5) | |
| Immunomodulators, *N* (%) | 3 (3.7) | |
| Disease location (*N*) | | |
| Extensive colitis/left-sided colitis/proctitis | 37/30/15 | |
| CRP, mg/dL, mean (SD) | 0.884 (1.967) | 8.47 (7.69) |
| WBC cells/μl, mean (SD) | 6716 (2317) | 12307 (3603) |
| CAI, mean (SD) | 4.71 (4.89) | |
| Matts's score, mean (SD) | 2.27 (0.89) | |

disease activity, only modest to absent CRP responses are observed in systemic lupus erythematosus (SLE), dermatomyositis, Sjogren's syndrome, or UC, although active inflammation is present.[9–11] In UC, endoscopic disease activity may predict future clinical symptoms,[12] but direct endoscopic or radiologic visualization of the degree of inflammation is rarely performed in outpatients with inactive or mild disease. Therefore, alternative biomarkers, which can conveniently and precisely monitor disease activity during therapy in inflammatory diseases, are required for the determination of adequate treatment.

By using a quantitative proteomic approach, we have previously reported that serum levels of leucine-rich alpha-2 glycoprotein (LRG) were elevated in patients with active RA and serum LRG levels were correlated with disease activity of not only RA but also CD, suggesting that serum LRG is a serological biomarker for monitoring disease activity.[13] LRG is an ≈50 kDa glycoprotein and contains repetitive sequences with a leucine-rich motif, first purified from human serum.[14,15] LRG has been reported to be expressed by the liver cells and neutrophils[16,17]; however, its function remains unclear. To date, the relationship between serum LRG levels and disease activity in UC has not been assessed. In this study we investigated serum LRG expression levels in UC patients and evaluated their correlation with clinical disease activity. Serum LRG levels were significantly increased in the active UC patients. LRG expression was upregulated in the inflamed colonic mucosa of UC possibly through stimulation by various cytokines including tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and IL-22, the expression of which are increased in active UC. Moreover, we show that serum LRG correlates more strongly than CRP with disease activity in UC. Therefore, serum LRG may be a useful disease activity biomarker for UC.

## MATERIALS AND METHODS

### Patients and Sera

Sera were obtained from patients with UC ($n = 82$), appendicitis ($n = 13$), and diverticulitis ($n = 4$) and surgical or biopsy samples were obtained from patients with UC ($n = 10$) from Osaka University Hospital (Osaka, Japan) and the Department of Surgery, Osaka Rosai Hospital, respectively. Sera from healthy controls (HCs) ($n = 50$), age/sex-matched with UC patients, were used. Diagnosis of UC was based on conventional clinical, radiological, endoscopic, and histopathological criteria. Clinical activities were determined using the Clinical Activity Index (CAI) for UC.[18] Clinical remission was defined as CAI $<6$.[19] In addition to CAI, the endoscopic findings were also graded according to Matts' criteria.[20] Endoscopic remission was defined as Matts' score $\leq 2$. Detailed patient characteristics are presented in Table 1. For Caucasian patients with UC, sera ($n = 30$) were obtained from the Department of Medicine, University of North Carolina Hospital (Chapel Hill, NC). Sera from HCs ($n = 19$), age/sex-matched with UC patients, were used. Detailed patient characteristics are presented in Table 2, while data of disease activity of UC is not available.

### Quantification of Serum LRG and Cytokines

Human serum LRG and mouse serum LRG were quantitated by human LRG assay kit (IBL, Fujioka, Japan) and mouse LRG assay kit (IBL, Fujioka, Japan). These enzyme-linked immunosorbent assay (ELISA) assays were performed

TABLE 2. Characteristics of Patients with UC in a Caucasian Cohort

| Characteristics | Patients with UC |
|---|---|
| Number (male:female) | 30 (18:12) |
| Age, yr, mean (SD) | 42.9 (17.9) |
| Age at diagnosis, yr, mean (SD) | 33.2 (15.7) |
| Treatment | |
| Salazosulfapyridine or mesalazine, *N* (%) | 14 (46.7) |
| Steroids, *N* (%) | 9 (30.0) |
| Immunomodulators, *N* (%) | 11 (36.7) |
| Anti-TNF therapy | 3 (10.0) |
| Disease location (*N*) | |
| Extensive colitis/left-sided colitis/proctitis | 16/11/3 |

in duplicate. The intraassay coefficients of variations for human LRG and mouse LRG were $\leq$7.98% and $\leq$8.93%, respectively. For the quantification of IL-6, TNF-$\alpha$, and IL-22 in human serum samples, the human IL-6 Ultra Sensitive ELISA (Biosource International, Camarillo, CA), human TNF-$\alpha$ UltraSensitive ELISA kit (Invitrogen, Carlsbad, CA), and human IL-22 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) were used following the manufacturer's guidelines.

## Western Blot Analysis

Frozen colon tissue samples were lysed in RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 × protease inhibitor cocktail; Nacalai Tesque, Kyoto, Japan) and 1 × phosphatase inhibitor cocktail (Nacalai Tesque) followed by centrifugation (13,200 rpm, 4°C, 15 minutes), after which the supernatants were stored at −80°C until use. Extracted proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously.[21] Samples transferred onto PVDF membranes were treated with a rabbit antihuman LRG polyclonal antibody (Proteintech Group, Chicago, IL) or a rabbit anti-GAPDH polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used as described previously.[21]

## Immunohistochemistry

Immunohistochemical analyses were performed according to a method described in our previous report.[22] Briefly, rabbit antihuman LRG polyclonal antibodies were used as the primary antibody. After incubation with the primary antibodies, the sections were treated with biotin-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and avidin-biotin-peroxidase complexes (Vector Laboratories). Immunoreactive cells were visualized with a diaminobenzidine substrate (Merck, Darmstadt, Germany) and were counterstained with hematoxylin.

## Mice

C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan). C57BL/6-background IL-6-deficient mice were kindly provided by Professor Yoichiro Iwakura (Laboratory of Molecular Pathogenesis, Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan). Mice were maintained under specific pathogen-free conditions. C57BL/6 and IL-6-deficient mice were used at 7–9 weeks of age. All experiments were conducted according to the institutional ethical guidelines for animal experimentation.

## LPS-mediated Acute Inflammation

To induce acute inflammation, wildtype (WT) mice and IL-6-deficient mice were injected intraperitoneally with 0 or 10 mg/kg LPS (*Escherichia coli* LPS, Sigma, St. Louis, MO) dissolved in 500 $\mu$L phosphate-buffered saline (PBS). Blood was collected at before and 24 hours after LPS injection and the serum was separated by centrifugation and stored at −30°C until used for ELISA analysis.

## Induction of Colitis

For induction of colitis, WT mice and IL-6-deficient mice were given 3% dextran sodium sulfate (DSS) (m/w 36,000–50,000; MP Biomedicals, Solon, OH) dissolved in drinking water provided ad libitum for 5 days, followed by provision of ordinary water for 20 days.

## Assessment of Severity of DSS-induced Colitis

WT mice were weighed daily from day 0 to day 25. Changes in body weight were calculated as follows: body weight change (%) = [(weight on a given day (days 0–13) − weight on day 0)/weight on day 0] * 100. Blood was collected from WT mice on days 5, 7, 10, 15, and 25 after DSS administration or day 0 by cardiac puncture under anesthesia and on days 0 and 10 from IL-6-deficient mice. The serum was separated by centrifugation and stored at −30°C until used for ELISA analysis.

## Cell Culture

The human colonic adenocarcinoma COLO205 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) and 1% penicillin–streptomycin (Nacalai Tesque) at 37°C under a humidified atmosphere of 5% $CO_2$.

For the analysis of LRG protein induction, COLO205 cells were stimulated with various concentrations of cytokines for 24 hours and culture supernatant were concentrated using Amicon Ultra-4 10K MWCO (Millipore, Bedford, MA). Concentrated supernatants were used for western blot analysis. Full-length human LRG cDNA was inserted into pcDNA3.1/V5-His-TOPO vector (Invitrogen) and designated pcDNA3.1-LRG-V5-His. pcDNA3.1-LRG-V5-His vector was transfected

into COS7 cells using Lipofectamine 2000 reagent (Invitrogen) and culture medium were used for the positive control.

## Quantitative Real-time Reverse-transcription Polymerase Chain Reaction (RT-PCR) Analysis

For the quantification of mRNA levels of LRG, various mouse organs were analyzed by real-time RT-PCR as described previously.[23] Levels of mouse LRG and mouse hypoxanthine phosphoribosyltransferase (HPRT) levels were determined by the 7900HT Real-time PCR system (Applied Biosystems, Foster City, CA) using specific primers: murine LRG forward 5′-ATCAAGGAAGCCTCCAGGAT-3′; reverse 5′-CAGCTGCGTCAGGTTGG-3′ and murine hypoxanitine phosphoribosyltransferase (HPRT) forward 5′-TCAGTCAACG GGGGACATAAA-3′; reverse 5′-GGGGCTGTACTGCTT AACCAG-3′.

## Statistics

The Mann–Whitney $U$-test or one-way analysis of variance (ANOVA) followed by a Scheffe's test were used for statistical analyses. Two-tailed Student's $t$-test was used for significant differences in LRG expression between identical patients with UC in active and remission disease stage. One-way ANOVA followed by a Dunnett's test was used for multiple comparison of the difference of serum LRG levels at various timepoints after DSS treatment in mice. Pearson's test was used to analyze the relationship between LRG and CRP, IL-6, or CAI. For drawing of receiver operating characteristic (ROC) curves and estimation of the area under the ROC curve (AUC) statistics, the software Excel Statistics 2010 (Social Survey Research Information, Tokyo, Japan) was used to quantify the ability to differentiate between remission and active by CAI. $P < 0.05$ was considered significant.

## Ethical Considerations

Informed consent was obtained from all donors and all studies involving human subjects were approved by the Institutional Review Boards of the National Institute of Biomedical Innovation, Osaka University Hospital, the Department of Surgery, Osaka Rosai Hospital, and the University of North Carolina.

# RESULTS

## Serum LRG Levels Are Increased in Active UC Patients

We quantified serum LRG concentrations by ELISA using sera from patients with UC. Serum LRG concentrations were significantly elevated in the active UC patients (CAI $\geq$6) (14.24 $\pm$ 8.08 $\mu$g/mL) compared with HC (3.07 $\pm$ 1.42 $\mu$g/mL; $P < 0.0001$) (Fig. 1A). There was also a significant difference between LRG serum levels in patients with active UC (CAI $\geq$6) (14.24 $\pm$ 8.08 $\mu$g/mL) compared with UC in remission (CAI <6) (5.34 $\pm$ 2.60 $\mu$g/mL; $P <$ 0.0001) (Fig. 1A). To determine whether serum LRG levels are increased in non-IBD disease controls, we quantified serum LRG levels in patients with appendicitis and

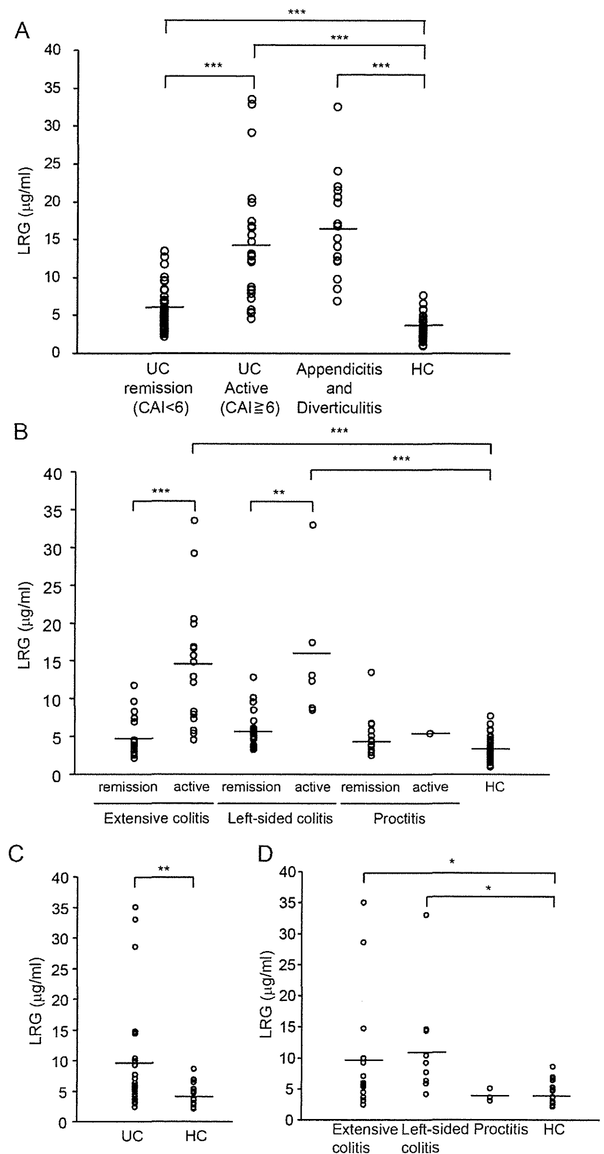


FIGURE 1. Serum LRG levels are increased in patients with active UC. (A) Serum levels of LRG were determined in 82 patients with UC (57 patients in remission [CAI <6], 25 patients in active [CAI $\geq$6] stage), appendicitis ($n$ = 13), diverticulitis ($n$ = 4) and 50 healthy controls (HC). ***$P < 0.0001$ by one-way ANOVA followed by Scheffe's post-hoc test. (B) Disease extension in UC was grouped into three categories: In UC patients in remission, extensive colitis ($n$ = 19), left-sided colitis ($n$ = 24), and proctitis ($n$ = 14); in active patients, extensive colitis ($n$ = 18), left-sided colitis ($n$ = 6), and proctitis ($n$ = 1) and HC ($n$ = 50). **$P < 0.005$, ***$P < 0.0001$ by one-way ANOVA followed by Scheffe's post-hoc test. (C) Serum levels of LRG were determined in patients with UC ($n$ = 30) and HC ($n$ = 19) in a Caucasian cohort. **$P < 0.005$ by Mann–Whitney $U$-test. (D) In a Caucasian cohort, disease extension in UC was grouped into three categories: extensive colitis ($n$ = 16), left-sided colitis ($n$ = 11), and proctitis ($n$ = 3) and HC ($n$ = 19). *$P < 0.05$ by one-way ANOVA followed by Scheffe's post-hoc test.

diverticulitis. Elevated serum LRG levels were also observed in appendicitis and diverticulitis (16.83 ± 6.50 μg/mL) compared with HC (3.07 ± 1.42 μg/mL; $P < 0.0001$) (Fig. 1A), suggesting that serum LRG levels are also increased in acute intestinal inflammation.

When UC were classified into three categories based on disease extent, significantly higher serum LRG concentrations were observed in active patients with extensive colitis (14.34 ± 7.89 μg/mL) compared with in remission (4.96 ± 2.68 μg/mL; $P < 0.0001$) and HC (3.07 ±1.42 μg/mL; $P < 0.0001$) and active patients with left-sided colitis (15.41 ± 9.16 μg/mL) compared with in remission (5.91 ± 2.41 μg/mL; $P = 0.0003$) and HC (3.07 ±1.42 μg/mL; $P = 0.001$) (Fig. 1B). Nonetheless, there was no clear difference between active patients with proctitis and HC, possibly due to the low number of patients in this group. In patients with UC in remission, serum LRG levels in all of three disease extent categories were comparable with HC (Fig. 1B). Significantly elevated serum LRG levels were also detected in a Caucasian UC cohort (9.46 ± 8.44 μg/mL) compared with HC (4.42 ± 1.91 μg/mL; $P < 0.005$) (Fig. 1C). In this Caucasian UC cohort, serum LRG levels were also significantly elevated in patients with extensive colitis (9.54 ± 8.05 μg/mL) compared with HC (4.42 ± 1.91 μg/mL; $P < 0.05$) and left-sided colitis (10.90 ± 9.16 μg/mL) compared with HC (4.42 ± 1.91 μg/mL; $P < 0.02$) (Fig. 1D). However, a clear difference was not observed between patients with proctitis and HC (Fig. 1D). These results suggest that serum LRG levels were elevated in active UC.

### Serum LRG Levels Are Correlated with Disease Activity in UC Patients

We investigated the correlation between serum LRG levels and disease activity (CAI) in UC patients. A positive correlation was observed between LRG and CAI ($r = 0.731$, $P < 0.00001$) (Fig. 2A). This correlation was stronger than that observed between CRP and CAI ($r = 0.654$, $P < 0.00001$) (Fig. 2A). When patients with UC were classified into active and remission according to the endoscopic findings, significantly elevated serum LRG levels and CRP levels were observed in patients with active UC compared with patients in remission ($P < 0.005$, respectively) (Supporting Fig. 1A). While serum LRG levels were significantly correlated with CRP levels in patients with UC ($r = 0.850$, $P < 0.00001$, $n = 82$) (Supporting Fig. 2A), such a correlation was not found when a CRP-negative subgroup (CRP <0.2, $n = 51$) was analyzed ($r = 0.101$, $P = 0.481$) (Supporting Fig. 2B). In this CRP-negative group, serum LRG levels were significantly correlated with CAI ($r = 0.416$, $P = 0.00241$) (Supporting Fig. 2C); however, significant correlation was not found between CRP and CAI ($r = -0.0896$, $P = 0.532$) (Supporting Fig. 2D). Additionally, in the CRP-negative group elevated serum LRG levels were detected in patients with endoscopically active UC compared with patients with UC in remission ($P = 0.0442$) (Supporting Fig. 1B). These findings in patients with low CRP may explain a better correlation of CAI with LRG than that with CRP.

When UC was classified by disease extent, a significantly higher positive correlation was detected between LRG and CAI than CRP and CAI both in extensive colitis ($r = 0.690$, $P < 0.000001$ and $r = 0.580$, $P = 0.000168$) and left-sided colitis ($r = 0.840$, $P < 0.000001$ and $r = 0.759$, $P < 0.000001$), but not in proctitis (Fig. 2B). Importantly, by analyzing sera obtained at active (CAI ≥6) and remission (CAI <6) disease stages from 10 identical UC patients, a significant decrease in serum LRG levels in remission was detected (Fig. 2C).

By generating an ROC curve, the sensitivity and specificity of serum LRG for remission and active by CAI were determined (Fig. 2D). The AUC for serum LRG levels was 0.901, whereas the AUC for CRP levels was 0.845. The cutoff value of serum LRG levels was 7.21 μg/mL (sensitivity = 84.0%, specificity = 82.5%). In contrast, when the cutoff value of CRP levels was set to 0.20, a maximum CRP value of normal range, the sensitivity was 80.0% and the specificity was 80.7%. These results emphasize the usefulness of monitoring serum LRG levels for the evaluation of the disease activity of UC.

### Expression of LRG Was Increased in Inflamed UC Colons

Next, to investigate whether local inflammatory sites in patients with UC are a potential source of increased serum LRG we first looked at the expression of LRG in the colon by western blot analysis on inflamed and noninflamed sites of surgically resected full-thickness colon specimens from patients with UC. Western blot analysis showed that LRG expression in colon tissues was increased in inflamed sites of active UC patients compared with noninflamed colon tissues (Fig. 3A). Next, we tried to examine the localization of LRG. By immunohistochemistry, increased expression of LRG was detected in the cytoplasm of intestinal epithelial cells (IECs) in inflamed tissues (Fig. 3B–E). In contrast, expression of LRG was lower in noninflamed tissues (Fig. 3B–E). These data suggest that inflamed colon tissue is a potential source of increased serum LRG in patients with UC.

### LRG Is Induced by Stimulation with TNF-α, IL-6, or IL-22

It has been reported that IL-6 is an inducer of LRG expression.[16] However, it is not clear whether LRG is induced by cytokines other than IL-6. At first we investigated the serum levels of IL-6, IL-22, and TNF-α, known

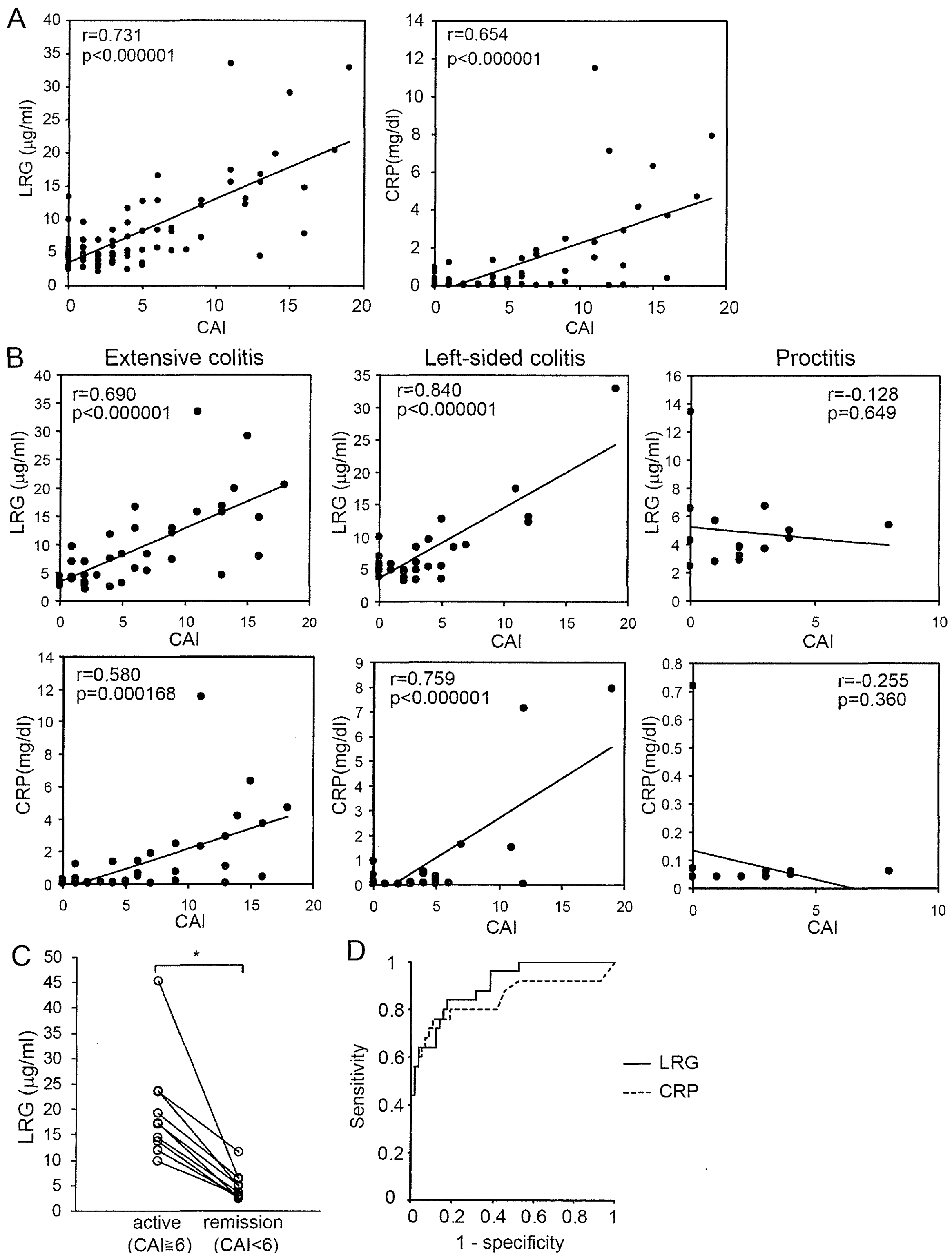


FIGURE 2. Serum LRG levels are correlated with disease activity better than CRP in patients with UC. (A) Serum levels of LRG correlated with CAI ($n = 82$; $P < 0.000001$; $r = 0.731$) better than CRP ($n = 82$; $P < 0.000001$; $r = 0.654$) in patients with UC. (B) Serum levels of LRG correlated with disease activity in extensive colitis ($n = 37$; $P < 0.000001$; $r = 0.690$) and left-sided colitis ($n = 30$; $P < 0.000001$; $r = 0.840$) better than CRP in extensive colitis ($n = 37$; $P = 0.000168$; $r = 0.580$) and left-sided colitis ($n = 30$; $P < 0.000001$; $r = 0.759$), while neither LRG ($n = 15$; $P = 0.649$; $r = -0.128$) nor CRP levels ($n = 15$; $P = 0.360$; $r = -0.255$) were correlated with disease activity in proctitis. (C) Compared with 10 identical active patients with UC, serum levels of LRG were decreased in remission. *$P < 0.002$ by Student's $t$-test. (D) ROC curves for LRG and CRP for differentiation between UC patients with remission ($n = 57$) and active ($n = 25$) by CAI.

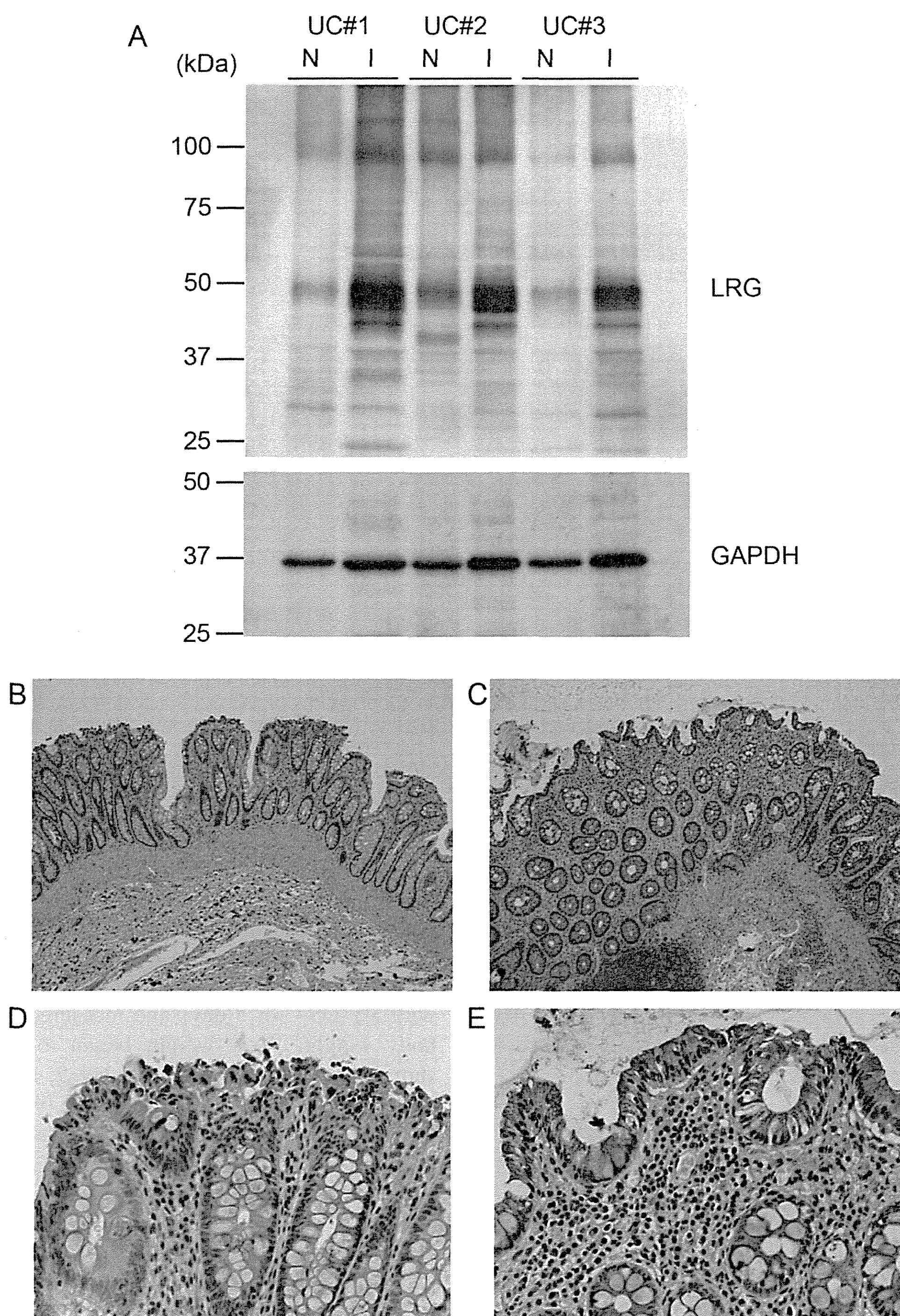


FIGURE 3. Expression of LRG is increased in lesion sites of ulcerative colitis. (A) Representative western blot analysis of three separate experiments for LRG using paired surgically resected full-thickness colon specimens from noninflamed (N) and inflamed (I) sites in patients with UC. GAPDH was used as a control of the relative amounts of proteins in each sample. Full-thickness colon tissues from UC in inflamed and noninflamed sites were evaluated by immunohistochemical analysis for LRG expression ($n = 10$ per experimental group). (B) Noninflamed mucosa (×42). (C) Inflamed mucosa from active UC (×42). (D) Noninflamed mucosa (×400). (E) Inflamed mucosa from active UC (×400). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

to be increased at the inflamed tissue in active UC.[24–26] Indeed, ELISA analysis using sera from 82 UC patients revealed that serum TNF-$\alpha$, IL-6, and IL-22 levels were significantly elevated in active UC patients compared with those patients in remission ($P = 0.0178$, $P = 0.00690$, and $P < 0.0001$, respectively) (Fig. 4A). Next, to investigate

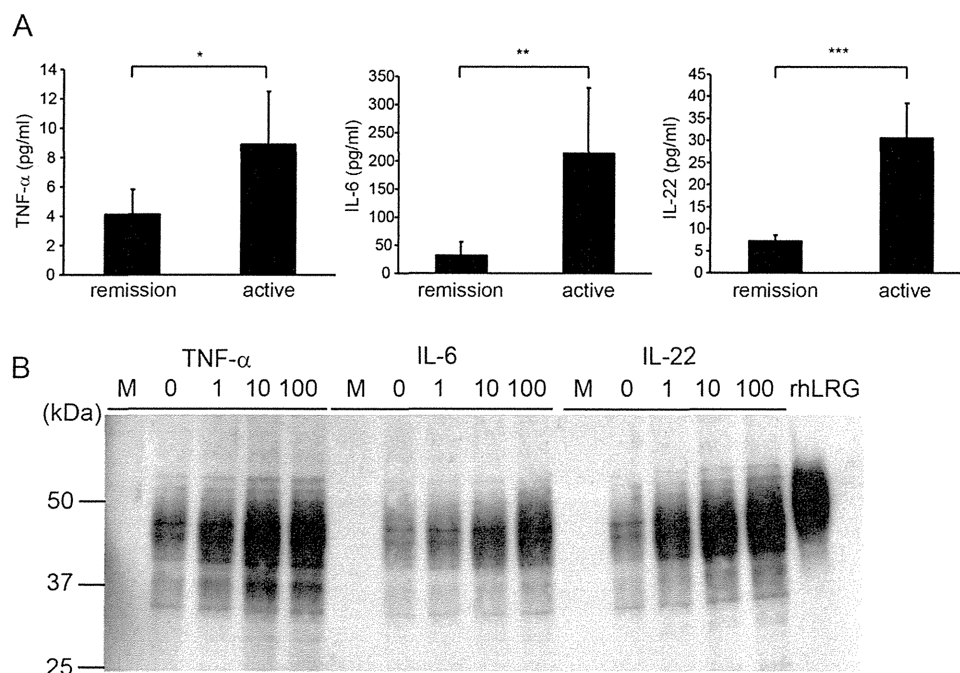


FIGURE 4. Expression of LRG was induced by TNF-α, IL-6, and IL-22. (A) Serum levels of TNF-α, IL-6, and IL-22 were determined in patients with UC (57 patients in remission [CAI <6] and 25 patients in active [CAI ≥6] stage). Data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$ by Mann–Whitney *U*-test. (B) LRG was determined in supernatants of COLO205 cells left untreated or stimulated with TNF-α, IL-6, and IL-22 at 1.0, 10, 100 ng/mL for 24 hours and analyzed by western blotting. There was a dose-dependent increase in LRG levels after treatment with TNF-α, IL-6, and IL-22.

which proinflammatory cytokines induce expression of LRG we stimulated human colonic adenocarcinoma COLO205 cells with TNF-α, IL-6, or IL-22 for 24 hours. After cytokine stimulation, secretion of LRG protein into the culture media was analyzed by western blotting. Interestingly, LRG was induced not only by stimulation with IL-6, but also by TNF-α and IL-22 in a dose-dependent manner (Fig. 4B). These results indicate that expression of LRG is induced by various proinflammatory cytokines including IL-6.

## Expression of LRG Through an IL-6-independent Pathway Is Demonstrated in LPS-mediated Acute Inflammation and DSS-induced Colitis

CRP is one of the representative acute phase proteins in humans and CRP production is primarily dependent on liver by circulating IL-6. To examine the possible differences in induction mechanisms between LRG and CRP, particularly with regard to the involvement of IL-6, we took advantage of murine models. We first assessed whether LRG is induced in WT mice by injecting LPS, an inducer of proinflammatory cytokines from macrophages, because CRP is poorly induced in mice during acute inflammation. At 24 hours after intraperitoneal injection of LPS, serum samples were prepared and serum LRG levels were determined by ELISA. Compared with WT mice, significant elevation of serum LRG levels were detected in LPS-administered WT mice (Fig. 5A), suggesting that LRG is induced during acute inflammation in mice as in humans.

We next used a murine IBD model to investigate induction mechanisms of LRG during colonic inflammation. DSS-induced colitis is often used as a murine model of UC.[27] We induced colitis in WT mice by treating them with 3% DSS for 5 days and measured changes in relative body weight. Body weight began to decrease at day 5, showed greatest reduction at day 9, and recovered at 18 days after DSS treatment (Fig. 5B). We analyzed changes in serum LRG levels by ELISA before and 5, 7, 10, 15, and 25 days after DSS treatment. Consistent with body weight loss, serum LRG levels were significantly elevated at 5 days after DSS treatment (Fig. 5C). Serum LRG levels remained high until day 15, but decreased at day 25. Delayed normalization of serum LRG levels is likely due to the prolonged inflammation at inflamed tissue sites. Additionally, a long half-life of serum LRG might also be involved in this, since our preliminary data suggest that the half-life of serum human LRG levels are about two times longer than that of CRP (data not shown). To investigate which organs produce LRG in DSS-induced colitis, RNA was extracted from colon, liver, and spleen before and 9 days after DSS treatment. By quantitative PCR analysis (Fig. 5D), expression of LRG was significantly increased in liver ($P = 0.00106$) and spleen ($P = 0.0376$);

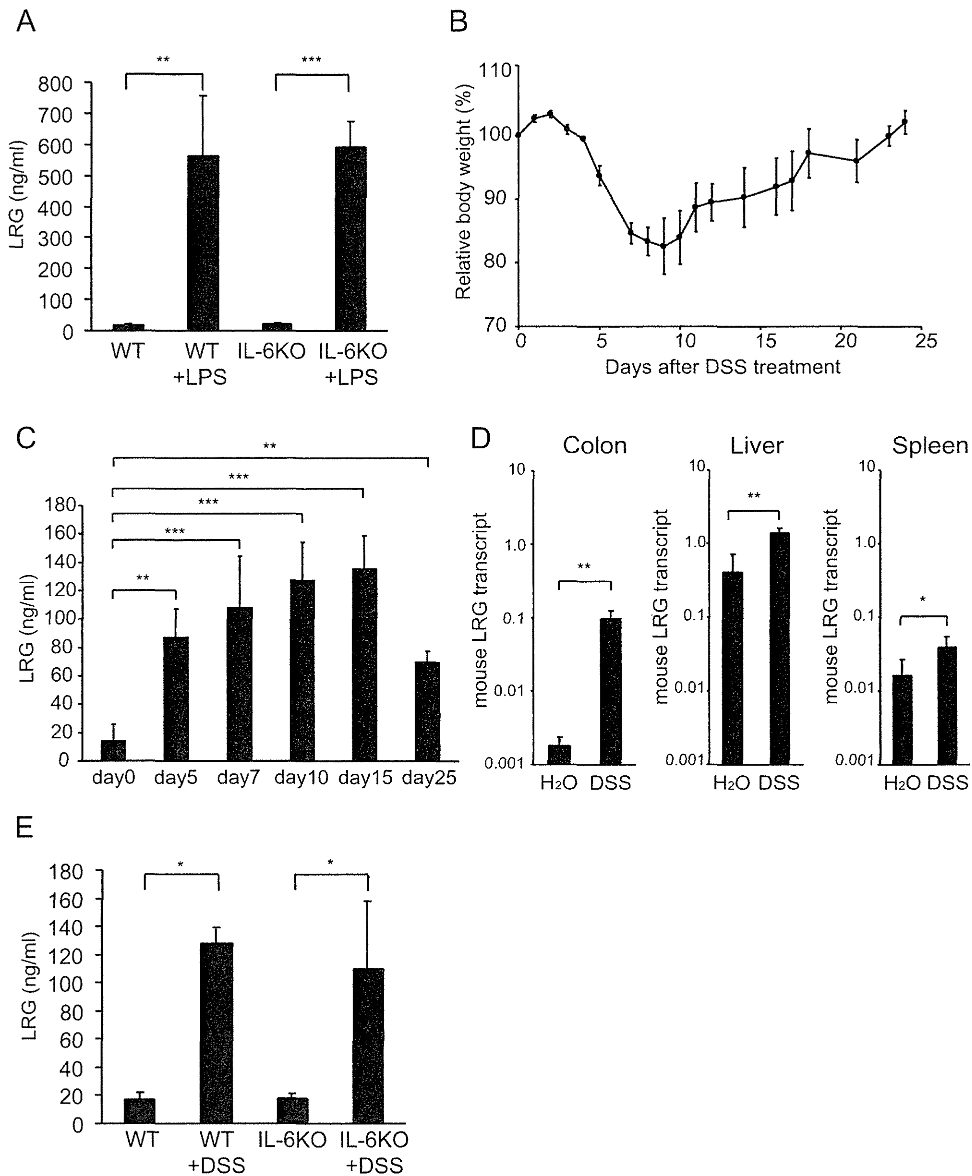


FIGURE 5. Induction of LRG has IL-6-independent pathway in LPS-mediated acute inflammation and active stage of DSS-induced colitis. (A) WT mice and IL-6-deficient mice were injected intraperitoneally with 0 or 10 mg/kg LPS dissolved in 500 $\mu$L PBS and serum LRG levels were measured after 24 hours. Data are expressed as mean ± SEM. $^{**}P < 0.005$, $^{***}P < 0.0001$ by one-way ANOVA followed by Scheffe's post-hoc test. (B) Relative body weight changes of mice with DSS-induced colitis in this study. Data are expressed as mean ± SEM ($n = 4$). (C) Expression of LRG is upregulated in murine DSS-induced colitis. At the indicated time, serum LRG levels were determined by ELISA analysis. $^{**}P < 0.005$, $^{***}P < 0.0001$ by one-way ANOVA followed by a by Dunnett's post-hoc test. (D) Nine days after control or DSS treatment, mice were euthanized and gene expression of LRG in the colon, liver, spleen, and kidney was determined by quantitative PCR analysis. Gene expression was calculated relative to HPRT. Data were expressed as mean ± SD ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.005$ by Student's *t*-test. (E) IL-6-deficient mice were used for DSS-induced colitis. Nine days after DSS administration, serum levels of mouse LRG was determined by ELISA analysis. $^{*}P < 0.05$ by one-way ANOVA followed by Scheffe's post-hoc test.

however, the strongest induction was observed in colon ($P = 0.000126$).

To investigate whether LRG induction is dependent on IL-6 or not, we analyzed serum LRG levels in IL-6-deficient mice. Interestingly, basal LRG levels in IL-6-deficient mice were similar to those in WT mice and LRG was robustly induced by LPS administration in IL-6-deficient mice (Fig. 5A). Moreover, increased serum LRG levels were also detected in the active stage (day 9) of DSS-induced colitis in IL-6-deficient mice (Fig. 5E). Importantly, the increase of serum LRG in IL-6-deficient mice was similar to that in WT mice (Fig. 5A,E). These findings indicate that LRG expression can be induced in the absence of IL-6.

## DISCUSSION

In this study we first demonstrated that serum LRG levels were significantly increased in sera of active UC patients compared with patients in remission and HC. Serum LRG is likely elevated in diverse racial groups, because we detected increased serum LRG levels not only in Japanese patients (Fig. 1A)[13] but also in Caucasian patients with UC (Fig. 1C,D) and CD (data not shown). In addition, levels of serum LRG were significantly correlated with disease activity in UC and the correlation was stronger than CRP. Moreover, by analyzing ROC curve and AUC, serum LRG levels showed higher AUC than CRP and serum LRG levels represented superior sensitivity and specificity to CRP for remission and active of UC by CAI (Fig. 2D), indicating that LRG is a useful marker to evaluate disease activity in UC. In the normal state, serum LRG is thought to be produced from liver and LRG is abundantly found in the sera of HC. In colonic inflammation, we found that the expression of LRG is increased in the inflamed mucosa of UC patients and mice with DSS colitis, suggesting that inflamed tissues can be a source for production of LRG (Fig. 3). The increased expression of LRG in inflamed tissue has previously been observed in appendix during acute appendicitis.[28] Moreover, in acute inflammatory disorders, including appendicitis and diverticulitis, increased expression of serum LRG was observed (Fig. 1A). These results indicate that the elevated expression of LRG at inflamed sites and in sera occurs in various acute and chronic inflammatory disorders. Therefore, increased serum LRG levels are not suitable for use as a specific diagnostic marker of IBD.

CRP is the most common serum marker used to evaluate disease activity in inflammatory diseases. However, serum CRP is primarily dependent on liver production induced by circulating IL-6. Compared with CD and RA, only modest to absent CRP responses are observed in UC, despite active inflammation in colon.[9] Indeed, our cohort of 82 UC patients, analyzed in this study, included five patients with normal value of CRP while having active disease (Fig. 2A). However, our study demonstrated that serum LRG levels were significantly increased in active UC patients' sera and correlated better with disease activity of UC than CRP levels (Figs. 1A, 2A). Particularly, in the group of patients with negative CRP (CRP $<0.2$), significant correlation was observed between serum LRG levels and CAI (Supporting Fig. 2C). Similarly, among CRP-negative patients serum LRG levels were significantly elevated in those with endoscopically active UC, compared with UC in remission (Supporting Fig. 1B). In addition, serum LRG levels were decreased after therapy (Fig. 2C), suggesting that LRG is a useful serological biomarker for evaluating disease activity and therapeutic effect in UC.

Better correlation of serum LRG levels with disease activity of UC than CRP might be explained in part by the differences in induction mechanisms between LRG and CRP. While the expression of CRP is essentially dependent on IL-6, several cytokines may compensate for the absence of elevated IL-6 in induction of LRG expression. Accordingly, expression of LRG in COLO205 cells was induced not only by IL-6 but also by TNF-$\alpha$ and IL-22 (Fig. 4B), all of which were increased in sera of UC patients (Fig. 4A). Expression of LRG was strongly induced by IL-22 in COLO205 cells, correlating with enhanced STAT3 (Tyr705) phosphorylation by IL-22 compared with IL-6 (data not shown). Thus, inflammatory cytokines such as TNF-$\alpha$ and IL-22 may mediate LRG expression in the absence of IL-6. Moreover, using DSS-induced colitis in IL-6-deficient mice we could demonstrate an IL-6-independent pathway for LRG induction (Fig. 5E). Because promoter regions of human and mouse LRG share high sequence homology and contain putative binding sites for transcription factors such as C/EBP, MZF1, and STAT,[17] it is conceivable that the similar IL-6-independent mechanisms of LRG induction are also involved in humans. Future studies are required to fully elucidate the induction mechanisms of LRG in both humans and mice.

In the three disease categories of UC based on extent of disease, serum LRG levels tended to be low in proctitis compared with extensive colitis and left-sided colitis (Fig. 1B). In addition, correlation between serum LRG levels and disease activity did not reach significance in proctitis (Fig. 2B). Although the low number of patients with active proctitis may preclude the proper evaluation of LRG levels, limited inflamed area of proctitis may also be a reason for slight increases of serum LRG levels in these patients. Given the increased production of LRG in inflamed colonic mucosa, fecal LRG might be a more sensitive disease biomarker for UC including proctitis. Optimization for the measurement of fecal LRG is currently under way in our laboratory.

This study also highlights the potential usefulness of LRG in evaluating murine colitis. Our results indicate that serum LRG levels increase as the disease progresses in a DSS-induced colitis model (Fig. 5B,C). In addition, the LRG expression is significantly upregulated in the colon with DSS-induced colitis (Fig. 5D). Thus, LRG in mice can be an objective disease activity marker for colitis models and may be useful for preclinical studies of IBD.

In conclusion, serum LRG levels reflect disease activity of UC better than CRP, especially in patients with low CRP. In the inflammatory condition, LRG is expressed in the inflamed tissue and expression of LRG is regulated by mechanisms different from that of CRP. These findings suggest that serum LRG is a novel and potential serologic biomarker for evaluating disease activity of UC.

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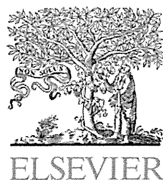

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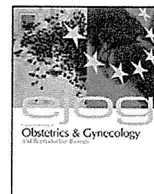


# Cervical non-squamous carcinoma: an effective combination chemotherapy of taxane, anthracycline and platinum for advanced or recurrent cases

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ABSTRACT

*Objective:* An effective salvage chemotherapy for advanced and recurrent non-squamous carcinoma of the uterine cervix has not yet been established. The aim of the present study was to analyze the safety and efficacy of a combination chemotherapy for this disease using taxane, anthracycline, and platinum.
*Study design:* This was a retrospective analysis of advanced and recurrent non-squamous cervical cancers treated at the Osaka University Hospital and the Osaka Medical Center for Cancer and Cardiovascular Diseases during a 10 year study period from 2000 to 2009. Single agent chemotherapies and combination chemotherapies for advanced and recurrent cervical cancer cases of non-squamous histology which were reported in the English literature were also reviewed.
*Results:* Salvage chemotherapy, using taxane, anthracycline and platinum, was performed for 5 advanced and 14 recurrent cases. Prior to the salvage chemotherapy, 15 (79%) of the 19 patients had already received either radiation or chemotherapy. A complete or partial tumor response was achieved in 8 (42%) of the 19 cases. The response rate for recurrent disease in a previously irradiated field was 40%. The median progression-free survival (PFS) and overall survival (OS) were 8 months (1–108) and 13 months (5–108), respectively. Grade 4 and febrile grade 3 neutropenia was observed in 6 cases (32%), but there was no case in which salvage chemotherapy had to be cancelled due to toxicity. According to previous reports, the cumulative response rate of combination chemotherapy (35%) was significantly higher than that of single agent chemotherapy (17%) ($p < 0.001$). OS tended to be longer in the combination chemotherapy cases (8.7 months to 18 months) than that of single agent chemotherapy cases (7.3+ months to 9.1+ months).
*Conclusion:* Combination chemotherapy of taxane, anthracycline, and platinum was found to have a survival benefit for advanced and recurrent cervical cancer patients of non-squamous carcinoma histology, with a tolerable toxicity.

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## 1. Introduction

Cervical cancer is the second most common cancer of women in the world, and nearly 4000 patients die of the disease annually in the United States [1]. Approximately 85–90% of these cervical cancers are squamous cell carcinomas and most of the remaining 10–15% are adenocarcinomas [2]. The incidence of adenocarcinoma, which has been demonstrated to be an independent prognostic factor for cervical cancer [3,4], has been on the rise, especially in younger women [5].

Cervical cancer is usually treated by surgery, radiation or chemoradiotherapy. It is estimated that approximately 35% of patients with cervical cancer will have recurrent or persistent disease after their initial treatment. The prognosis for advanced or recurrent cervical carcinoma is poor, with a 1-year survival rate between 10% and 15% [2]. In a fraction of these cases, the disease is well treated by radical surgery or radiation, but in most cases salvage chemotherapy must be performed. Recurrent or advanced squamous cell carcinoma was demonstrated to exhibit a better response to combination chemotherapy of cisplatin and paclitaxel than to single-agent chemotherapy of cisplatin in a randomized study [6]. Combination chemotherapy using paclitaxel and

*Abbreviations:* ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the plasma drug concentration versus time curve; CCRT, concurrent chemoradiotherapy; CR, complete response; G-CSF, granulocyte-colony stimulating factor; 5-$HT_3$, 5-hydroxytriptamine-3; MEP, mitomycin C, etoposide and cisplatin; NAC, neoadjuvant chemotherapy; OS, overall survival; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease; TEC, paclitaxel, epirubicin and carboplatin; TEP, paclitaxel, epirubicin and cisplatin; TPN, paclitaxel, pirarubicin and nedaplatin; TPP, paclitaxel, pirarubicin and cisplatin.

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carboplatin was also shown to be more effective for advanced or recurrent cervical cancer cases, most of which were squamous cell carcinoma [7].

Recently, Takekida et al. showed that combination chemotherapy of docetaxel and carboplatin was effective in a case of recurrent adenocarcinoma of the uterine cervix and also effective as a neoadjuvant chemotherapy (NAC) in 20 (69%) of 29 other non-squamous carcinoma cases [8].

To date, most large studies of salvage chemotherapy for advanced or recurrent cervical cancer have targeted the more common squamous cell carcinoma [6], whereas salvage chemotherapy for advanced and recurrent non-squamous carcinoma of the uterine cervix has not yet been established. In the present study, we performed a retrospective analysis of the efficacy and adverse effect of a salvage chemotherapy that uses taxane, anthracycline, and platinum for advanced and recurrent non-squamous carcinoma of the uterine cervix, and a literature review of salvage chemotherapy for those cases.

## 2. Materials and methods

Advanced or recurrent cases of non-squamous carcinoma of the uterine cervix, in which salvage chemotherapy using taxane, anthracycline, and platinum was performed at the Osaka University Hospital and the Osaka Medical Center for Cancer and Cardiovascular Diseases during the period of 2000–2009 (10-year period), were retrospectively analyzed. Locally advanced, bulky tumors were excluded from the present analysis, but persistent tumors after initial therapy, and primary tumors with distant metastasis, were included. Eligibility for salvage chemotherapies required that the patient have adequate findings in the following parameters: hematology (WBC $\geq 3000/\mu l$, platelets $\geq 100{,}000/\mu l$, granulocytes $\geq 1500/\mu l$ and hemoglobin $\geq 10$ g/dl), renal (creatinine $\geq 2$ mg/dl) and hepatic (bilirubin $\geq 3$ mg/dl, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) $\geq 2$ times the international normal value). A relative performance status of 0–2 was needed.

In the current study, the clinicopathological features of cases in which the specified salvage chemotherapy was performed, including the age of the patient, the histology and status of the disease, prior therapy and the adverse effects of each chemotherapy regimen, were retrospectively reviewed utilizing the clinical records, including physical examination notes, radiological reports, operative records, and histopathology reports. The histological diagnoses were made by authorized pathologists from the Departments of Pathology of the Osaka University and the Osaka Medical Center for Cancer and Cardiovascular Diseases, who were all trained at the Osaka University Hospital.

In order to evaluate the anti-tumor effect of the chemotherapies against the measurable diseases of advanced and recurrent cervical cancers of non-squamous histology, previously described standard criteria from the World Health Organization [9] and Pectasides et al. [10] were used. The tumors were assessed by CT scan and/or MRI at baseline and every three treatment courses thereafter. A complete response (CR) was defined as the disappearance of all known disease, determined by two observations not less than 4 weeks apart. We used RECIST (Response Evaluation Criteria in Solid Tumors, version 1.0) for evaluating the therapy response. A complete response (CR) required regression of all tumors. A partial response (PR) required >30% reduction in the largest diameter of the largest lesion. A progressive disease (PD) was defined as one in which new lesions appeared, or the diameter of the largest lesion enlarged more than 20%. All other conditions were considered to be stable disease (SD). The association of disease status (advanced versus recurrent) and response of the salvage chemotherapy was analyzed by Fisher's exact test.

In order to evaluate the survival effect of chemotherapies against advanced and recurrent cervical non-squamous carcinoma, progression-free survival (PFS) and overall survival (OS) were calculated. PFS was measured from the administration of chemotherapy to the date of the radiologic or pathologic diagnosis of relapse, or to the date of the last follow-up. OS was defined as the period from the start of chemotherapy to the patient's disease-specific death or to the date of the last follow-up. PFS and OS curves were constructed using the Kaplan–Meier method.

Adverse treatment effects were also analyzed. They were graded based on the National Cancer Institute's Common Toxicity Criteria (version 2.0). Granulocyte-colony stimulating factor (G-CSF), histamine H1 5-$HT_3$ antagonist and other antiemetic drugs were administered as needed.

Single agent chemotherapies and combination chemotherapies for advanced and recurrent cervical cancer cases of non-squamous histology which were reported in the English literature were also reviewed. Studies reporting the efficacy of salvage chemotherapy in fewer than 10 cases were excluded from the present analysis, as were studies reporting the efficacy of NAC. The response rates of combination chemotherapy and single agent chemotherapy for cumulative cases were compared by Fisher's exact test. The result was considered to be significant when the $p$-value was less than 0.05.

## 3. Results

### 3.1. Salvage chemotherapy using taxane, anthracycline and platinum

Salvage chemotherapy using taxane, anthracycline, and platinum was preformed in 19 advanced or recurrent cases of non-squamous carcinoma of the uterine cervix at the Osaka Medical Center for Cancer and Cardiovascular Diseases and the Osaka University Hospital during the period 2000–2009. TPP (150 mg/m$^2$ for paclitaxel, 40 mg/m$^2$ for pirarubicin and 50 mg/m$^2$ for cisplatin), TEC (150 mg/m$^2$ for paclitaxel, 50 mg/m$^2$ for epirubicin and AUC 4 for carboplatin), and TPN (150 mg/m$^2$ for paclitaxel, 40 mg/m$^2$ for pirarubicin and 70 mg/m$^2$ for nedaplatin) were performed every 3–4 weeks in 8, 7, and 4 cases, respectively.

**Table 1**
Clinical characteristics of the patients with measurable advanced or recurrent disease who received salvage chemotherapy using taxane, anthracycline, and platinum.

| Characteristics | Number of cases |
|---|---|
| Median age (years) | 50 (range: 21–67) |
| Performance status | |
| 0 | 5 |
| 1 | 12 |
| 2 | 2 |
| Status of the disease | |
| Recurrent | 14 |
| Advanced | 5 |
| Histology | |
| Mucinous | 9 |
| Adenosquamous | 5 |
| Endometrioid | 3 |
| Serous | 1 |
| Clear | 1 |
| Prior therapy | |
| Surgery + radiation | 12 |
| Surgery + chemotherapy | 2 |
| Surgery | 2 |
| CCRT | 1 |
| None | 2 |

**Table 2**
Anti-tumor effect of salvage chemotherapy using taxane, anthracycline, and platinum in patients with advanced or recurrent disease.

| Response | Number of cases | | |
|---|---|---|---|
| | Advanced | | Recurrent |
| CR | | 3 (16%) | |
| | 1 (20%) | | 2 (14%) |
| PR | | 5 (26%) | |
| | 1 (20%) | | 4 (29%) |
| SD | | 7 (37%) | |
| | 2 (40%) | | 5 (36%) |
| PD | | 4 (21%) | |
| | 1 (20%) | | 3 (21%) |

*3.2. Clinical characteristics of the patients with measurable advanced or recurrent disease who received salvage chemotherapy using taxane, anthracycline and platinum*

The clinicopathological features of the cases are shown in Table 1. These cases included 14 recurrent and 5 advanced cases. Performance status was 1 in 12 (63%) of 19 patients. Prior to the salvage chemotherapy using taxane, anthracycline, and platinum, 15 (79%) of 19 patients already had received radiation or chemotherapy.

*3.3. Anti-tumor and survival effects of salvage chemotherapy using taxane, anthracycline and platinum in patients with advanced or recurrent disease*

Complete response (CR) or partial response (PR) was achieved in 3 (16%) and 5 (26%) of 19 cases, respectively, indicating an overall response rate of 42% (Table 2). The response rates in advanced and recurrent cases were 40% and 43%, which did not indicate a significant difference ($p = 0.91$ by Fisher's exact test). OS and PFS curves are demonstrated in Fig. 1. The median PFS was 8 months (range 1–108), and the median OS was 13 months (range 5–108). The response rate of TEC therapy for recurrent disease in the previously irradiated field was 40% (4 out of 10 cases).

*3.4. Adverse effects of TEC and TC therapies*

Adverse effects of the salvage chemotherapy using taxane, anthracycline, and platinum in the patients with advanced or recurrent disease were evaluated in the 19 patients (Table 3).

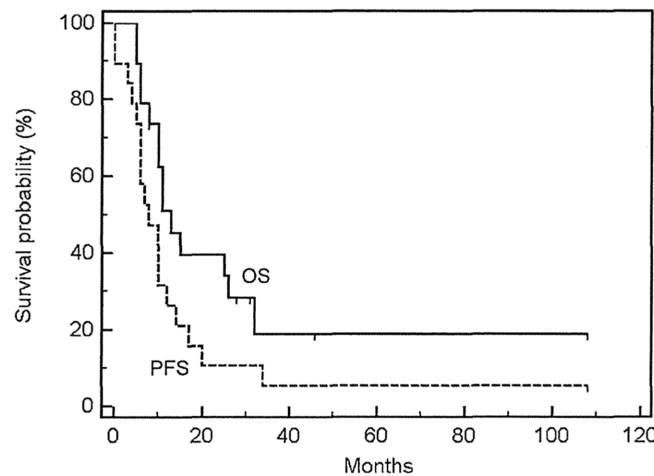


**Fig. 1.** Survival effects of salvage chemotherapy using taxane, anthracycline, and platinum in patients with advanced or recurrent disease.

**Table 3**
Adverse effects of TEC and TC therapies.

| Toxicity | Number of cases (%) |
|---|---|
| Hematological (grade 3 and grade 4) | 15 (78%) |
| Neutropenia (febrile grade 3 and grade 4) | 6 (32%) |
| Non-hematological (grade 3 and grade 4) | 0 (0%) |

**Table 4**
Literature review of the salvage chemotherapy for the advanced or recurrent cervical cancer cases of non-squamous histology (single agent chemotherapy).

| Author (year) | Drug | Dose | Response rate | PFS | OS |
|---|---|---|---|---|---|
| Thigpen et al. (1986) | Cisplatin | 50 mg/m$^2$ | 4/20 (20%) | – | 7.3+ |
| Sutton et al. (1993) | Ifosfamide (+ mesna) | 7.5 mg/m$^2$ | 6/40 (15%) | – | – |
| Look et al. (1997) | 5-FU (+ Leucovorin) | 1850 mg/m$^2$ | 6/43 (14%) | – | – |
| | | | rec: 6/43 (14%) | | |
| Curtin et al. (2001) | Paclitaxel | 170 mg/m$^2$ | 13/42 (31%) | – | – |
| Rose et al. (2003) | Etposide (oral) | 50 mg/m$^2$ | 5/42 (12%) | 3.1 | 9.1+ |
| Bigler et al. (2004) | Tamoxifen (oral) | 20 mg | 3/27 (11%) | 3.0 | 7.9 |
| | | Total | 37/214 (17%) | | |

Hematological toxicities of grade 3 or 4 were observed in 15 (78%) of 19 cases, but grade 4 and febrile grades 3 were detected in only 6 cases (32%). There was no case in which salvage chemotherapy had to be cancelled due to toxicity. Moreover, non-hematological toxicities of grades 3 or 4 were not observed in any cases.

*3.5. Literature review of salvage chemotherapy for advanced or recurrent cervical cancer cases of non-squamous histology*

The previous studies reporting single agent and combination chemotherapies which were performed for at least 10 cases of advanced or recurrent cervical cancer of non-squamous histology are listed in Table 4 (for single agent chemotherapy) and Table 5

**Table 5**
Literature review of the salvage chemotherapy for the advanced or recurrent cervical cancer cases of non-squamous histology (combination chemotherapy).

| Author (year) | Drug | Dose | Response rate | PFS | OS |
|---|---|---|---|---|---|
| Kavanagh et al. (1987) | Cisplatin | 50–60 mg/m$^2$ | 10/24 (42%) | – | 18 |
| | Doxorubicin | 40–50 mg/m$^2$ | | | |
| | 5-FU | 500–800 mg/m$^2$ | | | |
| Lissoni et al. (1997) | Cisplatin | 50 mg/m$^2$ | 10/19 (53%) | – | – |
| | Epirubicin | 70 mg/m$^2$ | rec: 1/2 (50%) | | |
| | Paclitaxel | 175 mg/m$^2$ | | | |
| Umesaki et al. (1999) | Cisplatin | 50 mg/m$^2$ | 5/31 (16%) | – | 8.7 |
| | Mitomycin C | 10 mg/m$^2$ | adv: 0/6 (0%) | | |
| | Etoposide | 100 mg/m$^2$ | rec: 5/25 (20%) | | |
| Present study (2011) | Cisplatin | 50 mg/m$^2$ | 8/19 (42%) | 8 | 13 |
| | or Carboplatin | AUC 4 | adv: 2/5 (40%) | | |
| | or Nedaplatin | 70 mg/m$^2$ | rec: 6/14 (43%) | | |
| | Epirubicin | 50 mg/m$^2$ | | | |
| | or Pirarubicin | 40 mg/m$^2$ | | | |
| | Paclitaxel | 150 mg/m$^2$ | | | |
| | | Total | 33/93 (35%) | | |

(for combination chemotherapy). The response rates ranged from 11% to 31% for single agent chemotherapies, and from 16% to 53% for combination therapies. The cumulative response rate of combination chemotherapy (35%) was significantly higher than that of single agent chemotherapy (17%) ($p < 0.001$ by Fisher's exact test). OS tended to be longer in the combination chemotherapy cases (8.7 months to 18 months) than that of single agent chemotherapy cases (7.3+ months to 9.1+ months).

## 4. Comment

The incidence of cervical adenocarcinoma has been increasing, especially in younger women [5]. Adenocarcinoma was demonstrated to be an independent prognostic factor [3,4]. In a randomized study, recurrent or advanced squamous cell carcinoma was demonstrated to exhibit a better response to combination chemotherapy of cisplatin and paclitaxel than to a single agent chemotherapy of cisplatin [6]. Bernett et al. demonstrated that the combination chemotherapy of gemcitabine and cisplatin was effective for advanced or recurrent squamous cell carcinomas [11]. A combination chemotherapy using cisplatin and topotecan was shown to be even more effective for advanced or recurrent cervical cancer cases, most of which were squamous cell carcinoma [12]. A combination chemotherapy using paclitaxel and carboplatin was also shown to be more effective for advanced or recurrent cervical cancer cases, most of which were squamous cell carcinoma [7].

A few small retrospective studies showed that combination chemotherapy using paclitaxel and carboplatin was effective in 4 (80%) of 5 recurrent adenocarcinomas [13] and that combination chemotherapy of docetaxel and carboplatin was effective in a case of recurrent adenocarcinoma of the uterine cervix [8]. Takekida et al. also showed that combination chemotherapy of docetaxel and carboplatin was effective as NAC in 20 (69%) of 29 non-squamous carcinoma cases.

To date, most large studies of salvage chemotherapy for advanced or recurrent cervical cancer targeted squamous cell carcinoma [6], and salvage chemotherapy for advanced and recurrent adenocarcinoma of the uterine cervix has not been established. In our prospective phase I/II study, TEC therapy (a combination chemotherapy using paclitaxel, epirubicin and carboplatin) was demonstrated to be a tolerable and effective treatment, not only as a remission-induction therapy for advanced and recurrent endometrial carcinomas, but also as the adjuvant therapy [14]. Lissoni et al. showed effectiveness of a combination therapy of paclitaxel, epirubicin and cisplatin (TEP therapy) which was effective not only in endometrial carcinoma, but also in advanced cervical adenocarcinoma [15]. A combination chemotherapy using taxane, anthracycline and platinum might be useful for treatment of advanced and recurrent cervical adenocarcinoma.

In the present study, we performed a retrospective analysis of the efficacy and the adverse effect of salvage chemotherapy using taxane, anthracycline, and platinum for advanced and recurrent non-squamous carcinoma of the uterine cervix. As expected, advanced and recurrent cervical carcinoma of the non-squamous histology type responded well to salvage chemotherapy using taxane, anthracycline, and platinum. Fifteen (79%) of 19 cases already had received chemotherapy, radiation, or concurrent chemoradiotherapy (CCRT). The response rate of TEC therapy for recurrent disease in the previously irradiated field was 40% (4 out of 10 cases). Febrile (grade 3) or grade 4 neutropenia was observed in 6 (32%) of 19 cases, but there was no case in which salvage chemotherapy had to be cancelled due to toxicity.

To date, there are several reports of salvage chemotherapy for advanced and recurrent cervical carcinoma of non-squamous histology [15–23]. The response rate of combination chemotherapies (35%) was significantly higher than that of a single agent chemotherapies (17%) in total ($p < 0.001$ by Fisher's exact test). For recurrent disease, 5-FU (with leucovorin) therapy exhibited a response in 14% of cases (6 of 43) [18], whereas TEP therapy showed 50%, although this was for a very limited set of cases (1 of 2 cases) [15], and MEP therapy (mitomycin C, etoposide and cisplatin) demonstrated a 20% (5 of 20 cases) response [23]. In our study, recurrent tumors responded to a combination chemotherapy of taxane, anthracycline and platinum in 43% (6 of 14 cases), implying that this chemotherapy has an effective potential for recurrent non-squamous carcinoma.

There are only limited data regarding the survival effect of salvage chemotherapy for advanced and recurrent non-sqiamous carcinoma. PFS and OS were 3.1 months and 9.1 months, respectively, for oral etoposide [20], and 3.0 months and 7.9 months, respectively, for oral tamoxifen [21]. Kavanagh et al. reported 18 months of OS by a combination chemotherapy of cisplatin, doxorubicin and 5-FU [22], and Umesaki et al. reported 8.7 months of OS by MEP therapy [23]. In our study, the PFS and OS of a combination chemotherapy of taxane, anthracycline and platinum were 8 months and 13 months, respectively. One patient was alive, without disease, for 108 month after salvage chemotherapy; in another patient, recurrence was detected 34 months after salvage chemotherapy for an initial recurrence and was still alive at 46 months. These results implied that a combination chemotherapy of taxane, anthracycline, and platinum might have significant survival benefit for advanced and recurrent non-squamous carcinoma patients.

Small studies have also shown the effectiveness of a combination chemotherapy using taxane and platinum [8,13]. The present study was a small retrospective study. In order to confirm the effectiveness of chemotherapy using taxane, anthracycline and platinum for advanced and recurrent non-squamous carcinoma of the uterine cervix, a large-scale prospective study is now required.

## Authors' contributions

Toshihiro Kimura and Takashi Miyatake: Data collection and analysis.

Yutaka Ueda: Study design, analysis of data and preparation of the manuscript.

Yukinobu Ohta: Data analysis.

Shoji Kamiura, Takayuki Enomoto, Tadashi Kimura: Data analysis and approval of the study.

## Conflict of interest statement

The authors have no conflicts of interest to declare.

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None.

## Ethical approval

All patients provided written informed consent before their treatments commenced. This study was approved by the Ethics Committee of Osaka University Hospital (#10302, March 11, 2011).

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