MINIREVIEWS

Role of nitric oxide in the pathogenesis of Barrett's-associated carcinogenesis

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Abstract

Barrett's esophagus (BE), a premalignant condition to Barrett's adenocarcinoma (BAC), is closely associated with chronic inflammation due to gastro-esophageal reflux. Caudal type homeobox 2 (CDX2), a representative marker of BE, is increased during the metaplastic and neoplastic transformation of BE. Nitric oxide (NO) has been proposed to be a crucial mediator of Barrett's carcinogenesis. We previously demonstrated that CDX2 might be induced directly under stimulation of large amounts of NO generated around the gastro-esophageal junction (GEJ) by activating epithelial growth factor receptor in a ligand-independent manner. Thus, we reviewed recent developments on the role of NO in Barrett's carcinogenesis. Notably, recent studies have reported that microbial communities in the distal esophagus are significantly different among groups with a normal esophagus, reflux esophagitis, BE or BAC, despite there being no difference in the bacterial quantity. Considering that microorganism components can be one of the major sources of large amounts of NO, these studies suggest that the bacterial composition in the distal esophagus might play an important role in regulating NO production during the carcinogenic process. Controlling an inflammatory reaction due to gastro-esophageal reflux or bacterial composition around the GEJ might help prevent the progression of Barrett's carcinogenesis by inhibiting NO production.

Key words: Barrett's esophagus; Nitric oxide; Epithelial growth factor receptor; Caudal type homeobox 2; Microbiome

Core tip: Barrett's carcinogenesis is closely associated with chronic inflammation caused by gastro-esophageal reflux of gastric acid, bile acid and intraluminal microorganisms. Caudal type homeobox 2 (CDX2) is a crucial biomarker of Barrett's esophagus. We previously demonstrated that a high amount of nitric oxide (NO) at the gastro-esophageal junction (GEJ) might directly induce CDX2 through phosphorylation of epidermal growth factor receptor (EGFR) in a ligand-independent manner. Together with a possible effect of anti-EGFR-targeting drugs in inhibiting Barrett's adenocarcinoma, future research for controlling NO production around the GEJ might provide a new insight for developing a management strategy of Barrett's carcinogenesis.

INTRODUCTION

Barrett's esophagus (BE) is defined as the premalignant condition which squamous epithelium is replaced by columnar epithelium, a well-known precursor of Barrett's adenocarcinoma (BAC)[1-3]. Because the incidence of Barrett's-associated neoplasm has increased sharply, it is important to elucidate its risk factors during the surveillance period. Many studies on the origin of Barrett's mucosa have proposed several theories, such as the differentiation of the stem cells in esophageal squamous tissues and a phenotypic switch and formation of metaplastic epithelium by gastro-esophageal refluxant[4-6].

Caudal type homeobox 2 (CDX2) is a member of the homeobox family of genes involved in the embryonic development of the alimentary tract and in the differentiation, proliferation, adhesion and apoptosis of the intestinal epithelium[7-9]. It is well-known that CDX2 is induced during the metaplastic and neoplastic transformation of BE, while it is not expressed in normal esophageal squamous epithelium and non-metaplastic gastric epithelium[8-11]. Thus, it has been reported that an immune-stain of CDX2 could become a useful biomarker of Barrett's-associated neoplasms[12-14]. Previous studies have revealed that reactive oxygen species (ROS) produced by the stimulation of acid, bile salts or major noxious agents in refluxed gastric juice might take a critical role for the induction of CDX2 during the sequential process of the metaplastic and neoplastic transformation of BE[7,11,15-28]. However, this mechanism is not fully understood.

ROLE OF NITRIC OXIDE IN BARRETT'S CARCINOGENESIS

Nitric oxide (NO) is a crucial regulatory mediator which has cytotoxic and cytostatic effects, depending on the environment[16,26]. A large amount of NO production is involved in cytotoxic processes, such as apoptosis, angiogenesis and DNA damage during cancer development[19]. In fact, it was reported that NO might work as a principle mediator in the induction of CDX2 during Barrett's carcinogenesis[7] and that reactive nitrogen oxide species might contribute to the progression from BE to BAC because of genotoxicity, such as DNA damage, strand breakage or modification[21,29,30].

From the point of view of NO generation, NO is classified into endogenous and exogenous[27] (Figure 1). Endogenous NO is derived from L-arginine by a family of related enzymes, which includes three different isoforms of NO synthase (NOS)[27,31]. Endothelial NOS

(eNOS) in endothelial cells and a neuronal NOS (nNOS) in neuronal cells were reported to be physiological mediators of NO production at low concentrations[27]. Inducible NO (iNOS) is an inflammation-induced enzyme, which can mediate harmful production of high amounts of NO[27,32]. The induction of iNOS might be critically involved in Barrett's carcinogenesis because its expression was shown to be gradually overexpressed during the sequential development toward BAC, but not in normal esophageal/gastric mucosa[19,27,32].

Exogenous NO is generated within the upper-gastrointestinal tract lumen from the reduction of salivary nitrite to NO in a reaction with acid refluxant[33-35]. After ingesting nitrate-containing food, human salivary nitrite concentrations increase to 2-5 mmol/L, which maintained for a few hours by entero-salivary recirculation of the dietary nitrate[35,36]. Swallowed saliva is the main source of nitrate. The gastric juice containing the acidity and ascorbic acid content might convert the salivary nitrite to NO at the gastro-esophageal junction (GEJ)[21,37]. Iijima *et al*[21] investigated the serum and saliva nitrate concentrations within the upper gastrointestinal tract lumen in healthy volunteers of *Helicobacter pylori*-negative under basal conditions and after nitrate ingestion using custom-made sensors. After ingesting 2 mmol of potassium nitrate, the nitrate concentration of the serum, salivary and intra-lumin increased from 30 μmol/L to 95 μmol/L, from 36 μmol/L to 252 μmol/L, and from 4.7 μmol/L to 23.2 μmol/L, respectively. The highest concentrations of NO within the upper gastrointestinal tract lumen were observed at the site of the neutral pH of esophageal fell to the acidic pH of gastric. This suggested that the luminal generation of NO from nitrate ingestion through salivary nitrite was maximal at the GEJ. Thus, the site of generation of luminal NO would move to the oral side of the distal esophagus in patients with gastro-esophageal reflux[38].

Additionally, luminal NO with a high concentration due to both exogenous and endogenous NO production around the GEJ is rapidly diffused across epithelial membranes, resulting in damage to the tissues surrounding tissues the GEJ[29,30,39-41]. Such a large amount of NO also causes the production of peroxynitrite, a representative genotoxic mediator, and can contribute to the development of Barrett's carcinogenesis *via* a rapid reaction with superoxide in an inflammatory/tumor microenvironment[20,21,26,27,37]. Of the plasma membranous receptors of the esophageal tissue, we previously demonstrated that during Barrett's carcinogenesis, a large amount of NO might directly activate epithelial growth factor receptor (EGFR)[42], a 170-kDa glycoprotein which is a member of the receptor tyrosine kinase family and is involved in cell proliferation, differentiation, angiogenesis, invasion and tumor progression[1,3,43-46]. Previous studies demonstrated that overexpression of EGFR was observed during histological progression in BE tissue, as well as during tumor infiltration and metastasis[1,45]. Although it was previously indicated that ROS might indirectly enhance the CDX2 expression by the exposure of bile
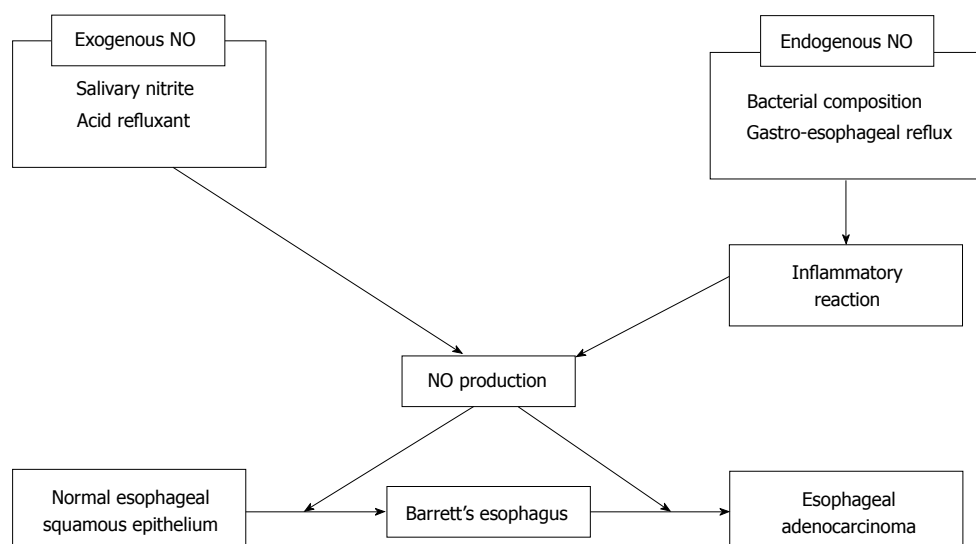
Figure 1 The process of nitric oxide generation. Exogenous NO is generated within the upper-gastrointestinal tract lumen from the reduction of salivary nitrite to NO in a reaction with acid refluxant. Endogenous NO is generated in chronic inflammation caused by gastro-esophageal reflux and bacterial composition. NO: Nitric oxide.

acid through EGFR activation in ligand-dependent and -independent manners[3,8,15,46], we investigated the direct role of NO through activation of EGFR during Barrett's carcinogenesis[42]. When we compared immune-staining of EGFR, CDX2 and nitrotyrosine, a marker of intra-cellular protein damaged by peroxynitrite, in normal human esophageal squamous epithelium specimens with those in BE specimens, we revealed the stronger expression of EGFR, CDX2 and nitrotyrosine in BE compared with those in normal human esophageal squamous epithelium with a positive correlation with BE progression. Second, we also investigated the effect of peroxynitrite, NOC7 which is NO donor and SIN-1 which is stimulant of peroxynitrite on the expression of phosphorylated EGFR and CDX2 by western blotting and reverse transcription-polymerase chain reaction. This showed that the phosphorylated EGFR and CDX2 were induced in dose- and time-dependent manners after expose to peroxynitrite, NOC7 and SIN-1. The expression of phosphorylated EGFR and CDX2 induced after expose to peroxynitrite, NOC7 and SIN-1 was diminished by EGFR-specific inhibitors AG1428 and EGFR-siRNA. Additionally, the induction of phosphorylated EGFR and CDX2 after exposure to NOC7 was diminished by carboxy-PTIO which is the NO scavenger in a dose-dependent manner. Taken together, these results suggest that the expression of CDX2 might be induced directly by the effect of extrinsic NO through the activation of EGFR in the progression of BE. Accordingly, these results indicate that the stimulation of high concentration of NO on the normal esophageal epithelium might directly induce the expression of CDX2 through EGFR activation and result in the progression of BE[7,8,27].

Notably, EGFR2 (HER2) is regarded as a useful target in controlling Barrett's cancer development, despite the uncertain treatment effect of HER2-targetting anti-cancer medications[47,48]. While researchers reported that the clinical trials targeting HER2 in the treatment of BAC were result it was not promising, another study showed that targeting erbB-2 in HER2-positive patients with adenocarcinoma at GEJ had better outcomes[49,50]. Gefitinib is another small molecule tyrosine kinase inhibitor (TKI) and the most common HER2-targetting anti-cancer medication that has been used to treat several malignancies. It is a representative oral TKI and inhibits EGF-induced phosphorylation and downstream signaling pathways. Janmaat et al[51] reported a phase Ⅱ study of 36 patients who had failed first-line treat-ment for advanced esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) and were administered gefitinib at 500 mg/d. In this report, gefitinib had modest activity as a second-line treatment with only 2.8% of the patients with a partial response (PR), 27.8% with stable disease and 47.8% with disease progression. The progression-free survival time and the median overall survival time were 59 d and 164 d, respectively. Javle et al[52] reported a phase Ⅰ/Ⅱ study of 6 patients who had locally advanced unresectable EAC. This study used a combination of gefitinib and chemoradiotherapy. The protocol were composed of administration of gefitinib orally at 250 mg/d for 1 year, oxaliplatin intravenously at 85 or 100 mg/m^2 on days 1, 15, 29, and preoperative radiotherapy. They reported that this combination study was tolerable, but had limited efficacy. The median overall survival time and the disease-free survival time were 10.8 and 8.4 mo, respectively. Ilson et al[53] reported a phase Ⅱ study of the treatment effect of Erlotinib, another oral TKI, in 30 patients who had failed first-line treatment for ESCC and EAC. The patients had a 6.7% PR and 10.3 mo of median overall survival. These results supported an important role for EGFR in Barrett's cancer development. Targeted inhibition of EGFR activity and inhibition of EGFR activity by controlling NO production around the

GEJ might be a new clue to improve the management of Barrett's-associated neoplasm.

POSSIBLE ORIGIN OF NO IN SWALLOWED MICROORGANISMS

Several studies have reported that the bacterial composition in the distal esophagus might play an important role in the carcinogenic process. Human microbiota is estimated to be composed of approximately 10^{14} bacterial cells. The relationship the bacteria and host can be commensal and pathogenic[54]. Human microbiota in the gastrointestinal tract are essential for human survival, aiding digestion, assisting in the synthesis of vitamins and maintaining a normal physiological environment[54,55]. The host provides these microbiota safe housing and nutrition in return for the benefits from the microbes. An imbalance in this relationship may cause chronic inflammation and inflammation-associated malignancies.

Previous report mentioned that the normal distal esophagus have 6 phyla and 140 species of bacterial compositions[56]. The impairment of the mucosal barrier in the distal esophagus by reflux of gastric juice might disrupt the microenvironmental homeostasis, resulting in progression of Barrett's-associated neoplasm with remarkable changes in esophageal bacterial biota[56-58]. In fact, it was reported that the characteristics of bacterial communities differed between groups with normal esophagus, reflux esophagitis (RE), BE or BAC, despite there being no significant differences in the amount of bacteria[55,58].

By investigating the composition of bacteria in the distal esophagus of 3 groups (group with normal esophagus, group with RE and group with BE) from endoscopic biopsy specimens using a histological examination and DNA extraction, Liu *et al*[55] reported that *Fusobacterium* was found not in the group with a normal esophagus, but in the group with RE and BE, and that the most common bacterium was *Streptococcus* in the groups with normal esophagus and RE and *Veillonella* in the group with BE. However, the total amount of the detected bacterial DNA was not different between the groups[55]. Narikiyo *et al*[59] investigated the bacteria in esophageal carcinomatous tissues, the surrounding non-cancerous tissues and saliva from healthy people to determine the correlation between microorganisms and inflammation/carcinogenesis of the esophagus. They showed that *Treponema denticola* and *Streptococcus anginosus* (*S. anginosus*) were detected in the saliva from patients with BAC. Both of these bacteria were in low numbers in the samples from healthy people. They also reported that *S. angiosus* and *Streptoccus mitis* infections induced inflammatory cytokines, including IL-8 and GROα, and suggested these bacteria might play a significant role in Barrett's carcinogenesis through chronic inflammation. Yang *et al*[54] reported a dramatic alteration of the esophageal microbiome, such as Gram-positive bacteria were decreased and Gram-negative bacteria were increased in patients with esophagitis and BE. In fact, Gram-negative bacteria have lipopolysaccharides (LPS) in their outer membrane, which can promote the induction of pro-inflammatory cytokines, including IL-1β, IL-8, COX-2 and iNOS, by activating Toll-like receptor 4 and its downstream cascade, which are related to proliferation, differentiation and migration[60,61]. In addition, LPS may also cause the relaxation of the lower esophageal sphincter by activating iNOS and delaying gastric employing by increasing the intra-gastric pressure through COX-2 activation[62,63]. This suggests that the alteration of Gram-negative bacteria in bacterial composition in the distal esophagus might contribute to the development of inflammation-associated Barrett's carcinogenesis. Thus, interventions with the microorganism in the distal esophagus provided novel insights for new preventive/treatment strategy of BE.

PERSPECTIVE

Many studies have contributed to a better understanding of the role of NO in Barrett's carcinogenesis. There may be three ways to control NO production in Barrett's mucosa, such as: (1) diminishing acidity of the gastro-esophageal refluxant by using a proton-pump inhibitor[64]; (2) medical intervention of intramucosal microorganisms; and (3) medical inhibition of iNOS activity. Recent studies have indicated a significant role for esophageal microbiology in Barrett's carcinogenesis because of the differences in the bacterial communities between groups with normal esophagus, RE, BE or BAC[55,58]. Therefore, it is likely that the eradication of these bacteria by antibiotics or probiotics might be effective in reducing Barrett's cancer risk, although limited data of suitable antibiotics combinations for the microbiome have been reported. Yang *et al*[54] reported that Gram-negative bacteria were increased in the patients with esophagitis and BE. LPS from Gram-negative bacteria can up-regulate gene expression of inflammatory cytokines, including COX-2 and iNOS, by activating the NF-κB pathway[54]. Several studies have suggested that NF-κB inhibitors, including nonsteroidal anti-inflammatory drugs (NSAIDs), sulfasalazine and immunosuppressive agents, could be effective in reducing Barrett's inflammation. Therefore, they were regarded as a potential medication for preventing Barrett's carcinogenesis[61]. Moreover, Hansel *et al*[62] demonstrated that the prodrug L-N⁶-(1-iminoethyl)lysine 5-tetrazole amide, an iNOS inhibitor, reduced the levels of NO in exhaled breath in healthy volunteers and patients with mild asthma without remarkable side-effects. These results suggest the therapeutic potential of iNOS inhibitors to reduce the risk of Barrett's cancer. Additionally, we previously demonstrated that the interaction between iNOS and COX-2 activities might play a crucial role in LPS-induced inflammation in a rat-model[65]. Therefore, controlling COX-2 activity can also regulate iNOS-derived NO

production in an inflammation reaction. Therefore, COX-2 inhibitors might have therapeutic potential in controlling the risk of neoplastic progression in patients with BE. Moreover, Calatayud *et al*[66] reported that the delay of gastric employing with an increase of the intra-gastric pressure from LPS stimulation could be blocked by down-regulating COX-2 activity using the selective COX-2 inhibitor NS398. In fact, in a meta-analysis of anti-cancerous effects of NSAIDs and aspirin for 1813 patients with EAC, Corley *et al*[67] suggested that the preventive role of COX-2 inhibitors in Barrett's carcinogenesis, although their clinical relevance against the development of Barrett's -associated neoplasm was unproven[67-69]. Accordingly, controlling enzymatic activity of iNOS and microorganisms in Barrett's tumor microenvironment may provide important insight into Barrett's carcinogenesis.

CONCLUSION

Barrett's carcinogenesis was associated with chronic inflammation as a consequence of gastro-esophageal reflux and microbiota in the gastrointestinal tract. This review focused on the role of NO in controlling inflammation during the Barrett's carcinogenic process. The control of NO production in chronic inflammation caused by gastro-esophageal reflux and microbiota might contribute to prevent Barrett's cancer development. Further studies are required to assess the therapeutic potential of controlling NO production.

REFERENCES

1   **Cronin J**, McAdam E, Danikas A, Tselepis C, Griffiths P, Baxter J, Thomas L, Manson J, Jenkins G. Epidermal growth factor receptor (EGFR) is overexpressed in high-grade dysplasia and adenocarcinoma of the esophagus and may represent a biomarker of histological progression in Barrett's esophagus (BE). *Am J Gastroenterol* 2011; **106**: 46-56 [PMID: 21157443 DOI: 10.1038/ajg.2010.433]
2   **Shaheen N**, Ransohoff DF. Gastroesophageal reflux, Barrett esophagus, and esophageal cancer: clinical applications. *JAMA* 2002; **287**: 1982-1986 [PMID: 11960541 DOI: 10.1001/jama.287.15.1982]
3   **Li Y**, Wo JM, Ray MB, Jones W, Su RR, Ellis S, Martin RC. Cyclooxygenase-2 and epithelial growth factor receptor up-regulation during progression of Barrett's esophagus to adenocarcinoma. *World J Gastroenterol* 2006; **12**: 928-934 [PMID: 16521222 DOI: 10.3748/wjg.v12.i6.928]
4   **Zhang HY**, Spechler SJ, Souza RF. Esophageal adenocarcinoma arising in Barrett esophagus. *Cancer Lett* 2009; **275**: 170-177 [PMID: 18703277 DOI: 10.1016/j.canlet.2008.07.006]
5   **Abdel-Latif MM**, Duggan S, Reynolds JV, Kelleher D. Inflammation and esophageal carcinogenesis. *Curr Opin Pharmacol* 2009; **9**: 396-404 [PMID: 19596608 DOI: 10.1016/j.coph.2009.06.010]
6   **Souza RF**, Krishnan K, Spechler SJ. Acid, bile, and CDX: the ABCs of making Barrett's metaplasia. *Am J Physiol Gastrointest Liver Physiol* 2008; **295**: G211-G218 [PMID: 18556417 DOI: 10.1152/ajpgi.90250.2008]
7   **Vaninetti N**, Williams L, Geldenhuys L, Porter GA, Guernsey DL, Casson AG. Regulation of CDX2 expression in esophageal adenocarcinoma. *Mol Carcinog* 2009; **48**: 965-974 [PMID: 19415720 DOI: 10.1002/mc.20549]
8   **Rahman FB**, Kadowaki Y, Ishihara S, Tobita H, Imaoka H,

Fukuhara H, Aziz MM, Furuta K, Amano Y, Kinoshita Y. Fibroblast-derived HB-EGF promotes Cdx2 expression in esophageal squamous cells. *Lab Invest* 2010; **90**: 1033-1048 [PMID: 20351696 DOI: 10.1038/labinvest.2010.71]
9   **Takahashi O**, Hamada J, Abe M, Hata S, Asano T, Takahashi Y, Tada M, Miyamoto M, Kondo S, Moriuchi T. Dysregulated expression of HOX and ParaHOX genes in human esophageal squamous cell carcinoma. *Oncol Rep* 2007; **17**: 753-760 [PMID: 17342311]
10   **McColl KE**. When saliva meets acid: chemical warfare at the oesophagogastric junction. *Gut* 2005; **54**: 1-3 [PMID: 15591495 DOI: 10.1136/gut.2004.047126]
11   **Burnat G**, Rau T, Elshimi E, Hahn EG, Konturek PC. Bile acids induce overexpression of homeobox gene CDX-2 and vascular endothelial growth factor (VEGF) in human Barrett's esophageal mucosa and adenocarcinoma cell line. *Scand J Gastroenterol* 2007; **42**: 1460-1465 [PMID: 17852856 DOI: 10.1080/00365520701452209]
12   **Johnson DR**, Abdelbaqui M, Tahmasbi M, Mayer Z, Lee HW, Malafa MP, Coppola D. CDX2 protein expression compared to alcian blue staining in the evaluation of esophageal intestinal metaplasia. *World J Gastroenterol* 2015; **21**: 2770-2776 [PMID: 25759548 DOI: 10.3748/wjg.v21.i9.2770]
13   **Freund JN**, Domon-Dell C, Kedinger M, Duluc I. The Cdx-1 and Cdx-2 homeobox genes in the intestine. *Biochem Cell Biol* 1998; **76**: 957-969 [PMID: 10392709 DOI: 10.1139/bcb-76-6-957]
14   **Silberg DG**, Swain GP, Suh ER, Traber PG. Cdx1 and cdx2 expression during intestinal development. *Gastroenterology* 2000; **119**: 961-971 [PMID: 11040183 DOI: 10.1053/gast.2000.18142]
15   **Avissar NE**, Toia L, Hu Y, Watson TJ, Jones C, Raymond DP, Matousek A, Peters JH. Bile acid alone, or in combination with acid, induces CDX2 expression through activation of the epidermal growth factor receptor (EGFR). *J Gastrointest Surg* 2009; **13**: 212-222 [PMID: 18854960 DOI: 10.1007/s11605-008-0720-7]
16   **Clemons NJ**, McColl KE, Fitzgerald RC. Nitric oxide and acid induce double-strand DNA breaks in Barrett's esophagus carcinogenesis via distinct mechanisms. *Gastroenterology* 2007; **133**: 1198-1209 [PMID: 17919494 DOI: 10.1053/j.gastro.2007.06.061]
17   **Feagins LA**, Zhang HY, Zhang X, Hormi-Carver K, Thomas T, Terada LS, Spechler SJ, Souza RF. Mechanisms of oxidant production in esophageal squamous cell and Barrett's cell lines. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G411-G417 [PMID: 18063706 DOI: 10.1152/ajpgi.00373.2007]
18   **Rantanen TK**, Räsänen JV, Sihvo EI, Ahotupa MO, Färkkilä MA, Salo JA. The impact of antireflux surgery on oxidative stress of esophageal mucosa caused by gastroesophageal reflux disease: 4-yr follow-up study. *Am J Gastroenterol* 2006; **101**: 222-228 [PMID: 16454822 DOI: 10.1111/j.1572-0241.2006.00420.x]
19   **Clemons NJ**, Shannon NB, Abeyratne LR, Walker CE, Saadi A, O'Donovan ML, Lao-Sirieix PP, Fitzgerald RC. Nitric oxide-mediated invasion in Barrett's high-grade dysplasia and adenocarcinoma. *Carcinogenesis* 2010; **31**: 1669-1675 [PMID: 20584750 DOI: 10.1093/carcin/bgq130]
20   **Iijima K**, Grant J, McElroy K, Fyfe V, Preston T, McColl KE. Novel mechanism of nitrosative stress from dietary nitrate with relevance to gastro-oesophageal junction cancers. *Carcinogenesis* 2003; **24**: 1951-1960 [PMID: 12970071 DOI: 10.1093/carcin/bgg168]
21   **Iijima K**, Henry E, Moriya A, Wirz A, Kelman AW, McColl KE. Dietary nitrate generates potentially mutagenic concentrations of nitric oxide at the gastroesophageal junction. *Gastroenterology* 2002; **122**: 1248-1257 [PMID: 11984511 DOI: 10.1053/gast.2002.32963]
22   **Jenkins GJ**, D'Souza FR, Suzen SH, Eltahir ZS, James SA, Parry JM, Griffiths PA, Baxter JN. Deoxycholic acid at neutral and acid pH, is genotoxic to oesophageal cells through the induction of ROS: The potential role of anti-oxidants in Barrett's oesophagus. *Carcinogenesis* 2007; **28**: 136-142 [PMID: 16905748 DOI: 10.1093/carcin/bgl147]
23   **Dvorak K**, Payne CM, Chavarria M, Ramsey L, Dvorakova B, Bernstein H, Holubec H, Sampliner RE, Guy N, Condon A, Bernstein

C, Green SB, Prasad A, Garewal HS. Bile acids in combination with low pH induce oxidative stress and oxidative DNA damage: relevance to the pathogenesis of Barrett's oesophagus. *Gut* 2007; **56**: 763-771 [PMID: 17145738 DOI: 10.1136/gut.2006.103697]

24   **Jenkins GJ**, Cronin J, Alhamdani A, Rawat N, D'Souza F, Thomas T, Eltahir Z, Griffiths AP, Baxter JN. The bile acid deoxycholic acid has a non-linear dose response for DNA damage and possibly NF-kappaB activation in oesophageal cells, with a mechanism of action involving ROS. *Mutagenesis* 2008; **23**: 399-405 [PMID: 18515815 DOI: 10.1093/mutage/gen029]

25   **Morelli MP**, Cascone T, Troiani T, De Vita F, Orditura M, Laus G, Eckhardt SG, Pepe S, Tortora G, Ciardiello F. Sequence-dependent antiproliferative effects of cytotoxic drugs and epidermal growth factor receptor inhibitors. *Ann Oncol* 2005; **16** Suppl 4: iv61-iv68 [PMID: 15923432 DOI: 10.1093/annonc/mdi910]

26   **Vaninetti NM**, Geldenhuys L, Porter GA, Risch H, Hainaut P, Guernsey DL, Casson AG. Inducible nitric oxide synthase, nitrotyrosine and p53 mutations in the molecular pathogenesis of Barrett's esophagus and esophageal adenocarcinoma. *Mol Carcinog* 2008; **47**: 275-285 [PMID: 17849424 DOI: 10.1002/mc.20382]

27   **Bae JD**, Jung KH, Ahn WS, Bae SH, Jang TJ. Expression of inducible nitric oxide synthase is increased in rat Barrett's esophagus induced by duodenal contents reflux. *J Korean Med Sci* 2005; **20**: 56-60 [PMID: 15716603 DOI: 10.3346/jkms.2005.20.1.56]

28   **Marchetti M**, Caliot E, Pringault E. Chronic acid exposure leads to activation of the cdx2 intestinal homeobox gene in a long-term culture of mouse esophageal keratinocytes. *J Cell Sci* 2003; **116**: 1429-1436 [PMID: 12640028 DOI: 10.1242/jcs.00338]

29   **Ara N**, Iijima K, Asanuma K, Yoshitake J, Ohara S, Shimosegawa T, Yoshimura T. Disruption of gastric barrier function by luminal nitrosative stress: a potential chemical insult to the human gastro-oesophageal junction. *Gut* 2008; **57**: 306-313 [PMID: 17965057 DOI: 10.1136/gut.2007.128074]

30   **Ito H**, Iijima K, Ara N, Asanuma K, Endo H, Asano N, Koike T, Abe Y, Imatani A, Shimosegawa T. Reactive nitrogen oxide species induce dilatation of the intercellular space of rat esophagus. *Scand J Gastroenterol* 2010; **45**: 282-291 [PMID: 20001645 DOI: 10.3109/00365520903469956]

31   **Ruano MJ**, Hernández-Hernando S, Jiménez A, Estrada C, Villalobo A. Nitric oxide-induced epidermal growth factor-dependent phosphorylations in A431 tumour cells. *Eur J Biochem* 2003; **270**: 1828-1837 [PMID: 12694196]

32   **Wilson KT**, Fu S, Ramanujam KS, Meltzer SJ. Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and associated adenocarcinomas. *Cancer Res* 1998; **58**: 2929-2934 [PMID: 9679948]

33   **Benjamin N**, O'Driscoll F, Dougall H, Duncan C, Smith L, Golden M, McKenzie H. Stomach NO synthesis. *Nature* 1994; **368**: 502 [PMID: 8139683 DOI: 10.1038/368502a0]

34   **Lundberg JO**, Weitzberg E, Lundberg JM, Alving K. Intragastric nitric oxide production in humans: measurements in expelled air. *Gut* 1994; **35**: 1543-1546 [PMID: 7828969 DOI: 10.1136/gut.35.11.1543]

35   **McKnight GM**, Smith LM, Drummond RS, Duncan CW, Golden M, Benjamin N. Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. *Gut* 1997; **40**: 211-214 [PMID: 9071933 DOI: 10.1136/gut.40.2.211]

36   **Pannala AS**, Mani AR, Spencer JP, Skinner V, Bruckdorfer KR, Moore KP, Rice-Evans CA. The effect of dietary nitrate on salivary, plasma, and urinary nitrate metabolism in humans. *Free Radic Biol Med* 2003; **34**: 576-584 [PMID: 12614846 DOI: 10.1016/S0891-5849(02)01353-9]

37   **Mukaida H**, Toi M, Hirai T, Yamashita Y, Toge T. Clinical significance of the expression of epidermal growth factor and its receptor in esophageal cancer. *Cancer* 1991; **68**: 142-148 [PMID: 2049734]

38   **Suzuki H**, Iijima K, Scobie G, Fyfe V, McColl KE. Nitrate and nitrosative chemistry within Barrett's oesophagus during acid reflux. *Gut* 2005; **54**: 1527-1535 [PMID: 16227357 DOI: 10.1136/gut.2005.066043]

39   **Ishiyama F**, Iijima K, Asanuma K, Ara N, Yoshitake J, Abe Y, Koike T, Imatani A, Ohara S, Shimosegawa T. Exogenous luminal nitric oxide exacerbates esophagus tissue damage in a reflux esophagitis model of rats. *Scand J Gastroenterol* 2009; **44**: 527-537 [PMID: 19172433 DOI: 10.1080/00365520802699260]

40   **Endo H**, Iijima K, Asanuma K, Ara N, Ito H, Asano N, Uno K, Koike T, Imatani A, Shimosegawa T. Exogenous luminal nitric oxide exposure accelerates columnar transformation of rat esophagus. *Int J Cancer* 2010; **127**: 2009-2019 [PMID: 20131319 DOI: 10.1002/ijc.25227]

41   **Asanuma K**, Iijima K, Sugata H, Ohara S, Shimosegawa T, Yoshimura T. Diffusion of cytotoxic concentrations of nitric oxide generated luminally at the gastro-oesophageal junction of rats. *Gut* 2005; **54**: 1072-1077 [PMID: 15860569 DOI: 10.1136/gut.2004.063107]

42   **Kusaka G**, Uno K, Iijima K, Endo H, Asano N, Koike T, Imatani A, Shimosegawa T. The role of nitric oxide in the induction of caudal-type homeobox 2 through epidermal growth factor receptor in the development of Barrett's esophagus. *Scand J Gastroenterol* 2012; **47**: 1148-1158 [PMID: 22834965 DOI: 10.3109/00365521.2012.703232]

43   **Lee HC**, An S, Lee H, Woo SH, Jin HO, Seo SK, Choe TB, Yoo DH, Lee SJ, Hong YJ, Park MJ, Rhee CH, Park IC, Hong SI. Activation of epidermal growth factor receptor and its downstream signaling pathway by nitric oxide in response to ionizing radiation. *Mol Cancer Res* 2008; **6**: 996-1002 [PMID: 18567803 DOI: 10.1158/1541-7786.MCR-08-0113]

44   **Andl CD**, Mizushima T, Nakagawa H, Oyama K, Harada H, Chruma K, Herlyn M, Rustgi AK. Epidermal growth factor receptor mediates increased cell proliferation, migration, and aggregation in esophageal keratinocytes in vitro and in vivo. *J Biol Chem* 2003; **278**: 1824-1830 [PMID: 12435727 DOI: 10.1074/jbc.M209148200]

45   **Hanawa M**, Suzuki S, Dobashi Y, Yamane T, Kono K, Enomoto N, Ooi A. EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus. *Int J Cancer* 2006; **118**: 1173-1180 [PMID: 16161046 DOI: 10.1002/ijc.21454]

46   **Goldman A**, Chen HD, Roesly HB, Hill KA, Tome ME, Dvorak B, Bernstein H, Dvorak K. Characterization of squamous esophageal cells resistant to bile acids at acidic pH: implication for Barrett's esophagus pathogenesis. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**: G292-G302 [PMID: 21127259 DOI: 10.1152/ajpgi.00461.2010]

47   **Gowryshankar A**, Nagaraja V, Eslick GD. HER2 status in Barrett's esophagus & amp; esophageal cancer: a meta analysis. *J Gastrointest Oncol* 2014; **5**: 25-35 [PMID: 24490040 DOI: 10.3978/j.issn.2078-6891.2013.039]

48   **Xu Y**, Sheng L, Mao W. Role of epidermal growth factor receptor tyrosine kinase inhibitors in the treatment of esophageal carcinoma and the suggested mechanisms of action. *Oncol Lett* 2013; **5**: 19-24 [PMID: 23255886 DOI: 10.3892/ol.2012.994]

49   **Clemons NJ**, Phillips WA, Lord RV. Signaling pathways in the molecular pathogenesis of adenocarcinomas of the esophagus and gastroesophageal junction. *Cancer Biol Ther* 2013; **14**: 782-795 [PMID: 23792587 DOI: 10.4161/cbt.25362]

50   **Rubenstein JH**, Dahlkemper A, Kao JY, Zhang M, Morgenstern H, McMahon L, Inadomi JM. A pilot study of the association of low plasma adiponectin and Barrett's esophagus. *Am J Gastroenterol* 2008; **103**: 1358-1364 [PMID: 18510610 DOI: 10.1111/j.1572-0241.2008.01823.x]

51   **Janmaat ML**, Gallegos-Ruiz MI, Rodriguez JA, Meijer GA, Vervenne WL, Richel DJ, Van Groeningen C, Giaccone G. Predictive factors for outcome in a phase II study of gefitinib in second-line treatment of advanced esophageal cancer patients. *J Clin Oncol* 2006; **24**: 1612-1619 [PMID: 16575012 DOI: 10.1200/JCO.2005.03.4900]

52   **Javle M**, Pande A, Iyer R, Yang G, LeVea C, Wilding G, Black J, Nava H, Nwogu C. Pilot study of gefitinib, oxaliplatin, and radiotherapy for esophageal adenocarcinoma: tissue effect predicts clinical response. *Am J Clin Oncol* 2008; **31**: 329-334 [PMID: 18845990 DOI: 10.1097/COC.0b013e318161dc04]

53   **Ilson DH**, Kelsen D, Shah M, Schwartz G, Levine DA, Boyd J, Capanu M, Miron B, Klimstra D. A phase 2 trial of erlotinib in patients with previously treated squamous cell and adenocarcinoma of the esophagus. *Cancer* 2011; **117**: 1409-1414 [PMID: 21425140 DOI: 10.1002/cncr.25602]

54   **Yang L**, Francois F, Pei Z. Molecular pathways: pathogenesis and clinical implications of microbiome alteration in esophagitis and Barrett esophagus. *Clin Cancer Res* 2012; **18**: 2138-2144 [PMID: 22344232 DOI: 10.1158/1078-0432.CCR-11-0934]

55   **Liu N**, Ando T, Ishiguro K, Maeda O, Watanabe O, Funasaka K, Nakamura M, Miyahara R, Ohmiya N, Goto H. Characterization of bacterial biota in the distal esophagus of Japanese patients with reflux esophagitis and Barrett's esophagus. *BMC Infect Dis* 2013; **13**: 130 [PMID: 23496929 DOI: 10.1186/1471-2334-13-130]

56   **Pei Z**, Bini EJ, Yang L, Zhou M, Francois F, Blaser MJ. Bacterial biota in the human distal esophagus. *Proc Natl Acad Sci USA* 2004; **101**: 4250-4255 [PMID: 15016918 DOI: 10.1073/pnas.0306398101]

57   **Yang L**, Chaudhary N, Baghdadi J, Pei Z. Microbiome in reflux disorders and esophageal adenocarcinoma. *Cancer J* 2014; **20**: 207-210 [PMID: 24855009 DOI: 10.1097/PPO.0000000000000044]

58   **Blackett KL**, Siddhi SS, Cleary S, Steed H, Miller MH, Macfarlane S, Macfarlane GT, Dillon JF. Oesophageal bacterial biofilm changes in gastro-oesophageal reflux disease, Barrett's and oesophageal carcinoma: association or causality? *Aliment Pharmacol Ther* 2013; **37**: 1084-1092 [PMID: 23600758 DOI: 10.1111/apt.12317]

59   **Narikiyo M**, Tanabe C, Yamada Y, Igaki H, Tachimori Y, Kato H, Muto M, Montesano R, Sakamoto H, Nakajima Y, Sasaki H. Frequent and preferential infection of Treponema denticola, Streptococcus mitis, and Streptococcus anginosus in esophageal cancers. *Cancer Sci* 2004; **95**: 569-574 [PMID: 15245592 DOI: 10.1111/j.1349-7006.2004.tb02488.x]

60   **Pikarsky E**, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, Ben-Neriah Y. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004; **431**: 461-466 [PMID: 15329734 DOI: 10.1038/nature02924]

61   **Yamamoto Y**, Gaynor RB. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. *J Clin Invest* 2001; **107**: 135-142 [PMID: 11160126 DOI: 10.1172/JCI11914]

62   **Hansel TT**, Kharitonov SA, Donnelly LE, Erin EM, Currie MG, Moore WM, Manning PT, Recker DP, Barnes PJ. A selective inhibitor of inducible nitric oxide synthase inhibits exhaled breath nitric oxide in healthy volunteers and asthmatics. *FASEB J* 2003; **17**: 1298-1300 [PMID: 12738811 DOI: 10.1096/fj.02-0633fje]

63   **Buchholz BM**, Chanthaphavong RS, Bauer AJ. Nonhemopoietic cell TLR4 signaling is critical in causing early lipopolysaccharide-induced ileus. *J Immunol* 2009; **183**: 6744-6753 [PMID: 19846874 DOI: 10.4049/jimmunol.0901620]

64   **Islami F**, Kamangar F, Boffetta P. Use of proton pump inhibitors and risk of progression of Barrett's esophagus to neoplastic lesions. *Am J Gastroenterol* 2009; **104**: 2646-2648 [PMID: 19806109 DOI: 10.1038/ajg.2009.369]

65   **Uno K**, Iuchi Y, Fujii J, Sugata H, Iijima K, Kato K, Shimosegawa T, Yoshimura T. In vivo study on cross talk between inducible nitric-oxide synthase and cyclooxygenase in rat gastric mucosa: effect of cyclooxygenase activity on nitric oxide production. *J Pharmacol Exp Ther* 2004; **309**: 995-1002 [PMID: 14988416 DOI: 10.1124/jpet.103.061283]

66   **Calatayud S**, García-Zaragozá E, Hernández C, Quintana E, Felipo V, Esplugues JV, Barrachina MD. Downregulation of nNOS and synthesis of PGs associated with endotoxin-induced delay in gastric emptying. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G1360-G1367 [PMID: 12433667 DOI: 10.1152/ajpgi.00168.2002]

67   **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 [PMID: 12512029 DOI: 10.1053/gast.2003.50008]

68   **Heath EI**, Canto MI, Piantadosi S, Montgomery E, Weinstein WM, Herman JG, Dannenberg AJ, Yang VW, Shar AO, Hawk E, Forastiere AA. Secondary chemoprevention of Barrett's esophagus with celecoxib: results of a randomized trial. *J Natl Cancer Inst* 2007; **99**: 545-557 [PMID: 17405999 DOI: 10.1093/jnci/djk112]

69   **Gatenby PA**, Ramus JR, Caygill CP, Winslet MC, Watson A. Aspirin is not chemoprotective for Barrett's adenocarcinoma of the oesophagus in multicentre cohort. *Eur J Cancer Prev* 2009; **18**: 381-384 [PMID: 19620873 DOI: 10.1097/CEJ.0b013e]

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