Attenuation of acid induced oesophagitis in VR-1 deficient mice

K Fujino, S G de la Fuente, Y Takami, T Takahashi, C R Mantyh

Background and aims: Activation of the vanilloid receptor subtype 1 (VR-1) results in release of proinflammatory peptides which initiate an inflammatory cascade known as neurogenic inflammation. We investigated its role in an acute model of surgically induced oesophagitis.

Methods: Oesophagitis was induced by pyloric ligation in wild-type and VR-1 deficient mice. A subset of animals were administered the VR-1 antagonist capsazepine, famotidine, or omeprazole one hour before surgery. Five hours after surgery, myeloperoxidase activity (MPO), histological damage scores, intragastric pH, and immunocytochemical analysis of substance P (SP) receptor endocytosis were determined.

Results: Oesophagitis induced knockout mice exhibited significantly lower levels of MPO activity, histological damage scores, and SP receptor endocytosis than wild-type mice. Inflammatory parameters were significantly reduced by acid inhibition and capsazepine in wild-type mice.

Conclusions: We conclude that acute acid induced oesophagitis is reduced in animals lacking VR-1. This suggests that acid induced oesophagitis may act through VR-1 and that inhibition of the receptor may reduce inflammation.
MATERIALS AND METHODS

Animals

These studies were conducted at the Durham Veterans Affairs Medical Center, Durham, North Carolina, USA. All aspects of the research were reviewed and approved by the institution’s animal care and use committee. Male 8–12 week C57BL/6 wild-type (VR-1 +/+ ) and VR-1 deficient (VR-1 −/− ) mice were purchased from an authorized vendor (Charles Rivers Laboratories, Raleigh, North Carolina, USA) or obtained by Dr Sidney Simon and housed under standard laboratory conditions until used. Mice were genotyped using standard polymerase chain reaction (PCR) technique. Genotypic screening of offspring was done as previously described by PCR using tail clip DNA. Briefly, tails were cut into 0.4–0.6 cm pieces and DNA isolated using a QIAamp kit from Qiagen (Valencia, California, USA). Samples were incubated in 180 μl of buffer ATL and 20 μl proteinase K, vortexed, and incubated at 55˚C until the tissue was completely lysed. After lysis was completed, a 400 μl buffer AL-ethanol mixture was added to the sample and mixed vigorously by vortexing. The mixture was then pipetted into a DNeasy mini column setting in a 2 ml collection tube and centrifuged at 6000 g (8000 rpm) for one minute; 500 μl of buffer AW1 and 500 μl of buffer AW2 were mixed with the sample and centrifuged for three minutes at full speed to dry the DNeasy membrane. The DNeasy mini column was placed in a clean 2 ml microcentrifuge tube and 200 μl of buffer AE were pipetted directly onto the DNeasy membrane. The final mixture was incubated at room temperature for one minute, and then centrifuged for one minute at 6000 g.

PCR amplification was carried out with primers (0.5 μl), 10 nM dNTPs (0.5 μl), MgCl2 (1.5 μl), and Taq polymerase (0.1 U) for 34 cycles with an annealing of temperatures of 94˚C for 30 seconds, 55 ˚C for 30 seconds, and 72˚C for one minute. Primers for wild-type mice were CGA GGA TGG GAA TAA CTC ACT, Mw 7435.8 μg/μmol and GGA TGA TGA AGA CGA CCT TGA AGT, Mw 7466.8 μg/μmol from Invitrogen (Carlsbad, California, USA). Primers for knockout screen consisted of AAT GGG CAG GTA GCC GGA TGA TCA AGC G, Mw 7782.0 μg/μmol and AAC AGA CAA TCG GCT GCT CGT ATG C, Mw 7632.0 μg/μmol from Invitrogen. PCR products were then separated on an agarose gel and products were visualised with ethidium bromide.

Induction of acid reflux oesophagitis

Animals were deprived of food but not water 18 hours prior to the studies. On the day of surgery, animals were weighed and anaesthesia was induced by injecting an intraperitoneal solution containing 90 mg/kg ketamine:10 mg/kg xylazine. Each procedure started once the animal exhibited no response to a toe pinch. Wild-type and VR-1 deficient animals received either an acid inducing oesophagitis (n = 6 each genotype) procedure or a sham operation (n = 6 each genotype). The abdominal area was clipped of fur with an electric razor and prepped with three washes of betadine. A small upper midline incision was made and both the pylorus and junction between the forestomach and corpus were ligated as previous reported.24 The non-glandular corpus region of the mouse stomach is achlorhydric with a pH of 6–7, and therefore to increase the acid contact the forestomach was ligated with an additional suture. For the sham operation, animals had an identical procedure except that the ligatures were loosely applied around the pylorus and corpus, respectively, and not tied. The animals were kept under anaesthesia for five hours, and then the entire oesophagus and stomach were removed and fixed in paraformaldehyde for further examination. The animals were then euthanised by cervical dislocation.

Because of our provocative results from these experiments, we elected to further study the effects of antisecretory agents and a VR-1 antagonist on two sets of mice. Thus a subset of wild-type and VR-1 (−−) mice were randomized to receive capsaizepin (100 μmol/kg, n = 6; Tocris, Ellisville, Missouri, USA), famotidine (10 mg/kg, n = 6; Sigma St Louis, Missouri, USA), or omeprazole (400 μmol/ kg, n = 6; Sigma). Administration of drugs was performed in all cases subcutaneously one hour prior to the operation. The dose of each drug was extrapolated from other mouse studies where acid secretion25 or VR-1 inhibition26 were dose dependently studied.

Histological examination

The removed distal oesophagus and proximal stomachs were paraffin embedded and subsequently cut into 5 μm sections. Sections were stained with haematoxylin-eosin and graded according to a previously validated scale (Table 1).27 The scale takes into account the presence of mucosal injury as well as infiltration of inflammatory cells within the lamina propria or submucosa. All specimens were scored by observers unaware of the treatment groups.

Intragastric pH measurement

Intragastric content was extracted from the removed specimens and centrifuged at 4˚C for two minutes. Intragastric pH was measured using a Corning 430 pH meter (Corning, New York, USA).

Myeloperoxidase (MPO) activity

Segments of the distal oesophagus were removed immediately after euthanasia and stored frozen at −80˚C for MPO activity measurement, as previously described.28 Specimens were weighed, placed in a plastic tube on ice, and 0.5% of hexadecyltrimethylammonium bromide (HTAB) in 50 mM KH2PO4 (pH 6) (HTAB buffer) were added to each sample. Samples were homogenised on ice using a Polytron tissue homogeniser for 15 seconds followed by three cycles of freeze/thawing. All samples were fortified with additional HTAB buffer to equal 1 ml HTAB/50 mg wet weight. Samples were then vortexed and 500 μl of each were transferred to microfuge tubes. Tubes were centrifuged in an Eppendorf microfuge (12 000 g) at 4˚C for two minutes and absorbance of each supernatant was read at 460 nm at 0, 30, and 60 seconds after addition of 2.9 ml of 0.167 mg/ml O-dianisidine dihydrochloride to 0.1 ml of supernatant. One unit of MPO activity was defined as degradation of 1 mol of peroxide per minute at 25˚C; the results are expressed in units per gram of protein.
Immunocytochemical analysis of SP release and quantification of NK-1 receptor endocytosis

SP release was assessed by analysis of NK-1 receptor endocytosis, as described previously. Briefly, portions of distal oesophagus from VR-1 (+/−), VR-1 (+/+), VR-1 (+/−) treated with famotidine, VR-1 (+/+) treated with omeprazole, VR-1 (+/−) treated with capsaicin, and VR-1 (−/−) mice were fixed overnight in ice cold freshly depolymerised paraformaldehyde (4% in phosphate buffered saline) at 4°C and then placed in ice cold phosphate buffered saline-30% sucrose for 24 hours. Tissue was then embedded in Tissue Tek OCT (Sakura, Torrance, California, USA), frozen, sectioned at 20 μm, mounted on Superfrost Plus glass slides (Fisher, Pittsburgh, Pennsylvania, USA), and dried with desiccant at room temperature for four hours. After being washed, sections were stained overnight at room temperature using a rabbit antiserum (No 11886-5 from Steven Vigna) specific for the COOH terminal 15 amino acids of the rat NK-1 receptor (SPR393–407) at a dilution of 1:3000. Sections were then washed and incubated with cyanine 3-conjugated IgG secondary antibody (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) at a dilution of 1:200 for the COOH terminal 15 amino acids of the rat NK-1 receptor (SPR393–407) at a dilution of 1:3000. Sections were then washed and coverslipped using one drop of Aquamount (Lerner Laboratories, Pittsburgh, Pennsylvania, USA) and dried with desiccant at room temperature for 24 hours. Tissue was then embedded in Tissue Tek OCT (Sakura, Torrance, California, USA), frozen, sectioned at 20 μm, mounted on Superfrost Plus glass slides (Fisher, Pittsburgh, Pennsylvania, USA), and dried with desiccant at room temperature for four hours. After being washed, sections were stained overnight at room temperature using a rabbit antiserum (No 11886-5 from Steven Vigna) specific for the COOH terminal 15 amino acids of the rat NK-1 receptor (SPR393–407) at a dilution of 1:3000. Sections were then washed and incubated with cyanine 3-conjugated IgG secondary antibody (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) at a dilution of 1:200 for the COOH terminal 15 amino acids of the rat NK-1 receptor (SPR393–407) at a dilution of 1:3000. Sections were then washed and coverslipped using one drop of Aquamount (Lerner Laboratories, Pittsburgh, Pennsylvania, USA). Immunostained sections were analysed using a Zeiss LSM-410 inverted krypton-argon confocal laser scanning system coupled to a Zeiss Axiosvert 100 microscope. Optical sections (0.5 μm) of 512×512 pixels were obtained and processed using Adobe PhotoDeluxe. Quantification of NK-1 receptor endocytosis was performed by analysing 10 NK-1 receptor immunoreactive oesophageal myenteric plexus neurones per mouse and determining the number of these cells containing >50 NK-1 receptor immunoreactive endosomes. Cytoplasmic endosomes were distinguished from plasma membrane associated NK-1 receptor immunoreactivity by ensuring that the nucleus of the cell was in the same optical section as the NK-1 receptor immunoreactive endosomes.

Figure 1: Myeloperoxidase (MPO) activity in sham operated and acid induced animals given famotidine, omeprazole, and capsaicin one hour prior to surgery. MPO activity was significantly increased in both vanilloid receptor subtype 1 (VR-1) (+/−) and VR-1 (−/−) acid exposed groups compared with sham operated animals (p<0.05). VR-1 (−/−) mice exhibited lower levels of MPO compared with VR-1 (+/−) (p<0.05). MPO activity was also significantly reduced by famotidine, omeprazole, and capsaicin (p<0.05). Data are presented as mean (SEM).

Figure 2: Pathological scores in sham operated and acid induced animals given famotidine, omeprazole, and capsaicin one hour prior to surgery. Scores were significantly increased in acid induced operated animals compared with sham operated mice (p<0.05). In acid induced operated mice, vanilloid receptor subtype 1 (VR-1) (+/−) scores were significantly higher than in VR-1 (−/−) mice, famotidine treated animals, omeprazole treated animals, and capsaicin treated mice (p<0.05). Data are presented as mean (SEM).

Statistical analysis

Data are presented as mean (SEM), except where indicated. Statistical analyses were performed using ANOVA followed by Dunnett’s or Tukey-Kramer post tests as indicated. Statistical analysis was done using GraphPad Instat version 3.00 for Windows 95 (GraphPad Software, San Diego, California, USA). Significance was assumed at p<0.05.

RESULTS

The acid induction model used produced significant levels of inflammation characterised by increased MPO activity, pathological scores, and histology compatible with oesophagitis. Both wild-type and VR-1 deficient mice exposed to the acid induction operation exhibited significantly higher levels of MPO activity compared with those within the sham operation group (fig 1). Figure 1 also illustrates the significantly higher MPO activity in VR-1 (+/+) compared with VR-1 (−/−) mice in acid exposed animals.

Similar to MPO activity, pathological scores were significantly increased by the acid induced operation in both wild-type and knockout mice (fig 2) but VR-1 (−/−) mice demonstrated significantly less pathological damage than VR-1 (+/+) animals.

Microscopic architecture was maintained and only a few inflammatory cells were observed in the lamina propria and mucosa of animals in the sham operated group (fig 3A, B) in both VR-1 (+/+) and (−/−) mice. Conversely, VR-1 (+/+) mice exposed to acid by the surgical ligation technique exhibited consistent erosions with infiltration of inflammatory cells, and disorganised architecture (fig 3C). However, VR-1 (−/−) mice exposed to the acid induction operation showed histological patterns similar to those seen in the sham operated group, with minimal to no inflammatory changes (fig 3D).
To test the effect of acid suppression in this model of acute oesophagitis, a subset of wild-type and VR-1 deficient mice were pretreated with famotidine, a histamine antagonist, or omeprazole, a proton pump inhibitor. Additionally, pretreatment with capsazepine, a VR-1 antagonist, was compared with animals in which antisecretory therapy was administered. Figure 1 illustrates MPO activity in sham operated mice and mice instrumented with an acid induction operation treated with capsazepine, famotidine, or omeprazole. MPO activity was significantly increased in wild-type mice exposed to acid; MPO levels were significantly reduced by pharmacological inhibition of acid with famotidine or omeprazole as well as the VR-1 antagonist capsazepine. The inhibition in MPO activity levels observed after treatment with capsazepine was statistically similar to that present in omeprazole treated animals. Similar to MPO, the pathological damage score was also significantly reduced with famotidine, omeprazole, or capsazepine pretreatment in wild-type animals (fig 2). There was no significant difference in pathological score between VR-1 (-/-) animals and wild-type animals treated with omeprazole, famotidine, or capsazepine.

Intragastric pH was also measured in all groups tested (table 2). Intragastric pH was significantly lower in both VR-1 (+/+ and (-/-) mice undergoing surgically induced oesophagitis. However, wild-type mice pretreated with famotidine, omeprazole, or capsazepine had significantly elevated pH levels in the oesophagitis model. Capsazepine pretreatment resulted in intragastric pH levels comparable with those of antisecretory therapy.

**Confocal microscopy and SP release**

Confocal microscopy of the myenteric plexuses of the lower oesophagus demonstrated normal distribution of the NK-1 receptor localised in the plasma membrane of neurones in animals in the sham operated group (fig 4A). However, in wild-type mice exposed to acid, the NK-1 receptor was observed in multiple intracellular cytoplasmic endosomes.

### Table 2 Intragastric pH in mice with either a sham operation or oesophagitis

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Operation</th>
<th>Drug</th>
<th>Intragastric pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>VR-1 (+/+)</td>
<td>Sham</td>
<td></td>
<td>6.12 (0.89)</td>
</tr>
<tr>
<td>VR-1 (-/-)</td>
<td>Sham</td>
<td></td>
<td>5.98 (0.95)</td>
</tr>
<tr>
<td>VR-1 (+/+)</td>
<td>Oesophagitis</td>
<td>Famotidine</td>
<td>2.15 (0.43)</td>
</tr>
<tr>
<td>VR-1 (-/-)</td>
<td>Oesophagitis</td>
<td></td>
<td>2.28 (0.33)</td>
</tr>
<tr>
<td>VR-1 (+/+)</td>
<td>Oesophagitis</td>
<td>Omeprazole</td>
<td>5.81 (0.35)**</td>
</tr>
<tr>
<td>VR-1 (+/+)</td>
<td>Oesophagitis</td>
<td>Capsazepine</td>
<td>4.33 (0.80)**</td>
</tr>
</tbody>
</table>

VR-1, vanilloid receptor subtype 1.

A subset of mice were administered famotidine, omeprazole, or capsazepine. Note that sham operated mice did not have a significant reduction in intragastric pH, while both (+/+) and (-/-) mice with oesophagitis had a significant reduction in pH. Note the increase in pH levels following antisecretory and VR-1 antagonism therapy. Values are mean (SD).

**p<0.01 compared with VR-1 (+/+).
indicating SP release and NK-1 receptor binding and subsequent internalisation (Fig 4B). We have previously demonstrated that this represents accurate SP release and receptor activation. Capsazepine pretreatment inhibited SP release and subsequent endocytosis, as seen by the majority of NK-1 receptors found on the plasma membrane (Fig 4C).

Quantification of internalisation was performed using a previously established method of internalised NK-1 receptor positive endosomes (Fig 5). Minimal endocytosis was found in sham operated animals but significant elevation in NK-1 endocytosis was seen in surgically induced oesophagitis. However, VR-1 (-/-) animals and VR-1 (+/+ plus capsazepine) animals with oesophagitis pretreated with famotidine, omeprazole, and capsazepine demonstrated a significant reduction in NK-1 endocytosis in comparison with sham operated wild-type animals.

DISCUSSION

In this report we investigated the role of VR-1 in an acute model of oesophagitis. Our results indicate that acid induced oesophagitis is significantly attenuated in animals lacking VR-1 or by pharmacologically antagonising the receptor in wild-type mice. The magnitude of the reduction in inflammation was similar to that produced by standard antisecretory therapy. Additionally, reduction of SP release and subsequent NK-1 receptor activation (as measured by NK-1 receptor endocytosis) mirrored the decrease in inflammation indexes in mice either deficient in VR-1 or wild-type mice provided with antisecretory drugs. These results strongly suggest that in this model of oesophagitis, acid exposure stimulates VR-1, which subsequently releases SP to initiate an inflammatory cascade.

VR-1 is a non-selective cation channel with six transmembrane domains that is found on primary sensory neurones and provides sensory information of chemical, physical, and inflammatory input. The receptor has been localised using immunohistochemistry in numerous locations throughout the gastrointestinal tract, including the oesophagus, stomach, small intestine, and colon. VR-1 immunoreactivity is found predominantly in nerve fibres of the myenteric plexus, with lesser staining in the lamina propria and nerve endings that innervate the mucosa. Interestingly, such nerve endings seem not to penetrate the entire extent of the mucosa, suggesting that the luminal stimulants that act on VR-1 either pass across the mucosa or enhance the production of secondary mediators to operate on the receptor. Recent studies indicate that VR-1 containing primary sensory neurones are present in the lower portions of the oesophagus and that the number of immunoreactive fibres increase in patients with oesophagitis.

Stimulation of VR-1 containing primary sensory neurones release neuropeptides, including SP, that convey nociceptive information to the spinal cord and also modulate inflammation in peripheral tissues via an axon reflex. Known agonists of VR-1 include heat, acid, and capsaicin. Additionally, a variety of endogenous ligands have emerged as potential agonists of VR-1. Work from our group and others have demonstrated that inhibition of VR-1 using selective antagonists, or surgical sensory denervation, attenuates several animal models of intestinal inflammation. Other investigators have reported similar findings in models of pancreatitis and cystitis. In addition to the reduction in inflammatory parameters to basal levels in these models, sensory nerve ablation or inhibition of VR-1 using selective VR-1 antagonists also significantly decreased NK-1 receptor internalisation, indicating diminution of SP release. This suggested that in these models, sensory nerves containing VR-1 are stimulated which results in SP release and subsequent inflammation.

In this study we intended to examine the effect of endogenous acid, a known agonist of VR-1, in mice either deficient in VR-1 or mice provided with an antagonist to VR-1 prior to acid exposure. In this model, ligation of the pylorus and forestomach results in rapid infusion of acid into the
osophagus (as demonstrated by the significant reduction in intragastric pH (table 2)) and in a robust inflammatory response with a significant increase in MPO activity and pathological damage. Mice genetically deficient in VR-1 (VR-1 (−/−)) demonstrated a significant reduction in MPO and pathological damage compared with wild-type animals (figs 1–2). This reduction in MPO and pathological scoring in VR-1 (−/−) mice was similar to mice that were preoperatively treated with standard antisecretory agents, famotidine and omeprazole, indicating that acid was the causative agent in initiating inflammation. Intragastric pH was elevated in these animals, strongly suggesting that inhibition of acid production prevented inflammation. Furthermore, pretreatment with capsazepine, an antagonist of VR-1, in VR-1 (+/+ or −/−) animals, demonstrated a nearly identical inhibition of inflammation as famotidine and omeprazole. Interestingly, capsazepine pretreatment resulted in an increase in intragastric pH. To date, there is no reported effect of capsazepine on acid secretion. The cause of this is not clear but several reports indicate that capsazepine may inhibit nicotinic acetylcholine receptors and non-specifically block voltage activated calcium channels.23 24

We also examined the effects of genetic deletion or antagonism of VR-1 on SP receptor internalisation in acid exposed mice. Quantification of the SP receptor using confocal microscopy has been shown to correspond to SP release from sensory neurones and has been shown to directly correlate with inflammation. Similar to previous work, SP release (as measured by SP receptor internalisation) mirrored the inflammatory parameters of MPO and pathological damage (figs 1, 2). Wild-type mice and mice pretreated with famotidine, omeprazole, and capsazepine demonstrated a significant reduction in SP receptor internalisation, suggesting that without acid, VR-1 is not stimulated to release SP. Furthermore, VR-1 deficient animals with acid exposure (pH 2.15) demonstrated a significant reduction in NK-1 endocytosis, indicating that without VR-1, acid cannot stimulate VR-1 to release SP.

Clinically, these results may provide insight into the pathophysiology of the presence of chronic cough and asthma in patients suffering form reflux disease. Previously, this association has been explained by several physiopathological mechanisms, including: (1) a vagal-oesophageal-bronchial reflex; (2) sensitive bronchial reactivity; and (3) microaspiration of gastric contents (reviewed by Hardinge).25 Hamamoto and colleagues26 proposed an alternative pathway in which C fibres contained in the vagal nerve elicit tachykinins release that contract the bronchial smooth muscle, increase bronchial secretion,27 and increase vascular permeability.28 29 The use of specific tachykinin receptor antagonists has been used to prevent bronchoconstriction and airway oedema in different animal models of acid induced reflux disease.30 31 To date, the involvement of VR-1 on this oesophageal-pulmonary circuit is unknown but the availability of potent VR-1 antagonists and animals deficient in VR-1 may clarify the role of the receptor in this inflammatory pathway.

Although these results are provocative, direct clinical correlation with GORD patients must be tempered on the basis of two arguments. Firstly, the animal model used in our experiments represents an acute (five hours) oesophagitis model that might not reflect the chronic process present in the majority of patients presenting with severe oesophagitis. Secondly, we used a model in which only acid was refluxated into the oesophagus. Although acid is a primary factor in the inflammatory process associated with oesophagitis, the role of biliary components in oesophagitis has been emphasised by many authors.32 Further studies are warranted to determine the effect of VR-1 inhibition in chronic acid exposure or alkaline reflux. Finally, the effects of genetic deletion of VR-1 or receptor antagonism do not to completely inhibit the effects of acid induced inflammation. As demonstrated in figs 1, 2, and 5, VR-1 (−/−) animals exposed to acid were found to have a significant reduction in inflammatory parameters and NK-1 endocytosis compared with wild-type animals but levels did not return to sham operated levels, indicating that other inflammatory processes are occurring.

In conclusion, this study presents direct evidence that acid induced oesohagitis is attenuated in mice deficient in VR-1. The reduction in inflammatory indices and pathological scoring mirrored the effects of standard antisecretory therapies. Additionally, SP release (and receptor endocytosis) was also reduced in VR-1 (−/−) mice and wild-type mice given capsazepine, indicating that acid stimulates VR-1 containing sensory neurones which in turn release SP. These results suggest a link between VR-1 and acid induced oesophagitis that might help elucidate the physiopathology of oesophagitis and provide a novel therapeutic target for patients with gastro-oesophageal reflux disease.

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Conflict of interest: None declared.

REFERENCES
EDITOR’S QUIZ: GI SNAPSHOT

Answer

From question on page 15

The biopsy was consistent with desmoid tumour. Desmoid tumours are one of the extracolonic manifestations of familial adenomatous polyposis (FAP). They represent the most common cause of death in FAP after colorectal cancer. The incidence of desmoid tumours in patients with FAP varies between 10% and 18%. Three major factors seem to play a role in the development of desmoid tumours: surgical trauma, oestrogen, and genetic factors. Despite their benign histopathological appearance, these tumours can be very aggressive and locally invasive. The clinical presentation of desmoid tumours varies from an asymptomatic abdominal wall mass, to bowel and ureteral obstruction, perforation, or haemorrhage. Medical treatment with anti-oestrogens, steroids, colchicine, interferon α, and warfarin has been tried with minimal success. Chemotherapy has been used with promising results. Surgery should be limited to acute or chronic small bowel obstruction because of the high rate of recurrence.

doi: 10.1136/gut.2005.065664

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