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Densities of rectal peptide YY and somatostatin cells as biomarkers for the diagnosis of irritable bowel syndrome

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ABSTRACT

Irritable bowel syndrome (IBS) is a common chronic disorder. IBS diagnosis is a diagnosis of exclusion since there are no blood tests, radiological or endoscopic examinations for this disorder. Although several attempts have been made to develop a symptoms-based diagnosis, such systems are not widely used in clinics. Several tests and examinations measuring pathological findings in IBS have been considered for the diagnosis of IBS, but none of them has proved useful as a biomarker. Abnormalities in the cell densities of rectal peptide YY (PYY) and somatostatin cells have been reported in IBS patients. The aim of the present study was to determine the utility of these abnormalities as biomarkers for the diagnosis of IBS. Patients with IBS established according to Rome III criteria ($n = 101$) were included in this study (71 females and 30 males with a mean age of 35 years; range 18–61 years), and 62 healthy subjects (38 females and 24 males with a mean age of 41 years; range 18–65 years) were recruited as controls. Both the patients and controls underwent colonoscopy during which rectal biopsy samples were taken. The tissue samples were immunostained for PYY and somatostatin, and the number of stained cells was quantified relative to both the area of epithelial cells and per microscopic field. The density of PYY cells was significantly lower in IBS patients than in the healthy controls ($P < 0.0001$); receiver operator characteristic (ROC) analysis revealed an area under the ROC curve (AUC) of 0.99. The somatostatin cell density in IBS patients was higher than in the controls ($P < 0.0001$); ROC analysis revealed an AUC of 0.86. The densities of the rectal PYY and somatostatin cells appear to be clinically effective biomarkers for IBS. Furthermore, measurement of these parameters is inexpensive, rapid and does not require considerable experience or sophisticated equipment.

Introduction

Irritable bowel syndrome (IBS) is a common chronic disorder that affects between 5% and 20% of the population of the Western world [1,2], and yet there are currently no blood tests, radiological or endoscopic examinations for diagnosing the disorder. Although several attempts have been made to obtain a diagnosis based on symptom assessments similar to those used in psychiatry and rheumatology [3–9], IBS diagnosis in clinical practice remains a process of exclusion [1,10–12]. Thus, a diagnosis of IBS can only be reached by conducting extensive expensive examinations and tests to exclude organic causes for the patient's symptoms.

Several abnormalities in the gastrointestinal endocrine cells have been described in IBS patients [13–27]. These abnormalities are believed to play an important role in the pathophysiology of IBS and represent a potential tool for the treatment of this disorder [28,29]. A previous study [30] found the cell density of rectal peptide YY (PYY) cells to be decreased, and that of somatostatin to be increased relative to healthy subjects. It has been suggested that these abnormalities can be used in the diagnosis of IBS [30]. However, that study included 50 patients and 27 controls, and the subtypes of IBS of the included patients were only those with diarrhea or constipation as the predominant symptom. The present study was undertaken to investigate the efficacy of the densities of rectal PYY and somatostatin cells as biomarkers for the diagnosis of IBS in a larger cohort of IBS patients and with a larger group of healthy controls for comparison than was used in the previous study. Furthermore, IBS patients with any

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of the IBS subtypes—constipation-predominant (IBS-C), diarrhea-predominant (IBS-D) or mixed (IBS-M)—were included.

Material and methods

Patients and controls

One hundred and one patients suffering from IBS according to Rome III criteria [9] were included in this study. Pregnant or lactating women, patients with clinically significant systemic diseases, drug abuse, serious psychiatric diseases, and abdominal surgery, with the exception of appendectomy, cesarean section and hysterectomy were excluded. The cohort comprised 71 females and 30 males with a mean age of 35 years (range 18–61 years); 45 patients had IBS-D, 14 had IBS-M and 42 had IBS-C. All of the patients had a long duration of IBS symptoms *i.e.* greater than 10 years, and their onset of IBS symptoms was not associated with a bout of gastroenteritis. The patients submitted to a physical examination and blood tests to exclude inflammation, liver and pancreas diseases, and thyroid dysfunction. Celiac disease was excluded by histopathological examination of duodenal biopsy samples. Forty-five of these patients used proton-pump inhibitors on demand, 18 were medicated with angiotensin II-receptor antagonist against hypertonia, 15 with statins against high blood cholesterol levels, and 2 with levothyroxine against hypothyroidosis.

The controls comprised 62 subjects (38 females and 24 males; mean age 41 years, range 18–65 years) who underwent colonoscopy with rectal biopsy sampling. The reasons for colonoscopy in these subjects were gastrointestinal bleeding, where the bleeding source was identified as hemorrhoids ($n = 36$), or angiodysplasia ($n = 4$), and otherwise healthy subjects with health worries caused by a relative(s) being diagnosed with colon carcinoma ($n = 22$). Five of these subjects were treated for hypertonia with angiotensin II-receptor antagonist, 4 with statins and 2 with levothyroxine.

The study was performed in accordance with the Declaration of Helsinki and was approved by the Regional Committee for Medical and Health Research Ethics West, Bergen, Norway. All subjects provided verbal and written consent to participate.

Colonoscopy, histopathology and immunohistochemistry

A standard colonoscopy procedure was performed on all of the patients and controls, and four biopsy samples were taken from the dorsal wall of the rectum about 15 cm from the anus in each patient. The biopsy samples were fixed overnight in 4% buffered paraformaldehyde, embedded in paraffin and then sectioned at 5 μm. The sections were stained with hematoxylin–eosin and immunostained with ultraView universal DAB detection kit (v1.02.0018, Venata Medical Systems, Basel, Switzerland) using the BenchMark Ultra IHC/ISH staining module (Venata Medical Systems). The sections were incubated with the primary antibodies for 32 min at 37 °C. The primary antibodies were polyclonal anti-porcine peptide YY (PYY; code no. PYY11A, Alpha Diagnostic, San Antonio, TX, USA) and polyclonal rabbit anti-synthetic human somatostatin (code no. A566, Dako, Glostrup, Denmark). Negative and positive controls were performed as described elsewhere [31,32].

Quantification of immunoreactive cell densities

The densities of rectal PYY and somatostatin cells were quantified by computerized image analysis and counting the number of positive cells in a microscopic field. Measurements using computerized image analysis were performed on a computer linked to a microscope (BX 43, Olympus, Tokyo, Japan) equipped with a digital camera (DP 26, Olympus), using Olympus cellSens imaging (version 1.7) software. The numbers of PYY- and somatostatin-immunoreactive cells were counted in ten randomly chosen microscopic fields, and the area of epithelial cells was measured. The number of endocrine cells in each field was counted manually by pointing and clicking the computer mouse, and the area of the epithelium containing these cells was drawn manually using the computer mouse. A ×40 objective was used, for which each frame (field) on the monitor represented a tissue area of 0.035 mm^2. Immunostained sections from the patients and controls were coded and mixed, and measurements were made by the same person (M.E.) who was blind to the identity of the sections (*i.e.*, IBS or control).

Statistical analysis

Differences in gender between patients and controls were tested using Fisher's exact test. Differences in the age profile were tested using the Mann–Whitney non-parametric test. Differences between controls and all IBS patients (IBS-total), and between IBS-D, IBS-M and IBS-C patients were tested using the Kruskal–Wallis non-parametric test with Dunn's post-test. The data are presented as mean ± SEM values, and differences with $P < 0.05$ were considered to be statistically significant.

Results

Patients and controls

The gender and age distributions did not differ significantly between the patients and the controls ($P = 0.2$ and 0.7, respectively).

Colonoscopy, histopathology and immunohistochemistry

The endoscopic and histopathological appearance of the colon and rectum was normal in both the patients and controls. PYY- and somatostatin-immunoreactive cells were found mostly in the crypts of Lieberkühn in both the patients and the controls, and were basket- or flask-shaped, sometimes with a long basal cytoplasmic process.

Quantification of immunoreactive cell density

PYY cell density

The densities of PYY cells as determined by computerized image analysis and as counted per microscopic field are summarized in Tables 1 and 2 and depicted graphically in Figs. 1 and 2. The Kruskal–Wallis test revealed significant differences between the IBS patients and the controls ($P < 0.0001$). Dunn's post-test revealed that PYY cell density was lower in IBS-total and all three of the IBS subgroups relative to the controls ($P < 0.0001$ for all). The results of receiver operator characteristic (ROC) analysis with the area under the ROC curve (AUC), 95% confidence intervals (95% CIs) and P values for PYY cell density are shown in Figs. 3 and 4. The sensitivity and specificity for cut-off values of <188 cells/mm^2 and <6 cells/microscopic field for a diagnosis of IBS are given in Table 3.

Somatostatin cell density

The somatostatin cell density was significantly higher in IBS patients than in the controls (Tables 1 and 2; Figs. 5 and 6; Kruskal–Wallis test, $P < 0.0001$). Dunn's post-test revealed that the increased somatostatin cell density was statistically significant in IBS-total and all of the IBS-subgroups relative to the controls ($P < 0.0001$ for all). ROC curves (Figs. 7 and 8) for somatostatin cell density, and the sensitivity and specificity for cut-off values

Table 1
Densities of PYY and somatostatin cells in controls and patients (measured by computerized image analysis), expressed as the number of cells relative to the area of epithelial cells. Data are mean ± SEM values.

Endocrine cell type	Controls Cells/mm^2	IBS-total Cells/mm^2	IBS-D Cells/mm^2	IBS-M Cells/mm^2	IBS-C Cells/mm^2
PYY	430 ± 9	144 ± 4***	145 ± 6***	128 ± 6***	148 ± 5***
Somatostatin	156 ± 7	251 ± 8***	265 ± 2***	257 ± 1***	234 ± 7***

*** $P < 0.0001$ *vs.* controls.

Table 2
Densities of PYY and somatostatin cells in controls and IBS patients expressed as the number of cells per microscopic field. Data are mean ± SEM value.

Endocrine cell type	Controls Cells/field	IBS-total Cells/field	IBS-D Cells/field	IBS-M Cells/field	IBS-C Cells/field
PYY	8.4 ± 0.3	4.5 ± 0.1***	4.6 ± 0.2***	4.5 ± 0.3***	4.6 ± 0.2***
Somatostatin	5.0 ± 0.2	9.8 ± 0.8***	8.9 ± 0.2***	8.3 ± 0.3***	8.4 ± 0.2***

*** $P < 0.0001$ *vs.* controls.

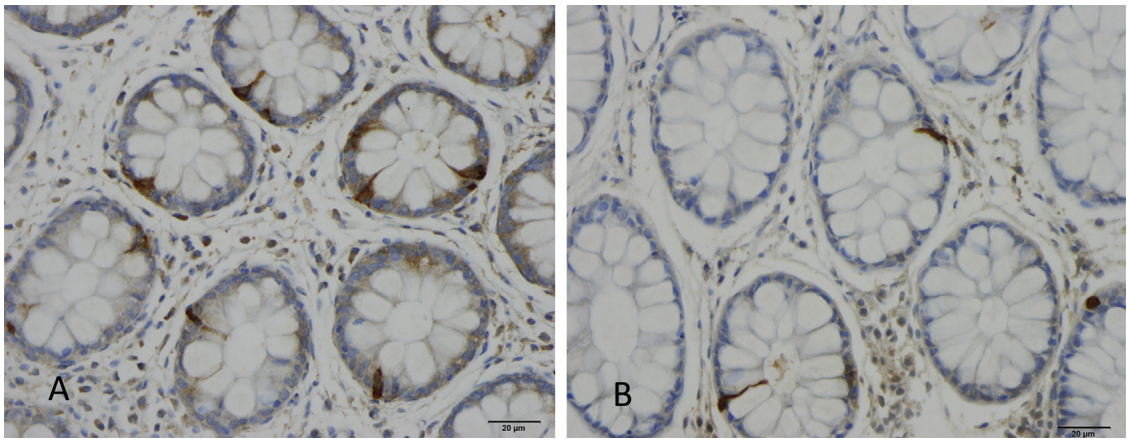

Fig. 1. Rectal PYY-immunoreactive cells in (A) a control subject and (B) a patient with IBS.

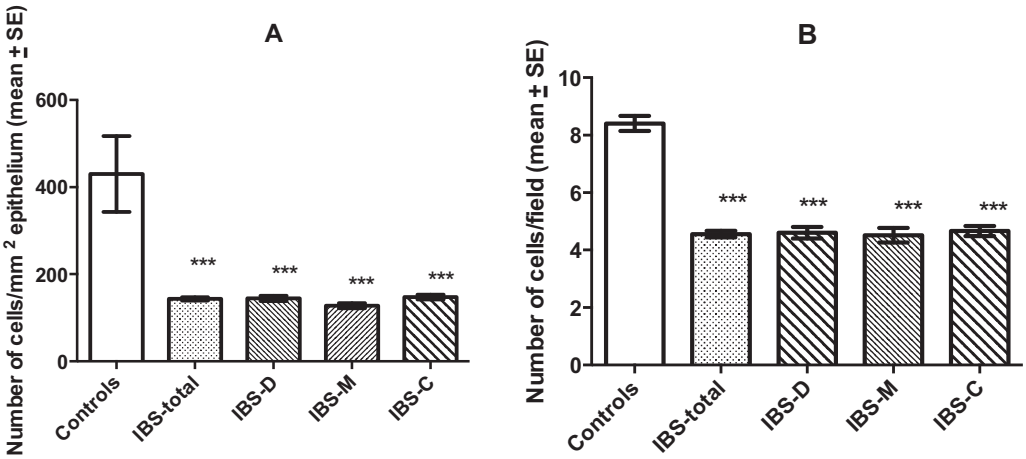

Fig. 2. Density of PYY cells expressed relative to the area of epithelial cells (A) and as the number of cells per microscopic field (B) in 10 randomly chosen fields in tissue samples taken from the controls and IBS-total, IBS-D, IBS-M, and IBS-C patients. *** $P < 0.0001$ *vs.* control group.

Table 3
Sensitivity and specificity of the PYY cell density as a biomarker for IBS at cut-off values of <188 cells/mm^2 epithelium and <6 cells/microscopic field.

	IBS-total		IBS-D		IBS-M		IBS-C	
	Cells/mm^2	Cells/field	Cells/mm^2	Cells/field	Cells/mm^2	Cells/field	Cells/mm^2	Cells/field
Sensitivity (%)	89	90	87	87	100	100	88	88
Specificity (%)	87	95	87	89	87	89	87	89

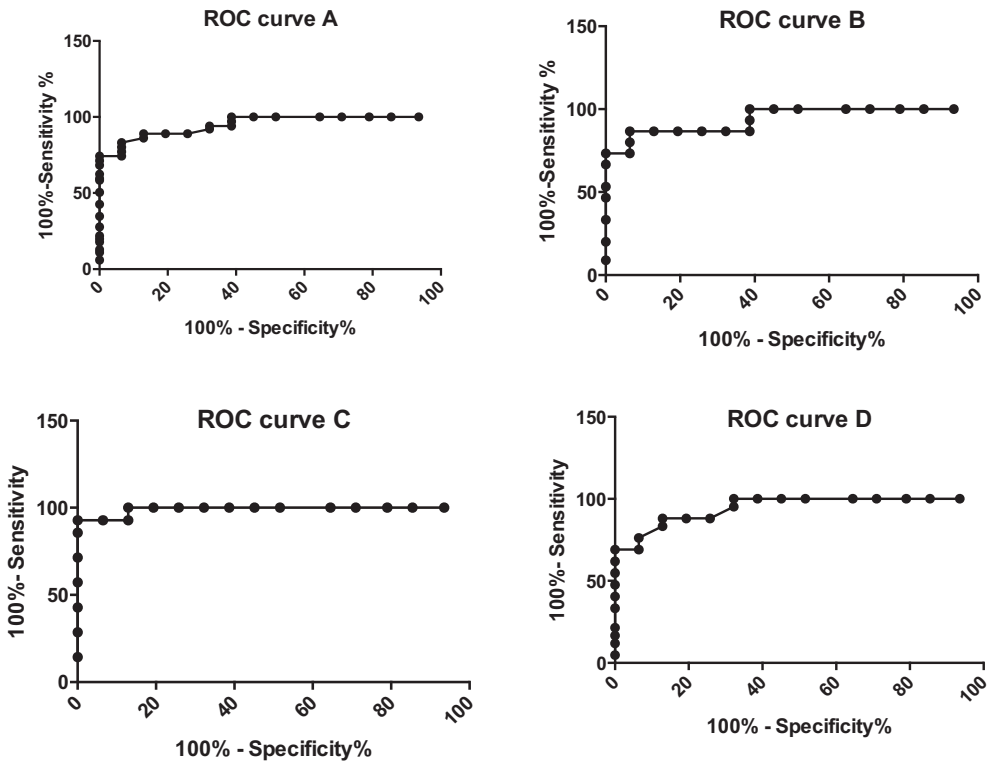

Fig. 3. Results of ROC analysis for PYY cell density as measured relative to the area of epithelial cells in (A) IBS-total (AUC = 0.95, 95% CI = 0.92–0.98, $P < 0.0001$), (B) IBS-D (AUC = 0.94, 95% CI = 0.90–0.98, $P < 0.0001$), (C) IBS-M (AUC = 0.99, 95% CI = 0.97–1.00, $P < 0.0001$) and (D) IBS-C (AUC = 0.95, 95% CI = 0.91–0.98, $P < 0.0001$). AUC = 1 represents a perfect test, and AUC = 0.5 represents a non-discriminating test.

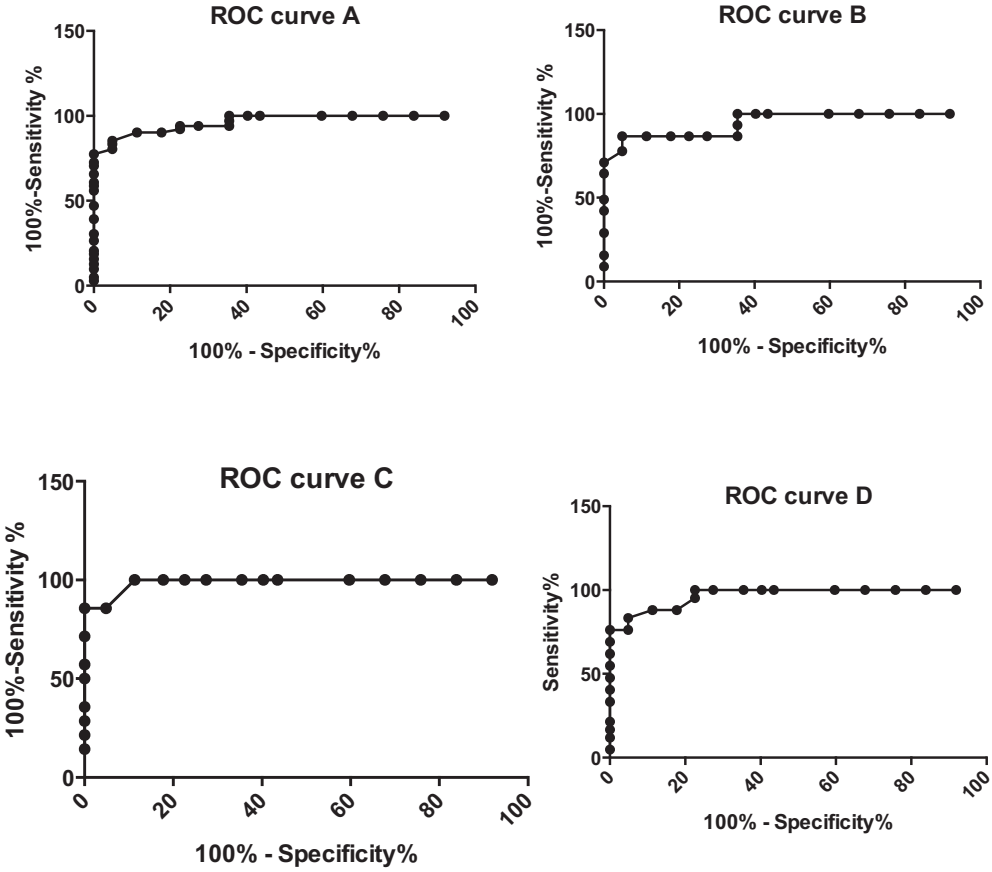

Fig. 4. Results of ROC analysis for PYY cell density measured in 10 randomly chosen microscopic fields in (A) IBS-total (AUC = 0.99, 95% CI = 0.98–1.00, $P < 0.0001$), (B) IBS-D (AUC = 0.99, 95% CI = 0.97–1.00, $P < 0.0001$), (C) IBS–M (AUC = 1.0, 95% CI = 1.00–1.00, $P < 0.0001$) and (D) IBS-C (AUC = 0.99, 95% CI = 0.98–1.00, $P < 0.0001$).

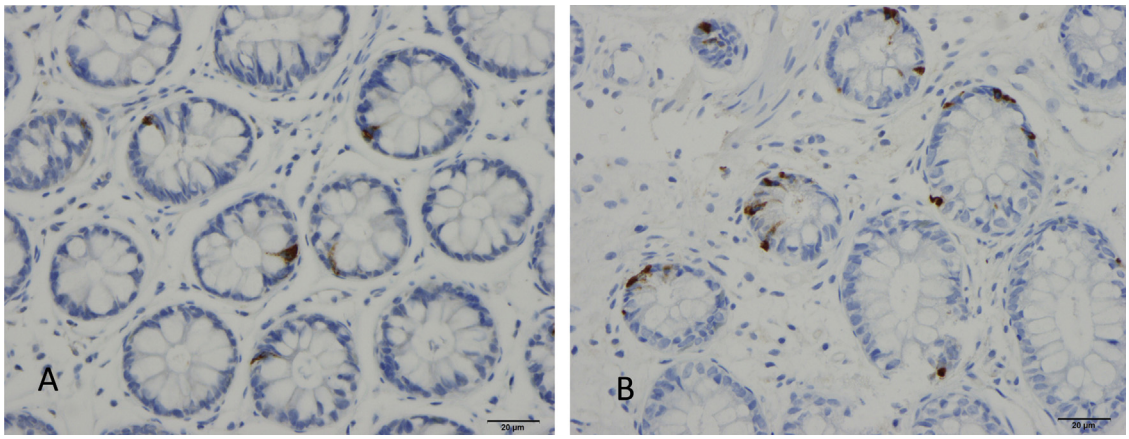

Fig. 5. Somatostatin-immunoreactive cells in rectal tissue from a control subject (A) and a patient with IBS (B).

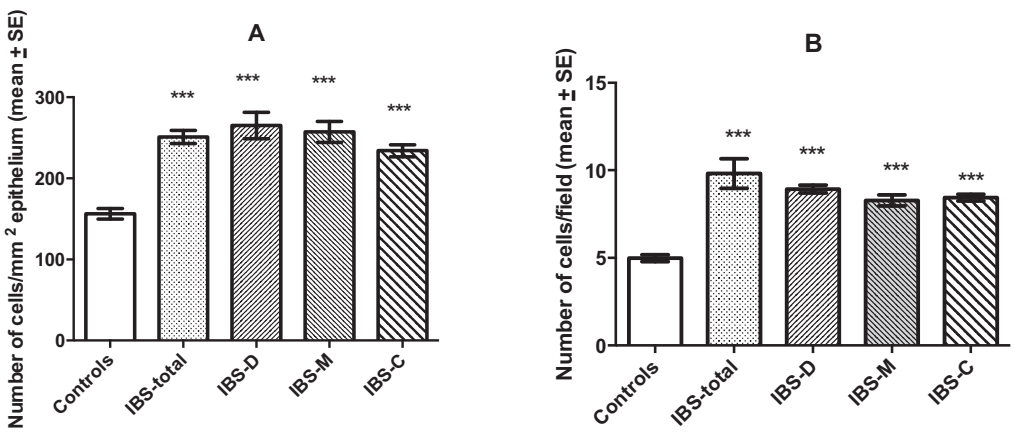

Fig. 6. Somatostatin cell density expressed relative to the area of epithelial cells (A) and (B) as the number of cells per microscopic field in IBS-total, IBS-D, IBS-M and IBS-C patients. $^{***}P < 0.0001$ *vs.* control group.

Table 4
Sensitivity and specificity of the somatostatin cell density as a biomarker for IBS at cut-off values of >182 cells/mm^2 epithelium and >6 cells/microscopic field.

	IBS-total		IBS-D		IBS-M		IBS-C	
	Cells/mm^2	Cells/field	Cells/mm^2	Cells/field	Cells/mm^2	Cells/field	Cells/mm^2	Cells/field
Sensitivity (%)	91	97	87	96	100	93	93	92
Specificity (%)	81	90	81	90	81	90	81	90

of >182 cells/mm^2 and >6 cells/microscopic field for a diagnosis of IBS are given in Table 4.

Discussion

The diagnosis of IBS is performed *via* a process of exclusion, whereby extensive examinations and tests are conducted to exclude other organic diseases [1,10–12]. Attempts have been made to achieve a positive symptom-based diagnosis similar to those used in psychiatry [3–9,33], which culminated in the establishment of the symptoms-based Rome I diagnostic criteria for IBS in 1988 by an international group of gastroenterologists. These criteria were succeeded by refinements in 1999 (Rome II criteria) and 2006 (Rome III criteria). The Rome criteria are not widely used in current clinical practice [9,11,34] because of the potential to miss organic diseases that mimic IBS symptoms but have a different pathophysiology and treatments [10,11,34,35]. Several tests and examinations measuring gut motility, visceral hypersensitivity, autonomic reactivity, mucosal inflammation, fecal proteases, gut flora, serum antibodies, gene expression and food allergies have been considered for the diagnosis of IBS, but none has proven useful as a biomarker for IBS diagnosis [10–13,36,37].

A panel of ten serological biomarkers that are commonly considered in the differential diagnosis for IBS patients, such as those for inflammatory bowel disease and coeliac disease, were examined by Lembo et al. as biomarkers for the diagnosis of IBS [38]. These authors used the smart Diagnostic Algorithm and found an AUC of 0.763 for this panel. Jones et al. added a further 24 serological biomarkers to that panel (thus totalling 34), and examined them together with 4 psychological markers [39]. The combination of these 34 markers has been reported to be good for differentiating IBS from health (AUC = 0.81), and was improved by adding the four psychological markers (AUC = 0.92) [39]. However, the use of serological biomarkers common to other gastrointestinal diseases cannot be considered as an IBS-specific biomarker; rather, it is yet another method of exclusion diagnosis. Furthermore, combining these serological markers with psychological markers, with

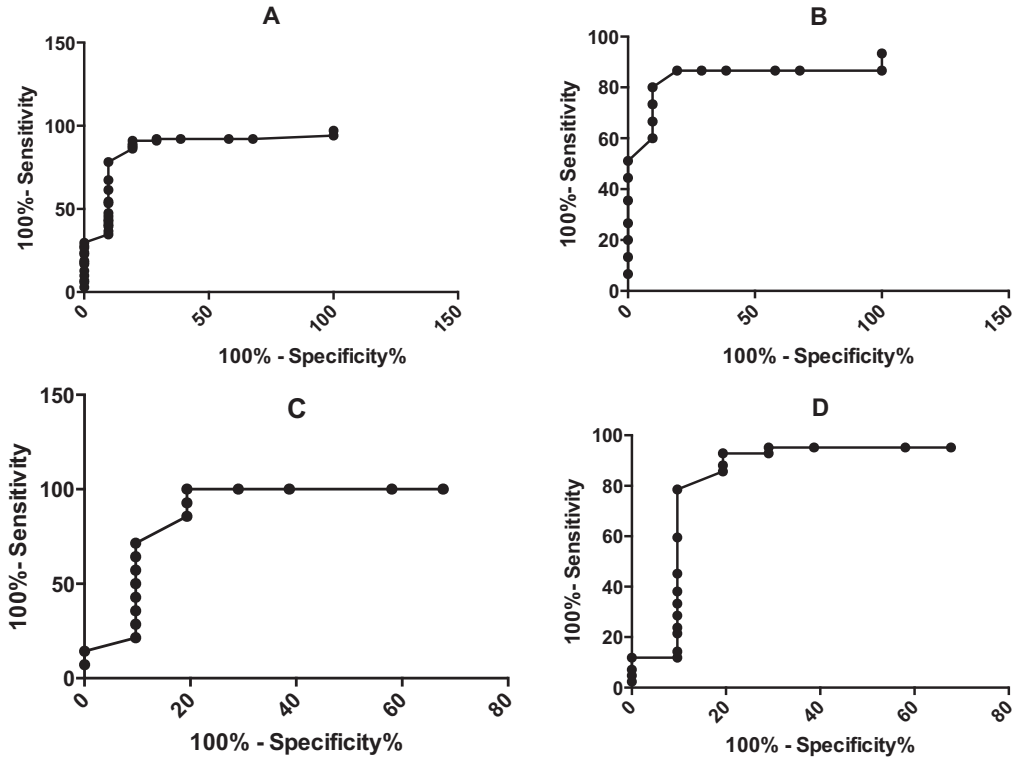

Fig. 7. ROC curves for somatostatin cell density expressed relative to the area of epithelial cells in (A) IBS-total (AUC = 0.86, 95% CI = 0.79–0.92, $P < 0.0001$), (B) IBS-D (AUC = 0.83, 95% CI = 0.73–0.93, $P < 0.0001$), (C) IBS-M (AUC = 0.90, 95% CI = 0.83–0.97, $P < 0.0001$) and (D) IBS-C (AUC = 0.86, 95% CI = 0.78–0.94, $P < 0.0001$).

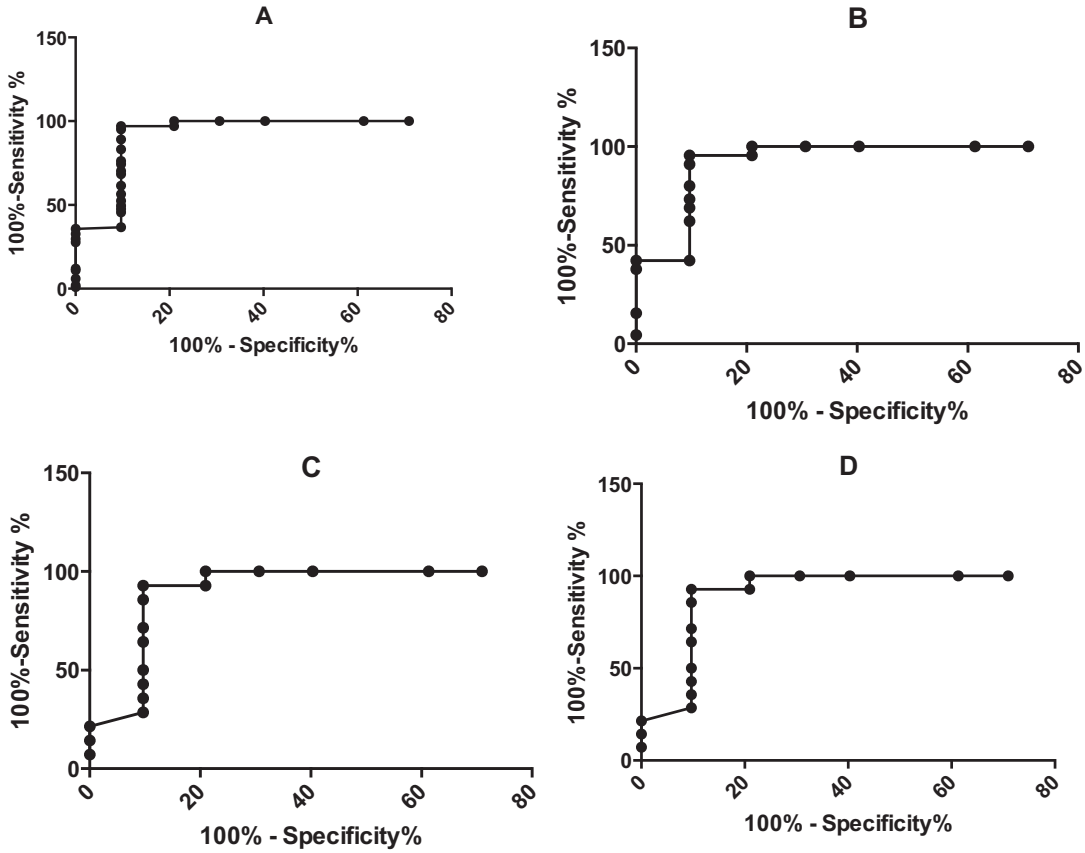

Fig. 8. ROC analysis for somatostatin cell density expressed as the number of cells per microscopic field in (A) IBS-total (AUC = 0.93, 95% CI = 0.89–0.98, $P < 0.0001$), (B) IBS-D (AUC = 0.92, 95% CI = 0.86–0.98, $P < 0.0001$), (C) IBS-M (AUC = 0.94, 95% CI = 0.90–0.98, $P < 0.0001$) and (D) IBS-C (AUC = 0.93, 95% CI = 0.88–0.98, $P < 0.0001$).

the hypothesis that anxiety and depression are more likely to result from the symptoms of the disorder [40], is not logical. Ultimately, this approach is complicated, time consuming, expensive and difficult to apply in everyday clinical practice.

Several abnormalities in the gastrointestinal endocrine cells, including in the stomach and the small and large intestines, have been reported in IBS patients [1,29,41], and these abnormalities appear to play an important role in the pathophysiology of the disease [28,29]. The density of duodenal chromogranin A cells has been proposed as a biomarker for the diagnosis of IBS [42], and is reportedly effective at differentiating IBS from the healthy condition (AUC = 97). The findings of the present study suggest two new biomarkers for the diagnosis of IBS: the cell densities of rectal PYY and somatostatin cells.

PYY cell density is a good biomarker for differentiating IBS from the healthy condition, with AUCs of 0.95 and 0.99 when expressed relative to the area of epithelial cells and per microscopic field, respectively. When analyzed according to the various IBS subtypes, the AUCs were 0.94, 0.99 and 0.95 for the density of PYY cells expressed relative to the areas of epithelial cells in IBS-D, IBS-M and IBS-C, respectively, and 0.99, 1.0 and 0.99, respectively, when expressed per microscopic field. Thus, the density of rectal PYY cells appears to be a good biomarker for all IBS subtypes. Can a blood-based biomarker assay for circulating PYY and somatostatin be used? PYY cells occur in the ileum, colon and rectum [30,43,44]. Similarly Somatostatin cells are localized in the stomach, small- and large intestine [15,30,43–46]. Hence circulating somatostatin and PYY would reflect the release of these hormones from the cells in all these segments. There was no difference between IBS patients and controls regarding fasting and postprandial PYY plasma levels [47]. Similarly, there was no significant difference in fasting somatostatin plasma level between IBS patients and controls [48]. Thus, measuring the circulating PYY and somatostatin is of no use as a marker for the diagnosis of IBS.

Similarly, the density of rectal somatostatin cells appears to be a good biomarker for differentiating between IBS and the healthy condition, with AUCs of 0.86 and 0.93 when expressed relative to the area of epithelial cells and per microscopic field, respectively. This parameter is equally good as a biomarker for all of the IBS subtypes, with AUCs of 0.83, 0.90 and 0.86 for IBS-D, IBS-M and IBS-C, respectively, expressed relative to the area of epithelial cells, and 0.93, 0.92 and 0.93, respectively, when expressed per microscopic field.

These findings establish that PYY and somatostatin cell densities can be used as biomarkers for IBS, clearly differentiating disease from the healthy condition, but the question as to whether they differentiate IBS from other gastrointestinal diseases that mimic IBS symptoms has yet to be answered definitively. The most commonly missed gastrointestinal diseases during symptom-based diagnosis are celiac disease, inflammatory bowel diseases including microscopic colitis and colorectal cancer [49,50]. It has been reported that plasma levels of PYY are increased in celiac disease [51], and the density of large-intestinal PYY cells is increased in lymphocytic colitis, unchanged in ulcerative colitis and in colon and rectal cancers, and decreased in Crohn's disease [32,44,52,53]. It therefore appears that with the exception of Crohn's disease, the density of rectal PYY cells can potentially be used to differentiate IBS from these gastrointestinal diseases. Large-intestinal somatostatin cell density is not changed in lymphocytic colitis or ulcerative colitis, and is decreased in colon and rectal cancers [32,44,52,53]. Therefore, as with PYY cell density, the density of rectal somatostatin cells seems likely to be able to differentiate IBS from some other gastrointestinal diseases.

The rectum is easily accessible for biopsy sampling and is generally well tolerated by patients, and immunohistochemistry is inexpensive and used routinely in all pathology laboratories. Furthermore, manually counting the PYY and somatostatin cells in ten microscopic fields is rapid and does not require sophisticated equipment or considerable experience.

Conclusion

This study has revealed two new biomarkers for the diagnosis of IBS, namely the densities of the rectal PYY and somatostatin cells. These two biomarkers fulfill the requirements for clinically useful biomarkers in that they have been shown to possess a good ability to differentiate IBS from the healthy condition, may be able to differentiate IBS from other gastrointestinal diseases that mimic IBS symptoms, and are inexpensive, simple, easy to perform and do not require considerable experience or sophisticated equipment.

Conflicts of interest

The authors have no conflicts of interest to report.

Author contributions

M.E. planned the study, recruited the patients and control subjects, performed the colonoscopy and morphometry, analyzed the data and drafted the manuscript. J.G.H., O.H.G. and T.H. contributed equally to the planning of the study and evaluation of the results, and commented upon the manuscript.

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