Yokoyama Keiji (Orcid ID: 0000-0003-1320-6545)

Takata Kazuhide (Orcid ID: 0000-0002-0255-2904)

Morihara Daisuke (Orcid ID: 0000-0003-3267-6235)

Hirai Fumihito (Orcid ID: 0000-0002-5493-5675)

Irsogladine maleate alters expression of a tight junction protein in portal hypertensive gastropathy

Keiji Yokoyama[1], Makoto Irie[2], Naoaki Tsuchiya[1], Eri Yamauchi[1], Motoko Kawashima[1], Takashi Miyayama[1], Hiromi Fukuda[1], Ryo Yamauchi[1], Kaoru Umeda[1], Kazuhide Takata[1], Takashi Tanaka[1], Shinjiro Inomata[1], Daisuke Morihara[1], Yasuaki Takeyama[1], Satoshi Shakado[1], Shotaro Sakisaka[1] and Fumihito Hirai[1]

[1]Department of Gastroenterology and Medicine, Fukuoka University Faculty of Medicine, Fukuoka, Japan.

[2] Department of Gastroenterology and Medicine, Fukuoka University Nishijin Hospital, Fukuoka, Japan.

Correspondence: Keiji Yokoyama, Department of Gastroenterology, Fukuoka University Faculty of Medicine

7-45-1 Nanakuma, Jonan-ku, Fukuoka-si, Fukuoka 814-0180, Japan

Phone: +81-92-801-1011 (Ext.3355)

Fax: +81-92-874-2663,

E-mail: yokotin@fukuoka-u.ac.jp

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Irsogladine maleate changes a tight junction in PHG

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Abstract

Background and Aim: Portal hypertensive gastropathy (PHG) is characterized by noninflammatory edema and vasodilatation of the lamina propria of the mucosal epithelium. In addition, the alterations of intercellular junction proteins and dilatation of the endothelial gaps have been reported. In this study, we examined whether irsogladine maleate (IM), a gastric mucosal protective agent, has the potential to improve PHG by restoration of tight junctions (TJs).

Methods: Twenty-four patients with PHG were registered and randomly assigned into 2 groups: 12 patients in the IM-administration group and 12 patients in the non-administration group. In the administration group, IM (4 mg/day) was administered orally for 12 weeks. Gastric mucosa with a red color in patients with PHG were obtained endoscopically on the registration day and 12 weeks later. The endoscopic findings were evaluated, an immunohistochemical analysis of claudin-3 (a TJ protein) expression in gastric mucosal tissues by a laser microscope was performed, and claudin-3 expression was quantified by western blot analysis.

Results: IM improved the degree of PHG in 2/12 patients endoscopically, in contrast to none of the 12 patients in the non-administration group. Immunohistochemical analysis showed that expression of claudin-3 increased in 8/12 patients in the IM-administration group, and 2/12 patients in the non-administration group ($p=0.036$). Western blot analysis revealed that the increase in claudin-3 after 12 weeks was significantly higher in the IM-administration group than in the non-administration group ($p=0.010$).

Conclusions: The present pilot study suggested that IM might improve the gastric mucosa in PHG through restoration of TJ-protein claudin-3.

Keywords: portal hypertension; tight junctions; gastrointestinal diseases; gastric mucosa; claudin-3

Introduction

Portal hypertensive gastropathy (PHG) due to portal hypertension (PH) is characterized by noninflammatory edema and vasodilatation of the lamina propria of the mucosal epithelium [1,2]. PHG is considered to be positive in 50–90% of portal hypertension cases, and sometimes acute or chronic refractory bleeding may occur [3,4]. The McCormack classification (MC) is widely used, classifying findings as either mild or severe [1]. Furthermore, alterations of intercellular junction proteins and the dilatation of the endothelial gaps have been reported [5,6]. Tight junction (TJs), which function in cell-cell adhesion, play a role in controlling the barrier function of epithelial cells; an important component of which are the claudin proteins.TJ injuries are known to cause various systemic diseases [7–9].

Irsogladine maleate (IM) was developed for the treatment of peptic ulcer disease and acute gastritis. IM has a protective mucosal effect based on a distinct mechanism, different to antisecretory drugs [10]. The primary function of IM is facilitation of gap junction intracellular communication (GJIC), through an increase in cyclic adenosine 3', 5'-monophosphate content via the inhibition of phosphodiesterase [11,12]. Increased GJIC by IM suppresses increased mucosal permeability by maintaining and fortifying the TJs, and through restoration of TJ proteins [11]. IM is anticipated to be effective not only in the stomach but also in other parts of the digestive tract [13–16].

The aim of the present pilot study was to examine whether IM has the potential to improve PHG by restoration of TJs.

Methods

Study population

The study enrolled 28 patients with PHG, confirmed by endoscopic observations, from July 2012 to December 2015 at our hospital. The inclusion criteria for the enrolled patients was that they were 20 years of age or older, and of whom written informed consent had been obtained. We confirmed that the enrolled patients did not meet the following exclusion criteria: patients with gastrointestinal bleeding, severe organ failure with complications such as heart disease (EF<40%) and lung disease, drug allergy, and/or portal vein thrombosis; patients taking anticoagulants, nonsteroidal anti-inflammatory drugs, and/or beta blockers; patients equivalent to Child-Pugh grade C (for the invasive inspection that biopsies the PHG

site of the gastric mucosa); patients who had been treated for liver cancer, who had recurrence within 12 weeks after treatment for gastroesophageal varices, who were pregnant or breastfeeding, or who wished to become pregnant during the study; and other patients who were judged inappropriate by the doctor responsible for the study. Further, the concomitant use with the following drugs were prohibited for the period from 1 week before IM administration to the end of administration: gastric ulcer treatments other than test drugs (e.g., defense factor enhancer, prostaglandin preparation, or muscarinic receptor antagonist). 15 patients had been administered Proton pump inhibitor (PPI) and 7 patients had been administered Histamine H2-receptor antagonist (H2RA) for more than 1 month and could not be excluded in this study, therefore we allowed them to enter this study, and did not change the dosage during the study period. This study has been conducted as an exploratory study because there were no previous studies on the effects of PHG on gastric lesions. Glutamine, which has an antioxidant effect as in the case of irsogladine, has an activity reduction of about 50% in the treatment group compared to the control group in the PHG model rat. Therefore, the effective rate was assuming 60% in the treatment group and 5% in the control group, and the significance level was 5% and the power was 80%, and the target number of cases was 24 patients (11 patients in each group, a total of 22 patients, with an estimated 2 patient dropout). Finally, 4 patients were excluded and 24 patients were registered and randomly assigned into two groups of 12 patients; the groups were the IM administration group and the non-administration group, as per the random number table at the Clinical Research Support Center of Fukuoka University Hospital. The CONSORT (Consolidated Standards of Reporting Trials) flow of this study is shown in Fig. 1.

The background characteristics of the study population are shown in Table 1. Administration group: sex is male/female: 8/4, mean age (± SD) is 65.1 ± 2.5, etiology is alcohol/ hepatitis c virus (HCV) / primary biliary cholangitis (PBC)/ cryptogenic: 4/3/3/2, and the MC is mild/severe: 9/3. Non-administration group: sex is male/female: 8/4, mean age (± SD) is 58.9 ± 2.57, etiology is alcohol/ HCV/ non-alcoholic steatohepatitis (NASH) / idiopathic portal hypertension (IPH): 3/5/3/1, and the MC is mild/severe: 10/2. 23 patients except for one patient with IPH were diagnosed with liver cirrhosis based on liver morphology, clinical and laboratory findings. IPH was diagnosed by percutaneous liver biopsy. The background characteristics of both groups are listed in Table 2. There were no clear significant differences in age, sex, and severity of PHG between the two groups.

Study design

The present study was a prospective, hepatologist-blinded, randomized controlled trial (RCT) conducted in accordance with the Declaration of Helsinki. The name of the registry and the registration number is JapicCTI-194993. And this study protocol was approved by the Ethics Committee of our hospital (Approval No.12-07-07).

In the administration group, IM (4 mg/day) was administered orally for 12 weeks. Gastric mucosa with red color in patients with PHG were obtained endoscopically on the registration day and 12 weeks later. The following tests were performed: a) McCormack classification of endoscopic observations, b) an immunohistochemical expression of claudin-3 protein in gastric mucosal tissues using a laser microscope, and c) quantification of the claudin-3 protein expression in gastric mucosal tissues by western blot analysis.

The definition of "improvement of endoscopic findings" was the improvement of McCormack classification (from severe to mild). The definition of "Imuunohistochemical expression of claudin-3 protein in gastric mucosal tissues by a laser microscope" was a blind evaluation of laser stability before and after administration was conducted by three hepatologists. The significantly increased expression of claudin-3 was observed; two of the three hepatologists judged that there was improvement, and all of them judged that the condition did not become worse.

Immunohistochemistry

Immunohistochemistry for TJ-constituent proteins expressed in the stomach was performed on formalin fixed gastric mucosal tissues obtained from endoscopic biopsies. In brief, fixed gastric mucosal tissues were immunostained with an antibody of TJ-constituent proteins. We performed preliminary experiments on TJ-constituent proteins expressed in the stomach, including claudin-1, -3, -4, -7, -18, occludin, and zonula occludens (ZO)-1. Fig. 2 shows the immunofluorescent staining of the preliminary experiments of claudin-3, -4, -18. Claudin-3 exhibited good laser-stability and high protein expression levels, and was as such selected for an immunohistochemical investigation (Sigma-Aldrich, St. Louis, MO) (1:100). The slides were viewed using a conventional light microscope (Carl Zeiss, Oberkochen, Germany) at × 200 magnifications.

Western blotting

Gastric mucosal tissues on 4.67-cm filters were washed with ice-cold phosphate-buffered saline and lysed in ice-cold NP-40 buffer, with 25 mM bicine buffer (pH 7.6) containing 1% NP-40, protease inhibitor (leupeptin, antipain, chymostatin, phosphoramidon, pepstatin A, elastatinal, and aprotinin, 10 μg/ml each), and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher, Waltham, MA). Further, the lysate was centrifuged at 1,000 g for 30 min, and then the pellet was resuspended using a homogenizer. Lysate proteins (2 μg per lane) were subjected to 14% polyacrylamide gel electrophoresis (100 V, 120 min) and then transferred (15 mA, 17 h) onto a polyvinylidene difluoride membrane. The specific claudin-3 proteins were detected using the different anti-claudin-3 antisera, polyclonal rabbit anti-claudin-3 (Sigma-Aldrich, St. Louis, MO) (1:2500) and Goat anti-rabbit HRP conjugated secondly antibody (Zymed, South San Francisco, CA) (1:1000). The immunoreactive proteins were visualized using an ECL-Prime Western Detection Reagents (GE Healthcare, Piscataway, NJ) by the ImageQuant analyzer LAS-3000 (FUJIFILM, Tokyo, Japan).

Statistical analyses

The results were analyzed using JMP version 13.0 (SAS Institute, Cary, NC, USA). Statistical analysis of the changes in each parameter and comparisons between groups included the Welch's t-test and Fisher’s exact test. P values less than 0.05 were considered significant.

Results

McCormack classification of endoscopic findings

IM improved the degree of PHG in 2/12 patients (case 3 and 12) in the administration group, as observed endoscopically, while no improvement was observed in the non-administration group (Fig. 3A). The endoscopic picture and immunohistochemical image of case 3 were shown (Fig. 4A). The endoscopic picture showed improvement of PHG in IM administration group.

Immunohistochemical expression of claudin-3 in gastric mucosal tissues

We performed immunohistochemical investigation on TJ-constituent proteins expressed in the stomach, and claudin-3 was selected as described above. The results of immunohistochemistry analysis demonstrated that expression of claudin-3 increased in 8/12 patients in the administration group, and in 2/12 patients in the non-administration group (p=0.036) (Fig. 3A). The endoscopic picture and immunohistochemical image of case 11 were shown (Fig. 4B). The immunohistochemical image showed increased claudin-3 expression before and after IM administration.

Quantification of claudin-3 expression in gastric mucosal tissues

Quantification of claudin-3 expression levels was performed by western blot analysis (Fig.5). We compared the increase/decrease from the baseline claudin3/β-actin value at registration to the claudin3/β-actin value after 12 weeks (⊿claudin3/β-actin; 12 weeks later - at registration). The administration group vs the non-administration group (±SD) were 0.17±0.08 and -0.12±0.07, respectively. After statistical analysis, Western blot analysis revealed that the increase in claudin 3 expression after 12 weeks was significantly higher in the treated group than in the non-treated group (p = 0.010) (Figure 3B).

Discussion

TJ proteins include claudin, occludin, tricelluline, junctional adhesion molecules, zonula occludens (ZO)-1–3, and PAR3, PAR6, and PALS1 as scaffold proteins [17-19]. The elucidation of the claudin family is advancing, with claudin-1–27 currently confirmed [20]. They show tissue specificity in various organs. Substance transportation via TJs is a passive process; TJs act as a hard barrier to large molecules such as proteins, yet comprise holes through which small molecules such as inorganic ions or water can move through.

The mechanism by which the permeability of TJs is regulated has yet to be fully elucidated [21-24]. When TJs are damaged, their barrier function disappears due to the increase in membrane permeability. As a result, foreign substances irrupt into the inside of the mucosa from the outside, or substances leak from the inside to the outside, which may cause diseases. Damaged TJs cause various disorders throughout the body including multiple sclerosis and ichthyosis, as well as inflammatory bowel disease and cholangitis [7-9,25-27].

For example, an abnormal skin barrier occurs in claudin-1-knockout mice. In addition, it has been reported that the TJs of the skin and/or liver of humans are affected by claudin-1 genetic mutations, as well as the inner ear by claudin-14 genetic mutations, resulting in difficulty in hearing [9]. Claudin proteins are expressed in the stomach and include claudin-1, -3, -4, -7, and -18. Among them, claudin-3 was selected for this study as it exhibited good stability under laser excitation and a high level of protein expression in the preliminary experiment. In order for claudin-3 to be expressed, the gastric mucosa must be atrophic mucosa or mucosa with intestinal metaplasia [28], and all cases in this study were associated with atrophic mucosa with intestinal metaplasia, at least in the antrum of the stomach. It might be related to the good dyeability of claudin-3.

Furthermore, observing the claudin-3 staining pattern in Fig. 2A and Fig. 4 in this study, it was more strongly stained at apical borders of lateral membranes. The staining pattern of claudin-3 was reported that claudin-3 was strongly stained at apical lateral membranes and was also stained at lateral membranes [29]. We considered that was consistent with the results of this study.

The role of mucin protein (MUC) having a sugar chain structure has been clarified as cell protection and cell-cell interaction due to the sugar chain structure. Currently, nine mucin genes (MUC1-4, MUC5B, MUC5AC, MUC6-8) were cloned [30], and their biochemical structures were clarified. As a result, it has been found that the abnormal mucosa and the normal gastrointestinal mucosa differ quantitatively or qualitatively. MUC1, MUC5AC and MUC6 are mainly expressed in the gastric mucosa, but MUC2 and MUC3 are expressed in the intestinal metaplasia mucosa [31]. There are still many unclear points regarding their production, secretion. Furthermore, Immunostaining of CD10, a brush border marker, is used as an index of intestinal differentiation of cells [32]. We did not evaluate the types of mucin (especially MUC5AC, MUC6, MUC2, and CD10) in this pilot study. However, it was considered to be an important future research topic.

There are various new reports on the relationship between gastric mucosa and TJs. Recently, claudin-5, -11, -23 have reported as members of the claudin family regarding gastric mucosa. It has been reported that claudin-5 in gastric mucosa was restored by nitrate under dysbiosis [33]. Furthermore, it has been reported that expression of claudin-11, -23 disrupt the structure and function of TJs, leading to damage of the barrier function of

epithelial cells [34]. However, because there is also an opinion that claudin-5 is not expressed in the epithelial cells [29], further verification of these new reports will be necessary.

In the present pilot study, we mainly evaluated claudin-3, however, future investigation of these claudin families may elucidate the effect of IM on TJs. We consider they will be one of the future research topics.

The intercellular communication activation effect of IM on gap junctions (GJs) pertains to the intercellular communication of ions, nutrition, metabolites, signals, etc. [35,36]. Currently, 12 different GJ connexin proteins have been established, namely connexin-1–12 [37-42], and similarly to the claudin proteins, these are thought to exhibit tissue specificity. A structural change involving connexin creates an opening in GJs, and molecules of 1 kDa or smaller move among cells, a process termed gap junction intercellular communication (GJIC). Morita et al. reported that activated GJs strengthened TJs, based on the fact that claudin-4 was up-regulated when connexin-26 was overexpressed in Caco-2 cells, a model of the epithelium of the small intestinal mucosa [11].

The effects of IM on intercellular communication were examined in the gastric mucosal epithelium of a rabbit, using a fluorescent dye called Lucifer Yellow CH, which propagates into cells via GJs. Lucifer Yellow CH was administered one minute after administering IM, and the cell population into which the dye propagated was studied using a fluorescence microscope three minutes later. A dose-dependent increase in the number of cells stained with Lucifer Yellow CH was observed, indicating that IM activated GJIC [12]. It has been reported that IM activates GJIC and up-regulates claudin-4 without changing the expression of connexin-26, and strengthens the barrier function of TJs [11,43-46]. Therefore, it is suggested that IM administration may potentially contribute to improvement of the disease state of PHG by remedying TJ collapse and enhancing the mucous membrane repair ability.

Out of 12 samples in the IM-administered group, improvement of PHG was recognized in two cases endoscopically, yet there was no overall statistically significant difference. In this study, microscopic improvement of PHG was indicated, but not macroscopic improvement. Since various factors such as vascular change and gastric mucosa change are related to the disease state of PHG, a single administration of IM was thought to be insufficient to provide macroscopic improvements.

For 4/12 cases in the IM-administered group, IM administration was continued in consenting patients subsequent to the study period. The average observation period was 458

days for this continued administration, and PHG did not worsen in any case. On the other hand, 6 out of 8 patients who discontinued IM administration were evaluated for PHG one year later, and the exacerbation of PHG was recognized in 1/6. However, there is no statistically significant difference and further long-term evaluation is required. To demonstrate further effects, some contrivance seems to be required, such as concomitant use with agents that act through different mechanisms, for example β-blockers or octreotide, which lead to PHG improvement by a lowering of portal blood pressure [47-50].

To conclude, the present pilot study suggested that IM administration might improve the gastric mucosa in PHG through restoration of TJs. This study had a number of limitations. First, it was a single center study and an exploratory study which the number of samples was small; second, in this exploratory study, other claudin families that did not stain well in the preliminary experiments, and the aforementioned these MUCs, CD10 and claudin-5,-11,-23 were not analyzed; third, Helicobacter pylori, which could affect TJ expression, was not analyzed. However, the promising results obtained still provide a valuable treatment candidate for PHG.

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Table 1. The background characteristics of the study population.

No.	IM	Sex	Age	Etiology	CPS	Co-administration of PPI/H2RA	MC
1	Ad	M	79	LC-Alcohol	A	PPI	mild
2	Ad	M	50	LC-Alcohol	B	PPI	mild
3	Ad	M	54	LC-Alcohol	A	H2RA	severe
4	Ad	M	71	LC-Alcohol	B	PPI	mild
5	Ad	M	62	LC-HCV	B	PPI	mild
6	Ad	M	72	LC-HCV	A	None	mild
7	Ad	M	62	LC-HCV	B	H2RA	severe
8	Ad	F	55	LC-PBC	B	PPI	mild
9	Ad	F	66	LC-PBC	B	PPI	mild
10	Ad	F	70	LC-NASH	A	H2RA	mild
11	Ad	M	72	LC-cryptogenic	B	PPI	mild
12	Ad	F	68	LC-cryptogenic	B	PPI	severe
13	N-Ad	M	41	LC-Alcohol	B	PPI	severe
14	N-Ad	F	49	LC-Alcohol	A	PPI	mild
15	N-Ad	M	66	LC-Alcohol	B	H2RA	mild
16	N-Ad	F	58	LC-HCV	A	PPI	mild
17	N-Ad	M	66	LC-HCV	B	H2RA	mild
18	N-Ad	M	64	LC-HCV	B	PPI	mild
19	N-Ad	M	64	LC-HCV	B	H2RA	mild
20	N-Ad	M	51	LC-HCV	A	PPI	mild
21	N-Ad	F	58	LC-NASH	B	None	severe
22	N-Ad	M	63	LC-NASH	B	PPI	mild
23	N-Ad	F	73	LC-NASH	A	PPI	mild
24	N-Ad	M	54	IPH	-	H2RA	mild

IM, irsogladine maleate; Ad, administration; N-Ad, non-administration; M, male; F, female; LC, liver cirrhosis; HCV, hepatitis c virus; PBC, primary biliary cholangitis; NASH, non-alcoholic steatohepatitis; IPH, idiopathic portal hypertension; CPS, Child-Pugh class; PPI, Proton pump inhibitor; H2RA, Histamine H2-receptor antagonist; MC, McCormack classification.

Table 2. The background characteristics between the administration and non-administration groups.

	Ad group	N-Ad group	P-value
Sex: male/female	8/4	8/4	NS
Age (±SD)	65.1±2.5	58.9±2.57	NS
MC: mild/severe	9/3	10/2	NS

SD, standard deviation; MC, McCormack classification; Ad, administration; N-Ad, non-administration; NS, not significant.

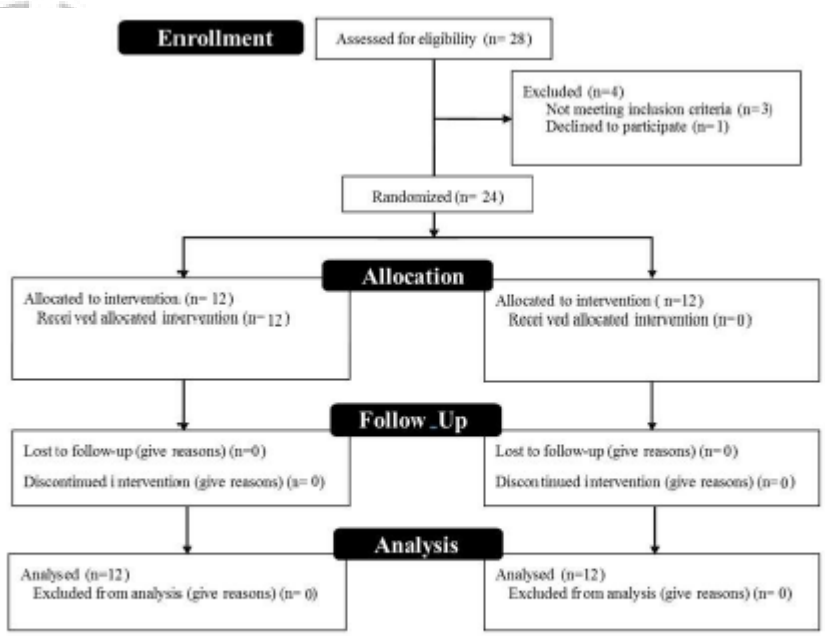

Fig. 1. The CONSORT flow diagram of this trial.

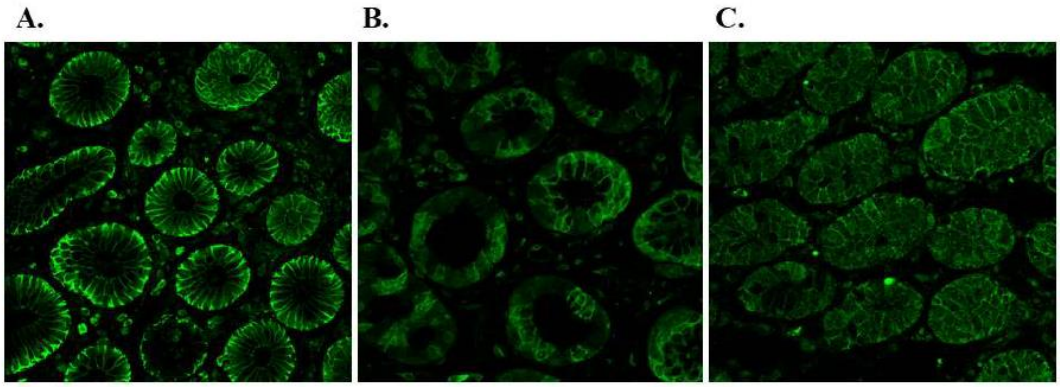

Fig. 2A. The immunostaining of the claudin-3 preliminary experiments.

Fig. 2B. The immunostaining of the claudin-4 preliminary experiments.

Fig. 2C. The immunostaining of the claudin-18 preliminary experiments.

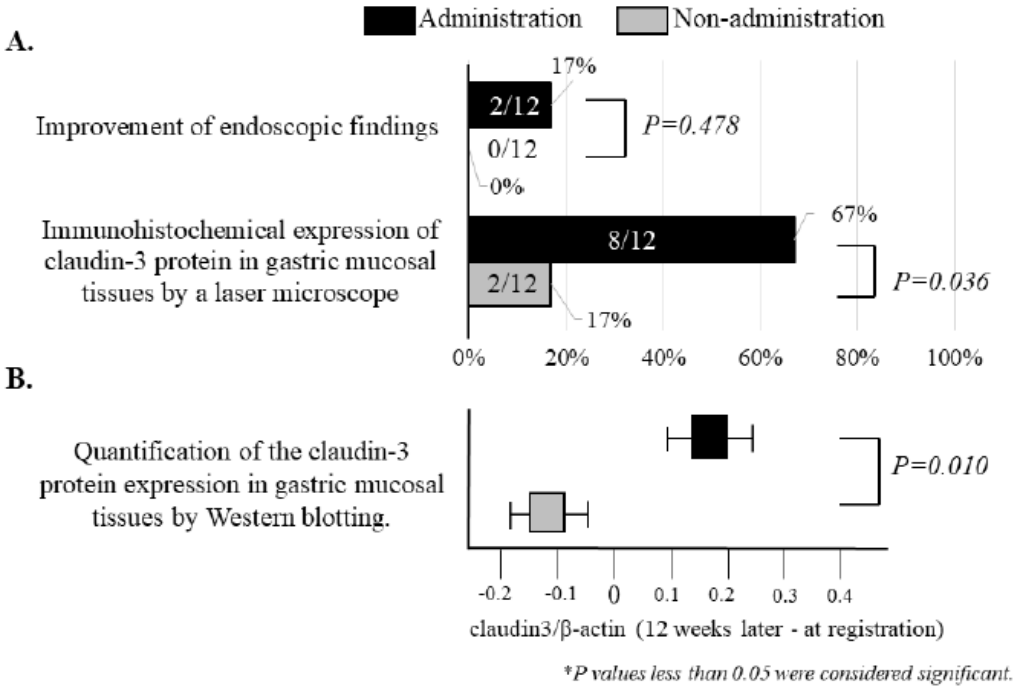

Fig. 3A. Graphic depicting the no significant improvement of endoscopic findings (p=0.478) and the significant increase immunohistochemical expression of claudin-3 protein in gastric mucosal tissues by a laser microscope (p=0.036) in the administration group relative to the non-administration group.

Fig. 3B. Graphic depicting the significant increase in Quantification of the claudin-3 protein expression (Western blotting analysis) observed in the administration group relative to the non-administration group (p=0.010).

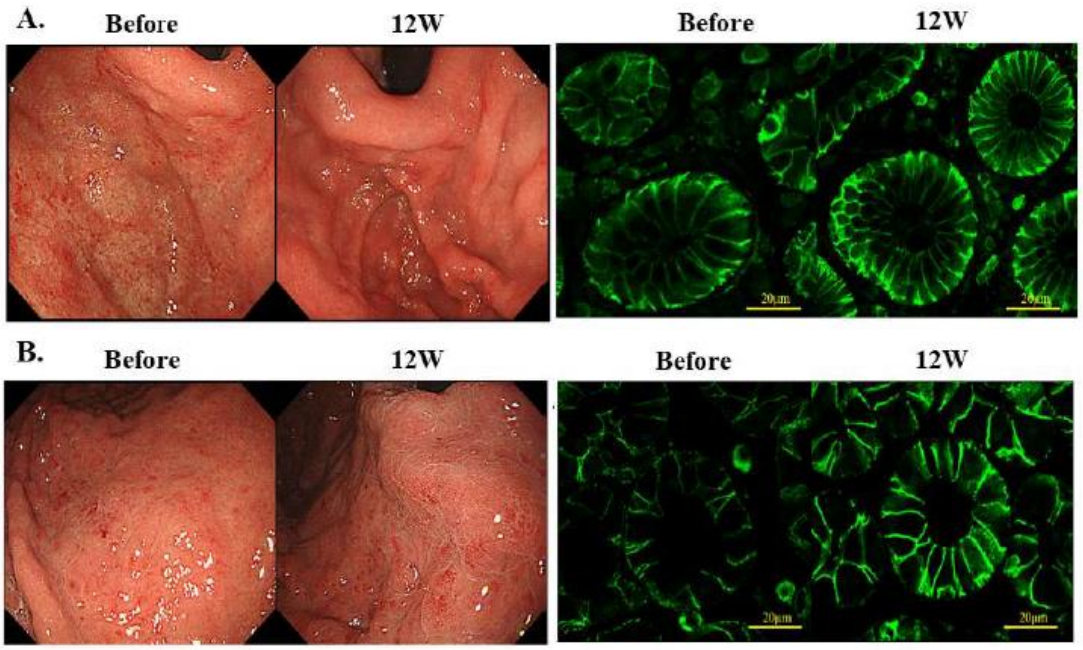

Fig. 4A. The endoscopic picture and immunohistochemical image of case 3. Representative endoscopic picture showing improvement of portal hypertensive gastropathy (PHG) in irsogladine maleate (IM) administration group.

Fig. 4B. The endoscopic picture and immunohistochemical image of case 11. Representative immunohistochemical image showing increased claudin-3 expression before and after IM administration.

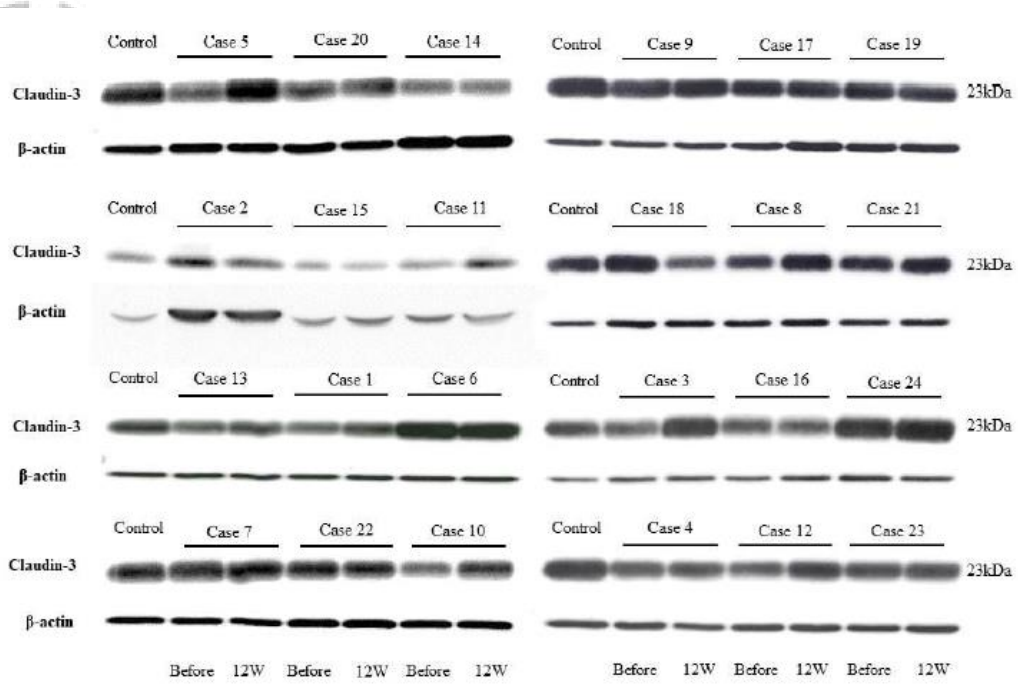

Fig. 5. Western blot analysis demonstrating increased claudin-3 expression before and after irsogladine maleate (IM) administration. Case 1-12 are IM administration groups, and case 13-24 are IM non-administration groups. β-actin expression is slightly strong in case2, but it is not a comparison between cases, but a comparison of claudin3 expression before and after IM administration in this study.