PROTECTIVE EFFECTS OF DIETARY AND PHARMACEUTICAL AGENTS AGAINST GASTROESOPHAGEAL REFLUX INDUCED ESOPHAGEAL CANCER

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ABSTRACT OF THE DISSERTATION

Protective effects of dietary and pharmaceutical agents against gastroesophageal reflux induced esophageal cancer

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There are two primary goals of this study. One is to establish a mouse esophageal adenocarcinoma (EAC) model and the other is to develop chemopreventive strategies to prevent EAC. We performed esophagogastroduodenal anastomosis (EGDA) on wild-type, $p53^{A135V}$ transgenic, and *INK4a/Arf*^{+/-} mice of A/J strain. Wild-type mice with EGDA were also treated with iron (50 mg/kg/m, *i.p.*) or gastrectomy plus iron to enhance carcinogenesis. At week 20 and week 40, we observed metaplasia in wild-type mice (1/20 at week 20; 6/37 at week 40), $p53^{A135V}$ mice (1/19 at week 20; 2/42 at week 40) and wild-type mice that also receiving gastrectomy and iron (1/15 at week 40). Esophageal squamous cell carcinoma (ESCC) developed in *INK4a/Arf*^{+/-} mice (1/14) and wild-type mice receiving gastrectomy and iron (3/14) at week 40. Twelve (92.3%) wild-type EGDA mice which were given iron from week 40 to 80 developed ESCC at week 80. None of these mice developed Barrett's esophagus (BE) or EAC.

We investigated the possible chemopreventive effects of α -tocopherol (389 ppm and 778 ppm), *N*-acetylcysteine (NAC, 500 ppm and 1,000 ppm), their combination (389 ppm α -tocopherol and 500 ppm NAC), omeprazole (1,400 ppm), Licofelone (1,000 ppm), the combination of omeprazole (250 ppm) and celecoxib (500 ppm) and the combination of zileuton (1,000 ppm) and celecoxib (500 ppm) in our EGDA rat model. All the esophagi of rats were harvested for histopathological examination. α -Tocopherol dosedependently decreased the incidence of EAC. The combination of α -tocopherol 389 ppm and NAC 500 ppm significantly reduced the incidence of EAC. Both omeprazole and Licofelone did not show inhibitory effect at the dose given. The combination of zileuton and celecoxib significantly reduced the tumor incidence, while omeprazole in combination with celecoxib did not show any effect on tumor incidence.

We concluded that under gastroesophageal reflux A/J mice are prone to develop ESCC but not EAC. α -Tocopherol can inhibit the development of EAC in our EGDA model with rats and stronger effects can be achieved when used in combination with NAC. Licofelone, omeprazole and omeprazole in combination with celecoxib did not show any chemopreventive effect on our EGDA rat model.

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DEDICATION

I dedicate my dissertation as a humble gift to my parents,

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LIST OF ABBREVIATIONS

4-HNE	4-Hydroxynonenal
5-Lox	5-lipoxygenase
8-OHdG	8-hydroxydeoxyguanosine
Arf	alternative reading frame
AUS	Antigen Unmasking Solution
BE	Barrett esophagus
COX	cyclooxygenase
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
DFMO	α –difluoromethylornithine
DMNM	2,6-dimethylnitrosomorpholine
DOC	deoxycholic acid
EAC	esophageal adenocarcinoma
EDA	esophagoduodenal anastomosis
EGDA	esophagogastroduodenal anastomosis
EGFR	epidermal growth factor receptor
ESCC	esophageal squamous cell carcinoma
GERD	gastroesophageal reflux disease
IC ₅₀	half-maximal inhibition
INK4A	inhibitor of kinase 4A
iNOS	inducible nitric oxide synthase

LOH	loss of heterozygosity
LOX	lipoxygenase
LTB4	Leukotriene B4
MBN	N-methyl-N-benzylnitrosamine
MDA	Malonyldialdehyde
Mdm2	murine double minute gene
MnTBAP	Mn (III) tetrakis (4-benzoic acid) porphyrin
NAC	N-acetylcysteine
NDGA	nordihydroguaiaretic acid
NF-κB	nuclear factor-к В
PPI	proton pump inhibitor
RNS	reactive nitrogen species
ROS	reactive oxygen species
SD rat	Sprague–Dawley rat
SOD	superoxide dismutase
TLESR	transient lower esophageal sphincter relaxation
TPRO	Thiazolidine-4-carboxylic acid

I. INTRODUCTION

A. Cancer

In the United States, cancer is the second leading cause of death in all populations (1). Accumulative human and animal studies show that cancer presents as an autonomous growth of tissue with genetic alterations (2). Many risk factors work alone or in combination to induce cancer in a certain period of time, these risk factors include tobacco and alcohol consumption, diet and nutrition, chronic infection, chronic inflammation, radiation, occupational carcinogen exposure, and environmental pollution (2).

It has been documented that there are four critical steps in carcinogenesis: initiation, promotion, malignant conversion and progression (1). Initiation often involves cellular DNA damage such as carcinogen induced DNA adduct formation or epigenetic changes such as DNA hypermethylation (3). Cell division is essential for the genetic changes occurring during the initiation stage to be passed to the daughter cells. The initiated cell clones are expanded in the promotion stage, and are susceptible to further genetic changes and malignant transformation. Malignant conversion is the step during which pre-neoplastic cells gain more genetic changes, acquire various malignant phenotypes during proliferation and finally form a neoplastic cell clone (4). If tumor promotion is stopped before the malignant conversion appears, it may result in premalignant lesions or benign tumors. Tumor progression is the final stage of carcinogenesis. At this stage, malignant cells will gain more aggressive characteristics over time and may become invasive (5). Once malignant cells spread beyond the primary tumor location, metastasis has occurred. Metastasis is the ultimate cause of most cancer death (1).

During carcinogenesis, normal tissues gain new features to be transformed into cancer. The most recognized new features are self-sufficiency in growth signals, insensitivity to growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, invasion and metastasis, and genome instability (6).

There are two general approaches about cancer management. One is to treat cancer after it has been diagnosed by appropriate surgery, chemotherapy, radiation therapy and other managements aimed to eradicate any detectable neoplasm. Due to the metastatic nature of many cancer types, this approach has not dramatically reduced the mortality of cancer patients except in the earliest stages of cancer formation. Another is to screen chronic diseases and risk factors associated with cancer and treat the premalignant diseases, to avoid risk factors and to prevent cancer from developing. The latter approach is more effective and beneficial to the general population (1).

B. Esophageal Carcinogenesis: Natural History, Clinical Presentation, Treatment, Etiology and Epidemiology

B.1 Natural history, Clinical Presentation and Treatment of Esophageal Cancer

There are a number of different types of primary cancer involving the esophagus. They can be categorized into two major types according to the site of origin and whether it is epithelial or non-epithelial. Epithelial cancers include squamous cell carcinoma, adenocarcinoma, adenoid cystic carcinoma, mucoepidermoid carcinoma, adenosquamous carcinoma, and undifferentiated carcinoma (small-cell carcinoma); non-epithelial cancers include leiomyosarcoma, carcinosarcoma and malignant melanoma (7). Among all these cancers, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) account for more than 95% of all primary esophageal malignancies (2).

Patients with esophageal cancer usually present with complaints of dysphagia, painful swallowing, pressure or pain in chest, and weight loss. Patients with advanced disease will also present with hoarseness, hiccups, pneumonia, and high levels of calcium in the blood (1). Symptoms of heartburn or gastroesophageal reflux disease (GERD) are usually more common with EAC patients. Barrett esophagus (BE), featuring the intestinal metaplasia of esophageal epithelium, is considered a pre-neoplastic lesion of EAC. The location of cancer differs between the two major histological types of esophageal cancer: EAC often occurs at the lower esophagus while ESCC at the cervical, upper or middle part of the esophagus. Because esophageal cancer is usually detected at an advanced stage, the five year survival rate is less than 15% (2).

Due to the popularity of endoscopic surveillance for GERD or BE and high through-put screening in high ESCC incidence areas, it is possible to detect a malignant lesion in an early stage. Therefore the managements of esophageal cancer, which relies mainly on extended resection and lymphadenectomy, now also include photodynamic therapy, laser ablation, mucosal destruction by argon beam or electrocautery, and endoscopic mucosal resection (8).

B.3 Epidemiology of Esophageal Cancer

Esophageal cancer is the sixth most frequent cancer world-wide, with more than 40,000 cases per year (2). The incidence of esophageal cancer varies dramatically in distinct geographical areas. Areas with high ESCC incidence include northern Iran, central Asian republics to north-central China, which was characterized as the esophageal cancer belt, parts of South America and southern and eastern Africa. The incidences are as high as 200 per 100,000 in these locations (2), whereas in the USA the total esophageal cancer incidence is 7 per 100,000 (1). The vast majority of esophageal cancer in the high incidence areas is ESCC. With high risk population screening, the incidence of ESCC has been stable or declining but that of EAC has been increasing during the last two decades in western countries (9). Johns Hopkins tumor registry data from 1959 to 1994 showed that new cases of EAC increased sharply after 1978. Patients with EAC exceeded that of patients with ESCC for the first time in 1994 (10). The increased EAC incidence features high male to female ratios at about 7:1 and higher incidence in the white population than that of black (11,12). The most affected sub-populations in order are white males, white females and Afro-American males in EAC (2,9). EAC generally affects people over 50 years old and peaks at ages 55 to 65 (13). Accordingly, the increasing trend is more prominent among older males: EAC rate doubled among people 65 years old or younger and increased three to four fold among people older than 65 years (14). As strikingly noteworthy as the increased incidence and disease patterns can be, the underlying reasons are still elusive.

B.3 Etiology

Even though the reasons for the increased EAC incidence are not clear, there are several risk factors related to population-based high EAC incidence. They will be discussed below.

B.3.1 Gastroesophageal Reflux Disease (GERD)

Gastroesophageal reflux is a common event in the human population. Everyone has the experience of reflux at some point in their lives (7,15,16). It becomes a disease only when people have persistent symptoms and complications. The term GERD is used to describe individuals who are exposed to the risk of physical complications from gastro-esophageal reflux, or who experience clinically significant impairment of health-related well-being (quality of life) due to reflux-related symptoms, after adequate reassurance of the benign nature of their symptoms (17).

The most popular complaints of patients are heartburn and regurgitation (7). Endoscopic examination may find esophagitis and esophageal mucosa breaks, including erosion and ulceration (15).

GERD is a common disease. Some population-based studies showed that about 20% of the general population experiences the symptoms of GERD (18-21). Pathophysiologically, GERD occurs when gastric contents reflux repeatedly into the esophageal lumen for a few months to a life time. It is believed that transient lower esophageal sphincter relaxations (TLESR) are the major mechanisms underlying GERD (22-25). Other possible mechanisms include hiatal hernia (26), gastric emptying abnormalities (27), and visceral hypersensitivity (28). Risk factors that contribute to GERD are genetic susceptibility (29-31) and obesity (32,33). The influence of alcohol

intake and smoking is unclear (30). A recent report analyzed the relationship of GERD with esophageal atresia, the most commonly seen congenital abnormality of esophagus (34). Patients who had a history of surgery for the treatment of esophageal atresia are more likely to develop GERD, GERD-related esophagitis and BE.

The treatments of GERD include anti-reflux surgery, acid suppressant therapies, and agents targeting TLESR reduction (17,35).

Population-based studies provide convincing data that GERD is a risk factor for EAC (36). The odds ratio of EAC among patients with recurrent GERD symptoms versus those who have none is 7.7 (37). Recent human studies and animal experiments indicate that GERD is also a risk factor in ESCC development. Studies which evaluated ESCC and laryngeal squamous cell carcinoma incidence in gastrectomy and partial gastrectomy patients found increased ESCC and laryngeal squamous cell carcinoma, indicating surgery-induced GERD is associated with squamous cell carcinoma (38-41). Sammon *et al.* believed that a maize-based diet which is in short of riboflavin and high in linoleic acid leads to GERD and may be the reason for local high ESCC incidence in Africa (42). They did not observe any food contamination. Animal studies also support this nutrient-deficiency hypothesis (43,44).

B.3.2 Barrett's Esophagus (BE)

Chronic GERD will induce an adaptive response in the squamous epithelium of the esophagus and transform the squamous epithelium into columnar epithelium by the appearance of glandular structures and goblet cells (45,46). These special pathological changes are defined as Barrett's esophagus (BE) (47). The incidence of BE in patients with GERD is about 15% (47,48). The appearance of specialized columnar epithelium in the esophagus is the trademark of this disease. This type of pathological change is acknowledged as a pre-malignant lesion of the esophagus. The risk of BE patients developing EAC has been estimated to be 0.5% per year. Therefore, these patients are at 30-125 times higher risk of developing EAC than the general population (46,49,50).

The incidence of BE has increased dramatically since the 1970s and this is most likely due to improved diagnostic techniques with widely applied flexible endoscopy (51). The mean age with a confirmed diagnosis is about 63 years old and the prevalence of BE reaches its peak at 70 years of age (52). Population-based studies suggest that BE is a disease that occurs predominantly in Caucasians. In a recent cross-sectional study, African-Americans, Caucasian-Americans and other races in the US were found to have a similarly high prevalence of reflux symptoms. However, African-Americans had a lower prevalence of erosive disease than Caucasian-Americans (53). Other independent crosssectional studies among patients referred for endoscopy have also reported that erosive esophagitis, esophageal strictures and BE are uncommon in African-Americans compared with Caucasians (54-56). Despite having similar proportions of subjects suffering GERD symptoms, it is obvious that Caucasian-Americans tend to have more GERD complications than African-Americans. BE appeared in 8.9% (194/2174) of white patients with GERD symptoms, but only 2.4% (6/249) of black patients (55). Another population-based study showed that BE has a surprisingly high prevalence in Hispanics living in the USA (57). Similarly it was reported that there is a 2% incidence of BE in all patients who underwent endoscopic examination in Taiwan (58). The investigators

believe that accelerated modernization and adoption of Western customs may be one of the reasons of the changed disease patterns.

Because BE is the known pre-malignant disease of EAC, it is important to identify high risk patients for further management and surveillance. BE is more frequent in GERD patients who get the disease at an earlier age, present nocturnal reflux symptoms more severely and with more complications such as esophagitis, stricture and ulceration (59). A study in a community-based practice compared patients with GERD symptoms for less than one year with patients with GERD symptoms for 1-3 years, the odds ratio of BE was 1:3 and increased to 1:6.4 if patients had the symptoms for more than 10 years (60). Interestingly, prolonged GERD symptoms, nocturnal reflux and more complications are shown to increase EAC incidence as well (37).

Other risk factors involved in BE are obesity (61-63), life style factors (alcohol consumption, smoking and diet) (64,65), and genetic factors (66,67). *H. pylori* infection is believed to be a protective factor for BE (68,69).

BE is not considered a risk factor of ESCC. Rosengard *et al.* reported 3 cases (2%) of ESCC in BE patients from 1980 to 1986 (70). All ESCC lesions were located in the squamous epithelium above the segment of Barrett's mucosa, and the patients had substantial use of tobacco and alcohol (70,71). Natural history of the patients suggests that BE and ESCC are more likely two different events. Another report described adenosquamous carcinoma (coexistent adenocarcinoma and squamous cell carcinoma) arising in BE in a 72 years old male (72), which is a rare situation.

B.3.3 Esophageal Cancer and Dietary Factors

Dietary factors have been evaluated in several studies of both cancer types (73-77). In a recent prospective cohort study, it was reported that total fruit and vegetable intake was significantly associated with decreased risk of ESCC but not EAC. When the authors examined fruit and vegetable consumption separately, the association with ESCC remained the same with fruits but not vegetables, although both contributed to the risk reduction. When they examined ESCC and EAC risk in subgroups, significant associations were observed between ESCC risk and the intake of two fruit groups, Rosacea (apples, peaches, nectarines, plums, pears and strawberries) and Rutaceae (citrus fruits). They also found a significant protective association between EAC risk and the intake of Chenopodiaceae (spinach) (78). Their findings are consistent with previous reports (76,79,80). One case-control study showed red meat, salted meat and boiled meat consumption is associated with increased incidence of ESCC (81). It also showed a positive but non-statistically significant association with EAC (82). This observation may be due to the relatively small number of EAC cases in the study. Healthy diets (diets rich in fruits, vegetables, poultry, fish) showed protective effects in both cancer types (77,83).

The protective effects of fruits and vegetables are generally credited to antioxidant vitamins and trace mineral elements. There are several population-based studies evaluating the risk of esophageal cancer and vitamin intake. Vitamin E, vitamin C, and beta-carotene are the most intensely studied vitamins. Antioxidant vitamins showed possible protective effects on both EAC and ESCC (84-89). One study suggested that the protective effects of antioxidant vitamins may be stronger among subjects under more oxidative stress due to smoking or GERD (88). To date, there are no interventional studies showing the chemopreventive effect of these antioxidant vitamins on EAC and ESCC (90,91). Two other reports showed that patients with head and neck cancer undergoing radiation therapy had a surprisingly higher second cancer incidence or mortality rate when given 400 IU α -tocopherol supplement (92,93). These findings may be due to the pro-oxidant effects of high α -tocopherol doses which increased the vulnerability of the patients to the damage of radiation therapy.

The discrepancy between the observational studies and interventional studies may be because: 1) the doses of the agents used in intervention studies were too low; 2) the interventional trials did not include proper target populations; 3) the combination of different antioxidants should be considered in future clinical trials.

B.3.4 Esophageal Cancer and Tobacco, Alcohol Consumption and Socioeconomic Level

Smoking is generally accepted as a risk factor for both ESCC and EAC. Population-based studies conducted in the United States, Europe, Taiwan and mainland China all agreed that increased duration or intensity of smoking significantly enhances the incidence of ESCC with a relative risk 5 to 10 times higher than non-smokers (73,94-96). Smoking is not as strong a risk factor for EAC as it is for ESCC, but it still increases EAC risk by over 3 fold (97). The different influence of smoking on EAC and ESCC may be because of different carcinogenic mechanisms in these two cancers. EAC is probably more inflammation driven, while ESCC is more likely carcinogen driven (98).

Considering alcohol consumption, it is not only recognized that alcohol is a risk factor but also that it synergizes with smoking to increase the risk of ESCC (95,99). Smoking will increase the production of acetaldehyde production from ethanol and

ethanol also serves as a solvent for carcinogens in tobacco (100,101). However, so far there is no convincing evidence to consider alcohol as a risk factor for EAC (9,97,102).

Both ESCC and EAC are related to low socioeconomic levels (103). The association remains even with multiple adjustments for established risk factors, such as reflux symptoms, body mass and smoking. It is reported that EAC cases are more likely to be found in populations with people of higher socioeconomic levels and senior age when compared with ESCC (98,104).

B.4 Chronic Inflammation and EAC

Inflammation is a protective response intended to eliminate exogenous and endogenous stimuli which cause cell injury, as well as necrotic cells and tissues resulting from the original insult. It is a complex reaction of vascularized connective tissues, featuring increased blood flow and vascular permeability, as well as emigration of the leukocytes from capillaries to the spot of injury (105). Inflammation closely interacts with repair, during which damaged tissue is replaced by the regenerated parenchymal cells (105). Although the primary goal of inflammation is to clear exogenous and endogenous pathogens and to help the system to go back to homeostasis, both inflammation and repair can cause harm. This is true in both acute and chronic inflammation (105). Chronic inflammation is an inflammatory response that lasts weeks to years. During this period of time, active inflammation, tissue injury and the healing process occur simultaneously (105). Chronic inflammation is characterized by: 1) tissue infiltration with macrophages, lymphocytes and plasma cells (inflammatory cells); 2) persistent tissue destruction caused by inflammatory cells; and 3) regeneration of destructed tissue (105). Chronic inflammation may be caused by the following reasons: 1) infections caused by virus or microorganisms; 2) long time exposure to potentially toxic agents; and 3) autoimmune diseases such as rheumatoid arthritis or multiple sclerosis (105).

Back in 1863, Virchow hypothesized a link between inflammation and cancer (106). It is reported that about 25% of all cancer cases arise from infection and chronic inflammation (107). Activated inflammatory cells are an important link between the two different lesions. There are numerous reports demonstrating that leukocytes, macrophages and other inflammatory cells can induce DNA, RNA and protein damage and modification in proliferating cells found in inflammatory tissues by the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (107-111). Besides direct damage of biological molecules, there are some key molecules which have been reported to connect inflammation and cancer such as cytokines, chemokines, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-Lox), signal transducers and activators of transcription 3 and nuclear factor erythroid 2-related factor 2 (112).

B.5 Oxidative Stress and EAC

Oxidative stress is a term to describe the imbalance of the production of free radicals such as reactive oxygen species and the neutralization of reactive intermediates. Under normal conditions, the leakage of activated oxygen from the mitochondria during oxidative respiration is a major source of reactive oxygen *in vivo* (105). The major free radicals include \cdot O₂-(superoxide anion), H₂O₂ (hydrogen peroxide), \cdot OH (hydroxyl

radical), ROOH (organic hydroperoxide), RO• (alkoxy) and ROO• (peroxy radicals), HOCl (hypochlorous acid), and OONO-(peroxynitrite). Among them, •OH is extremely reactive and will attack almost all of the cell components. One of the most recognized reactions which produces •OH is the Fenton reaction catalyzed by iron (113).

(1)
$$\operatorname{Fe}^{2+} + \operatorname{H}_2\operatorname{O}_2 \to \operatorname{Fe}^{3+} + \operatorname{OH}^{-} + \operatorname{OH}^{-}$$

(2) $\operatorname{Fe}^{3+} + \operatorname{H}_2\operatorname{O}_2 \to \operatorname{Fe}^{2+} + \operatorname{OOH}^{\cdot} + \operatorname{H}^{+}$

Xanthine oxidase, NADPH oxidases and cytochromes P450 are enzymes capable of producing superoxide. Hydrogen peroxide can be produced by a wide range of enzymes including some oxidases. There are some well studied antioxidant enzymes in cells as superoxide dismutase (SOD), catalase, and glutathione peroxidase. They catalyze the reactions below (105).

SOD catalyzes the dismutation of superoxide:

(1)
$$M^{(n+1)+} - SOD + O_2^{-} \rightarrow M^{n+} - SOD + O_2$$

(2)
$$M^{n+} - SOD + O_2^{-} + 2H^+ \rightarrow M^{(n+1)+} - SOD + H_2O_2$$
.

Where M = Cu (n=1); Mn (n=2); Fe (n=2); Ni (n=2).

Catalase catalyzes the decomposition of hydrogen peroxide:

$$2 \text{ H}_2\text{O}_2 \rightarrow 2 \text{ H}_2\text{O} + \text{O}_2$$

One example of glutathione peroxidase catalyzed reaction is illustrated below:

$$2GSH + H_2O_2 \rightarrow GS-SG + 2H_2O$$

Oxidative stress plays complex roles in the immune defense mechanisms. Once the immune system is initiated, the activated phagocytes will produce both ROS and RNS. The non-specific cytotoxic response will destroy most of the pathogen-hosting cells to prevent the escape of the pathogen from immune defense (105).

Many studies support the idea that oxidative stress is a driving force of cancer. By knocking out copper- and zinc- containing superoxide dismutase, mice developed liver cancer later in life (114). Another report showed that heterozygous manganese-containing superoxide dismutase knockout animals have an increased risk of lymphomas, adenocarcinomas and pituitary adenomas as they grow old (115). In esophageal cancer, oxidative stress is possibly a driving force as well (116,117). One component of refluxate, bile acid, can induce oxidative stress in several ways. 1) Direct detergent effect of bile acids will activate membrane enzyme as phospholipase A2, which releases arachidonic acid. Another two enzymes, cyclooxygenase (COX) and lipoxygenase (LOX), will produce ROS by partial reduction of molecular oxygen along with the production of prostaglandins and leukotrienes from arachidonic acids (118). 2) Several reports demonstrated that mitochondria can be damaged directly by lipophilic bile acids resulting in elevated level of ROS (119-121). 3) By activation of nuclear factor- κ B (NF- κ B), bile acid can induce the transcription of iNOS which will generate RNS (118,122,123). Our laboratory has developed two EAC models, esophagoduodenal anastomosis (EDA) and esophagogastroduodenal anastomosis (EGDA) (Figure 1A). In both models oxidative stress is possibly one of the driving forces of esophageal adenocarcinogenesis (116,124,125). The supplementation of iron induces higher tumor yield in both models. In a preliminary chemoprevention study of α -tocopherol acetate, a marginal protective effect was observed (126). .

C. Pathological Progression and Molecular Changes of EAC

C.1 Pathological Progression of EAC

Pathologically, there are four major morphological changes in the progression of EAC, which are chronic GERD induced reflux esophagitis, BE, BE with dysplasia and finally EAC.

The typical reflux-induced esophagitis features hyperplasia of esophageal squamous epithelium, infiltration of inflammatory cells in lamina propria and ulceration. Events leading esophagitis to BE are not clear yet. Stem cell hypothesis is one of the well-accepted theories (127). It is likely that the reepithelialization of esophagitic ulceration activates the multi-potential stem cells in esophageal epithelium. In the presence of acid, bile acids and duodenal contents multi-potential stem cells differentiate into a variety of epithelial cells found in BE (128). Although there are other theories that explain the appearance of BE, including submucosal gland migration and congenital rests of gastric epithelium, both human and animal data are not conclusive (129). The epithelium of BE is an incomplete form of intestinal metaplasia. The characteristic cells of this lesion are goblet cells. They are usually positioned between intermediate mucous cells in glandular structures of Barrett's mucosa. Paneth cells may be present too. Mature absorptive intestinal cells with a well-developed brush border are rare to be seen (47).

BE with dysplasia is especially important clinically due to its precancerous nature. It can be found in all parts of BE and is unrelated to the duration of the disease (128). Dysplasia can be located either at the upper half of the BE glandular crypt or the lower portion. Dysplastic glands have a combination of cytological and architectural abnormalities, presenting with nuclear crowding, stratification, loss of cellular polarity, and enlarged, elongated hyperchromatic nuclei and budding, branching, crowding and irregular shapes of glandular structures (128).

In EAC cases, back-to-back glands are a new feature accompanying with budding and branching. The nucleus may display bizarre chromatin patterns, pronounced pseudostratification with nuclei reaching the crypt luminal surface and markedly enlarged, abnormal nuclei and nucleoli. The definitive diagnosis can be made when neoplastic cells break the basement membrane and invade into the lamina propria (128).

C.2 Major Molecular Changes in EAC Carcinogenesis

Carcinogenesis is a multi-genetic procedure which leads to unregulated cell proliferation. The altered function of a small set of genes will lead to malignant transformation. These genes can be divided into two categories: proto-oncogenes and tumor suppressor genes (1). Proto-oncogenes regulate cell proliferation and differentiation. Point mutations, deletion of a negative regulatory sequence, promoter deregulation or gene amplification are found to be the mechanisms to activate proto-oncogenes into oncogenes. Oncogenes have gained a function of promoting cell proliferation, differentiation, motility or survival to transform susceptible cells into neoplastic phenotype (1). It is a dominant mechanism of carcinogenesis since the alteration of one gene allele is enough to initiate or promote cancer. Proteins encoded by tumor suppressor genes are involved in growth inhibition and tissue differentiation (1). Since only inactivation of both alleles can switch off the function of the gene, the loss of function of a tumor suppressor gene is a recessive mechanism of carcinogenesis (130).

The molecular evolution of EAC is a complicated network of genetic events. It is suggested that the combination of clonal expansion and genetic instability may be the mechanism underlining the progression from BE to EAC (131). Several genes are activated or inactivated during EAC carcinogenesis. The most frequently detected genes are as follows.

1) P53 is located on chromosome 17 (17p13.1) of the human genome. It encodes a phosphoprotein of 53,000 daltons. P53 protein will accumulate in the nucleus under different forms of stress, especially those leading to DNA damage. It acts as a transcriptional factor to regulate genes involved in the cell cycle, apoptosis, DNA repair and differentiation (2). Generally, when the cells are subjected to DNA damage, p53 will be activated, hold the cell cycle at G_1/S point and initiate DNA repair. But if the DNA damage is irreparable, p53 will activate the apoptosis program to avoid permanent DNA changes (2). As a tumor suppressor gene, both alleles need to be inactivated to lose its function. One allele of p53 gene is frequently inactivated by loss of heterozygosity (LOH). This genetic change of p53 gene has been found in 75% to 80% of EAC (132). LOH of p53 is developed by multiple chromosome mechanisms such as DNA deletion, LOH without copy number change and tetraploidy followed by genetic loss (133). BE patients with p53 LOH have increased risk of cancer (134). p53 mutation rates also increase in BE, low grade dysplasia, high grade dysplasia and EAC sequence, which increase from 29% to 66% in BE to low grade dysplasia and 40% to 88% in high grade dysplasia to EAC (132). Even though p53 mutation does not guarantee the presence of p53 protein accumulation, nuclear p53 staining is detected in most of p53 mutant samples (135).

2) *P16* is located at chromosome 9p21 of the human genome. It is also called cyclin dependent kinase inhibitor 2, inhibitor of kinase 4A (*INK4A*), and multiple tumor suppressor (*MTS1*). This gene contains two reading frames which encode two distinctive proteins without a single amino acid in common. One of the reading frames encodes *p16* which is an inhibitor of cyclin-dependent kinases 4 and 6. The loss of the p16 protein results in increased cell proliferation, escape of senescence and longer life span. Another reading frame is named $p14^{4RF}$ which means alternative reading frame. The function of $p14^{4RF}$ is closely related to p53 by way of murine double minute gene (*Mdm2*). The activation of $p14^{4RF}$ blocks *Mdm2* which leads to the accumulation and activation of p53 (2). The loss of 9p21 allele in EAC has been reported (136,137). Point mutations of the *p16* gene is rare in EAC (138). Promoter methylation with or without LOH is a common phenomenon in EAC and BE. It occurs in 85% of dysplasia cases of BE (139). Promoter hypermethylation is also a mechanism to silence $p14^{4RF}$ in EAC carcinogenesis (140).

3) Epidermal growth factor receptor (EGFR) is located at chromosome 7p12-13 (132). It is also know as ErbB1/HER1. EGFR is a receptor tyrosine kinase on cell surface and can be activated by several ligands including epidermal growth factor and transforming growth factor- α (141). The activation of EGFR will trigger several pathways, among which are RAS-RAF-MEK-ERK and P13K-AKT pathways (141). Abnormal behavior of EGFR has been reported in human EAC. EGFR protein is overexpressed in 30% to 55% of EAC cases (142,143). The expression of EGFR increases along with the development from BE to esophagus adenocarcinoma (EAC) and is related to poor prognosis of the disease (144-146). An EGFR receptor inhibitor, gefitinib, is on clinical trial for the treatment of EAC (147).

D. Animal Models of Esophageal Cancer

As discussed above, reflux induced BE is the major risk factor of EAC while carcinogen exposure (tobacco and alcohol) is more likely to induce ESCC. The animal models for EAC and ESCC should mimic the pathogenesis of these two diseases. Having this in mind, both reflux and carcinogens are used to develop EAC and ESCC animal models.

D.1 Rat Models

EAC Most widely used and BE models are surgery-induced gastroduodenoesophageal reflux rat models. In 1989, Pera et al. developed a chronic reflux-induced EAC model on Sprague-Dawley rats (SD rat) by esophagojejunostomy with gastric preservation plus 2, 6-dimethylnitrosomorpholine (DMNM) injection weekly after surgery. Rats receiving only esophagojejunostomy with gastric preservation did not show EAC 17 weeks after the surgery. Surgery combined with carcinogen induced both EAC and ESCC at 18 to 19 weeks after surgery with 24% to 38% EAC incidence and 13% to 2% ESCC incidence, respectively (148,149). Attwood et al. made some adjustments of Pera's procedure and developed duodenoesophageal anastomosis, which is also known as esophagoduodenostomy or esophagoduodenal anastomosis (EDA) (150). EDA itself produced EAC in 1/14 rats and benign diffuse papillomatosis in 7/14 rats, but did not induce ESCC at 22 weeks after surgery. EDA plus DMNM or methyl-namylnitrosamine increased the incidences of EAC to about 30% and ESCC to 40%. Most rat tumors showed both ESCC and EAC pathological change with nests of cells
producing keratin in one area and mucin in another. Only a small percentage of tumors were pure, well-differentiated EAC. Their experiment showed that reflux alone can produce EAC in rat's esophagus although the tumor incidence is low (1/14). Reflux and carcinogen combination increased both EAC and ESCC formation than reflux alone.

Our laboratory adapted the EDA procedure to study EAC. We observed BE and EAC in SD rats with EDA. But the EAC yield was only about 10% and rats had to be sacrificed early because of anemia. When EDA animals were supplemented with iron (50 mg/kg/month) to compensate for surgery induced iron malabsorption, the EAC yield increased to 73% (151,152). ESCC was not found with or without iron supplementation. Further sample analysis revealed that iron deposited in the stroma tissue under the esophageal epithelium and oxidative stress was more prominent in these samples. We later modified the EDA procedure by making an anastomosis between the gastroesophageal junction and duodenum, which is known as esophagogastroduodenal anastomosis (EGDA) (124). EGDA is a better choice to induce BE and EAC in rats by avoiding major nutritional complications and severe large-area esophagitis, which are unwanted effects of the EDA procedure. It is noteworthy that ESCC was not found in our EDA or EGDA model with or without iron supplementation.

Miwa *et al.* tried a new strategy to develop esophageal carcinoma (153). Instead of inducing reflux of gastric and duodenal contents directly into the esophagus, they did duodeno-forestomach reflux which induced the duodenal reflux into the forestomach of the rats and left the esophagus intact. At 50 weeks after the surgery they did not find any EAC in the animals but they did observe 18% BE lesion in the animals (153). The advantage of their procedure is that the esophagus was left intact similar to reflux in humans. The biggest problem about this model is that they did not see any EAC 50 weeks after the surgery, which largely limits the application of the model.

Recently a novel esophageal perfusion model was reported by Yan Li *et al.* (154). They buried a subcutaneous osmotic micro-pump to deliver desired agents to the upper esophageal lumen through a catheter inserted into the upper esophagus. This procedure avoids surgical complications that the earlier models may have encountered and enables precise control of the agents for perfusion. This model has the advantage of studying the primitive damage induced by different reflux components.

D.2 Mouse Models

Mouse esophagus is very similar to rat esophagus in histology. It is necessary to develop a mouse EAC and BE model to take advantage of genetic modified mouse strains for mechanistic studies. Several groups tried different strategies to develop a mouse EAC or BE model. Xu *et al.* performed esophagojejunostomy on Swiss–Webster mice and found 4 cases of BE (12%) and 4 cases of EAC (12%) in the surgical group (155). The surgical mice which received N-methyl-N-benzylnitrosamine (MBN) treatment had a higher BE incidence (7 cases, 20%) but EAC incidence remained the same. They found more ESCC and adenosquamous carcinoma in the carcinogen plus surgery group (155). Later this group introduced *p27* knockout genotype into Swiss–Webster mice and treated the mice with carcinogen plus surgery. They found 86% BE and 23.3% EAC in the *p27* knockout group (156). The majority of cancer they found in this model is still ESCC, which is 62%. Their study showed that it is possible to apply a surgical procedure, which successfully induces EAC in the rat, to the mouse and induce EAC as well. The mice in

all their studies were kept 18 to 20 weeks after the surgery. Therefore, the mouse esophagi were exposed to reflux and carcinogen in a shorter period of time compared with our rat model (30 to 40 weeks). This may be a partial reason for the low EAC incidence they observed. Another shortcoming of their study is that they did not give iron supplementation to the surgery mice, which might develop iron deficiency after the surgery. The high adenosquamous carcinoma and ESCC yield in this model are not desirable. The detailed mechanisms of this observation are still unknown. It is necessary to improve this model for higher EAC yield.

D.3 Canine Models

A dog EAC and BE model was reported by Kawaura *et al.*, using cardiectomy to induce gastric reflux and total gastrectomy plus esophagojejunostomy to induce alkaline reflux (157). BE was developed in 14 of the 26 dogs in the gastric reflux group and 10 of the 24 dogs in the intestinal reflux group 18 - 39 months after the surgery. The two groups showed one EAC case each after 63 and 66 months respectively. Because dogs have non-keratinized stratified squamous epithelium and submucosal glands in their esophagi similar to humans, reflux induced BE and EAC in dogs may be more relevant to the human condition.

E. EAC Chemoprevention

Since GERD and BE are major risk factors of EAC, it is possible to identify a population at high risk and to treat the pre-malignant disease. So far endoscopic surveillance and surgical managements are major strategies of BE management applied

clinically (158). Chemopreventive agents based on different mechanisms are tested in both clinical trials and animal models to prevent EAC development.

E.1 Anti-oxidative Treatment

Different antioxidants were used in the chemoprevention study of EAC. Piazuelo *et al.* treated rats that underwent esophagojejunal anastomosis with 3 mg/kg SOD *s.c.* once every three days for one to four months and found that SOD treatment significantly reduced EAC incidence 3 and 4 months after the surgery (159). Their data support the chemopreventive role of antioxidants in EAC. But SOD is not an ideal chemopreventive agent considering the cost. Another group followed the same idea and treated their EDA rats with Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP), which is a non-peptidyl mimic of SOD. EAC incidence was significantly reduced in the MnTBAP treated group (160). Due to the extremely small sample size (n=5), their results need confirmation.

Other antioxidants have been tried in animal experiments. Thiazolidine-4carboxylic acid (thioproline, TPRO) is a RNS scavenger. Two studies targeting RNS used this agent to prevent EAC. TPRO significantly decreased EAC incidence in EDA rats treated with 0.5% TPRO in diet (161,162).

Vitamin E and selenium are considered safe and effective antioxidants for longterm clinical use. The chemoprevention study in Linxian, China showed that the combination of beta-carotene, vitamin E and selenium significantly reduced total mortality, total cancer mortality and gastric cardia cancer mortality (163). Our laboratory used both vitamin E and selenium to prevent EAC development in our EDA model (126). To our surprise selenium (in the form of sodium selenate, 1.7 mg/kg in diet) significantly increased EAC incidence from 67.9% in the surgical control group to 90.3% in the selenium treated group. It is known that selenium is a component in many antioxidative enzyme systems, such as the glutathione peroxidase family and thioredoxin reductase family (164). But selenium plays a more complicated role in cancer. Sodium selenate was found to induce chromosome breaks and spindle disturbance in mouse bone marrow after oral administration (165). It was also carcinogenic by increasing tumor incidence from 16.9% to 41.7% in Long-Evans rats (166). It is likely that sodium selenate is also carcinogenic in our EGDA rat model. Selenium is usually in the form of organoselenium compounds in food. They are considered safer and more potent and may be a better choice for chemoprevention studies (167). Vitamin E in diet (10 fold of basal diet level) did not reduce EAC incidence compared to the surgical control group and only elevated plasma vitamin E to the same level as the non-operated control. But the vitamin E plus selenium group showed significantly reduced tumor incidence compared with selenium only treated group. Due to the nutritional deficiency induced by EDA surgery, it is possible that a higher dose of vitamin E, which can significantly increase the plasma vitamin E level, may have a better chemopreventive effect.

E.2 Anti-inflammatory treatment

Because reflux-induced chronic inflammation is a prominent pathological process in EAC development, anti-inflammatory treatment has long been considered an attractive approach to prevent EAC. It is known that arachidonic acid metabolism pathway is more active in reflux-induced inflammation of the esophagus (117,168). Epidemiological studies showed that long-term use of nonsteroidal anti-inflammatory drugs can inhibit the development of EAC (169,170). The chemoprevention studies therefore have focused on the arachidonic acid metabolism pathways.

Targeting arachidonic acid pathway, our laboratory first tested sulindac (a cyclooxygenase inhibitor), nordihydroguaiaretic acid (NDGA, a lipoxygenase inhibitor) and α -difluoromethylornithine (DFMO, an ornithine decarboxylase inhibitor) for their chemopreventive effect in our EGDA rat surgical model. Sulindac 300 ppm in diet alone or in combination with NDGA or DFMO reduced tumor incidence significantly. NDGA alone only showed slight chemopreventive effect in this model and DFMO alone did not affect tumor incidence (171). The results were also confirmed by another group (172); supporting the hypothesis that arachidonic acid metabolism pathway is one of the major pathological process leading to EAC.

COX-2 and 5-Lox are two enzymes which are overexpressed in BE and EAC (173,174). MF-Tricyclic and celecoxib are COX-2 inhibitors and were tested in the EDA rat model for their chemopreventive effect. These two agents significantly inhibited the formation of EAC in EDA rat model (172,175). The major concern about using COX-2 inhibitors in EAC chemoprevention is that lipoxygenase-derived products may increase by inhibiting COX-2 alone due to shunting of arachidonic acid pathway (176). In order to achieve a better chemoprevention effect, we used both COX-2 and 5-Lox inhibitors (celecoxib and zileuton) in our EGDA rat model. As we expected, the combination of the two agents (500 ppm each) significantly decreased the EAC incidence in EGDA rats. These two agents had additive but not synergistic effect between them (177).

E.3 Antacid treatment

PPIs (proton pump inhibitors) are widely used antacid treatments for management of gastric reflux. Since PPI treatments effectively control the GERD symptoms, it is expected that long-term acid suppression may also prevent EAC development, but the role of the PPI treatment in esophageal adenocarcinogenesis is still controversial (178,179).

Cooper et al. observed squamous re-epithelialization in 48% patients under 1-13 years PPI treatment even though the length of Barrett's esophagus did not change (180). But one case report showed EAC arising under the squamous re-epithelialized BE, which gives rise to concern that PPI treatment may hide advanced malignancies (179,181). Another study showed that long-term acid suppression increased the risk of EAC significantly, but this group of patients also had more cases of reflux symptoms, oesophagitis, BE, or hiatal hernia than the control (182). Peters et al. observed significantly reduced length and area of BE in PPI treated patients under endoscope (183), which gives the expectation of stabilizing the histological condition of BE by PPI. The chemopreventive effect of PPI was also evaluated in rat models with gastroesophageal reflux induced by different surgical procedures. These studies failed to show any benefit of PPI in the prevention of esophageal cancer, but surprisingly showed an early appearance of abnormality in esophageal epithelium (184,185). The lack of a strictly selected control is the major confounding factor to draw a conclusion from PPI chemoprevention human data. The insufficient exposure of the rats to both reflux and PPI treatment and low EAC incidence make the animal study data less convincing.

The major side effect of proton pump inhibitors is hypergastrinemia. After long term treatment, gastrin level may exceed 400 pmol/L (normal range 10-59pmol/L) (186).

Gastrin has a proliferative effect on Barrett's esophagus by activating cholecystokinin 2 receptor and inducing COX-2 expression (187-189). PPI may also promote EAC by allowing bile acid-induced mutagenesis in a neutral environment (190,191). Based on current understanding, the role of PPI in EAC carcinogenesis needs to be further explored.

F. Agents Used in This Study

F.1 Antioxidants: Vitamin E and *N*-acetylcysteine (NAC)

Vitamin E describes 8 different compounds. These compounds include 4 tocopherols and 4 tocotrienols (designated as α -, β -, γ -, and δ -) (Figure 2A). The tocotrienols share the same ring structure with tocopherols but have an unsaturated tail. The most abundant source of vitamin E in food is vegetable oil (192). All the vitamin E forms have similar antioxidant function (193). α -Tocopherol transfer protein is found in the liver cytosol to help transfer vitamin E between membranes (194). This protein prefers nature *SRR*- α -tocopherol, synthetic 2*R*- α -tocopherols and *all*-*rac*- α -tocopherol to other vitamin E forms. Therefore, α -tocopherol is preferably maintained in human plasma and tissue and meets human vitamin E requirements (193).

NAC is a derivative of amino acid L-cysteine (Figure 2B). It is the precursor of glutathione. The thiol (sulfhydryl) group in NAC can neutralize endogenous and exogenous oxidants. One chemoprevention study showed that the application of NAC significantly reduced the incidence of lymphoma in ROS sensitive ataxia-telangiectasia mutated (Atm) mice (195). Another cell culture study showed that NAC can decrease tumor cell invasive capacity by inhibiting the transdifferentiation of skin fibroblasts to

myofibroblasts (196). But in EUROSCAN trial, NAC 600mg daily treatment for 2 years did not prevent the recurrence or the occurrence of second primary tumor in patients with head and neck or lung cancer (197). The population in the study may not be the appropriate target population for NAC chemoprevention. The dose used in the study may be too low, considering the 1,200 mg NAC used in clinic as a mucolytic agent (198).

F.2 Antacid Agent: Omeprazole

Omeprazole (5-methoxy-2-[(4-methoxy-3, 5-dimethyl-pyridin-2-l) methylsulfonyl] -3H-benzimidazole), also known by its common trade name Prilosec or Losec, is one of the most widely used PPIs (Figure 3). It covalently binds to the hydrogen/potassium adenosine triphosphatase enzyme system (the H⁺/K⁺ ATPase enzyme, which is called proton pump) of the gastric parietal cell resulting in long lasting reduction of gastric acid production (199,200). The inhibition is irreversible, therefore it requires parietal cells to produce new proton pumps or to activate the resting ones to regain function (201). Omeprazole is absorbed primarily in small intestine after oral dosage and completely metabolized by P450 system in liver. A dose of 20 mg daily or greater is able to virtually abolish intragastric acidity in most individuals (202). Because of its efficiency and safety, omeprazole is used world wide to treat gastric ulcer, GERD and gastritis. Despite 20 years of clinical application of PPIs, the rising incidence of EAC did not slow down. In the current study the chemopreventive issues of omeprazole were addressed using our EGDA rat model.

F.3. Anti-inflammatory Agents: Celecoxib, Zileuton and Licofelone

The targets of the anti-inflammatory agents celecoxib, zileuton and Licofelone, are two important enzymes in inflammatory cascades. They are COX-2 and 5-Lox. Their major substrates are arachidonic acids. The arachidonic acid pathways with which COX and 5-Lox enzymes are involved were illustrated (Figure 4).

There are three COX isozymes found in mammalian cells, COX-1, which is constitutively expressed, COX-2, which is inducible and COX-3 which is found primarily in the central nervous system (203-206). COX-1 is detected in the gastrointestinal system, kidneys, vascular smooth muscle and platelets. COX-2 is undetectable in most tissues and can be induced by inflammatory mediators such as growth factors and cytokines (207). Hence COX-2 is responsible for the rising prostaglandin E2 (PGE2) level in inflammation-induced pathological condition. PGE2 promotes tumor growth by EP receptor signaling to stimulate cell proliferation and angiogenesis (208). Selective COX-2 inhibitors, such as celecoxib, were developed to reduce PGE2 production in pathological conditions.

Celecoxib is the brand name of 4-[5-(4-methylphenyl)-3-(trifluoromethyl) pyrazol-1-yl]benzenesulfonamide (209) (Figure 5A). This selective COX-2 inhibitor has a half-maximal inhibition (IC₅₀) on COX-1 from 4 to 19 μ M and 0.003 to 0.006 μ M on COX-2 (210). After oral administration, celecoxib will reach the peak plasma level within 2 hours and be extensively metabolized by liver. Only about 2% of celecoxib is excreted intact from urine and feces. The majority of celecoxib is metabolized by oxidation and conjugation (211). It is reported that celecoxib significantly inhibited EAC development in rat models (175,177). Celecoxib is also used as a part of adjuvant therapy

in EAC patients or chemopreventive treatment in BE patients. Even though more cases are required to confirm the efficacy, the drug is very well tolerated (212-214).

Leukotriene B4 (LTB4) is an important inflammatory mediator produced in 5-Lox pathway which has chemotactic activity on neutrophils and eosinophils (215). The activated eosinophils will release singlet oxygen and hydrogen peroxide to promote oxidative stress. 5-HETE and 5-oxo-ETE also have been reported to have inflammatory potential (216). There are several cancers with 5-Lox overexpression, such as colon cancer (217), pancreatic cancer (218), oral cancer (219), and esophageal cancer (174).

Zileuton (N-(1-benzo[b]thien-2-ylethyl)-N-hydroxyurea) (Figure 5B) is the specific inhibitor of 5-Lox. It inhibits the production of LTB4 in both human and rat polymorphonuclear leukocytes with IC₅₀ of 0.4 μ M (220). In vivo, zileuton has shown rapid and sustained inhibition of LTB4 in both dogs and rats at oral doses of 0.5 to 5 mg/kg (220). Zileuton is metabolized by liver and excreted in urine as a conjugate (221,222). Zileuton has chemopreventive effects in EAC, lung and oral cancer models (177,223-225).

Blocking either COX pathway or 5-Lox pathway will lead to another concern, the "shunting effect". Theoretically an ideal inhibition will be achieved by a COX-2/5-Lox dual inhibitor. Licofelone (1H-pyrrolizine-5-acetic acid, 6-(4-chlorophenyl)-2,3-dihydro-2,2-dimethyl-7-phenyl-) is a new anti-inflammatory agent with COX-2/5-Lox inhibitory activity (226) (Figure 5C). Its anti-inflammatory effect has been shown in down-regulated polymorphonuclear leukocyte function (227) and antithrombotic activity (228). Licofelone has potent COX-2 and 5-Lox inhibitory efficiency. The IC₅₀ of COX-2 and 5-Lox inhibitory activitien are 0.21 μ M and 0.18 μ M in bovine thrombocyte intact cell assay and

intact bovine polymorphonuclear leukocytes, respectively (229). Licofelone inhibited the synthesis of PGE 2 and LTB4 in a dose-dependent manner ($IC_{50} = 3.9 \mu M$ and 3.8 μM , respectively) in a human whole blood assay and basophilic leukemia cell assay using RBL-1 cells (230). Animal study data are consistent with in vitro data. Licofelone inhibited PGE2 and LTB4 secretion in a carrageenan-induced rat paw edema model, with ED₅₀ value of 17 mg/kg p.o. (231). Plasma concentrations of Licofelone peaked at 0.7 to 4 hours after oral administration in human or rats. Most metabolites are excreted in feces and the highest tissue levels of Licofelone are detected in the lung, liver, kidney, heart and intestine, suggesting enterohepatic circulation (226,232). Recently it is reported that Licofelone showed anticancer effects in colon and prostate cancer cell lines by inducing apoptosis (233,234).

II. GOALS AND SPECIFIC AIMS

The goal of this thesis research was to study esophageal adenocarcinogenesis and its prevention. Our previous studies utilizing the rat EGDA model demonstrated that esophageal reflux-induced inflammation, associated with oxidative stress and arachidonic acid metabolism, is a major contributory factor for the development of BE and EAC. Based on our previous results, we hypothesized that some antioxidative nutrients, inhibitors of COX and LOX enzymes, and antacid agents can protect EGDA rats from developing EAC. In order to test this hypothesis, we thought it would be prudent to develop a mouse model. The mouse model should be more economical than rat models in the expenses for diet, chemopreventive agents, and animal per diem charge. A mouse model for EAC would facilitate mechanistic studies, as the involvement of different pathways in esophageal adenocarcinogenesis can be studied in genetically modified mice. In developing the mouse EGDA model, we hypothesized that mice with mutated p53 or defective INK4a/Arf would develop EAC earlier than wild-type mice. These models would also enable us to mimic the p53 mutation or $p16^{INK4a}$ alterations present in the development of human EAC.

Specific Aims

A. To Develop EGDA Surgery Models for EAC in Wild-type A/J, $p53^{A135V}$ Transgenic and *INK4a/Arf^{+/-}* Heterozygous Mice. These defective genes combined with surgery induced chronic reflux are expected to result in an earlier tumorigenesis and higher tumor yield than the wild-type mice. These may enable us to develop an EAC model that more closely mimics molecular alterations of the human disease.

B. To Investigate the Chemopreventive Effect of α -Tocopherol and NAC, Alone or in Combination, as Well as Omeprazole on the EGDA Model for EAC. Three different doses of α -tocopherol will be provided in the diet to produce different antioxidative nutritional statuses. The effect of α -tocopherol nutrition on oxidative damage and tumorigenesis will be analyzed. NAC will also be studied alone or in combination with α -tocopherol to further test the oxidative stress hypothesis. The effect of the PPI, omeprazole, on EAC carcinogenesis will also be evaluated in the EGDA model. The existing rat model will be used in this study if a suitable EGDA mouse model cannot be developed.

C. To Investigate the Chemopreventive Effect of the Dual COX-LOX Inhibitor Licofelone, in Comparison to the Effects of a Combination of Celecoxib and Zileuton as Well as to Investigate the Combined Chemopreventive Effects of Celecoxib and Omeprazole. Licofelone will be administered from 3 to 41 or 20 to 41 weeks after surgery to evaluate the effects of treatment duration on tumorigenesis. Zileuton and celecoxib will be used together to serve as a comparison to evaluate the effect of Licofelone. Omeprazole and celecoxib will be used together to examine the combined effects of the anti-inflammatory agent with an antacid agent.

III. RATIONALE AND RESEARCH DESIGN

A. To Establish an EGDA-induced Esophageal Adenocarcinogenesis Model in Wildtype, *p53*^{A135V} Transgenic and *INK4a/Arf*^{+/-} Heterozygous A/J Mice. (*Specific aim 1*)

A.1. EGDA Mouse Model in EAC Study

Several mouse esophageal adenocarcinoma models have been reported. Duncan et al. reported an E1A/E1B transgenic mouse model (235). All transgenic mice developed adenocarcinoma at the squamocolumnar junction in the gastric cavity at 12 to 17 weeks old without surgery or carcinogen treatment. But the animals can not breed to keep a stable strain and the tumor was in the forestomach instead of the esophagus. Fein et al. reported a *p53* knockout mouse model with gastrectomy and esophagojejunostomy (236). Out of 12 p53 knockout mice, 4 survived after 24 weeks of observation and two of them had EAC and another one had squamous cell carcinoma. Due to the short life span of p53 knockout mice, the application of this model is limited. Another research group reported a mouse EAC model achieved by esophagojejunostomy and the carcinogen MBN using both wild-type and p27 knockout mice (156,236,237). Animals developed both adenocarcinoma and squamous carcinoma. With p27 mutation they observed more adenocarcinoma but it was still only 23.3% of total tumor cases. Another attempt of developing mouse EAC model was designed by feeding C57BL/6 mouse with zinc deficient diet, 0.2% deoxycholic acid (DOC) diet, and zinc deficient plus 0.2% DOC diet. After 152 days of feeding, 5 out of 8 mice on zinc deficient plus 0.2% DOC diet and 1 out of 11 mice on zinc deficient diet developed mucinous metaplastic epithelium in

esophagus. However neither BE nor EAC was found in any of the animals (188). There are no reports of EGDA mouse surgery models so far. The anatomy of EGDA and EGDA plus gastrectomy model is illustrated in Figure 1. The arrows indicate the direction of the reflux. The EGDA procedure is to make two 0.5 cm incisions on the gastroesophageal junction and the duodenum on the anti-mesenteric border and then anastomose together with accurate mucosa to mucosa opposition.

A.2. Research Design for Part A

Six to eight week old A/J mice with or without $p53^{A135V}$ transgene and $INK4a/Arf^{+/-}$ were divided into 7 groups:

Group A: non-operated control (10 male, 10 female)
Group B: EGDA control (49 male, 52 female)
Group C: p53A135V transgenic mouse with EGDA (36 male, 42 female)
Group D: INK4a/Arf+/-mouse with EGDA (24 male, 5 female)
Group E: EGDA plus omeprazole treatment plus iron supplementation (36 male)
Group F: EGDA plus iron dextran supplementation (30 male)
Group G: EGDA plus gastrectomy plus iron supplementation (26 male)

Female A/J mice (6-8 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME) as breeders. Two genetic modified mouse strains were generous gifts from Dr. Ming You (238). The mice were bred to A/J mice for at least 5 generations in our animal facility before we started the experiment. All the mice were housed 10 per cage in plastic cages with hardwood bedding and dust covers before surgery. They were given

water ad libitum and were fed lab chow before the surgery and AIN-93M purified diet (Research Diet Inc., New Brunswick, NJ) or AIN-93M purified diet added with 1,400 ppm omeprazole after the surgery. Surgeries were performed when the mice were 6-8 weeks old by Dr. Xiaoxin Chen. Body weights were monitored once per four weeks in the duration of the studies. All mice were euthanized by CO₂. The esophagus was removed, opened longitudinally, fixed in 10% buffered formalin for 24 hours and transferred to 80% ethanol.

B. The Chemopreventive Effect of α-Tocopherol, NAC Alone or in Combination and Omeprazole on EGDA Rat Model (*Specific aim 2*).

B.1. Rationale of the Study: Oxidative Stress is One of the Driving Forces of EAC Carcinogenesis

The carcinogenic role of oxidative stress in esophageal adenocarcinogenesis has been reported in both human and animal studies. In samples from human patients, it has been reported that glutathione content was progressively decreased in the GERDesophagitis-metaplasia-dysplasia-adenocarcinoma sequence while myeloperoxidase activity was higher than in controls, plateauing at Barrett's epithelium without dysplasia. Glutathione content was inversely correlated with levels of DNA adducts (239). Another study showed that an oxidative DNA damage marker, 8-hydroxydeoxyguanosine (8-OHdG), was significantly increased in the distal esophagus with Barrett's epithelium and high-grade dysplasia as well as in EAC (240). The expression of manganese superoxide dismutase was significantly reduced in esophageal tissue of patients with specialized intestinal metaplasia, low-grade dysplasia, high-grade dysplasia, and esophageal adenocarcinoma when compared with normal esophagus. The expression was similar for esophageal adenocarcinoma and high-grade dysplasia (241). These findings indicated that oxidative stress is associated with EAC carcinogenesis.

In our EDA rat model we observed increased staining of iNOS and nitrotyrosine in macrophages with the progression of the disease (152). In a later study iron injection, which increased oxidative stress, increased the EAC incidence in our EGDA rat model (116,117,126). Vitamin E supplementation at 778 ppm in diet showed a chemopreventive effect (126).

B.2. Research Design for Part B

Six to eight weeks old male SD rats from Taconic Farms (Germantown, NY) were housed three per cage, separated into eight groups.

Group A: non-operated control (n=9) Group B: EGDA control (n=36) Group C: EGDA rats treated with 389 ppm α -tocopherol (5 times basal diet level) (n=36) Group D: EGDA rats treated with 778 ppm α -tocopherol (10 times basal diet level) (n=36) Group E: EGDA rats treated with 500 ppm NAC (n=36) Group F: EGDA rats treated with 1,000 ppm NAC (n=36) Group G: EGDA rats treated with 389 ppm α -tocopherol plus 500 ppm NAC

(n=36)

Group H: EGDA rats treated with 1,400 ppm omeprazole (n=36)

The rats were given water *ad libitum*, maintained on a 12 h light/dark cycle (6 am - 6 pm), and allowed to acclimate for 2 weeks on lab chow prior to surgery. Solid food was withdrawn from one day before to one day after surgery. The rats were fed with respective AIN-93M based diets according to the protocol after the surgery. EGDA was performed according to the procedure described previously (124), which was approved by the Institutional Animal Care and Use Committee at Rutgers University (protocol no. 94-017). The surgeries were performed by Dr. Xiaoxin Chen and Dr. Bin Zhang. The EGDA animals were given iron dextran *i.p.* at 50 mg Fe/kg once every month, starting 2 weeks after surgery and continuing for the duration of the experiment to increase oxidative stress (124). The animals were weighed weekly. At the termination of the experiment, all the rats were euthanized with CO₂, and blood was collected by retro-orbital sinus bleeding. The esophagus was removed, opened longitudinally and examined for gross abnormalities. It was then fixed in 10% buffered formalin for 24 h and transferred to 80% ethanol.

C. The Chemopreventive Effect of Anti-inflammatory Agents Licofelone, Celecoxib, Zileuton and Antacid Agent Omeprazole on EGDA Rat Model (*Specific aim 3*).

C.1. The Rationale of the Study: Chronic Inflammation Is a Driving Force of EAC Carcinogenesis.

The most important risk factor for EAC in humans is GERD (242). A mixed refluxate containing acid, bile acid and digestive enzymes induces inflammation at the

esophageal epithelium (243). The pathologic progression of EAC is characterized by GERD-induced esophageal hyperplasia, intestinal metaplasia, columnar dysplasia and EAC (244). A large body of evidence shows significant increase of inflammation-related cytokines, enzymes and transcriptional factors during the progression of the conditions. COX-2 is the rate-limiting enzyme in the production of inflammatory mediator PGE2. COX-2 expression is low or absent in normal esophageal epithelium but increased in BE and EAC (245-249). Interleukin-1β, interleukin-6, interleukin-8 and tumor necrosis factor- α are significantly over-expressed in cancer samples compared with the healthy control (250-255). NF- κ B is a pleiotropic transcription factor that regulates the expression of the inflammatory cytokines mentioned above (256). The activation of NF- κB is a common event in EAC (252-254,257). This evidence stimulated a chemoprevention study targeting inflammatory pathways. COX-2 inhibition-oriented chemoprevention study showed promising results (175,257). Our laboratory demonstrated that 5-Lox is another important enzyme contributing to inflammation following GERD by producing LTB4 (171,177). COX-2 and 5-Lox inhibitors celecoxib and zileuton showed a chemopreventive effect on our EGDA rat model (177). As an inhibitor of both COX-2 and 5-Lox, Licofelone was expected to reduce tumor incidence in our EGDA model by inhibiting the inflammation pathway. In this study we investigated the chemopreventive effect of Licofelone administered three to forty-one weeks after the surgery or twenty to forty-one weeks after the surgery. We also investigated the combined effect of antacid agent omeprazole and COX-2 inhibitor celecoxib.

C.2. Research design for Part C

Six to eight weeks old male SD rats from Taconic Farms (Germantown, NY) were housed three per cage, separated into eight groups.

Group A: non-operated control (n=9) Group B: EGDA control sacrifice at 15 and 20 weeks after surgery (n=17) Group C: EGDA control sacrifice with treated groups (n=34) Group D: EGDA rats treated with 1,000 ppm Licofelone (n=34) Group E: EGDA rats treated with 1,000 ppm zileuton and 500 ppm celecoxib (n=34) Group F: EGDA rats treated with 1,000 ppm Licofelone starting at 20 weeks after the surgery (n=34) Group G: EGDA rats treated with 500 ppm celecoxib (n=34) Group H: EGDA rats treated with 250 ppm omeprazole (n=34) Group I: EGDA rats treated with 250 ppm omeprazole plus 500 ppm celecoxib (n=34)

The rats were given water *ad libitum*, maintained on a 12 h light/dark cycle (6 am – 6 pm), and allowed to acclimate for 2 weeks on lab chow prior to surgery. Solid food was withdrawn from one day before to one day after surgery. The rats were fed with respective AIN-93M based diets according to the protocol after the surgery. The surgeries were performed by Dr. Xiaoxin Chen. EGDA was performed according to our previous procedure (124). The procedure, animal care and sample processing were the same as described in Section B.

IV. MATERIAL AND METHODS

A. Animals

Female A/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) as breeders. Two genetically modified mouse strains were generous gifts from Dr. Ming You (Washington University School of Medicine). One has 99.9% A/J mouse background carrying three copies of a transgene containing an *Ala-135-Val p53* mutation (258). Another has 98% A/J mouse background with heterozygous *Ink4a/Arf* knockout. Mouse strains were obtained by backcrossing UL53-3 mice (FVB/J mice carrying three copies of the p53 transgene) to A/J mice for 10 generations and 129-B6 mixed mice (*Ink4a/Arf^{KO}*) to A/J mice for 6 generations, respectively (259). Before we started the experiment, we bred the two mouse strains to A/J background for at least 5 generations in our animal facility.

We included 151 male and 62 female A/J mice, 36 male and 42 female $p53^{A135V}$ transgenic mice, and 24 male and 5 female *Ink4a/Arf*^{+/-} mice in our study when they were 6-8 weeks old. All the mice used in the study were bred in our animal facility. The mice were housed 10 per cage in plastic cages with hardwood bedding and dust covers, in a high efficiency particulate air (HEPA)-filtered, environmentally controlled room (24 ± 1°C, 12/12 h light/dark cycle). They were given lab chow and water ad libitum before the surgery and switched to the respective AIN-93M based diet according to the protocol.

Male SD rats at 6-8 weeks old were obtained from Taconic Farms (Germantown, NY) for all the studies with the rat EGDA model. They were housed 3 per cage in plastic cages with hardwood bedding and dust covers, in a high efficiency particulate air (HEPA)-filtered, environmentally controlled room ($24 \pm 1^{\circ}$ C, 12/12 h light/dark cycle 6 am – 6 pm). They were given lab chow and water ad libitum before the surgery and switched to respective AIN-93M based diet after the surgery according to the protocol. The rats were allowed to acclimate for 2 weeks prior to surgery. Animals were weighed once every week.

B. Surgery Procedure

The animals were anesthetized with ketamine-xylazine. Both ketamine and xylazine were diluted and pre-mixed in normal saline. Rat ketamine-xylazine mix was composed with 8 ml ketamine 100 mg/ml, 1.2 xylazine 100 mg/ml and 90.8 ml normal saline and was given in 1 ml per 100 g body weight through *i.p.* injection. Mouse ketamine-xylazine mix was composed with 2.4 ml ketamine 100 mg/ml, 0.36 xylazine 100 mg/ml and 97.2 ml normal saline and was given in 1 ml per 30 g body weight through *i.p.* injection. The expected duration of anesthesia was one hour. EGDA was performed through an upper midline incision. Two 1 cm incisions (1.5 cm for rat model) were made on the gastroesophageal junction and the duodenum on the anti-mesenteric border, and then were anastomosed together with accurate mucosal to mucosal opposition (Figure 1A). EGDA plus gastrectomy were performed following EGDA procedure with a total gastrectomy (Figure 1B). As shown in Figure 1B, the whole stomach was removed

and the esophageal end and the duodenal end were closed respectively after the anastomosis. Surgery procedures were approved by the Animal Care and Facilities Committee at Rutgers University (protocol no. 94-017). The surgery procedures in mice were performed by Dr. Xiaoxin Chen; the surgeries in part B were performed by Dr. Xiaoxin Chen and Dr. Bin Zhang and surgeries in part C were performed by Dr. Xiaoxin Chen. After the animals recovered from the anesthesia, they were given Buprenex s.c. (Henry Schein Inc, 0.25 mg/kg for rats and 0.5 mg/kg mice) and Baytril *i.p.* (Henry Schein Inc, 10 mg/kg for rats and 85 mg/kg for mice) to relieve surgical pain and prevent infection, respectively. During first three days after the surgery, soft food was provided in a Petri dish in the cage. The surgical animals were checked twice every day for the recovery of surgical wound, vomiting and dead bodies. All the surgical animals were randomized into groups one week after the surgery.

C. Iron Supplementation

EGDA mice were given iron dextran (Henry Schein, Inc. Catalog No. 1063291) 50 mg/kg supplementation through *i.p.* injection. Mice in group F (EGDA plus 50 mg/kg iron dextran supplementation) and group G (EGDA plus Gastrectomy plus 50 mg/kg/m iron dextran supplementation) received iron supplementation 2 weeks after surgery. Mice in group E (EGDA plus 1,400 ppm) received iron supplementation 20 to 40 weeks after surgery. The EGDA control mice accepted iron supplementation from 40 to 80 weeks after the surgery.

The EGDA rats were given iron dextran through *i.p.* injection at 50 mg/kg once every month, starting 2 weeks after surgery and continuing for the duration of the experiment.

D. Experimental Diets

All animals were given lab chow before the surgeries. Solid food was withdrawn from one day before to one day after surgery to keep an empty GI tract. All the diets were made by Research Diets, Inc. (New Brunswick, NJ). The diets were made once every month and kept at 4°C until use.

In part A, all mice were on AIN-93M purified diet after the surgeries except group E (EGDA plus 1,400 ppm omeprazole), which received AIN-93M purified diet added with 1400 omeprazole.

In part B AIN-93M purified diet containing 77.8 ppm vitamin E (in the form of α -tocopherol acetate) was used as the basic diet. The levels of α -tocopherol acetate were increased to 5 and 10-fold to contain 389 ppm and 778 ppm α -tocopherol acetate in the supplemented diet. The other diets are also AIN-93M based containing 500 ppm NAC, 1,000 ppm NAC, 389 ppm α -tocopherol acetate plus 500 ppm NAC, and 1,400 ppm omeprazole.

In part C, the diets were made to contain 1,000 ppm Licofelone, 1,000 ppm zileuton plus 500 ppm celecoxib, 500 ppm celecoxib, 250 ppm omeprazole, and 250 ppm omeprazole and 500 ppm celecoxib.

E. *p53*^{A135V} Transgenic and *Ink4a/Arf*^{+/-} Mouse Genotype

All mice were genotyped for p53 mutation (Ala135Val) and Ink4a/Arf knockout using the procedures reported previously (259). Briefly, genomic DNA was extracted from tail clippings from each mouse by DNeasy Blood & Tissue Kit (QIAGEN, USA, Catalog No. 69504). For *p53*^{A135V} transgenic mouse, PCR primers were designed from the regions of mouse p53 exon 5 that contained the Ala-135-Val mutation. The primer sequences were as follows: (a) 5'-TAC TCT CCT CCC CTC AAT AAG-3'; and (b) 5'-CTC GGG TGG CTC ATA AGG TAC CAC-3'. Standard PCRs were performed. The total PCR reaction volume was 20 µl containing 2 µL DNA template (10-40 mg/L), 1 mM dNTP, 2.5 mM Mg²⁺ solution, 2 µl 10x PCR buffer, 2 µM of each primers and 0.75U/µl Taq DNA polymerase. The initiation step was held for 3 minutes at 94 °C. The thermal cycle was programmed as follows: 94 °C for 30 seconds, 50 °C for 1 minute and 72 °C for 1 minute in total 35 cycles. The final elongation step was held for 7 minutes at 72 °C. PCR primers generated a 190-bp amplified exon 5 fragment from both wild-type *p53* allele and transgene allele. After amplification, the fragment was incubated with the restriction endonuclease HphI at a final concentration of 500 U/ml. HphI cleaves once within the amplified transgene and does not cleave the wild-type allele. The cleaved fragments (150 bp and 40 bp) were then subjected to electrophoresis on 2% polyacrylamide gel along with a 50 bp DNA size marker and visualized by UV light after staining with ethidium bromide. This procedure was repeated at least once for each mouse for confirmation (260) (Figure 6).

For $Ink4a/Arf^{/+}$ genotype, PCR primers were derived from the regions of mouse p16INK4a exon 2 and the neo cassette. The primer sequences were as follows: (a) *p16INK4a* exon2F: 5'TTA ACA GCG GAG CTT CGT AC 3'; and (b) *p16INK4a* exon2R: 5'GAA TCT GCA CCG TAG TTG AG 3'. Together, p16INK4a exon2F and p16INK4a exon2R amplify a 159-bp product from the exon 2 of *p16INK4a*. The other pair of primers is: neoF: (a) 5'CTT GGG TGG AGA GGC TAT TC 3' and neoR; and (b) 5'AGG TGA GAT GAC AGG AGA TC 3'. Together, neoF and neoR amplify a 280-bp product from the neo insert. Standard PCRs were performed. The total PCR reaction volume was 20 µl containing 2 µL DNA template (10-40 mg/L), 1 mM dNTP, 2.5 mM Mg²⁺ solution, 2 µl 10x PCR buffer, 2 µM of each primers and 0.75U/µl Taq DNA polymerase. The initiation step was held for 5 minutes at 94 °C. The thermal cycle was programmed as follows: 94 °C for 30 seconds, 65 °C for 1 minute and 72 °C for 1 minute in total 30 cycles. The final elongation step was held for 7 minutes at 72 °C. PCR products were then subjected to electrophoresis on 1% agarose gel along with a 50 bp DNA size marker and visualized by UV light after staining with ethidium bromide. The DNA having both wild-type *p16INK4a/ARF* alleles (*Ink4a/Arf*^{+/+}) displayed only a single 159-bp fragment; DNA with a wild-type p16INK4a/ARF and target mutation allele ($Ink4a/Arf^{+/-}$) showed 159- and 280-bp bands, whereas DNA with both target mutation alleles $(Ink4a/Arf^{-})$ showed only a single 280-bp fragment (Figure 7). This procedure was repeated at least once for confirmation (259).

F. Tissue Preparation

At the termination of the experiment, all animals were euthanized by CO_2 asphyxiation. Serum samples were taken by cardiac puncture or the orbital venous sinus. The esophagus was removed, opened longitudinally and examined for gross abnormalities. The success of the EGDA was evaluated by putting a probe through the gastroesophageal junction to duodenum. If the probe could not pass through, the procedure was considered unsuccessful and the rat was considered as invalid. If a visible tumor was observed, the length, width and height were measured. The average was calculated as tumor diameter. Tumor volume was calculated by the formula: volume = $4/3\pi r^3$. The esophagus was fixed in 10% buffered formalin for 24 h and then transferred to 80% ethanol. The formalin-fixed esophagus was swiss-rolled, processed and embedded in paraffin. The suture line was used as a reference to distinguish between the esophagus and duodenum. Five-micrometer sections were mounted onto glass slides and used for pathological analyses.

G. Histopathology

G.1 Pathological Diagnosis

Histopathological analysis was carried out on the 1st and 30th H&E-stained slides. EAC was diagnosed when neoplastic columnar epithelial cells invaded through the basement membrane. Neoplastic columnar cells were characterized by the partial loss of cell polarity and maturation, nuclear atypia and an increase in mitotic figures. ESCC was diagnosed when squamous epithelium cells lost their normal orientation, the nuclear/cytoplasm ratio was increased and chromatin pattern was altered and some of those cells broke basement membrane and invaded into lamina propria (244).

G.2 Specific Staining

G.2.1 Alcian Blue Staining for Mucin

Paraffin slides were deparaffinized in xylene, 2 changes, 5 minutes each followed by rehydration in 100%, 95%, 70% and 50% gradient ethanol, for 2 minutes each. After washed with running tap water for 5 minutes, the slides were treated with 3% acetic acid (3 ml glacial acetic acid plus 97 ml distilled water) for 2 minutes. They were then stained in Alcian blue solution (1 g Alcian blue 8GX, in 3 ml glacial acetic acid and 97 ml distilled water) for 30 minutes. Excessive Alcian blue was washed off by running tap water for 2 minutes then the slides were stained with Harris Hematoxylin (Sigma, Catalog No. HS16-500ML) for 1 minute. Excessive Hematoxylin was washed off by distilled water, 2 changes, and 5 minutes each. The slides were then decolorized with 5 dips in acid alcohol (15 ml 1M hydrochloric acid in 500 ml 70% ethanol) and washed with running tap water for 20 minutes. The cytoplasm was stained with 6 dips in Eosin solution (Sigma, Catalog No. HT110316-500ML) following by decolorization and dehydration in 50%, 70%, 95% and 100% gradient ethanol, for 2 minutes each. The slides were cleared by xylene, 2 changes and 5 minutes each and mounted with coverglass using Clarion mounting medium (Sigma, Catalog No. C0487-100ML).

G.2.2. Perl's Method for Iron Staining

The slides were deparaffinized with xylene, 2 changes, 5 minutes each followed by dehydration in 100%, 95%, 70% and 50% gradient ethanol, and 2 minutes each. After rinsed with distilled water for 5 minutes, the slides were placed in stock potassium ferrocyanide solution (10 g potassium ferrocyanide in 100 ml distilled water) for 5 minutes. Prepare working potassium ferrocyanide-hydrochloric acid solution by mixing 70 ml stock potassium ferrocyanide solution and 30 ml 10% hydrochloric acid solution (10ml concentrated hydrochloric acid in 90 ml distilled water). The working solution has to be mixed just before use. The slides were merged in working potassium ferrocyanidehydrochloric acid solution for 20 minutes. Excessive working potassium ferrocyanidehydrochloric acid solution was washed off in distilled water (3 changes 3 minutes each). The slides were then stained in nuclear fast red solution (Sigma, Catalog No. N3020-100ML) for 5 minutes. Excessive dye was washed off in running tap water for 5 minutes. The slides were dehydrated and mounted as described before. Nine samples from each group were scored and analyzed.

G.2.3. Immunohistochemistry

Immunohistochemistry was performed on esophageal tissue sections using antibodies to detect the expression pattern and the quantity of these markers. The slides were deparaffinized and dehydrated as described before. After rinse in running tap water and distilled water, endogenous peroxidase on the slides was quenched by 3% hydrogen peroxide (Fisher, Catalog No. H325-100). The slides were washed well in PBS buffer and the antigens on the tissue were unmasked with Antigen Unmasking Solution (AUS) (Fisher, Catalog No. NC9401067, 3 ml AUS in 200 ml distilled water) by boiling the slides in AUS with a microwave (10% power) for 20 minutes. The container with the slides was cooled in a cold water bath until the slides reached the room temperature. After washing in PBS buffer, the tissue on the slides was then incubated in normal animal serum in PBS solution (10% vol/vol) in a humidified chamber for 30 minutes. Mouse as well as sheep, rabbit and goat antibodies require horse, goat and rabbit normal serum, respectively. All normal serum was bought from Vector Laboratories under catalog No. S-1000, S-2000 and S-5000). The slides were then incubated with the first antibody in PBS in humidified chamber at 4 °C overnight immediately after removing the normal serum by aspirating. The slides were washed well in PBS the second day and incubated in 1:200 biotinylated second antibody (Vector Laboratories) diluted in 10% normal serum PBS solution under room temperature for 30 minutes. ABC reagent (Vector Laboratories, Catalog No. PK-7200. 5 ml PBS with 2 drops of reagent A and 2 drops of reagent B, mix 30 minutes before use) was prepared right after the application of second antibody. The slides were washed well in PBS following ABC reagent incubation for 30 minutes at room temperature. After washing well with PBS, the slides were then developed with DAB chromagen (Vector Laboratories, Catalog No. SK-4100). The slides were washed well with running tap water, counterstained with Mayer's hematoxylin (Sigma, Catalog No. MHS16-500ML) for 1 minute and rinsed with running tap water for 15 minutes. The slides were then dehydrated in gradient ethanol, cleared in xylene and mounted as described previously. Antibodies used in the studies were listed in Table 1.

H. TBAR Assay

Serum levels of malondialdehyde (MDA) were measured by $OxiSelect^{TM}$ TBAR Assay Kit (Cell Biolabs, Inc., Catalog No. STA-330) in six serum samples from each group. The experiment was performed following the protocol accompanying the kit. The results were expressed as μM for MDA.

I. pH Measurement

The stomach and duodenum content of normal control rats (n=8) and the stomach content of EGDA rats (Group B, n=24; Group H, n=30) were collected in labeled centrifuge tubes. Samples were centrifuged at 10,000 rpm for 10 minutes. The supernatants (about 100 μ l) were collected and the pH values were measured by a pH meter.

J. Analysis of Fat-soluble Vitamins

Serum samples (n=9 in group A and n=10 in other groups) were used for the measurement of retinol, α -tocopherol and γ -tocopherol by HPLC according to our previous methods (116). In brief, fat-soluble vitamins were extracted from 150 µl of serum with ethanol and hexane. The hexane phase was dried, dissolved in a mixture of

ethanol and acetonitrile (1:1 ratio) and injected onto the HPLC, which used a LC18 column (4.6x15 mm, 100 A; Supelco, Bellefonte, PA). The nutrients were eluted isocratically using a mixture of ethanol:acetonitrile (1:1 ratio) and detected with a Waters 490 multiwavelength detector (Waters-Millipore, Milford, MA) with a wavelength setting at 300, 325 and 450 nm (for tocopherols, retinoids, and carotenoids, respectively).

K. PGE2 and LTB4 Measurement

For analyzing serum levels of PGE2 and LTB4, rat serum samples (n=8 for PGE2; n=7 for LTB4) were extracted by ethyl acetate with a ratio of 1:5. Extractions were dried by vacuum centrifuge. The dried samples were resuspended and analyzed by enzyme immunoassay for PGE2 and LTB4 (kits from Cayman Chemical, Ann Arbor, MI). The results were expressed as ng/ml for PGE2 and pg/ml for LTB4.

L. Statistical Analysis

The tumor incidence results were analyzed by the Fisher's exact test. The doseresponse effect was analyzed by Extended Mantel-Haenszel x^2 test. The tumor volume data were analyzed by the Mann–Whitney test, body weight, immunohistochemistry staining scores, iron deposition scores, MDA levels, PGE2 and LTB4 levels and fat soluble vitamin levels were measured by one-way ANOVA with Tukey's post-hoc test. Other data were analyzed by the Student's *t*-test using the computer software Statview 4.2. All continuous numeric variables were expressed as Mean \pm SD.

V. RESULTS AND DISCUSSION

A: Mouse Surgical Model of Esophageal Adenocarcinoma

A.1. Results

A.1.1. General Condition

Most mice (85%, 255/300) survived the surgery. The rest died of anesthesia, bleeding or unknown reasons during the surgery. The animals lost about 3 to 5 g body weight due to the stress of the surgery during the first month. They then started to gain weight but not as fast and remained lighter compared to the non-operated control (Figure 8). EGDA plus iron supplement group (Group F) had significantly higher body weight than the EGDA plus gastrectomy plus iron supplement group (Group G) since week 24. It suggested that gastrectomy significantly reduced body weight of EGDA mice. At week 20, the mice in EGDA plus omeprazole 1,400 ppm group had a significantly lower body weight compared with the surgical control group (Group B) and EGDA plus iron supplement group (Group F). We started to give iron supplement to the mice in the EGDA plus omeprazole 1,400 ppm group from week 21. The mice in this group gained 6 gram body weight in average from week 20 to week 32. The body weight of the surgical control group was not significantly different compared with the *p53*^{4/35V} transgenic mice group (Group C) and *INK4a/Arf* heterozygous knockout mice group (Group D).

Six mice were treated as invalid samples due to unsuccessful surgeries. Fortyseven mice were excluded from this study (Table 2). Among them, 16 were sacrificed due to sickness and 31 died before the end of the experiment (11 due to blockage of the gastrointestinal tract, 4 due to infection subsequent to iron injection and 16 due to unknown reasons). Autopsy of these 16 mice failed to find any noticeable abnormalities due to decomposition of the bodies.

A.1.2. Histopathological Findings at Week 20 and 40 after the Surgery

Normal mouse esophagus is covered by stratified keratinized squamous epithelium consisting of several layers of squamous epithelial cells (Figure 9A). Reflux induced hyperplasia of the squamous epithelium with infiltration of inflammatory cells in the epithelium and submucosa of all the mice, suggesting esophagitis (Figure 9B). We found one sample with metaplasia in both the wild-type and $p53^{A135V}$ transgenic mice group at 20 weeks after EGDA but not in INK4a/Arf heterozygous knockout mice. At 40 weeks after EGDA we found 6 out of 37, 2 out of 42 and 1 out of 15 metaplasia samples in wild-type, $p53^{A135V}$ transgenic and EGDA plus gastrectomy with iron supplementation groups, respectively. Metaplasia was confirmed by Alcian blue staining as scattered mucinous cells in the middle of hyperplastic squamous epithelium (Figure 9E, 9F). We did not find any EAC at both time points. We found one sample with ESCC in INK4a/Arf heterozygous knockout mice with EGDA group and another three in EGDA plus gastrectomy with the iron supplementation group (Figure 9D). The tumor incidence in EGDA plus gastrectomy with iron supplementation group was significantly higher than the EGDA control group (p<0.05). No cases of intestinal metaplasia and EAC were observed in those samples (Table 3).

A.1.3 Immunochemistry of P53
In order to examine the expression pattern of mutant p53 protein in the esophageal epithelium, we performed p53 immunohistochemistry using a polyclonal p53 antibody. In the wild-type esophageal epithelium, we observed only background staining. In contrast, we observed strong positive nuclear staining in basal cells of $p53^{A135V}$ transgenic mouse esophageal epithelium, indicating the accumulation of mutant p53 protein (Figure 10).

A.1.4. ESCC observed in EGDA mice at 80 weeks

We observed 12 examples of ESCC in 13 EGDA mice at 80 weeks after the surgery. The tumors were generally located at the distal esophagus and invaded into the muscle layer (Figure 11A). Under higher magnification, it was shown that neoplastic cells were squamous epithelial cells and surrounded by muscle fibers (Figure 11B). Some neoplastic cells formed cystic structures and secreted keratin. The only one mouse without ESCC had mild squamous dysplasia. Metaplasia was not observed in these mice (Table 3).

A.2 Discussion

This study was primarily designed to develop a surgical model of BE and EAC in mice. The EGDA procedure was first used in our rat model (124). Considering the smaller body size of the mice, extra efforts were made to develop the mouse EGDA model. In addition to choosing smaller sized sutures and surgical needles, we used a heating pad to maintain the body temperature of the mice after the surgery. The animal care after the surgery was focused on the signs of GI track blockage, including vomiting, white bubbles around the mouth and nausea. Once we found mice with the signs described above, we used a gavage needle to push the food blocking the esophageal passage into the stomach.

EGDA was performed on wild-type, $p53^{A135V}$ transgenic and *INK4a/Arf*^{+/-} mice of A/J background. In addition, omeprazole (1,400 ppm in diet), iron (50 mg/kg/m, *i.p.*), or gastrectomy plus iron, were given to some of these mice in order to modulate disease progression. Unfortunately, we observed only metaplasia as scattered mucinous cells in a small percentage of mice, but not as typical intestinal metaplasia. Moreover, ESCC but not EAC, was induced at a low incidence, and long-term gastroesophageal reflux in combination with iron (Group B at week 80) did produce a high incidence of ESCC (92.3%, 12/13).

Human studies and animal experiments had repeatedly indicated that reflux was indeed a risk factor of ESCC and incidence of ESCC and laryngeal SCC was higher in patients after total or partial gastrectomy (38-41). Animal studies also supported this hypothesis (43,44). Reflux either directly induced ESCC or promoted ESCC induced by carcinogen treatment in rat models (44). In a previous study using the EDA procedure in rats, reflux increased the incidence of ESCC induced by 2,6-dimethylnitrosomorpholine or methyl-n-amylnitrosamine by 40% (150).

It was interesting that A/J mice were not susceptible to BE and EAC after the same surgical procedure as rats. The difference we observed between the rats and mice suggested that rat and mouse esophageal epithelium might response to gastroesophageal reflux differently, even though they are very similar in histology. The exact underlying mechanism remains puzzling. It is possible that mouse and rat esophageal epithelium

may follow different malignant transformation mechanisms. The experimental data about this hypothesis is scarce. What we already know is that mouse and human cells have different mechanisms regarding immortalization (261). Normal, non-immortalized human cells usually do not express telomerase and progressively lose telomere after successive rounds of cell division, while telomerase is constitutively expressed in some somatic tissues in inbred mouse strains and the length of telomere of mouse is 3-10 times longer than that of human (261). As suggested by comparison of mouse and human cell, the different patterns of rat and mouse esophageal cancer we observed in the current study may be answered by comparative genomic and proteomic studies with rat and mouse esophageal epithelium.

Scattered mucinous cells were observed in mouse esophagus after surgery, suggesting that BE and EAC in mouse esophagus is still possible (189). Our recent study on the rat model and human BE have suggested squamous de-differentiation (i.e., loss of squamous transcription factors, p63 and sox2) and columnar differentiation (i.e., gain of intestinal transcription factors) were two essential aspects of intestinal metaplasia (262). Since embryonic esophageal epithelium of p63^{-/-} mice and hypomorphic Sox2 mice showed metaplastic changes and gene expression (263,264), we speculate that p63 or Sox2 knockout mice may be more susceptible to BE and EAC after surgery. It is likely that proper combinations of genetic manipulation, surgery, or carcinogen treatment may be needed to induce BE and EAC in mouse esophagus.

Rodents have keratinized squamous epithelium without submucosal glands in the esophagus, whereas humans have non-keratinized squamous epithelium with submucosal glands in the esophagus. When histological and physiological resemblance to humans is

considered, a model with pigs may offer many advantages over rodent models. Pigs also suffer from GERD and stress ulceration of the esophagus (265). Just as human, pigs have non-keratinized stratified squamous epithelium and submucosal glands in their esophagi (65). A pig model has advantages as follows: 1) similarity to humans in gastrointestinal physiology and anatomy; 2) suitability for surgery (266); 3) better acceptance by the public than canine models; and 4) lower cost of maintenance.

In conclusion, EGDA failed to induce BE and EAC in wild-type, $p53^{A135V}$ transgenic and *INK4a/Arf^{+/-}* mice on the A/J background. The underling reason may be revealed by the comparison of the genomics or proteomics of esophageal epithelium in mouse and rat as well as cell transformation studies with mouse and rat esophageal epithelium cells.

B: α-Tocopherol, NAC and Antacid Treatment in the Chemoprevention of EAC

B.1. Results

B.1.1. General Observations

The animals tolerated the surgery procedure, with a survival rate of 87%. We started the experiment with 251 EGDA rats and 9 non-operated control rats. At the end of the experiment we had 211 valid surgical rats and 9 non-surgical control rats for analysis. All animals were sacrificed at 40 weeks after the surgery. There were 26 invalid rats due to unsuccessful surgery. Fourteen rats died during the experiment due to sickness. Before the death, rats showed symptoms of weight loss, lethargy and fluffiness. The average body weight of EGDA rats was 8% lower than that of the non-operated control rats but the difference was not statistically significant. Treatment with α -tocopherol, NAC and omeprazole did not significantly affect the body weight of the rats (Figure 12).

B.1.2. Fat Soluble Vitamin Levels

EGDA rats appeared to have lower serum α -tocopherol, γ -tocopherol, and retinol levels than the non-operated control (Table 4), but the results are not statistically significant. Supplementation with α -tocopherol at 389 ppm and 778 ppm increased the serum α -tocopherol concentration by 57% (from 30.7 µmol/L in surgical control group to 48.2 µmol/L) and 79.8% (to 55.2 µmol/L, p<0.05), respectively; but the supplement decreased the serum γ -tocopherol level to 17% and 15% of surgical control (from 0.76 µmol/L to 0.12-0.13 µmol/L, p<0.05), respectively. The serum concentration of retinol was not affected by α -tocopherol supplementation. NAC alone did not affect the serum level of α -tocopherol (27.0 µmol/L). When NAC was administered together with α - tocopherol, the serum concentration of α -tocopherol was increased to 49.4 µmol/L. γ -Tocopherol level was decreased in the combination group compared with NAC 500 ppm treatment group. But neither of them was statistically significant. Retinol concentration remained almost the same regardless the treatment. Omeprazole did not affect the serum levels of the three nutrients.

B.1.3. Effects on Tumorigenesis

In the EGDA model, well-differentiated mucinous adenocarcinomas developed at the squamocolumnar junction. Most of the rats had only one esophageal tumor per rat, but 13 rats had two tumors each. We did not observe any cases with more than 2 tumors. An example of invasive tumor is shown (Figure 13A). The neoplastic tissue invaded muscle layer and formed a massive mucinous tumor at the distal part of the esophagus. The mucin secreted by the tumor cells formed pools of various sizes. There was more stroma tissue than neoplastic tissue in the tumor. Neoplastic cells formed glandular structure of different sizes surrounded by stroma tissue. At the edge of the tumor, we observed intestinal metaplasia in squamous epithelium (Figure 13D). The squamous epithelium was transformed into columnar epithelium, which also contained occasional mucin secreting goblet cells.

 α -Tocopherol, NAC and omeprazole reduced number of visible tumors except the 500 ppm NAC treatment group; however, the reductions were not statistically significant compared with the surgical control (Table 5). We also measured the visible tumor volume in all the groups (Figure 14). The α -tocopherol 778 ppm group (D), α -tocopherol 389 ppm plus NAC 500 ppm group (G) and the omeprazole 1,400 ppm group (H) appeared to

decrease the number of tumors over 1,000 mm³, but not statistically significant and tumors larger than 2,500mm³ were not observed in these groups.

The standard EGDA procedure induced EAC in 84% of the rats. Supplementation of α -tocopherol to 5 and 10 times of the AIN-93M diet level (389 and 778 ppm, respectively) decreased the EAC incidence rate in a dose-dependant manner (P=0.03 for trend) (Table 6). Supplementation with NAC at 1,000 ppm caused a slight non-significant decrease in tumor incidence. However, the combination of low doses of NAC (500 ppm) and α -tocopherol (389 ppm) produced a significant decrease in tumor incidence compared to the surgical control group. Omeprazole also appeared to decrease tumor incidence, but the results were not statistically significant.

In order to assess the physiological effects of omeprazole in the surgical rats, we measured pH value of the stomach and duodenal juice of the non-operated control, as well as the mixed duodenal and gastric juice in the surgical control and omeprazole treated group. We found that, as expected, the surgery-induced refluxate was more acidic than the duodenal juice and more alkaline than the gastric contents of the non-operated control. It suggested that surgery successfully produced a mixed refluxate was higher than that in the surgical control (Figure 15), and suggesting omeprazole functioned as an antacid agent.

B.1.4. 4-Hydroxynonenal (4-HNE) Immunohistochemistry and TBAR Assay

In normal control esophageal tissue (Figure 16A), we observed only non-specific background staining. We observed strong positive staining of 4-HNE adducts in the infiltrating macrophages in surgical control samples (Figure 16B), but not in lymphocytes. The strongest staining was observed in the region of the esophageal anastomosis. Areas more distal to the anastomosis had fewer stained cells. α -Tocopherol (778 ppm) treatment decreased the number of positively stained cells compared with the surgical control (Figure 16D), but the effect was not statistically significant by Tukey's post-hoc test. The combination of α -tocopherol 389 ppm and NAC 500 ppm significantly reduced the number of positively stained cells control (Figure 16G).

The MDA serum levels were measured by TBAR assay. MDA concentration was higher in all the surgical groups than the normal control, with group G (α -tocopherol 389 ppm plus NAC 500 ppm) showing a significant difference (p<0.05). The antioxidant treatment did not affect MDA serum levels compared with all surgical groups (Figure 17).

B.1.5. Caspase-3 and Proliferating Cell Nuclear Antigen (PCNA) Staining

We failed to observe any positive caspase-3 staining in the esophageal epithelium of the normal rats. The surgical control group, α -tocopherol 778 ppm group and α tocopherol 389 ppm plus NAC 500 ppm group all showed positive nuclear and perinuclear caspase-3 staining in the hyperplastic, metaplastic, dysplastic and neoplastic tissues and their staining scores were significantly higher than the normal control group (Figure 18). The deviation in the surgical group of samples was high and resulted in statistically non-significant difference between surgical groups. PCNA immunohistochemistry resulted in positive nuclear staining of the basal cells in normal esophageal squamous epithelium. The hyperplastic, metaplastic, dysplastic and neoplastic cell nuclei in the surgery samples were also stained positively. Surgical control group, α -tocopherol 778 ppm group and α -tocopherol 389 ppm plus NAC 500 ppm group had significant higher staining scores than the normal control group. But the antioxidant treatments did not show any influence on the quantity of PCNA positively stained cells compared with the surgical control group (Figure 19).

B.1.6. EGFR Immunohistochemistry

EGFR immunohistochemistry was performed to evaluate its staining pattern in the EGDA rat model. We only observed sporadically positive membrane staining in the metaplastic and dysplastic tissues (Figure 20). We did not observe any positive staining in hyperplastic and neoplastic cell membranes, which was reported staining positively in human samples (Figure 21). The staining pattern was the same in all the surgical groups.

B.2 Discussion

A contributory role of oxidative stress in esophageal adenocarcinogenesis has been observed in both human and animal studies (117). In patient samples, it has been reported that glutathione content is progressively decreased in the esophagitis-metaplasiadysplasia-adenocarcinoma sequence, while myeloperoxidase activity is higher than in controls, plateauing at the stage of BE. Glutathione content is negatively correlated with DNA adducts (239). An oxidative DNA damage marker, 8-hydroxydeoxyguanosine, is significantly increased in the distal esophagus with Barrett's epithelium and high-grade dysplasia, as well as in EAC (240). Expression of manganese superoxide dismutase is significantly reduced in esophageal tissues of BE, low-grade dysplasia, high-grade dysplasia, and EAC when compared with normal esophagus (241). These findings imply strongly that oxidative stress is an important event in esophageal adenocarcinogenesis.

Antioxidants have been studied as potential cancer chemopreventive agents. One population-based case-control study in Sweden suggest that subjects with a high intake of vitamin C, beta-carotene and α -tocopherol have 40-50% reduced risk of EAC (88). We chose α -tocopherol and NAC as our chemopreventive agents in the present study based on the different antioxidative mechanisms these two utilize. α -Tocopherol functions as a lipid peroxidation inhibitor in cell membrane by virtue of its chain-breaking and free radical scavenger actions. In contrast, NAC is a small, water soluble molecule directly providing SH-groups for adduction or oxidation, and is a precursor of glutathione (198). With possible clinical applications in mind, we chose these agents because of their low toxicity and low cost. α-Tocopherol alone or in combination with NAC showed significant tumor inhibitory effect on EAC in our EGDA model. The highest doses in diet are equivalent to the commonly used supplementation dose of vitamin E and NAC on market (400 IU and 500 mg, respectively). The calculation is based on allometric scaling (267). For example, for a rat that consumes 20 g of diet daily, the diet contains 80 kcal and 20 mg NAC (for a diet containing 1,000 ppm NAC). The calorie-based dosage equals to 20 mg/80 kcal or 0.25 mg/ kcal. For a person with a caloric requirement of 2,000 kcal/day, this is equivalent to $0.25 \times 2,000 = 500 \text{ mg/day}$. The daily dose of NAC ranges from 250 mg to 1,500 mg clinically for patients with chronic pulmonary diseases. Due to the extensive first-pass metabolism, oral administration of NAC results in low plasma

and tissue levels, but plasma levels are dose dependent (198,268). Considering the dose used in clinical trial, the doses of NAC used in this study are relatively low (197).

 α -Tocopherol supplementation significantly increased the serum level of α tocopherol, but reduced the serum level of γ -tocopherol. This phenomenon is consistent with previous reports (269). The tumor inhibitory effect of α -tocopherol we observed in this study was dose-dependent with significant inhibition (from 84% to 59%) at the highest dose (778 ppm). NAC alone did not significantly reduce tumor incidence. The highest dose of NAC (1,000 ppm) achieved less inhibition than low dose α -tocopherol. In contrast to NAC treatment alone, the combination of 500 ppm NAC and 389 ppm α tocopherol inhibited tumor incidence from 84% to 55%. It is possible that α -tocopherol played a major role in the combination treatment.

Omeprazole at 1,400 ppm reduced EAC incidence from 84% to 64% in the current study, which is statistically non-significant. We did not observe any unusual weight loss or high death rate in this group of animals. The pH data showed that omeprazole effectively inhibited acid secretion at the dose given. These data support that omeprazole does not promote EAC formation. There have been concerns on the side effects of long term PPI treatment related side effects. Hypergastrinemia is the major side effect of PPI treatment, which may induce epithelial proliferation. At the same time, bile acids in a neutral refluxate may induce DNA mutations in esophageal epithelium (190,191). PPI treatment may also induce squamous re-epithelialization which covers more advanced malignancies in Barrett's glands located in submucosa (179,181). Our data showed that PPI did not have chemopreventive effect on EAC. In order to achieve chemopreventive effects, PPI may be applied in combination with other agents. It is

reported that the combination of omeprazole 40 mg twice daily plus aspirin 325 mg once daily for 10 days significantly reduced the production of mucosal PGE2 level and PCNA expression (270). The ongoing ASPECT Trial (Aspirin Esomeprazole Chemoprevention Trial) using PPI in combination with aspirin may provide more information about this matter (271).

4-HNE is an extensively studied lipid peroxidation product, which is diffusible and can react with DNA bases and proteins (272). It is also an inducer of cyclooxygenase-2 and a mediator of oxidative stress (273,274). It has been reported that enhanced lipid peroxidation and 4-HNE production may play a role in the recruitment of inflammatory cells (275). 4-HNE has also been reported to form DNA adducts at codon 249 of the p53 gene and to inhibit nucleotide excision repair through interaction with cellular repair proteins (276,277). In the present study, we found strong positive staining in the infiltrating inflammatory cells. We only found background staining in the nearby epithelial cells. This phenomenon may be explained by: 1) lipid peroxidation level is higher in the infiltrating inflammatory cells than the epithelial cells; 2) the method of immunohistochemistry we used is not sensitive enough to detect the 4-HNE adduct in the epithelial cells; 3) high level of 4-HNE in the infiltrating inflammatory cells may act on the nearby epithelial cells by diffusion.

The EGFR expression in our animal model showed only sporadic positive staining in metaplastic and dysplastic tissues. This staining pattern is totally different from the staining patterns reported in human EAC cases, in which positive staining of EGFR is found in more than 30% of all cases (142). The histopathological and molecular differences between human and rat esophagus and EAC were reported (262,278). The

results suggest rat esophageal epithelial cells tend to maintain a stronger squamous differentiation than humans. Bonde *et al.* reported an adenocarcinoma cell line they established from their surgery-induced rat EAC sample and planted it into nude mice. The xenograft tumor showed well-developed squamous cell carcinoma phenotype (264). It is suggesting that change of growing environment, from cell culture media to nude mouse, will cause the surgery-induced adenocarcinoma in rat to switch back to squamous phenotype. Considering the differences of human and rat EAC, it is possible EGFR may play different roles in human and rat EAC.

Caspase-3 staining and PCNA staining showed no statistical differences among surgical control (Group B), α -tocopherol 778 ppm (Group D) and α -tocopherol 389 plus NAC 500 ppm group (Group G). It is likely that both α -tocopherol and NAC will not cause extensive apoptosis or proliferation in esophageal epithelium. This finding partly explains the safety of these two agents.

In conclusion, our results lend support to the oxidative stress hypothesis of esophageal adenocarcinogenesis. α -Tocopherol, alone or in combination with NAC, significantly reduced tumor yield. Tocopherols and NAC appear to be promising chemopreventive agents of EAC. Tocopherols alone or in combination with NAC should be further investigated in GERD and BE patients to assess their value as chemopreventive agents for EAC. Omeprazole did not promote EAC and did not have chemopreventive effect in our EGDA rat model.

C: the Chemopreventive Effect of Anti-inflammatory Agents Licofelone, Celecoxib, Zileuton and Antacid Agent Omeprazole on EGDA Rat Model

C.1 Results

C.1.1 General Condition

We performed EGDA surgery on 291 rats, 256 of which survived. The survival rate was 88%. Three of the rats died during the surgery. The anatomic structure was changed at the gastrointestinal junction in the rats after the surgery. The chances of gastrointestinal junction blockage were higher than in the non-operated control. Eight rats died of GI track blockage during the experiment, 1 died of stomach tumor, 2 died of sarcoma, 2 were sacrificed early (27 weeks and 35 weeks) due to sickness, 2 died of intestinal damage caused by iron injection and 17 died without obvious reasons. We collected the esophagus when we found the dead body and found the tissues were partly decomposed under the microscope and we considered them as invalid samples.

Starting from one week after the surgery, animals had gained weight in all groups (Figure 22). The growth rate of group F reduced slightly after receiving the Licofelone 1,000 ppm diet. At 40 weeks, there were no significant differences in body weight among surgical groups. The difference between normal control and the surgical control groups is non-significant.

C.1.2. Pathological Finding of 15 and 20 Weeks Samples

We sacrificed 7 rats in group B on 15 weeks after the surgery. We observed hyperplasia in all the samples and we had one sample with metaplasia. On week 20, we

sacrificed the remaining 10 rats in this group and we observed under the microscope 1 rat with esophageal adenocarcinoma, 2 rats with metaplasia, and all rats with hyperplasia.

C.1.3. Effect of Treatment on Tumorigenesis

At the termination of the experiment on 41 weeks after surgery, we collected the esophagus and the number of esophageal tumors was scored (Table 7). There were 24 non-informative samples due to unsuccessful surgery. We observed 5 visible tumors out of 30 samples in control group (Group C), 3 out of 29 samples in Licofelone (1,000 ppm) early treatment group (Group D), 2 out of 27 samples in zileuton 1,000 ppm plus celecoxib 500 ppm group (Group E), 4 out of 30 samples in Licofelone 1,000 ppm later treatment group, zero out of 30 samples in celecoxib 500 ppm group (Group G), 6 out of 30 samples in omeprazole 250 ppm treatment group (Group H), and 4 out of 28 samples in omeprazole 250 ppm plus celecoxib 500 ppm group (Group I). In all the samples only a single tumor was observed in each tumor-bearing rat. The visible tumor incidence did not show any statistical significance between groups. There was no significant difference on tumor volume data among groups C, D, F, H, and I (Figure 23).

The histopathological changes were the same as what we observed in α tocopherol study. We found hyperplasia, intestinal metaplasia, dysplasia and EAC lesions in our samples (Figure 24). The histopathological diagnosis showed the highest tumor incidence in surgical control (10/28, 35.7%) and omeprazole plus celecoxib group (10/28, 35.7%), which was lower than our α -tocopherol chemoprevention study (Table 8). Statistical analysis indicated that the cancer incidence of the zileuton (1,000 ppm) plus celecoxib (500 ppm) group, which had only one EAC case (1/26, 3.8%) in all the 26 samples, was significantly lower than the surgical control group (10/28, 35.7%). However, this is the only group that showed a statistical significant reduction in EAC vield compared with surgical control in all the treatment groups. Zileuton and celecoxib combination also significantly reduced the tumor incidence compared with two Licofelone treated groups. But the combination treatment only showed marginal effect compared with celecoxib 500 ppm group. Even though we did not observe any visible tumor samples in the celecoxib 500 ppm group, there were 7/29 (24.1%) incidences of histopathological adenocarcinomas. Licofelone 1,000 ppm in the diet was not effective in preventing esophageal adenocarcinoma regardless the time of intervention. Omeprazole 250 ppm alone or in combination with celecoxib 500 ppm had no significant effect on carcinogenesis. It was noteworthy that the omeprazole 250 ppm plus celecoxib 500 ppm group had 10 tumors out of 28 samples, which yielded a tumor rate of 35.7%, while the celecoxib 500 ppm group and omeprazole 250 ppm group had tumor rates of 24.1% and 27.6%, respectively. It suggests that the combination treatment of PPI and COX-2 inhibitor may not be more effective than either single treatment.

C.1.4 PGE2 and LTB4 in Serum

Compared to the surgical control group (Group C, Figure 25), Licofelone early treatment group (Group D) had marginally lower serum PGE2 levels. Licofelone treatment significantly reduced PGE2 levels compared to zileuton 1,000 ppm and celecoxib 500 ppm treatment. There were no significant differences between normal control, surgical control, and zileuton 1,000 ppm plus celecoxib (500 ppm) treatment group on serum LTB4 concentrations (Figure 26).

C.1.5. Iron Deposition

The iron staining was done to evaluate the iron deposition pattern in the esophageal epithelium of normal control and surgical control rats used in this study and also in normal control and surgical control rats of previous α -tocopherol chemoprevention study. There was only one in all 9 samples of the normal control group (Figure 27, normal control A) of Licofelone chemoprevention study showed iron staining. Iron supplementation significantly increased the iron deposition in the surgical control group (Figure 27, surgical control A) of this study. All the samples in this group showed iron staining in the macrophages and fibroblasts in lamina propria of the esophagus. The staining was mainly located at the distal part of the esophagus. In α -tocopherol chemoprevention study, the esophageal samples of the normal control group all showed iron deposition in the lamina propria (Figure 27, normal control B). Iron supplementation significantly increased iron deposition in the surgical control group (Figure 27, surgical control B) compared with normal control in the α -tocopherol chemoprevention study. When the two normal controls and surgical controls were compared, normal control and surgical control group in α -tocopherol chemoprevention study showed significant more positively stained cells than their counterparts in Licofelone chemoprevention study.

C.2. Discussion

The experimental rats developed esophageal adenocarcinoma as our previous studies. The only significant tumor reduction was observed in the group that received

treatment with zileuton and celecoxib. This result is consistent with our previous observations (177,262).

This study was designed to achieve two goals: 1) to test the chemopreventive effect of Licofelone; 2) to test the chemopreventive effect of the combination of omeprazole and celecoxib. Based on previous studies on COX-2 and 5-Lox inhibitors, we were expecting a better chemopreventive effect of Licofelone on EAC than either celecoxib or zileuton alone. To our surprise, Licofelone 1,000 ppm in diet, either given from 3 to 40 or 20 to 40 weeks after the surgery, did not prevent EAC development. It is known that Licofelone inhibited the PGE2 and LTB4 secretion in a carrageenan-induced rat paw edema model, with ED₅₀ value of 17 mg/kg p.o. by gavage (231). In our study we fed the rats with standard AIN-93M diet with 1,000 ppm Licofelone. Based on the daily diet consumption of rat (20 g/day) and average body weight range of rats (300 to 600 g) during the entire study, the dose range of Licofelone on the rats is about 33 to 67 mg/kg, which is about 2 to 4 times as reported ED_{50} dose. The pharmacological studies of Licofelone in rats were done by oral gavage. Vidal et al. reported their Licofelone efficacy study using rabbit femoral artery injury model (279). Licofelone 10 mg/kg/day given in diet relieved the inflammation, inhibited COX-2 and 5-LOX protein expression in vascular lesions and attenuated PGE2 and LTB4 production in plasma. Their results suggest that Licofelone in diet is also effective, although there is no data to compare the efficacy of Licofelone by gavage or in diet. In our serum PGE2 and LTB4 enzyme assay, Licofelone 1,000 ppm in diet did not significantly inhibit PGE2 or LTB4 production. This contradiction may due to the difference of animal models used in our and other studies. The reflux in our EGDA model may not be a strong insult to stimulate PGE2 and

LTB4 peak in plasma as other models. As an analgesic and anti-inflammatory agent, Licofelone is designed to treat osteoarthritis. Licofelone efficiently relieved the symptoms and reduced the clinical progression of osteoarthritis in several animal models (279-283). Licofelone 200 mg twice daily also showed more efficiencies over naproxen with less cartilage loss in a human phase III clinical trial (284). Having these pre-clinical data and clinical data in mind, the dose we chose in our study is reasonable and we expected Licofelone to function as an anti-inflammatory agent in our study. Considering our experiment and the reports of others, we think Licofelone is an effective antiinflammatory agent but can not prevent EAC development in our EGDA rat model. Our results suggest there may be other mechanisms involved other than COX-2 and 5-Lox inhibition in EAC chemoprevention. Recently it was reported that celecoxib, a COX-2 inhibitor, also inhibits tumor progression and angiogenesis in a colon cancer xenograft model or cell line by COX-2 independent ways (285,286). It is reported that celecoxib impaired phosphorylation of substrates for the receptor tyrosine kinases c-Met and insulin-like growth factor receptor and increased β -catenin phosphorylation (286).

Omeprazole, a proton pump inhibitor, also failed to produce a significant inhibitory effect when given alone or in combination with celecoxib (500 ppm). Given that omeprazole 1,400 ppm in diet did not significantly inhibit EAC formation in our α tocopherol chemoprevention study, the result we have with 250 ppm omeprazole in diet is reasonable. When we compare the omeprazole plus celecoxib treatment group with omeprazole treatment or celecoxib treatment group, the combination treatment was not more effective than either treatment alone. It suggests that the combination of antacid and anti-inflammatory treatment may not enhance the chemopreventive effect of either treatment. The combination of omeprazole and celecoxib is not a new idea. It is a common clinical formulation to treat GI tract ulcers. In an omeprazole and celecoxib combination clinical trial, it is reported that the combination is more effective than celecoxib alone in preventing the recurrence of ulcer bleeding during one year treatment (287). No obvious side effects were found in this trial. In a population-based retrospective cohort study, patients who were more than 75-years old treated with omeprazole plus celecoxib had shorter GI hospitalization time than celecoxib alone (288). Clinical EAC chemopreventive data about the combination of omeprazole and celecoxib are lacking. Based on the results of our study, this combination may not have better chemopreventive effects than either of the agents alone.

In previous studies, celecoxib at 500 ppm demonstrated an inhibitory effect on EAC in our EGDA rat model (177,262). In the present study, no visible tumor was observed with 500 ppm of celecoxib. Based on histological analysis, the EAC incidence rate of the celecoxib group was lower than the positive control group. However, the difference was not statistically significant. The lack of a statistic difference may due to the relative low EAC yield in this study. The tumor incidence (35.7%) in the control group of the current study is lower than the tumor incidence that we observed in previous α -tocopherol study (84%).

There are two major factors that influence the tumor yield in our EGDA rats. One is iron supplementation. In our previous study iron supplementation was found to be an enhancing factor for EAC development. Iron dextran 4 mg/kg/week *i.p.* promoted EAC incidence from 25.6% (11/43) to 53.7% (22/41) in SD rats with EGDA (124). We did iron deposition staining with the samples in this experiment and the α -tocopherol

chemoprevention study to compare the iron deposition in the esophagus of two normal control and two surgical control groups. We found more iron staining positive cells in both normal control and surgical control groups in α -tocopherol chemoprevention study than in Licofelone chemoprevention study (P<0.05). It suggests that there is a difference in iron metabolism in those animals, even though we used the same strain of rats from the same animal farm (SD rats, Taconic Farms) at the same age in our experiments. The exact reasons are elusive at this moment. The possible reasons may be: 1) the basal diet we used in α -tocopherol study contained more iron than the diet we used in Licofelone study (since we do not have the diet samples, we are not able to test this hypothesis); 2) relatively low expression or activity of critical proteins in iron absorption and transportation such as duodenal cytochromes B, divalent metal transporter 1 and ferroportin in rats of Licofelone study; and 3) relatively high expression or activity of transferrin receptor on the cells in stroma tissue of esophagus in the rats of α -tocopherol study.

Another reason contributes to EAC yield is the reflux induced by the surgery. A stronger reflux will induce more tumors. We previously observed that 73% EDA rat with iron supplementation had EAC at 31 weeks after the surgery, while 53.7% EGDA rat with iron supplementation had EAC at 40 weeks after the surgery (124,151). The reflux-induced esophagitis in EDA model was more severe than the EGDA model. Is it possible that we might induce more severe reflux in one experiment than another? In the α -tocopherol chemoprevention study we have two surgeons to perform surgeries, while in Licofelone chemoprevention study we only have one surgeon performing the surgeries. It is possible that the other surgeon might tend to make larger anastomotic openings and

induce more severe reflux in the rats. Because we did not measure the anastomosis in these two studies, there is no direct evidence to prove this assumption.

In conclusion, Licofelone 1,000 ppm failed to prevent EAC development in our EGDA rat model. Zileuton 1,000 ppm plus celecoxib 500 ppm showed significantly chemopreventive effect toward EAC, which is consistent with our previous findings. Omeprazole 250 ppm alone or in combination with celecoxib 500 ppm can not prevent EAC. Further studies are needed to investigate a proper combination with omeprazole in chemoprevention.

VI. GENERAL DISCUSSION AND FUTURE DIRECTION

Two important aspects of EAC research are involved in the current study. One is the attempt to establish a mouse EAC model and another is chemoprevention. EGDAinduced gastroesophageal reflux, which successfully induces EAC in SD rat, failed to induce EAC in A/J mice, but produced ESCC instead. It is interesting that rat and mouse esophagi respond to EGDA in different ways even though these two species share both genetic and histological similarities. In chemoprevention studies, our results showed that some antioxidants and anti-inflammatory agents significantly decreased tumor incidences in our EGDA rat model.

There are two important morphological phases in EAC carcinogenesis. One is the formation of intestinal metaplasia after long term exposure of reflux; the other is the neoplastic transformation of intestinal metaplasia to EAC. In our mouse model, we did not observe any intestinal metaplastic glands. The metaplasia we observed is scattered mucinous cells positioned among squamous epithelial cells. The molecular events that lead to the metaplastic change in human is still not clear (289). By comparing the changes of mouse and rat esophagi after reflux, we may be able to obtain more information about the molecular changes that result in metaplastic lesions in rat. Suitable cell lines from mouse and rat esophagi may be better tools to conduct genetic manipulation and to yield information in this area.

The chemoprevention study in the current work provides us new perspectives on future EAC prevention strategy. A current ongoing clinical trial is the "Aspirin Esomeprazole Chemoprevention Trial (AspECT)", which tests the chemopreventive effect of the combination of anti-inflammatory agent and PPI (271). Since PPIs are commonly used clinically, it is reasonable to consider these agents as part of chemopreventive strategy. However, our results suggest that PPIs may not be an effective EAC prevention agent. Our study suggests that anti-inflammatory agents and antioxidants may be beneficial. The combination PPI, anti-inflammatory agents and antioxidants should be considered in future clinical trials.

In future clinical trial, the target population of chemoprevention study could be those with pre-malignant diseases, such as BE patients. Among all GERD patients, only about 12% will develop BE (289). It is challenging to identify those patients and provide them proper care. To fulfill this goal, we need to apply cell biology into our study and try to screen the cells in esophageal epithelium and find those with the potential of morphological change. We also need to draw the genetic map which leads BE to EAC. We are hoping to find some important molecules and pathways to help the management of this fatal disease.

Antigen	Antibody source	Catalogue number	Concentration
P53	Vision BioSystems Inc.	NCL-p53-CM5p	1:500
Cleaved Caspase-3 (Asp175)	Cell Signaling Technology, Inc.	9664 (rabbit mAb)	1:200
PCNA	Dako North America, Inc.	M0879 (mouse mAb)	1:100
EGFR	Sigma-Aldrich, Inc.	E2760 (mouse mAb)	1:100
4-HNE	Cosmo Bio Co., LTD	NNS-MHN-020P-EX (mouse mAb)	1:50

Table 1. The antibodies used in the experiments.

Group	Genotype	Treatment	Total animal	Surgical mortality	Death during the
			number		experiment
А	Wild-type	Non-operated control	20	-	-
В	Wild-type	EGDA and	101	16	12
		50 mg/kg/m Fe, <i>i.p.</i> (from Wk 41 to 80)			
С	р53 ^{А135V}	EGDA	78	13	4
D	INK4a/Arf ^{+/-}	EGDA	29	2	3
Е	Wild-type	EGDA, omeprazole (1,400 ppm) and	36	6	17
		50 mg/kg/m Fe, <i>i.p.</i> (from Wk 21 to 40)			
F	Wild-type	EGDA and	30	3	4
		50 mg/kg/m Fe, <i>i.p.</i> (from Wk 2 to 40)			
G	Wild-type	EGDA, gastrectomy and	26	5	7
		50mg/kg/m Fe, <i>i.p.</i> (from Wk 2 to 40)			

Table 2. Mortality rates of the mouse experiment

	1	1 1 0	Week 20	Week	40	Week 80
Group	Genotype	Treatment	Metaplasia	Metaplasia	ESCC	ESCC
А	Wild-type	Non-operated control	0/10	0/10	0/10	-
В	Wild-type	EGDA and	1/20	6/37	0/37	12/13
		50 mg/kg/m Fe, <i>i.p.</i> (from Wk 41 to 80)	(5%)	(16.2%)		(92.3%)
С	р53 ^{А135V}	EGDA	1/19	2/42	0/42	-
			(5.3%)	(4.8%)		
D	INK4a/Arf ^{+/-}	EGDA	0/10	0/14	1/14	-
					(7.1%)	
Е	Wild-type	EGDA, omeprazole (1,400 ppm) and	-	0/11	0/11	
		50 mg/kg/m Fe, <i>i.p.</i> (from Wk 21 to 40)				
F	Wild-type	EGDA and	-	0/22	0/22	-
		50 mg/kg/m Fe, <i>i.p.</i> (from Wk 2 to 40)				
G	Wild-type	EGDA, gastrectomy and	-	1/14	3/14 ^a	-
		50mg/kg/m Fe, <i>i.p.</i> (from Wk 2 to 40)		(6.7%)	(21.4%)	

Table 3. Development of metaplasia and ESCC in mouse esophagus after EGDA

^a Compared with Group B (P<0.05)

			a-tocopherol	γ-tocopherol	Retinol
Group	Treatment	Diet	(µmol/L)	(µmol/L)	(µmol/L)
	Non-operated				
А	control	AIN-93M (77.8 ppm α-tocopherol)	41.1±8.1	1.21±0.7	1.97±0.63
В	EGDA	AIN-93M (77.8 ppm α-tocopherol)	30.7±10.9*	0.76±0.81	1.65±0.53
С	EGDA	AIN-93M-5xT (389 ppm α-tocopherol)	48.2±23.2#	0.13±0.23#	1.69±0.69
D	EGDA	AIN-93M-5xT (778 ppm α-tocopherol)	55.2±17.7#	0.12±0.16#	1.61±0.47
Е	EGDA	AIN-93M + 500 ppm NAC	27.0±8.2	0.34±0.47	1.76±0.28
F	EGDA	AIN-93M + 1,000 ppm NAC	30.6±6.7	0.58±0.31	1.61±0.37
		AIN-93M-5xT (389 ppm α-tocopherol) +	49.4±28.0		
G	EGDA	500 ppm NAC		0.18±0.24#	1.49±0.39
H	EGDA	AIN-93M + 1,400 ppm omeprazole	25.2±4.5	0.24±0.25	1.76±0.53

Table 4. Rat serum concentrations of α -tocopherol, γ -tocopherol and retinol at 40 weeks after EGDA.

Compared with group A, *P<0.05, Compared with group B, #P<0.05

Group	Diet	Treatment	Incidence Rate
А	AIN-93M-1xT (77.8 ppm α-tocopherol)	Non-operated control	- (0/9)
В	AIN-93M-1xT (77.8 ppm α-tocopherol)	EGDA	65% (20/31)
С	AIN-93M-5xT (389 ppm α-tocopherol)	EGDA	45% (15/33)
D	AIN-93M-10xT (778 ppm α-tocopherol)	EGDA	52% (14/27)
Е	AIN-93M + 500 ppm NAC	EGDA	69% (22/32)
F	AIN-93M + 1,000 ppm NAC	EGDA	50% (14/28)
G	AIN-93M-5xT + 500 ppm NAC	EGDA	44% (12/27)
Н	AIN-93M + 1,400 ppm omeprazole	EGDA	45% (15/33)

Table 5. Visible tumor incidence of α -tocopherol, NAC and omeprazole chemoprevention study with EGDA rats

There were no statistic differences between groups.

Group	Diet	Treatment	Incidence Rate
А	AIN-93M (77.8 ppm α-tocopherol)	Non-operated control	- (0/9)
В	AIN-93M (77.8 ppm α-tocopherol)	EGDA	84% (26/31)
С	AIN-93M-5xT (389 ppm α-tocopherol)	EGDA	61% (20/33)
D	AIN-93M-10xT (778 ppm α-tocopherol)	EGDA	59% (16/27)*
Е	AIN-93M + 500 ppm NAC	EGDA	78% (25/32)
F	AIN-93M + 1,000 ppm NAC	EGDA	68% (19/28)
G	AIN-93M-5xT(389 ppm α -tocopherol) + 500 ppm NAC	EGDA	55% (15/27)*
Н	AIN-93M + 1,400 ppm omeprazole	EGDA	64% (21/33)

Table 6.Histopathological tumor incidence of α-tocopherol, NAC and omeprazole chemoprevention study with EGDA rats

Compared with group B, *P<0.05, Fisher's exact test Group B, C and D showed dose-response effect (Extended Mantel-Haenszel chi square test, P=0.03).

Group	Treatment	Animals/group	Time of Intervention	Incidence rate
А	Non-operated control	9		-
В	Surgical control (Week 15&20) ^a	15		-
С	Surgical control	28		17.8%(5/28)
D	Licofelone (1,000 ppm)	26	3 to 40 weeks	11.5%(3/26)
E	Zileuton (1,000 ppm) + Celecoxib (500 ppm)	26	3 to 40 weeks	3.8%(1/26)
F	Licofelone (1,000 ppm)	29	20 to 40 weeks	13.8%(4/29)
G	Celecoxib (500 ppm)	29	3 to 40 weeks	0(0/29)
Н	Omeprazole (250 ppm)	29	3 to 40 weeks	20.7%(6/29)
I	Omeprazole (250 ppm) + Celecoxib (500 ppm)	28	3 to 40 weeks	14.3%(4/28)

Table 7. Visible tumor incidence of Licofelone, zileuton, celecoxib and omeprazole chemoprevention study with EGDA rats

^aSeven rats were sacrificed on Week 15 and another 8 on Week 20.

Group	Treatment	Animals/Group	Time of Intervention	Tumor Incidence
А	Non-operated control	9		-
В	Surgical control	15		1/8 (12.5%) ^a
С	Surgical control	28		10/28 (35.7%)
D	Licofelone (1,000 ppm)	26	3 to 40 weeks	8/26 (30.7%)
E	Zileuton (1,000 ppm) + Celecoxib (500 ppm)	26	3 to 40 weeks	1/26 (3.8%)*
F	Licofelone (1,000 ppm)	29	20 to 40 weeks	10/29 (34.4%)
G	Celecoxib (500 ppm)	29	3 to 40 weeks	7/29 (24.1%)
Н	Omeprazole (250 ppm)	29	3 to 40 weeks	8/29 (27.6%)
Ι	Omeprazole (250 ppm) + Celecoxib (500 ppm)	28	3 to 40 weeks	10/28 (35.7%)

Table 8. Histopathological findings of Licofelone, zileuton, celecoxib and omeprazole chemoprevention study with EGDA rats

^a One EAC bearing rat was found among 8 rats in group B sacrificed on Week 20, all the other animals sacrificed at week 41. * Compare with group C, P=0.004; compare with group D, P=0.02; compare with group F, P=0.006; compare with group, P=0.05.



Figure 1. The anatomy of EGDA (A) and EGDA plus gastrectomy (B)







N-acetylcysteine

Figure 2. The structure of vitamin E family (A) and NAC (B)



Figure 3. The structure of omeprazole



Figure 4. COX and 5-Lox pathway of arachidonic acid metabolism (207)


Figure 5. The structure of celecoxib (A), zileuton (B) and Licofelone (C)



Figure 6. $P53^{A135V}$ transgenic mice genotyping. Mice carrying the transgene produced two extra bands (150 bp and 40 bp, T: transgenic mouse) besides the 190 bp (WT: wild type mouse).



Figure 7. $INK4a/Arf^{+/-}$ mice genotyping. Wild type mouse (WT) showed a 159 bp band and heterozygous knockout mice (+/-) produced both 159 bp and 280 bp bands.



Figure 8. Body weight changes of mice in 40 weeks. A: Non-operated control group; B: EGDA plus iron (50 mg/kg/m, *i.p.* from week 41 to 80) group; C: $p53^{A135V}$ EGDA group; D: *INK4a/Arf*^{*/-} EGDA group; E: EGDA plus omeprazole (1,400 ppm) plus iron (50 mg/kg/m, *i.p.* from week 21 to 40) group; F: EGDA plus iron (50 mg/kg/m, *i.p.* from week 2 to 40) group; and G: EGDA plus gastrectomy plus iron (50 mg/kg/m, *i.p.* from week 2 to 40) group. Group F had significant higher body weight than Group G since week 24. It suggested that gastrectomy significantly compromised body weight of EGDA mice. At week 20, Group E had significant lower body weight compared with Group B and Group F. We started to give the mice in Group E iron supplement from week 21 and the mice in this group gained 6 gram body weight in average from week 20 to week 32. The body weight of surgical control group was not significantly different compared with Group C and Group D.



Figure 9. Histopathology of mouse esophagus after EGDA. A: In the non-operated control group, the basal cell layer of the epithelium was smooth and the nuclei were in a single line (x400); B: The epithelium responded to surgery-induced reflux with hyperplasia. Layers of the squamous epithelium increased and papillae were enlarged (x400); C: After long-term reflux, the epithelial cells lost their polarity with condensed nuclei and increased mitosis (x400); D: Later, the squamous epithelium lost its normal architecture. Neoplastic cells penetrated the basement membrane and invaded into the stroma tissue (x400); E: At 20 weeks after the surgery, mucin-producing cells were observed in the parabasal layer of the squamous epithelium (x200); and F: Alcian blue staining confirmed mucin secretion in these scattered blue cells. (x200)



Figure 10. p53 immunohistochemistry staining. A: wild-type mouse; and B: $p53^{A135V}$ transgenic mouse (x400). p53 expression in the esophagi of wild-type and $p53^{A135V}$ mice. Strong nuclear accumulation appeared in the esophageal epithelial cells of $p53^{A135V}$ mice, suggesting the mutant form of p53 protein (x400).



Figure 11. Esophageal squamous cell carcinoma in A/J mouse 80 weeks after EGDA. A: In a swiss-roll of 80 weeks esophageal sample, the tumor located at the lower 1/3 of the esophagus (x25); and B: A high magnification picture of a well-differentiated squamous carcinoma was found in the swiss-roll (x400). The neoplastic cells invaded into the muscle layer.



Figure 12. The bodyweight changes of EGDA rats in α -tocopherol, NAC and omeprazole chemoprevention study. A: Non-operated control group; B: EGDA plus iron (50 mg/kg/m *i.p.*) group; C: EGDA plus 389 ppm α -tocopherol plus iron (50 mg/kg/m *i.p.*) group; D: EGDA plus 778 ppm α -tocopherol plus iron (50 mg/kg/m *i.p.*) group; E: EGDA plus 500 ppm NAC plus iron (50 mg/kg/m *i.p.*) group; F: EGDA plus 500 ppm NAC plus iron (50 mg/kg/m *i.p.*) group; G: EGDA plus 778 ppm α -tocopherol plus iron (50 mg/kg/m *i.p.*) group; G: EGDA plus 778 ppm α -tocopherol plus iron (50 mg/kg/m *i.p.*) group; and H: EGDA plus 1,400 ppm omeprazole plus iron (50 mg/kg/m *i.p.*) group. The body weight changes were not significant different between groups during the experiment.



Figure 13. Histopathology of EGDA rats (H&E staining) in α -tocopherol, NAC and omeprazole chemoprevention study. A: a swiss-roll of a tumor bearing esophagus showing an invasive tumor in distal part of esophagus (25x); B: normal esophageal epithelium (400x); C: hyperplastic esophageal epithelium (200x); D: metaplastic lesion of esophageal epithelium (400x); E: dysplastic lesion of esophageal epithelium (400x); and F neoplastic gland (400x).



Figure 14. Tumor volume in EGDA rats in α -tocopherol, NAC and omeprazole chemoprevention study. High dose α -tocopherol (group D), the combination (group G) and omeprazole (group H) appear to decrease the number of large tumors. Tumors larger than 2,500 mm³ were not observed in these groups. The average volume of visible tumors did not show any significant difference between groups.



Figure 15. pH values of gastric and duodenal contents of EGDA rats in α -tocopherol, NAC and omeprazole chemoprevention study (Mean \pm SD). The gastric and duodenal contents of normal control rats (Group A, n=8), the refluxate of EGDA rats (Group B, n=24) and omeprazole-treated EGDA rats (Group H, n=30) were collected and centrifuged. The pH of the supernatant was measured by a pH meter.



Figure 16. 4-HNE immunohistochemistry in α -tocopherol, NAC and omeprazole chemoprevention study (Mean ± SD, x400). Monoclonal 4-HNE antibody was used to detect 4-HNE-His/Lys/Cys adducts. Positively stained cells were counted in 3 high magnification fields (x400) around the metaplastic lesion and the average numbers of positive cells were calculated. A: normal control group (n=5); B: surgical control group (n=9); D: α -tocopherol 778 ppm treatment group (n=9); and G: α -tocopherol 389 ppm plus NAC 500 ppm treatment group (n=9). Letters of lower-case (a, b and c) were used to show the statistical difference between groups. Groups shared at least one same letter were those that were not statistically different. α -Tocopherol alone or in combination with NAC significantly reduced the number of positively stained infiltrating inflammatory cells compared with Group B.



Figure 17. MDA serum level in α -tocopherol, NAC and omeprazole chemoprevention study (Mean ± SD, n=6). A: non-operated control; B: operation control; D: α -tocopherol 778 ppm; and G: α -tocopherol (389 ppm) plus NAC 500 ppm group. Letters of lower-case (a and b) were used to show the statistical difference between groups. Groups shared at least one same letter were those that were not statistically different. Groups without a single letter in common were those that were statistically different. α -tocopherol alone or in combination with NAC did not show any effect on serum MDA levels compared with Group B.



Figure 18. Cleaved caspase 3 expression in α -tocopherol, NAC and omeprazole chemoprevention study (Mean \pm SD, x400). A: Normal control (n=5) was stained negatively for caspase 3; B: Surgical control (n=6); D: α -Tocopherol 778 ppm group; and G: α -Tocopherol 389 ppm plus NAC 500 ppm (n=6) showed positive staining. Letters of lower-case (a) were used to show the statistical difference between groups. Groups shared at least one same letter were those that were not statistically different. Groups without a single letter in common were those that were statistically different. There was no significant difference between groups.



Figure 20. PCNA expression in α -tocopherol, NAC and omeprazole chemoprevention study (Mean ± SD, x400). A: Normal control (n=5); B: Surgical control group (n=6); D: α -Tocopherol 778 ppm group (n=6); and G: α -Tocopherol 389 ppm plus NAC 500 ppm group (n=6). Letters of lower-case (a and b) were used to show the statistical difference between groups. Groups shared at least one same letter were those that were not statistically different. Groups without a single letter in common were those that were statistically different. Group B, D and G showed significantly increased staining compared with normal control group. There were no significant differences between surgical groups.



Figure 20. EGFR staining pattern in EGDA rats in α -tocopherol, NAC and omeprazole chemoprevention study (x400). EGFR staining was negative in hyperplasia squamous epithelium (A); sporadically positive in metaplastic and dysplastic epithelium from mild (B) to strong (C) and the staining was negative in esophageal adenocarcinoma (D).



Figure 21. EGFR expression pattern in human esophageal tissue. A: EGFR staining in human esophageal squamous epithelium; B: negative EGFR staining in human esophageal adenocarcinoma; and C: positive EGFR staining in human esophageal adenocarcinoma. (Wang KL. *et al.* Expression of epidermal growth factor receptor in esophageal and esophagogastric junction adenocarcinomas: association with poor outcome. *Cancer*, Volume 109, Issue 4, 2007. Pages: 658-667. Copyright@ 2007, Wiley-Liss, Inc. reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc.)



Figure 22. Body weight changes of EGDA rats in Licofelone, zileuton, celecoxib and omeprazole chemoprevention study. A: Non-operated control group; B: EGDA plus iron (50 mg/kg/m *i.p.*) group(sacrificed at week 15 and 20); C: EGDA plus iron (50 mg/kg/m *i.p.*) group; D: EGDA plus 1,000 ppm Licofelone plus iron (50 mg/kg/m *i.p.*) group (Licofelone diet was given from week 3 to 40); E: EGDA plus 1,000 ppm zileuton and 500 ppm celecoxib plus iron (50 mg/kg/m *i.p.*) group; F: EGDA plus 1,000 ppm Licofelone plus iron (50 mg/kg/m *i.p.*) group; F: EGDA plus 1,000 ppm Licofelone plus iron (50 mg/kg/m *i.p.*) group; H: EGDA plus 250 ppm omeprazole plus iron (50 mg/kg/m *i.p.*) group; and I: EGDA plus 250 ppm omeprazole plus 500 ppm celecoxib plus iron (50 mg/kg/m *i.p.*) group. The body weight changes were not significantly different between groups during the experiment.



Figure 23. Visible tumor volume in Licofelone, zileuton, celecoxib and omeprazole chemoprevention study. The visible tumor volume was plotted and the bar in the plot showed the group mean of the tumor volume. The difference was not statistically significant comparing the tumor volume in group C, D, F, H and I. Group E had only one tumor.



Figure 24. Histopathology of EGDA rats in Licofelone, zileuton, celecoxib and omeprazole chemoprevention study (x400). A: Normal mucosa of esophageal epithelium (H&E); B: Hyperplastic lesion of esophageal epithelium (H&E); C: Intestinal metaplastic lesion of esophageal epithelium. The glandular structure was highlighted with Alcian Blue; D: Dysplastic lesion of metaplastic esophageal epithelium (H&E); E: EAC of EGDA rat (H&E); and F: neoplastic cells metastasized to a lymph nodule.



Figure 25. PGE2 level in serum in Licofelone, zileuton, celecoxib and omeprazole chemoprevention study (Mean \pm SD, n=8). A: normal control group; C: surgical control group; D: Licofelone 1,000 ppm group; and E: zileuton 1,000 ppm and celecoxib 500 ppm combination. Letters of lower-case (a and b) were used to show the statistical difference between groups. Groups shared at least one same letter were those that were not statistically different. Groups without a single letter in common were those that were statistically different. The difference between Groups C and Group D was not statistically significant. But Group C showed significantly reduced PGE2 production compared with Group E.



Figure 26. LTB4 level in serum in Licofelone, zileuton, celecoxib and omeprazole chemoprevention study (Mean \pm SD, n=7). A: normal control group; C: surgical control group; D: Licofelone 1,000 ppm group; and E: zileuton 1,000 ppm and celecoxib 500 ppm combination. Letters of lower-case (a) were used to show the statistical difference between groups. Groups shared at least one same letter were those that were not statistically different. Groups without a single letter in common were those that were statistically different. The variance we observed in LTB4 serum level was not statistically significant.



Figure 27. Iron deposition (Mean \pm SD, n=9, x400). Only one sample in the normal control group (normal control A) of the study in specific aim 3 (Licofelone study) showed positive staining, while all the samples in the normal control group (normal control B) of the study in specific aim 2 (α -tocopherol study) showed positive staining. Iron supplementation significantly increased positively stained cells in all surgical control groups. Surgical control B showed significantly more iron positive cells than surgical control group A. Letters of lower-case (a, b, c and d) were used to show the statistical difference between groups. Groups shared at least one same letter were those that were not statistically different. Groups without a single letter in common were those that were statistically different.

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Publications

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Awards

Jing Hao. Awarded Graduate Student Travel Award of 2008 annual SOT (society of toxicology) meeting.

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