

Increased Expression of VEGF, COX-2, and Ki-67 in Barrett's Esophagus: Does the Length Matter?

Evanthia Zampeli · George Karamanolis · George Morfopoulos ·
Elias Xirouchakis · Vasiliki Kalampoki · Spyros Michopoulos · Sotiria Savva ·
Vasilios Tzias · Irene Zouboulis-Vafiadis · Dimitrios Kamberoglou ·
Spiros D. Ladas

Received: 15 September 2011 / Accepted: 15 November 2011
© Springer Science+Business Media, LLC 2011

Abstract

Background Barrett's esophagus (BE) is a major complication of gastroesophageal reflux disease due to its neoplastic potential. The length of the metaplastic epithelium has been associated with cancer risk. Angiogenesis, inflammation, and increased cell proliferation are early events in the malignant sequence. Vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2) and Ki-67 are indirect markers of these complex mechanisms. **Aims** To examine the expression of VEGF, COX-2 and Ki-67 in BE and investigate whether there is an association to Barrett's length.

Methods Immunohistochemistry for VEGF, COX-2, and Ki-67 was performed in well-characterized Barrett's samples, evaluated using a qualitative scale and compared between long (LSBE) and short (SSBE) segments.

Results The study population consisted of 98 patients (78 men). LSBE and SSBE was diagnosed in 33 (33.7%) and 65 (66.3%) cases, respectively. VEGF was expressed in vascular endothelium of all Barrett's specimens. COX-2 and Ki-67 expression in metaplastic epithelia was strong in 81.6 and 61.2% of the samples, respectively. Ki-67

expression was significantly stronger in LSBE ($p = 0.035$), whereas VEGF expression was significantly increased in SSBE ($p = 0.031$). COX-2 expression was not associated with Barrett's length.

Conclusions VEGF, COX-2, and Ki-67 were overexpressed in the majority of Barrett's samples. The length was inversely associated with VEGF expression and directly associated with Ki-67 expression.

Keywords Barrett's esophagus · Length · VEGF · COX-2 · Ki-67

Introduction

Barrett's esophagus (BE) is considered a complication of gastroesophageal reflux disease (GERD). It entails the replacement of squamous epithelium in the distal esophagus by intestinal epithelium, which is characterized by the presence of goblet cells [1]. The clinical significance of BE rests on its undisputed association with esophageal adenocarcinoma [2].

The prevailing hypothesis regarding BE pathogenesis is that esophageal mucosa's injury by the refluxate results in a flawed healing process. The contact of the lower esophageal mucosa with the gastro-duodenal juice generates an inflammatory microenvironment [3]. In this context, genetic, molecular, and immune responses are activated towards diverse directions: esophagitis and/or intestinal metaplasia in a few cases and dysplasia and/or advanced neoplasia on rare occasions [4–7]. Carcinogenesis is a multistep process during which cells acquire specific biological properties called “the hallmarks of cancer” [8, 9]. Barrett's metaplastic cells are predisposed to develop these capabilities and in that sense, relevant molecular markers have long been the subject of investigation [4].

E. Zampeli (✉) · V. Kalampoki · S. Michopoulos
Gastroenterology Department, Alexandra General Hospital,
80 Vas. Sofias Ave., 11528 Athens, Greece
e-mail: evazamb@yahoo.com

G. Karamanolis · I. Zouboulis-Vafiadis · S. D. Ladas
Gastroenterology Division, First Department of Propaedeutic
and Internal Medicine, Athens University Medical School,
Laikon Hospital, Athens, Greece

G. Morfopoulos · E. Xirouchakis · S. Savva · V. Tzias ·
D. Kamberoglou
Gastroenterology and Pathology Department, 1st IKA Hospital,
Athens, Greece

Until now, concurrent evaluation of the molecular markers vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), and Ki-67 in Barrett's epithelium has not been performed. Marked expression of VEGF has previously been shown [10–13]. COX-2 is an inducible enzyme with multiple activities that has been implicated in Barrett's neoplastic sequence. A progressive increase in COX-2 expression has been demonstrated from normal squamous to metaplastic epithelium, dysplasia and invasive neoplasia [14, 15]. Several studies indicated that COX-2 exerts a regulatory effect on VEGF production [10, 12, 16, 17]. Factor Ki-67 is a nuclear protein that is associated with cellular proliferation [18]. As a proliferation marker, Ki-67 has been applied in the immunohistochemical study of various malignancies, where the percentage of positive cancer cells is of prognostic value [19]. The study of Ki-67 in BE has been restricted and complementary to other factors as for example “minichromosome maintenance proteins” and cyclin D1 [20, 21].

Barrett's variable length has been an intriguing issue as several studies have shown that long BE (LSBE) is correlated to increased malignant risk [22–26], whereas others concluded that short BE (SSBE) carries the same risk [27, 28]. Although this remains unresolved, clinical practice guidelines do not take into account Barrett's length. However, probable divergence between LSBE and SSBE in terms of clinical and/or histological parameters would point towards different pathogenesis and/or neoplastic risk.

The aims of our study were to investigate: (1) the immunohistochemical expression of VEGF, COX-2 and Ki-67 in Barrett's biopsy samples and (2) whether there was a correlation between these markers and the length of metaplastic epithelium.

Patients and Methods

Patients

Patients with an endoscopic diagnosis of BE were retrospectively identified through the endoscopic database of a tertiary center in Athens for a period of 7 years. Histologically confirmed cases were included in the study. We excluded patients with cirrhosis, malignancy, heart and respiratory failure, as well as chronic renal disease on the assumption that the “inflammation” that underlies these systemic conditions may affect the expression of the studied molecules. The use of proton pump inhibitors (PPIs) and/or H₂-blockers prior to Barrett's diagnosis and systemic treatment with aspirin and/or non-steroidal anti-inflammatory drugs (NSAIDs) were recorded in the context of the association of COX-2 expression to the consumption of these medications.

Barrett's was endoscopically diagnosed when squamocolumnar junction (Z-line) was displaced proximal to esophagogastric junction (EGJ) either circumferentially or in the form of salmon-colored flames. Long and short BE were defined according to whether the visualized segment was longer or shorter than 3 cm, respectively. In accordance with the department's standard practice, biopsy specimens have been obtained according to the Seattle protocol [29]. All samples were reviewed by two experienced gastrointestinal pathologists: the presence of intestinal metaplasia with goblet cells confirmed the diagnosis of BE. Hematoxylin and eosin (H&E) was the standard stain used, whereas Alcian blue at pH 2.5 was applied in doubtful cases. Pathologists were blinded with respect to Barrett's length.

In order to determine immunohistochemical expression of VEGF, COX-2 and Ki-67 in squamous esophageal epithelium, 33 patients who were referred to our department for various indications and had endoscopically normal esophagus were consecutively recruited and sampled. None of the 33 subjects was on PPIs before undergoing endoscopy. Biopsy specimens from normal esophagus of the control group were placed in 10% formalin and then embedded in paraffin. Sections were prepared for H&E stain and immunohistochemical study.

Informed consent from the control group as well as ethics committee's approval for biologic material use was obtained.

Immunohistochemistry

All tissues had been fixed in neutral buffered formaldehyde and processed into paraffin wax. Three-micrometer-thick sections were cut, dewaxed, and rehydrated in ethanol solutions, then treated with 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase.

Antigen retrieval was performed by heating in a 1-mM EDTA buffer solution (pH 8.0) in a microwave, followed by blocking of non-specific reactivity. Sections were then stained using the following antibodies:

1. Monoclonal antibody Ab7 to VEGF (clone VG1), which recognizes 121,165 and 189 isoforms of VEGF (NeoMarkers/LabVision Corporation, USA, dilution 1:200).
2. Polyclonal antibody to COX-2 protein (Santa Cruz Biotechnology, USA, dilution 1:200).
3. Monoclonal antibody to Ki-67 (clone MIB-1) (Dako-Cytomation, Denmark, dilution 1:500).

All slides were incubated at room temperature with the primary antibodies for 30 min and the immunohistochemical reaction was visualized using the Envision-ChemMate kit (DakoCytomation). Specimens were counterstained

with Mayer's hematoxylin. Negative controls involved the omission of the primary antibody.

Based on an initial review of the VEGF-stained slides and a previously applied method by Lanás et al. [30], immunohistochemical expression of VEGF was evaluated in microvessels' endothelial cells. The distinction between small and large vessels was achieved by using the microscope's ruler (30 μ m): when the vessel's diameter was ≤ 30 μ m the vessel was considered small. The evaluation was qualitative and based on the percentage of positive microvessels: no staining, mild (<33% of the total number of microvessels stained positive for VEGF), moderate (33–66% positive microvessels) and strong staining (>66% positive microvessels).

Methods that have been applied in published studies were minimally revised and used for the evaluation of COX-2 and Ki-67 expression [14, 31, 32]. The assessment was based on the percentage of positive metaplastic epithelia according to the following scale: no staining, mild (<20% positive epithelial cells), moderate (20–40% positive cells), and strong staining (>40% positive cells).

Statistical Analysis

All data were analyzed using the SAS software (Version 9.1, SAS Institute Inc., Cary, NC). Immunohistochemical parameters were univariately compared between the two groups of patients (LSBE and SSBE). The Chi-square test and Fisher's exact test were used as necessary. Significance testing was two-sided and set to <0.05.

Results

Patient Characteristics

Out of 156 cases of endoscopically detected metaplasia, 58 were excluded due to either lack of histological confirmation or severe co-morbidities. The study population

consisted of 98 patients (78 men, mean age 58.3 ± 13.3 years). Long and short BE were diagnosed in 33 (33.7%) and 65 (66.3%) cases, respectively. The mean length for LSBE and SSBE was 5.875 ± 2.156 cm and 1.844 ± 0.507 cm, respectively. Seventy-five (76.5%) patients used PPIs and/or H2-blockers and 12 (12.2%) patients were systemically taking aspirin and/or NSAIDs prior to Barrett's diagnosis. One patient was diagnosed with adenocarcinoma, whereas dysplasia of any grade was found in seven cases (7.1%). We did not study these cases on a separate basis because the overall number was small.

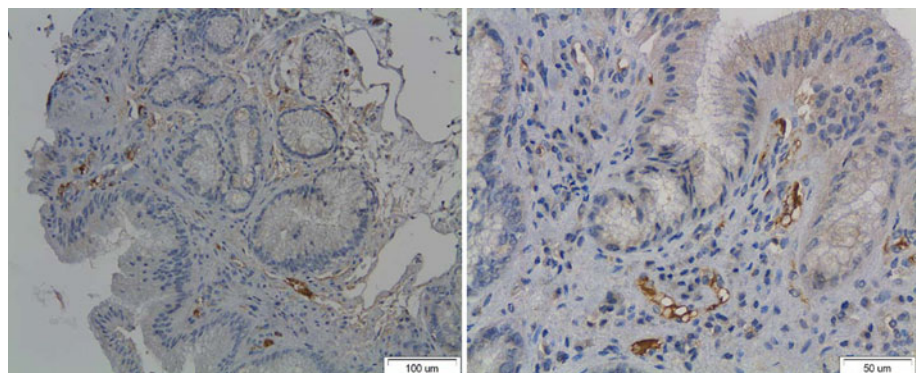
Immunohistochemical Analysis

Squamous epithelium did not stain for VEGF in any sample of the control group. A weak staining of the columnar epithelial cells was observed, whereas muscularis mucosa did not stain for VEGF. Inflammatory cells and especially plasma cells stained positive. The strongest staining, compared to other structures, was observed in endothelial cells and this was the case for all Barrett's samples (Fig. 1). VEGF expression was graded as mild, moderate, and strong in 34 (34.7%), 40 (40.8%), and 24 (24.5%) samples, respectively. Among the eight specimens with dysplasia and cancer VEGF expression was strong only in two.

Immunohistochemistry for COX-2 produced more distinct results with less background staining. Squamous epithelial cells stained weakly only at the basal layer. Stromal mesenchymal cells (fibroblasts, macrophages etc.), endothelia and smooth muscle cells were all negative for COX-2. The intensity of staining for glandular epithelia was moderate to intense in all samples (Fig. 2). COX-2 expression was mild, moderate, and strong in three (3.1%), 15 (15.3%), and 80 (81.6%) specimens, respectively. In all but one sample with dysplasia, COX-2 expression was strong, whereas in the case of cancer it was moderate.

The expression of Ki-67 in squamous esophageal epithelium was limited to the compartment above the basal membrane, which exhibits high proliferation properties.

Fig. 1 Immunohistochemical expression of VEGF. Note the intense staining of endothelia (original and $\times 100$ magnification of the same field)



The quality of staining in metaplastic epithelium was satisfactory and the intensity was moderate or strong in all samples (Fig. 3). The positive nuclei were documented at the base of crypts and glands. Ki-67 expression was mild, moderate, and strong in 38 (38.8%), 50 (51.0%), and ten (10.2%) samples, respectively. In six out of seven cases with dysplasia, Ki-67 expression was moderate.

Differences Between LSBE and SSBE

The results of the comparative study between LSBE and SSBE are shown in the Table 1. VEGF expression was mild, moderate, and strong in 13 (39.3%), 17 (51.5%), and three (9.0%) cases of LSBE, respectively. The relevant values for SSBE were: 21 (32.3%), 23 (35.3%), and 21 (32.3%). The comparative analysis demonstrated that VEGF expression was significantly stronger in SSBE ($p = 0.031$).

Cyclooxygenase-2 expression was mild, moderate, strong in one (3.0%), seven (21.2%), 25 (75.7%) cases of LSBE and in two (3.0%), eight (12.3%), 55 (84.6%) cases of SSBE, respectively. Due to the low frequencies, two

groups were included in the final comparative analysis: mild/moderate and strong. No differences between LSBE and SSBE were observed.

Ki-67 expression was mild, moderate, strong in eight (24.2%), 22 (66.6%), eight (24.2%) cases of LSBE and in 30 (46.1%), 28 (43.0%), two (3.0%) cases of SSBE, respectively. Two groups were included in the final analysis: mild and moderate/strong. Ki-67 expression was significantly stronger in LSBE ($p = 0.035$).

Discussion

To the best of our knowledge, this is the first study that jointly evaluated VEGF, COX-2, and Ki-67 expression in Barrett's epithelium. VEGF expression in small vessels' endothelia was evenly distributed among our samples, whereas COX-2 and Ki-67 expression in metaplastic epithelia was marked in the majority of the specimens. The comparative analysis revealed that LSBE was associated with milder VEGF and stronger Ki-67 expression.

Vascular endothelial growth factor is a growth factor with a potent regulatory effect on angiogenesis. The most widely applied method for the evaluation of angiogenesis is counting the microvessel density (MVD), which is based on the immunohistochemical spotting of the endothelia [33, 34]. It has been shown that there is an association between MVD and VEGF expression [12, 35]. Thus, the immunohistochemical expression of VEGF is considered an indirect marker of angiogenesis. However, it should be mentioned that there is no validated method for the evaluation of angiogenesis in BE.

Lanas et al. [30] evaluated the effect of COX-2 inhibitors on VEGF expression in Barrett's mucosal vessels. We similarly assessed VEGF expression by measuring small, positive vessels in BE samples. As in Lanas' study, we used a monoclonal antibody as opposed to the polyclonal that is commonly employed. Squamous epithelium did not stain in our samples and this was in agreement with published data [10]. The weak staining of metaplastic cells could have been attributed to technical issues, but the

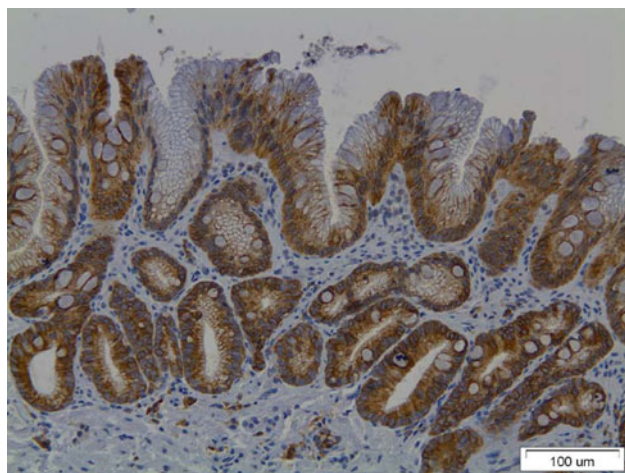


Fig. 2 Immunohistochemical expression of COX-2. The staining of glandular epithelium was marked

Fig. 3 Immunohistochemical expression of Ki-67. Marked staining was noticed at the base of crypts and glands (original and $\times 100$ magnification of the same field)

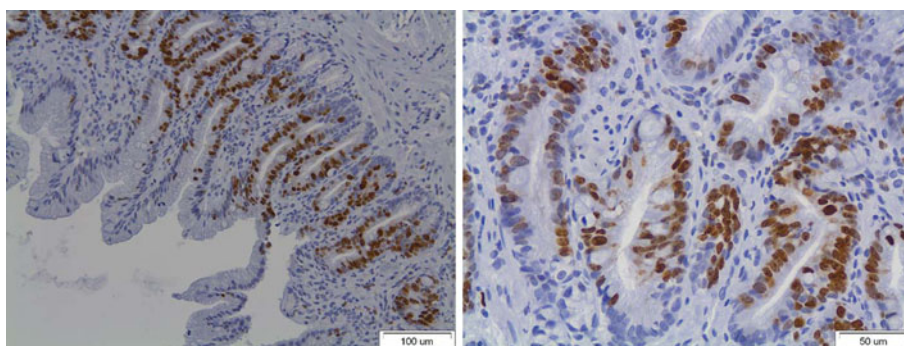


Table 1 Comparative analysis according to Barrett's length

Variables	SSBE N = 65	LSBE N = 33	p value
VEGF expression			0.031
Mild	21 (32.3)	13 (39.4)	
Moderate	23 (35.4)	17 (51.5)	
Strong	21 (32.3)	3 (9.1)	
COX-2 expression			0.285
Mild/moderate	10 (15.4)	8 (24.2)	
Strong	55 (84.6)	25 (75.8)	
Ki-67 expression			0.035
Mild	30 (46.1)	8 (24.2)	
Moderate/strong	35 (53.9)	25 (75.8)	

Statistically significant values are shown in bold

Values are expressed as N (%)

LSBE long-segment Barrett's esophagus, SSBE short-segment Barrett's esophagus, VEGF vascular endothelial growth factor, COX-2 cyclooxygenase-2

satisfactory staining of the endothelial cells rendered our technique reliable.

All Barrett's samples stained positive for VEGF and the distribution was rather uniform among the specimens. VEGF expression seems to be a stable and solid finding in the early stages of Barrett's sequence. We hypothesized that VEGF production may be linked to the pathogenesis of metaplasia. Our results are in accordance with previous studies that have shown increased VEGF expression in metaplastic epithelium [10–13]. It is worth noting that most work has been done in esophagectomy specimens, because the primary goal was to assess the prognostic significance of VEGF expression and angiogenesis in Barrett's neoplastic sequence.

The immunohistochemical expression of VEGF was significantly stronger in patients with SSBE. In other words, the presence of small, VEGF-positive vessels in the sample rendered short Barrett more probable. When we planned our study, we expected the exact opposite result, based on Auvinen's [10] speculation that the microvessels' network is responsible for the salmon-red color of metaplastic epithelium. On the condition that VEGF expression does reflect angiogenesis we thought that it would be more extensive in cases of LSBE. On the other hand, given that VEGF is secreted under the inflammatory conditions at the lower esophagus and that angiogenesis contributes to the healing process, it may be that inflammation in the studied cases of SSBE was more intense. Guided by our results, we assumed that increased VEGF expression and angiogenesis in SSBE partly explains the malignant potential of short segments. If the extent of metaplasia is a risk factor for long segments, it may be that early and increased formation of new vessels is a risk factor for short ones.

Ki-67 is a nuclear protein, exclusively encountered in proliferating cells. The study of Ki-67 in BE is pointed towards its probable prognostic role in combination with other factors, like chromosomic proteins and cyclin D1 [20, 21]. Sikkema et al. [32] found that increased expression of Ki-67 and protein p53 predicted progression to high-grade dysplasia and/or adenocarcinoma. Other studies underline the importance of the part of the epithelium that expresses Ki-67. Deep layers are expected to have high proliferating properties, whereas the expression of the protein in differentiated chambers has been associated with dysplasia [20]. In our study, immunohistochemistry for Ki-67 at the base of glandular epithelium was moderate/strong in more than half of our Barrett's samples. In about 2/3 of our Barrett's specimens, metaplastic cells maintained moderate to high-growth capacity. This could be related to the "inflammatory microenvironment" in the distal esophagus of patients with GERD [3].

We found a correlation between Ki-67 and Barrett's length. Ki-67 expression was significantly increased in LSBE, even though the clinical significance of this finding is not clear. An explanation could be that increased proliferation characterizes long Barrett's, which therefore bears high malignant risk. However, the latter is still debatable and although the general impression exists that LSBE is indeed "worse", current surveillance guidelines do not separate the two groups [22, 23, 27]. On the other hand, inflammation due to the trauma in the lower esophagus could alternatively explain the increased expression of Ki-67. It denotes intensive restoration process of the damaged epithelium. On the rare occasion of neoplastic evolution, the activated proliferation process is modified or reinforced.

Cyclooxygenase-2 expression has been extensively studied on the basis of epidemiologic observations regarding reduced frequency of Barrett's and, more importantly, of Barrett's adenocarcinoma among aspirin/NSAIDs users [36–38]. Published studies showed a step-wise increase of COX-2 expression along the sequence "injured squamous epithelium"—dysplastic Barrett—cancer [14, 39, 40]. COX-2 production is induced, under inflammatory circumstances, by cytokines and oncogenes [41]. When cultures of metaplastic cells were exposed to acid and bile salts, COX-2 expression was significantly increased [39].

In accordance with previous studies [14, 15], we showed that COX-2 expression in the metaplastic epithelia was strong and universal. We found no association to Barrett's length. The skepticism in regards to the increased expression is whether COX-2 is a causative factor for intestinal metaplasia or merely a consequence of inflammation and the special conditions at the lower esophagus. We may hypothesize that COX-2 overexpression in BE is a

secondary phenomenon, taking into account the increased frequency of prior antisecretory treatment and the low frequency of aspirin/NSAIDs use in our group. Early COX-2 up-regulation in metaplastic epithelium has been correlated with increased gastrin, in the context of chronic PPIs consumption [42]. Gastrin induced COX-2, prostaglandin E₂, and cell proliferation in biopsies and cell lines. On the other hand, the suppression of COX-2 by aspirin/NSAIDs is well established. The observed protective effect of COX-2 inhibition on the occurrence of Barrett's metaplasia has not been adequately explained.

In conclusion, we found that VEGF, COX-2, and Ki-67, which reflect angiogenesis, inflammation, and cell proliferation, respectively, were overexpressed in the majority of Barrett's samples. Our results indicate that these processes are active in the early stages of the neoplastic sequence. Whether their role is causative remains to be elucidated. The finding of strong Ki-67 expression in long segments may be related to increased malignant potential. On the other hand, strong VEGF expression in short segments was unexpected and may also represent a potentially ominous factor.

Conflict of interest No conflict of interest declared.

References

1. Shaheen NJ, Richter JE. Barrett's oesophagus. *Lancet*. 2009;373:850–861.
2. Spechler SJ, Goyal RK. The columnar-lined esophagus, intestinal metaplasia, and Norman Barrett. *Gastroenterology*. 1996;110:614–621.
3. Souza RF, Huo X, Mittal V, et al. Gastroesophageal reflux might cause esophagitis through a cytokine-mediated mechanism rather than caustic acid injury. *Gastroenterology*. 2009;137:1776–1784.
4. Morales CP, Souza RF, Spechler SJ. Hallmarks of cancer progression in Barrett's oesophagus. *Lancet*. 2002;360:1587–1589.
5. Fitzgerald RC, Abdalla S, Onwuegbusi BA, et al. Inflammatory gradient in Barrett's oesophagus: implications for disease complications. *Gut*. 2002;51:316–322.
6. Jankowski J. Gene expression in Barrett's mucosa: acute and chronic adaptive responses in the oesophagus. *Gut*. 1993;34:1649–1650.
7. Fitzgerald RC, Onwuegbusi BA, Bajaj-Elliott M, Saeed IT, Burnham WR, Farthing MJ. Diversity in the oesophageal phenotypic response to gastro-oesophageal reflux: immunological determinants. *Gut*. 2002;50:451–459.
8. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100:57–70.
9. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646–674.
10. Auvinen MI, Sihvo EI, Ruotula T, et al. Incipient angiogenesis in Barrett's epithelium and lymphangiogenesis in Barrett's adenocarcinoma. *J Clin Oncol*. 2002;20:2971–2979.
11. Mobius C, Stein HJ, Becker I, et al. The 'angiogenic switch' in the progression from Barrett's metaplasia to esophageal adenocarcinoma. *Eur J Surg Oncol*. 2003;29:890–894.
12. Couvelard A, Paraf F, Gratio V, et al. Angiogenesis in the neoplastic sequence of Barrett's oesophagus. Correlation with VEGF expression. *J Pathol*. 2000;192:14–18.
13. Sihvo EI, Ruotula T, Auvinen MI, Koivisto A, Harjula AL, Salo JA. Simultaneous progression of oxidative stress and angiogenesis in malignant transformation of Barrett esophagus. *J Thorac Cardiovasc Surg*. 2003;126:1952–1957.
14. Lagorce C, Paraf F, Vidaud D, et al. Cyclooxygenase-2 is expressed frequently and early in Barrett's oesophagus and associated adenocarcinoma. *Histopathology*. 2003;42:457–465.
15. Morris CD, Armstrong GR, Bigley G, Green A, Attwood SE. Cyclooxygenase-2 expression in the Barrett's metaplasia-dysplasia-adenocarcinoma sequence. *Am J Gastroenterol*. 2001;96:990–996.
16. Mobius C, Stein HJ, Spiess C, et al. COX2 expression, angiogenesis, proliferation and survival in Barrett's cancer. *Eur J Surg Oncol*. 2005;31:755–759.
17. Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell*. 1998;93:705–716.
18. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol*. 2000;182:311–322.
19. Seo HK, Cho KS, Chung J, et al. Prognostic value of p53 and Ki-67 expression in intermediate-risk patients with nonmuscle-invasive bladder cancer receiving adjuvant intravesical mitomycin C therapy. *Urology*. 2010;76:512.e1–7.
20. Going JJ, Keith WN, Neilson L, Stoeber K, Stuart RC, Williams GH. Aberrant expression of minichromosome maintenance proteins 2 and 5, and Ki-67 in dysplastic squamous oesophageal epithelium and Barrett's mucosa. *Gut*. 2002;50:373–377.
21. Sarela AI, Verbeke CS, Pring C, Guillou PJ. Is symptom control the correct end point for proton pump inhibitor treatment in Barrett's oesophagus? *Gut*. 2004;53:1387–1388.
22. Avidan B, Sonnenberg A, Schnell TG, Chejfec G, Metz A, Sontag SJ. Hiatal hernia size, Barrett's length, and severity of acid reflux are all risk factors for esophageal adenocarcinoma. *Am J Gastroenterol*. 2002;97:1930–1936.
23. Weston AP, Sharma P, Mathur S, et al. Risk stratification of Barrett's esophagus: updated prospective multivariate analysis. *Am J Gastroenterol*. 2004;99:1657–1666.
24. Menke-Pluymers MB, Hop WC, Dees J, van Blankenstein M, Tilanus HW. Risk factors for the development of an adenocarcinoma in columnar-lined (Barrett) esophagus. The Rotterdam Esophageal Tumor Study Group. *Cancer*. 1993;72:1155–1158.
25. Thomas T, Abrams KR, De Caestecker JS, Robinson RJ. Meta analysis: cancer risk in Barrett's oesophagus. *Aliment Pharmacol Ther*. 2007;26:1465–1477.
26. Anandasabapathy S, Jhamb J, Davila M, Wei C, Morris J, Bresalier R. Clinical and endoscopic factors predict higher pathologic grades of Barrett dysplasia. *Cancer*. 2007;109:668–674.
27. Yousef F, Cardwell C, Cantwell MM, Galway K, Johnston BT, Murray L. The incidence of esophageal cancer and high-grade dysplasia in Barrett's esophagus: a systematic review and meta-analysis. *Am J Epidemiol*. 2008;168:237–249.
28. Rudolph RE, Vaughan TL, Storer BE, et al. Effect of segment length on risk for neoplastic progression in patients with Barrett esophagus. *Ann Intern Med*. 2000;132:612–620.
29. Levine DS, Blount PL, Rudolph RE, Reid BJ. Safety of a systematic endoscopic biopsy protocol in patients with Barrett's esophagus. *Am J Gastroenterol*. 2000;95:1152–1157.
30. Lanas A, Ortego J, Sopena F, et al. Effects of long-term cyclooxygenase 2 selective and acid inhibition on Barrett's oesophagus. *Aliment Pharmacol Ther*. 2007;26:913–923.
31. Benamouzig R, Uzzan B, Martin A, et al. Cyclooxygenase-2 expression and recurrence of colorectal adenomas: effect of aspirin chemoprevention. *Gut*. 2010;59:622–629.

32. Sikkema M, Kerkhof M, Steyerberg EW, et al. Aneuploidy and overexpression of Ki67 and p53 as markers for neoplastic progression in Barrett's esophagus: a case-control study. *Am J Gastroenterol*. 2009;104:2673–2680.
33. Doukas CN, Maglogiannis I, Chatziioannou A, Papapetropoulos A. Automated angiogenesis quantification through advanced image processing techniques. *Conf Proc IEEE Eng Med Biol Soc*. 2006;1:2345–2348.
34. Simpson JF, Ahn C, Battifora H, Esteban JM. Endothelial area as a prognostic indicator for invasive breast carcinoma. *Cancer*. 1996;77:2077–2085.
35. Kitadai Y, Haruma K, Tokutomi T, et al. Significance of vessel count and vascular endothelial growth factor in human esophageal carcinomas. *Clin Cancer Res*. 1998;4:2195–2200.
36. Corley DA, Kerlikowske K, Verma R, Buffler P. Protective association of aspirin/NSAIDs and esophageal cancer: a systematic review and meta-analysis. *Gastroenterology*. 2003;124:47–56.
37. Vaughan TL, Dong LM, Blount PL, et al. Non-steroidal anti-inflammatory drugs and risk of neoplastic progression in Barrett's esophagus: a prospective study. *Lancet Oncol*. 2005;6:945–952.
38. Nguyen DM, Richardson P, El-Serag HB. Medications (NSAIDs, statins, proton pump inhibitors) and the risk of esophageal adenocarcinoma in patients with Barrett's esophagus. *Gastroenterology*. 2010;138:2260–2266.
39. Shirvani VN, Ouatu-Lascar R, Kaur BS, Omary MB, Triadafilopoulos G. Cyclooxygenase 2 expression in Barrett's esophagus and adenocarcinoma: ex vivo induction by bile salts and acid exposure. *Gastroenterology*. 2000;118:487–496.
40. Vallbohmer D, DeMeester SR, Oh DS, et al. Antireflux surgery normalizes cyclooxygenase-2 expression in squamous epithelium of the distal esophagus. *Am J Gastroenterol*. 2006;101:1458–1466.
41. Jones DA, Carlton DP, McIntyre TM, Zimmerman GA, Prescott SM. Molecular cloning of human prostaglandin endoperoxide synthase type II and demonstration of expression in response to cytokines. *J Biol Chem*. 1993;268:9049–9054.
42. Abdalla SI, Lao-Sirieix P, Novelli MR, Lovat LB, Sanderson IR, Fitzgerald RC. Gastrin-induced cyclooxygenase-2 expression in Barrett's carcinogenesis. *Clin Cancer Res*. 2004;10:4784–4792.