Membrane-bound mucins and mucin terminal glycans expression in idiopathic or \textit{Helicobacter pylori}, NSAID associated peptic ulcers

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Abstract

\textbf{AIM:} To determine the expression of membrane-bound mucins and glycan side chain sialic acids in \textit{Helicobacter pylori} (\textit{H. pylori})-associated, non-steroidal inflammatory drug (NSAID)-associated and idiopathic-gastric ulcers.

\textbf{METHODS:} We studied a cohort of randomly selected patients with \textit{H. pylori} (group 1, \textit{n} = 30), NSAID (group 2, \textit{n} = 18), combined \textit{H. pylori} and NSAID associated gastric ulcers (group 3, \textit{n} = 24), and patients with idiopathic gastric ulcers (group 4, \textit{n} = 20). Immunohistochemistry for MUC1, MUC4, MUC17, and staining for \textit{Erythrina cristagalli} agglutinin and \textit{Sambucus nigra} agglutinin (SNA) lectins was performed on sections from the ulcer margins.

\textbf{RESULTS:} Staining intensity of MUC17 was higher in \textit{H. pylori} ulcers (group 1) than in idiopathic ulcers (group 4), 11.05 ± 3.67 vs 6.93 ± 4.00 for foveola cells, and 10.29 ± 4.67 vs 8.00 ± 3.48 for gland cells, respectively \((P \leq 0.0001)\). In contrast, MUC1 expression was higher in group 4 compared group 1, 9.89 ± 4.17 vs 2.93 ± 5.13 in foveola cells and 7.63 ± 4.60 vs 2.57± 4.50 for glands, respectively \((P < 0.0001)\). SNA lectin staining was increased in group 4, in parallel to elevated MUC1 expression, indicating more abundant α2-6 sialylation in that group.

\textbf{CONCLUSION:} Cytoplasmic MUC17 staining was sig-
Peptic ulcers are diverse in origin, and the pathogenesis of peptic ulcer disease (PUD) is related to infection with Helicobacter pylori or to the use of aspirin or non-steroidal inflammatory drug (NSAID)-type medications. A growing number of patients have ulcers not associated with these or other causes, termed idiopathic ulcers. Idiopathic ulcers have been shown to have unique clinical characteristics and are important for understanding of ulcer pathogenesis and subsequently treatment. Major findings of this paper include the observation that MUC17 staining was significantly decreased in idiopathic ulcer cases, whereas MUC1 mucin expression was increased. Idiopathic ulcers also demonstrated higher sialic acid mucin residue staining. These alterations in mucin core proteins and sialylation patterns may be associated with differences in epithelial cytoprotection, and therefore may play a role in ulcer pathogenesis.

INTRODUCTION

The pathogenesis of the majority of peptic ulcers is related to infection with Helicobacter pylori (H. pylori) or to the use of aspirin or non-steroidal inflammatory drug (NSAID)-type medications. A growing number of patients have ulcers not associated with these or other causes, termed idiopathic ulcers. Idiopathic ulcers have been shown to have unique clinical characteristics and are associated with higher morbidity and mortality[1-7].

Mucins, heavily glycosylated high-molecular-weight glycoproteins, are expressed at high levels in gastric epithelial cells and help form the gastric mucous unstirred layer that protects the mucosa from acid and pepsin. There are 2 groups of mucins encoded by 21 genes: secreted and membrane-bound[8-10]. Alterations of gastric mucins may play a primary or secondary role in the pathogenesis of peptic ulcer disease[11]. In a previous study of patient cohort with gastric ulcer we did not observe any significant changes in secretory mucin expression (MUC5AC and MUC6) between various pathogenic types of ulcer (idiopathic, H. pylori-associated, or aspirin/NSAID-associated ulcer)[12].

The pattern of membrane-bound mucins and sialic acid expression in peptic ulcer disease has never been examined. The membrane-bound mucins MUC1, MUC4 and MUC17 have been previously described as part of the normal glycocalyx and forms the mucin barrier of the stomach[12,13]. MUC1 is a ubiquitous epithelial cell surface mucin that exhibits altered expression and glycosylation during inflammation and neoplastic development. It has been shown to be important for limiting the inflammation and attachment of H. pylori in gastric mucosa[14]. MUC4 is a trans-membrane mucin whose expression is altered in gastrointestinal neoplasia[15]. MUC17 has been shown to inhibit apoptosis, promote intestinal epithelial migration, and to be cytoprotective in animal models of colitis[16-18]. In addition, peripheral glycans residues on mucin glycoproteins have been shown to play a role in mucosal barrier function and bacterial populations[19].

Since membrane-bound mucins may be a significant factor in gastric epithelial cytoprotection, we choose to study the expression pattern of gastric membrane-bound mucins in various types of peptic ulcer. Comparisons of the relative expression of these cytoprotective proteins in idiopathic vs other ulcer types may help elucidate pathogenic factors unique to this type of ulcer.

MATERIALS AND METHODS

Patients

The study cohort was described in details in our previous paper examining secreted-mucins, MUC5AC and MUC6, in the mucosa of gastric ulcer patients[20]. In the present study we examined specimens from the same patients for the expression of membrane-bound mucins and peripheral sialic acid side-chains. As described previously, after approval of the protocol by the IRB of Rabin Medical Center, consecutive patients with gastric ulcers who underwent endoscopy and gastric biopsy were included and stratified according to etiology: H. pylori-associated (group 1), NSAID-associated (group 2), both H. pylori and NSAID-associated (group 3), or no etiologic factor (idiopathic, group 4). Patients with aspirin exposure were included in the NSAID groups. Cases where no biopsies could be found, with proven malignancy, or with a specific gastrointestinal disease diagnoses other then peptic ulcer, were excluded[21]. As described previously, standard clinical practice was used for H. pylori diagnosis for this study, which used standard clinical criteria based on both histology using hematoxylin and eosin staining and rapid urease testing. If available, breath tests for H. pylori diagnosis were also used[22]. Special stains such as Giemsa and toluidine blue could be used at the discretion of the pathologist. One positive test was required for the diagnosis of H. pylori infection, and two negative tests (e.g., histology and rapid urease testing) was required for a patient to be categorized as negative.
**Tissue samples and mucin immunohistochemistry**

We used the same methods as described previously.[31] In brief: paraffin embedded blocks was cut into 4 µm thick sections, deparaffinized in xylene and rehydrated using a graded ethanol series. Antigen was retrieved by boiling the slides in a microwave oven for 15 min in 0.01 mol/L citrate buffers (pH = 6.0). Exogenous peroxidase was blocked with a 3% H₂O₂-methanol solution, and the slides were incubated in 10% normal goat serum for 30 min to prevent nonspecific staining. The tissue sections were incubated overnight at 4°C with primary antibody. The standard anti-mouse Ig HRP-DAB and biotin-streptavidin-peroxidase methods were then used, and the sections were lightly counterstained with hematoxylin. Histologically normal gastric biopsies were used as positive controls for MUC1, MUC4 and MUC17. The sections incubated with phosphate-buffered saline (0.01 mol/L, pH = 7.4) instead of primary antibody were used as negative controls. Primary antibodies included anti-MUC1 used at 1:100 dilution, (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal anti-MUC4 clone 8G7, used as described previously,[29] and anti-MUC17 polyclonal antibody against a synthetic peptide corresponding to a portion of the MUC17 tandem repeat sequence (PTTAEGTSMPTSTPSE) was used as described previously.[28]

**Sambucus nigra agglutinin and Erythrina cristagalli Agglutinin fluorescent and histochemical staining**

The lectins studied included *Sambucus nigra* agglutinin (SNA), to detect sialic acid in α2-6 glycosidic linkage to underlying glycans, most commonly galactose,[23]; and *Erythrina cristagalli* agglutinin, or *Erythrina cristagalli* agglutinin (ECA), to detect N-acetyllactosamine (Galβ1-4GlcNAc)[23], the most common glycan structure underlying sialic acids in α2-6 linkage. N-acetyllactosamine is exposed when the sialic acid is removed. For fluorescent staining the tissues were blocked with 1% BSA/PBS (bovine serum albumin, Sigma-Aldrich) for 10 min, and incubated with fluorescein-conjugated *Sambucus nigra* agglutinin (SNA-FITC, 1:1000 dilution, Vector Labs) in HEPES/NaCl buffer (10 mmol/L HEPES, 150 mmol/L NaCl pH 7.5) for 1 h at room temperature. The nuclei were counterstained with DAPI, and tissues were mounted with aqueous mounting medium (Vector Labs).

For lectin histochemical staining, endogenous peroxidase was first blocked with H₂O₂, and endogenous biotin was blocked with Avidin-Biotin blocking kit (Vector labs) according to manufacturer’s instructions. Tissues were further blocked with 1% BSA/TBST (0.05 mol/L Tris HCl, 150 mmol/L NaCl pH = 8.0, 0.1% Tween 20) for 10 min, and incubated for 30 min with biotinylated-SNA (1:1000 dilution, Vector Labs) or with biotinylated-ECA (1:2500 dilution, Vector labs) in 1% BSA/TBST supplemented with 10 mmol/L CaCl₂ and 10 mmol/L MnCl₂ at room temperature. Tissues were washed and incubated for 30 min with horseradish peroxidase conjugated streptavidin (Streptavidin-HRP, 1:500 dilution, Jackson ImmunoResearch), followed by 5 min incubation with Vector Blue substrate (Vector Labs) in 0.1 mol/L Tris/levimisol. Nuclei were counterstained with nuclear fast red for 30 min, and tissues were mounted with aqueous mounting medium (Vector Labs).

**Staining Interpretation**

All slides were scanned by the NanoZoomer and digitalized (NanoZoomer 2.0 series, Hamamatsu, Japan). Whole slide scans allow complete review, examination and analysis of all parts of the tissue, accurately assigned identical low or high power microscopy fields. Cytoplasm staining was assessed in at least 10 high-power fields by two observers at 2 sites, the foveola and the glands. Range of cytoplasmic staining included 0: 0%; 1: < 10%; 2: 11%-25%; 3: 26%-50%; 4: 51%-75%; 5: > 75%. Intensity of staining included: 0: no staining; 1: weak staining; 2: intermediate staining; 3: Strong staining. The averages of the grades were calculated and the final staining score was defined as the product of scores for the range and intensity of cytoplasmic staining, as described previously.[31] All specimens were scored blinded to clinical data.

**Statistical analysis**

We performed statistical analysis using Statistical Package for the Social Sciences software 19.0 (SPSS, Inc.). Patient groups were compared using the Pearson χ² test, Fisher’s exact test and Duncan test. P values were considered significant when ≤ 0.05.

**RESULTS**

**Patient characteristics**

Patient characteristics and clinical data were detailed previously.[31] In brief: the study group included a total of 92 patients; Group 1: *H. pylori*-associated gastric ulcer (n = 30), Group 2: aspirin and/or NSAID-associated gastric ulcer (n = 18), Group 3: combined *H. pylori and NSAID*-associated gastric ulcer (n = 24), and Group 4: *H. pylori/NSAID* negative or idiopathic gastric ulcer (n = 20). The average age was 66.6 ± 15.5 years (range 18-95 years), with 47.8% women. Group 4 patients (idiopathic ulcer) were predominantly hospitalized inpatients compared with the other groups (80% vs 45.8%, P = 0.007). In addition, mortality was greater in idiopathic ulcer patients compared with other groups (25% vs 9.7%, P = 0.04). No significant differences between the groups were found in origin, primary indication for endoscopy, haemoglobin level, ulcer number, size or location within the stomach.[31]

**Mucin staining scores**

MUC1 protein was strongly expressed on the apical membrane of the glands and mucosal surface foveola epithelial cells. The staining score for the surface epithelium was generally higher than gland cells but did not reach significance (Table 1). MUC1 expression was significantly higher for both superficial foveola epithelium and glands, in patients with *H. pylori/NSAID* negative
(idiopathic) ulcers (Group 4) than in *H. pylori* positive/NSAID negative (Group 1, \( P < 0.0001 \)) (Figure 1, Table 2). MUC1 was not expressed in ulcers with *H. pylori* negative/NSAID positive status (Group 2), and this finding was statistically significant when compared to the other 3 groups. MUC4 expression was not significantly different between the glands and surface epithelium, nor between Group 1 (*H. pylori* positive/NSAID negative ulcers) and patients with Group 4 (idiopathic ulcer) patients (Figure 1A, Tables 1 and 2). MUC17 protein was strongly expressed on the apical membrane of the mucosal epithelial cells. MUC17 was also expressed in small vacuoles within the foveola surface cells (Figure 1B). The staining intensity was similar between the foveola and glands (Figure 1, Table 1). Staining score was higher in Group 1 (*H. pylori* positive/NSAID negative) than in Group 4 (idiopathic ulcer) patients, with mean score of 11.05 ± 3.67 vs 6.93 ± 4.00 for foveola, and 10.29 ± 4.67 vs 8.00 ± 3.48 for gland cells, respectively (\( P < 0.0001 \)). In contrast, the opposite was observed with MUC1, with higher MUC1 expression in idiopathic ulcers (Group 4) vs vs Group 1 (Figure 1, Table 2). Patients with *H. pylori* positive/NSAID negative ulcers had multiple co-morbidities, were more often inpatients at the time of endoscopy, had fewer subacute presentations, and had poorer survival[11]. This concurs with Chan et al[1], who noted that three quarters of patients with acutely bleeding *H. pylori*/NSAID-negative ulcers have significant co-morbidity including major organ failure and malignancy.

**DISCUSSION**

Peptic ulcers arise from a variety of pathogenic pathways and differ in their clinical characteristics[20]. We have observed that patients with *H. pylori*/NSAID-negative ulcers had multiple co-morbidities, were more often inpatients at the time of endoscopy, had fewer subacute presentations, and had poorer survival[11]. This concurs with Chan et al[1], who noted that three quarters of patients with acutely bleeding *H. pylori*/NSAID-negative ulcers have significant co-morbidity including major organ failure and malignancy.

**Gastric membrane-bound mucin expression has not been previously studied in the setting of peptic ulcer disease. In the present study, the distribution of immunohistochemical staining for MUC1, MUC4 and MUC17, and lectin binding to representative glycan residues in the margins of gastric peptic ulcer was studied. We compared the staining intensity between 4 patient groups: *H. pylori* positive, NSAID positive, either positive or both negative. The MUC1 gene has 1201 nucleotides, is located on chromosome 1q21, and has a short intracellular domain, a transmembrane domain, and a large glycosylated extracellular domain[24]. The other membrane-bound mucins such as MUC4 and MUC17 have general structural similarity, but with the addition of extracellular cysteine-rich EGF-like domains[10]. The EGF-like domains of MUC17 have been shown to inhibit intestinal cell apoptosis and stimulate cell migration, contributing to cell restitution[25]. The functions of MUC4 and MUC17 in normal gastric mucosa are unknown.

In the present study, cytoplasmic MUC17 staining associated with *H. pylori* infection was significantly increased, and was higher at the surface (foveola) and glands areas than in the cases with idiopathic ulcer. The opposite was demonstrated for MUC1 that significantly increased in the foveola and glands in the group of idiopathic ulcer patients. This observation of MUC1 up regulation might be important, since the protection

**Table 1 MUC1, MUC4 and MUC17 mucin and lectin expression scores**

| Patient group      | MUC1 | MUC4 | MUC17 | SNA | ECA |
|--------------------|------|------|-------|-----|-----|
|                     | Surface foveola | Gland cell | Surface foveola | Gland cell | Surface foveola | Gland cell | Surface foveola | Gland cell | Surface foveola | Gland cell |
| *H. pylori*/NSAID-  | 2.93 ± 5.13 | 2.57 ± 4.50 | 5.50 ± 3.14 | 11.05 ± 3.67 | 10.29 ± 4.67 | 2.00 ± 2.63 | 2.69 ± 4.01 | 13.27 ± 3.08 | 5.62 ± 4.37 |
| n = 28             | n = 28 | n = 14 | n = 14 | n = 21 | n = 21 | n = 16 | n = 16 | n = 26 | n = 26 |
| *H. pylori*/NSAID+  | 0.00 ± 0  | 0.00 ± 0  | 3.38 ± 3.07 | 9.23 ± 3.64 | 9.57 ± 4.73 | 5.06 ± 5.52 | 3.44 ± 4.70 | 14.07 ± 2.01 | 4.47 ± 4.32 |
| n = 14             | n = 14 | n = 13 | n = 13 | n = 20 | n = 20 | n = 18 | n = 18 | n = 15 | n = 15 |
| *H. pylori*/NSAID+  | 11.00 ± 3.78 | 7.33 ± 4.13 | 3.75 ± 2.93 | 5.93 ± 2.99 | 8.80 ± 3.76 | 4.18 ± 5.57 | 5.94 ± 5.30 | 13.78 ± 2.73 | 4.61 ± 4.64 |
| n = 18             | n = 18 | n = 16 | n = 16 | n = 19 | n = 19 | n = 17 | n = 17 | n = 18 | n = 18 |
| *H. pylori*/NSAID-  | 9.89 ± 4.17 | 7.63 ± 4.60 | 4.59 ± 3.77 | 6.93 ± 4.00 | 8.00 ± 3.48 | 6.17 ± 4.69 | 3.94 ± 5.45 | 13.06 ± 4.06 | 8.00 ± 5.15 |
| n = 19             | n = 19 | n = 17 | n = 17 | n = 22 | n = 22 | n = 18 | n = 18 | n = 18 | n = 18 |

\[ P = 0.04, P < 0.0001, P < 0.0001 \text{ between Group 2 to Group 1, 3, and 4, respectively.} \]

ECA: *Erythrina cristagalli* agglutinin; *H. pylori*: *Helicobacter pylori*; NSAID: Non-steroidal anti-inflammatory drug; SNA: *Sambucus nigra* agglutinin.
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A

Staining scores

| Staining intensity score | gECA | fECA | gSNA | fSNA | gMUC17 | fMUC17 | gMUC4 | fMUC4 | gMUC1 | fMUC1 |
|--------------------------|------|------|------|------|--------|--------|-------|-------|-------|-------|
| Group 1                  |      |      |      |      |        |        |       |       |       |       |
| Group 4                  |      |      |      |      |        |        |       |       |       |       |

B

Group 1

Group 4

C

Group 1: H. pylori+ Aspirin

Sialidase treated
efficiency against acid, pepsin and bacteria provided by different mucins is probably not equal. The decrease of MUC17 expression in the idiopathic ulcer group, even though partially compensated by higher expression of MUC1, may be insufficient for induction of effective protection. We also found a significant decrease in MUC1 expression in H. pylori negative/NSAID positive ulcers. This finding was statistically significant when compared to the other 3 groups, and cannot easily be explained. We speculate that NSAID therapy decreased MUC1 expression through decrease of prostaglandin E synthesis. The presence of H. pylori infection may mask this phenomenon through other causes of mucin synthesis and secretion. Interestingly, foveolar expression of sialic acids in α2-6 glycosidic linkage was significantly higher in these cases, a finding that cannot be attributed towards a specific mucin, but may be a global phenomenon that particularly belongs to idiopathic ulcer disease. The increase in sialic acid residues may affect mucins’ protection against aggressive luminal agents or bacteria[26,27]. The distance between adjacent carbohydrates side-chains may increase due to the negative charge of sialic acid at the end of each chain, leading to increased exposure of the mucin backbone to acid and pepsin.

The interactions of pathogenic factors such as aspirin, NSAIDs and H. pylori with gastric mucins is complex. Aspirin and NSAIDs inhibit mucosal cyclooxygenase, which is responsible for homeostatic mechanisms including gastric mucin secretion and accumulation[28]. H. pylori disrupts the assembly of the mucin molecule via inhibition of galactosyltransferase responsible for synthesis of mucin O-glycans[29,30]. Furthermore, H. pylori reduces gastric mucous viscosity by elevating pH through urease secretion, thereby enhancing its motility within gastric mucous[31]. Kobayashi et al[32] demonstrated how BabA and SabA adhesins on H. pylori bind to Lewis B and sialyl Lewis X (a tetrasaccharide) blood group antigens on H. pylori, Helicobacter pylori.

Figure 1 Membrane bound mucin and lectin expression in different gastric ulcer groups. A: Staining scores in the different gastric ulcer groups (mean ± SD). I = group 1 = H. pylori positive/NSAID negative; II = group 2 = H. pylori negative/NSAID positive; III = group 3 = H. pylori positive/NSAID positive; IV = group 4 = H. pylori positive/NSAID negative (f = foveola, g = gland). Statistical significance was determined by two-tail T test: SNA foveola group I vs IV (P < 0.05), MUC1 foveola and gland group I vs IV (P < 0.0001), MUC17 foveola and gland group I vs IV (P < 0.0001); B: MUC1 stain is stronger in group 4 compared to group 1 in both gland (thick arrows) and foveola cells (thin arrows), in contrast, MUC17 stain is stronger in group 1 compared to group 4 in foveola (thin arrows) and to a lesser extent in gland cells (thick arrows). Scale bars = 1 mm; C: SNA-FITC lectin staining of sialic acid in α2-6 glycosidic linkage in four representative gastric ulcer samples from Group 1 (top panels) and Group 4 (lower panels). SNA-FITC staining (green color) is stronger in group 4 compared to group 1 in foveola cells (thin arrows) and in gland cells (thick arrows). No staining is observed in sialidase treated tissue, confirming SNA binding specificity. Scale bars = 250 μm. NSAID: Non-steroidal anti-inflammatory drug; SNA: Sambucus nigra agglutinin; H. pylori: Helicobacter pylori.
prospective well controlled studies. Idiopathic peptic ulcers are an increasingly encountered entity, with unique clinical and endoscopic features. Future efforts should focus on identifying genetic and epigenetic factors which regulate mucin secretion and mucin glycan modifications in this setting, especially related to MUC1 and MUC17 membrane-bound mucins.

**COMMENTS**

**Background**

Peptic ulcers arise from a variety of pathogenic pathways and differ in their clinical characteristics. Mucin-type proteins are cytoprotective proteins that are highly expressed in the gastrointestinal tract, and are grouped in secreted and membrane-bound groups. The different types of membrane-bound mucins and glycosides that are associated with different types of peptic ulcer have not previously been studied.

**Research fronts**

Membrane-bound mucins include MUC1, MUC3, MUC4, and MUC17 and differ in terms of structure and biologic properties. Differences in mucin expression may reflect different pathophysiological pathways of peptic ulcer subtypes.

**Innovations and breakthroughs**

This is the first description of membrane bound mucins in different types of peptic ulcer. Major findings included that cytoplasmic MUC17 staining was significantly decreased in the cases with idiopathic ulcer, whereas the opposite was observed for both MUC1 and SNA lectin.

**Applications**

The study results suggest that different types of peptic ulcer may be associated with different membrane bound mucin patterns. Further studies are needed to determine if this is a cause or effect of the specific pathology, and whether determination of gastric mucin expression can help predict susceptibility to specific types of ulcer. In addition, further studies are needed to determine if manipulation or augmentation of specific gastric mucins may prevent ulcer formation or improve healing.

**Peer review**

In this search the authors reported significant variations of mucin levels in different ulcers and support rationality the results. The search appears of clinical and speculative interest. Although the study is retrospective on small sample of patients, it is able to lead significant speculations about mucosal damage in course of Helicobacter pylori infection and non-steroidal inflammatory drug intake.

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Table 2 Comparison of mucin and sugar residues staining score between group 1 and group 4

| Mucin/sugar | Group 1 | n | Group 4 | n | P value |
|------------|---------|---|---------|---|---------|
| MUC1 foveola | 2.93 ± 5.13 | 28 | 9.89 ± 4.17 | 19 | < 0.0001 |
| MUC1 glands | 2.57 ± 4.50 | 28 | 7.63 ± 4.60 | 17 | < 0.0001 |
| MUC4 foveola | 2.79 ± 4.22 | 14 | 4.59 ± 3.77 | 17 | 0.221 |
| MUC4 glands | 5.50 ± 3.14 | 14 | 4.00 ± 4.12 | 17 | 0.286 |
| MUC17 foveola | 11.05 ± 3.65 | 21 | 6.93 ± 4.00 | 22 | < 0.0001 |
| MUC17 glands | 10.29 ± 4.67 | 21 | 8.00 ± 3.48 | 22 | < 0.0001 |
| SNA foveola | 2.00 ± 2.63 | 16 | 6.17 ± 4.69 | 18 | 0.004 |
| SNA glands | 2.69 ± 4.01 | 16 | 3.94 ± 5.45 | 18 | 0.457 |
| ECA foveola | 13.27 ± 3.08 | 26 | 13.06 ± 4.06 | 18 | 0.846 |
| ECA glands | 5.62 ± 4.37 | 26 | 8.00 ± 5.15 | 18 | 0.107 |

ECA: Ecteinascidinae agglutinin; SNA: Sambucus nigra agglutinin.
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