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Down-regulation of galectin-3 in the pathogenesis of intestinal Behçet’s disease: Implication of proteomics and functional studies

Hyun Jung Lee

Department of Medicine

The Graduate School, Yonsei University
Down-regulation of galectin-3 in the pathogenesis of intestinal Behçet’s disease: Implication of proteomics and functional studies

Directed by Professor Jae-Hee Cheon

The Doctoral Dissertation
Submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Hyun Jung Lee

June 2016
This certifies that the Doctoral Dissertation of Hyun Jung Lee is approved.

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I’d like to thank to my parents and parents-in-law. They’ve always encouraged me with love. At last, I want to give my love to my husband, Chan Ho Kim and my lovely child, Se Ryung Kim, who have always been beside me whenever I needed them.

I’d like to dedicate this paper to all of you with all of my heart.

Thanks everyone.

Hyun Jung Lee
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ABSTRACT

Down-regulation of galectin-3 in the pathogenesis of intestinal Behçet’s disease: Implication of proteomics and functional studies

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(Directed by Professor Jae-Hee Cheon)

Background: Although intestinal Behçet’s disease (BD) has an unpredictable disease course with exacerbations and remission like inflammatory bowel disease (IBD), data concerning diagnosis, treatment, and prognosis are yet to be determined and the pathogenesis of intestinal BD are poorly understood. Therefore, we aimed to discover biomarkers by investigating the differentially expressed proteins in the intestinal tissues from patients with intestinal BD using proteomics analysis and validate the markers associated with disease pathogenesis by functional studies.
**Methods:** Intestinal tissue samples were obtained from intestinal BD patients who underwent surgery due to disease exacerbation for the screening study. Two-dimensional electrophoresis (2-DE) was performed to characterize the total proteins of intestinal BD patients. Candidate protein spots were identified using matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF/TOF) and bioinformatics analysis. Various *in vitro* and *in vivo* functional validation studies were applied to validate the results of 2-DE and MS.

**Results:** Proteomic profiles of tissue samples were compared, and approximately 550 protein spots were observed in each of the gels. Mass spectrometric analysis identified seven differentially expressed proteins in intestinal BD patients, of which 4 proteins were up-regulated including heat shock protein 27, transgelin, manganese superoxide dismutase, and calprotectin, and 3 were down-regulated including heat shock protein 60, selenium binding protein, and galectin-3. In the inflamed mucosa of intestinal BD patients, galectin-3 expressions were reduced compared with controls, which was consistent with the proteomic results. The mRNA expressions of anti-inflammatory cytokines, such as *TGFB* and *IL10*, were markedly decreased in shGal-3 cell lines. Moreover, caspase-1 activation and IL-1β production were significantly increased in galectin-3−/− BMDMs compared with WT BMDMs upon *S.typhimurium* infection which facilitates the activation of NLRC4 inflammasome. In addition, epithelial cell death rate was increased in shGal-3 cell lines, along with that *XBP1s* are decreased and *GRP78* was increased in shGal-3 HT-29 cells compared with control cells.
Conclusions: A distinct proteomic profile of the intestinal tissues in intestinal BD patients was found that 4 up-regulated and 3 down-regulated proteins were identified and galectin-3 expressions were decreased in the inflamed mucosa of intestinal BD patients. Anti-inflammatory cytokine productions were decreased and NLRC4 inflammasomes were activated by down-regulating galectin-3. In addition, galectin-3 was associated with autophagy-induced cell death. Our data indicate that galectin-3 might play a protective role in the pathogenesis of intestinal BD.

Key words: Intestinal Behçet’s disease (BD), Biomarkers, Proteomics, Galectin-3
I. INTRODUCTION

Intestinal Behçet’s disease (BD) is a chronic, relapsing inflammatory disorder of the gastrointestinal tract which is diagnosed when patients with BD have predominant gastrointestinal symptoms and intestinal ulcerations documented by objective measures. The incidence of intestinal involvement in patients with BD shows remarkable geographic variations, ranging 0 – 60%, and appears to be more common in East Asian countries, including Korea and Japan, but less common in the Middle East. Intestinal BD has an unpredictable disease course characterized by
exacerbations and remission and often requires surgery due to high rates of complications despite medical treatment.\(^3\)\(^-\)\(^5\)

Although the pathogenesis of inflammatory bowel disease (IBD), including intestinal BD is still unclear, both genetic and environmental factors are considered to be attributable to the disease development. A growing body of evidence demonstrated that epithelial barrier defects and dysregulated mucosal immune responses to intestinal microbes in genetically susceptible individuals might result in the sustained intestinal inflammation.\(^6\) Dysregulated enterocyte shedding and apoptosis is known to cause intestinal barrier instability\(^7\) and inappropriate innate immune responses to commensal flora and defective autophagy function are observed in patients with IBD.\(^8\),\(^9\) To date, genome-wide association (GWA) studies revealed approximately 200 genetic biomarkers susceptible for the development of IBD including \textit{CARD15/NOD2}, \textit{ATG16L1}, and \textit{IRGM}.\(^8\)\(^-\)\(^11\) However, although our previous study indicated that \textit{IL17A}, \textit{IL23R}, and \textit{STAT4} polymorphisms were associated with susceptibility to intestinal BD,\(^12\) little is known about disease pathogenesis of intestinal BD.

Recently, because of the limitations of the currently available biomarkers, researchers are interested in using proteomics to discover IBD biomarkers to better understand a disease and to help in the diagnosis, disease activity measurement, and prediction of treatment response.\(^13\)\(^-\)\(^15\) To the best of our knowledge, no studies on biomarkers in intestinal BD using proteomics analysis have not yet been performed.
Therefore, we aimed to search differentially expressed proteins of intestinal BD and discover biomarkers associated with the disease pathogenesis. We characterized a number of potential biomarkers through two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS)-based proteomics. Furthermore, our data demonstrated that galectin-3 might play a protective role in intestinal BD which is down-regulated in the inflamed intestinal epithelium using various *in vitro* and *in vivo* functional studies.

II. MATERIALS AND METHODS

1. **Patients and sample collection**

Forty eight patients with intestinal BD seen at Severance Hospital, Yonsei University, Seoul, Korea were enrolled in this study. The diagnosis of intestinal BD was made according to the previously established criteria based on colonoscopic features and clinical manifestations using a modified Delphi process. Patients were classified into definite, probable, and suspected types of intestinal BD. Tissue samples were obtained from surgical specimens of intestinal BD patients who underwent surgery because of bowel complications, intractability with medical treatment, and/or presence of an abdominal mass. Fifty two healthy persons were included as the control group and control samples were obtained from the normal colon tissues of subjects who underwent colorectal surgery other than inflammatory bowel disease. Samples were immediately frozen in liquid nitrogen and subsequently stored at -80°C until proteomic analyses. Venous blood from patients
with intestinal BD and healthy controls were collected in 10 ml glass tubes and centrifuged at 1,500 g for 15 min. Serum aliquots were stored at –80°C until use.

Intestinal tissue samples from two intestinal BD patients who underwent surgery due to disease exacerbation were used for the screening study. To verify the differentially expressed proteins, 15 intestinal BD and 16 normal intestinal tissue samples were used for immunohistochemistry (IHC) assessment and serum samples from 31 intestinal BD and 35 healthy controls were collected for ELISA.

Informed consent was obtained from all patients and control subjects. This study was approved by the Institutional Review Board of Yonsei University College of Medicine and was conducted in accordance with the Declaration of Helsinki.

2. **Two-dimensional electrophoresis (2-DE)**

Two-dimensional electrophoresis (2-DE) was carried out essentially as described. Aliquots in sample buffer (7 M urea, 2 M thiourea, 4.5% CHAPS, 100 mM DTT, 40 mM Tris, pH 8.8) were applied to immobilized pH 3–10 nonlinear gradient strips (Amersham Biosciences, Uppsala, Sweden). Isoelectric focusing (IEF) was performed at 80,000 Vh. The second dimension was analyzed on 9–16% linear gradient polyacrylamide gels (18 cm x 20 cm x 1.5 mm) at constant 40 mA per gel for approximately 5 h. After protein fixation in 40% methanol and 5% phosphoric acid for 1 h, the gels were stained with Coomassie G-250 solution for 12 h. The gels were destained with H₂O, scanned in a Bio-Rad GS710 densitometer (Richmond, CA, USA) and converted into electronic files, which were then analyzed with Image
Master Platinum 5.0 image analysis program (Amersham Biosciences).

3. **Protein identification by MALDI-TOF/TOF MS**

Protein spots were excised from the gels with a sterile scalpel and placed into Eppendorf tubes. Proteins were digested using trypsin (Promega, Madison, WI, USA) as previously described. For MALDI-TOF/TOF MS analysis, the tryptic peptides were concentrated by a POROS R2, Oligo R3 column (Applied Biosystems, Fostercity, CA, USA). After washing the column with 70% acetonitrile, 100% acetonitrile and then 50 mM ammonium bicarbonate, samples were applied to the R2, R3 column and eluted with cyano-4-hydroxycinamic acid (CHCA) (Sigma-aldrich, St. Louis, MO, USA) dissolved in 70% acetonitrile and 0.1% TFA before MALDI-TOF/TOF MS analysis. Mass spectra were acquired on a 4800 Proteomics Analyzer (Applied Biosystems) operated in MS and MS/MS modes. Peptide fragmentation in MS/MS mode was by collision-induced dissociation (CID) using atmosphere as the collision gas. The instrument was operated in reflectron mode and calibrated using the 4700 calibration mixture (Applied Biosystems) and each sample spectrum was additionally calibrated using trypsin autolysis peaks. For MS analysis, 800–4000 m/z mass range was used with 1000 shots per spectrum. A maximum of 15 precursors with minimum S/N of 50 were selected for MS/MS analysis. Collision energy of 1 kV was used for CID, and 2000 acquisitions were accumulated for each MS/MS spectrum. Peptide mass fingerprinting was carried out using the Mascot search engine included in the GPS Explorer software and mass spectra used for manual *de novo* sequencing were
annotated with the Data Explorer software (Applied Biosystems).

4. **Mascot database search**

The mascot algorithm (Matrixscience, Boston, MA, USA) was used to identify peptide sequences present in a protein sequence database. Database search criteria were, taxonomy; *homo sapiens* (NCBI nr database downloaded on Mar 24 2013), fixed modification; carboxyamidomethylated (+57) at cysteine residues; variable modification; oxidized (+16) at methionine residues, maximum allowed missed cleavage, 1. Mass tolerances of 100 ppm, 0.1 Da were used for precursor and fragment ions, respectively. Only peptides resulting from trypsin digests were used for protein identification.

5. **Enzyme-linked immunosorbent assay (ELISA)**

The concentrations of Gal-3 in the serum were determined using commercial ELISA kits (R&D systems, Minneapolis, MN, USA) according to the manual.

6. **Immunohistochemistry (IHC) assessment**

IHC staining was performed with a Vecastain ABC kit (Vector Labs, Burlingame, CA, USA). Tissue sections were incubated first with the primary anti-galectin-3 antibody (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C, then with a biotinylated secondary linking antibody, and finally with a streptavidin-peroxidase complex for 1h. The final color product was developed
using aminoethylcarbazole (Dako, Carpinteria, CA, USA). Sections were counterstained with hematoxylin and mounted and the tissues were photographed using an Olympus photomicroscope (Olympus Corp., Tokyo, Japan). To quantify galectin-3 expression, we randomly selected 3 fields for each sample at x200 magnification and scored range from 0-3.

7. **Cell culture and treatment**

The HT-29 cell line (KCLB 30038, Korean Cell Line Bank, Seoul, Korea) was maintained at 37°C in Roswell Park Memorial Institute medium (RPMI medium) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics in a humidified atmosphere of 5% CO₂. To generate galectin-3 knock down colon cancer HT-29 cell lines, transfections with human galectin-3 small hairpin RNA (shRNA, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) plasmids and, as a control a nonspecific shRNA or scramble RNA, were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). shRNA transfected cells were isolated by selection with 20 μg/ml puromycin (Santa Cruz Biotechnology, Inc., CA, USA) for 4 weeks. After purification of puromycin-resistant cell lines, galectin-3 expression was evaluated using Western blot analysis. Subsequently, stable galectin-3-silenced cell lines (shLGALS3) and control cell lines (SCR) were selected for further analyses. Cells were incubated with TNF-α (40 ng/mL, R&D systems) and LPS (1–10 g/mL, Sigma-aldrich) for the indicated time with or without recombinant human galectin 3 (100ng/mL, Prospec, NJ, USA).
8. **Immunofluorescence**

Cells were fixed with 4% paraformaldehyde solution (pH 7.4) and washed them in PBS. The cells were blocked in 5% BSA or 1% BSA with normal serum in 0.1% Triton X-100, respectively, washed, and incubated with primary antibodies (LC3B, 1:1000, Cambridge, MA, USA; Gal3, 1:1000). Fluorescence was visualized by secondary antibodies (Alexa Fluor 555-conjugated antibodies and Alexa Fluor 488-conjugated antibodies, 1:500, Invitrogen, Waltham, MA, USA). Nuclei of cells were stained with DAPI solution (blue, Invitrogen). Images were obtained using light microscopy (Olympus BX41; Olympus Optical, Tokyo, Japan) at a magnification of × 200 or × 400.

9. **Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)**

Total RNA extraction and reverse-transcription was performed as previously described. PCR primer information is described in Supplementary Table 1 excepting for TNFA (AccuTarget qRT-PCR primer, Bioneer, Daegeon, Korea). Samples were amplified in a StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA) for 45–55 cycles using the following PCR variables: 95℃ for 30 sec, 60–63℃ for 30 sec, and 72℃ for 40 sec, respectively. Finally, quantitative analysis was performed using the relative standard curve method. The results were reported as a relative expression or fold change compared
to the calibrator after normalization of the transcript level to the endogenous control, BACTIN.

10. **Western blotting**

The proteins extracted from the tissues were lysed in a buffer containing 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1mM EDTA, 1% Triton X, 0.05% Sodium deoxycholate and protease inhibitors. The protein samples were fractionated on 8% or 12% SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes for 70 min at 100 V (Bio-Rad). Blots were incubated with primary anti-galectin-3 antibody (1:500, sc-20157, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), caspase-1 (1:2000, Adipogen, San Diego, CA, USA), and IL-1β (1:2000, R&D system, MN, USA) followed by horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL) reagents using the ECL kit (Thermo Scientific, Hudson, NH, USA). Anti-beta-actin (Santa Cruz Biotechnology, Inc.) was used as a loading control.

11. **Flow cytometric analysis**

Necrosis and apoptosis were examined by flow cytometry using Annexin V-FITC/PI kit (BD Biosciences, San Diego, CA, USA). The samples were analyzed with a FACSverse flow cytometer (BD Biosciences) with 10,000 events collected and further analyses were performed with Flow Jo software (Tree Star, San Carlos, CA, USA).
12. **Bone marrow derived macrophage preparation**

Mouse primary bone marrow-derived macrophages (BMDMs) were prepared from C57BL/6 mice as described previously. All mice were maintained under specific pathogen-free conditions. Protocols for the animal experiments were approved by the Institutional Ethical Committee, Yonsei University College of Medicine. Mouse BMDMs stably expressing non-targeting (scramble) or targeting Gal-3 shRNA were prepared by lentiviral infection and cloned by puromycin selection. All BMDMs were maintained in L929-conditioned DMEM supplemented with 10% FBS and 100 U/mL penicillin/streptomycin.

13. **Statistical analysis**

Data are expressed as means ± standard error of mean (SEM). Parametric and nonparametric analyses were performed using Student’s *t*-test and Mann-Whitney *U* test, respectively. A value of *P* < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS V20.0 for Windows (SPSS Inc., Chicago, IL, USA).
III. RESULTS

1. Galectin-3 is down-regulated in the inflamed intestine of patients with intestinal Behçet’s disease

The clinical characteristics of 48 intestinal BD patients and 52 control groups enrolled in this study are shown in Table 1. There were no statistically significant differences between the two groups regarding age and sex. Of the 48 patients with intestinal BD, 15 patients (31.2%) showed moderate disease activity and 33 (68.8%) showed severe disease activity.

We first performed a tissue proteomics analysis to identify proteins with differential expressions between the intestinal BD and control groups. Approximately 550 protein spots were observed in each of the gels (Figure 1a) and 30 protein spots in the intestinal BD patients were noted to have qualitative or quantitative differences compared with controls, including 12 up-regulated, 15 down-regulated, 3 unique spots. Our study mainly focused on the highly different expressed proteins, and therefore, the spots with 5-fold change or more were defined as the differential spots in the 2-DE gel. After the images were compared, 7 differentially expressed proteins were incised from the gels and analyzed by MALDI-TOF/TOF MS. The results revealed that 4 proteins were up-regulated including heat shock protein 27 (2.2-fold), transgelin (4.7-fold), superoxide dismutase (3.3-fold), and calprotectin (9.3-fold), and 3 were down-regulated including heat shock protein 60 (7.0-fold), selenium binding protein (9.0-fold), and
galectin-3 (5.8-fold) (Table 2). Figure 1b shows a typical MS/MS spectrum of a down-regulated protein in intestinal BD patients, galectin-3. These 7 proteins were known to be involved in stress response, oxidative stress, and inflammation\textsuperscript{20-22} and, among them, galectin-3 is considered to regulate immune response in chronic inflammatory disorders including IBD.\textsuperscript{23-25} Thus, we next focused on galectin-3 for further investigation.

To verify the proteomic results, IHC analyses for galectin-3 were performed using the colon tissues from intestinal BD patients and control tissue specimens. The protein expressions of galectin-3 were significantly reduced in the intestinal crypt epithelium of patients with intestinal BD compared to control tissues, which was consistent with previous results using IBD biopsy samples (Figure 2a).\textsuperscript{26-28} However, mRNA levels of galectin-3 were significantly higher in the inflamed colon of TNBS- or DSS-administrated mice than in the normal colon (Supplementary Figure 1), similar to recent data investigating galectin-3,\textsuperscript{29} which is speculated to be related with an increase in compensation for an inflammatory condition. Together, these data support our proteomic observation that galectin-3 might play a protective role and be involved in the pathogenesis of intestinal BD which is down-regulated in the inflamed intestinal epithelium.

It has been known that Gal-3 is elevated in the sera of patients with BD\textsuperscript{30,31} as well as those of IBD.\textsuperscript{32,33} Thus, we measured serum galectin-3 levels in healthy controls, patients with systemic BD, and patients with intestinal BD. Serum galectin-3 levels were significantly higher both in systemic BD patients (mean 9.42 ± 0.92) and intestinal BD patients (mean 7.11 ± 0.59) than in healthy controls (mean 3.95 ±
0.235) as shown in Figure 2b. When we classified the 31 cases intestinal BD into moderate and severe groups based on DAIBD score, galectin-3 mRNA level was numerically more up-regulated in severe group of intestinal BD compared with those in moderate group, but with no statistical significance (6.81 ± 3.51 vs 7.93 ± 3.15; \( p = 0.353 \)) (Figure 2b).

Table 1. Characteristics of healthy controls and patients with intestinal Behçet’s disease

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<th>Control (n = 52)</th>
<th>Intestinal BD (n = 48)</th>
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<tr>
<td>Male (%)</td>
<td>20 (38.5%)</td>
<td>21 (43.8%)</td>
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<td>Age (yrs, mean ± SD)</td>
<td>40.90 ± 10.51</td>
<td>44.36 ± 11.24</td>
</tr>
<tr>
<td>Disease duration (months)</td>
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<td>112.6 ± 64.71</td>
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<tr>
<td>Clinical activity</td>
<td></td>
<td></td>
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<td>Quiescent (≤19)</td>
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<td>0</td>
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<tr>
<td>Mild (20–39)</td>
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<td>Moderate (40–74)</td>
<td></td>
<td>15 (31.2%)</td>
</tr>
<tr>
<td>Severe (≥75)</td>
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<td>33 (68.8%)</td>
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Figure 1. Proteomic profiles of the intestinal tissues from intestinal BD and the controls, examples of up or down-regulated spots in intestinal BD

(a) Seven significantly differentially expressed proteins were incised and analyzed by MALDI-TOF/TOF MS (arrows on 2-DE gels). (b) Typical MS/MS spectrum from a down-regulated protein in intestinal BD patients, galectin-3.

Table 2. Protein identification by MALDI-TOF/TOF

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<th>Spot no</th>
<th>Protein description</th>
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<td>61187/5.70</td>
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<tr>
<td>503</td>
<td>Selenium binding protein1</td>
<td>gi</td>
<td>14290607</td>
<td>52928/5.93</td>
<td>DGLIPLEIR(41) LVLPSLISSR(18) HEIVQTSLK(87) IYVVVDGEPR(51) QYDISDPQPR(30) GGFVLLDGETFEVK(35) VAGGPQMIQSLDGKR(32) HNVMIETEWAAPNVLR(33) GTWERPAGAAPGLYDFWYQPR(22) DGFNPADVEAGLYGSHLTVWDWQR(38)</td>
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<td>Accession</td>
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<tr>
<td>890</td>
<td>Galectin-3</td>
<td>2385452</td>
<td>26193/8.57</td>
<td>-5.8</td>
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Figure 2. Galectin-3 expression in intestinal BD patients

(a) Immunohistochemistry of Gal-3. Colon tissues were stained with antibodies against Gal-3. Right panel shows expression scores. Sections were counterstained with hematoxylin. (b) Serum Gal-3 levels. The levels of circulating Gal-3 were assessed by ELISA in the serum of normal healthy controls (HC, n = 35), patients with systemic BD (sysBD, n = 17), and patients with intestinal BD (intBD, n = 31). Results are shown as individual values (symbols) and SD (lines) for each group of patients. *P < 0.05 vs. HC, ***P < 0.005 vs. HC, #P < 0.05 vs. sysBD. ns, not significant.
2. Galectin-3 down-regulation suppresses anti-inflammatory cytokine production in intestinal epithelial cells in response to TNF-α and LPS

To explore the specific role of galectin-3 in regulating cytokine secretion, we established stably galectin-3 knock-downed HT-29 cells as intestinal epithelial cells (IEC), using shRNA (shown as shGal-3). Decreased galectin-3 levels were confirmed using quantitative RT-PCR and western blotting (Figure 3a, b, respectively). To investigate the effect of galectin-3 knockdown on the mRNA expression, the cells were treated for 4 h with or without TNF-α or LPS and analyzed by quantitative RT-PCR. The results show that LPS-stimulated HT-29 cells produced more significantly reduced mRNA expression of LGALS3 than controls, while TNF-α-stimulated HT-29 cells produced significantly higher mRNA expression of LGALS3 than controls (Figure 3c, d). Exogenous recombinant galectinl-3 reduced mRNA expression of TLR4 and IL8 by LPS and elevated mRNA expression of IL10 (Figure 4a, b). Furthermore, mRNA expression of TGFB and IL10 were markedly decreased in shGal-3 cell lines compared with control cells when stimulated by LPS or TNF-α (Figure 4c, d).
Figure 3. Confirmation of galectin-3 expression in HT-29 / shGal-3 HT-29 cells

(a) Transcript levels of *LGALS3* were quantified by real-time quantitative reverse transcription polymerase chain reaction and normalized to *BACTIN*. (b) Galectin-3 levels were analyzed using western blotting. Cells were treated with LPS (5 μg/ml) for 72 h and protein levels were analyzed using western blotting. (c, d) *LGALS3* expression in cells treated with TNF-α or LPS. Cells were treated with TNF-α or LPS for 4 h and the transcript levels were quantified by real-time quantitative reverse transcription polymerase chain reaction and normalized to *BACTIN*. *P < 0.05 vs. Veh, **P < 0.01 vs. Veh, ***P < 0.05 vs. Veh, #P < 0.05 vs. TNF-α or LPS, ##P < 0.01 vs. TNF-α or LPS, ###P < 0.005 vs. TNF-α or LPS. Veh, treated with PBS; rhGal, treated with recombinant human galectin-3; SCR, scramble control HT-29 cell; shLGALS3, shLGALS3 HT-29 cells.
Figure 4. Galectin-3 affects cytokine expressions.

(a, b) Gene expression of proinflammatory cytokine (TLR4 and IL8). (c, d) Gene expression of antiinflammatory cytokine TGFB, and IL10. Cells were treated with LPS (5 μg/ml) for 4 h and the transcript levels were quantified by real-time quantitative reverse transcription polymerase chain reaction and normalized to BACTIN. *P < 0.05 vs. Veh, **P < 0.01 vs. Veh, ***P < 0.05 vs. Veh, #P < 0.05 vs. LPS, ##P < 0.01 vs. LPS, ###P < 0.005 vs. LPS. Veh, treated with PBS; rhGal, treated with recombinant human galectin-3; SCR, scramble control HT-29 cell; shLGALS3, shLGALS3 HT-29 cell.
3. **Loss of galectin-3 increases bacteria-induced inflammasome activation**

Recently, accumulating evidence has shown that inflammasome plays a pivotal role in host defense against intestinal microbes and intestinal inflammation.\(^{35}\) Inflammasome activation results in the recruitment and activation of caspase-1, a key enzyme in the processing of pro-IL-1β into the mature IL-1β. As shown in Figure 5a, mRNA levels of *NLRP3* were not different in the colon tissues between intestinal BD patients and healthy controls, whereas *NLRC4* expression was significantly increased in the inflamed colon tissues of intestinal BD patients when compared with controls. Consistently, mRNA expression of inflammasome genes, such as *TLR5, IL1B, and NLRC4* related to inflammasome excepting for *NLRP3* were significantly increased in shGal-3 HT-29 cells (Figure 5b).

Accordingly, we sought to investigate the differential role of galectin-3 in NLRP3- or NLRC4-induced caspase-1 activation in macrophages using BMDMs from WT and galectin-3\(^{-/-}\) mice. No significant difference in caspase-1 activation and IL-1β production between WT and galectin-3\(^{-/-}\) mice was observed when BMDMs were treated with LPS plus ATP, a well-known NLRP3 inflammasome stimulator (Figure 6a). However, caspase-1 activation and IL-1β production were significantly increased in galectin-3\(^{-/-}\) BMDMs compared with WT BMDMs upon *S.typhimurium* infection facilitating the activation of NLRC4 inflammasome (Figure 6b), which may be due to higher basal mRNA levels of *NLRC4* in galectin-3\(^{-/-}\) BMDMs than in WT BMDMs (Figure 6c). These observations suggest that galectin-3 might be a negative regulator for the activation of NLRC4 inflammasome, but not
of NLRP3 inflammasome. Thus, our data suggest that galectin-3 may be involved in anti-inflammatory cytokine production and regulating inflammasome pathway in response to TNF-α and LPS stimulation.
Figure 5. Galectin-3 affects inflammasome

(a) mRNA levels in colon tissues. Transcript levels of LGALS3, IL1B, NLRP3, and NLRC4 from human colon tissues were quantified by real-time quantitative reverse transcription polymerase chain reaction and normalized to BACTIN. (b) Gene expression related to inflammasome (IL1B, NLRP3, NLRC4, TLR5) in cells treated with TNF-α or LPS. Data represent means ± S.D. values (n=3). Cells were treated
with TNF-α (40 ng/ml) or LPS (1 μg/ml) for 4 h in mRNA analysis and for 24 h in protein analysis. The transcript levels were quantified by real-time quantitative reverse transcription polymerase chain reaction and normalized to BACTIN (mRNA) and the protein levels were analyzed using western blotting. *P < 0.05 vs. Veh, **P < 0.01 vs. Veh, ***P < 0.05 vs. Veh, #P < 0.05 vs. TNF-α or LPS, ##P < 0.01 vs. TNF-α or LPS, ###P < 0.005 vs. TNF-α or LPS. Veh, treated with PBS; rhGal, treated with recombinant human galectin-3; SCR, scramble control HT-29 cell; shLGALS3, shLGALS3 HT-29 cell.
Figure 6. Loss of galectin-3 increases NLRC4 in macrophages

(a) BMDMs were untreated or treated with LPS, ATP, and Nigericin. Cultural supernatants or soluble lysates were immunoblotted with anti-caspase 1. Levels of IL-1β in culture media were measured by ELISA. (b) BMDMs were infected with *S. typhimurium*. Supernatants or lysates were immunoblotted with the appropriate antibodies as indicated. Levels of IL-1β in culture media were measured by ELISA. (c) mRNA levels of NLRC4 in the colon tissue of WT and galectin-3 KO mice were quantified by real-time quantitative reverse transcription polymerase chain reaction and normalized to *BACTIN*. 
4. **Loss of galectin-3 induces autophagy-induced cell death by ER-stress**

Barrier integrity is important to keep immune tolerance towards intestinal microbiota and prevent chronic inflammation in IBD. Recent studies reported that defect in autophagy and increased ER stress could trigger epithelial cell apoptosis which were linked to IBD pathogenesis. In addition, galectin-3 is known to have the dual role in apoptosis that intracellular galectin-3 acts as an anti-apoptosis factor, therefore, we evaluated whether epithelial barrier function is maintained in shGal-3 cells.

First, we evaluated the role of galectin-3 in cell death in response to various stimuli. LPS-induced cell death was increased in shGal-3 cell lines and thapsigargin treatment and serum starvation which induced ER stress also increased cell death in shGal-3 cells compared with control cells (Figure 7a-c). In addition, we investigated the effects of Gal-3 on ER-stress and autophagy in HT-29 cells in the presence or absence of LPS. Levels of \( XBP1s \), an active spliced form of \( X-box–binding \) protein 1 (\( XBP1 \)) and the ratio of \( XBP1s/unspliced \) \( XBP1 \) (\( XBP1u \)) was significantly down-regulated in shGal-3 HT-29 cells compared with control cells, whereas glucose-regulated protein 78 (\( GRP78 \)) was markedly increased as shown in Figure 7d-f.
Figure 7. Galectin-3 activates ER-stress/autophagy and leads cell death

(a-c) Effects of galectin-3 on cell death. Representative data of Annexin V/PI stain analysis. Cells were treated with LPS for 72h and thapsigargin for 24h. Annexin V staining corresponds to early apoptosis, Annexin V + PI staining to late apoptosis, and PI staining alone to necrotic cells, which were performed and analyzed by flow cytometry. (d-f) Expression of GRP78, XBP1s, and ratio of XBP1s/XBP1u in cells treated with LPS. Cells were treated with LPS (1 μg/ml) for 4 h and the transcript levels were quantified by real-time quantitative reverse transcription polymerase chain reaction and normalized to BACTIN. *P < 0.05 vs. Veh, **P < 0.01 vs. Veh, ***P < 0.05 vs. Veh, #P < 0.05 vs. LPS, ##P < 0.01 vs. LPS, ###P < 0.005 vs. LPS.

Veh, treated with PBS; rhGal, treated with recombinant human galectin-3; SCR, scramble control HT-29 cell; shLGALS3, shLGALS3 HT-29 cell. SCR, scramble
control HT-29 cell; shLGALS3, shLGALS3 HT-29 cell; TH, treated with thapsigargin (5 M).
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*Supplementary Table 1. Nucleotide sequence of primers used in the study*
Supplementary Figure 1. Galectin-3 expression in murine colitis models

mRNA levels in colon tissues of mice with trinitrobenzenesulfonic acid (TNBS)- and dextran sulfate sodium (DSS)-induced colitis (a, b, respectively). Total RNAs were isolated from mouse colon tissues and converted into complementary DNA. Transcript levels of LGAL3, IL1B, NLRP3, and NLRC4 from mice were quantified by real-time quantitative reverse transcription polymerase chain reaction and normalized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each data point represents mean ± SD of data (n=3–4).
IV. DISCUSSION

In the present study, I identified distinct protein profiles of intestinal BD by proteomic analysis which showed 4 upregulated, including heat shock protein 27, transgelin, superoxide dismutase, and calprotectin, and 3 downregulated proteins, including heat shock protein 60, selenium binding protein, and galectin-3. Especially, galectin-3 expressions were markedly decreased in the inflamed mucosa of intestinal BD patients, suggesting its potential as a biomarker. I further demonstrated that galectin-3 might play a protective role in the pathogenesis of intestinal BD through regulating inflammatory cytokine production, inflammasome activation, and epithelial cell apoptosis. To the best of our knowledge, this is the first study to identify biomarkers in intestinal BD using proteomics analysis.

IBD is thought to result from an inappropriate and continuing inflammatory response to commensal microbes in a genetically susceptible host. To date, GWAS have been successful in identifying genetic loci that contribute to IBD pathogenesis, including barrier function (GNA12, HNF4A), innate and adaptive immune regulation (NOD2, CARD9, IL23R, TNFSF15), autophagy (ATG16L1, IRGM, LRRK2), and ER stress (XBP1). However, genetic changes do not always translate into protein activity and genomics does not take into account posttranslational modification. Thus, in recent decades, proteomics is highlighted as a complementary method to genomics for biomarker discovery in IBD and some markers have been revealed to differentiate IBD subtype (serum antibody anti-saccharomyces cerevisiae antibody; ASCA, perinuclear anti-neutrophil cytoplasmic
antibody; pANCA), while others to predict relapse (fecal calprotectin). Similar to IBD, intestinal BD is known to have a wax and wane disease course and relapse frequently even after surgery; however, data regarding disease pathogenesis are extremely limited. Because we have recently reported that genetic variants in IL17A, IL23R, and STAT4 are associated with intestinal BD pathogenesis by polymorphism analysis, in this study, I aimed to investigate differentially expressed proteins of intestinal BD by proteomics approach.

In the present study, 7 differential protein spots were identified using MALDI-TOF/TOF MS. These observations are in agreement with previous findings in IBD showing that protein profiles were involved in stress response (heat shock protein 27; HSP 27, HSP 60), oxidative stress (superoxide dismutase; SOD, selenium binding protein; SBP), and inflammation (calprotectin, galectin-3). Among the potential candidate proteins, decreased level of galectin-3 was confirmed by IHC analysis in the inflamed mucosa of intestinal BD patients, which is consistent with prior studies showing that galectin-3 was reduced in the inflamed tissue in IBD patients. In addition, galectin-3 significantly ameliorates intestinal inflammation in a DSS colitis model. Therefore, I speculated a protective role of galectin-3 in intestinal BD and sought to further elucidate the underlying mechanism of galectin-3 in the intestinal immune responses.

Galectin-3, a 31-kDa chimeric lectin, is a member of a large family of S-type lectin characterized by binding affinity for β-galactoside structure. It is expressed by various cell types, including epithelial cell of gastrointestinal tract and activated immune cells and present both intracellularly and extracellularly. By binding to
its glycan ligand at the cell surface, galectin-3 regulates inflammatory responses through its function on cytokine secretion, cell adhesion and migration, and cell apoptosis and controls immune responses through regulating damage-associated molecular pattern (DAMP) and pathogen-associated molecular pattern (PAMP) pathways. Galectin-3 is considered to play a critical role in the modulation of chronic inflammatory disorders such as IBD and other autoimmune diseases; however, the results are highly controversial so far. In IBD, several studies suggested galectin-3 as a negative regulator of inflammatory response that diminished interaction between galectin-3 and laminin could cause increased intestinal permeability and downregulation of galectin-3 might result in inappropriate T cell proliferation. On the contrary, others demonstrated the pro-inflammatory role of galectin-3 that might lead to a longer cell survival and promote activation of NLRP3 inflammasome in macrophages. Our findings support a protective role of galectin-3 in the development of intestinal inflammation. Our data show that galectin-3 is able to inhibit inflammation by inducing anti-inflammatory cytokines, such as TGFβ and IL10 and regulating the inflammasome activation. Previous studies report that galectin-3 negatively regulates IL-17A responses through inhibition of IL-23/IL-17 axis cytokine production by dendritic cells and suppresses Th1-mediated inflammation in a colitis model. In addition, consistent with our observation, galectin-3 downregulation in dendritic cells also reduced IL-10 expressions, indicating the possibility of regulatory T cell induction. As well as adaptive immune responses, the innate immune system plays an essential role for the host defense responses and recent findings suggest that
inflammasomes, such as NLRP3, NLRC4, and NLRP6, are critical in maintaining intestinal homeostasis.\textsuperscript{35,46} In this study, galectin-3 showed a distinct effect on the regulation of inflammasomes that galectin-3 might be a negative regulator for NLRC4 inflammasome but not for NLRP3. It is important that the innate immune system selectively promotes host defense response against pathogenic bacteria, but not commensal bacteria. Since intracellular invasion is a property that generally distinguishes pathogenic bacteria from commensal bacteria, intracellular sensing of PAMP by NLRC4 inflammasome effectively triggers a production of IL-1\(\beta\) and inflammatory responses.\textsuperscript{35,46}

Besides the role of galectin-3 in the regulation of immune response, galectin-3 is also known to be involved in apoptosis by intracellular association with anti-apoptotic pathways, compared with the extracellular signaling of pro-apoptotic stimuli.\textsuperscript{39,41} In this study, I demonstrated that epithelial cell death was increased by down-regulating galectin-3. Moreover, \textit{GRP78} expression, a marker of ER stress, was markedly induced and \textit{XBP1} splicing was reduced in galectin-3 knockdown cell lines. These findings are in accordance with previous data that uncompensated ER stress due to autophagy defects promotes intestinal inflammation and leads to cell death in inflammatory conditions.\textsuperscript{47,48} In addition, autophagy negatively regulates inflammasome activity by degrading and removing assembled inflammasomes,\textsuperscript{49} and by downregulating pro-IL-1\(\beta\),\textsuperscript{50} implicating that defect in autophagy could exacerbate intestinal inflammation.

On the other hand, the mechanisms of regulation of galectin-3 expression are yet to be studied. Here, I report down-regulation of galectin-3 protein by LPS and
upregulation by TNF-α in epithelial cell lines. Although it has been reported that LPS down-regulates galectin-3,11 TNF-α also reduced the level of galectin-3.27,28 The reasons of discrepancy between our observations and previous data are unclear, but could be due to difference in treatment time that galectin-3 levels temporarily increased within the first 4 h of culture with TNF-α and then rapidly dropped during overnight culture.27 Because both TNF-α and LPS are important mediators of intestinal inflammation, the effect of cytokine stimulation on galectin-3 expression requires further investigation.

In summary, I performed proteomic analysis and identified that galectin-3 expressions were significantly reduced in the inflamed intestinal epithelium of intestinal BD patients. Moreover, down-regulation of galectin-3 decreased anti-inflammatory cytokine production, increased the activation of NLRC4 inflammasome, and promoted epithelial cell death by autophagy defects and ER stress. Our data suggest that galectin-3 might play a protective role in the pathogenesis of intestinal BD which could translate into new therapeutic opportunities, although further studies are needed to confirm our findings in a colitis model specific for intestinal BD.
V. CONCLUSION

In the present study, I aimed to investigate differentially expressed proteins of intestinal BD by proteomic approach.

1. I identified distinct protein profiles of intestinal BD by proteomic analysis which showed 4 upregulated, including heat shock protein 27, transgelin, superoxide dismutase, and calprotectin, and 3 downregulated proteins, including heat shock protein 60, selenium binding protein, and galectin-3.

2. Galectin-3 expressions were markedly decreased in the inflamed mucosa of intestinal BD patients.

3. Exogenous recombinant galectin-3 reduced mRNA expression of TLR4 and IL8 by LPS and mRNA expression of TGFβ and IL10 were markedly decreased in shGal-3 cell lines compared with control cells when stimulated by LPS or TNF-α.

4. mRNA expression of inflammasome genes, such as TLR5, IL1B, and NLRC4 related to inflammasome excepting for NLRP3 were significantly increased in shGal-3 HT-29 cells.

5. No significant difference in caspase-1 activation and IL-1β production between WT and galectin-3−/− mice was observed when BMDMs were treated with LPS plus ATP.

6. However, caspase-1 activation and IL-1β production were significantly increased in galectin-3−/− BMDMs compared with WT BMDMs upon S.typhimurium infection, which may be due to higher basal mRNA levels of
*NLRC4* in galectin-3−/− BMDMs than in WT BMDMs.

7. In addition, LPS-induced cell death was increased in shGal-3 cell lines and thapsigargin treatment and serum starvation which induced ER stress also increased cell death in shGal-3 cells compared with control cells.

8. Levels of *XBP1s*, an active spliced form of X-box–binding protein 1 (*XBP1*) and the ratio of *XBP1s*/unspliced *XBP1* (*XBP1u*) was significantly down-regulated in shGal-3 HT-29 cells compared with control cells, whereas glucose-regulated protein 78 (*GRP78*) was markedly increased.

In conclusion, I demonstrated that galectin-3 might play a protective role in intestinal BD through regulating inflammatory cytokine production, inflammasome activation, and epithelial cell apoptosis which could translate into new therapeutic opportunities.
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25. Pejnovic NN, Pantic JM, Jovanovic IP, Radosavljevic GD, Milovanovic MZ,


ABSTRACT (IN KOREAN)

Galectin-3 발현 감소가 베체트 장염 병태생리에 미치는 영향:
프로테오믹스와 기능 연구를 통한 규명

<지도교수 천 재 회>

연세대학교 대학원 의학과

이 현 정

배경: 베체트 장염은 만성 재발성 면역 질환으로, 염증성 장질환과 유사하게 반복적인 관해와 재발의 임상 경과를 보이는 것으로 알려져 있다. 하지만 베체트 장염은 혼란 질환이 아니므로 이에 대한 관심이 상대적으로 적어, 진단과 치료방법 및 병태생리에 대한 자세한 연구가 부족한 상황이다. 따라서 본 연구에서는 프로테오믹스 방법을 이용하여 베체트 장염 환자의 조직에서 특이적으로 발현되어 있는 단백질을 분석하고 이를 통하여 질병의 병태생리와 관련된 바이오마커를 찾아보고자 하였다.

방법: 베체트 장염의 질병 악화로 수술을 시행한 환자의 조직을 이용하여 프로테오믹스 연구를 진행하였다. 2-dimensional electrophoresis (2-DE)
분석을 통하여 베체트 장염 환자의 단백질을 분리, matrix-assisted lase desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF MS)와 bioinformatics를 이용하여 후보단백질을 동정, 분석하였다. 면역 조직화학적 염색과 효소면역측정법을 이용하여 2-DE와 MS를 통하여 발굴한 후보단백질의 발현을 확인하였다. shGal-3 cell line에서 염증성 사이토카인, inflammasome의 발현은 실시간 중합효소연쇄반응을 이용하여 분석하였으며, caspase-1의 활성과 IL1β의 분비 정도는 western blot과 효소면역측정법으로 확인하였다. 또한, 세포사멸은 유세포 분석기를 이용하여 관찰하였다.

결과: 2-DE와 MS분석을 통하여, 정상 대조군과 비교한 베체트 장염 환자의 조직에서 특이적으로 발현되어 있는 7개의 후보단백질을 동정하였으며, 이중 4개의 단백질 (heat shock protein 27, transgelin, manganese superoxide dismutase, and calprotectin)은 베체트 장염에서 증가된 소견을 보이고, 3개의 단백질 (heat shock protein 60, selenium binding protein, and galectin-3)은 감소된 소견을 보였다. 이중 galectin-3는 베체트 장염 환자의 염증 조직에서 대조군에 비하여 의미 있게 감소되어 있었으며, 이는 프로테오믹스 연구 결과와 일치하는 소견이었다. shGal-3 cell line에서 대조군에 비하여 LPS 자극에 의하여 TLR4와 IL8은 증가하였으며, TFGβ와 IL10은 유의하게 감소되었다. 또한 shGal-3 cell line에서 TNFα 자극을 주었을 때, TLR5, IL1β, and
NLRC4의 발현은 의미있게 증가하였으나, NLRP3의 발현은 차이를 보이지 않았다. 마찬가지로 LPS와 ATP에 의하여 NLRP3 inflammasome의 활성을 자극하였을 때에는 caspase-1의 활성이나 IL1β의 분비가 증가되지 않았으나, S.typhimurium 감염을 통하여 NLRC4 inflammasome의 활성을 자극하였을 때에는 대조군에 비하여 galectin-3/− 마우스의 BMDM에서 caspase-1 활성이 증가되었으며, IL1β의 분비가 의미있게 증가하였다. 마지막으로 shGal-3 cell line에서 LPS 자극에 의한 세포사멸이 유의하게 증가된 소견을 보였으며, 이는 XBP1s의 감소와 GRP78의 증가와 연관성을 보였다.

결론: 본 연구에서 프로테오믹스 방법을 이용하여 베체트 장염 환자에서 특이적으로 발현되어 있는 7개의 단백질을 발굴하였으며, 이중 galectin-3는 베체트 장염 환자의 염증 조직에서 현저히 감소되어 있었다. Galectin-3의 감소는 염증성 사이토카인의 분비, inflammasome의 활성화, 장관 상피세포 사멸을 조절함으로써 장관의 염증 반응을 억제함을 알 수 있었으며, 이상의 결과를 종합하여 볼 때 galectin-3는 베체트 장염의 발병에 있어서 보호역할을 할 수 있을 것으로 생각된다.