厚生労働科学研究費補助金（難治性疾患克服研究事業）
好酸球性食道炎 / 好酸球性胃腸炎の疾患概念確立と治療指針作成のための臨床研究
総合分担研究報告書

プロトンポンプ阻害薬抵抗性非びらん性胃食道逆流症の原因としての好酸球性食道炎の頻度および食道への好酸球性侵潤が食道運動機能に及ぼす影響に関する研究

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研究要旨

Part-1（22 年度）：好酸球性食道炎は、プロトンポンプ阻害薬 (PPI) 抵抗性の非びらん性胃食道逆流症（NERD）の原因の一つとして考えられているが、PPI 倍量抵抗性 NERD の原因である好酸球性食道炎の頻度は明らかでない。今回、PPI 倍量抵抗性 NERD23 症例の原因について食道生検を含む上部消化管内視鏡検査、食道内圧検査、食道 pH・インピーダンス検査を行い検討した。PPI 倍量抵抗性 NERD 患者の原因は好酸球性食道炎 1 例 (4.3%)、びまん性食道痙攣 1 例（4.3%）、液体逆流 11 例（47.8%）、空気逆流 1 例（4.3%）であり、原因不明は 9 例 (39.1%) であった。

Part-2（23 年度）：食道への好酸球性侵潤の食道運動機能に及ぼす影響は明らかではない。今回、食道に好酸球侵潤を有する症例に対する治療前後の検討から、食道への好酸球侵潤が下部食道括約筋（LES）弛緩不全および食道体部の運動異常の原因となっている症例が存在することが判明した。

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A. 研究目的

Part-1（22 年度）：

好酸球性食道炎が PPI 抵抗性 NERD の原因である頻度は明らかでないため、2007 年から当科における PPI 倍量抵抗性 NERD 患者の原因について、食道生検を含めた上部消化管内視鏡検査、high resolution manometry（HRM）による食道内圧検査、食道 pH・インピーダンス検査を行い検討した。

Part-2（23 年度）：

食道に好酸球侵潤を有する好酸球性食道炎患者および食道・胃・十二指腸に好酸球侵潤を認めた好酸球性胃腸症患者の治療前後の食道運動機能を評価し、食道への好酸球性侵潤が食道運動機能に及ぼす影響を明らかにする。

B. 研究方法

Part-1（22 年度）：

対象は消化管運動機能改善薬や漢方薬に加え PPI 倍量分割投与を行うも逆流症状（胸やけ、つかえ感、胸痛）が残存した PPI 倍量抵抗性 NERD 患者 23 症例（男性 10 人、女性 13 人、平均年齢 52.6 歳）である。PPI 倍量抵抗性 NERD 患者と診断後、好酸球性食道炎の存在を確認するため上部消化管内視鏡検査を行い、食道所見に関わらず中部～下部食道から 3 個の生検を行った。内視鏡検査において異常がみられない場合には HRM による食道内圧検査を施行した。食道内圧検査施行も一次性食道運動障害の所見が得られない場合には、すべてのタイプの逆流を捉えることが可能である食道 pH・インピーダンス検査を施行し逆流と症状の関連を検討した。食道 pH・インピーダンス検査時には PPI（倍量分割投与）のみを内服した状態で行った。液体逆流症状は液体逆流発生後 5 分以内の症状とした。逆流と症状の関連は symptom index（液体逆流に伴う症状 / 総液体逆流回数）により評価を行い、symptom index（SI）が 50% 以上であった

場合に症状は液体逆流に関連するものと判定した。空気単独逆流と症状の関連に関しては、個々の空気逆流と症状について検討を行い判定した。

Part-2（23年度）：

対象は「つかえ感」を主訴に来院し精査の結果、食道に20個以上/HPFの好酸球侵潤を認めた好酸球性食道炎（71歳、女性）および食道・胃・十二指腸に20個以上/HPFの好酸球侵潤を認めた好酸球性胃腸炎（73歳、男性）の2例である。治療前後にHRMによる食道内圧検査を行った。

C. 研究結果

Part-1（22年度）：

23例中の1例は内視鏡検査により好酸球性食道炎と診断された。この症例は内視鏡を食道内に挿入した直後に食道内に多数の輪状溝が数秒間出現した。またヨード散布直後にも同様な輪状溝が出現し好酸球性食道炎を疑い食道中部～下部の生検を行った。すべての生検部位より25個以上/high power field（HPF）の好酸球が確認され好酸球性食道炎と診断した。好酸球性食道炎の1例を除く22例に対して食道内圧検査が行われ、1例が嚥下後に正常な蠕動波がみられるが、嚥下後の多くの収縮波は連発する同期性収縮波であり、びまん性食道痙攣と診断した。残りの21例に対して24時間食道pH・インピーダンス検査を行った。21人中11人（47.8%）が液体逆流に対してSI陽性であり、4人（17.4%）は酸逆流（pH4未満）に対してSI陽性であり、7人（33.3%）は酸以外（pH4以上）の液体逆流に対してSI陽性であった。また1例は空気単独逆流による胸やけと診断した。以上食道pH・インピーダンス検査により21人中12人（57.1%）の症状は逆流に関連するものであり、23人中の14人(60.9%)の症状の原因が明らかとなった。しかし、23人中9人（39.1%）の症状の原因は未だ不明であった。

Part-2（23年度）：

症例1（71歳の女性。好酸球性食道炎症例）

「つかえ感」を主訴に来院。食道に好酸球侵潤を認め好酸球性食道炎と診断した。

食道内圧検査での水嚥下の結果は、10回ともほぼ同様であり、30mmHg未満の弱い収縮波の蠕動を何とか確認できるが、30mmHg以上の収縮波を有効収縮波とした場合には、蠕動波は下部食道においてのみ観察された。LES圧も低値（10mmHg前後）であった。治療はPSL30mgの経口投与を行った。PSL内服後翌日より症状改善し、3日目には症状は完全に消失した。血液データにおいても末梢の好酸球も2日目には正常化した。その後、PSLの減量を行ったが、症状、好酸球数の再燃は認めなかった。4週後に内視鏡検査を再施行した所、治療前に観察された多数の輪状溝は消失し、また治療前には十分に観察されなかった粘膜の血管透見も観察されるようになった。中部～下部食道にかけて3個の生検を行ったが好酸球は確認できなかった。その後の食道内圧検査では、水嚥下後の収縮波の評価では、30mmHg未満の蠕動波は治療前より圧の上昇がみられ、蠕動があることは治療前に比べ容易に確認できるが有効収縮波である30mmHg以上の収縮波での評価では明らかな違いは認めなかった。

症例2（73歳の男性。好酸球性胃腸炎：好酸球侵潤を食道、胃、十二指腸に認める）

「食事摂取困難、つかえ感」の精査のため当科紹介。上部消化管内視鏡検査では中部食道より肛側の狭小化を認め、また収縮も強く送気しても十分に伸展しない状況であった。また深吸気時にも食道胃接合部の伸展は得られず、柵状血管は観察されず、アカラシア患者において観察される下部食道の全周性の襞集中像（Esophageal Rosette）が観察された。狭小部の生検より好酸球20個以上/HPFが確認された。胃、十二指腸にも散在する発赤を認め、同部位の生検より好酸球20個以上/HPFが確認された。胸部CT検査においても中部食道から下部食道における全周性の壁肥厚を認めた。

食道内圧検査では、ほとんどの嚥下に伴い中部食道から下部食道において100mmHg以上の強い同期性収縮およびLES弛緩不全が観察された。この強収縮波が存在する部位は内視鏡での狭小部位と一致していた。内圧所見からはvigorous achalasiaの所見であった。

治療としてPSL30mgの経口投与を行った。PSL投与後の翌日より症状改善を認め、3日目に完全消失となった。血液検査においても末梢の好酸球数は3日目には正常化した。その後、PSL漸減を行うが症状の再燃は認めなかった。約4週後の内視鏡検査では、中部食道にやや狭小化している部分が観察されたが、治療前と比べ明らかな改善が認められた。中部食道から肛側の血管透見もみられた。下部食道では深吸気時に柵状血管の下端を含めた、ほぼ全体像が観察されるようになった。中部から下部食道の生検においても好酸球は認めなかった。治療後約1カ月の食道内圧検査では嚥下後のLES弛緩不全は認めず、食道上部においては蠕動波の出現不良であったが平滑筋領域では蠕動波を認め、ほぼ正常な食道運動であった。

D. 考察

Part-1（22年度）：

PPI抵抗性NERD患者のうち逆流（液体単独、液体＋空気、空気単独）に関連するものが12例（52.1%）にみられたが、逆流とは関連のない好酸球性食道炎が1例（4.3%）、また一次性食道運動障害であるびまん性食道痙攣も1例（4.3%）みられた。当科における好酸球性食道炎は未だ1例のみであり、好酸球性食道炎がPPI抵抗性NERDの原因としての頻度を言及することは時期尚早であるが、今回の検討から、好酸球性食道炎や食道運動障害がPPI抵抗性NERD患者の原因疾患の一つであることを確認できた。PPI投与も症状の改善が得られない場合には、好酸球性食道炎を疑い内視鏡検査を施行し、好酸球性食道炎患者において観察されることが多い縦走溝、輪状溝、白斑等が認められない場合においても食道生検を行い好酸球侵潤の有無を確認すること、また食道内圧検査により食道運動障害の有無を確認することが重要であると考えられた。

Part-2（23年度）：

食道への好酸球侵潤を有する症例に対する治療前後の検討から、食道への好酸球侵潤がLES弛緩不全および食道体部の運動異常の原因となる症例が存在することが明らかとなった。特に症例2においては好酸球侵潤によりLES弛緩不全、蠕動異常を呈し、アカラシア症状を認めた症例である。アカラシアの発症はLES弛緩、一次蠕動波に関連する中枢、外来迷走神経、食道壁在神経叢のどこか、または複数個所の異常により発症すると考えられているが、原因は未だ明らかではなく、アカラシア発症を考える上で大変興味ある症例である。症例2においてはPSLによる治療が遅れた場合には、LES弛緩不全が不可逆的であった可能性も否定できない。

E. 結論

Part-1（22年度）：

当科での好酸球性食道炎がPPI抵抗性NERDの原因である頻度は23例中1例（4.3%）にみられた。PPI抵抗性NERDの原因の一つとして好酸球性食道炎を念頭に置き診療する必要がある。

Part-2（23年度）：

食道への好酸球侵潤が食道運動異常を引き起こす可能性がある。日常診療において原因が明らかでない「つかえ感」症例の診療において、食道への好酸球侵潤による食道運動異常が原因である可能性も念頭に置き診療する必要がある。

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G. 知的所有権の取得状況

1. 特許取得
   特になし
2. 実用新案登録
   特になし
3. その他
   特になし

Ⅱ. 研究成果の刊行に関する

一覧表

研究成果の刊行に関する一覧

雑　誌

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Ⅲ. 研究成果の刊行物・別刷

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CASE REPORT

A case of eosinophilic esophagitis with atypical clinical course

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Abstract Eosinophilic esophagitis is a rare chronic disease that mainly occurs in middle-aged males. Treatment with a glucocorticoid and/or proton pump inhibitor is usually necessary to relieve unpleasant symptoms. An 83-year-old female patient with dysphagia and heartburn was diagnosed with eosinophilic esophagitis based on endoscopic findings, while histological examination identified dense infiltration of intraepithelial eosinophils. The symptoms and eosinophil infiltration spontaneously disappeared without any treatment approximately 2 months later. No obvious lifestyle or dietary changes to explain elimination of possible antigens were identified in this case. We report an atypical case of eosinophilic esophagitis with spontaneous regression.

Keywords Eosinophilic esophagitis · Endoscopy · Glucocorticoid · Dysphagia

Introduction

Eosinophilic esophagitis is a disease characterized by chronic esophageal mucosal inflammation with dense infiltration of eosinophils in esophageal squamous epithelium [1]. It is considered to be caused by local allergic reactions to food or airborne antigens, and affected patients have reported various esophageal symptoms, including food impaction, dysphagia, and heartburn [2]. For diagnosis of eosinophilic esophagitis, characteristic endoscopic findings and identification of dense eosinophilic infiltration in endoscopic biopsy specimens are considered to be important. Eosinophilic esophagitis is reported to be a chronic disease, with a risk of esophageal stenosis caused by long-standing inflammation-induced fibrosis in the esophageal submucosal layer [3, 4]. Fewer than 20 cases have been previously reported in Japan, and all of those patients required drug administration for remission induction, based on a MEDLINE search using "eosinophilic esophagitis" and "Japanese" as keywords. Herein, we report a case of eosinophilic esophagitis in an elderly female, whose symptoms and esophageal eosinophilic infiltration spontaneously regressed without treatment.

Case report

An 83-year-old female periodically visited the outpatient clinic of Okuizumo Hospital for postsurgery follow-up examinations. She had been treated in 2002 for pituitary adenoma by surgical resection and was administered hydrocortisone at 10 mg/day for subclinical postsurgery hypopituitarism. She began to report dysphagia and heartburn when eating food in February 2010. The symptoms gradually worsened, and endoscopic examination was performed in September 2010. Although endoscopy failed to show any organic esophageal diseases, including reflux esophagitis and neoplastic diseases, narrow-band imaging (NBI) revealed shallow linear furrows in the mid-esophagus area (Fig. 1a, b). Other endoscopic findings characteristic of eosinophilic esophagitis, such as multiple rings,

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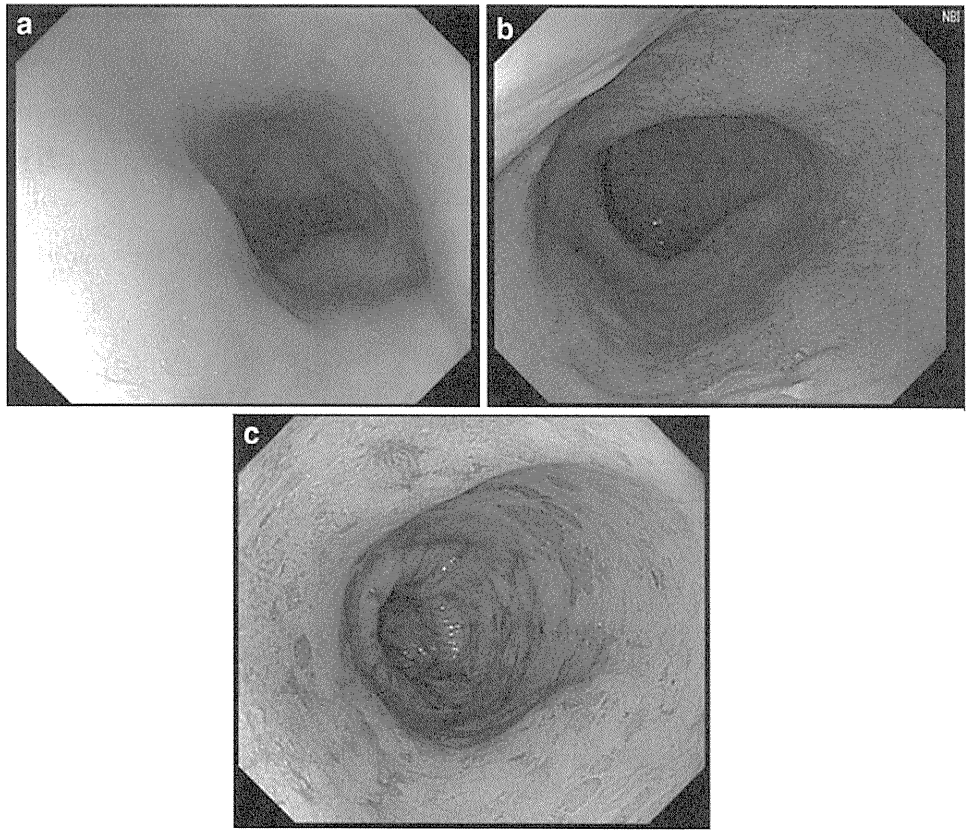

Fig. 1 Endoscopic images obtained with standard white-light (**a**) and narrow-band imaging (**b**). In narrow-band images, longitudinal linear furrows were identified. Lugol staining showed uneven staining of esophageal mucosa (**c**)

white stipple-like exudates, wrinkled pattern, and corrugated esophagus, were not found. Following Lugol staining, brownish change of esophageal mucosa was weak and uneven, suggesting possible diffuse inflammation (Fig. 1c). Two esophageal biopsy specimens taken from the middle and lower esophagus showed dense infiltration of more than 20 eosinophils in a high-power field of esophageal squamous epithelium (Fig. 2). A diagnosis of eosinophilic esophagitis was established, since other clinical conditions related to possible esophageal eosinophilic infiltration were not found.

The patient had no allergic diseases including bronchial asthma and no family history of allergic diseases. Laboratory tests were all within normal ranges, including peripheral blood leukocytes (6750/μl), eosinophils (122/μl), and IgE (22.2 IU/ml). In addition, plasma interleukin (IL)-5, IL-13, IL-15, eotaxin3, and thymic stromal lymphopoietin (TSLP) were all normal. The results of a skin prick test, patch test, and radioallergosorbent test (RAST) for standard allergens were all negative.

Computed tomography (CT) and endoscopic ultrasonography (EUS) showed normal nonthickened esophageal wall. Esophageal high-resolution manometry detected normal esophageal body peristaltic contractions and normal lower esophageal sphincter resting pressure with appropriate relaxation during ingestion (Fig. 3).

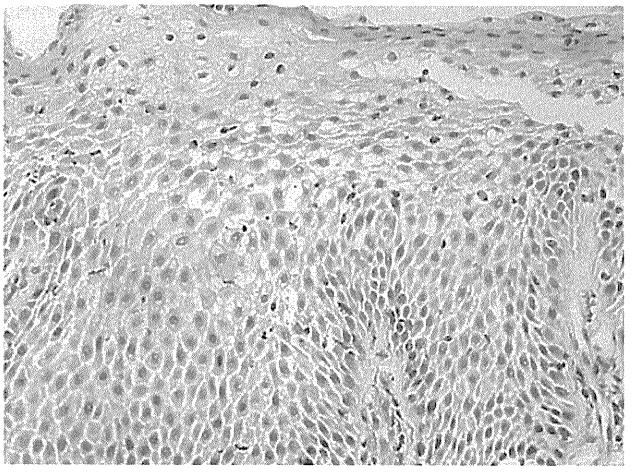

Fig. 2 Photomicrograph of biopsy specimen showing dense infiltration of eosinophilic leukocytes in esophageal squamous epithelium (>20 eosinophils in ×400 high-power field), hematoxylin–eosin staining

Locally active glucocorticoid administration was planned for relieving esophageal symptoms by depressing inflammation caused by eosinophilic infiltration. However, before starting the administration, the symptoms began to gradually decline and nearly disappeared 2 months later. Endoscopic examination performed in November 2010 did not show characteristic findings of eosinophilic esophagitis

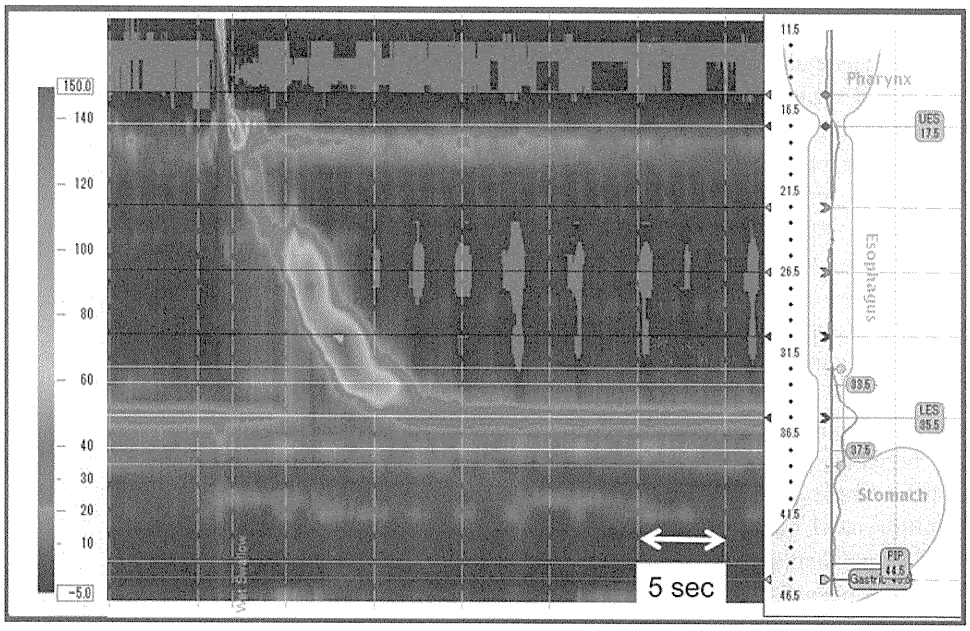

Fig. 3 Esophageal high-resolution manometry findings revealing pressure activity in the esophagus from the pharynx to the stomach. Contractile pressure, peristaltic velocity, and lower esophageal sphincter function were within normal limits. *Reddish color* shows higher levels of pressure

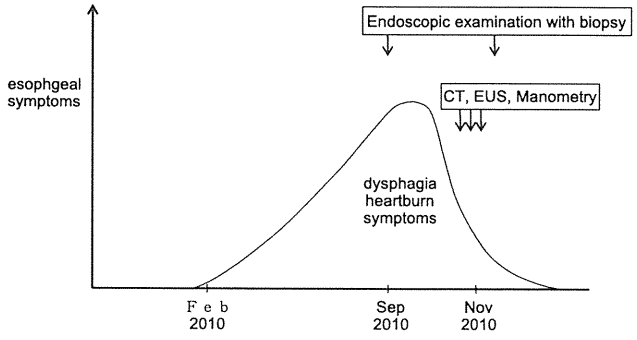

Fig. 4 Illustration of the clinical course. Esophageal symptoms spontaneously disappeared without specific treatment

even with NBI imaging and Lugol staining. Endoscopic biopsy specimens obtained in November 2010 did not show any abnormal infiltration by intraepithelial eosinophils. Thereafter, the patient was regularly examined in the outpatient clinic and confirmed to have no esophageal symptoms without drug administration targeting eosinophilic esophagitis. The clinical course is shown in Fig. 4.

Discussion

Eosinophilic esophagitis is a recurrent long-lasting chronic disease, and cases of spontaneous remission are rare [7]. Straumann et al. [8] reported the rarity of spontaneous remission in adult cases with eosinophilic esophagitis followed for up to 12 years, while Spergeal et al. [9] reported that only 2% of pediatric patients with eosinophilic esophagitis showed spontaneous remission during observation periods as long as 14 years.

Food antigens, especially nuts, soy, wheat, milk, eggs, and seafood, are reported to be related to development of eosinophilic esophagitis, and their elimination from the diet frequently relieves symptoms caused by eosinophilic esophagitis, especially in pediatric patients [10–12]. In animal studies as well as human case studies, airborne allergens such as *Aspergillus* have been reported to be important allergens that cause esophageal infiltration by eosinophils [13–15]. Therefore, elimination of food and/or airborne allergens appropriate for each patient is expected to relieve esophageal eosinophil infiltration with symptom resolution. However, identification of possible allergens in individual patients is difficult, even following skin prick and skin patch tests, or RAST [16], and elimination of a specific food from the diet based on results of those tests has often been reported to be not adequately effective to relieve related symptoms [17].

For treatment of eosinophilic esophagitis, oral glucocorticoid administration is considered to be the standard therapy, with high rates of success reported [1, 18]. To minimize possible adverse effects of glucocorticoid administration, locally active glucocorticoids that are systematically inactive because of rapid catabolism in the liver, such as budesonide and fluticasone, are now used as first-line therapeutic drugs for the disease [19, 20].

Because of wider distribution of information concerning symptomatic and endoscopic characteristics of eosinophilic esophagitis, the number of reports in Japan is increasing [5, 6]. The majority of reported patients are middle-aged males with some allergic complications. In addition, they frequently show ring-like multiple strictures in the esophagus, and require glucocorticoid or proton pump inhibitor administration to control esophageal symptoms.

The present case report is of an atypical elderly female patient. Generally, presence of endoscopic findings characteristic of eosinophilic esophagitis, such as liner farrows, multiple rings, white stipple-like exudates, and wrinkled pattern, are found frequently on diagnostic endoscopic study. However, endoscopic characteristics that facilitated diagnosis were only evident when NBI imaging, which can clarify the superficial structure of the esophageal mucosa, was employed in this case. In addition, her symptoms and eosinophilic infiltration of esophageal epithelium spontaneously disappeared without treatment. Although follow-up examinations of this patient are important, therapeutic intervention is considered not to be necessary, since the purpose of such treatment is to relieve unpleasant esophageal symptoms and normalize health-related quality of life.

The mechanism of spontaneous remission of eosinophilic esophagitis in the present case is not clear, though it is possible that an unintended change of lifestyle or dietary habit may have decreased antigen exposure. We were unable to identify a possible antigen, or an obvious change of lifestyle or dietary habit. The second possible mechanism is related to the chronic administration of hydrocortisone in this patient for postsurgical hypopituitarism. However, the dose of hydrocortisone administered is only 10 mg/day, which is not a pharmacological dose but rather a physiological replacement dose. In addition, administration of hydrocortisone had already been started 8 years before the appearance of symptoms and the diagnosis of the disease. Therefore, it is difficult to consider administration of hydrocortisone as a possible mechanism for spontaneous remission of eosinophilic esophagitis in this case. Nevertheless, reports of atypical cases are important to avoid unnecessary treatment and minimize therapy-related adverse effects.

Finally, in this case, thickened esophageal wall was not found even by CT/EUS examinations. According to the literature, in only part of the patients, thickened esophagus was found in patients with eosinophilic esophagitis. The absence of thickened esophageal wall may represent weak disease activity in this patient. In addition, as a second possible reason, disease activity may have at least partly regressed spontaneously at the time point when the CT/EUS examinations were done, since CT/EUS were done 1 month after the pathological diagnosis of this case. In future study, the relationship between possible spontaneous remission and absence of esophageal wall thickening should be investigated to predict clinical course of patients with eosinophilic esophagitis.

In summary, we diagnosed an atypical elderly female with eosinophilic esophagitis, in whom esophageal symptoms and esophageal eosinophilic infiltration spontaneously disappeared.

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RAPID COMMUNICATION

Eosinophilic esophagitis investigated by upper gastrointestinal endoscopy in Japanese patients

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Abstract
Background The prevalence of eosinophilic esophagitis (EE) is increasing rapidly in Western countries. Several case series of EE have also been reported in Japan. However, the prevalence of EE in Japanese patients as investigated by upper gastrointestinal endoscopy is unknown. Therefore, we carried out a prospective multicenter study to address this issue.
Methods From July to December 2010, 23,346 patients who had undergone routine upper gastrointestinal endoscopy in 17 institutions were enrolled. In patients with symptoms suggesting EE, such as dysphasia, food impaction, and heartburn, and/or in patients in whom endoscopic findings suggested pathology, esophageal biopsy samples were collected, and the numbers of eosinophils in the squamous epithelium were counted.
Results During the study period of 6 months, 4 patients were endoscopically and histologically diagnosed with EE. The prevalence of EE was calculated to be 17.1/100,000.
Conclusion The prevalence of EE in Japanese patients by upper gastrointestinal endoscopy has now been documented.

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Keywords Eosinophilic esophagitis · Prevalence · Japanese population

Introduction

Eosinophilic esophagitis (EE), first described in 1977 [1], was recognized as a new disease in which patients experience esophageal eosinophilia and gastroesophageal reflux disease (GERD)-like symptoms that do not respond to the usual GERD management methods, such as the administration of proton pump inhibitors (PPIs) [2]. The prevalence of EE has been reported to be increasing rapidly in Western countries. Straumann et al. [3] reported that the proportion of patients with EE increased from 2/100,000 in 1989 to 23/100,000 in 2004 in Switzerland. Similarly, in the US, Prasad et al. [4] found that the prevalence of EE was 55/100,000 in 2006 and that the incidence of clinically diagnosed EE had increased markedly over the last 3 decades.

In Japan, the first case of EE was reported in 2006 [5], and other cases of EE have been reported since then [6, 7]. The prevalence of EE has not yet been investigated in the Japanese population. The prevalence of EE in endoscopy-examined cases was recently reported in the USA as 6.5% [8]. Therefore, to determine the prevalence of EE in Japanese patients investigated by routine upper gastrointestinal endoscopy, we analyzed patients in a prospective multicenter study.

Methods

In 17 institutions that ranged from primary medical clinics to tertiary-care referral centers in the San-In district of

western Japan, patients who underwent upper gastrointestinal endoscopy, either due to symptoms or as part of an annual medical check-up, were enrolled from January to December 2010. In patients who suffered from symptoms suggesting EE, such as dysphasia, food impaction and heartburn, and/or who had endoscopic findings suggestive of EE, such as wrinkle patterns, linear fissures, and white stipple-like exudates [9, 10], biopsy samples were taken from the upper, middle and lower esophagus, and the numbers of eosinophils in the squamous epithelium were counted. Photographs of typical endoscopic EE findings were provided beforehand to all participating endoscopists. All upper gastrointestinal endoscopies were performed by expert endoscopists. EE was histologically confirmed by the presence of more than 20 eosinophils in every high-power field [2]. In patients with histologically confirmed eosinophilic infiltration, the absence of other diseases that may cause esophageal eosinophilic infiltration was clinically confirmed.

Results

During the study period of 6 months, upper gastrointestinal endoscopy was carried out in 23,346 patients at 17 institutions, and 4 cases of EE were found. The prevalence of EE in Japanese patients investigated by upper gastrointestinal endoscopy was calculated to be 17.1/100,000. The characteristics of the 4 EE patients are listed in Table 1. None of the 4 cases had gastrointestinal eosinophilia. In two cases, PPI administration was at least partially effective for symptom resolution, although the long-term observation period was somewhat limited. In one case, fluticasone administration was markedly effective, but PPI administration was not effective. The remaining case experienced spontaneous disappearance of the subjective symptoms. These 4 EE patients were not critically ill and had no serious complications, such as stricture of the esophagus. In the present study, no endoscopy-negative EE patients had esophageal symptoms suggesting EE.

Discussion

EE is a chronic allergic disorder of the esophageal mucosa, possibly caused by antigens in the air and in food, with marked eosinophilic infiltration in the esophageal squamous epithelium [11, 12]. The prevalence of EE in the Japanese population is unknown, although it may be increasing, as is the case in Western countries. In the present study, the prevalence of EE was found to be 17.1/100,000 in patients investigated by upper gastrointestinal endoscopy. This prevalence of EE was higher than expected, since the reported prevalence of EE in Western countries was 23–55/100,000 in recent etiological studies [3, 4]. Our study is not an epidemiological one, and strong inclusion bias was present in the enrollment. However, the presence of EE in approximately one in 5,000 endoscopy-investigated cases is high enough to encourage endoscopists to carefully consider the possible presence of minute endoscopic findings suggesting EE in patients they see in their daily clinical practice.

Abe et al. [7] reported that the endoscopic recognition of EE is not difficult, based on the identification of characteristic findings, such as linear furrows, transient and constant concentric rings, and white exudates. Molina-Infante et al. [13] also reported that the specificity of endoscopy-identified multiringed esophagus for the diagnosis of EE was only 37%, whereas that of furrows or exudates was 90% in Spanish adults. In the present study, all of the patients with EE had characteristic endoscopic findings. These endoscopic findings and symptoms may be good markers for the diagnosis of EE, although calculations of their sensitivity and specificity were difficult because of the small number of patients with EE in our study. Future studies of larger numbers of patients will be necessary to confirm the value of these markers. In two cases found in the present study, PPI administration partially improved symptoms, as previously reported [7, 9, 14, 15]. Although one diagnostic guideline for EE published in the USA required a lack of responsiveness to high-dose PPI or normal pH monitoring [2], many investigators did not follow this, and used variable diagnostic criteria for EE [16]. In the presence of acid reflux, eosinophilic infiltration

Table 1 Characteristics of 4 cases of eosinophilic esophagitis

Case	Age	Gender	Symptoms	Endoscopic findings	Treatment
1	83	F	Dysphasia, heartburn	Wrinkle pattern, white stipple-like exudates	None
2	58	M	Heartburn, epigastric pain	Linear fissures, mucosal edema	PPI partially effective
3	51	M	Food impaction	White stipple-like exudates, small ulcerations	PPI partially effective
4	61	F	Chest pain, epigastric pain	Linear fissures, small ulcerations, white stipple-like exudates	PPI not effective, fluticasone effective

in the esophageal mucosa is reported to be aggravated though several mechanisms, including augmented production of the cytokine eotaxin-3 [17–19]. Therefore, as a first-line treatment of EE, PPIs may be considered for their safer drug profile than standard treatment with glucocorticoids.

Considering the results of the present study, EE should be considered more readily in clinical practice when patients are suffering from dysphagia, food impaction, or reflux symptoms. EE may be common in Japanese patients, since its prevalence in endoscopy-investigated cases was 17.1/100,000 patients.

Conflict of interest The authors declare that they have no conflict of interest.

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Roles of Krüppel-like factor 4 in oesophageal epithelial cells in Barrett's epithelium development

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► An additional figure is published online only. To view this file please visit the journal online (http://gut.bmj.com).

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ABSTRACT

Objectives The mechanism of transformation to intestinal metaplasia in Barrett's oesophagus has not been clarified. We previously reported that bile acids activate the Cdx2 promoter via nuclear factor kappa B (NF-κB) and stimulate production of Cdx2 protein in oesophageal keratinocytes, resulting in production of intestinal-type mucin. Krüppel-like factor 4 (KLF4) is an important transcription factor in the development of intestinal mucosa and has similar functions as Cdx2. In the present study, we investigated the direct effects of bile acids on KLF4 expression as well as the precise mechanisms of expression in cultured oesophageal squamous epithelial cells.

Methods We investigated the expression of KLF4 in rat and human Barrett's epithelium specimens, while the response of that expression to bile acids was studied using a KLF4 promoter luciferase assay. In addition, oesophageal squamous epithelial cells were transfected with a KLF4 expression vector, after which their possible transformation into intestinal-type epithelial cells was investigated.

Results In both rat and human tissues, Barrett's epithelium strongly expressed KLF4. Furthermore, a bile acids mixture increased KLF4 promoter activity, and mRNA and protein expression in oesophageal epithelial cells. Results from mutation analysis of the KLF4 promoter suggested that the NF-κB binding site is responsible for bile acid-induced activation of the KLF4 promoter. In addition, KLF4 and Cdx2 stimulated each other by directly binding to the promoter of the other, while transfection of the KLF4 expression vector in oesophageal epithelial cells induced production of MUC2 protein.

Conclusion Bile acid-induced sequential expression of KLF4 followed by MUC2 production may have an important role in the development of Barrett's epithelium.

Significance of this study

What is already known about this subject?

- Cdx2 is a key mediator in the development of Barrett's oesophagus.
- Bile acids directly augment Cdx2 via NF-κB.
- Cdx1 is also an important molecular mediator of Barrett's oesophagus.

What are the new findings?

- KLF4 is expressed in Barrett's epithelium.
- The expression of KLF4 in oesophageal keratinocytes in response to bile acids induces metaplastic changes during Barrett's epithelium development.
- The transcriptional network related to KLF4 and Cdx2 has important roles in development of this disease.

How might it impact clinical practice in the foreseeable future?

- Molecular targets for treatment of Barrett's oesophagus will be defined.

INTRODUCTION

Barrett's oesophagus is an acquired condition, in which stratified squamous epithelium is replaced by metaplastic columnar epithelium in the distal oesophagus.[1] The condition is associated with chronic gastro-oesophageal reflux disease (GORD)[2] and reflux of duodenal contents with bile acids is generally considered to be one of the most important risk factors in its development.[3] In association with tissue damage and regeneration, one or a few stem cells may attempt to adapt to this new environment by altering the patterns of some gene expressions, and thus undergo profound phenotypic changes that lead to a different type of epithelium that is more resistant to such a novel environment. The causal link between bile acid reflux and alterations of some transcription factors has been studied; however, the precise mechanism of promotion of Barrett's epithelium formation by bile acid reflux remains to be characterised.

A number of studies have found that Cdx2 is a key mediator in the development of Barrett's oesophagus.[4 5] We previously reported a two-step mechanism involved in the development of Barrett's epithelium, in which bile acids activate the Cdx2 promoter via nuclear factor kappa B (NF-κB) and stimulate the production of Cdx2 protein in oesophageal immature keratinocytes, with a resulting production of intestinal type mucin.[6] In addition to Cdx2, we also recently showed that bile acids induce the expression of Cdx1 in oesophageal immature keratinocytes, and demonstrated an interplay mechanism between Cdx1 and Cdx2 that causes upregulation of each other by directly binding to the promoter of the other, stimulating the development of Barrett's epithelium.[7]

Krüppel-like factors (KLFs) are zinc finger-containing transcription factors that exhibit homology to the *Drosophila melanogaster* segmentation gene product Krüppel. KLFs comprise a family of evolutionarily conserved zinc finger transcription factors that regulate numerous biological processes, including proliferation, differentiation, development

and apoptosis.[8] Among them, KLF4 (gut-enriched Krüppel-like factor) is highly expressed in epithelial cells of the small and large intestines.[9] The expression pattern of KLF4 is similar to that of Cdx2 in the intestine, as it is expressed mainly in non-proliferating differentiating and differentiated cells of the upper crypt and villus/surface mucosa, where Cdx2 is also expressed.[8] During embryogenesis, the expression of KLF4 begins to rise, which correlates with a critical period of gut epithelium morphogenesis, similar to that of Cdx2.[10] Furthermore, colonic goblet cells in KLF4−/− mice do not show a normal goblet cell morphology and the goblet cell marker MUC2 exhibits patchy expression throughout the colonic epithelium.[11] These findings suggest that KLF4 plays a fundamental role in the development of intestinal mucosa.

With regard to carcinogenesis, it was reported that KLF4 expression is downregulated in human adenomatous polyps and cancer of the colon.[12] Notably, in colorectal adenomas and adenocarcinomas, the level of Cdx2 protein is markedly reduced.[13] Also, Cdx2 was shown to activate a KLF4 promoter construct in Chinese hamster ovary (CHO) cells and colon cancer related RKO cells,[14 15] while an inter-regulation mechanism between KLF4 and Cdx2 has been speculated.

In the present study, we investigated whether alterations of KLF4 expression in response to bile acids in oesophageal keratinocytes induce metaplastic changes during Barrett's epithelium development. Furthermore, we investigated the transcriptional network connecting KLF4 and Cdx2 in development of the disease.

MATERIALS AND METHODS

Rat model of Barrett's oesophagus

To induce Barrett's oesophagus, we employed Levrat's model with minor modifications, as previously described.[6 7 16] In brief, the gastro-oesophageal junction was cut and the oesophageal end separated. The distal end of the oesophagus was then reimplanted 2 cm beyond the ligament of Treitz in an end-to-side fashion into a loop of the jejunum and the proximal end of the stomach was ligated. Six months after formation of oesophageal–jejunal anastomoses, the rats were killed and their oesophagi removed.

Patients and tissues

Human oesophageal tissues were collected after obtaining informed, written consent from all subjects. During endoscopy procedures, biopsy specimens of normal squamous mucosa from the distal oesophagus (n=6) and Barrett's oesophagus without dysplasia (n=6) were taken, then snap-frozen in liquid nitrogen. Barrett's oesophagus was histologically defined as the presence of columnar epithelium containing goblet cell metaplasia.

Cell culture and bile acid treatment

Five cell lines, including Het-1A (a human normal oesophageal cell line immortalised by viral SV40 transfection; American Type Culture Collection, ATCC, Manassas, Virginia, USA), OE33 (a human oesophageal adenocarcinoma cell line; European Collection of Cell Cultures, ECACC, Salisbury, Wiltshire, UK), OE19 (a human cell line established from an adenocarcinoma obtained from the gastric cardia/oesophageal gastric junction; ECACC), SW480 (a human colorectal adenocarcinoma cell line, ATCC) and HeLa (a human cervical adenocarcinoma cell line, ATCC), were used in this study. Primary cultures of oesophageal keratinocytes from normal rat oesophagi were established, as previously described.[6]

A mixture of bile acids (Sigma Chemicals, St. Louis, Missouri, USA), which included cholic acid, glycocholic acid and taurocholic acid, was used as a stimulant, as previously described.[7]

Vector construction and luciferase assay

We amplified 1700 bp of the KLF4 promoter (accession No. AF117109) by PCR, then cloned that into the MluI and BglII sites of a pGL3-basic luciferase vector (Promega, Madison, Wisconsin, USA) to generate pKLF4/1700-Luc (−1735 to −36), which generated pKLF4/1080-Luc (−1115 to −36), pKLF4/425-Luc (−460 to −36), pKLF4/233-Luc (−268 to −36), and pKLF4/35-Luc (−70 to −36). The position +1 refers to the major transcription start site identified in the KLF4 gene.[9] We also amplified 1541 bp of the Cdx2 promoter (accession No. NC_000071) by PCR, then cloned that into the KpnI and BglII sites of a pGL3-basic luciferase vector to generate pCdx2/1541-Luc (−1415 to +125), which generated pCdx2/1014-Luc (−888 to +125), pCdx2/631-Luc (−506 to +125), pCdx2/438-Luc (−313 to +125), pCdx2/319-Luc (−194 to +125), pCdx2/219-Luc (−94 to +125), and pCdx2/74-Luc (+52 to +125), as previously described.[6] Furthermore, 1750 bp of the MUC2 promoter (accession No. AF221746) was amplified by PCR, then cloned into the MluI and BglII sites of a pGL3-basic luciferase vector to generate pMUC2/1750-Luc (−1804 to −55), which generated pMUC2/823-Luc (−877 to −55), pMUC2/463-Luc (−517 to −55), pMUC2/214-Luc (−268 to −55), pMUC2/80-Luc (−134 to −55), and pMUC2/39-Luc (−93 to −55). The position +1 refers to the major transcription start site identified in the MUC2 gene.[17] As an internal control for the dual luciferase assay, pRL-TATA-*Renilla*-Luc was used.[6] To produce mutated KLF4 promoter constructs for pM/KLF4-Luc, 5′-ggcggccgcc**agtacttca**ccggccgagagagcgagcgcggctcc-3′ was used (nucleotide substitutions indicated in bold), to produce mutated Cdx2 promoter constructs for pM/Cdx2-Luc, 5′-cggcgggtcat**tc-caagtctc**tacagcttactggcaaggaggtgggaggaaa-3′ was used, and to produce mutated MUC2 promoter constructs, for pM/MUC2-Luc, 5′-cttggcaaat**aatacgtgaatat**ttcgcacctccctcgtcctccgccctcg-3′ was used.

cDNA encoding full-length mouse KLF4 (NCBI NM-010637) was amplified by PCR and cloned into a pcDNA5/FRT/V5-His-TOPO Vector (Invitrogen, Carlsbad, California, USA). Vector DNA without KLF4 sequences was used as a negative control. A Cdx2 expression vector was also constructed, as previously reported.[6]

Het-1A, OE33, and OE19 cells were separately cultured and transfected with 0.5 μg of each promoter vector and 0.02 μg of pRL-TATA-*Renilla*-Luc in each well, with Lipofectamine 2000 (Invitrogen). At 24 h after transfection of the luciferase vectors, the cells were stimulated with various concentrations of the bile acids mixture or the vehicle alone for 3 h, then cell lysates were used to determine luciferase activity. Also, Het-1A cells were cultured and transfected with 0.2 μg of each indicated promoter vector and a total of 0.2 μg of each indicated expression vector or an empty vector, along with 0.02 μg of pRL-TATA-*Renilla*-Luc in each well for 24 h, then the cell lysates were used for measurement of luciferase activity.

Immunohistochemistry

Immunohistochemistry was performed as previously described.[6] To identify KLF4-expressing cells, tissue sections were incubated with the anti-KLF4 antibody (1:100; Medical & Biological Laboratories, Nagoya, Japan), followed by incubation with secondary biotinylated anti-rabbit immunoglobulin (DAKO, Carpinteria, California, USA). Bound antibodies were detected

using a 3-amino-9-ethylcarbazole substrate–chromogen system (DAKO). The sections were counter-stained with haematoxylin.

RNA extraction and real-time PCR

Extraction of total RNA was performed as previously described.[6] DNase I-treated RNA was reverse transcribed into cDNA using a ReverTra Ace α kit (Stratagene Toyobo, Tokyo, Japan). A real-time fluorescence PCR assay based on SYBR Green (Applied Biosystems, Foster City, California, USA) was then performed using the primers described in table 1.

Primary cultured cells were transfected with control non-specific siRNA (Qiagen, Hilden, Germany), p50 siRNA (Santa Cruz Biotechnology, Santa Cruz, California, USA), or p65 siRNA (Santa Cruz Biotechnology) using Lipofectamine 2000. The reduced levels of p50 or p65 mRNA expression induced by transfection of each siRNA were determined using siRNA specific primers (Santa Cruz Biotechnology).

Protein extraction and western blot analysis

Protein extraction and western blot analysis were performed as previously previously.[6] The membranes were incubated with anti-KLF4 (1:200; Abnova, Taipei, Taiwan), anti-p50 (1:200; Santa Cruz Biotechnology), anti-p65 (1:200; Santa Cruz Biotechnology), or anti-β-actin (1:3000; Sigma Chemicals) antibodies, followed by horseradish-peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin (DAKO).

Immunofluorescence cytochemistry

Immunofluorescence cytochemistry was performed as previously described.[6] The cells were labelled with anti-KLF4 (1:100), anti-Cdx2 (1:50; BioGenex, San Ramon, California, USA), anti-MUC2 (1:100; Santa Cruz Biotechnology), anti-p50 (1:100), anti-p65 (1:100), and anti-cytokeratin (CK) 20 (1:200; Santa Cruz Biotechnology) antibodies. Binding of the primary antibodies was detected using FITC-conjugated anti-mouse, anti-rabbit, or anti-goat immunoglobulin, or rhodamine-conjugated anti-mouse or anti-rabbit immunoglobulin (DAKO). The cells were nuclear counter-stained with 4′,6-diamidino-2′-phenylindole dihydrochloride (DAPI) (Pierce Biotechnology, Rockford, Illinois, USA).

Table 1 The sequences of oligonucleotide primers used in this study

Primer		Sequence (5′ to 3′)
Human GAPDH	Fw	gaaggtgaaggtcggagtca
	Rv	aatgaaggggtcattgatgg
Human KLF4	Fw	tcccatctttctccacgttc
	Rv	agtcgcttcatgtgggagag
Human MUC2	Fw	ctcccagacaggagaacgag
	Rv	gggatcgcagtggtagttgt
Rat GAPDH	Fw	gtgaaggtcggtgtgaacg
	Rv	cttgccgtgggtagagtcat
Rat KLF4	Fw	caggctgtggcaaaacctat
	Rv	cggtagtgcctggtcagttc
KLF4 promoter, including the NF-κB binding site	Fw	gcaagcgagcgagaagttat
	Rv	gagtcctggggactgtgc
KLF4 promoter, including GC boxes	Fw	gtgcgcggagtttgtttatt
	Rv	ccgcgcgcttcttacttat
Cdx2 promoter, including the Sp-1-binding site	Fw	cagccattggtgtctgtgtc
	Rv	ttctttcctcccacctcctt
KLF4 promoter including Cdx2-binding site	Fw	tggccatcggacatacttatc
	Rv	gccccaaagtcaacgaagta
MUC2 promoter, including the CACCC/Sp-1 element	Fw	tagttcacctggggtgtgtg
	Rv	gacgagggaggtgcgaag

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) analysis was performed using an EpiQuik Chromatin Immunoprecipitation Kit (Epigentek Group, Brooklyn, New York, USA), according to the method reported by D'Amico *et al*.[18] Het-1A cells were transiently transfected with a KLF4 promoter vector and stimulated with a bile acids mixture for 3 h, after which ChIP analysis was performed. Also, Het-1A cells were transiently transfected with a KLF4 promoter vector, Cdx2 promoter vector, MUC2 promoter vector and KLF4 expression vector, Cdx2 expression vector, or empty vector, after which ChiP analysis was performed. Total DNA prior to immunoprecipitation was used as the input value. Chromatin was immunoprecipitated with anti-KLF4, anti-Cdx2, anti-p50, and anti-p65 antibodies, or IgG as a negative control. Immunoprecipitated DNA–protein complexes were isolated and a real-time PCR assay was then performed using the primers described in table 1.

Statistical analysis

All data are expressed as the mean ± SEM. Multiple comparisons were performed with ANOVA, followed by a Dunnett test. Statistical comparisons between two groups were done with a Mann–Whitney U test. p values less than 0.05 were considered to be statistically significant.

RESULTS

Expression of KLF4 mRNA in adult rat tissues

KLF4 mRNA was found to be expressed throughout the gastrointestinal tract of adult rats, with high levels of expression observed in the jejunum, ileum, proximal, and distal colon, while a lower level of expression was found in the oesophagus (figure 1A).

Immunohistochemistry examinations of rat Barrett's epithelium

Six months after the procedure, columnar-lined epithelia consisting of absorptive cells and goblet cells were observed above the oesophageal–jejunostomy in the rats. KLF4-positive cells with nuclear staining were observed in the columnar epithelia above the oesophageal–jejunostomy, mainly in the surface villi, whereas there was a small number of cells in the crypts (figure 1B).

Expression of KLF4 mRNA in human normal oesophagus and Barrett's oesophagus

We also determined KLF4 mRNA expression levels in endoscopic biopsy specimens of normal oesophagus and Barrett's oesophagus obtained from the human subjects. KLF4 mRNA expression levels of Barrett's oesophagus were significantly higher than those of normal squamous epithelium (figure 1C).

Effects of bile acids on KLF4 promoter activity

The bile acids mixture had a stimulatory effect on KLF4 promoter activity in a dose-dependent manner, with an approximately twofold increase in transcriptional activation in Het-1A, OE33, and OE19 cells (figure 2A,B,C). We constructed a series of reporter plasmids containing different lengths of the KLF4 promoter. The transcriptional activity of these constructs was analysed in Het-1A cells with 200 μm of the bile acids mixture. The plasmid pKLF4/1080-Luc exhibited a similar level of activation by the bile acids mixture as that shown by pKLF4/1700-Luc. However, the plasmids pKLF4/425-Luc, pKLF4/233-Luc, and pGL3-basic without the KLF4 promoter showed no activation response to bile acid stimulation (figure 2D). These results revealed that bile acid-induced activation of the KLF4-promoter is controlled by a site located between −1155

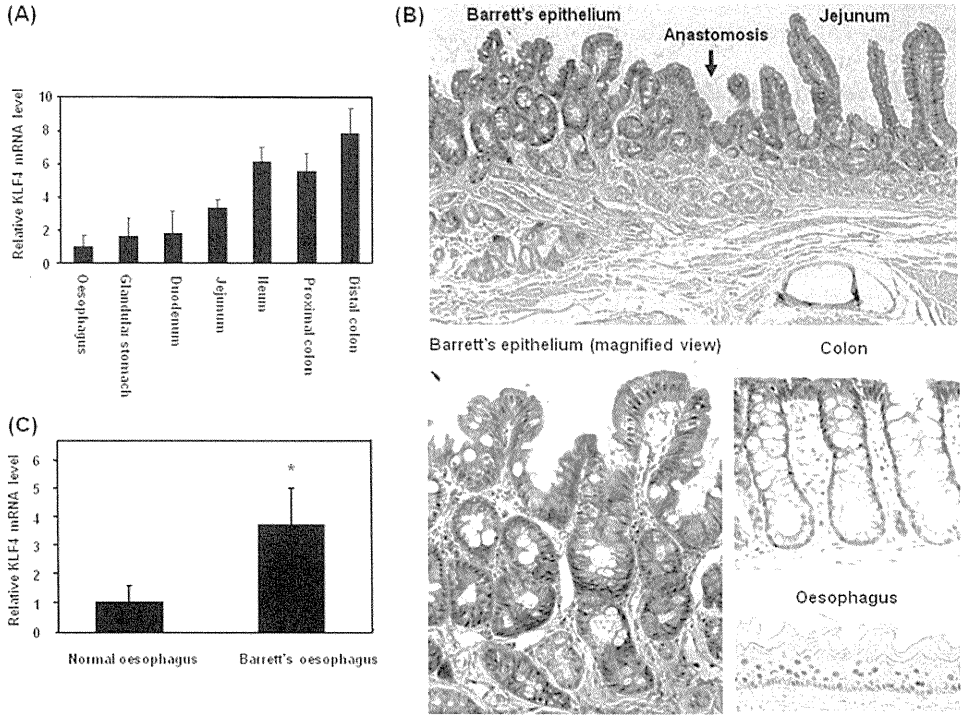

Figure 1 (A) Tissue distribution of KLF4 mRNA in gastrointestinal tissues. RNA was extracted from various rat tissues and analysed by real-time PCR for KLF4 expression. Data were normalised to GAPDH mRNA. Results are expressed as the mean ± SEM of three experiments. (B) Immunohistochemistry findings for KLF4. Barrett's epithelium was examined 6 months after performance of oesophageal–jejunal anastomosis. KLF4-positive cells were mainly observed in surface villi, though a small number of cells were observed in the crypts in Barrett's epithelium. Normal oesophagus and colon specimens are shown. KLF4-positive cells in the normal colon were localised predominantly on the surface epithelium. In the oesophagus, KLF4-positive cells were localised on the cells of the suprabasal layer, though the level was low. (C) Expression of KLF4 mRNA in human normal oesophagus and Barrett's oesophagus. Results are expressed as the mean ± SEM of six samples. *p<0.05 vs. normal oesophagus.

and −460. To determine whether bile acids function as a direct transcriptional activator of KLF4, we examined the KLF4 promoter region for the putative NF-κB binding site using the computational program TESS (http://www.cbil.upenn.edu/). We identified a putative NF-κB binding site from −605 to −596 (ggcagttccc) and speculated that bile acids might bind to the KLF4 promoter in this region. Therefore, to investigate the role of the NF-κB site following bile acid-induced stimulation of KLF4 expression, the element of the putative NF-κB binding site was mutated, which completely abolished bile acid-induced activation of the KLF4 promoter (figure 2D).

To confirm whether bile acids bind to the KLF4 promoter, a ChIP assay was performed using Het-1A cells. Real-time PCR analysis was performed to amplify the promoter region of KLF4 from −650 to −424 that contains the NF-κB binding site. The amount of transcript in the bile acid-treated samples was significantly higher than that in the vehicle-treated samples of DNA immunoprecipitated with the anti-p50 and anti-p65 antibodies (figure 2E).

Direct effects of bile acids on KLF4 mRNA and protein expressions in oesophageal epithelial cells

To determine whether bile acids augment KLF4 mRNA expression, we investigated the direct effect of a bile acids mixture on KLF4 mRNA expression using Het-1A and OE33 cells, and found that the bile acids augmented KLF4 mRNA expression in

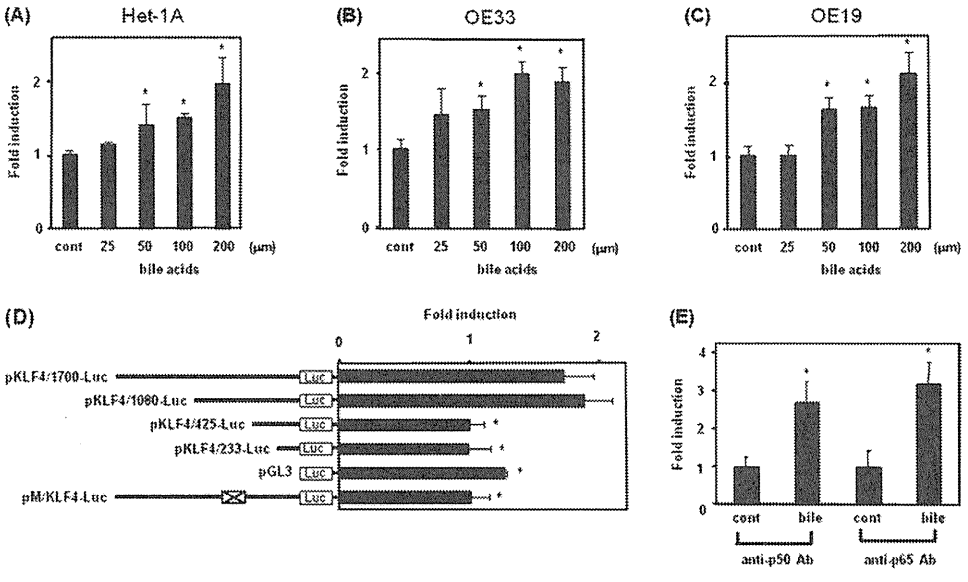

Figure 2 Effects of bile acids on transcriptional activation of KLF4 in (A) Het-1A, (B) OE33, and (C) OE19 cells. Twenty-four hours after transfection with the KLF4 promoter vector, cells were stimulated with various concentrations of the bile acids mixture or vehicle alone for 3 h, then cell lysates were used to determine luciferase activity. Results are expressed as the mean ± SEM of four experiments. *p<0.05 vs. control. (D) Reporter gene analysis of KLF4 promoter deletion and mutation constructs in Het-1A cells. Twenty-four hours after transfection with the indicated KLF4 promoter vectors, cells were stimulated with the bile acids mixture (200 μM) or vehicle alone for 3 h. Results are expressed as the mean ± SEM of four experiments. *p<0.05 vs. pKLF4/1700-Luc. (E) Chromatin immunoprecipitation assay. At 24 h after transfection with the KLF4 promoter vector, Het-1A cells were treated with the bile acids mixture (200 μM) or vehicle alone for 3 h. Anti-p50 and anti-p65 antibody immunoprecipitated DNA was purified and analysed by real-time PCR for the KLF4 promoter, including the NF-κB binding site. The amount of precipitated DNA was normalised to input DNA. Results are expressed as the mean ± SEM of three experiments. *p<0.05 vs. control.