

Original article

Dysregulation of the Wnt pathway in adult eosinophilic esophagitis

M. Giannetti,¹ H. A. Schroeder,¹ A. Zalewski,² N. Gonsalves,² P. J. Bryce¹

Divisions of ¹Allergy-Immunology and ²Gastroenterology, Department of Medicine, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, USA

SUMMARY. Eosinophilic esophagitis (EoE) is a chronic inflammatory disease characterized pathologically by eosinophil infiltration. In addition to loss of barrier integrity, a dominant T Helper 2-associated immune response and strong allergic connection, the esophagus tissue undergoes dramatic changes, with frequent presence of mucosal rings, strictures, linear furrows, and trachealization. Although the inflammatory mechanisms behind this disease are being increasingly well understood, the structural features remain unexplained. We examined the expression of key members of the Wnt-signaling pathway in biopsies from patients with EoE. This pathway has been shown to be critically important in regulating cellular homeostasis, growth, and differentiation and to be dysregulated in several disease conditions. Biopsies from adult EoE patients were collected by endoscopy and mRNA extracted. After cDNA synthesis, the relative gene expression from key upstream (secreted frizzled-related protein 1) and downstream (c-myc and Cyclin D1) molecules in the Wnt pathway, as well as several Wnt pathway members (Wnt1, Axin1, low-density lipoprotein receptor-related protein 6, glycogen synthase kinase 3 beta, and β -catenin), were determined. Biopsies from patients with EoE displayed significantly higher expression of secreted frizzled-related protein 1 than controls, as well as reductions in Cyclin D1 and c-myc. In contrast, there were no differences in the Wnt pathway molecules. The levels of expression of Cyclin D1 and c-myc, as well as β -catenin, in EoE patients showed strong correlations with the frequency of esophageal eosinophils. Our findings suggest that although there are no changes in the overall levels of key Wnt pathway genes in adult EoE, there is evidence for dysregulation of upstream and downstream regulators of Wnt signaling. Importantly, the associations with eosinophilia suggest that these may participate in the pathogenesis of this disease and be markers of disease severity.

KEY WORDS: eosinophilic esophagitis, remodeling, signaling.

INTRODUCTION

Eosinophilic esophagitis (EoE) is a chronic inflammatory disorder, characterized by the presence of eosinophils in the esophageal tissue that is seen in both children and adults. Clinically, EoE is associated with significant esophageal dysfunction that can cause dysphagia, food impaction, failure to thrive, heartburn, or abdominal pain.¹ A characteristic feature seen on endoscopy that likely explains these responses are the presence of macroscopic changes in the esophageal tissue that cause narrowing of the

diameter, including the formation of rings, strictures, or furrowing.² Although there has been much interest in EoE in children, the adult form remains less understood.³ There is evidence both for and against childhood and adult EoE being a single disease, yet a key difference seems to be the presence and severity of these structural changes.⁴

The inflammatory processes underlying EoE are being increasingly defined with key roles identified for allergen-mediated triggering, pathology driven by mast cells, interleukin-13, and eotaxin chemokines.⁵⁻⁸ Epithelial barrier integrity is thought to be a key component in protection against allergen. Prior studies have demonstrated downregulation of molecules important in maintaining the epithelial barrier, such as filaggrin and involucrin.^{6,9} Furthermore, allergen avoidance is sufficient to diminish esophageal eosinophils in many EoE patients.¹⁰ In contrast, the pathogenesis of the structural changes remains unclear.

Address correspondence to: Dr Paul J. Bryce, PhD, Division of Allergy-Immunology, Northwestern University Department of Medicine, 240 E. Huron Avenue, Chicago, IL 60611, USA.
Email: p-bryce@northwestern.edu

Financial support: This work was supported by funding from the CURED Foundation (to PJB and NG) and The Sunshine Charitable Foundation (to PJB).

The Wnt pathway consists of a family of proteins known to exert potent control over proliferation and differentiation of healthy intestinal tissues – dysregulation of this pathway has been extensively studied in the setting of cancer.¹¹ In the esophagus, Wnt family members are expressed widely and are dysregulated in Barrett's esophagus.^{12,13} For example, secreted frizzled-related protein 1 (SFRP1), an important upstream inhibitor of Wnt signaling at high concentrations,¹⁴ is significantly downregulated in Barrett's esophagus.¹⁵ The outcome of such changes in Wnt pathway responses in esophageal cancer is the enhanced expression of several growth-promoting genes, including c-Myc and Cyclin D1.¹⁶ Interestingly, microarray data from pediatric EoE biopsies reported a fourfold increase in SFRP1 above control levels,¹⁷ but this previous study focused on other key genes.

Here we examined the expression profiles of several key Wnt pathway components, focusing on adult EoE, because these patients exhibit more structural changes. Surprisingly, rather than increased activation of this pathway such as seen in Barrett's esophagus, adult EoE had significant upregulation in SFRP1 expression, similar to the pediatric EoE microarray. There were no significant alterations found in the Wnt family proteins, but there was downregulation in the Wnt-signaling target genes Cyclin D1 and c-myc. These negatively correlated with the levels of eosinophils present in the esophageal tissues of these patients, suggesting that the loss of Wnt pathway signals may relate to the severity of EoE disease.

METHODS

Patients

All patients were enrolled in a prospective clinical trial assessing EoE responses to food elimination and consented to gene expression analysis on biopsy tissues taken during esophagogastroduodenoscopy. Additional biopsies were taken for pathological assessment of eosinophil frequency, which is standard of care and used to diagnose EoE based on greater than 15 eosinophils per high-powered field (Eos/hpf). Patients were all over 18 years old and had undergone an unsuccessful treatment period of 6–8 weeks of proton pump inhibitor therapy prior to assessment, as necessitated by current consensus guidelines.¹ Controls were determined as those patients with less than 15 Eos/hpf. The control group and EoE group did not significantly differ in sex or age but differed in biopsy eosinophil counts (0 ± 0 vs. 57.2 ± 6.8 , $P < 0.005$ by Student's *t*-test). The Northwestern University institutional review board committee approved this work.

Biopsy processing

Esophageal biopsies were taken and immediately placed in RNAlater reagent (Qiagen, Valencia, CA, USA) to stabilize mRNA and stored until processing. After homogenization using a TissueMiser hand-held homogenizer (Fisher Scientific, Pittsburgh, PA, USA), mRNA was extracted using an RNeasy extraction kit (Qiagen), as per the manufacturers protocol. The quality and quantity of the mRNA was determined using a Nanodrop (NanoDrop Products, Wilmington, DE, USA).

Real-time reverse transcription polymerase chain reaction (RT-PCR)

After cDNA synthesis using qScript cDNA synthesis kit (Quanta BioSciences, Gaithersburg, MD, USA), gene expression was determined by polymerase chain reaction (PCR) using an ABI 7500 Thermal Cycler (Applied Biosystems, Grand Island, NY, USA) and inventoried, specific Taqman primer/probe reagents (Applied Biosystems) for each gene of interest (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]: Hs02758991, SFRP1: Hs00610060, WNT: Hs01011247, β -catenin: Hs00394718, Axin1: Hs00394718, GSK-3 β : Hs01047719, CyclinD: Hs00765553, c-Myc: Hs00153408). The PCR reaction was run for 50 cycles of 30 seconds at 90°C, 60 seconds at 56°C, 120 seconds at 72°C. Expression of GAPDH was utilized as a housekeeping gene and used to determine changes in the cycle threshold values, as previously shown.⁸

Statistical analysis

Statistical differences between groups were determined using Graphpad Prism (San Diego, CA, USA) software and unpaired *t*-test comparisons with Welch's correction to not assume equal variance.

RESULTS

SFRP1 expression is enhanced in adult EoE

Although not a primary focus of the study, because existing microarray data from pediatric EoE had reported a fourfold increase in SFRP1,¹⁷ we initially sought to determine the expression levels of this important Wnt regulator in our adult sample collection. Using real-time RT-PCR and Taqman-based specific primer and probe combinations, mRNA expression of *sfrp1* was determined in EoE positive (greater than 15 Eos/hpf by pathological assessment) versus controls (patients demonstrating less than 15 Eos/hpf by pathological assessment). As shown in Figure 1, *sfrp1* expression was significantly elevated in EoE versus controls. Interestingly, this increase

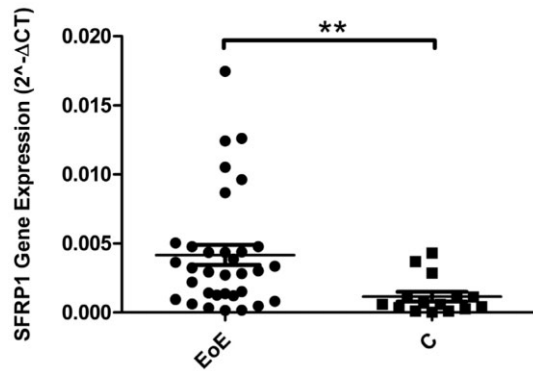


Fig. 1 Expression of secreted frizzled-related protein 1 (SFRP1) is elevated in adult eosinophilic esophagitis (EoE) patients. Biopsy samples from patients with EoE or controls (C) were analyzed by real-time reverse transcription polymerase chain reaction for gene expression of mRNA for SFRP1. The difference in cycle threshold (CT) was determined for each sample compared with the expression of glyceraldehyde-3-phosphate dehydrogenase. ** $P < 0.01$ by Student's t -test.

was also around fourfold, suggesting that the dysregulation of this upstream Wnt-pathway regulator is similarly affected in both adult and pediatric EoE.

Gene expression for many Wnt pathway members are not altered

Since the gene expression for SFRP1 was altered, we next examined if the Wnt pathway was also similarly affected. As shown in Figure 2, the mRNA expression levels for several key Wnt pathway members (Wnt1, β -catenin, Axin1 and GSK-3 β) were unaltered in EoE versus controls (Fig. 2).

Downstream Wnt response genes are downregulated in EoE

We next queried if there were evidence for alterations in the expression levels of genes that are known to be regulated by signaling through the Wnt pathway. *Cyclin D1*, upregulation of which has been shown to be prognostic in esophageal carcinoma studies,¹⁸ showed significantly lower expression in EoE patient biopsies than that in control patient biopsies (Fig. 3a). The gene expression of *c-Myc*, another Wnt target gene in the esophagus,¹⁶ also trended toward less than control, with several EoE patients showing undetectable levels, but this did not meet statistical significance when compared with control biopsy expression levels (Fig. 3b).

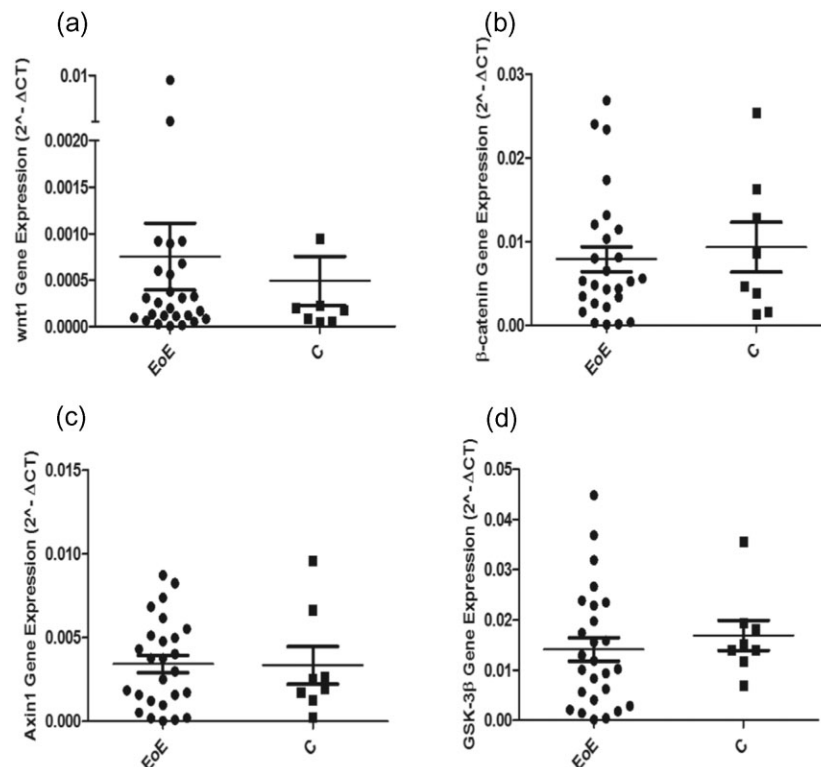


Fig. 2 Gene expression for Wnt pathway members are not altered in adult eosinophilic esophagitis (EoE) patients. Real-time reverse transcription polymerase chain reaction analysis of the expression of mRNA for (a) Wnt1, (b) β -catenin, (c) Axin1 and (d) GSK-3 β was performed on biopsy samples from adult patients with EoE or controls (C). The difference in cycle threshold (CT) was determined for each sample compared to expression of the housekeeping gene, GAPDH.

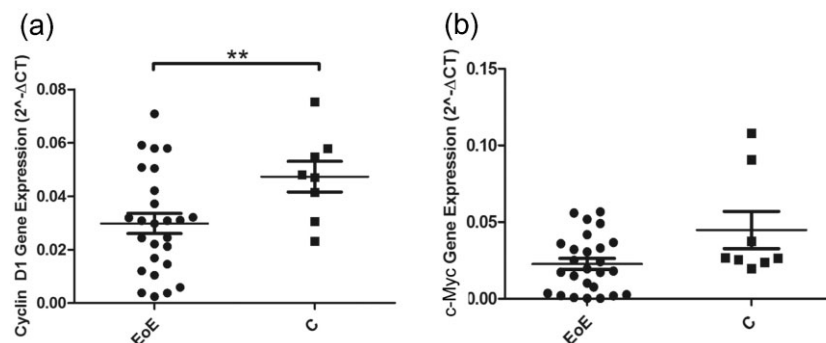


Fig. 3 Downstream Wnt response genes are downregulated in eosinophilic esophagitis (EoE). The expression of mRNA for (a) Cyclin D1 and (b) c-Myc was determined by real-time reverse transcription polymerase chain reaction on biopsy samples from adult patients with EoE or controls (C). The difference in cycle threshold (CT) was determined for each sample compared to expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase. ** $P < 0.01$ by Student's t -test.

Expression of Wnt response genes correlate with clinical pathology

Because many of the gene expression profiles displayed a range of levels within the EoE patient group, we queried if this is related to disease-related parameters. We compared the gene expression values for each patient with the reported frequency of esophageal eosinophils, used for clinical diagnosis. As shown in Figure 4, we observed significant correlations between the expression levels of β -catenin (Fig. 4a), c -Myc (Fig. 4b), and *Cyclin D1* (Fig. 4c) with the eosinophil frequency, used as a surrogate marker for disease severity and response to therapy.¹ This association was particularly strongest with β -catenin and c -Myc, which showed a highly significant correlation.

DISCUSSION

EoE represents an increasingly prevalent disorder in both children and adults,⁴ and there are few treatment options available.¹⁹ In recent years, studies have begun to elucidate mechanisms likely behind the

pathology, including role of allergic triggers and Th2-associated immune responses.²⁰ Additionally, links to epithelial barrier integrity, specifically filaggrin, an important junctional protein, have been demonstrated⁶ and suggest a deficiency in barrier function in EoE, similar to that observed in other allergic diseases.²¹ EoE also presents with several hallmark endoscopic features, including rings, furrows, and strictures, that we postulated might be due to changes in tissue homeostasis through the Wnt pathway because this has been previously shown to be dysregulated in a number of esophageal diseases.^{12,13,15} Surprisingly, our data demonstrates that the Wnt pathway is downregulated, as evident by increased SFRP1, an upstream inhibitor, and decreased Cyclin D1 and c-Myc, downstream response genes.

While the Wnt pathway is often unregulated in aberrant tissue growth of many cancers, including Barretts esophagitis,^{13,18,22} it is also a critically important mechanism that regulates epithelial homeostasis through self-renewal and differentiation.²² It has been suggested that continual stimulation of the Wnt pathway is required for normal proliferation of transit-amplifying cells,²² such as esophageal epithe-

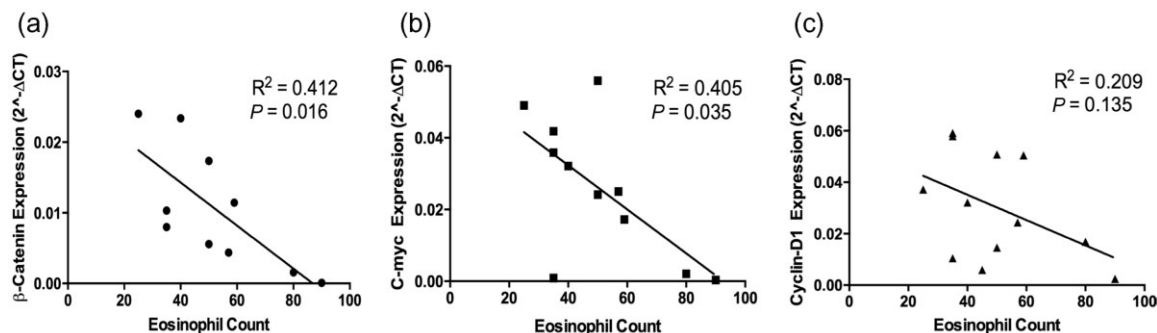


Fig. 4 Expression of Wnt-related genes correlates with clinical pathology. For each individual patient, the reported frequency of eosinophils in paired biopsies was determined by pathological assessment and these numbers correlated with the gene expression values for (a) β -catenin, (b) c-Myc, and (c) Cyclin D1. Linear regression best-fit analysis was performed using GraphPad Prism, and R^2 values and P values were calculated.

lium. Indeed, experimental overexpression of the Wnt upstream inhibitor, Dickkopf Wnt signaling pathway inhibitor 1 (Dkk-1), led to ablation of normal intestinal epithelial cell proliferation.²³ Although we did not observe any significant differences in Dkk-1 (data not shown), our findings show a significant upregulation of SFRP1, another upstream inhibitor, which has been previously shown to be decreased in esophageal cancer.^{13,16} Consequently, loss of epithelial homeostasis via this mechanism may be a contributing factor in the loss of barrier integrity in EoE.

Altered differentiation of epithelial cells, as determined by epithelial-mesenchymal transition of cells from biopsies treated with transforming growth factor beta (TGF β) *ex vivo*, has been demonstrated in EoE and to respond to therapy.²⁴ Mast cell activation is upregulated in EoE⁸ and these cells are a potent source of TGF β ,²⁵ as can be eosinophils.²⁶ The TGF β pathway has been shown to extensively interact with the Wnt pathway,²⁷ but the cross talk between these in EoE remains unclear. Potentially, the upregulation of SFRP1 could be a feedback response designed to limit the effects of TGF β in the esophageal tissue. Alternatively, the responses we observe on the Wnt family pathway may be more directly related to esophageal inflammation, since SFRP1 has been shown to be upregulated in periodontal inflammation and its inhibition led to reductions in inflammatory cell infiltrate and other pathological features.²⁸ Importantly, our data clearly demonstrate that the alterations in the Wnt-associated responses are not at the level of expression of the Wnt-signaling proteins and, instead, would appear to be dysregulation of an upstream regulator that impacts downstream responses.

The strong correlation of the genes we investigated with eosinophil frequency suggests that the Wnt family pathway might be predictive of EoE severity. Clinically, the presence of eosinophils in EoE has been used as a diagnostic marker for therapeutic efficacy in both steroid or dietary therapy studies.^{1,19} Our findings, demonstrating a clear correlation between Wnt-associated pathway gene expression and the frequency of eosinophils in the biopsy from the same patient would suggest that these genes are potential biomarkers for EoE. Currently, there are no clinical biomarkers for EoE, although a recent report described a predictive panel of miRNA molecules that distinguished none-EoE from EoE.²⁹ Interestingly, the two key miRNA molecules from this study, miR-21 and miR-223, have been previously connected to dysregulation of Wnt-signaling pathways in colorectal cancer.³⁰

In conclusion, our findings demonstrate a dysregulation in the Wnt-signaling pathway of the adult EoE patients we studied. While a previous microarray had suggested altered expression of the gene for SFRP1 in EoE,¹⁷ this pathway had not been

fully interrogated, despite being previously shown to be altered in other esophageal diseases.^{2,7,9} In contrast to our prediction, that Wnt pathway responses may be increased and associate with the structural changes characteristic of EoE, our findings show increased expression of SFRP1, an upstream regulator, and decreased expression of Wnt-dependent genes. These link to the severity of eosinophilic inflammation and suggest that Wnt signals are contributing to the inflammatory events in the esophagus.

Acknowledgments

We thank Drs Terrence Barrett (University of Kentucky) and Ikuo Hirano (Northwestern University) for advice and opinions.

References

- 1 Liacouras C A, Furuta G T, Hirano I *et al*. Eosinophilic esophagitis: updated consensus recommendations for children and adults. *J Allergy Clin Immunol* 2011; 128: 3–20, e6; quiz 1–2.
- 2 Hirano I, Moy N, Heckman M G, Thomas C S, Gonsalves N, Achem S R. Endoscopic assessment of the oesophageal features of eosinophilic oesophagitis: validation of a novel classification and grading system. *Gut* 2013; 62: 489–95.
- 3 Gonsalves N, Kahrilas P J. Eosinophilic oesophagitis in adults. *Neurogastroenterol Motil* 2009; 21: 1017–26.
- 4 Straumann A, Aceves S S, Blanchard C *et al*. Pediatric and adult eosinophilic esophagitis: similarities and differences. *Allergy* 2012; 67: 477–90.
- 5 Blanchard C, Mingler M K, Vicario M *et al*. IL-13 involvement in eosinophilic esophagitis: transcriptome analysis and reversibility with glucocorticoids. *J Allergy Clin Immunol* 2007; 120: 1292–300.
- 6 Blanchard C, Stucke E M, Burwinkel K *et al*. Coordinate interaction between IL-13 and epithelial differentiation cluster genes in eosinophilic esophagitis. *J Immunol* 2010; 184: 4033–41.
- 7 Blanchard C, Stucke E M, Rodriguez-Jimenez B *et al*. A striking local esophageal cytokine expression profile in eosinophilic esophagitis. *J Allergy Clin Immunol* 2011; 127: 208–17, 17 e1–7.
- 8 Hsu Blatman K S, Gonsalves N, Hirano I, Bryce P J. Expression of mast cell-associated genes is upregulated in adult eosinophilic esophagitis and responds to steroid or dietary therapy. *J Allergy Clin Immunol* 2011; 127: 1307–8, e3.
- 9 Matoso A, Mukkada V A, Lu S *et al*. Expression microarray analysis identifies novel epithelial-derived protein markers in eosinophilic esophagitis. *Mod Pathol* 2013; 26: 665–76.
- 10 Gonsalves N, Yang G Y, Doerfler B, Ritz S, Ditto A M, Hirano I. Elimination diet effectively treats eosinophilic esophagitis in adults; food reintroduction identifies causative factors. *Gastroenterology* 2012; 142: 1451–9, e1; quiz e14–5.
- 11 de Lau W, Barker N, Clevers H. WNT signaling in the normal intestine and colorectal cancer. *Front Biosci* 2007; 12: 471–91.
- 12 Ali I, Rafiee P, Zheng Y *et al*. Intramucosal distribution of WNT signaling components in human esophagus. *J Clin Gastroenterol* 2009; 43: 327–37.
- 13 Clement G, Braunschweig R, Pasquier N, Bosman F T, Benhattar J. Alterations of the Wnt signaling pathway during the neoplastic progression of Barrett's esophagus. *Oncogene* 2006; 25: 3084–92.
- 14 Zhong X, Desilva T, Lin L *et al*. Regulation of secreted Frizzled-related protein-1 by heparin. *J Biol Chem* 2007; 282: 20523–33.
- 15 Zou H, Molina J R, Harrington J J *et al*. Aberrant methylation of secreted frizzled-related protein genes in esophageal adenocarcinoma and Barrett's esophagus. *Int J Cancer* 2005; 116: 584–91.

- 16 Brown J, Bothma H, Veale R, Willem P. Genomic imbalances in esophageal carcinoma cell lines involve Wnt pathway genes. *World J Gastroenterol* 2011; 17: 2909–23.
- 17 Blanchard C, Wang N, Stringer K F *et al.* Eotaxin-3 and a uniquely conserved gene-expression profile in eosinophilic esophagitis. *J Clin Invest* 2006; 116: 536–47.
- 18 Zhao J, Li L, Wei S *et al.* Clinicopathological and prognostic role of cyclin D1 in esophageal squamous cell carcinoma: a meta-analysis. *Dis Esophagus* 2012; 25: 520–6.
- 19 Vashi R, Hirano I. Diet therapy for eosinophilic esophagitis: when, why and how. *Curr Opin Gastroenterol* 2013; 29: 407–15.
- 20 Rothenberg M E. Biology and treatment of eosinophilic esophagitis. *Gastroenterology* 2009; 137: 1238–49.
- 21 Irvine A D, McLean W H, Leung D Y. Filaggrin mutations associated with skin and allergic diseases. *N Engl J Med* 2011; 365: 1315–27.
- 22 Gregorieff A, Clevers H. Wnt signaling in the intestinal epithelium: from endoderm to cancer. *Genes Dev* 2005; 19: 877–90.
- 23 Kuhnert F, Davis C R, Wang H T *et al.* Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. *Proc Natl Acad Sci U S A* 2004; 101: 266–71.
- 24 Kagalwalla A F, Akhtar N, Woodruff S A *et al.* Eosinophilic esophagitis: epithelial mesenchymal transition contributes to esophageal remodeling and reverses with treatment. *J Allergy Clin Immunol* 2012; 129: 1387–96, e7.
- 25 Aceves S S, Chen D, Newbury R O, Dohil R, Bastian J F, Broide D H. Mast cells infiltrate the esophageal smooth muscle in patients with eosinophilic esophagitis, express TGF-beta1, and increase esophageal smooth muscle contraction. *J Allergy Clin Immunol* 2010; 126: 1198–204, e4.
- 26 Wong D T, Elovic A, Matossian K *et al.* Eosinophils from patients with blood eosinophilia express transforming growth factor beta 1. *Blood* 1991; 78: 2702–7.
- 27 Lam A P, Gottardi C J. β -catenin signaling: a novel mediator of fibrosis and potential therapeutic target. *Curr Opin Rheumatol* 2011; 23: 562–7.
- 28 Li C H, Amar S. Inhibition of SFRP1 reduces severity of periodontitis. *J Dent Res* 2007; 86: 873–7.
- 29 Lu T X, Sherrill J D, Wen T *et al.* MicroRNA signature in patients with eosinophilic esophagitis, reversibility with glucocorticoids, and assessment as disease biomarkers. *J Allergy Clin Immunol* 2012; 129: 1064–75, e9.
- 30 Fu J, Tang W, Du P *et al.* Identifying microRNA-mRNA regulatory network in colorectal cancer by a combination of expression profile and bioinformatics analysis. *BMC Syst Biol* 2012; 6: 68.