ENVIRONMENTAL AND GENETIC INTERACTIONS OF ESOPHAGEAL AND LIVER CANCER IN TWO HIGH-RISK AREAS, CHINA

by

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(Under the Direction of Jia-Sheng Wang)

ABSTRACT

Two case-control studies were carried out in this dissertation research to explore environmental and genetic interactions of esophageal and liver cancer in two high-risk populations. In the esophageal cancer case-control study, including 190 esophageal squamous cell carcinoma (ESCC) patients and 380 age-, gender- and residency- matched controls from Huaian, urinary free fumonisin B_1 (FB_1), sphinganine (Sa), sphingosine (So), Sa/So ratio, oxidative stress biomarker 8-hydroxy-2'-deoxyguanosine (8-OHdG), and genetic polymorphisms of genes, GSTT1, GSTM1, hOGG1, XPD, COX-2, NF-κB, TNF-α, and iNOS were evaluated. Analysis of questionnaires revealed that esophageal lesion, preference for salty food (ten-years-ago), preference for hot food (ten-years-ago), frequent pickled/salty food intake, mildewed food intake (ten-years-ago), and first-degree family history of cancer were associated with increased ESCC risk, while body mass index, frequent garlic intake, and tea drinking were protective factors. High levels of 8-OHdG, free FB_1, and polymorphic XPD 751 Gln genotype were associated with increased risk for ESCC. Additive effects were observed for XPD 751 Gln genotype and high free FB_1 (OR=9.35; 95% CI: 4.16–21.04), as well as XPD 751 Gln genotype and

frequent pickled/salty food intake (OR=5.70, 95%CI: 2.42–13.43). The association between free FB_1 level and ESCC risk suggests urinary free FB_1 as a good FB_1 exposure biomarker for future human epidemiological studies. In the hepatocellular carcinoma (HCC) case-control study, including 60 HCC patients and 120 age-, gender- and residency- matched controls from Southern Guangxi area, hepatitis B virus (HBV) genotype and basic core promoter (BCP) mutations, as well as serum level of aflatoxin B_1 (AFB_1)-lysine adduct were determined. The $1762^T/1764^A$ double mutations, 1753^V mutations, and 1752^V mutations were associated with HCC risk. Additive effects were observed for high serum AFB_1 -lysine adduct levels and $1762^T/1764^A$ double mutations (OR=6.94, 95% CI: 1.68–27.78), as well as 1753^V mutations (OR=5.13, 95% CI: 1.79–14.71). These data confirmed the association of BCP mutations and their interactions with dietary AFB_1 exposure on HCC risk in this high-risk population. Overall, strong environmental (chemical and viral) and genetic interactions were found to play important roles in HCC and ESCC formation in these two high-risk populations.

INDEX WORDS: hepatocellular carcinoma; hepatitis B virus; HBV genotype; HBV gene mutations; aflatoxin; AFB_1-lysine adduct; esophageal squamous cell carcinoma; fumonisin B_1; sphingosine; sphinganine; oxidative stress; 8-hydroxy-2’-deoxyguanosine; biomarker; genetic polymorphism

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DEDICATION

To my parents, for their endless love and support

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Chapter 1

INTRODUCTION

Cancer is the second and the third leading causes of death in developed and developing countries, respectively [1]. The global burden of cancer is expected to keep rising in the coming decades. Although progress has been made in treatment and early diagnosis, the prognosis remains unfavorable in most types of cancer. These facts remain cancer as a severe public health problem of great research interest. Carcinogenesis is a long-term process involving multiple stages and factors. These factors can be categorized into external (environmental) and internal (individual susceptibility or genetic) factors, which may act together or in sequence to initiate or promote carcinogenesis.

Esophageal cancer is one of the leading causes of cancer death worldwide, especially in East and South Africa, Central Asia and China [1]. Esophageal squamous cell carcinoma (ESCC) is the dominant type of esophageal cancer in many high risk areas. Multiply risk factors, including excessive use of alcohol and tobacco, consumption of salt-pickled, salt-cured, and moldy foods, and conjunction of hot food and beverages, have been associated with development of ESCC [2-4]. In addition, other potential risk factors, especially dietary fumonisins exposure, awaiting validation, have been suggested in some high risk areas. Huaian City is one of the high risk areas for ESCC in China. The high-incidence in this area has been proposed partly associated with the heavy dietary exposure to fumonisins in corn [5-8]. Molecular biomarkers, including urinary

sphingolipid metabolites, sphinganine (Sa) and sphingosine (So), and free fumonsin B_1 (FB_1), have been evaluated as promising biomarkers of dietary FB_1 exposure [9]. 8-hydroxy-2'-deoxyguanosine (8-OHdG) has been demonstrated as a potential biomarker of oxidative DNA damage related to multiple environmental exposures, including smoking and fumonisin exposure [10-12]. However, its application in ESCC development awaits validation. Although the exact mechanism of ESCC formation remains to be elucidated, individual susceptibility factors have been suggested to play an important role in ESCC development. Genetic polymorphism of xenobiotic-metabolizing genes, DNA repair genes and infection associated inflammatory genes are of great interests in this dissertation study.

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer death worldwide, especially in Southeast Asia and sub-Sahara Africa [1]. Infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) and long-term dietary exposure to aflatoxins are the major risk factors of HCC in these high-risk areas [1]. Southern Guangxi area is one of the high risk areas of HCC in China [13]. Previous studies by others and our laboratory found that high incidence of HCC in this area was partly due to heavy dietary exposure to aflatoxin via high level of corn and groundnut consumption [14-17]. Serum aflatoxin B_1-albumin adduct has been applied as molecular biomarker to evaluate aflatoxin exposure and quantitate actual level of aflatoxin exposure in high-risk populations [18-20]. Besides aflatoxin exposure, HBV infection is a potent risk factor in etiology of HCC in this area [16, 17]. HBV has at least 8 genotypes with distinct geographical distribution [21]. Specific HBV genotype and gene mutations have been found to be associated with HCC risks, especially for mutations within the basic core

promoter (BCP) region of HBV X-gene which have been reported commonly in HCC [22].

The working hypothesis of this dissertation research is that environmental carcinogen exposure, genetic susceptibility, and their interactions are critical in human esophageal and liver cancer formation in high risk areas. Specific aims for the dissertation project include:

1) To evaluate dietary FB_1 exposure level in a population-based case-control study carried out in Huaian, China, including 190 ESCC cases and 380 matched healthy controls by measuring FB_1 biomarkers, including urinary free FB_1, Sa and So.

2) To assess the role of oxidative stress in development of ESCC through measuring urinary 8-OHdG in cases and controls.

3) To assess the role of genetic polymorphisms of xenobiotic metabolizing enzymes, DNA repair enzymes, and inflammation-regulating proteins in modulating the individual susceptibility to ESCC by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analyses.

4) To detect HBV genotypes and BCP mutations in a population-based case-control study of HCC carried out in Southern Guangxi area, China, including 60 cases and 120 matched controls through the nested PCR followed by direct sequencing.

5) To assess the association of aflatoxin exposure with development of HCC by measuring serum AFB_1-lysine adducts levels in both case and control samples.

The major findings in ESCC case-control studies include medical history of esophageal lesion, preference for salty food (ten-years-ago), preference for hot food (ten-

years-ago), frequent pickled/salty food intake, mildewed food intake (ten-years-ago), and first-degree family history of cancer were associated with increased risks of ESCC. Frequent garlic intake, tea drinking, and body mass index (BMI) were protective factors for ESCC development. High levels of urinary 8-OHdG and free FB_1, as well as XPD 751 Lys/Gln or Gln/Gln genotype were significantly associated with increased risk for ESCC. Also, urinary 8-OHdG level was positively correlated with free FB_1 level in controls, suggests the potential role of oxidative DNA damage caused by environmental toxin exposure in ESCC development. The association between urinary free FB_1 level and increased ESCC risk suggests urinary free FB_1 as a good FB_1 exposure biomarker for future epidemiological studies. Additive effects were observed for XPD 751 Lys/Gln or Gln/Gln genotype and higher urinary free FB_1 level (OR=9.35, 95% CI: 4.16–21.04), as well as XPD 751 Lys/Gln or Gln/Gln genotype and frequent pickled/salty food intake (OR=5.70, 95%CI: 2.42–13.43). Overall, strong environmental (chemical and viral) and genetic interactions were found to play important roles in ESCC formation in this high-risk population.

The major findings in HCC case-control studies include that $1762^T/1764^A$ double mutations (OR=3.89, 95% CI: 1.40–10.77), 1753^V mutations (OR=2.87, 95% CI: 1.49–5.49), and 1752^V mutations (OR=5.96, 95% CI: 1.75–20.25) were associated with HCC risk. Additive effects were observed for high serum AFB_1-lysine adduct levels and $1762^T/1764^A$ double mutations (OR=6.94, 95% CI: 1.68–27.78), as well as 1753^V mutations (OR=5.13, 95% CI: 1.79–14.71). Data from this study confirmed association of BCP mutations and their interactions with dietary AFB_1 exposure on HCC risk in this high-risk population.

Chapter 2

LITERATURE REVIEW

Cancer is one of the leading causes of death in the world. It accounted for 7.9 million deaths worldwide (around 13% of all deaths) in 2007 and is anticipated to continue rising. In 2050 there will be 27 million new cancer cases and 17.5 million cancer death [1]. The three strategies developed by World Health Organization (WHO) in controlling and reducing cancer are cancer prevention, early detection, and management of cancer patients. More than 30% of cancer can be prevented by modifying or avoiding key environmental risk factors [23]. Therefore, identification of major risk factors in cancer development is of great interest in cancer prevention.

This chapter provides a literature overview on epidemiology and etiology of esophageal squamous cell carcinoma (ESCC) and hepatocellular carcinoma (HCC).

ESOPHAGEAL CANCER

Esophageal cancer is a malignancy of the esophagus. There are two main types of esophageal cancer – ESCC and esophageal adenocarcinoma (EAC) – that account for more than 95% of all esophageal cancer. These two types of esophageal cancer have distinct pathological and etiological characteristics.

2.1 Epidemiology of esophageal cancer

2.1.1 Incidence and mortality

Esophageal cancer is one of the most common cancers worldwide. An estimated 529,000 new cases (361,931 in males and 167,352 in females) were expected to occur according to global cancer statistics of 2007 [1]. It ranks sixth in males and ninth in females in terms of new cases in the world [1]. The incidence rate of esophageal cancer shows significant variation in geographic distribution, varying internationally by more than 50-fold in terms of incidence. High incidences are found in East and South Africa, Northern France and Central Asia, with age-standardized incidence greater than 11/100,000 population, and even higher than 100/100,000 population in several regions in Asia. In Asia, there is a notorious "central Asia esophageal cancer belt", which extends from northeastern China westward northern Iran along the path of the Silk Road, consisting of several countries, including China, Turkmenistan, Uzbekistan, Karakalpakstan, Kazakhstan and Iran [24]. However, since very few well-established cancer registries have been established in these areas, the presence of such a belt is not well studied and entirely confirmed. Low incidences are found in Western Africa, Southeast Asia and North America, with age-standardized incidence less than 6/100,000 population in males and 2.5/100,000 population in females.

In general, the prognosis of esophageal cancer is poor. The estimated five-year relative survival rate was around 15% in the United States in 1996–2002. In Europe, this rate was 8.5% and 10.5% in male and female patients, respectively, in 1990–1994 [1]. In developing countries, the survival rate is anticipated to be worse. There were estimated 442,000 people died from esophageal cancer in 2007, which accounted for 5.8% of

cancer death worldwide, and 85% of these death was anticipated to occur in developing countries [1]. It is noteworthy that these cancer statistics provide data regardless histological type of esophageal cancer. In general, in esophageal cancer high incidence areas, most cancer cases are ESCC, whereas in low incidence areas, especially developed countries, EAC is more frequently found.

The incidences of esophageal cancer also show variations among different race/ethnicity in the same region. For instance, although the overall esophageal cancer incidence rates were comparable between Black and non-Hispanic white males (5.4/100,000 and 4.4/100,000 population, respectively) in Los Angeles County of California, incidence rates by histological type showed obvious difference [25]. Age-standardized incidence of ESCC was 3.9/100,000 in Black males, which was more than 3-fold of the rate in non-Hispanic white males (1.2/100,000 population). With respect to EAC, the age-standardized incidences were 0.9/100,000 and 2.7/100,000 population in Black and non-Hispanic white males, respectively.

2.1.2 Age and gender distribution

The overall incidence of esophageal cancer is three to four times higher in males than females. The male to female ratio is lower in high-incidence areas whereas higher in low-incidence areas. In the high incidence populations with incidences greater than 100/100,000 population, including Gonbad City, Iran [24], and Linxian, China [26, 27], incidence rates were as high in females as in males, or even higher in females than in males. Esophageal cancer is not commonly found in children and young adults. The incidence increases progressively with age. ESCC occurs more frequently in people of

age between 60 and 70 years. EAC occurs in younger age group than ESCC, mostly found in age greater than 50 years.

2.1.3 Time trend of esophageal cancer incidence

In a comparison of time trends of esophageal cancer incidence (1973–1997) among areas and ethnic groups in East Asia, Europe and USA, patterns of time trend differed among populations [28]. In general, a decreased trend was found in populations with relative high incidence rate, whereas an increased trend was observed in populations with low incidence. For instance, in Shanghai, China, age-standardized rate in males decreased from around 25/100,000 population in 1973–1977 to 15/100,000 in 1983–1987 and furthermore, around 7/100,000 in 1993–1997. Bas-Rhin in France had a incidence rate around 17/100,000 population in males in 1973–1977, which increased slightly to 19/100,000 till period of 1983–1987 and decreased obviously to around 13/100,000 in 1993–1997. Similar pattern is also found in Hong Kong Chinese males and Africa American males. On the contrary, age-standardized rate rises moderately from 1978–1982 to 1993–1997 in male populations from Japan, Denmark, England, and American white. Consistently, females followed the similar pattern in all investigated populations, but more gradually [28]. Since ESCC is more frequent in high-incidence populations and EAC is more frequent in low-incidence populations, the change of incidence over time is believed attributing to decreased rate in ESCC and increased rate in EAC overall.

In recent decades, the incidence of EAC has been rising rapidly in Western countries where had low incidence of esophageal cancer traditionally, mostly due to increased incidence of overweight/obesity, chronic gastric reflux, and Barrett esophagus [1]. In the

United States, it was estimated that new esophageal cancer cases in 2008 was 16,470 totally, increasing by 6% from 15,560 new cases in 2007 [29, 30].

2.1.4 Esophageal cancer in China and Huaian City

China is one of the high risk areas of esophageal cancer in the world. Around 250,000 cases were diagnosed each year in China, which contributes to more 40% of new esophageal cancer cases worldwide [1]. The prevalence of esophageal cancer distributes unevenly in China. According to nation-wide population-based registries in China in 1998–2002, age-standardized incidence of esophageal cancer ranged from 0.3/100,000 to 132.7/100,000 population/year and age-standardized mortality ranged from 2.7/100,000 to 110.6/100,000 population/year [31]. The high incidence areas in China remain in a few demarcated areas which belong to the "central Asia esophageal cancer belt", including Taihang Mountains area and northern Jiangsu area.

In 1998–2002, there were 5,964 new esophageal cancer cases reported in Huaian city, with an age-standardized incidence of 98.5/100,000 population [31]. It is one of the seven areas (city, town) in China with age-standardized incidence greater than 80/100,000 population. The incidences of esophageal cancer in Huaian were 113.4/100,000 and 84/100,000 population in males and females, respectively. Opposite to developed countries, ESCC remains the predominant type of esophageal cancer in the high risk areas in China, accounting for more than 90% of esophageal cancer [2, 31].

2.2 Etiology and risk factors of esophageal cancer

Although the exact mechanism(s) of esophageal carcinogenesis remain unclear, as a

complex disease, it is widely accepted that multiple risk factors are involved in the development of esophageal cancer. The fact that esophageal cancer incidence varies dramatically over the space and time indicates that environmental factors play important roles in the etiology of esophageal cancer in addition to individual susceptibility factors. The risk factors for esophageal cancer differ between different histological types of cancer and show among population heterogeneity. Table 2.1 summarizes the major risk factors that have been suggested or identified in ESCC and EAC development, while their contributions to cancer development vary by populations and studies [3, 4].

For ESCC, no single predominating risk factor has been identified. Instead, several risk factors may co-exist and vary among populations [2]. In high-incidence areas, such as China and Iran in the central Asia esophageal cancer belt, the predominating factors are highly related to diet, including consumption of food containing mycotoxins and other carcinogens, i.e. nitrosamines, vitamin and micronutrient deficiencies, consumption of very hot beverage/food. In low-incidence areas, such as the United States, the major risk factor is alcohol abuse, often companied by dietary deficiencies. In areas with intermediate incidence, such as Japan and several Africa countries, several risk factors including alcohol drinking, smoking, consumption of hot tea, have been found.

2.2.1 Dietary fumonisin exposure

Fumonisins (FNs) are a group of mycotoxin produced mainly by the fungus *Fusarium verticillioides*, *Fusarium proliferatum*, and more rarely, other *Fusarium* species [32]. They are found in corn and corn-based foods worldwide. FNs were first isolated from culture of *F. verticillioides* strain MRC 826, which was isolated in corn samples collected

Table 2.1 Common risk factors for esophageal cancer

Squamous cell Carcinoma (ESCC)	Adenocarcinoma (EAC)
Age (60-70 years of age)	Age (50-60 years of age)
Gender	Gender
Race	Race
Achalasia	Barrett's esophagus
Alcohol abuse	Gastroesophageal reflux disease
Tobacco smoking	Hiatal hernia
Salt-pickled, salt-cured and moldy foods	Overweight/obesity
Antioxidant vitamins	
Tract mineral deficiencies	
HPV serotype 16 and 18	
Helicobactor pylori infection	
Hot food and beverages	
Fungal toxins	
Radiation therapy	

from an esophageal cancer high-risk area in South Africa in 1988 [33]. In the same year, the chemical structures of the FNs were also identified [34]. Till now, at least 15 FNs have been isolated, characterized and designated into four main categories (A, B, C, and P). Only fumonisin B_1 (FB_1) (Figure 2.1) and FB_2 appear to be biologically important, and FB_1 is the most abundant naturally occurring fumonisn [32].

Consumption of FNs-contaminated corn has been associated with a variety of fatal animal diseases, including leukoencephalomalacia in horses, pulmonary edema in swine, and hepatotoxicity in horses, swine and rats [35]. Also, FNs induced liver and kidney cancer in rodents in several studies [32, 36]. Lifetime studies in BD IX rats fed FNs-contained diets yielded a high incidence (80%) of liver tumors, with no lesions in control animals [37, 38]. Long-term intake of 50 ppm FB_1 resulted in HCC in 66% of treated BD

IX rats [39]. The two-year feeding study conducted by US Food and Drug Administration (FDA) found a significantly increased incidence of kidney tumors in F344 male rats (26% at 50 ppm FB_1-treated group, 38% at 100 ppm FB_1-treated group) and HCC in B6C3F1 female mice (43% at 50 ppm FB_1-treated group, 88% at 80 ppm FB_1-treated group) [40]. Based on then available evidences in 2002, International Agency for Research on Cancer (IARC) announced that there is sufficient evidence in experimental animals for the carcinogenicity of FB_1, but inadequate evidence in humans, and hence classified FB_1 as a type 2B human carcinogen (possibly carcinogen to humans) [32].

Although the health effect of FB_1 in human populations has not been well proven, consumption of FNs-contaminated food has been associated with esophageal cancer in several high-incidence populations in South Africa and China [5, 7, 41-43]. Also, it has been associated with increased risk for neural tube defects along the Texas-Mexico border [44].

2.2.1.1 Absorption, distribution, metabolism, and excretion of FB_1

The kinetics and metabolism of FB_1 are characterized by poor absorption, rapid elimination and no metabolism *in vitro* or *in vivo*. After oral administration of [^{14}C] FB_1, the radioactivity of FB_1 in plasma and tissues was low (2-<6% of dose) in rats, vervet monkeys, pigs and cows, indicating that the absorption of FB_1 was negligible [45-48]. When administered by intraperitoneal or intravenous injection to rats, the radioactivity of [^{14}C] FB_1 was distributed widely and subsequent elimination was rapid with half-life time of approximately 10 to 20 minutes [49]. Most of the FB_1 excrete through feces with over 90% of recovered in the feces and less than 1% recovered in urine after oral dose in rats.

Similar to rats, in vervet monkeys, FB_1 was widely distributed and rapidly eliminated with a mean half-life time of 40 minutes after intravenous injection, and recovered in 61% of feces (61%) and 1.2% of urine excretion in a 3-day period [46].

The liver and kidney are the two major organs retaining most of the absorbed FB_1. In male Wistar rats fed FB_1 by gavages, considerable concentrations of FB_1 were found in the liver and kidney tissues, and FB_1 persisted in liver and kidney much longer than in plasma, evidenced by elimination half-lives of 4 hour, 7 hour, and 3 hour, respectively [50].

Both *in vivo* animal models and *in vitro* cell studies found little evidence of metabolism of FB_1. A study in [^{14}C] FB_1-treated primary rat hepatocytes showed that there were a tiny few (approximately 0.01%) FB_1 bounded to hepatocytes, and no metabolites were detected after incubation for 44 hours [51]. The latter aspect was confirmed by the fact that FB_1 was not a substrate for cytochrome P450 and microsomal esterase after incubation with liver microsomal preparations. In vervet monkeys, along with FB_1, partially hydrolysed metabolites of FB_1 were recovered in feces but not in urine and bile, indicating that the hydrolysis probably occur in gut, and by microbial degradation [46, 47].

There are no studies on absorption, distribution, metabolism and excretion of FB_1 in human in literature. However, because the rate of elimination of FB_1 is anticipated to be a function of body weight, FB_1 is predicted to be retained in human body much longer than in rodents: in a 70-kg human being the half-life following FB_1 administration was estimated to be 128 minutes [52].

2.2.1.2 Interruption of sphingolipid metabolism

FB_1 is a reportedly inhibitor of ceramide synthase. FB_1 consists of a long hydroxylated hydrocarbon chain, to which tricarballylic acid, methyl, and amino acid groups are added. As shown in Figure 2.1, it is structurally similar to the long-chain base backbones of sphingolipids, which play an important role in membrane and lipoprotein structure as well as in cell regulation as second messengers for growth factors, differentiation factors, and cytokines [53]. Therefore, FB_1 is anticipated to function through interruption of sphingolipid metabolism. The mechanism and pathway involved in this process was reviewed and summarized by Cai and colleagues in Figure 2.2 [54]. Incubation of primary rat hepatocytes with FB_1 resulted in inhibition of serine incorporation into sphingosine (So) moiety of cellular sphingolipids with an IC50 of 0.1 μM [55]. On the contrary, the amount of the biosynthetic intermediate, sphinganine (Sa) increased, suggesting that FB_1 inhibited the conversion of Sa to N-acetyl-Sa. Furthermore, FB_1 inhibited the activity of sphingosine N-acyltransferase (ceramide synthase) in rat liver microsomes with 50% inhibition at approximately 0.1 μM. Also, FB_1 reduced the conversion of So to ceramide by intact hepatocytes. In agreement with this mechanism, a number of animal studies found that FB_1 feeding cause an increase of free Sa and usually to less extent, So in urine, serum and tissues [54, 56-58]. Thus, disruption of the *de novo* sphingolipid biosynthesis and interference with sphingolipids-mediated signal transduction may be the critical mechanisms in FB_1 toxicity.

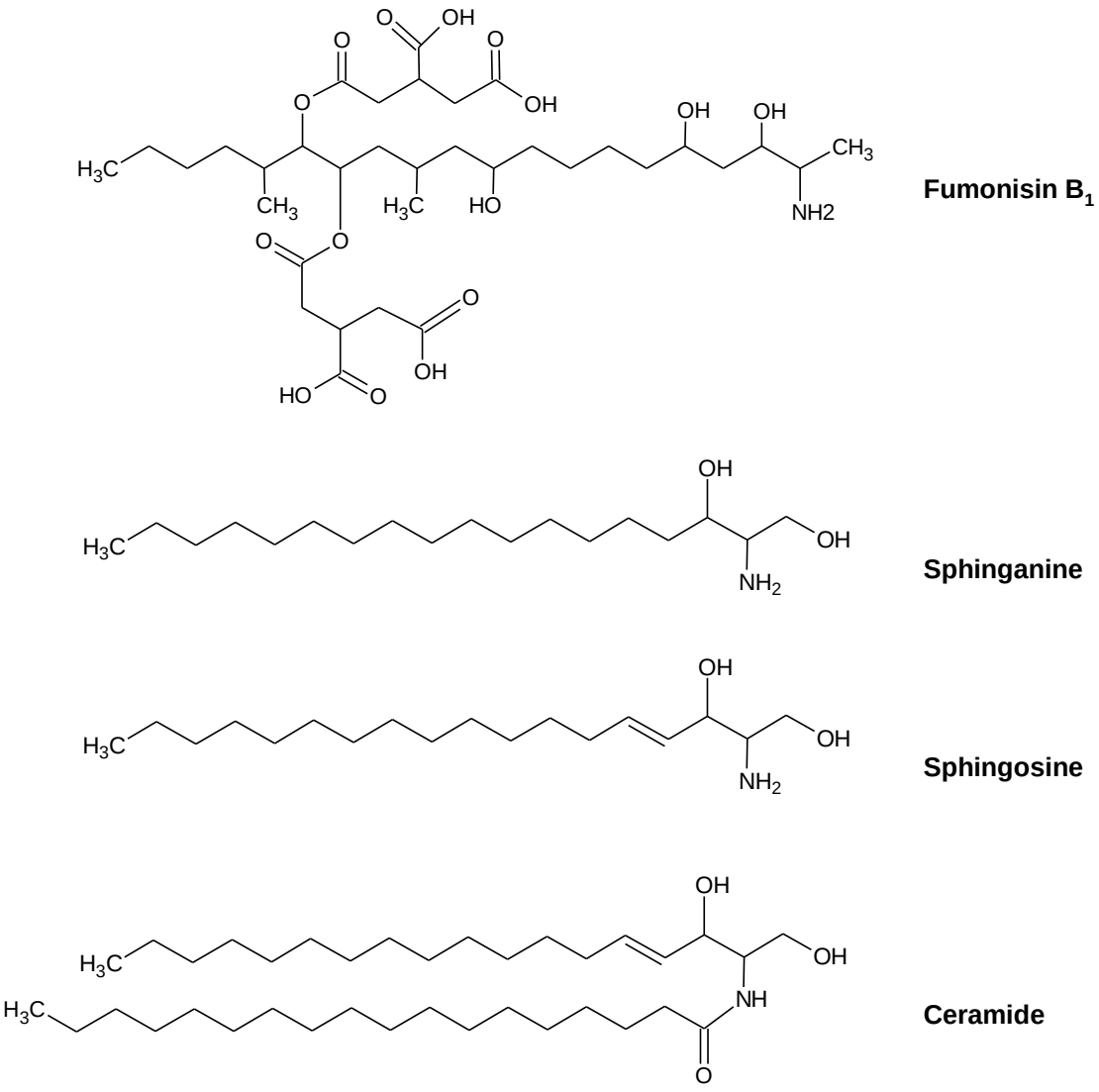

Figure 2.1 Molecular structures of Fumonisin B_1, sphinganine, sphingosine, and ceramide (Modified from figures in a review by Stockmann-Juvala and Savolainen [35])

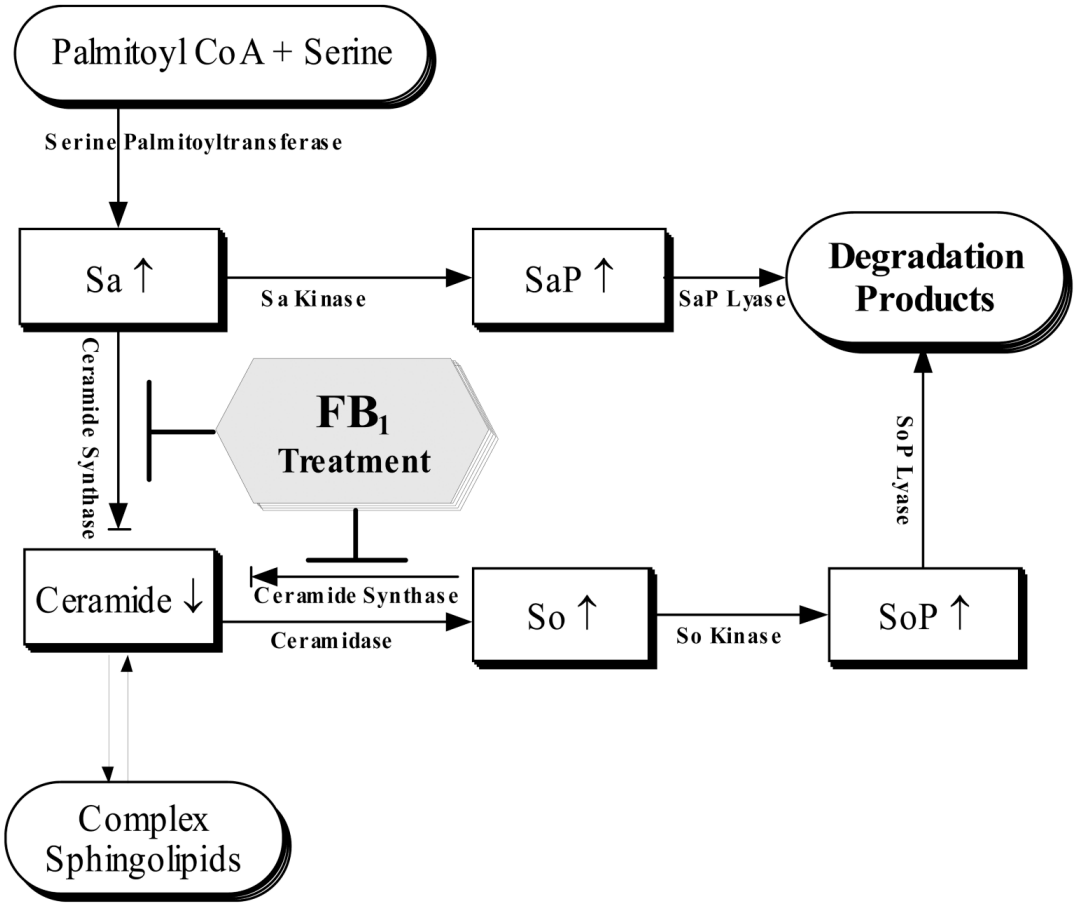

Figure 2.2 Disruption of *de novo* sphingolipid biosynthetic and turnover pathways by FB_1 treatment (adapted from figures by Cai et. al [54])

2.2.1.3 Occurrence of FB_1

FNs are ubiquitous contaminants of cereal grains (corn) over the world. Although the contents of FB_1 in corn products vary depending on geographical location and local weather, FNs contamination is a severe global problem. Dutton reviewed the occurrence of FB_1 in corn in different countries in 1996 and found FB_1/FB_2 contamination was commonly detected in countries and areas all over the world [59], which has been further confirmed by later on surveys in corn and corn-based products from different regions of the world [60-63]. For instance, an investigation in Transkei area of South Africa,

showed that out of 10 households, an average of 63% of corn kernels were infected with *F. verticillioides* [33]. A study of commercially grown corn in South Africa showed that 187 (75.1 %) of 249 maize samples were contaminated with FB_1 with the highest level of 7100 μg/kg [64]. Food products, including corn meal, canned corn, dog and cat food purchased in Iowa state were contaminated with FB_1/FB_2 (44/160, 27.5%) with a concentration up to 4140 μg/kg [65]. A survey reported that 35 (36.1%) of 97 corn product from USA retail market were positive for FB_1 with maximum levels of 235 μg/kg and 350 μg/kg in canned and frozen corn, respectively [66]. A recent study collected 156 samples of breakfast cereals from Canadian retail market and detected FB_1/FB_2 in over 30% of samples [60].

FB_1 contamination in corn is also common in China. In esophageal cancer high risk counties of Cixian and Linxian, China, FB_1 has been detected in all corn samples collected from local households: half of these corn samples showed heavy mold contamination with a mean FB_1 level of 74 mg/kg (range: 18–155 mg/kg), another half without visible mold contamination showed an average FB_1 level of 35.3 mg/kg (range: 20–60 mg/kg) [42]. Another study conducted in Cixian County achieved a consistent result: FB_1 was detected in 100% (10/10) moldy corn samples and 90.9% (10/11) apparently health corn samples, with average FB_1 level of 88.9 mg/kg and 1.4 mg/kg, respectively [61]. In an investigation in Huaian, FB_1 was detected in 95.7% (112/117) of corn samples, with an average of 2.84 mg/kg (range: 0.1–25.5 mg/kg) [62]. A survey of 282 corn samples from 6 provinces in China found detectable FB_1 in 99.6% samples with 25.2% of samples having a level higher than 5 mg/kg [63].

2.2.1.4 Human health effect of FB_1

An outbreak of acute foodborne disease caused by FB_1 was reported in south India in 1995 with 1,424 people affected [67]. These patients consumed corn and sorghum harvested and left in the fields during unseasonable rains. The main symptoms included transient abdominal pain, borborygmus and diarrhea, which happened within one hour after consumption of the bread from the moldy corn or sorghum [67]. Analysis of the food samples collected from the affected households showed a significantly higher FB_1 level than from controls.

Neural tube defects (NTDs) are among the most common human birth defects, while the etiological basis remains poorly understood. In 1990–1991, a cluster of NTD was reported in Mexican-American women along the south Texas border [68]. During the same period, corn crops registered in this area showed unusually high level of FB_1. A follow-up case-control study in this area suggested that FB_1 exposure was associated with increased risk of NTDs [44]. Globally, NTDs was also related to consumption of FB_1 contaminated food. In northern China and Transkei of South Africa rural area where corn is the principal staple food commonly contaminated with FNs, the incidence of NTDs is often 6–10 times higher than the average global rates [69].

Although IARC declared that there is inadequate evidence to classify FB_1 as human carcinogen related to esophageal cancer in 2002, progress has been made in evaluation of etiological role of FB_1 in esophageal cancer. In South Africa, Marasas and colleagues conducted a cross-sectional study in evaluating FB_1 exposure in esophageal cancer risk [5]. Homegrown corn was collected from 12 households each in low-, intermediate-, and high-esophageal cancer incidence areas in Transkei during two continuous years. The

prevalence of *F. verticilloides* in corn was different among these areas, suggesting a correlation between FB_1 contamination and esophageal cancer. A follow-up study reported significantly higher proportion of corn kernels infected by *F. verticilloides* in the high-risk area than the low-risk area regarding both apparently healthy and moldy maize samples (42% vs. 8%, 68% vs. 35%, respectively) [6]. Similarly, in homegrown corn samples from 12 households each in the Bizana and Kentani districts, low- and high-incidence areas of esophageal cancer in South Africa, respectively, FB_1 levels were 6.5 ± 5.3 μg/g and 23.9 ± 14.6 μg/g in moldy corns from the Bizana and Kentani, respectively ($p<0.001$); FB_1 levels were 0.06 ± 0.2 μg/g and 1.6 ± 2.1 μg/g in healthy corns from the Bizana and Kentani, respectively ($p<0.001$) [7]. An exposure assessment was performed by combing the corn consumption with FB_1/FB_2 contamination in home-grown corn in subjects from the Bizana (n=229) and Transkei (n=178) area, South Africa [8]. Consistently, the individual daily intake of FB_1 based on corn consumption and FB_1 level in corn in the high-risk area was significantly higher than that in the low-risk area.

Similar correlation was also observed in cross-sectional studies conducted in Chinese population. In Cixian County, China, the frequency of FB_1 contamination in the high risk area was about twice than in the low risk area, and the average content of FB_1 in corn samples from high-risk area was approximately 3-fold of the level in the low risk area [61]. In a study comparing the FBs contamination in corn samples collected from Linxian and Shangqiu Counties, high- and low-risk areas for esophageal cancer in Henan Province, China, respectively, contamination rate of FNs in Linxian corn (48%) was about 2-fold of the rate in Shangqiu corn (25%) [43]. Consistent with these studies, the detectable rate and average level in corn samples collected from households of Huaian,

was significantly higher than those from Huaitai, a esophageal cancer low risk area [62]. These cross-sectional studies, through comparing FB_1 contamination in corn samples from different areas with different incidence of esophageal cancer, suggested a possible link between FB_1 exposure and esophageal cancer risk.

2.2.1.4 Biomarkers of FB_1 exposure

Although FB_1 contamination in corn or corn products have been suggested in correlation with high rates of esophageal cancer in several populations, the direct evidence of FB_1 intake and esophageal cancer risk is inadequate, largely due to fewer validated FN biomarkers available for epidemiological study and for quantitative evaluation of human exposure and disease risk.

As described previously, inhibition of *de novo* sphingolipid biosynthesis and disruption of sphingolipid metabolism are known biological effects of FB_1, therefore, sphingolipid metabolites, such as Sa, So, and the Sa/So ratio were proposed as biomarkers for FNs exposure [9]. Application of these sphingolipid as exposure biomarker has been supported by evidence from animal studies. Elevated level of Sa and Sa/So ratio in serum and urine were found in different animal species treated with FNs-contaminated food or FB_1, including vervet monkey, horse, pig, rat, mouse, trout, rabbit, duck and chicken, and occurred in a dose-dependent manner [9]. Nevertheless, application of these biomarkers was failed in human studies in finding their correlation with FN exposure or disease risks [70-73].

Based on the absence of measurable metabolites of FB_1, FB_1 itself was proposed as a biomarker and analytical methods were developed [9]. Although animal studies indicated

a rapid elimination of orally dosed FB_1 from circulation as described previously, which hinders the interest in measurement of free FB_1 in biological fluids as biomarkers of exposure, efforts have been made on monitoring free FB_1 in human urine [74], plasma [75], feces [76] and hair [77] in recent years. Available data from these studies suggested feasibility of using free FB_1 as a human exposure biomarker. Our previous study in Fisher 344 rats established a dose-response correlation between FB_1 dosing and free FB_1 excreted in urine and feces [54]. A recent study in three groups of Mexican female subjects who had different levels of Tortilla consumption found that subjects with high Tortilla consumption had a 3-fold higher mean urinary FB_1 level compared to low Tortilla consumption subjects [78]. Our previous study investigated food contamination with FB_1 as well as urinary free FB_1 as exposure biomarkers in two Chinese populations from Huaian and Fusui, high risk areas of esophageal cancer and primary liver cancer, respectively [79]. Consistent with estimated daily intake level of FB_1, urinary free FB_1 level was significantly higher in Huaian subjects than those in Fusui subject, suggesting urinary FB_1 as a potential biomarker to assess FN exposure in human populations.

2.2.2 Smoking and drinking

Alcohol drinking and tobacco smoking, alone or in combination, are associated with an increased risk of esophageal cancer, including both ESCC and EAC. They have long been known as the major risk factors of ESCC in Western countries. In 1970s and 1980s, a serial of ecologic and case-control studies conducted in North America and Western Europe have established a strong association between alcohol consumption, as well as tobacco smoking and esophageal cancer [80-86]. A case-control study of esophageal

cancer in Africa American found a relative risk of 6.4 associated with use of alcoholic beverages, and a relative risk of 1.5 associated with cigarette smoking after adjustment for ethanol consumption [80]. A prospective study in 474,606 US participants found hazard ratios of 9.27 in current smokers and 4.93 in alcohol drinkers of more than three alcoholic beverages per day [81]. And ever smoking was estimated to attribute 77% for ESCC risk in this US population. An early investigation estimated that alcohol drinking was responsible for 75% of esophageal cancer incidence in the United States [82]. A serial of ecologic and case-control studies conducted in France population by Tuyns and colleagues reported that alcohol drinking and cigarette smoking interacted to increase esophageal cancer risk in a multiplicative manner [83-85]. In a case-control study in US population, the odds ratio for combined use of alcohol and tobacco was 35.4 in white males and 149.2 in black males, which were higher than the odds ratios in those exposed to single factor [86]. Meanwhile, alcohol consumption and/or tobacco smoking were estimated to account for 93% of ESCC in blacks and 86% in whites. In population with overall heavy alcohol consumption, the risk of ESCC increased more strongly with the alcohol consumption than with tobacco use [87]: the relative risk was higher in subjects with the highest level of alcohol consumption than those with the highest level of smoking. Furthermore, an obvious increased risk for ESCC was observed with increased amount of alcohol consumption, while no consistent correlation between duration of alcohol use or starting age of alcohol use and ESCC risk. On the contrary, both duration and daily dose of cigarette smoking are major determinants of ESCC risk associated with tobacco use [87].

Although alcohol drinking and tobacco smoking are definitely involved in the

etiology of ESCC in Western countries, the evidence is less conclusive and to a less extent in ESCC development in the high incidence areas of Asia and South Africa. In Linxian high-risk population, China, only 15% of ESCC cases were attributed to smoking while alcohol drinking rate did not differ significantly between cases and controls [88, 89]. A cohort study following up 29,584 adult in Linxian for 15 years found a weak association between smoking and ESCC with relative risks of 1.33 (95% CI, 1.15–1.53) and 1.32 (95% CI, 1.15–1.51) for ever and current smokers [27]. They failed to detect significant association of ESCC with alcohol drinking, with a relative risk of 0.92 (95% CI, 0.82–1.03). In northeastern Iran high risk area, no much difference was observed in smoking status between cases and controls, and alcohol drinking was rare in this population [90].

2.2.3 Dietary factors and behavior

Dietary factors also play a causal role in ESCC carcinogenesis. Consumption of salt-pickled, salt-cured and moldy foods has been widely suspected as risk factors of ESCC [91]. Pickled vegetables have been a type of popular traditional food in some areas in China. They were prepared by placing tightly packed moist vegetables in a large ceramic container. The vegetables were then fermented in water for several months and often stored for more than one year [91]. During the storage period, nitrite formed by bacteria from nitrate in water or in the vegetables can form nitrosamines with amines in the vegetables. In additional, molds usually found growing on the surface of the pickles, not only produce mycotoxins but also facilitate formation of N-nitroso compounds. Besides, salt-cured and moldy foods are often found contaminated by N-nitrosamines and/or

fungal toxins [92]. N-nitrosamines and other nitroso compounds are proven to induce ESCC in animal studies [93]. Their potential role in development of ESCC in human was further confirmed in high-risk areas, especially in China. N-nitrosamines and their precursors were found in significant higher level in water and food samples in Linxian County, compared to an ESCC low-risk area in China [91]. The total N-nitroso compounds daily intake and its urinary excretion level were significantly higher in subjects from Nan'ao County, China, another ESCC high-risk area, in comparison with a low-risk area [94]. Several nitrosamines, including *N*-nitrosomethylbenzylamine (NMBA), have been isolated in the diets and gastric juice collected from studied subjects in Linxian County [91, 95]. O^6-methylguanine has been detected in DNA of normal esophageal tissue from ESCC patients in China, which provide substantial evidence that methylating nitrosamines may play an important role in ESCC development [96].

Besides toxicants in diet, antioxidants in food may protect damage caused by environmental risk factors, resulting in protective effect in cancer development. The dietary protective factors that have been studied in ESCC development include fruits, vegetables, meat and soy [97-100]. The antioxidant vitamins, including β-carotene, vitamin A, vitamin C and vitamin E, have been evaluated for their protective role for ESCC development in both epidemiological investigation and chemoprevention studies, especially in high-risk populations (reviewed in [101]). Although not totally consistent, most of the studies found a weak decreased risk of esophageal cancer associated with intake of β-carotene, vitamin C and vitamin E [101]. And these protective factors act most likely in combination with each and other micronutrients [101]. For vitamin A, although several studies indicated its protective effect in esophageal cancer risk, the role

of vitamin A remains unclear because of conflict findings [101].

Trace element deficiencies were also linked to the ESCC development, especially in high-risk areas. An early study in Henan Province, China, indicated a correlation between esophageal cancer mortality and deficiencies of multiply trace elements, such as molybdenum, zinc, manganese, magnesium, silicon, nickel, iron, and iodine [91]. Among these elements, molybdenum received more attention. Its low intake as a contributing factor of esophageal cancer has been proved by follow-up studies in this area and other populations [102-104]. Zinc is another element of research interest. Its deficiency increased nitrosamine-induced esophageal cancer rate in rats and has been proved in human [102, 105]. An investigation in South Africa reported an association of low blood selenium level with esophageal cancer [106], which was further confirmed by studies in Linxian County, China [104, 107].

Recurrent thermal injury from consumption of hot foods and drinks has long been suspected to be a risk factor of esophageal cancer. However, it is still unclear whether this association exists. Epidemiologic studies between 1974 and 2008 on association of high-temperature food or drink were reviewed recently by Uslami and colleagues [108], showing little consistency among studies and most of the supporting evidence comes from case-control studies. For instance, in the 18 case-control studies investigating the association between high temperature food and ESCC risk, 12 reported an increased risk of ESCC, while 2 studies reported a decreased risk, and 4 found no statistical significant association. No association was found in the prospective study. Therefore, although increased risk of ESCC associated with higher food/drink temperature, the evidence was weak and limited. This conclusion is consistent with the declaration by a working group

of IARC in 1991, which stated that there was no sufficient evidence to recognize tea, coffee or mat é, as risk factors of human cancer, and drinking hot mat é was a probable risk factor in humans [109].

2.2.5 Oxidative stress

Reactive oxygen species (ROS) are produced endogenously as part of the physiological processes and metabolic reactions, as well as exogenously by environmental factors, such as UV radiation, tobacco smoking, and other carcinogens [110]. Under normal conditions, there is a balance between oxidants and antioxidant defenses. Disturbances of this balance can cause toxic effects by damage of components of the cell, including proteins, lipids, and DNA.

Hydroxyl radical is the most important oxygen-free radical that can cause damage to bio-molecular (proteins, DNA, lipids). It attacks DNA strands and forms a variety of oxidation products. Among them, the 8-hydroxy-2'-deoxyguanosine (8-OHdG) is the most abundant DNA lesion which is widely accepted as the most common marker of oxidative DNA damage [10]. Through keto-enol tautomerism, 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodG) is formed from 8-OHdG. Therefore, in literature, the 8-OHdG and 8-oxodG are usually used for the same compound.

The mutagenic potential of 8-OHdG has been supported by experiments showing loss of base-pairing specificity, misreading of adjacent pyrimidine, or insertion of adenine opposite the lesions induced by 8-OHdG [111]. Also, the oxidized guanosine can yield G:C→T:A transversions in bacteria and human cells, further proving its mutagenic potential [112]. Numerous studies have analyzed 8-OHdG level in human biological

samples in relation to oxidative stress, diet, cancer risk, and aging. As a result of these studies, 8-OHdG has been established as an important biomarker of oxidative stress, as well as a biomarker of the effect of lifestyle and diet, aging process by the free radical theory, and cancer risks (reviewed in [10]).

The major long-term effect of extensive oxidative stress is damage on DNA, which serve as an important mechanism in carcinogenesis by initiating cancer and/or contributing to a cancer prone microenvironment [113]. Alternation of 8-OHdG level has been related to multiply occupational and environmental carcinogen exposures, such as smoking, benzene, asbestos, silica and polycyclic aromatic hydrocarbons (PAHs) (reviewed in [11]). Elevation of 8-OHdG levels in relation to tobacco smoking was most studied. One obvious example is the study conducted by Kiyosawa and colleagues, who reported significantly elevated 8-OHdG levels in peripheral leukocytes of health humans after smoking [114] . Statistically significant higher levels of 8-OHdG was also observed in current smokers compared to non-smokers [115]. Besides, a previous study found that incubation with FB_1 resulted in elevation of 8-OHdG in rat C6 glioma cells [12].

Although the relationship between 8-OHdG level and carcinogen exposures is biological convincible, human epidemiological studies in relation of 8-OHdG with cancer risk were limited and showed inconsistent results [116-119]. For instance, the analysis of 8-OHdG in liver tissue from chronic HCV carriers showed that 8-OHdG level was associated with increased HCC risk with a relative risk of 2.48 ($p=0.023$), and the 8-OHdG level was correlated with severity of hepatic inflammation [116]. In a study conducted by Thanan and colleagues, urinary 8-oxodG level was about 2-fold higher in cholangiocarcinoma patients than in health controls [117]. A recent chemoprevention

study found that urinary 8-OHdG level is reduced after intake of green tea polyphenols (GTP), a naturally occurring antioxidant, in south Guangxi HCC high risk area [118]. Opposite evidence also exists. For instance, in a HCC case-control study in Taiwan, although not statistically significant, higher 8-oxodG levels was found associated with decreased risk of HCC [119].

Regarding esophageal cancer, although data is limited, current evidence supported a possible role of 8-OHdG level in esophageal cancer risk [120-122]. An analysis of 8-OHdG level in esophagus samples revealed that, compared to normal tissue, 8-OHdG level was significantly increased in the distal esophagus in both Barrett's epithelium and high-grade dysplasia, which were considered as precursor lesions of EAC [120]. In a study conducted by Breton and colleagues, 17 patients with cancers of the esophagus and cardia had a significantly higher levels of 8-oxodG in peripheral lymphocytes than controls [121]. Similarly, the study conducted by Diakowska and colleagues found a significantly higher serum 8-OHdG level in ESCC patients than healthy controls [122].

2.2.6 Individual susceptibility

It has been well accepted that genetic difference plays an important role in determining individual susceptibility to environmental carcinogens and developing of cancer as well. It has been well recognized that most cancer, including esophageal cancer, is the result of complex interaction of individual susceptibility and environmental factors. Genetic difference can lead to sensitivity (susceptibility) to carcinogen, which is involved in multiple steps in carcinogenesis, including carcinogen activation/detoxification, DNA damage and repair, microenvironment stability and other known or unknown

processes/factors.

2.2.6.1 Metabolic pathway

Xenobiotic-metabolizing enzymes are classified into two categories: phase I and phase II enzymes. Glutathione S-transferase (GST) is a family of enzymes involved in phase II metabolism which can interact with a variety of endogenous and exogenous substrates. At present, six isoenzyme classes of human GST have been identified – alpha (A), mu (M), pi (P), theta (T), Zeta (Z), and Omega (O). Previous studies suggested that mutations in GSTs can change individual susceptibility to a broad range of carcinogens and toxins as well as affect the toxicity and efficacy of certain drugs [123]. In the GST family, only GSTM1 and GSTT1 in mu and theta classes have gene deficiency (null genotype) with the consequence of reduced or no conjugation activity [124, 125]. Homozygous deletion of GSTM1 and GSTT1 has been associated with increased risk of certain cancers, including gastric and esophageal cancers [126-129], while conflicting result exists [130].

2.2.6.2 Inflammation pathway

Inflammation is a key event in cancer development. In the process of carcinogenesis, a serial of reactions caused by chronic inflammation, including persistent tissue damage, cell proliferation, and enrichment of reactive oxygen and nitrogen species, contribute to a cancer prone microenvironment [110]. Numerous factors act as mediators in the process of carcinogenesis in such a microenvironment with several key molecules participating in regulation of inflammation and cancer development [110].

Cytokines are secreted or membrane-bound molecules, including interleukins (IL), tumor necrosis factor-alpha (TNF-α), some growth factors, and differentiation factors (colony-stimulating factors), which mediate important cellular function in growth, differentiation, and activation of immune cells. Accumulating studies suggest that inflammatory cytokines facilitate cancer development [131]. Among them, TNF-α is a proinflammatory cytokine produced by activated macrophages. It locates in the upside part of inflammation interactive pathway by initiating an inflammatory cascade consisting of other inflammatory cytokines [110]. It is a critical mediator in host antiviral defense and a major inducer for Nuclear Factor-κB (NFκB) activation, which is highly linked to cancer development [132]. Besides, TNF-α can induce DNA damage, inhibit DNA repair, and even be produced by tumor cells to act as a tumor growth factor [133, 134]. Increased TNF-α expression after short-term exposure to FB_1 has been observed in rats and mice [135-137]. Furthermore, knockout of TNF-α inhibited FB_1-induced hepatotoxicity in mice suggested that TNF-α plays a role in mediating FB_1 toxicity [138, 139]. Recently, a case-control study conducted in North China high incidence area for esophageal cancer (both ESCC and EAC) found a possible relationship between TNF-α polymorphism and esophageal cancer risk [140].

NF-κB is a transcription factor acting as a critical mediator in inflammation progress. Its inactive form exists in the cytoplasm and is activated by inflammatory stimuli, including infectious agents (viruses and bacteria), necrotic cell products, and inflammatory cytokines. After activation, it translocates to the nucleus, where it binds to the promoter regions of its target genes and activate/promote expression of cell-cycle genes, apoptosis inhibitors and invasive proteases [110, 141, 142]. Recent studies showed

that NF-κB can regulate inflammative cell apoptosis and phagocytosis, which has been considered the most important mediator in inflammation and infection related tumorigenesis as its constitutive activation is commonly observed in several cancers [143, 144]. NF-κB signaling pathway has been found constitutively activated in the ESCC cell lines [145]. Also, overexpression of NF-κB was associated with aggressive pathologic features of esophageal cancer, resistance to chemoradiotherapy, and poor survival rate in human studies [146, 147]. Recently, NF-κB polymorphisms have been investigated in relation to chronic inflammation disease as well as cancer risk, and some achieved positive result [148-156].

Inductible Nitric Oxide Synthase (iNOS) is an enzyme catalyzing production of nitric oxide (NO), which is an important regulatory molecule in modulating inflammation response and carcinogenesis. iNOS was found to be overexpressed in chronic inflammatory disease and various types of cancers [110]. Because iNOS is induced by proinflammatory cytokines, such as TNF-α and IL-1β, and transactivated by NF-κB, it may act as a downstream effecter of cytokines and NF-κB in linking inflammation reaction to cancer [157]. In addition, NO produced by iNOS signaling can enhance the activity of cyclooxygenase -2 (COX-2), which is another promising link between inflammation and cancer [158]. Overexpression of iNOS has been detected in esophageal cancer patients and genetic polymorphisms of iNOS have been associated with increased risks of cancer, while the studies are limited [159-161].

COX-2 is an enzyme responsible for converting arachidonic acid to prostaglandins, which are key mediators of inflammation. COX-2 is undetectable in most normal tissues and inducible by a wide range of stimuli, such as TNF-α and iNOS [158]. The abundance

of COX-2 is found in activated macrophages and other inflammation cells. Overexpression of COX-2 was found in various types of cancer and involved in cellular proliferation, anti apoptosis, angiogenesis, and metastasis [110]. Instead of acting as driving force in carcinogenesis, COX-2 is suggested as enhancer in cancer development within the period of chronic inflammation process [110]. COX-2 overexpression has been observed in ESCC and found associated with deep invasion, tumor stage and poor survival [162, 163].

2.2.6.3 DNA repair genes

The DNA repair system is the major defense of the body against DNA damages and gene alterations caused by environmental exposure to carcinogens. Hereditary genetic defects in DNA repair system can lead to increased risk of developing cancer, which has been demonstrated in numbers of epidemiologic studies [164].

Base excision repair (BER) is the major mechanisms for removal of oxidative DNA damage. BER is a multi-step process involving four major enzymes (DNA glycosylase, apurinic/apyrimidinic endonuclease, DAN polymerase and ligase), in which 8-oxoguanine DNA glycosylase 1 (OGG1) initiates the cycle [165]. Several studies revealed a possible relationship between gene expression variations in human hOGG1 and reduced cellular survival [165, 166], while the role of functional hOGG1 Ser326Cys polymorphism in cancer development remain controversially [167-170].

Nucleotide excision repair (NER) is another important defense pathway against DNA damage. The xeroderma pigmentosum group D (XPD) gene encodes a helicase that act as a key component of the core transcription factor TFIIH. Point mutations of XPD play a

causative role in xeroderma pigmentosum, which is characterized by high ultraviolet-light hypersensitivity, high mutation frequency and cancer proneness [171]. Meta analysis suggested that XPD, the key gene, involved in the early steps of NER, was a cancer susceptibility gene regardless of environmental factors [172] and may associate with ESCC development [173].

HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC, also called hepatoma) is a primary malignancy of the liver. Originally, primary liver cancer was divided into carcinoma of hepatic cells and carcinoma of the bile duct, which were termed as hepatoma and cholangioma later on, respectively [174]. HCC is the most common primary liver cancer in adult, accounting for 70% to 85% of primary liver cancer cases [1]. It ranks fifth most common cancer in males and eighth in females in the world [1]. There are significant geographical variations in HCC prevalence, sex and age distribution, as well as etiology and risk factors. Despite the progress in early diagnosis and medical treatment, HCC prognosis is poor, and room for further improvement in survival rate is limited [175]. Therefore, identification and control of etiology factors associated with HCC development is the most practical strategy in HCC prevention.

2.3 Epidemiology of HCC

2.3.1 Geographic variation in incidence and mortality

Worldwide, a total of 711,000 new cases was estimated to occur according to 2007's global cancer statistics [1]. The geographical variation of cancer incidence has been noted over past decades. More than 80% of new cases are in developing countries, mostly in Asia and sub-Saharan Africa. Counties in East Asia (China, Mongolia, Korea and Japan), Southeastern Asia (Thailand, Singapore and Philippines), and sub-Saharan Africa (Gambia, Guinea, Mali and Mozambique), have age-standardized incidence over 20/100,000 population [25]. Several European countries (Italy, France and Switzerland) and Latin American countries have moderate risk with age-standardized incidence of 11–20/100,000 population [25, 176, 177]. Countries with intermediate risk (5–10/100,000 population) include Germany, Spain, Slovak and Croatia [25]. The incidence of HCC is low (less than 5/100,000 population) in the United States, Canada, Austria and the United Kingdom [25, 176]. However, there are still large areas in the world where the incidence data is incomplete or unknown [176].

The incidences of HCC also vary among different race/ethnicity in the same area. The typical example is the immigrant Chinese populations. In Los Angeles County of California, the age-standardized incidence of HCC was 17.2/100,000 in Chinese, higher than 9.0/100,000 in Black, 10.2/100,000 in Hispanic White, and 5.0/100,000 in non-Hispanic White [25].

HCC is one of the most fatal diseases. The estimated five-year relative survival rate was less than 11% even in developed countries [1, 178]. Due to poor prognosis, it is the 3rd most common cause of death from cancer with almost the same deaths (680,000 vs. 711,000) as new cases [1].

2.3.2 Gender and age distribution

Males are more prone to HCC than females, the male/female ratio being 2.4:1 worldwide [1]. In low incidence areas, the male predominance is less marked, with the ratio being 2:1. In high incidence areas, the ratio increases to 3: 1 or even higher [177].

In all populations, the incidence of HCC increases progressively with age, and the highest prevalence is among those with age over 65 years [176]. In high-incidence areas, there is a shift toward younger age groups, that is, the incidences of HCC peaks earlier than low-incidence areas [177]. HCC patients under 40 years of age are more frequent in the high-incidence populations than in the low-incidence populations. In the high-risk populations, incidence increases after 30 years of age and peaks at the age interval 50 to 60 years. Actually, HCC is not rare to be found at any age over 20 years, especially in the high risk populations [175].

2.3.3 Time trend in HCC incidence

Each population/country has its own unique pattern of changes in HCC incidence over the decades. Time trends of HCC incidence (1973–1997) showed different patterns among areas with different prevalence and between males and females [179]. For instance, Shanghai and Hong Kong Chinese male populations (high-risk areas) showed an increased trend of HCC incidence from 1973 to 1982 and 1973 to 1987, respectively, and then decreased. Varese (Italy) males (moderate-risk area) showed an increased trend continuously in the same period, while the HCC incidence in several other intermediate and low-risk areas in European kept stable among males. In the United State, increased time trends in both White and Black males were observed. Age-standardized incidence of

HCC in White males increased from 2.4/100,000 to 3.0/100,000 and 3.8/100,000 in 1983–1987, 1988–1992 and 1993–1997, respectively. In Black males, the incidence increased from 4.9/100,000 to 6.5/100,000 and 7.1/100,000 within the same intervals [25, 180, 181]. Females trend tended to follow the males' pattern, but more gradually [179]. The overall death rates of liver cancer in USA increased by 47.3% from 1990 to 2005 among males and by 27.1% from 1991 to 2005 among females [182]. In brief, in high-risk populations of developing countries, incidence of HCC decreased in both males and females, especially in recent decades. However, in Western countries where the incidence is relatively low, HCC incidence is rising or keeps stable. Other than the United States, the increase of HCC incidence has been observed in Italy, the United Kingdom, Canada, Japan and Australia [176]. The increased incidence in these areas has been considered variously attributed to prevalence of hepatitis C virus infection [183], and increased alcohol abuse [175]. Also, increase of hospitalization and mortality in immigrants from areas with high incidence may play a contributable role [184].

2.3.4 HCC in China and Southern Guangxi high risk area

China is one of the HCC high incidence countries. A total of 350,000 new liver cancer cases were diagnosed each year, accounting for more than 55% of the world's new cases (age-standardized incidence in 2007: 35.2/100,000 in males and 13.3/100,000 in females) [1]. It is the third most common cancer and the second major cause of cancer death in the Chinese population. According to national disease surveillance in 1991–2000, the average mortality of HCC in China was around 20/100,000 population, showing an ascendant time trend [185]. The incidence of HCC within China has

considerable geographical variation. The high-incidence areas in China are located along the country's southeastern coastline, in the provincial level areas of Jiangsu, Zhejiang, Fujian, Guangdong, and Guangxi [17].

In Guangxi Zhuang Autonomous Region, mortality of HCC ranked the first among all malignant tumors in both males and females, accounting for 30.7% of all cancer death in 2004–2005 [13]. Crude mortality of HCC was 34.4/100,000 population, with mortality of 55.3/100,000 in males and 13.2/100,000 in females. The crude mortality in 2004–2005 was higher than the crude mortality in 1990–1992 (27.3/100,000 in all, 41.8/100,000 in males, 11.7/100,000 in females), while age-standardized mortalities were comparable between years of 1990–1992 and 2004–2005 (24.4/100,000 in 1990–1992 and 22.2/100,000 in 2004–2005). The male to female ratio of mortality was 4.19:1. In 2004–2005, age-specific mortality rose significantly from 30 years old and peaked at 75 years old, which was later about 10 years than that in 1990s.

In Guangxi Region, geographical variation was also observed with a higher incidence in the south Guangxi area compared to the north Guangxi area. Fusui County within the south Guangxi area is a traditional HCC endemic area that has been monitored for a long period. According to surveillance result in 1974–2003 in Fusui County, a total of 5968 HCC cases were diagnosed within this period, accounting for 61.5% of all diagnosed malignant tumors [186]. The average incidence rates were 53.3/100,000 (age-standardized incidence: 54.3/100,000), 85.9/100,000 (age-standardized incidence: 87.3/100,000), and 18.3/100,000 (age-standardized incidence: 18.6/100,000) in whole population, males, and females, respectively. Incidence rates increased sharply from 25 years of age in females and 20 years of age in males, peaked around 45 years old, and

then declined slowly. A shift of incidence toward the age group older than 45 years was observed in recent years (1994–2003). The male to female ratio was 4.7:1. The age-standardized incidence of HCC decreased by 13.3% from 72.5/100,000 population in 1974 to 62.8/100,000 population in 2003, showing a downward trend.

2.4 Etiology and risk factors of HCC

The major known risk factors of HCC are chronic infection of hepatitis B virus (HBV) and hepatitis C virus (HCV), and consumption of aflatoxin-contaminated food. Generally, in developing countries, 59% of liver cancers are attributable to HBV and 33% are attributable to HCV [1]. In developed countries, 23% of liver cancers are attributable to HBV while 20% are attributable to HCV [1]. Aflatoxin contamination in food is mostly found in the high-risk areas in China and sub-Saharan Africa. Excessive alcohol drinking is also an important risk factor of HCC worldwide, especially in developed countries in Europe, North America, and Oceania [177]. Tobacco smoking has been considered as a potential risk factor of HCC in several studies while the results were inconclusive [177]. Accumulative evidence in recent years shows that obesity, as well as diabetes mellitus, are possible risk factors of HCC, which are independent of viral infection, alcohol abuse or other known risk factors [1].

2.4.1 Dietary aflatoxin exposure

Aflatoxins (AFs) are a group of closely related difuranocoumarin compounds (Figure 2.3) produced by the common fungi *Aspergillus flavus* and *Aspergillus parasiticus*, which frequently contaminate agricultural commodities, including corn, peanuts, cottonseed and

other oilseeds [187]. AFs were first discovered in peanut meal that were responsible for the deaths of over 1 million turkeys in England in 1961, known as “turkey X disease” [188]. Since then, AFs contamination in food has been reported worldwide and considered as a severe public health issue, especially in developing countries where long-term food storage in warm and moist conditions promote mold growth. Causal relationship between dietary AFs exposure and HCC development has been well demonstrated in both animal studies and epidemiological studies. In 1993, IARC announced that there was sufficient evidence to classify AFB_1 and mixture of AFs as Group 1 human carcinogen [14].

The structures of AFs were first identified in early 1960s [188]. Since then, there are a total of 18 AFs identified, while six of them studied most and found frequently, as shown in Figure 2.3 [14]. AFB_1 and AFB_2 are naturally occurring AFs, which are identified in physicochemical assays as intensely blue fluorescent compounds under long-wave ultraviolet light. AFG_1 and AFG_2 are naturally occurring AFs that are identified in physicochemical assays as yellow-green fluorescent compounds. AFB_1 and AFB_2 are produced by both *A. flavus* and *A. parasiticus*, while AFG_1 and AFG_2 are produced exclusively by *A. parasiticus*. AFM_1 and AFM_2 are the major metabolites of AFB_1 and AFB_2, respectively, which were originally discovered in milk of animals which consumed feed contaminated with AFs.

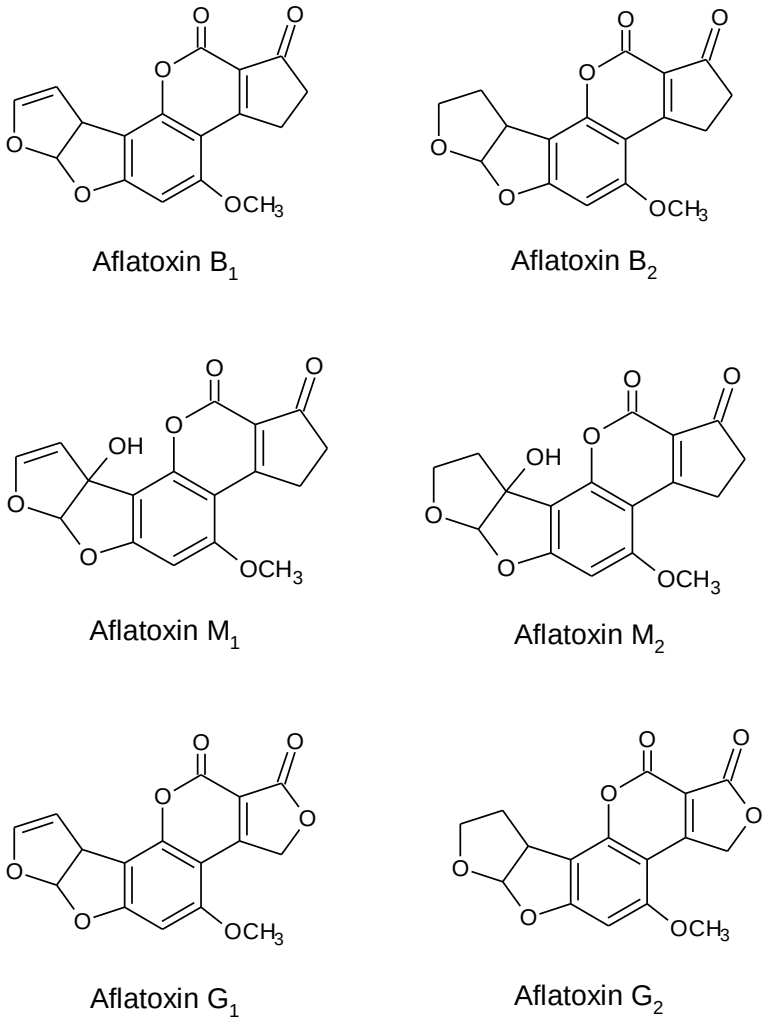

Figure 2.3 Structure of common aflatoxins

2.4.1.1 Metabolism of AFB_1

AFB_1 is the predominant and most toxic form of naturally occurring AFs, but it is not carcinogenic by itself without phase I metabolic activation via cytochromes P450 (CYP 450) to form AFB_1-8,9-epoxide to exert its DNA binding activity and hepatocarcinogenic effect (Figure 2.4) [187]. The major CYP enzymes involved in phase I metabolism of AFB_1 in human include CYP 1A2, 2A6 and 3A4 [189, 190]. CYP450 3A4 appears to be the dominant CYP450 involved in 8, 9-epoxide formation at all AFB_1 concentrations

[189]. CYP450 1A2 has a high affinity to AFB_1, while it is less expressed in human liver than 3A4 [189]. Besides, CYP450 enzymes can oxidize AFB_1 to other deactivated products expressing less mutagenic, carcinogenic and toxic effect than AFB_1: CYP450 1A2 can readily oxidize AFB_1 to AFQ_1 and AFM_1, 3A4 can oxidize AFB_1 to AFQ_1 [189, 190].

The 8,9-epoxide of AFB_1 is short-lived but highly reactive and can bind to cellular DNA, RNA, and protein [189]. The extent of covalent binding of AFB_1-8, 9-epoxide to DNA (AFB_1-DNA adduct) is highly correlated to the carcinogenic effect of AFB_1, which has been demonstrated in both animal and human studies [187, 191]. The primary AFB_1-DNA adducts formed in liver is 8, 9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB_1 (AFB_1-N^7-Gua) adducts, which is unstable and can decompose to a more stable form rapidly. The major decomposed derivative is imadozole ring-opened AFB_1 formamido-pyrimidine adducts (AFB_1-N^7-FAPY), which is the most stable form of all AFB_1-DNA adducts and is relatively resistant to DNA repair process [192]. In humans, the reactive 8, 9-epoxide of AFB_1 can undergo phase II transformation through conjugation mediated by phase II enzymes, including GSTs, α-glucuronidase, and sulfate transferase. GST-mediated conjugation is the critical pathway for AFB_1 detoxification, which yield stable, non-toxic AFB_1-epoxide-GSH-conjugates that is excreted in the bile [193] (shown in Figure 2.4). The GSH conjugates can also undergo sequential metabolism to be excreted as mercapturic acid (AFB_1-N-acetylcysteine) in the urine [18]. Besides, the 8, 9-epoxides of AFB_1 can hydrolyze rapidly through a non-enzymatic process to 8, 9-dihydrodiol and then in turn to dialdehydes, which yields albumin adduct with primary amine groups such as lysine [194]. Ultimately, the amount of genotoxic AFB_1-DNA adducts is determined

by the proportion of AFB_1 activated to AFB_1-8, 9-epoxide and AFB_1-8, 9-epoxide detoxified mainly through GST-mediated conjugation, with the whole process controlled by phase I and II enzyme involvement [194].

There are significant variations in response to AFB_1 between animal species, which may be material to the proportion of AFB_1 metabolized to the 8,9-epoxide, while the pharmacokinetics are still not well known [189]. All species of animals are susceptible to AFs, but the susceptibility varies depending on the species, age, gender and the nutritional status of the animals [195]. Rainbow trout is generally considered the most sensitive species to carcinogenicity of AFs [196]. Also, rat is extremely sensitive whereas mouse appears highly resistant to tumor formation induced by AFs except under conditions of liver injury through HBV infection [194]. The species variation in sensitivity to AFs is paralleled by differences in activity of aflatoxin metabolizing enzymes. In mouse, constitutive hepatic expression of an GST isoform (mGSTA3-3) with high affinity for AFB_1-8, 9-epoxide was observed, whereas rat does not constitutively express the GST isoform with high epoxide conjugating activity [194]. Cross-species comparison indicated that human and rat may have similar level of sensitivity to AFs-induced carcinogenic effect partly due to the limited GST-mediated conjugation activity in both human and rat [193, 197, 198].

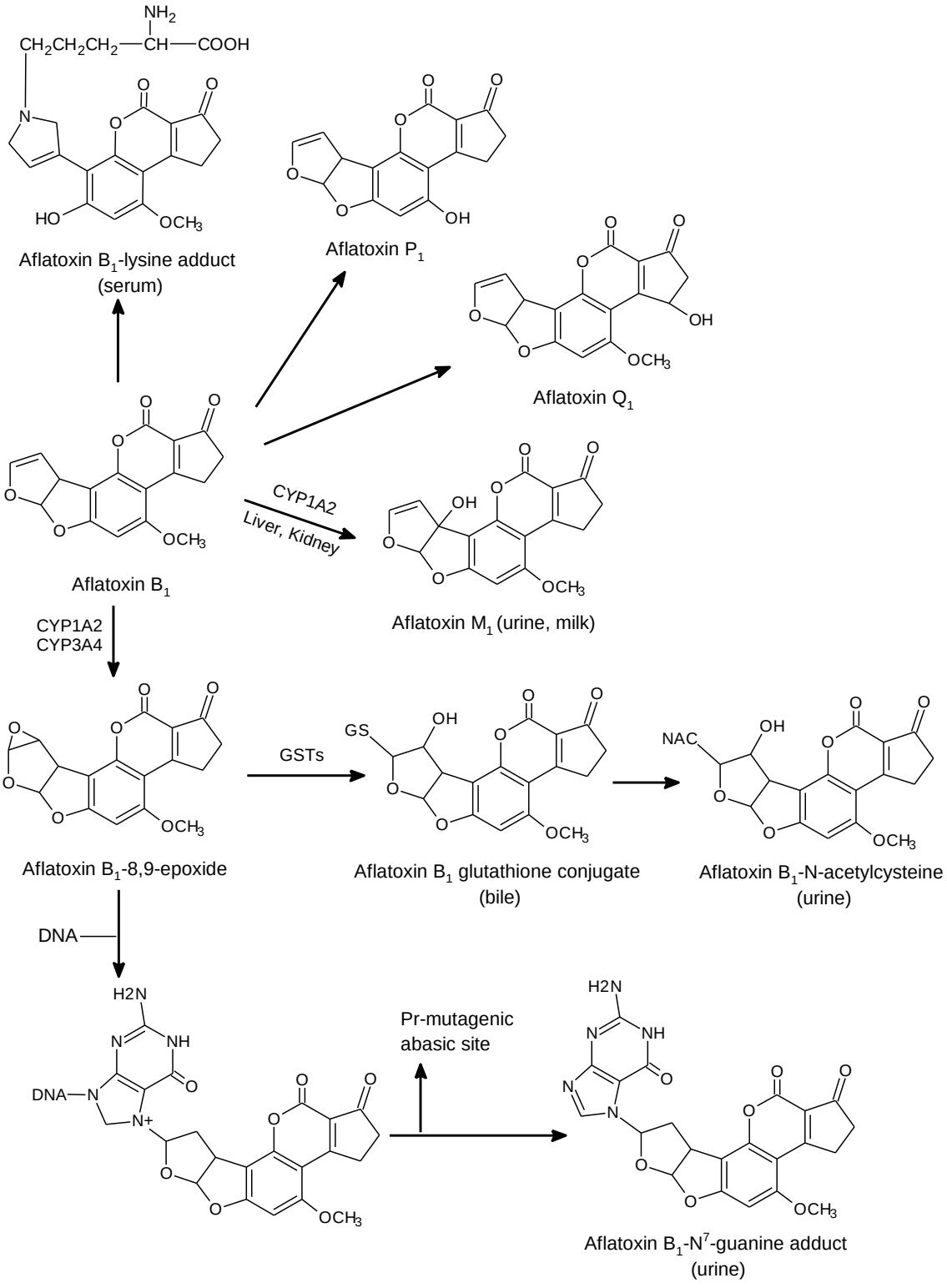

Figure 2.4 Metabolic pathway of aflatoxin B_1

(modified from figure in a review by Kensler et al. [18])

2.4.1.2 Occurrence of AFB_1

AFs are produced by *A. flavus* and *A. parasiticus* fungi when the temperatures are between 24 and 35 ℃ in crops prior to harvest [199]. The content of AFs will increase within crops and agriculture commodities during storage and food processing whenever the moisture content exceeds 7%. Extensive reviews of AFs contamination levels in agricultural commodities in the world have been provided in IARC monograph in 1993 and 2002 [194, 200]. As reviewed, AFs are more frequently found within the tropics zone in the world than within the temperate zone. However, due to the effect of global warming, the fungal invasion and AFs contamination in crops outside the tropics zone have been observed frequently in recent years [199]. Also, globalization makes AFs detected in agricultural commodities all over the world. Contamination in foods used as staples such as corn is a severe problem, especially in the developing countries in Asia and Africa. For instance, investigation of AFs in peanuts imported into Japan (1994–2000) showed that 355 (6.9%) of 5108 samples were contaminated with AFB_1 and 145 (2.8%) samples contained AFs over 10 μg/kg [201]. With regard to AFs contamination in staples in other Asian countries, relatively higher detection frequencies and high levels of AFs have been found in various staples. Investigation of AFs contamination in corn and flour in China, India, Indonesia, Philippines and Thailand, found that 1263 (49.7%) of 2541 samples were contaminated with AFB_1 at levels ranging from 0.11 to 4030 μg/kg [194]. In peanut products, oil and butter in local markets of China, Malaysia and Philippines, 235 (39.6%) of 594 samples were contaminated with AFB_1 at levels of 1–244 μg/kg [202-204]. In consistent with these findings, our previous study in Fusui County indicated that AFB_1 was detected in 23 (76.7%) of 30 ground corn samples

(range, 0.4–128.1 μg/kg), 20 (66.7%) of 30 cooking peanut oil samples (range, 0.1–52.5 μg/kg) and 7 (23.3%) of 30 rice samples (range, 0.3–20 μg/kg) [15].

2.4.1.3 Health effect of AFB_1

Aflatoxicosis is the poisoning caused by consumption of AFs contaminated food. There are two forms of aflatoxicosis, acute severe intoxication and chronic subsymptomatic intoxication [199]. Consequences of acute aflatoxicosis include direct liver damage and subsequent illness, characterized by jaundice, rapidly developing ascites, portal hypertension, and even death. Chronic subsymptomatic aflatoxicosis results in immunologic suppression and nutritional deficit [199].

Acute lethal aflatoxicosis in human has been reported in developing countries sporadically. In 1988, 13 Chinese children were reported died of acute hepatic encephalopathy in Malaysia [205]. Later on, the deaths were associated with consumption of AFs contaminated rice noodles shortly before the outbreak [206]. More recently, outbreak of acute aflatoxicosis in Kenya in 2004 was one of the most severe aflatoxicosis in history, causing more than 125 human deaths with a case fatality rate of 39% [207, 208]. This outbreak was linked to inadequately stored homegrown corn infected with *A. flavus* fungi [209].

It has been well established that AFs feed reduced growth rate and weight gain in animal studies [199]. this effect of AFs on productivity has been confirmed in human studies recently, in which a dose-response relationship between AFB_1 exposure level and the degree of stunting and underweight was observed in children less than 5 years old in Benin and Togo, where AFB_1-albumin adducts were detectable in 99% of the studied

children [210]. Another study found an association between dietary AFs exposure and prevalence of malnutrition status in children of 3–36 months in Kenya [211]. Children suffering from protein malnutrition (kwashiorkor) in developing countries may also be associated with AFs exposure. In a study conducted in South Africa, compared to aflatoxin-negative children, the children with detectable aflatoxin in both serum and urine showed a significantly lower haemoglobin level, a longer duration of oedema, an increased number of infections, and a longer duration of hospital stay [212], which confirmed the earlier studies that suggested AFs exposure as a factor modulating the recovery rate from kwashiorkor and resulting in increased risk of frequent infections [213, 214]. Although related human data is limited, dietary AFB_1 exposure has been linked to multiple nutritional statuses. Our previous study in 507 Ghanaian adults established inverse correlations between serum AFB1-albumin adducts level and both vitamin A ($r = -0.232$, $p = 0.003$) and E ($r = -0.178$, $p = 0.023$) [215]. In a Chinese population, the inverse association between plasma selenium concentrations and AFB1-albumin adducts was statistically significant [216].

Immunosuppressive effects of AFs have been widely studied in animals and extended reviewed by IARC [194, 200]. In general, exposure to AFs-contaminated food results mainly in suppression of cell-mediated immunity in different animal species, as well as other possible effects, including impairment of humoral immune functions, decrease of antibody formation, and modification of cytokine expression [194, 199, 200]. Although extension of these animal results to human is uncertain, it was suggested that these animal responses are relevant to humans, while the dose-response relationship is not known [199]. Our previous study (unpublished data) detected significant suppression of

select cellular immune components, including CD8+ T cells, CD8+CD45RA+CD27- T cells, and CD3-CD56+ NK cells in Ghanaian adults with high AFB_1-albumin adducts level. In a earlier study in Gambian children, secretory immunoglobulin A (IgA) was significantly lower in subjects with detectable AFB_1-albumin adducts comparing to those without detectable AFB_1-albumin adducts in serum [217]. Although progress has been made, evidence in direct linkage between AFB_1 exposure and suppression of immune functions in humans is limited and inconsistent [199].

The possible role of chronic dietary AFs exposure in human health effect has been researched mostly in its role as a carcinogen related to liver cancer development. Beginning from 1960s, a large number of epidemiological studies have been carried out to study the possible association between dietary AFs exposure and risk of primary liver cancer [200]. Most of these studies were conducted in high-risk populations in the developing countries of sub-Saharan Africa and Asia. One of the pioneer research published in 1971 is the cross-sectional study in Uganda by Alpert and his colleagues, in which the highest incidence of HCC occurred in areas with highest level of aflatoxin contamination in food [218]. Later on, numerous cross-sectional studies and case-control studies have been conducted in different populations. In 1980s and 1990s, several well organized cohort populations have been established in Shanghai (China) [219, 220], Qidong (China) [221, 222], and Taiwan (China) [20, 223-225]. In the Shanghai cohort recruiting 18,244 male residents of 45–64 years old, 55 were diagnosed with primary liver cancer with followup to 1992 [219]. A nested case-control study was conducted using 50 of the cases and 267 matched controls. Urinary AFs biomarkers, including AFB_1, AFP_1, AFM_1, and AFB_1-N^7-guanine adducts, and HBsAg status, were analyzed in

study subjects. Presence of any of these urinary AFs biomarkers was significantly associated with increased risk of HCC. Furthermore, a supra-multiplicative combinative effect of AFs exposure and HBV infection on HCC was observed: compared with individuals having no detectable urinary AFs biomarkers and HBsAg-negative, adjusted relative risks were 3.4 (95% CI, 1.1-10), 7.3 (95% CI, 2.2-2.4), and 59 (95% CI, 17-212) in individuals positive for urinary AFs biomarkers only, HBsAg only, and both factors, respectively. In a cohort study enrolling 25,618 subjects from Taiwan, China, 79 HBsAg-positive HCC cases were identified in 1991–1997 and matched to 149 HBsAg-positive controls. A significantly increased risk for HCC was observed in subjects with detectable AFB_1-albumin adducts (OR=2.0, 95% CI, 1.1–3.7) [223]. A nested case-control study of an extended follow-up of this Taiwan cohort analyzed AFB_1-albumin adducts in serum samples (174 cases and 832 controls) and AFB_1 metabolites (AFB_1, AFP_1, AFM_1 and AFG_1) in urine samples (142 cases and 684 controls). HBsAg-positive subjects with both high-than-median AFB_1-albumin adducts and urinary AFB_1 metabolite levels had a significantly increased HCC risk (OR=17.3, 95% CI, 7.0–42.8), which was higher than HBsAg-positive subjects regardless AFB_1 status (OR=7.5, 95% CI, 5.1–10.9) [20]. These studies, regardless of study design (cross-sectional study, case-control study, cohort study) and assessment method of AFs exposure (estimated AFs consumption via dietary questionnaires and AFs level in food, AFs exposure biomarker measurements), provided consistent results and thereby established a substantial causal relationship between AFs exposure and HCC development in a dose-response manner. In an extended review by IARC in 1993 and re-evaluated in 2002 [194, 200], AFB_1 and mixture of AFs were classified as known human carcinogens causing primary liver cancer.

2.4.1.4 Biomarkers of AFB_1

It is inaccurate to assess AFs exposure at the individual level either by dietary questionnaire or by direct measurement of AFs in foods. Therefore, AFs biomarkers were developed and increasingly used to assess AFs exposure in epidemiology study. As mentioned previously, AFB_1 undergo different metabolic pathway to form various AFs derivatives that exists in circulatory system or excreted via bile or urine. Measurement methods of these AFs derivatives in biological fluids haven been developed, including AFB_1 metabolites in the urine, such as AFB_1, AFP_1, AFM_1, AFB_1-mercapturic acid and AFB_1-N^7-guanine adducts, and AFB_1-albumin adducts in the blood. Validation and application of these biomarkers in both animal models and human epidemiological studies has been performed over past 20 years and dose-response relationships have been well established [226, 227]. These biomarkers result from different biochemical processes in the body and thereby have different characteristic half-lives. Urinary AFM_1 and other products of detoxification process reflect recent exposure to AFs, usually within days. Although associations between these short-term biomarkers and HCC risk have been found, as described previously [20, 219, 220], large variation has been observed from day to day, that may due to changes in intake of AFs contaminated food and collection time of biological samples [19]. Therefore, using of these urinary AFs biomarker on a single day may not be a reliable indicator of chronic exposure of AFs in assessment of HCC risk. The AFB_1-albumin adducts measured in the blood (serum or plasma) have a half-life of 30 to 60 days. It reflects AFB_1 accumulating exposure status over a period of 2 to 3 months and hence has been considered as the most reliable biomarker of chronic exposure applied in human studies [18-20].

2.4.2 Hepatitis B virus

The association between chronic infection of hepatitis B virus (HBV) and the development of HCC has been supported by substantial evidence, including epidemiological studies, molecular studies of tissues and cell line, and animal models. In 1994, HBV has been listed as a known human carcinogen (Group I) by IARC [228]. Although the exact mechanism(s) remains largely unknown, progress has been made in HBV-induce hepatocarcinogenesis. Since the HBV-induce hepatocarcinogenesis involves long-term interplay between HBV and host hepatocytes, it can be argued that HBV genetic variations (genotypes, mutations) resulting in persistence or severity of HBV infection might predispose to HCC development.

2.4.2.1 Virology of human HBV

Human HBV is a small hepatotropic enveloped DNA virus belongs to the *Hepadnaviridae* family [229]. There are three distinct morphological forms [230]. The most abundant is the small, spherical, noninfectious particle, measuring 17 to 25 nm in diameter. Tubular, filamentous form of various lengths is also observed. The third is the 42 nm Dane particle (virion) which consists of an outer lipid member envelope studded with envelope proteins, a hollow-shell nucleocapsid core formed of capsid proteins (HBcAg), and enclosed DNA genome and reverse transcriptase enzyme [231]. The envelope proteins are large (L), middle (M), and small (S) surface proteins (also called pre-S1, pre-S2 and HBsAg) that are involved in searching and binding of host hepatic cell.

HBV DNA is a circular partially double-stranded molecule with approximately 3,200

nucleotides (nt). It is composed of one complete DNA strand (minus strand) with fixed length that is held in a big loose circle by a particle second strand (plus strand) with variable length [177]. Minus strand DNA is the template for the synthesis of the viral mRNA transcripts [230]. The circular genome is very compact, with four partially overlapping open reading frames (ORFs) known as gene S, C, P and X, which encode the envelope (Pre-surface 1, pre-surface 2, surface), core (core, pro-core), polymerase and X protein, respectively. Additionally, there are two enhancers (EnhI and EnhII) that regulate transcription of HBV promoters, including basic core promoter (BCP) that controls the transcription of the pre-core and core regions [232].

2.4.2.2 Natural history of HBV infection

Human HBV is transmitted by intimate person-to-person contact or direct contact with infectious blood or blood-derived body fluids [1]. The transmission mode differs between areas as well as age at which the transmission takes place. The person transmitting HBV may have an acute infection or be a carrier, which is defined as a person who is seropositive for HBV surface antigen (HBsAg) on at least two occasions six months apart [228]. The natural history and clinical manifestations of HBV infection are variable [228]. The lag between exposure to HBV and onset of B hepatitis is 2-26 weeks, and the clinical expression of this infection is heterogeneous. Subclinical episodes of acute hepatitis are common. Chronic HBV infection may follow acute symptomatic or asymptomatic infection but is more frequent after asymptomatic infection.

Age at infection is the major determinant of chronic infection [228]. Perinatal transmission confers the highest probability of chronic infection, with around 80% to

100% of infected children developing chronic infection. In children aged 1–10, the risk is 20-40% and appears to decline dramatically in adolescence and adult with the probability of chronic infection in the range of 0–10%.

2.4.2.3 Epidemiology of HBV infection and HCC development

According to data from World Health Organization (WHO), approximately 350 million people are chronically infected with HBV [233]. Hepatitis B endemicity has been classified by the proportion of the adult population who are chronic HBV carriers [1]. Populations with prevalence of less than 2%, 2–7% and over 8% are classified as low, intermediate and high endemic, respectively [1, 234]. Worldwide, the low endemic areas are North America, Western and Northern Europe, Australia, and New Zealand; intermediate endemic areas are eastern and southern Europe, Middle East, Central Asia and Japan; high endemic areas include China, Southeast Asia, sub-Saharan Africa. It was estimated that lifetime risk of HBV infection was over 60% in high endemic area, compared to less than 20% in low endemic areas [234]. Whereas adult transmission (e.g., parenteral and sexual transmission) is predominant in areas of low endemicity, childhood infection plays an important role in both intermediate and high endemic areas, and more marked in high endemic areas [1]. It's noteworthy that, although the reason is unknown, proportion of perinatal transmission in childhood infection is significantly higher in China (30 – 40%) than other high endemic areas, such as Sub-Saharan Africa (15 – 20%).

The association between chronic HBV infection and development of HCC has been well documented in the past 30 years. As early as 1970, Sherlock and colleagues noted an association between chronic HBV infection and HCC patients [235]. Subsequent ecologic

studies found that the global distribution of chronic HBV infection and HCC were closely parallel [236]. Although bias and poor documented data exist, the correlation is obvious between prevalence of HBV infection and HCC incidence or mortality worldwide [177]. The high endemic areas of HBV chronic infection have high incidence areas of HCC, such as China, Southeast Asia and sub-Saharan Africa. In western countries where HBV carrier rate are usually less than 2%, HCC is uncommon, such as the United States.

Increased risk for HCC in chronic hepatitis B patients has been identified in a number of case-control and cohort studies. In 1981, Beasley and colleagues conducted a prospective study in 22,707 male Chinese in Taiwan [237]. At the time of recruitment, 15.2% of subjects showed positive for HBsAg. After an average follow-up period of 7 years, 194 HCC cases occurred, of which 184 (95%) were positive for HBsAg. The relative risk for HCC in HBV infected subjects was more than 100-fold higher than in non-infected subjects. The increased risk of HCC in HBsAg-positive subjects has been confirmed by several other cohort studies. Yeh and colleagues studies a cohort of 7917 males followed over an average period of 4 years in southern Guangxi, China [17]. Among 76 HCC patients observed, 69 (91%) were positive for HBsAg at enrollment in contrast to 23% of all study subjects, with a relative risk for HCC of 38.6. A 3-years follow-up in 12,222 male subjects from Chongming Island in China reported a 6.7-fold higher risk for HCC in HBsAg positive subjects than non-infected subjects [238]. The more recent cohort study is the Haiman City cohort in China [239]. Study showed that 15% of 58,545 males and 10.7% of 25,340 females were HBV carriers. They were followed up for 8 years and a total of 1092 deaths from HCC occurred. The relative risk for HCC in HBV carriers was 18.8 and 33.5 in males and females, respectively.

However, it is noteworthy that the relative importance of HBV in HCC etiology varies greatly in different population, and may change over time [240]. For instance, a prospective study in blood donors in Italy following over an average period of 29 years found that only 0.6% of HBsAg-positive blood donors developed HCC, which was not different than the rate of 0.6% in HBsAg-negative blood donors [241]. Although the exact reason is unknown, it may be due to the difference in clinical manifestations and outcome of hepatitis B between high- and low- endemic areas. Also, hepatitis B in low-risk areas mostly acquired during adolescence or adulthood, indicating a short-term persistence of HBV infection. These factors may modulate the risk of HCC [236]. Meta-analysis of HBV and HCC revealed summary odds ratios of 22.5 (95%CI: 19.5 – 26.0) for HBsAg positive carriers without anti-HCV or HCV RNA [242].

The association between HBV infection and HCC is also supported by prevention studies. HBV vaccination is a strategy to interrupt HBV transmission and proved of high immunogenicity and efficacy [240]. Declines in incidence and mortality of HCC have been observed in children and adolescents in Taiwan, where the hepatitis B vaccination program was introduced first in 1984 [243]. Also, the decrease was observed in a HBV vaccination trial conducted in Qidong, China, a high endemic area of HCC [244].

2.4.2.4 HBV genotypes

In 1980s, Okamoto and colleagues suggested to define HBV sequence genotype based on an inter-group divergence of equal or greater than 8% in the complete nucleotide sequence [245], which is widely accepted now. Till now, eight genotypes have been classified (A–H) [21]. The geographical distribution of HBV genotypes is

remarkably distinct [21, 22, 229]. HBV genotype A is mainly found in North America and Northwestern Europe. Genotype B and C are predominant in Southeast Asia. Their distribution in this area follows an intermixed pattern with more genotype C in northern regions and more genotype B in southern regions, especially in mainland China [246]. Genotype D is widely distributed over the world. It predominates in the Mediterranean area and in the Middle East up to India. Genotype E is genetically similar to genotype D and most found in West and South Africa. Genotype F is found in South and Central America. Genotype G is mostly detected in co-infection with other genotypes, mostly genotype A [247]. This genotype has no specific endemic area in the world. Genotype H was newly identified in Central America [248]. This genotype has been less studied. Recent years, hybrids of different genotypes are increasingly documented [22]. These hybrids were found more frequently in high immigrant populations.

Accumulating studies suggested differences in clinical manifestations and outcomes, including HCC, among HBV genotypes. Most of these studies were conducted in East Asia populations where genotype B and C are predominant. In a cohort of 4841 males in Taiwan follow-up for 14 years, the risk of HCC for subjects infected with genotype C was 5-fold of subjects infected with genotype A or B [249]. Increased risk of HCC in genotype C-carriers was also reported in another cohort study in 2762 HBsAg-positive subjects in Taiwan, which showed that HCC incidence in genotype C-carriers was more than twice as high as in genotype B-carries [250]. However, a study in Taiwan follow-up of pediatric patients with chronic HBV infection for 15 years and found that 74% of the children with HBV-associated HCC were identified as HBV genotype B carriers [251]. Another investigation in 1096 chronic HBV carriers did not report difference of severity

of liver disease between HBV genotype B- and C- carriers [252]. A HCC case-control study in Qidong high-risk population found that genotype C was predominant in both HBsAg positive cases and controls (91.4% vs. 93.0%), showing no significant difference [253]. The difference regarding increased risk of liver disease was also reported in genotypes other than C. For instance, in a study in India where genotype A and D are predominant, genotype D carriers were associated with severe liver disease, including HCC, compared to genotype A [254]. Genotype F was reported as a risk genotype in a cohort of 1176 Alaska Native people chronically HBV infected [255].

2.4.2.5 HBV mutations

HBV genetic mutations may occur spontaneously, or as the consequence of evolutionary pressure by endogenous immune system and/or exogenous factors, such as vaccination or drug therapy [22]. As reviewed by Pujol and colleagues, there are several frequently detected mutations that may have an impact on clinical manifestations and outcomes of HBV infection, including G1896A mutation at pre-core region, C1653T, A1753V, A1762T, G1764A and C1766T at BCP region, and several mutations/deletions at Pre-S/S and P genes [22]. The most studied mutations are those in basic core promoter (BCP) domain. BCP is an essential element in HBV genome that controls the transcription activity of HBV procore and core RNA. Also, it resides in X gene domain which encodes the functional X protein. Mutations in this critical region may influence outcome of HBV infection, including severe liver disease and HCC, which has been demonstrated in a number of studies [250, 256-261].

T1762A and T1764C mutations in BCP region usually occur together, also called as

$1762^{T}/1764^{A}$ double mutations. The $1762^{T}/1764^{A}$ double mutations result in substitutions of methionine for lysine at 130 (K130M) and isoleucine for valine at 131 (V131I) amino acid sequences of the HBV X protein. *in vitro* study demonstrated that these double mutations result in suppression of HBeAg expression and modest increase of viral replication rate [262]. Epidemiological studies conducted in several high-risk populations indicated that $1762^{T}/1764^{A}$ double mutations commonly existed in HCC patients and suggested it as a potential biomarker for studying hepatocarcinogenesis and early detection of HCC [250, 256-259]. Other than the double mutations, 1753^{V} mutations were also found to cause suppression of HBeAg expression and increase of viral DNA replication [263]. 1753^{V} mutations has been associated with progression of liver damage, including chronic hepatitis, liver cirrhosis and HCC [260, 261], while conflict results exist [264].

2.4.3 Hepatitis C virus

Hepatitis C virus (HCV), a member of the Flaviviridae, is a single-stranded RNA virus of positive polarity approximately 9500 nucleotides (9.5 kb) in length. It contains a single open reading frame (ORF) of ~9 kb that encodes a polyprotein of ~3000 amino acids. The HCV proteins include three structural proteins (core, envelope 1 and 2) and six nonstructural proteins (NS2, NS3, NS4B, NS5A and NS5B). Among them, the core protein is most likely contributing to hepatocarcinogenesis [175]. HCV was first discovered in 1989 as the etiological agent of parentally transmitted non-A, non-B hepatitis [265]. HCV is considered as a cytotoxic virus that does not integrate directly into the host genome [266]. The C hepatitis caused by HCV is considered a result of the

reaction of the host immune system against the virus infected cells.

The two risk factors most frequently responsible for the transmission of HCV infection are blood transfusion from unscreened donors and injected drug use [267]. However, the transmission patterns vary between areas with different prevalence of HCV. In HCV low prevalence areas, such as the United States, transfusions second to injected drug use are most responsible for HCV infection, whereas in moderate- or high-prevalence areas, such as Japan, Italy and Egypt, medical (dental) procedures and unsafe injections are the most important factors contributing to HCV infection [267].

HCV is a serious public health problem worldwide. According to WHO's estimation, 3% of the world's population, e.g., more than 170 million people, are chronic infected with HCV, with 3 to 4 million new infections every year [268]. Unfortunately, active or passive vaccination of HCV is still not available to date. The HCV infection rate also has great variations in geographical distribution. The rate of infection is around 1% in North America and Western Europe, while it is higher in Africa and Eastern Mediterranean where some countries have infection rate up to 10-20% [1].

HCV is a leading cause of chronic liver diseases including hepatic fibrosis, liver cirrhosis, and HCC. However, the proportion of HCV-related HCC varies considerably. In most high-incidence areas in developing countries, such as China, chronic HBV infection is the major risk factor of HCC. Whereas, in the intermediate- or low- incidence areas in developed countries, HCV infection appears to be the major risk factors of HCC, such as Japan, Spain, Italy and the United States [269, 270]. There are significant differences between HCV- and HBV- related HCC. First, HCV infection is easier to yield chronic infection than HBV infection (60-80% of HCV vs. 10% of HBV cases) [271].

The second difference is the greater propensity of HCV to promote liver cirrhosis compared with HBV: 5-10% of HCV carriers develop liver cirrhosis after 10 years of infection, approximately 10- to 20- fold higher than that of HBV [271]. In general, chronic HCV infection is typically acquired in patients in their 30s and first develop chronic inflammation that lead in some to liver cirrhosis (20-40%) in their 50s and subsequently (4-6%) to HCC in their 60s (usually 10-40 years after infection) [266]. Thus, HCV-related HCC patients are usually elder than those with HBV-related HCC, and HCC is almost exclusively found in cirrhotic HCV patients whereas HCC is more frequently found in HBV patients without significant liver cirrhosis [272].

Although the mechanism underlying the HCV-induced hepatocarcinogenesis remain unclear, the association between HCV infection and HCC development has been well established demonstrated by human epidemiological studies conducted throughout the world [273]. In 1994, IARC evaluated the HCV role in HCC development: infection with HCV, as indicated by presence of HCV antibodies, was associated with an increased HCC in all 3 available cohort studies; in 23 case-control studies, odds ratios ranged from 1.1 to 134 and were significant in 18 of these studies. Based on these human carcinogenicity data, IARC stated that there is sufficient evidence in humans for the carcinogenicity of chronic infection with HCV (group 1) [228]. Besides, successful clearance of chronic HCV infection through interferon treatment has been shown to reduce the HCC incidence and mortality related to liver disease, providing further supporting evidence for the etiology role of HCV in HCC [274, 275]. In general, the prevalence of HCV infection varies remarkably among countries. The attribution of HCV to HCC development is notably high in Japan with an estimation of 60-80% of HCC

related to HCV infection, whereas it is low in other areas, including China, especially in HCC high risk areas [276, 277].

2.4.4 Major risk factors in Southern Guangxi area, China

In Southern Guangxi area, China, dietary AFs exposure and chronic HBV infection are the two major environmental risk factors of HCC. A hospital-based case-control study recruiting 50 primary live cancer cases and 50 matched controls conducted in Guangxi reported positive HBsAg in 86% of cases and 22% of controls, associated with increased HCC risk (OR=17.0; 95% CI, 4.3-99.4) [278]. The persistence of HBV infection was estimated responsible for at least 80% of all HCC cases in this area. In 1982–1983, a cohort was assembled in five communities of Fusui and Wuming counties in southern Guangxi area, which enrolled 7917 male residents between ages of 25 and 64 years old [17]. Until 1986, there were 149 deaths occurred and 76 (51.0%) were due to primary liver cancer. Staple foods consumed in these communities were sampled and used to determine AFB_1 contamination on a population-based level. A positive and almost perfect linear relationship was observed between mortality rates of primary liver cancer and AFB_1 exposure level (mg/person/year) calculated based the AFB_1 contamination in food and food surveys ($r_{Pearson}$ = 1.00, p=0.004). Prevalence of HBsAg was observed in 69 (90.8%) of 76 cases and 68 (22.4%) of 304 controls, associating with significantly increased risk of primary liver cancer (RR=38.6, 95% CI, 16.0–117.1).

Although strong association between HCV infection and HCC risk was found in many populations, HCV did not seem to play an important role in the risk of HCC in southern Guangxi area. A study conducted in this area found that only 5.4% (10/186)

HCC patients had positive anti-HCV [279]. This result was confirmed by another study in the same area, which reported 0.7% (1/140) healthy controls and none of 39 HCC cases tested positive for anti-HCV [280].

2.5 Significance

Esophageal cancer is the most common cancer in Huaian, China, with an age-standardized incidence of 98.5/100,000 population, which is seven times higher than the national wide incidence (13/100,000 population) [31]. Despite the high incidence in past decades and distinct geographic environment, no systematic study has been published to characterize potential risk factors, especially environmental exposure factors for ESCC in this area. Therefore, we conducted a population-based case-control study using pre-designed questionnaire and carefully selected biomarkers to explore ESCC risk for exposure to environmental carcinogen, life styles, individual susceptibility factors, as well as their interactions. The aims of this study are to identify possible risk factors of ESCC in this high risk populstions and assess the potential gene-gene and gene-environment interaction. Upon completion, this research could provide useful information in a clear overview of both environmental and genetic risk factors in etiology of ESCC, as well as their contributions to high ESCC incidence in this population.

Southern Guangxi area is one of the HCC high risk areas in China with an age-standardized incidence of 54.3/100,000 population[186]. Consumption of foods contaminated with AFs is a risk factor secondary only to HBV chronic infection in HCC development in this area [17]. However, none of previous study has comprehensively incorporated risk-specific biomarkers of dietary AFs exposure and HBV infection into

the evaluation of HCC risk in this area. Therefore, we conducted a case-control study to investigate the risk specific biomarkers for HCC, including HBV genotypes and BCP mutations, as innovative HBV genetic biomarkers, and serum AFB_1-lysine adduct, as an AFB_1 long-term exposure biomarker, in HCC development. Upon completion, this study could generate useful data in evaluation of these biomarkers, and assessment of chemical/viral carcinogen exposures, as well as their interaction in the process of hepatocarcinogenesis in this high risk population.

Chapter 3

ESOPHAGEAL CANCER CASE-CONTROL STUDY

MATERIALS AND METHODS

3.1 Study design and selection of study population

This population based case-control study consists of 190 ESCC cases and 380 healthy controls. All study subjects were residents of five rural farming communities (township) belong to ChuZhou District which locates at the north side of Huaian City, Jiangsu Province, China. All eligible cases were identified through the malignant tumor registry of Huaian Center for Disease Control (CDC). These cases were newly diagnosed with primary ESCC in 2002–2003 and 2006–2007. Cases were diagnosed at local hospital by endoscopy, X-ray or clinical histopathology and reported to Huaian CDC malignant tumor registry. In total, 107 and 83 ESCC cases were randomly selected from these five townships in 2002–2003 and 2006–2007, respectively. Population-based controls were healthy individuals, randomly selected from local household registration office. Recruitment criteria included: 1) free of cancer history, 2) no use of prescribed medications at the time of recruitment, 3) no pregnancy or lactation for female subjects, 4) no more than one participant recruited in each household. Eligible controls were 1:2 matched to cases on age (± 5 years), gender and residency. Informed consent of questionnaire interview and biological samples collection was obtained from each subject. The study protocol was approved by Institutional Review Board for human

subjects at Texas Tech University and Southeast University.

3.2 Interview questionnaire and data collection

A unified questionnaire, administered by trained personnel, was used to collect information of study subjects through in-person interview. Study subjects were asked about their demographic and socioeconomic characteristics, personal medical history, dietary factors, smoking history, alcohol drinking history, tea drinking history, and family history of cancer.

3.2.1 Demographic and socioeconomic factors

Demographic and socioeconomic characteristics include age, gender, occupation, education attainment, marital status, and current and 10-years-ago family income. Individual income was then calculated by dividing family income by family member number for statistical analysis.

3.2.2 Health and medical history

Study subjects were asked about their height and weight 1 year prior (1 year before diagnosis for cases). We calculated body mass index (BMI) by dividing weight (kg) by squared height (m^2) and categorized for further analysis according to WHO standard (Underweight: <18.5; Normal weight: 18.5 – 24.9; Overweight: 25 – 29.9; Obese: 30 & Above). Study subjects were asked in detailed about their medical history of esophageal lesion and gastric lesion. Esophageal lesion was defined as one or more of the following esophageal diseases which was clinically diagnosed: 1) chronic esophagitis, 2) reflux

esophagitis, 3) epithelial proliferation, 4) epithelial metaplasia, 5) epithelial polypus, and 6) epithelial erosion. Gastric lesion was defined as one or more of the following esophageal diseases which was clinically diagnosed: 1) chronic atrophic gastritis, 2) chronic gastritis, 3) acute gastritis, 4) gastric ulcer, and 5) gastric polypus.

3.2.3 Dietary factors

Dietary information was collected from a 120-item semiquantitative food frequency questionnaire. Food items were assigned to eight categories: 1) staple diet, 2) fried food, 3) pickled and salty food, 4) animal meat, egg and milk, 5) bean and bean product, 6) vegetables, 7) fresh fruits, and 8) nuts and dried fruits. These food items were considered representative of regular diets of Chinese population, which was developed by Institute of Nutrition and Food Safety, Chinese CDC. Study subjects were asked about their usually dietary intake for the previous 12-month period. In addition, they were asked about their dietary habits, including whether they ate on time usually, eating speed, whether they favored salty (spicy, sweet, sour) food now and ten years ago, whether they favored high temperature of food now and ten years ago, and whether they consumed several specific food items frequently, including garlic, onion, soy bean products and pickled/salty food. Study subjects also provided habit and activities that might involve exposure to environmental toxin, including current and 10-years-ago moldy food intake, storage time of grain, and clean up interval of food storage utensils.

3.2.4 Smoking history

Study subjects were asked whether, over their lifetime before the diagnosis for cases

and before the time of recruitment for controls, they had ever smoked at least one cigarette per day for at least one year continuously. Subjects with positive response were defined as smoker. They were further asked about the age at which they started smoking, the number of years they had smoked, the method of tobacco smoking (cigarettes, pipes smoking, hookah, and others), and whether their family number were smoker. Subjects were asked whether they ever temporarily stopped smoking for more than three months and the duration of stopping smoking if the response was positive. Smokers were also asked to estimate the average number of cigarettes (piece/day), pipes or water pipes smoking (gram/day) in a typical day and during each decade of life. For those smoked pipes or hookah, the amount of smoking was converted into piece of cigarette (piece/day) by dividing amount of smoking per day (gram/day) by 0.8.

3.2.5 Alcohol drinking history

Study subjects were asked whether, over their lifetime before the diagnosis for cases and before the time of recruitment for controls, they had ever taken at least one drink of alcoholic beverage per week for at least six months continuously. Subjects with positive response were defined as alcohol drinker. They were further asked about the age at which they started drinking, the number of years they had drunk and the type of alcoholic beverage (hard liquor, low-alcohol liquor, beer, wine) they ever drank on a regular basis. Subjects were asked about the duration of temporarily stopping drinking for at least three months. We also asked study subjects to estimate the average consumption of each type of alcoholic beverage per week (gram/week) in a typical week and during each decade of life. To calculate drinking intensity (gram/week) on the basis of ethanol volume, we

multiplied the amount of alcoholic beverage per week (gram/week) by the beverage-specific volume percentage of pure ethanol (5% for beer, 12% for wine, 25% for low-alcohol liquor, and 54% for hard liquor).

3.2.6 Tea drinking history

Study subjects were asked whether, over their lifetime before the diagnosis for cases and before the time of recruitment for controls, they had ever drunk at least one cup of tea per week for at least six month continuously. Subjects with positive response were defined as tea drinker. They were further asked about the age at which they started drinking, the number of years they had drunk and the type of tea they usually drank (green tea, red tea, flower tea, wulong tea, other), preference for thickness of tea (thick, moderate, light), preference for water temperature of tea (hot, warm, cold), and average consumption of tea per month (gram/month).

3.2.7 Family history of cancer

Study subjects were asked to recall whether any of their relatives were clinically diagnosed with cancer. For those with positive response, they were further asked about the relationship of the relatives who were diagnosed with cancer, and correspondingly, the type of cancer diagnosed.

3.3 Biological sample collection

Morning urine samples of study subjects were collected at the time of recruitment and a fraction of 100 ml were stored at -70℃ before shipping to the US laboratory for

analysis. Blood samples of study subjects were drawn at village clinics at the time of recruitment using Vacutainer (10ml) containing 20 µl of 10% EDTA (Fisher Scientific, PA, USA). In the laboratory of Township hospital, 2 ml of blood samples were centrifuged at 2500g for 10 minutes to separate serum, layer of white blood cells, and red blood cell pellets, which were be fractioned and stored at -70℃ before shipping to the US laboratory for analysis..

3.4 Chemicals and Materials

Boric acid, o-phthaldialdehyde (OPA), 2-mercaptoethanol, FB_1 from *Fusarium verticilioides moniliforme* (~98%, TLC), D-erythro-sphingosine (Sa), D-erythro-sphinganine (So), 8-hydroxydeoxyguanosine (8-OHdG), 10× phosphate buffered saline (PBS), ammonium hydroxide, ammonium acetate, sodium chloride, sodium phosphate monobasic, hydrochloric acid, formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). D-erythro-C20-sphingosine (C20So) was obtained from Avanti Polar Lipids, Inc (Alabaster, AL, USA). Triethylammonium formate HPLC buffer (0.25N, pH 3.0) was from Regis Technologiesm Inc (Morton Grove, IL, USA). Other HPLC-grade solvents including water, methanol, 2-propanol, dimethyl chloride, acetonitrile, and ethyl acetate were from Honeywell Burdick & Jackson (Muskegon, MI, USA). OPA reagents were prepared by dissolving 10 mg of OPA and 30 µl of 2-mercaptoethanol in 250 µl of methanol and mixing with 4.75 ml of 3 % boric acid buffer (pH 10.5) and stored at 4 ℃ avoiding light before use. All PCR primers were synthesized by Integrated DNA Technology Inc. (Coralville, IA, USA). Restriction enzymes used in digestion of PCR products were purchased from New England Biolabs (Beverly, MA, USA).

3.5 Measurement of urinary free FB_1 level

3.5.1 Purification and Concentration of urinary free FB_1

Frozen morning urine samples were thawn in 4 ℃ and centrifuged at 1500g for 5 minutes before use. Aliquot of 10 ml supernatant was loaded onto a pre-equilibrated FumoniTest immunoaffinity column from VICAM (Watertown, MA, USA) by gravity. After washing with 10 ml of 1× PBS, 3×0.5 ml of 20% methanol in 10 mM hydrochloric acid was used to elute FB_1 fractions directly onto a previously conditioned primed Oasis HLB cartridge (3cc/60mg 30μm) from Waters Co. (Milford, MA, USA). The HLB cartridge was sequentially washed with 2 ml of water and 2 ml of 30% aqueous methanol, and then eluted with 3×0.6 ml of 2% formic acid in methanol. Finally, eluents containing FB_1 fractions were evaporated to dryness under a gentle stream of ultra-high-purity nitrogen gas at 35 ℃. Dry residues were reconstituted with 100 μl of 50% aqueous methanol.

3.5.2 OPA derivatization

The 100 μl of suspension was transferred into a glass HPLC vial for analysis. Because of the instability of OPA derivatives of FB_1, OPA florescent derivative of FB_1 was formed using an on-line (automatic) injector program in Agilent HPLC system, in which the 100 μl FB_1 solution was thoroughly mixed with 20 μl of OPA reagent for one minute immediately before injection.

3.5.3 HPLC analysis of FB_1

HPLC-fluorescence analysis was carried out on an Agilent 1100 liquid

chromatography system equipped with an on-line degasser, a quaternary gradient pump, an automatic injector, a fluorescence detector, and a ChemStation data system (Agilent Technologies, Wilmington, DE, USA). Chromatographic separations were performed in a Luna C18 (2) column (5 μm particle size, 250 × 4.6 mm) from Phenomenex (Torrance, CA, USA), which was maintained at 35 ℃. The mobile phase consisted of a linear gradient starting from 0.1 M sodium phosphate monobasic (pH 3.4)-methanol (35/65, v/v) to 0.1 M sodium phosphate monobasic (pH 3.4)-methanol (20/80, v/v) over 13 min. The flow rate was 1.0 ml/min and injection volume was 100 μl. OPA fluorescent derivative of FB_1 was then monitored at an emission wavelength of 440 nm (excitation wavelength 330 nm). FB_1 peak was identified by comparison with FB_1 standard based on peak retention time. Typical HPLC chromatograms of urinary free FB_1 are shown in Figure 3.1. Concentration of FB_1 was determined using external calibration curve generated from a serial of blank urine samples spiked with FB_1 standard.

The limit of quantitation for the HPLC method was 20 pg per injection of FB_1. When blank urine sample was spiked with 10, 20, 50, 100, 200, 500 pg of each FB_1 standard per ml urine, the mean recovery rate of FB_1 was 83.4 ± 0.8%. Method repeatability was evaluated by triplicates obtained on the same day. The RSD% was 4.42%. The concentration of urinary free FB_1 was adjusted with urinary creatinine level.

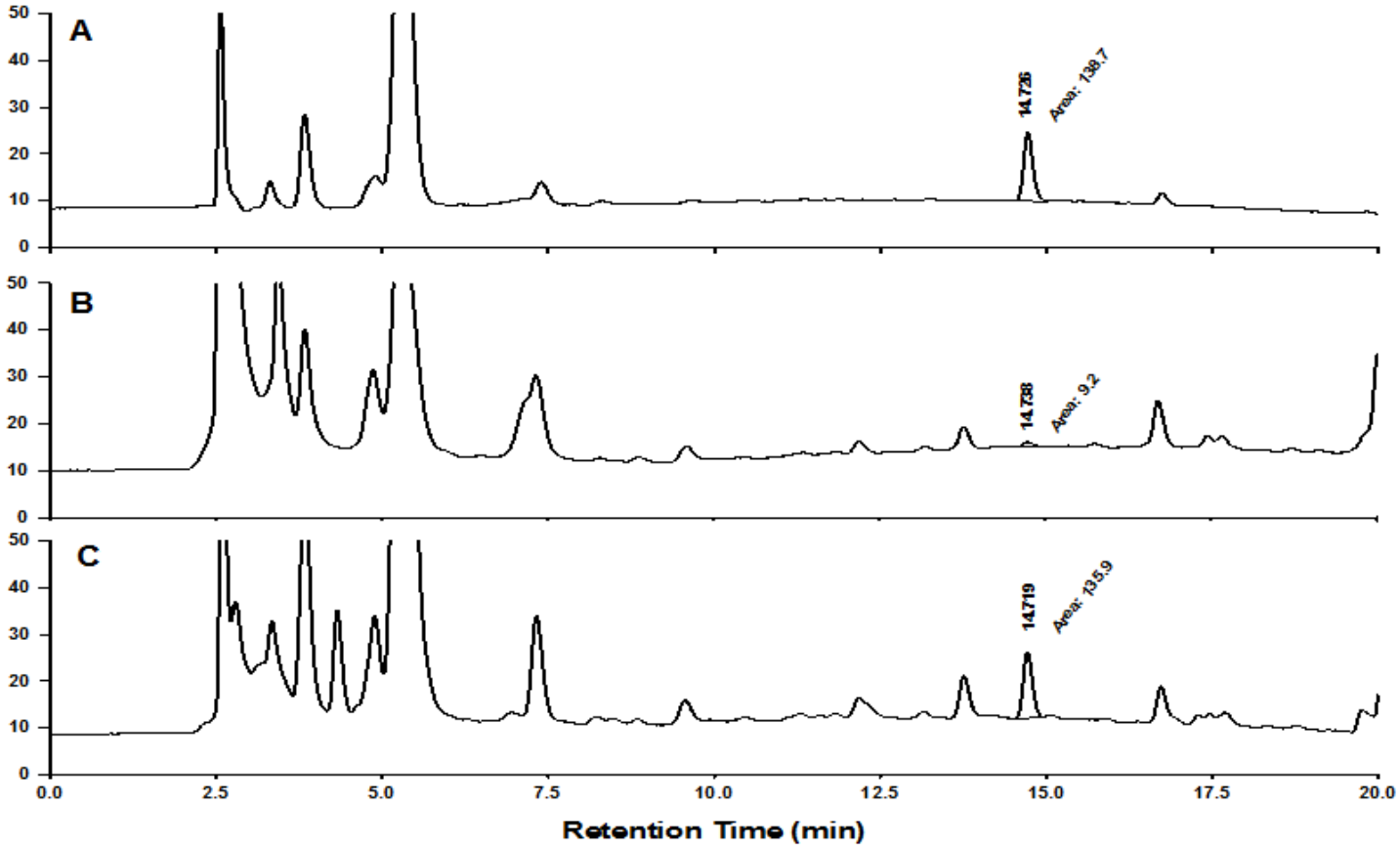

Figure 3.1 Chromatograms of OPA fluorescent derivative of FB_1 standard and samples. (A) Analytical standard containing 10 ng injection of FB_1 (retention time = 14.726 min) (B) Urine extracts from a control sample, showing OPA derivative of FB_1 (RT = 14.738 min). (C) Urine extract from a case sample, showing OPA derivative of FB_1 (RT = 14.719 min).

3.5.4 LC/MS/MS confirmation of urinary free FB_1

Human urine samples with > 10 ng/ml of FB_1 levels from above HPLC analysis were measured using a LC/MS/MS method. In brief, FB_1 fractions were resolved on a Surveyor LC system from Thermo Finnigan (San Jose, CA, USA) comprising a Surveyor MS pump, a Surveyor autosampler, and a Finnigan Xcalibur 1.3 data system. Chromatographic separations were performed in a BetaBasic C18 column (3 μm particle size, 150 × 2.1 mm, Thermo Finnigan) maintained at 30 ℃. The mobile phase consisted of a linear gradient starting from water-acetonitrile-formic acid (897/100/3, v/v/v) to water-acetonitrile-formic acid (100/897/3, v/v/v) in 15 min and held for 10 min before a 10-min equilibration period between each injection. The flow rate was 0.2 ml/min, and injection volume was 15 μl. A LCQ Advantage ion trap mass spectrometer (Thermo Finnigan) was operated in positive electrospray ionization mode with an inlet capillary temperature of 250 ℃ and source voltage of 4.0 kv. Both the sheath gas and the auxiliary gas were nitrogen gas set to 40 and 10 units, respectively. Mass fragment was scanned in a selected ion monitoring mode at m/z 722.3. In addition, tandem mass spectrometry was performed by scanning mass fragments from m/z 200 to 750, and selected reaction monitoring of the ion transition of m/z 722.3 to 352.3, both were achieved at normalized collision energy of 58%. Typical mean retention time (RT) was 13.5 min for FB_1 (Figure 3.2). The limit of quantitation of the LC/MS/MS method was 150 pg per injection of FB_1. Non-detectable was designated as one-half of the limit of detection (LOD) in continuous variable analysis. A good correlation between the HPLC and LC/MS/MS methods was observed in the 8 measured urine samples (r2=0.987).

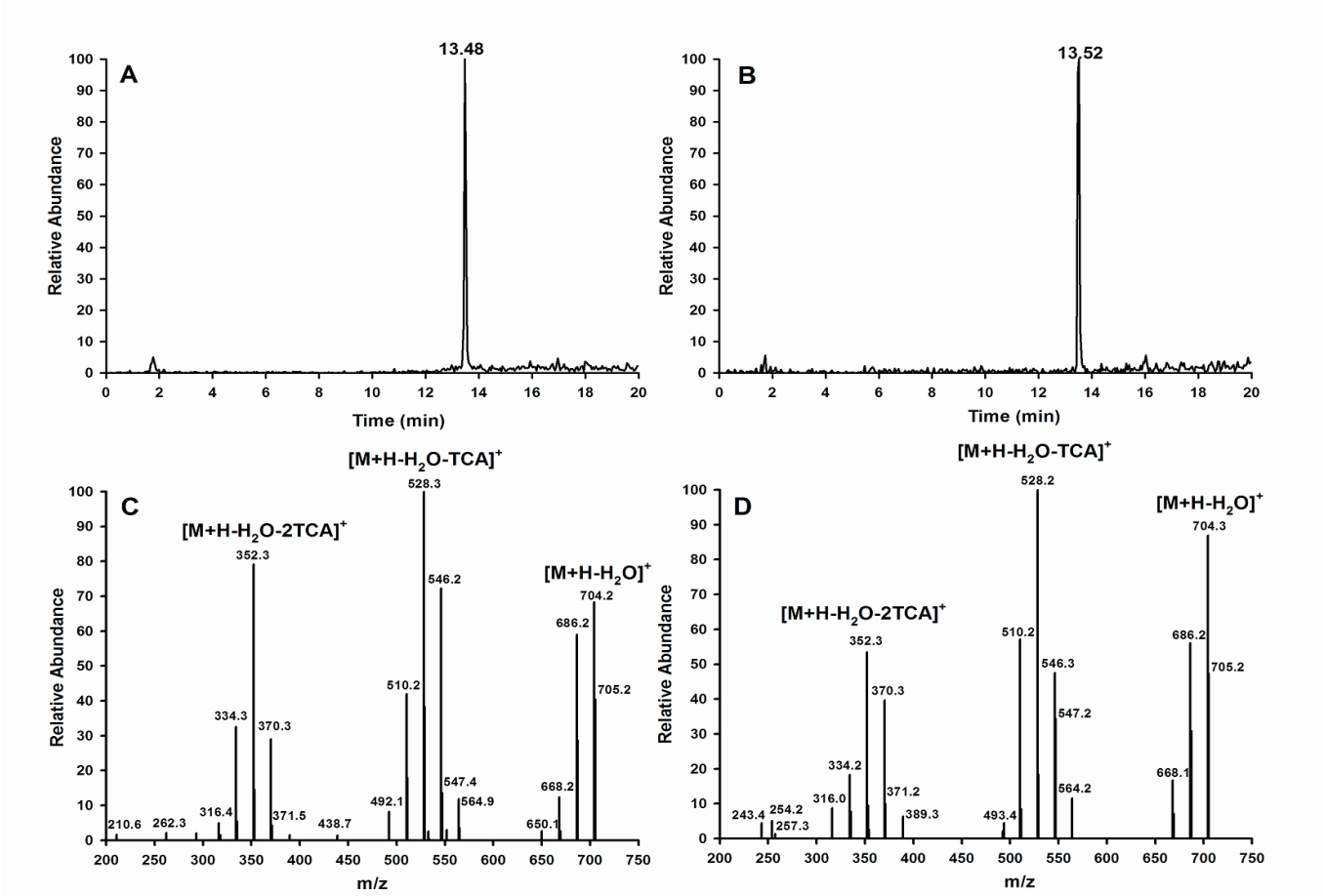

Figure 3.2 Identification of FB_1 by LC/MS/MS analysis. (A) Chromatogram of analytical standard containing 75 ng injection of FB_1 (RT=13.48 min). (B) Chromatogram of human urine extract showing FB_1 (RT=13.52 min). (C) MS/MS spectra of the FB_1 standard. (D) MS/MS spectra of the human urine extract.

3.6 Measurement of urinary sphingolipids metabolites (Sa and So)

3.6.1 Extraction and derivatization of urinary Sa and So

In brief, urinary pellets were harvested from urine sample (6 ml for females and 12 ml for males) via centrifugation at 1500 ×g for 10 min. A total of 500 µl of 1 ×PBS (pH 7.4), 20 µl of 1.5 µM C20So (internal standard), and 3 ml of ethyl acetate were added to each pellet sample. Samples were then mixed and gentle rotated for 30 min on a rugged rotator (GlasCol, Terre Haute, IN, USA). Then 2 ml of organic phase was evaporated to dryness at 35 ℃. The dry residue was then reconstituted in 275 µl of 80% aqueous methanol and derivatized by addition of 25 µl of OPA reagent and mix thoroughly for 5 minutes at room temperature before injection for HPLC-fluorescence analysis.

3.6.2 HPLC analysis of Sa and So

The fluorescent derivatives of So, Sa and C20So were resolved on an Agilent 1100 liquid chromatography system (Agilent Technologies, Wilmington, DE, USA). Chromatographic separations were performed on a Zorbax Eclipse XDB-C18 column (5 µm particle size, 250 × 4.6 mm, Agilent Technologies). The mobile phase consisted of a linear gradient starting from 5 mM triethylammonium formate (pH 4.3)-methanol-acetonitrile (15/45/40, v/v/v) to methanol-acetonitrile (60/40, v/v) over 20 min at a flow rate of 1.0 ml/min. The fluorescent derivatives were monitored with an excitation and an emission wavelength of 340 nm and 455 nm, respectively. The peaks of Sa and So were identified by comparison with retention time of Sa and So standard. Recovery status was monitored using the relative areas of C20So in measured samples versus reference standard. Concentration of Sa and So was determined using external calibration curves

generated from a serial of blank urine samples spiked with Sa and So standard. The limit of quantitation for the HPLC method was 0.03 pmol per injection.

3.7 Measurement of urinary 8-OHdG level

3.7.1 Extraction of urinary 8-OHdG

Urinary 8-OHdG level was extracted by a solid phase extraction (SPE) method, which was described in detail previously [118]. In brief, 0.5 ml urine sample was purified with a pre-conditioned Oasis HLB cartridge (3cc/60mg 30μm, Waters Co., Milford, MA, USA). After washed with 5% aqueous methanol, 8-OHdG fraction was eluted with 2% formic acid in 15% aqueous methanol. The eluents containing 8-OHdG were evaporated to dryness by high purity nitrogen gas at 45 °C and reconstituted with 150 μl of 10 mM ammonium acetate in 2% aqueous methanol (pH 4.3) for HPLC analysis.

3.7.2 HPLC analysis of urinary 8-OHdG

A description of measurement of urinary 8-OHdG by high performance liquid chromatography (HPLC)-electrochemical detection (ECD) method was provided in previous publication [118]. Briefly, HPLC-ECD analysis was carried out on an ESA HPLC CoulArray multi-channel ECD system equipped with a double solvent delivery module, an autosampler, a CoulArray detector with 8 channels, and a Coulochem data system (ESA-A Dionex Company, Chelmsford, MA, USA). Chromatographic separations were performed in an YMCbasic column (S3 μm particle size, 4.6 × 150 mm, Waters Co. Milford, MA, USA). The mobile phase consisted of a linear gradient starting from 10 mM ammonium acetate (PH 4.3)-methanol (98/2, v/v) to 10 mM ammonium

acetate (PH 4.3)-methanol (60/40, v/v) over 18 minutes. The flow rate was 0.8 ml/min and injection volume was 50 μl. The channels of CoulArray detector were set at 270mv, 300mv, 330mv and 360mv, with the main target peak appeared at 300mv. The peak of 8-OHdG was identified by comparison with retention time of 8-OHdG standard at channel 300mv. Concentration of 8-OHdG in urine samples was determined using external calibration curves generated from a serial of blank urine samples spiked with 8-OHdG standard. The limit of quantitation for the HPLC method was 1 ng/ml urine samples. The average recovery rate was 84.8%. The concentration of urinary 8-OHdG was adjusted with urinary creatinine level.

3.8 Measurement of urinary creatinine

Creatinine in urine was analyzed using the 96-well Creatinine Assay Kit from Cayman Chemical (Ann Arbor, MI, USA). The analysis is based on a modified Jaffe colorimetric method that measures the difference in absorbance (495 nm) of the creatinine-picrate complex before and after acidification. The assay was carried out according to manufacturer's instructions, and absorbance was measured using an ELx808 Absorbance Microplate Reader from BioTek Instruments, Inc. (Winooski, VT, USA).

3.9 Determination of genetic polymorphisms

3.9.1 Genomic DNA extraction

Genomic DNA was extracted from 200μl of white blood cell using Qiagen QIAamp DNA Blood Mini Kit in accordance with manufacturer's protocol (QIAGEN Inc. Valencia, CA, USA). Purified DNA was then stored at -20℃ for further analysis.

3.9.2 General procedure of PCR amplification

In general, PCR reactions were performed in a 25 μl system containing approximately 30 ng of genomic DNA template, 2.5 μl of 1 x PCR buffer, 1.5 mM MgCl2, 0.2 mM of each dNTP, 0.2 mM of each primer (table 3.1), and 4 units of Taq DNA polymerase. PCR amplification was conducted on Applied Biosystems 9700 96 well-plate PCR machine (Foster city, CA, USA). PCR amplification product was subjected to electrophoresis on 2% agarose gel stained with 0.5 μg/ml ethidium bromide (EB) and identified by comparing with PCR markers/ladders. Strict quality control procedures were implemented to ensure high genotyping accuracy. PCR mixture work was performed in a PCR station equipped with a UV lamp. Positive controls and negative controls were included in each PCR amplification reaction. Five percent of the samples were randomly selected to run PCR in duplicates to estimate accuracy of the genotyping.

3.9.3 Genotyping of GSTM1 and GSTT1

The genotyping for GSTM1 and GSTT1 gene was performed using a multiplex PCR reaction with modification [281], in which GSTM1 primers, GSTT1 primers and beta-globin primers (serving as internal standard) were included (table 3.1). The amplification conditions included an initial denaturation at 95 ℃ for 7 min, followed by 33 cycles at 95 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 45 s, then followed by a final extension at 72 ℃ for 5 min. PCR amplification products were visualized by electrophoresis on EB-stained 2% agarose gel. In the presence of the beta-globin band (bp), absence of PCR product bands of 215 bp (GSTM1) and 480 bp (GSTT1) were indicative of null genotypes of GSTM1 and GSTT1, respectively.

3.9.4 Genotyping of TNF-α G308A

A typical PCR-RFLP method was used to determine TNF-α G308A polymorphism in study subjects with minor modification [282]. The amplification conditions included an initial denaturation at 95 ℃ for 7 min, followed by 33 cycles at 95 ℃ for 30 s, 58 ℃ for 30 s, and 72 ℃ for 30 s, then followed by a final extension at 72 ℃ for 5 min. Five microliters of PCR product was digested in 20 μl of reaction system (2.0 μl 10 × buffer, 5 units of NcoI) overnight for 4 hours. The digestion product was then subjected to electrophoresis on EB-stained 3% agarose gel. Presence of two bands with size of 87 and 20 bp indicated wide type (G/G), and presence of uncut band of 107 bp indicated mutant type (C/C). Presence of all three bands (20, 87, and 107 bp) was indicative of heterozygous type (G/C).

3.9.5 Genotyping of NF-κB -94ins/del ATTG

Genotyping for NF-κB -94ins/del ATTG polymorphism was determined using a published PCR-RFLP method with modification [283]. The 25 μl system was amplified by an initial denaturation at 95 ℃ for 7 min, followed by 35 cycles at 95 ℃ for 30 s, 61 ℃ for 1 min, and 72 ℃ for 45 s, and followed by a final extension at 72 ℃ for 5 min. Five microliters of PCR product was digested in 20 μl of reaction system (2.0 μl 10 × buffer, 4 units of PflMl) at 37 ℃ for 4 hours. The digestion product was then subjected to electrophoresis on EB-stained 2% agarose gel. Presence of two bands with size of 240 and 45 bp indicated ins type (I/I), and presence of band of 285 bp indicated del type (D/D). Presence of all three bands (285, 240 and 45 bp) was indicative of heterozygous type (I/D).

3.9.6 Genotyping of iNOS Ser608Leu

Genotyping for iNOS Ser608Leu polymorphism was determined using a published PCR-RFLP method with modification [284]. A touch-down PCR technique was used to amplify target DNA template. After an initial denaturation at 95 ℃ for 7 min, 10 cycles of amplification was performed at 95 ℃ for 30 s, 61 ℃ for 1 min with decreasing of 0.5 ℃ per cycle, and 72 ℃ for 45 s. Then another 30 cycles' amplification was carried out at 95 ℃ for 30 s, 56 ℃ for 1 min, and 72 ℃ for 45 s. Finally, the extension step was at 72 ℃ for 5 min. the 288bp PCR product (5 μl) was digested in 20 μl of reaction system (2.0 μl 10 × buffer, 5 units of Tsp509I) overnight at 65 ℃. The digestion product was then verified by electrophoresis on EB-stained 2% agarose gel. Presence of two bands with size of 113 and 175 bp indicated wide type (Ser/Ser), and presence of three bands with size of 31, 113, 142 bp indicated mutant type (Leu/Leu). Presence of all four bands indicated heterozygous type (Ser/Leu).

3.9.7 Genotyping of COX-2 G765C

Genotyping for COX-2 G765C polymorphism was determined using a published PCR-RFLP method with modification [285]. A touch-down PCR technique was used. The PCR amplification condition started with an initial denaturation at 95 ℃ for 7 min, followed by 29 cycles of amplification at 95 ℃ for 30 s, 65 ℃ for 30 s and decreasing for 0.3 ℃ per cycle, and 72 ℃ for 30 s. Then An additional 19 cycles of amplification at 95 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 30 s, was followed by a final extension step at 72 ℃ for 5 min. Ten microliters of PCR product were digested with 5 units of AciI in 20 μl of digestion system overnight at 37 ℃. The digestion product was then visualized

by electrophoresis on EB-stained 2.5% agarose gel. Presence of two bands with size of 100 and 209 bp indicated wide type (G/G), and presence of uncut band of 309 bp indicated mutant type (C/C). Presence of all three bands (100, 209, and 309 bp) indicated heterozygous type (G/C).

3.9.8 Genotyping of XPD Lys751Gln

A typical PCR-HPLC analysis was performed to determine genotype of XPD Lys751Gln polymorphism in study subjects [286]. The 25 μl PCR reaction system was amplified by an initial denaturation at 95 ℃ for 7 min, followed by 38 cycles at 95 ℃ for 30 s, 60 ℃ for 1 min, and 72 ℃ for 45 s, and a final extension at 72 ℃ for 5 min. The 344 bp PCR product (10 μl) was digested by 5 units of restriction enzyme PstI in a 20 μl of digest system for 4 hours at 37 ℃. After electrophoreses in EB-stained 2.5% agarose gel, the wide type (Lys/Lys) had two bands with size of 111 and 234 bp, where the mutant type (Gln/Gln) had three bands with size of 63, 110, and 171 bp. The heterozygous type had all these four bands.

3.9.9 Genotyping of hOGG1 Ser326Cys

To determine the hOGG1 Ser326Cys polymorphism, genomic DNA of study subjects were amplified and digested with restriction enzyme using a published method with modification [287]. The 25 μl PCR reaction system was amplified by an initial denaturation at 95 ℃ for 7 min, followed by 30 cycles at 95 ℃ for 30 s, 60 ℃ for 1 min, and 72 ℃ for 1 min, and a final extension at 72 ℃ for 5 min. Four units of Fnu4HI were used to digest 10 μl of PCR product in 20 μl of digestion system at 37 ℃ for 4 hours.

Separation of digestion products was performed on EB-stained 3% agarose gel. Presence of uncut band (200 bp) indicated wild type (Ser/Ser), and presence of cut band (100 bp) indicated mutant type (Cys/Cys). Presence of both bands indicated heterozygous type (Ser/Cys).

3.10 Statistical analysis

In the analyses, we aimed to identify the associations between ESCC risk and the investigated factors obtained through questionnaire interview and laboratory measurement. In particular, we estimated the independent effect of each factor, along with the effect after adjustment of potential confounding factors.

Our approach was first to fit single factor obtained through questionnaire interview separately into logistic regression model to assess risk of ESCC. Differences between cases and controls of these factors were compared by using conditional logistic regression models. A weighted paired-sample t test was used to compare age difference between cases and controls. Gender difference was analyzed using unconditional logistic regression model with adjustment of age. Odds ratios (ORs) and 95 percent confidence interval (CI) for ESCC risk were calculated as well. Trend tests for ordinal categorical variables were performed using median values for each category, or assigning an integer to each layer of category e.g., 1-3, as appropriate, and then fitted the terms as continuous values in the model.

Cohen's kappa coefficients were calculated in assessment of agreement between several factors, including current and 10-years-ago individual income, current and 10-years-ago preference to salty food, current and 10-years-ago preference to spicy food,

Table 3.1 Primers and restrictive endonucleases used for PCR-RFLP analysis

Name	Primer sequence	PCR product size (bp)	RFLP endonuclease	RFLP site sequence	Reaction condition	Ref.
GSTM1	F: 5’-GAACTCCCTGAAAAGCTAAAGC-3’ R: 5’-GTTGGGCTCAAATATACGGTGG-3’	215	-	-	-	
GSTT1	F: 5’-CCTTACTGGTCCTCACATCTC-3’ R: 5’-TCACCGGATCATGGCCAGCA-3’	480	-	-	-	
β-globin	F: 5’-CAACTTCATCCACGTTCACC-3’ R: 5’-GAAGAGCCAAGGACAGGTAC-3’	268	-	-	-	
TNF-α G308A	F: 5′-TCCTCCCTGCTCCGATTCCG-3′ R: 5′-AGGCAATAGGTTTTGAGGGCCAT-3′	107	NcoI	C-CATGG	37 ℃ overnight	
NF-κB -94ins/del ATTG	F: 5’-TTAATCTGTGAAGAGATGTGAATG-3’ R: 5’-GTAGGGAAGCCCCCAGGA-3’	289	PflMl	CCANNNN-NTGG	37 ℃ 4 hr	
iNOS Ser608Leu	F: 5’-TGTAAACCAACTTCCGTGGTG-3’ R: 5’-GTCTCTGCGGGTCTGAGAAG-3’	288	Tsp509I	-AATT	65 ℃ overnight	
COX-2 G765C	F: 5'-AGGCAGGAAACTTTATATTGG-3' R: 5'-ATGTTTTAGTGACGACGCTTA-3'	308	AciI	C-CGC	37 ℃ overnight	
XPD Lys751Gln	F: 5’-TCAAACATCCTGTCCCTACT-3’ R: 5’-CTGCGATTAAAGGCTGTGGA-3’	344	PstI	CTGCA-G	37 ℃ 3 hr	
hOGG1 Ser326Cys	F: 5’-GGAAGGTGCTTGGGGAAT-3’ R: 5’-ACTGTCACTAGTCTCACCAG-3’	200	Fnu4HI	GC-NGC	37 ℃ 4 hr	

current and 10-years-ago preference to high temperature of food, and current and 10-years-ago mildewed food intake. For factors showing substantial to almost perfect agreement, only those representing exposure status 10 years ago were evaluated in multivariate analysis later on. Those factors showing only moderate agreement were all allowed into the regression model for further evaluation in multivariate analysis.

We then did multivariate analysis in which multiple factors were fitted into the conditional model simultaneously. Stepwise selection was used to determine which of these variables stayed in the final model. In the selection process, variables with p value less than 0.2 were allowed to enter the model and those with p value less than 0.05 were allowed to stay in the model. In the first multivariate model, factors investigated through questionnaire interview were evaluated. Those factors met the stepwise selection criteria were kept in the model. Also, alcohol drinking and smoking status were forced into the model regardless the significance. In the second multivariate model, all investigated factors, including those obtained through questionnaire interview and laboratory measurement, were evaluated using multivariate conditional logistic regression model. In this model, only those factors met the stepwise selection criteria were kept in the model.

In assessment of fumonisin biomarkers (urinary free FB_1, Sa, So, and Sa/So ratio) in cases and controls, our first approach was to fit these continuous variables into conditional logistic regression model with adjustment of potential confounding factors. Then gender (male, female) difference in cases and controls were analyzed using wilcoxon rank-sum test. We then categorized these variables in tertile, using the cutpoints calculated among controls. ORs and 95% CIs for ESCC risk were calculated using conditional logistic regression model with adjustment of potential confounding factors.

Test for linear trend of ORs were performed using median values for each category and then fitted the terms as continuous values in the model. Unconditional logistic regression model with adjustment of potential confounding factors plus age and gender was used to calculate OR and 95% CI after stratification of potential confounding factors. Likelihood ratio tests were used to evaluate interaction, e.g., comparing the log likelihood of a full regression model including the interaction term with a reduced model without the interaction term. Correlation between urinary fumonisin biomarkers (continuous variables) and possible related variables (continuous variables), including age, corn intake (gram/day) and corn meal intake (gram/day) were analyzed using spearman rank correlation test.

These analyses were repeated in evaluating association between ESCC risk and urinary 8-OHdG levels. Urinary 8-OHdG was divided into low, moderate and high levels using cutpoints calculated in controls. We also evaluated the correlation between urinary 8-OHdG (continuous variable) and possible related factors, including age and urinary free FB_1 concentration (pg/mg creatinine).

In analysis of genetic polymorphisms and ESCC risk, the Pearson's $\chi 2$ test or Fisher's exact test were used to test difference between cases and controls, as appropriate. ORs and 95% CIs were calculated using conditional logistic regression model without adjustment of possible confounding factors. After stratification of gender, unconditional logistic regression analysis with adjustment of age was performed to estimate ORs and 95% CIs in male subjects and female subjects. We also performed additional analysis of gene-gene interaction in modulating ESCC risk using conditional logistic regression model or unconditional regression model adjusted by age in male subjects and female

subjects. The combinative effects of mutant (adverse) alleles in inflammation-related pathway were analyzed as a categorical variable by grouping the subjects according to the total number of mutant (adverse) alleles in the pathway. Trend test was performed to test for a linear trend of the ORs using median values for each category. We also performed unconditional logistic regression analysis to assess specific genetic polymorphisms on ESCC risk stratifying by family history of cancer and possible environmental exposure risk factors, including smoking, alcohol drinking, fumonisin exposure (urinary free FB_1), and pickled food intake.

A p-value of less than 0.05 (two-tailed) was considered statistically significant. All data were analyzed by using SAS software version 9.1 (SAS Institute Inc., Cary, NC, USA).

Chapter 4

ESOPHAGEAL CANCER CASE-CONTROL STUY – RESULTS

4.1 Questionnaire data

4.1.1 Demographic and socioeconomic characteristics

Demographic and socioeconomic characteristics of study subjects are summarized in Table 4.1. Significant differences were found between cases and controls in education attainment, individual income per month at the time of recruitment and 10 years before the recruitment. Moderate agreements between current and 10-years-ago's individual income were observed in both cases and controls, as indicated by Cohen's kappa coefficient (κ) of 0.50 and 0.44 in cases and controls, respectively.

4.1.2 Health and medical history

Table 4.2 presents health status and medical history of esophageal and gastric lesion in study subjects. Control group had a significantly higher average weight and lower average height than case group. Correspondingly, average BMI in cases was significantly lower than in controls ($p<0.001$). A total of 12.2% of cases and 2.4% of controls reported a medical history of esophageal lesion, showing a significant difference. Chronic esophagitis was the most diagnosed esophageal lesion, reported in 70.6% (16/23) of cases and 88.9% (8/9) of controls. Reflux esophagitis was only reported by one case and three controls. In cases, 63.6% (21/33), 18.2% (6/23) and 0.6% (2/33) were ever diagnosed

with chronic gastritis, chronic atrophic gastritis and gastric ulcer, respectively. In controls, 70.5% (55/78), 7.7% (6/78) and 12.8% (10/78) of subjects were ever diagnosed with chronic gastritis, chronic atrophic gastritis and gastric ulcer, respectively. In general, medical history of gastric lesion was reported in 17.5% of cases and 20.7% of controls, with no significant difference.

4.1.3 Dietary factors

Table 4.3 presents dietary behaviors in ESCC cases and controls. Significant differences between cases and controls were observed in dietary habits, including both eating on time and eating speed. Compared with controls, significantly higher percentage of cases favored salty and spicy food now, as well as 10 years ago. Substantial agreements were detected in both cases and controls between current and 10-years-ago's preference for salty food (cases: κ=0.81; controls: κ=0.91), and between current and 10-years-ago's preference for spicy food (cases: κ=0.75; controls: κ=0.85). Similar, more than 60% of cases favored hot food, significantly higher than the percentage in controls now, as well as 10 years ago. Almost perfect agreement was observed between current and 10-years-ago's preference for high temperature of food in both cases and controls (cases: κ=0.87; controls: κ=0.93). Additionally, frequent intake of special foods differed significantly between cases and controls, including garlic, pickled/salty vegetable, staple cooking oil, corn, and corn meal.

Table 4.1 Demographic and socioeconomic characteristics of cases and matched controls

Characteristic	Cases (n = 190)	Controls (n = 380)	P-value[a]	OR (95%CI) [b]
	No. (%)[c]			
Gender				
Male	111 (58.4)	222 (58.4)	1.00	1.0
Female	79 (41.6)	158 (41.6)		1.00 (0.70 – 1.43)
Educational attainment				
Illiterate	113 (59.5)	185 (48.8)	<0.001	1.00
Primary school	55 (29.0)	106 (28.0)		0.74 (0.47 – 1.15)
Middle school	17 (9.0)	64 (16.9)		0.30 (0.15 – 0.60)
High school and above	5 (2.6)	24 (6.3)		0.25 (0.09 – 0.72)
P for trend				<0.001
Marital Status				
Married	146 (76.8)	314 (82.9)	0.07	1.0
Not Married (divorced, widowed, single)	44 (23.2)	65 (17.2)		1.54 (0.96 – 2.47)
Ten-years-ago income, yuan/month				
< 50	52 (27.4)	108 (28.4)	0.01	1.0
50 – 100	43 (22.6)	127 (33.4)		0.73 (0.46 – 1.18)
≥ 100	95 (50.0)	145 (38.2)		1.44 (0.92 – 2.26)
P for trend				0.07
Current income, yuan/month				
< 100	56 (29.5)	75 (19.7)	0.02	1.0
100 – 300	82 (43.2)	175 (46.1)		0.63 (0.41 – 0.97)
≥ 300	52 (27.4)	130 (34.2)		0.50 (0.30 – 0.83)
P for trend				0.008
	Mean ± SD			
Age, years	62.0 ± 7.9	61.9 ± 7.8	0.85	1.00 (0.98 – 1.03)

[a] weighted t test of mean difference between cases and controls or conditional logistic regression of difference between cases and controls, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression or unconditional logistic regression with adjustment of age and gender, as appropriate.
[c] percentage may not add to 100 because of rounding.

Table 4.2 Health and medical history of disease in cases and matched controls

Characteristic	Cases (n = 190)	Controls (n = 380)	P-value[a]	OR (95%CI) [b]
	No. (%)[c]			
BMI, kg/m^2				
Underweight (<18.5)	38 (20.0)	26 (6.8)	<0.001	1.0
Normal (18.5 – 24.9)	134 (70.5)	258 (67.9)		0.34 (0.19 – 0.60)
Overweight (≥25)	18 (9.5)	96 (25.3)		0.12 (0.06 – 0.25)
P for trend				<0.001
History of esophageal lesion				
No	166 (87.8)	368 (97.6)	<0.001	1.0
Yes	23 (12.2)	9 (2.4)		5.47 (2.44 – 12.27)
History of gastric lesion				
No	156 (82.5)	299 (79.3)	0.38	1.0
Yes	33 (17.5)	78 (20.7)		0.82 (0.53 – 1.28)
	Mean ± *SD*			
Weight, kg	56.1 ± 10.7	58.6 ± 10.6	0.007	0.97 (0.96 – 0.99)
Height, cm	162.0 ± 7.7	159.6 ± 8.3	<0.001	1.07 (1.04 – 1.10)
BMI, kg/m^2	21.4 ± 3.6	23.0 ± 3.5	<0.001	0.87 (0.82 – 0.92)

[a, b] p value and odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression.

[c] percentage may not add to 100 because of rounding.

Table 4.3 Dietary behaviors of cases and matched controls

Characteristic	Cases (n = 190)	Controls (n = 380)	P-value[a]	OR (95%CI) [b]
Eating on time				
No	20 (10.6) [c]	19 (5.0)	0.02	1.0
Yes	169 (89.4)	360 (95.0)		0.47 (0.25 – 0.89)
Eating speed				
Not fast	131 (70.1)[d]	290 (78.0)	0.03	1.0
Fast	56 (30.0)	82 (22.0)		1.53 (1.04 – 2.24)
Preference for Salty food				
No	113 (59.5)	270 (71.2)	0.005	1.0
Yes	77 (40.5)	109 (28.8)		1.71 (1.18 – 2.50)
Preference for spicy food				
No	135 (71.1)	299 (78.7)	<0.050	1.0
Yes	55 (29.0)	81 (21.3)		1.48 (1.00 – 2.19)
Preference for sour food				
No	165 (86.8)	338 (89.0)	0.44	1.0
Yes	25 (13.2)	42 (11.1)		1.24 (0.71 – 2.17)
Preference for sweet food				
No	138 (72.6)	246 (64.7)	0.06	1.0
Yes	52 (27.4)	134 (35.3)		0.69 (0.47 – 1.01)
Preference for Salty food (10-years-ago)				
No	101 (53.2)	264 (69.7)	<0.001	1.0
Yes	89 (46.8)	115 (30.3)		2.06 (1.42 – 2.99)
Preference for spicy food (10-years-ago)				
No	124 (65.3)	291 (76.6)	0.004	1.0
Yes	66 (34.7)	89 (23.4)		1.81 (1.21 – 2.70)
Preference for high temperature of food				
No	74 (39.0)	231 (61.0)	<0.001	1.0
Yes	116 (61.1)	148 (39.1)		2.38 (1.66 – 3.42)
Preference for high temperature food (10-years-ago)				
No	74 (39.0)	233 (61.5)	<0.001	1.0
Yes	116 (61.1)	146 (38.5)		2.34 (1.64 – 3.32)

Table 4.3 continued

Characteristic	Cases (n = 190)	Controls (n = 380)	P-value[a]	OR (95%CI) [b]
Garlic intake				
Infrequent (occasional or never)	157 (82.6)	252 (67.6)	<0.001	1.0
Frequent	33 (17.4)	121 (32.4)		0.44 (0.29 – 0.68)
Onion intake				
Infrequent (occasional or never)	178 (93.7)	344 (92.5)	0.48	1.0
Frequent	12 (6.3)	28 (7.5)		0.77 (0.38 – 1.59)
Soy bean product intake				
Infrequent (occasional or never)	56 (29.5)	102 (27.5)	0.64	1.0
Frequent	134 (70.5)	269 (72.5)		0.91 (0.62 – 1.35)
Pickled/Salty food intake				
Infrequent	97 (51.1)	266 (70.0)	<0.001	1.0
Frequent	93 (49.0)	114 (30.0)		2.26 (1.56 – 3.27)
Staple cooking oil				
Vegetable oil	100 (52.6)	279 (73.4)	<0.001	1.0
Lard	90 (47.4)	101 (26.6)		2.63 (1.78 – 3.89)
Corn intake				
No	142 (75.1)	332 (87.4)	<0.001	1.0
Yes	47 (24.9)	48 (12.6)		2.43 (1.50 – 3.91)
Corn meal intake				
No	50 (26.3)	134 (35.3)	0.03	1.0
Yes	140 (73.7)	246 (64.7)		1.51 (1.03 – 2.21)
Corn meal intake, gram/day				
No	50 (26.3)	134 (35.3)	0.10	1.0
0 – 50	65 (34.2)	110 (29.0)		1.55 (1.00 – 2.41)
≥ 50	75 (39.5)	136 (35.8)		1.47 (0.95 – 2.28)
P for trend				0.08

[a, b] p value and odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression.
[c] Number (%).
[d] percentage may not add to 100 because of rounding.

4.1.4 Staple food storage and mildew food intake

Table 4.4 presents food storage activities and intake of mildewed food. Current intake of mildewed food was reported in 14.2% cases and 8.7% controls, respectively, showing a significant difference. The difference was more obvious regarding 10-years-ago's intake of mildewed food, with positive response in 17.9% of cases and 8.4% of controls, respectively. A moderate agreement between current and 10-years-ago's intake of mildewed food was observed in both cases and controls (cases: κ=0.59; controls: κ=0.51). Cases and controls also differed significantly in whether using freezer for food storage. We did not find significant differences for clean-up status, interval of utensils for food storage and storage time of grain.

Table 4.4 Food storage habits and mildewed food intake of cases and matched controls

Characteristic	Cases (n = 190)	Controls (n = 380)	P-value[a]	OR (95%CI) [b]
	No. (%)[c]			
Clean-up of food storage utensils				
No	79 (41.8)	158 (42.1)	0.85	1.0
Yes	110 (58.2)	217 (57.9)		1.03 (0.72 – 1.49)
Mildewed food intake				
No	163 (85.8)	347 (91.3)	0.04	1.0
Yes	27 (14.2)	33 (8.7)		1.79 (1.02 – 3.12)
Mildewed food intake (10-years-ago)				
No	156 (82.1)	348 (91.6)	0.002	1.0
Yes	34 (17.9)	32 (8.4)		2.29 (1.37 – 3.82)
Use of freezer for food storage				
No	151 (80.8)	247 (65.7)	<0.001	1.0
Yes	36 (19.3)	129 (34.3)		0.48 (0.32 – 0.74)
	Mean ± *SD*			
Storage time of staple grain, month	8.1 ± 4.4	7.8 ± 5.1	0.49	1.01 (0.98 – 1.05)
Interval of utensil clean-up, month	5.9 ± 4.3	6.5 ± 5.1	0.33	0.98 (0.93 – 1.02)

[a, b] p value and odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression.

[c] percentage may not add to 100 because of rounding.

4.1.5 Alcohol drinking

Table 4.5 presents the ORs and 95% CIs for ESCC and alcohol drinking characteristics. No significant difference was observed between cases and controls regarding drinking status, along with all investigated drinking-related factors, including drinking start age, duration, intensity, and estimated cumulative consumption of ethanol. As shown in Table 4.6 and 4.7, drinking-related parameters differed between males and females, while no significant association with ESCC risk was found in both males and females.

4.1.6 Tobacco smoking

Table 4.8 presents the ORs and 95%CIs for ESCC and tobacco smoking characteristics. No significant difference was observed between cases and controls in smoking status, along with all investigated drinking-related factors, including smoking start age, duration, intensity of smoking (total tobacco, or cigarette only), and estimated cumulative dose of tobacco or cigarette only.

Table 4.9 and 4.10 present tobacco smoking-related measurements in male and female subjects, respectively. No significant association was found for these factors in both males and females.

Table 4.5 Alcohol drinking status in cases and matched controls

Variables	Cases (n = 190)	Controls (n = 380)	P-value[a]	OR (95%CI) [b]
	No. (%)[c]			
Drinking status				
Never drinker	102 (53.7)	196 (51.6)	0.54	1.0
Drinker	88 (46.3)	184 (48.4)		0.87 (0.56 – 1.36)
Drinking start age (years)				
Never drinker	102 (53.7)	196 (52.0)	0.20	1.0
≤ 25	40 (21.1)	102 (27.1)		0.70 (0.41 – 1.21)
>25	48 (25.3)	79 (21.0)		1.13 (0.68 – 1.89)
Drinking duration (years)	`			
Never drinker	102 (53.7)	194 (51.6)	0.09	1.0
≤ 30	50 (26.3)	79 (21.0)		1.14 (0.68 – 1.91)
>30	38 (20.0)	103 (27.4)		0.64 (0.37 – 1.10)
Drinking intensity (ethanol, gram/week)				
Never drinker	102 (54.3)	196 (52.0)	0.45	1.0
≤400	36 (19.2)	89 (23.6)		0.75 (0.45 – 1.27)
>400	50 (26.6)	92 (24.4)		1.02 (0.60 – 1.74)
Cumulative consumption (ethanol, kg-years)				
Never drinker	102 (54.3)	196 (52.0)	0.82	1.0
≤1.9	42 (22.3)	91 (24.1)		0.85 (0.51 – 1.41)
>1.9	44 (23.4)	90 (23.9)		0.89 (0.51 – 1.56)
	Mean ± SD			
Drinking start age, years	29.1 ± 8.7	28.4 ± 10.1	0.48	1.01 (0.98 – 1.04)
Drinking duration (years)	29.1 ± 11.2	30.9 ± 12.0	0.21	0.99 (0.96 – 1.01)
Drinking intensity, gram/week	601.5 ± 462.0	567.7 ± 615.0	0.80	1.00 (1.00 – 1.00)
Cumulative consumption, kg-years	2.6 ± 2.4	2.9 ± 3.3	0.36	0.96 (0.87 – 1.05)

[a, b] p value and odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression.

[c] percentage may not add to 100 because of rounding.

Table 4.6 Alcohol drinking status in male cases and controls

Variables	Cases (n = 190)	Controls (n = 380)	P-value[a]	OR (95%CI) [b]
Drinking status				
Never drinker	31 (27.9)	60 (27.0)	0.88	1.0
Drinker	80 (72.1)	162 (73.0)		0.96 (0.58 – 1.60)
Drinking start age (years)				
Never drinker	31 (27.9)	60 (27.0)	0.19	1.0
≤ 25	38 (34.2)	96 (43.6)		0.77 (0.43 – 1.36)
>25	42 (37.8)	64 (29.1)		1.27 (0.71 – 2.27)
Drinking duration (years)	`			
Never drinker	31 (27.9)	60 (27.3)	0.11	1.0
≤ 30	44 (39.6)	66 (30.0)		1.35 (0.75 – 2.43)
>30	36 (32.4)	94 (42.7)		0.74 (0.41 – 1.31)
Drinking intensity (ethanol, gram/week)				
Never drinker	31 (28.2)	60 (27.3)	0.62	1.0
≤400	30 (27.3)	71 (32.3)		0.82 (0.45 – 1.51)
>400	49 (44.6)	89 (40.5)		1.07 (0.61 – 1.88)
Cumulative consumption (ethanol, kg-years)				
Never drinker	31 (28.2)	60 (27.3)	0.95	1.0
≤1.9	36 (32.7)	70 (31.8)		1.00 (0.55 – 1.81)
>1.9	43 (38.1)	90 (40.9)		0.93 (0.53 – 1.64)

[a, b] p value and odds ratio (OR) and 95% confidence interval (CI) were calculated using unconditional logistic regression with adjustment of age.
[c] percentage may not add to 100 because of rounding.

Table 4.7 Alcohol drinking status in female cases and controls

Variables	Cases (n = 190)	Controls (n = 380)	P-value[a]	OR (95%CI) [b]
Drinking status				
Never drinker	71 (89.9)	136 (86.1)	0.41	1.0
Drinker	8 (10.1)	22 (13.9)		0.67 (0.30 – 1.64)
Drinking start age (years)				
Never drinker	71 (89.9)	136 (86.6)	0.76	1.0
≤ 25	2 (2.5)	6 (3.8)		0.64 (0.12 – 3.25)
>25	6 (7.6)	15 (9.6)		0.77 (0.29 – 2.06)
Drinking duration (years)	`			
Never drinker	71 (89.9)	136 (86.6)	0.64	1.0
≤ 30	6 (7.6)	13 (8.3)		0.89 (0.32 – 2.44)
>30	2 (2.5)	8 (5.1)		0.48 (0.10 – 2.31)
Drinking intensity (ethanol, gram/week)				
Never drinker	71 (91.0)	136 (86.6)	0.62	1.0
≤400	6 (7.7)	18 (11.5)		0.64 (0.24 – 1.68)
>400	1 (1.3)	3 (1.9)		0.64 (0.07 – 6.26)
Cumulative consumption (ethanol, kg-years)				
Never drinker	71 (91.0)	136 (86.6)	0.46	1.0
≤1.9	6 (7.7)	21 (13.4)		0.55 (0.21 – 1.42)
>1.9	1 (1.3)	0 (0.0)		-

[a, b] p value and odds ratio (OR) and 95% confidence interval (CI) were calculated using unconditional logistic regression with adjustment of age.
[c] percentage may not add to 100 because of rounding.

Table 4.8 Tobacco smoking status in cases and matched controls

Variables	Cases (n = 190)	Controls (n = 380)	P-value[a]	OR (95%CI) [b]
	No. (%)[c]			
Smoking status				
Non-smoker	75 (39.5)	155 (40.9)	0.71	1.0
Smoker	115 (60.5)	224 (59.1)		1.07 (0.74 – 1.56)
Smoking starting age (years)				
Non-smoker	75 (39.5)	155 (41.0)	0.46	1.0
≤ 25	58 (30.5)	128 (33.9)		0.92 (0.58 – 1.46)
>25	57 (30.0)	95 (25.1)		1.21 (0.80 – 1.84)
Smoking duration (years)				
Non-smoker	75 (39.5)	155 (40.9)	0.65	1.0
≤ 30	46 (24.2)	80 (21.1)		1.22 (0.76 – 1.96)
>30	69 (36.3)	144 (38.0)		0.97 (0.63 – 1.51)
Tobacco smoking intensity (cigarettes/day)				
Non-smoker	75 (39.5)	155 (40.9)	0.82	1.0
<20	49 (25.8)	89 (23.5)		1.15 (0.73 – 1.79)
≥20	66 (34.7)	135 (35.6)		1.02 (0.67 – 1.56)
Cumulative dose (pack-years, total tobacco)				
Non-smoker	75 (39.5)	155 (41.0)	0.86	1.0
≤ 30	62 (32.6)	115 (30.4)		1.12 (0.74 – 1.70)
>30	53 (27.9)	108 (18.6)		1.03 (0.65 – 1.63)
Passive smoking (source: family members)				
No	85 (44.7)	191 (50.4)	0.20	1.0
Yes	105 (55.3)	188 (49.6)		1.27 (0.88 – 1.83)
	Mean ± SD			
Smoking starting age (years)	28.6 ± 9.1	27.0 ± 9.8	0.12	1.02 (1.00 – 1.05)
Smoking duration (years)	33.2 ± 11.7	34.0 ± 11.6	0.17	0.98 (0.96 – 1.01)
Smoking intensity, cigarettes/day	23.1 ± 25.5	26.1 ± 35.9	0.42	1.00 (0.99 – 1.01)
Cumulative dose , pack-years	39.6 ± 47.4	47.0 ± 66.5	0.25	1.00 (0.99 – 1.00)

[a, b] p value and odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression.

[c] percentage may not add to 100 because of rounding.

Table 4.9 Tobacco smoking status in male cases and controls

Variables	Cases (n = 190)	Controls (n = 380)	P-value[a]	OR (95%CI) [b]
Smoking status				
Non-smoker	26 (23.4)	59 (26.6)	0.54	1.0
Smoker	85 (76.6)	163 (73.4)		1.18 (0.69 – 2.01)
Smoking starting age (years)				
Non-smoker	26 (23.4)	59 (26.6)	0.60	1.0
≤ 25	52 (46.9)	108 (48.7)		1.09 (0.62 – 1.92)
>25	33 (29.7)	55 (24.8)		1.36 (0.72 – 2.56)
Smoking duration (years)				
Non-smoker	26 (23.4)	59 (26.6)	0.45	1.0
≤ 30	30 (27.0)	48 (21.6)		1.48 (0.76 – 2.87)
>30	55 (49.6)	115 (51.8)		1.06 (0.60 – 1.87)
Tobacco smoking intensity (cigarettes/day)				
Non-smoker	26 (23.4)	59 (26.6)	0.50	1.0
<20	32 (28.8)	51 (23.0)		1.42 (0.75 – 2.69)
≥20	53 (47.8)	112 (50.5)		1.07 (0.61 – 1.89)
Cumulative dose (pack-years, total tobacco)				
Non-smoker	26 (23.4)	59 (26.6)	0.77	1.0
≤ 30	40 (36.0)	73 (32.9)		1.25 (0.68 – 2.28)
>30	45 (40.5)	90 (40.5)		1.13 (0.63 – 2.02)
Passive smoking (source: family members)				
No	54 (48.7)	120 (54.1)	0.36	1.0
Yes	57 (51.4)	102 (46.0)		1.24 (0.79 – 1.96)

[a, b] p value and odds ratio (OR) and 95% confidence interval (CI) were calculated using unconditional logistic regression with adjustment of age.
[c] percentage may not add to 100 because of rounding.

Table 4.10 Tobacco smoking status in female cases and controls

Variables	Cases (n = 190)	Controls (n = 380)	P-value[a]	OR (95%CI) [b]
Smoking status				
Non-smoker	49 (62.0)	96 (61.2)	0.91	1.0
Smoker	30 (38.0)	61 (38.9)		0.97 (0.55 – 1.70)
Smoking starting age (years)				
Non-smoker	49 (62.0)	96 (61.5)	0.43	1.0
≤ 25	6 (7.6)	20 (12.8)		0.59 (0.22 – 1.57)
>25	24 (30.4)	40 (25.6)		1.18 (0.64 – 2.21)
Smoking duration (years)				
Non-smoker	49 (62.0)	96 (61.2)	0.99	1.0
≤ 30	16 (20.3)	32 (20.4)		0.98 (0.49 – 1.96)
>30	14 (17.7)	29 (18.5)		0.95 (0.44 – 2.04)
Tobacco smoking intensity (cigarettes/day)				
Non-smoker	49 (62.0)	96 (61.2)	0.87	1.0
<20	17 (21.5)	38 (24.2)		0.88 (0.45 – 1.73)
≥20	13 (16.5)	23 (14.7)		1.11 (0.51 – 2.41)
Cumulative dose (pack-years, total tobacco)				
Non-smoker	49 (62.0)	96 (61.5)	0.95	1.0
≤ 30	22 (27.9)	42 (26.9)		1.03 (0.55 – 1.91)
>30	8 (10.1)	18 (11.5)		0.87 (0.35 – 2.19)
Passive smoking (source: family members)				
No	31 (39.2)	71 (45.2)	0.38	1.0
Yes	48 (60.8)	86 (54.8)		1.28 (0.74 – 2.23)

[a, b] p value and odds ratio (OR) and 95% confidence interval (CI) were calculated using unconditional logistic regression with adjustment of age.
[c] percentage may not add to 100 because of rounding.

4.1.7 Tea drinking

Table 4.11 presents the ORs and 95% CIs for ESCC and tea smoking characteristics. There were 21.1% of cases and 34.1% of controls defined as regular tea drinkers. A statistically significant inverse association between tea drinking status and ESCC risk was observed. In subjects drinking tea regularly for at least 6 month during their lifetime (tea drinker), green tea was the most common type consumed, reported in 91.4% of cases and 91.7% controls. No significant difference was observed between cases and controls in tea drinking start age, duration, intensity, and preference for thickness of tea. In subjects drinking tea regularly, 48.7% of cases and 27.3% of controls favored hot tea, respectively, showing a significant difference.

4.1.8 Family history of cancer

Table 4.12 presents family history of cancer in cases and controls. There were 55.3% of cases and 42.1% of controls reported cancer history in their relatives, showing a significant difference. The difference was more obvious if limited to cancer history in first-degree relatives, as well as family history of the specific cancer type, i.e. esophageal cancer. Among subjects having family history of cancer, the most common type of cancer reported was esophageal cancer (66% of cases with family history of cancer, 58% of controls with family history of cancer), followed by stomach cancer (21% of cases with family history of cancer, 21% of controls with family history of cancer) and cardia cancer (9% of cases with family history of cancer, 8% of controls with family history of cancer).

Table 4.11 Tea drinking status in cases and matched controls

Variables	Cases (n = 190)	Controls (n = 380)	P-value[a]	OR (95%CI) [b]
	No. (%)[c]			
Tea drinking status				
No	150 (79.0)	249 (65.9)	<0.01	1.0
Yes	40 (21.1)	129 (34.1)		0.44 (0.27 – 0.70)
Tea drinking start age (years)				
< 40	22 (61.1)	57 (49.1)	0.31	1.0
≥40	14 (38.9)	59 (50.9)		0.67 (0.31 – 1.46)
Tea drinking duration (years)				
< 23	17 (47.2)	55 (48.3)	0.60	1.0
≥23	19 (52.8)	59 (51.8)		0.81 (0.37 – 1.78)
Preference for thickness of tea				
Light/moderate tea	23 (59.0)	77 (59.7)	0.79	1.0
Thick	16 (41.0)	52 (40.3)		1.11 (0.52 – 2.35)
Preference for temperature of tea				
Cold/warm	20 (51.3)	93 (72.7)	0.01	1.0
Hot tea	19 (48.7)	35 (27.3)		2.58 (1.21 – 5.50)
Tea drinking intensity (gram/month)				
≤100	19 (48.7)	76 (61.3)	0.30	1.0
>100	20 (51.3)	48 (38.7)		1.51 (0.72 – 3.15)
	Mean ± *SD*			
Tea drinking intensity, gram/month	169.9 ± 129.7	136.3 ± 98.3	0.20	1.00 (1.00 – 1.01)

[a, b] p value and odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression.

[c] percentage may not add to 100 because of rounding.

Table 4.12 Family history of cancer in cases and matched controls

Characteristic	Cases (n = 190)	Controls (n = 380)	P-value[a]	OR (95%CI) [b]
Family history of cancer				
No	85 (44.7) [c]	220 (57.9)	0.003	1.0
Yes	105 (55.3)	160 (42.1)		1.71 (1.20 – 2.43)
First-degree family history of cancer				
No	95 (50.0)	246 (64.7)	<0.001	1.0
Yes	95 (50.0)	134 (35.3)		1.88 (1.31 – 2.71)
Family history of esophageal cancer				
No	121 (63.7)	287 (75.5)	0.004	1.0
Yes	69 (36.3)	93 (24.5)		1.75 (1.20 – 2.56)
Family history of stomach cancer				
No	168 (88.4)	347 (91.3)	0.27	1.0
Yes	22 (11.6)	33 (8.7)		1.37 (0.78 – 2.42)
Family history of cardia cancer				
No	181 (95.3)	367 (96.6)	0.44	1.0
Yes	9 (4.7)	13 (3.4)		1.41 (0.59 – 3.37)
Number of family member with cancer history				
0	85 (44.7)	220 (57.9)	0.007	1.0
1	68 (35.8)	114 (30.0)		1.55 (1.05 – 2.30)
≥1 (2 or more)	37 (19.5)	46 (12.1)		2.10 (1.27 – 3.49)
P for trend				0.002

[a, b] p value and odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression.
[c] Number (%).

4.1.9 Multivariate analysis of questionnaire data

Table 4.13 presents the multivariate analysis result for ESCC risk and host factors investigated through questionnaire interview. After adjustment of potential confounding factors, significant increased risks of ESCC were found in subjects having medical history of esophageal lesion (OR=7.32, 95% CI: 2.58–20.78), eating fast (OR=1.71, 95% CI: 1.03–2.86), favoring salty food 10 years ago (OR=2.02, 95% CI: 1.23–3.30), favoring high temperature food 10 years ago (OR=2.28, 95% CI: 1.44–3.63), consuming pickled/salty food frequently (OR=2.42, 95% CI: 1.46–3.95), consuming mildewed food frequently (OR=2.80, 95% CI: 1.43–5.50), and having family history of cancer in first-degree relative(s) (OR=2.10, 95% CI: 1.29–3.41). Protective effects on ESCC risk were found in subjects consuming garlic frequently (OR=0.47, 95% CI: 0.27–0.81), drinking tea regularly (OR=0.35, 95% CI: 0.18–0.65), and having high BMI (OR=0.84, 95% CI: 0.78–0.90). No statistically significant effects were observed in alcohol drinking and tobacco smoking status.

Table 4.13 Multivariate conditional regression analysis of host factors and ESCC risk

Characteristics	*P*-value [a]	OR_{ad} (95%CI) [b]
Esophageal lesion	<0.001	7.32 (2.58 – 20.78)
Eating fast	0.04	1.71 (1.03 – 2.86)
Preference for salty food (10-years-ago)	0.005	2.02 (1.23 – 3.30)
Preference for high temperature food (10-years-ago)	<0.001	2.28 (1.44 – 3.63)
Frequent pickled/Salty food intake	<0.001	2.42 (1.46 – 3.95)
Mildewed food intake (10-years-ago)	0.003	2.80 (1.43 – 5.50)
First-degree family history of cancer	0.003	2.10 (1.29 – 3.41)
Alcohol drinking	0.93	0.97 (0.52 – 1.82)
Tobacco smoking	0.44	1.23 (0.74 – 2.07)
Frequent garlic intake	0.007	0.47 (0.27 – 0.81)
Tea drinking	0.001	0.35 (0.18 – 0.65)
BMI	<0.001	0.84 (0.78 – 0.90)

[a, b] p value and adjusted odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression.

4.2 Fumonsin exposure and ESCC risk

In this study, dietary fumonsin exposure level was evaluated by measuring urinary FB_1 biomarkers, including urinary free FB_1, Sa, So, and the Sa/So ratio.

4.2.1 Urinary free FB_1 and ESCC risk

Urinary free FB_1 was detectable in 95.8% (181/189) of ESCC cases and 84.2% (319/379) of controls, showing a significant difference ($p<0.001$).

As illustrated in Figure 4.1, urinary free FB_1 level (pg/mg creatinine) was significantly higher in cases (mean ± SD: 470.41 ± 1215.95, median: 176.13) than in controls (mean ± SD: 213.56 ± 437.75, median: 56.92) ($p<0.001$). Gender difference was observed for urinary free FB_1 level in both cases ($p=0.008$) and controls ($p=0.003$), which female subjects had a significantly higher free FB_1 level than male subjects. In case group, the average levels (mean ± SD) of urinary free FB_1 were 337.91 ± 820.23 (median: 135.58) and 654.91 ± 1601.33 (median: 274.28) pg/mg creatinine in male and female subjects, respectively; in control group, the average levels of urinary free FB_1 were 167.98 ± 408.48 (median: 49.80) and 277.32 ± 469.60 (median: 80.37) pg/mg creatinine in male and female subjects, respectively. No age difference of urinary free FB_1 was found in both case and control group.

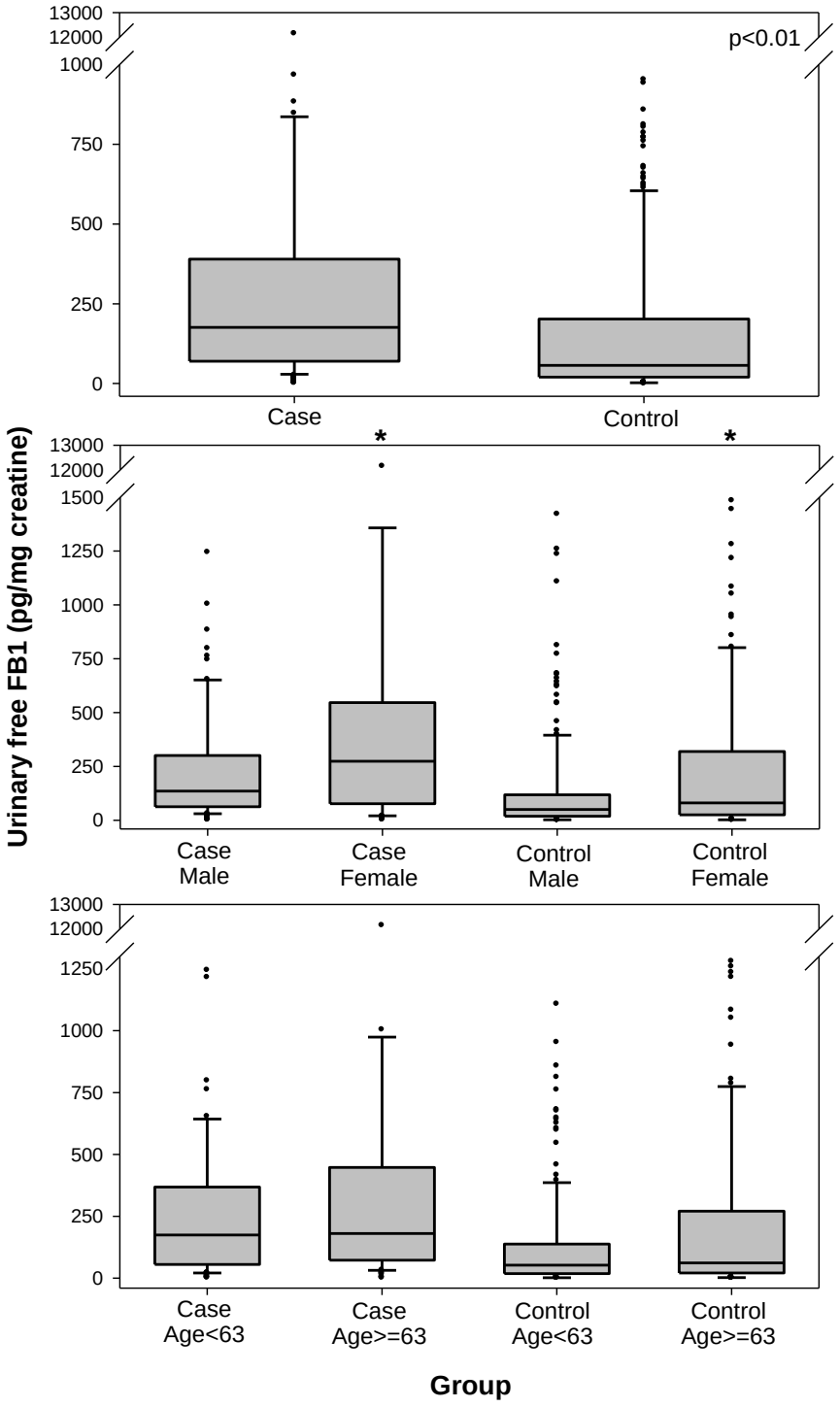

Figure 4.1 Urinary free FB_1 concentrations (pg/mg creatinine) in esophageal squamous cell carcinoma cases and controls. The dots, error bars and upper and lower ends of the box represent outliers, spread, and first and third quartiles, respectively. Star (*) mark represents p value less than 0.05 in comparison between males and females within cases or controls through Wilcoxon signed-rank test.

Table 4.14 presents the ORs and 95% CIs of urinary free FB_1 for ESCC risk. Significant associations were observed between urinary free FB_1 and ESCC risk, with or without adjustment of possible confounding factors. The associations remained significant after gender stratification. No interaction of urinary free FB_1 levels and gender was found.

We assessed the correlation between urinary free FB_1 levels and intake of corn meal. As illustrated in Figure 4.2, in subjects consumed corn meal, Significant correlations between urinary free FB_1 concentration and estimated corn meal intake (gram/week) were found in both controls ($r_{Spearman} = 0.20$, N = 245, $p<0.01$) and cases ($r_{Spearman} = 0.22$, N = 140, $p<0.01$). After stratified by gender, the correlation remained significantly in female controls, male cases and controls. We also examined the correlation between urinary free FB_1 and corn intake (gram/week) and found no significant results.

4.2.2 Urinary sphingolipids metabolites (Sa, So, and Sa/So ratio) and ESCC risk

Figure 4.3 shows urinary Sa level in cases and controls. The mean ± SD level of urinary Sa in cases was 1.07 ± 3.77 pmol/ml, with a median value of 0.20 pmol/ml. This did not differ significantly from urinary Sa level in controls (mean ± SD: 1.65 ± 14.44 pmol/ml, median: 0.19). In both case and control groups, urinary Sa level in females was significantly higher than in males ($p<0.001$ in both cases and controls). The average urinary Sa levels (pmol/ml) were 0.84 ± 3.85 (median: 0.11), 1.39 ± 3.66 (median: 0.61), 1.96 ± 18.71 (median: 0.13), 1.21 ± 3.16 (median: 0.47) in male cases, female cases, male controls and female controls, respectively. No age difference was observed in case and control groups.

Table 4.14 Esophageal squamous cell carcinoma risk by tertile of urinary free FB_1

	Category					*P* for trend
	Low	Moderate		High		
		OR	95% CI	OR	95% CI	
Range, pg/mg creatinine	<29.60	29.60 – 116.58		≥116.58		
Cases [No. (%)][a]	19 (10.1)	50 (26.5)		120 (63.5)		
Controls [No. (%)]	126 (33.3)	126 (33.3)		127 (33.5)		
Odds ratio	1	2.61	1.47 – 4.64	6.39	3.63 – 11.24	<0.001
Adjusted odds ratio[b]	1	5.21	2.40 – 11.33	9.51	4.33 – 20.87	<0.001
Gender						
Male (*n* = 333)						
Cases [No. (%)]	10 (9.1)	35 (31.8)		65 (59.1)		
Controls [No. (%)]	85 (38.5)	78 (35.3)		58 (26.2)		
Adjusted odds ratio[c]	1	6.72	2.61 – 17.29	14.63	5.76 – 37.18	<0.001
Female (*n* = 237)						
Cases [No. (%)][a]	9 (11.4)	15 (19.0)		55 (69.6)		
Controls [No. (%)]	41 (26.0)	48 (30.4)		69 (43.7)		
Adjusted odds ratio[c]	1	2.62	0.89 – 7.77	4.08	1.59 – 10.46	0.007
P Interaction						0.28

[a] percentage may not add to 100 because of rounding.
[b] conditional logistic regression with adjustment for possible confounding factors.
[c] logistic regression with adjustment for age and possible confounding factors.

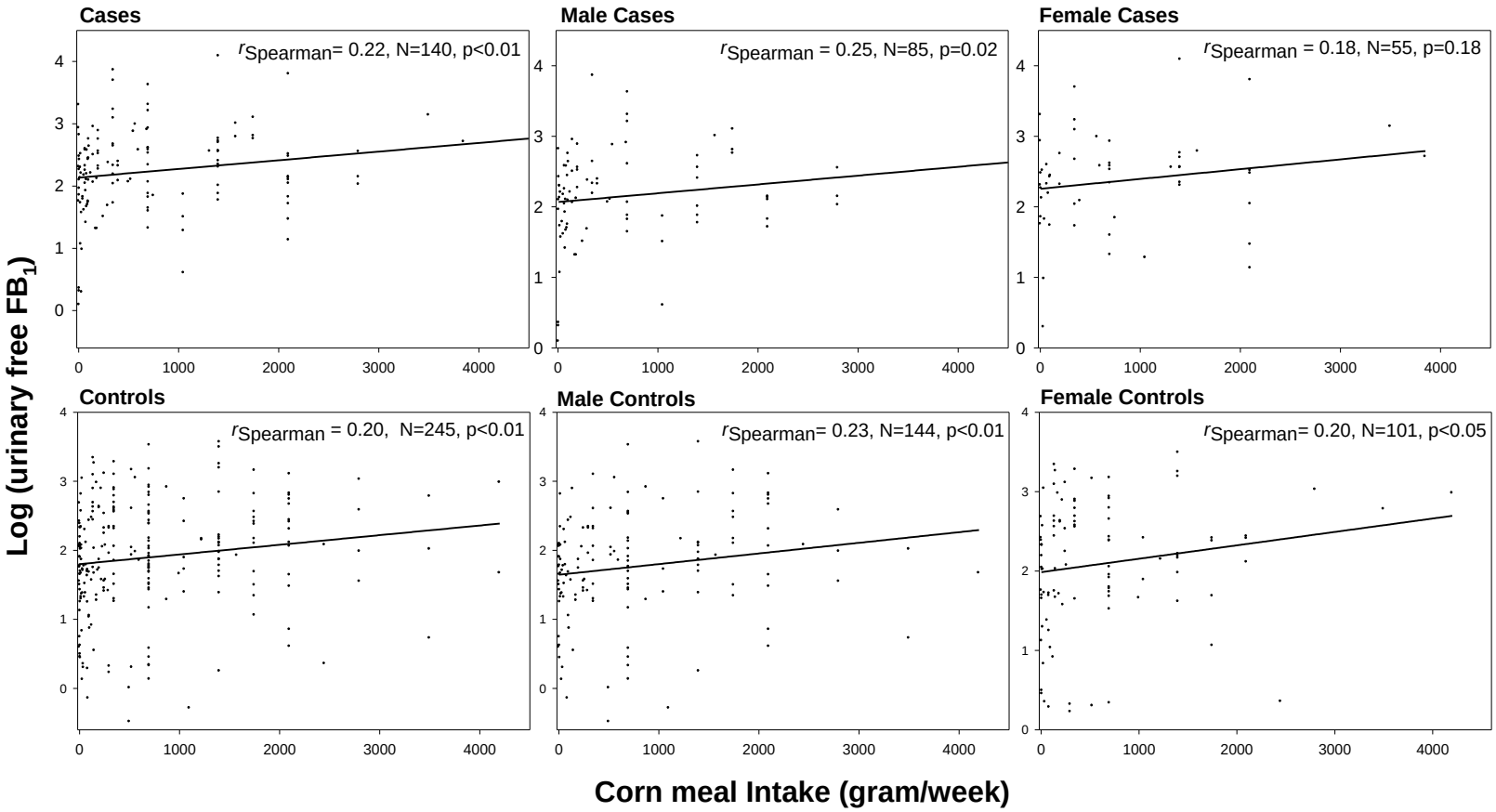

Figure 4.2 Correlation of corn meal intake (gram/week) and logarithm of urinary free FB_1 concentration [Log(pg/mg creatinine)] in esophageal squamous cell carcinoma cases and controls who consumed corn meal. P values were obtained using Spearman rank correlation test.

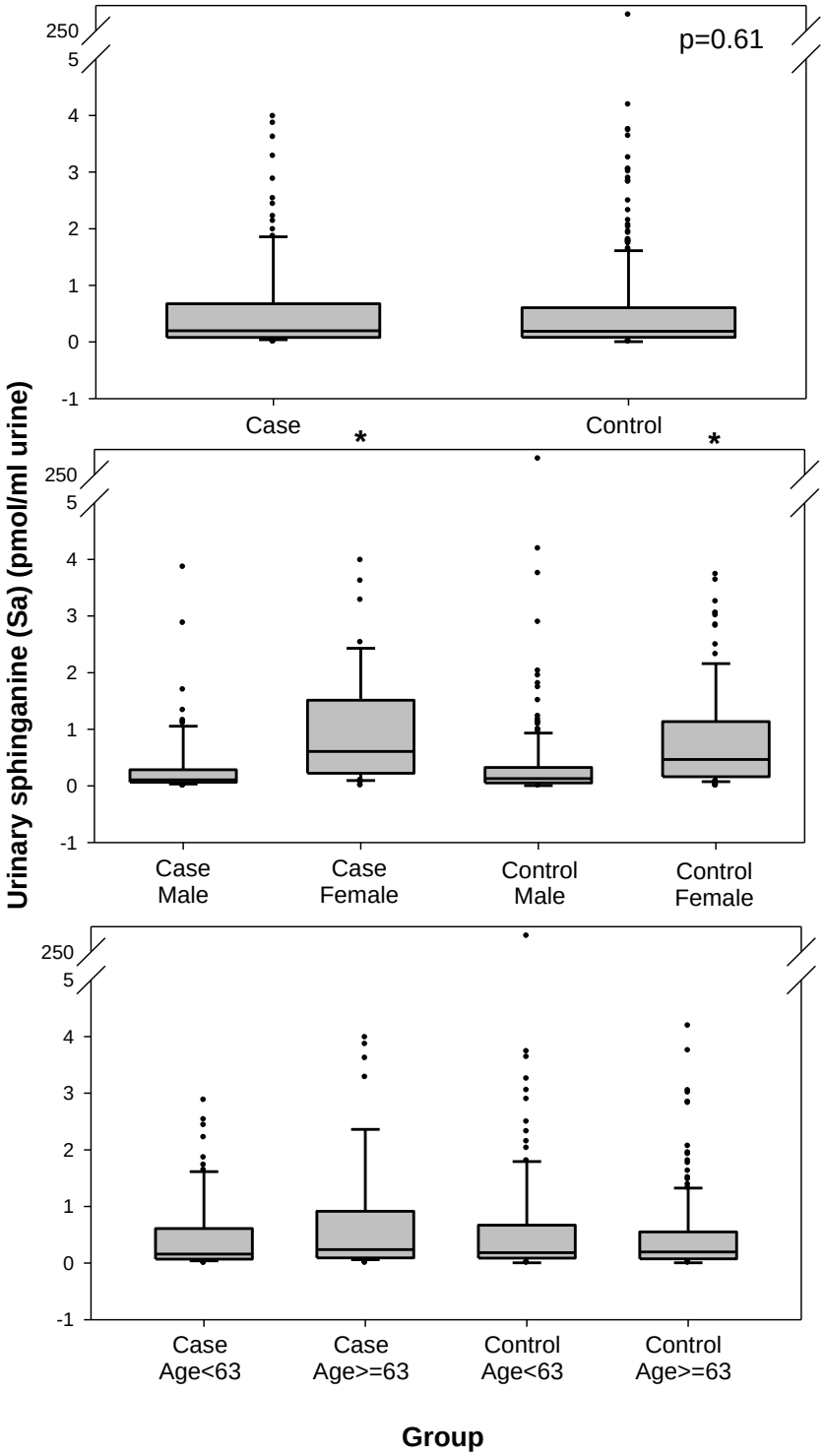

Figure 4.3 Urinary Sa (pmol/ml) in esophageal squamous cell carcinoma cases and controls. The dots, error bars and upper and lower ends of the box represent outliers, spread, and first and third quartiles, respectively. Star (*) mark represents p value less than 0.05 in comparison between males and females within cases or controls through Wilcoxon signed-rank test.

Figure 4.4 shows urinary So level in cases and controls. The mean ± SD level of urinary So in cases and controls were 2.62 ± 4.37 pmol/ml (median: 0.96) and 2.71 ± 6.39 pmol/ml (median: 1.10), respectively, showing no significant difference. In both case and control groups, urinary So level in females was significantly higher than males ($p<0.001$ in both cases and controls). The average urinary So levels (pmol/ml) were 1.48 ± 2.45 (median: 0.61), 4.21 ± 5.76 (median: 2.17), 1.64 ± 2.79 (median: 0.74), 4.21 ± 9.15 (median: 2.00) in male cases, female cases, male controls and female controls, respectively. No age differences in case and control group were found.

As shown in Figure 4.5, no significant difference of urinary Sa/So ratio was observed in cases (mean ± SD: 0.72 ± 2.90, median: 0.23) and controls (mean ± SD: 1.09 ± 5.41, median: 0.19). The urinary Sa/so ratio was significantly higher in females than males in both cases ($p<0.001$) and controls ($p<0.001$). In cases, the average Sa/So ratios were 0.90 ± 3.60 (median: 0.18) in male subjects and 0.47 ± 1.43 (median: 0.27) in female subjects; in controls, the average Sa/So ratios were 1.32 ± 6.53 (median: 0.17) in male subjects and 0.77 ± 3.25 (median: 0.23) in female subjects. There were no age difference of Sa/So ratio in both cases and controls.

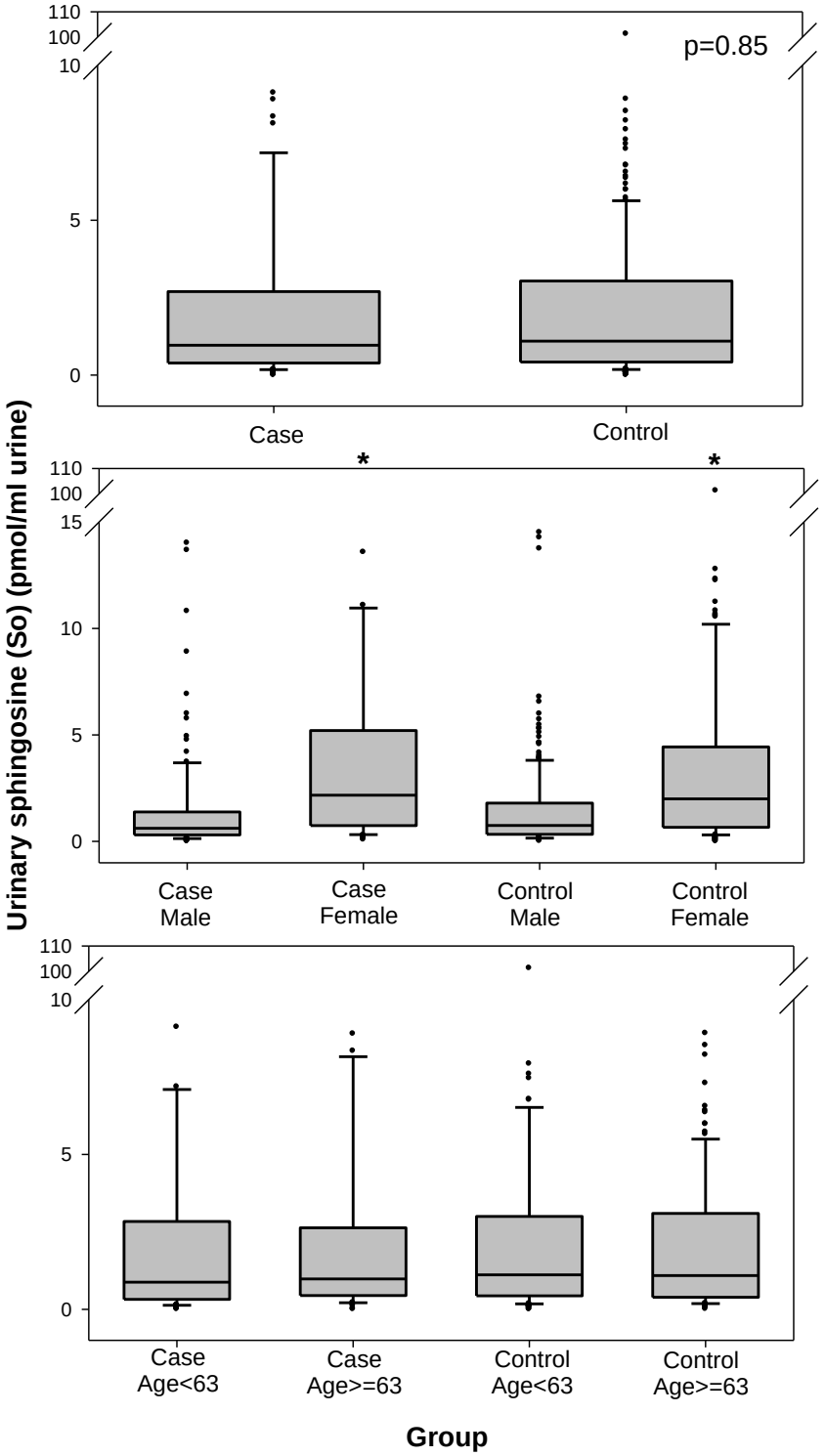

Figure 4.4 Urinary So (pmol/ml) in esophageal squamous cell carcinoma cases and controls. The dots, error bars and upper and lower ends of the box represent outliers, spread, and first and third quartiles, respectively. Star (*) mark represents p value less than 0.05 in comparison between males and females within cases or controls through Wilcoxon signed-rank test.

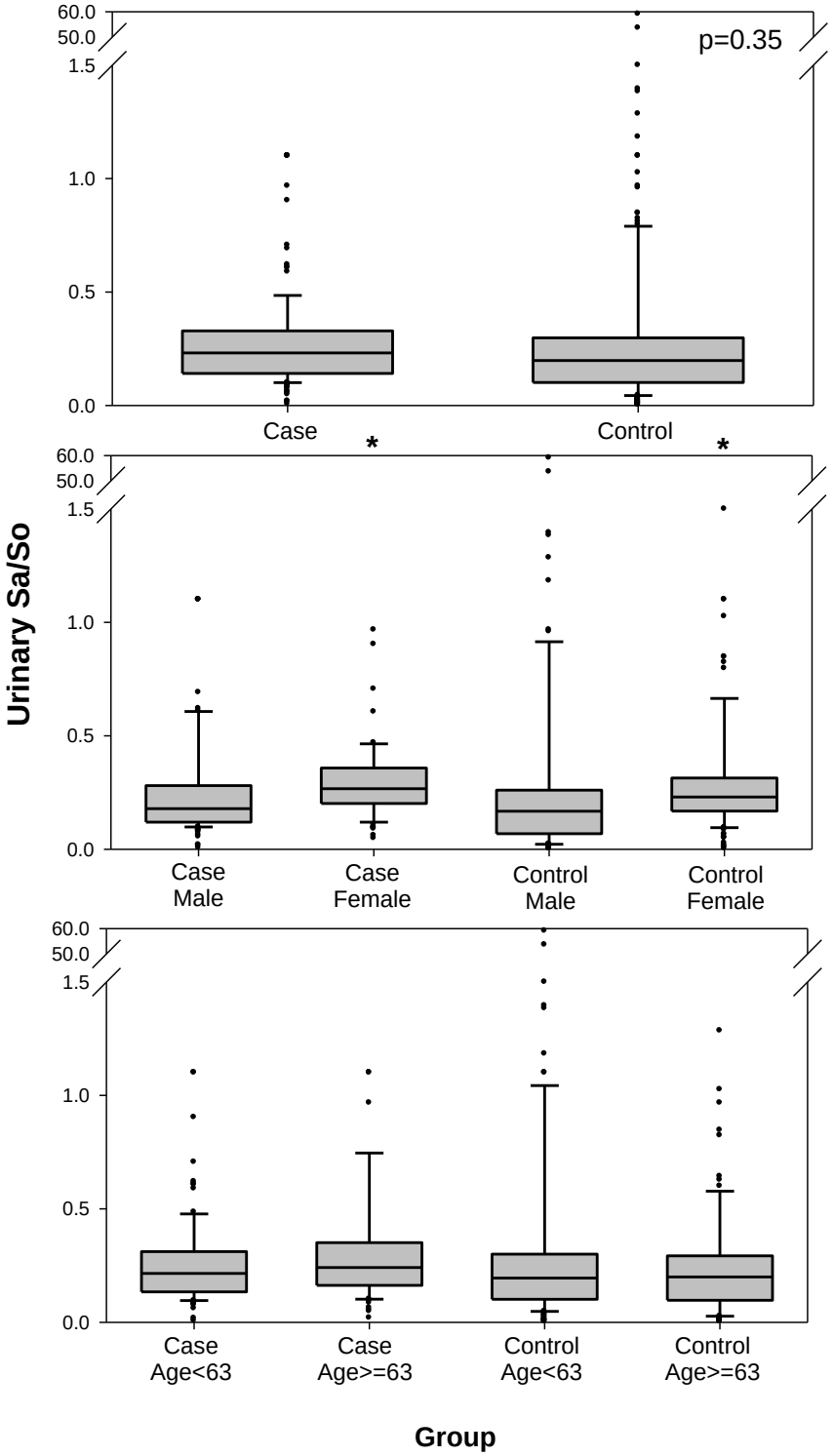

Figure 4.5 Urinary Sa/So ratio in esophageal squamous cell carcinoma cases and controls. The dots, error bars and upper and lower ends of the box represent outliers, spread, and first and third quartiles, respectively. Star (*) mark represents p value less than 0.05 in comparison between males and females within cases or controls through Wilcoxon signed-rank test.

As shown in table 4.15-4.17, we estimated ORs and 95% CIs by tertile of urinary sphingolipids biomarkers, including Sa, So and Sa/So ratio, in control group. No significant associations were observed between ESCC risk and levels of urinary Sa, as well as So, with or without adjustment of possible confounding factors. Further analysis after stratification with gender also found no significant association. Significant association between urinary Sa/So ratio and esophageal cancer was observed with or without adjustment of confounding factors. The adjusted ORs (95% CI) for moderate vs. low and high vs. low urinary Sa/So ratio level were 1.54 (0.83 – 2.88) and 2.01 (1.05 – 3.84), respectively (p for trend=0.04). We also examined ORs and 95% CIs after stratification of gender. Significantly increased risks for ESCC was only observed in males with moderate Sa/So ratio level (OR_{ad}=2.00, 95% CI: 1.04 – 3.83).

We assessed the correlation between urinary Sa/So ratio and urinary free FB_1 concentration, finding no significant correlation in both cases ($r_{Spearman}$ = -0.03, N = 189, p = 0.64) and controls ($r_{Spearman}$ = -0.03, N = 279, p = 0.53). Also, no significant correlation was found after stratified by gender. No significant correlation between urinary Sa/So ratio and other factors, including estimated corn meal intake and corn intake, were found in both cases and controls with or without stratified by gender.

Table 4.15 Esophageal squamous cell carcinoma risk by tertile of urinary Sa

	Category					*P* for trend
	Low	Moderate		High		
		OR	95% CI	OR	95% CI	
Range, pg/mg creatinine	<0.12	0.12 – 0.44		≥0.44		
Cases [No. (%)][a]	65 (34.4)	56 (29.6)		68 (36.0)		
Controls [No. (%)]	132 (34.7)	123 (32.4)		125 (32.9)		
Odds ratio	1	0.92	0.57 – 1.46	1.11	0.68 – 1.80	0.48
Adjusted odds ratio[b]	1	0.87	0.47 – 1.60	1.19	0.63 – 2.27	0.40
Gender						
Male (*n* = 333)						
Cases [No. (%)]	56 (50.9)	31 (28.2)		23 (20.9)		
Controls [No. (%)]	103 (46.4)	77 (34.7)		42 (18.9)		
Adjusted odds ratio[c]	1	0.64	0.34 – 1.20	0.95	0.45 – 2.01	0.88
Female (*n* = 237)						
Cases [No. (%)][a]	9 (11.4)	25 (31.7)		45 (57.0)		
Controls [No. (%)]	29 (18.4)	46 (29.1)		83 (52.5)		
Adjusted odds ratio[c]	1	2.69	0.81 – 8.95	1.94	0.63 – 5.97	0.98
P Interaction						0.22

[a] percentage may not add to 100 because of rounding.
[b] conditional logistic regression with adjustment for possible confounding factors.
[c] unconditional logistic regression with adjustment for age and possible confounding factors.

Table 4.16 Esophageal squamous cell carcinoma risk by tertile of urinary So

	Category					*P* for trend
	Low	Moderate		High		
		OR	95% CI	OR	95% CI	
Range, pg/mg creatinine	<0.55	0.55 – 2.02		≥2.02		
Cases [No. (%)][a]	65 (34.4)	62 (32.8)		62 (32.8)		
Controls [No. (%)]	125 (32.9)	130 (34.2)		125 (32.9)		
Odds ratio	1	0.93	0.60 – 1.44	0.95	1	0.93
Adjusted odds ratio[b]	1	0.92	0.52 – 1.62	1.07	1	0.92
Gender						
Male (*n* = 333)						
Cases [No. (%)]	51 (46.4)	38 (34.6)		21 (19.1)		
Controls [No. (%)]	91 (41.0)	83 (37.4)		48 (21.6)		
Adjusted odds ratio[c]	1	0.69	0.37 – 1.25	0.82	0.38 – 1.75	0.70
Female (*n* = 237)						
Cases [No. (%)][a]	14 (17.7)	24 (30.4)		41 (51.9)		
Controls [No. (%)]	34 (21.5)	47 (29.8)		77 (48.7)		
Adjusted odds ratio[c]	1	1.27	0.45 – 3.61	1.46	0.57 – 3.73	0.47
P Interaction						0.44

[a] percentage may not add to 100 because of rounding.
[b] conditional logistic regression with adjustment for possible confounding factors.
[c] unconditional logistic regression with adjustment for age and possible confounding factors.

Table 4.17 Esophageal squamous cell carcinoma risk by tertile of urinary Sa/So ratio

	Category					*P* for trend
	Low	Moderate		High		
		OR	95% CI	OR	95% CI	
Range, pg/mg creatinine	<0.14	0.14 – 0.26		≥0.26		
Cases [No. (%)][a]	44 (23.3)	67 (35.5)		78 (41.3)		
Controls [No. (%)]	130 (34.2)	127 (33.4)		123 (32.4)		
Odds ratio	1	1.58	1.00 – 2.50	2.02	1	1.58
Adjusted odds ratio[b]	1	1.54	0.83 – 2.88	2.01	1	1.54
Gender						
Male (*n* = 333)						
Cases [No. (%)]	35 (31.8)	42 (38.2)		33 (30.0)		
Controls [No. (%)]	103 (46.4)	63 (28.4)		56 (25.2)		
Adjusted odds ratio[c]	1	2.00	1.04 – 3.83	1.53	0.78 – 3.00	0.24
Female (*n* = 237)						
Cases [No. (%)][a]	9 (11.4)	25 (31.7)		45 (57.0)		
Controls [No. (%)]	27 (17.1)	64 (40.5)		67 (42.4)		
Adjusted odds ratio[c]	1	0.75	0.24 – 2.41	1.12	0.36 – 3.51	0.46
P Interaction						0.19

[a] percentage may not add to 100 because of rounding.
[b] conditional logistic regression with adjustment for possible confounding factors.
[c] unconditional logistic regression with adjustment for age and possible confounding factors.

4.3 Urinary 8-OHdG and ESCC risk

Figure 4.6 presents urinary 8-OHdG level in cases and controls. The average level (mean±SD) of urinary 8-HdG levels were 49.67 ± 235.07 and 24.04 ± 48.86 ng/mg creatinine, with median value of 22.04 and 14.70 ng/mg creatinine, in cases and controls, respectively. A borderline statistically significance was found in association urinary 8-OHdG level with ESCC (p=0.07) without adjustment of possible confounding factors. In cases, we observed a borderline significant higher urinary 8-OHdG level in females compared to males (p=0.08). In controls, urinary 8-OHdG level in females was significantly higher than in males (p=0.04). Although the urinary 8-OHdG level was higher in older age group (≥63 years) than in young age group (<63 years), the difference was not statistically significant. No significant age difference of urinary 8-OHdG level was found in case group.

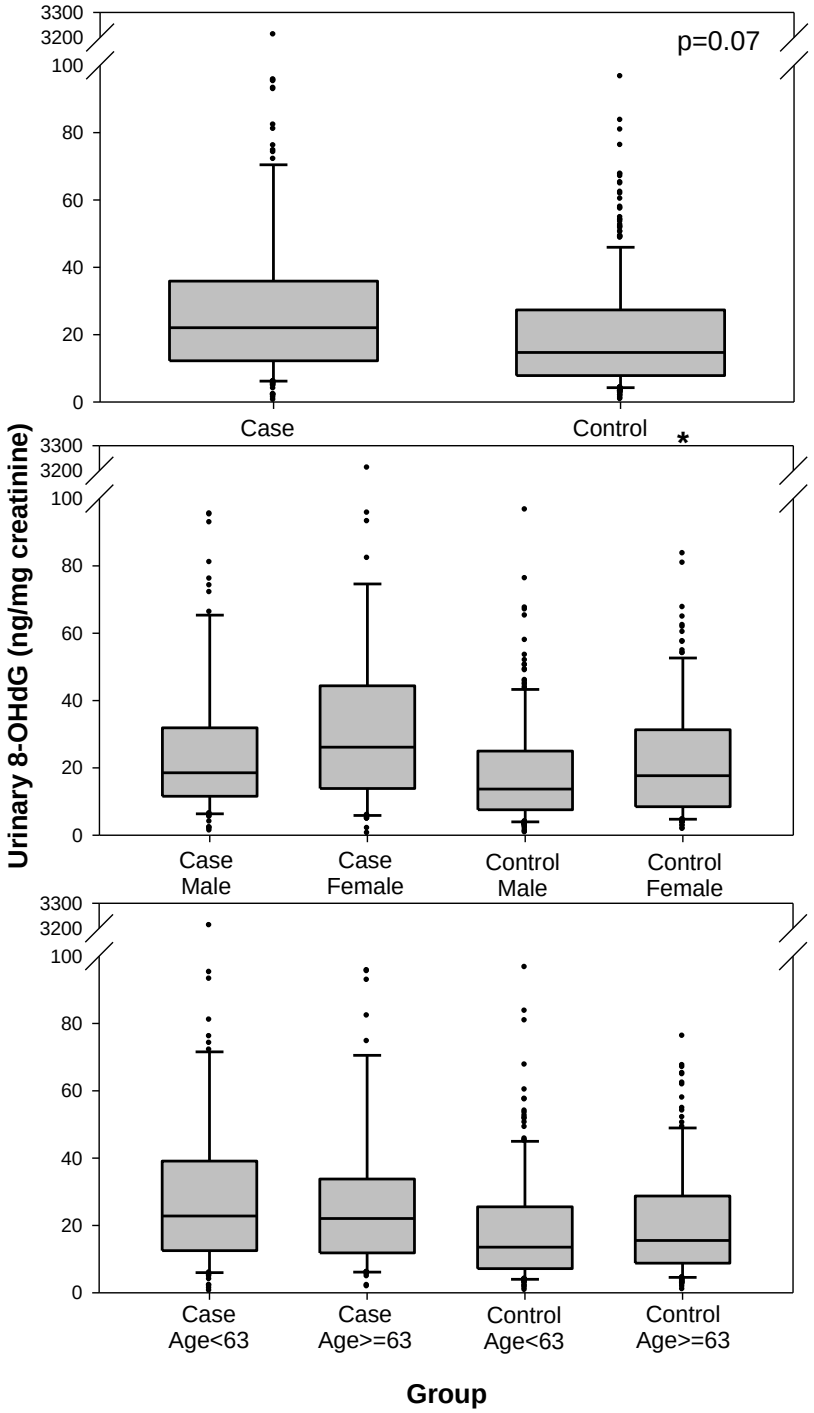

Figure 4.6 Urinary 8-OHdG levels in ESCC cases and controls. The dots, error bars and upper and lower ends of the box represent outliers, spread, and first and third quartiles, respectively. Star (*) mark represents p value less than 0.05 in comparison between males and females within cases or controls through Wilcoxon signed-rank test.

The ORs and 95% CIs for esophageal cancer by tertiles of urinary 8-OHdG in control groups are shown in table 4.18. Results showed significant associations between urinary 8-OHdG levels and ESCC risk, with or without adjustment of possible confounding factors. The adjusted ORs of ESCC for moderate vs. low and high vs. low urinary 8-OHdG level were 1.45 (95% CI: 0.77–2.71) and 2.49 (95% CI: 1.36–4.56), respectively (p for trend = 0.002). The association between ESCC and urinary 8-OHdG levels was also been examined after stratified by gender, age group, pickled/salty food intake, alcohol drinking, tobacco smoking, tea drinking and Hogg1 genotypes. Compared to low urinary 8-OHdG level, high urinary 8-OHdG level had significantly increased risk of ESCC in all strata except female, subjects who consumed pickled/salty food frequently, non-alcohol drinker, and carried hOGG1 326 Ser/Ser wide type. Tests for interactions of these variables and urinary 8-OHdG levels in ESCC risk found no significant difference.

Table 4.18 Esophageal squamous cell carcinoma risk by tertile of urinary 8-OHdG level

	Category of urinary 8-OHdG concentration					*P* for trend
	Low	Moderate		High		
		OR	95% CI	OR	95% CI	
Range, ng/mg creatinine	<10.16	10.16 – 22.83		≥22.83		
Cases [No. (%)][a]	34 (18.0)	64 (33.9)		91 (48.2)		
Controls [No. (%)]	125 (33.0)	129 (34.0)		125 (33.0)		
Odds ratio	1	1.84	1.13 – 2.99	2.67	1.66 – 4.29	<0.001
Adjusted odds ratio[b]	1	1.45	0.77 – 2.71	2.49	1.36 – 4.56	0.002
Gender[c]						
Male (*n* = 333)	1	1.86	0.89 – 3.91	3.66	1.75 – 7.68	<0.001
Female (*n* = 237)	1	0.53	0.19 – 1.50	1.21	0.49 – 2.94	0.24
P Interaction						0.12
Age (years) [c]						
≤63 (*n* = 300)	1	1.04	0.46 – 2.34	2.20	1.02 – 4.76	0.07
>63 (*n* = 270)	1	1.61	0.67 – 3.83	2.70	1.18 – 6.18	0.02
P Interaction						0.94
Pickled/salty food intake [c]						
Frequent (*n* = 207)	1	1.76	0.73 – 4.20	2.06	0.88 – 4.86	0.15
Infrequent (*n* = 363)	1	1.09	0.49 – 2.44	3.21	1.54 – 6.72	<0.001
P Interaction						0.20
Alcohol drinking status [c]						
Non-drinker (*n* = 298)	1	0.84	0.36 – 1.95	1.72	0.79 – 3.73	0.07
Drinker (*n* = 272)	1	1.83	0.79 – 4.25	3.43	1.47 – 8.00	0.004
P Interaction						0.54
Tobacco smoking status [c]						
Non-smoker (*n* = 230)	1	1.31	0.48 – 3.62	3.38	1.28 – 8.93	0.004
Smoker (*n* = 339)	1	1.36	0.64 – 2.87	2.27	1.11 – 4.64	0.02
P Interaction						0.78
Tea drinking status [c]						
No (*n* = 399)	1	1.38	0.71 – 2.72	2.35	1.24 – 4.47	0.006
Yes (*n* = 170)	1	0.95	0.28 – 3.18	3.13	1.02 – 9.60	0.02
P Interaction						0.73
hOGG1 Ser326Cys genotype [c]						
Ser/Ser (*n* = 98)	1	0.43	0.06 – 2.89	1.44	0.20 – 10.43	0.20
Ser/Cys (*n* = 273)	1	1.03	0.42 – 2.55	4.01	1.74 – 9.24	<0.01
Cys/Cys (*n* = 196)	1	2.40	0.95 – 6.11	2.70	1.07 – 6.82	0.07
P Interaction						0.85

[a] percentage may not add to 100 because of rounding and calculations of percentages excluding missing values.
[b] conditional logistic regression with adjustment for possible confounding factors.
[c] logistic regression with adjustment for gender, age and possible confounding factors, where appropriate.

As illustrated in Figure 4.7, we assessed the correlation between urinary 8-OHdG level and urinary free FB_1 concentration. Significant correlations between urinary 8-OHdG and urinary free FB_1 level were found in all controls ($r_{Spearman}$=0.18, N=379, p<0.001) and male controls ($r_{Spearman}$=0.19, N=221, p=0.005). A borderline significance was found of the correlation in female controls ($r_{Spearman}$=0.14, N=158, p=0.07). No significant correlations between urinary 8-OHdG and free FB_1 level were detected in cases, with or without stratification of gender (cases: $r_{Spearman}$=0.06, N=189, p=0.40; male cases: $r_{Spearman}$=0.06, N=110, p=0.55; female cases: $r_{Spearman}$=-0.004, N=79, p=0.97). We also assessed the correlation between urinary 8-OHdG level and age. A borderline significance was observed in correlation between urinary 8-OHdG level and age in all controls ($r_{Spearman}$=0.09, N=379, p=0.09). After stratification of gender, a significant correlation was only detected in male controls ($r_{Spearman}$=0.16, N=221, p=0.02). No significant correlations between urinary 8-OHdG and age were detected in cases, with or without stratification of gender (cases: $r_{Spearman}$=-0.008, N=189, p=0.91; male cases: $r_{Spearman}$=-0.05, N=110, p=0.60; female cases: $r_{Spearman}$=0.07, N=79, p=0.55).

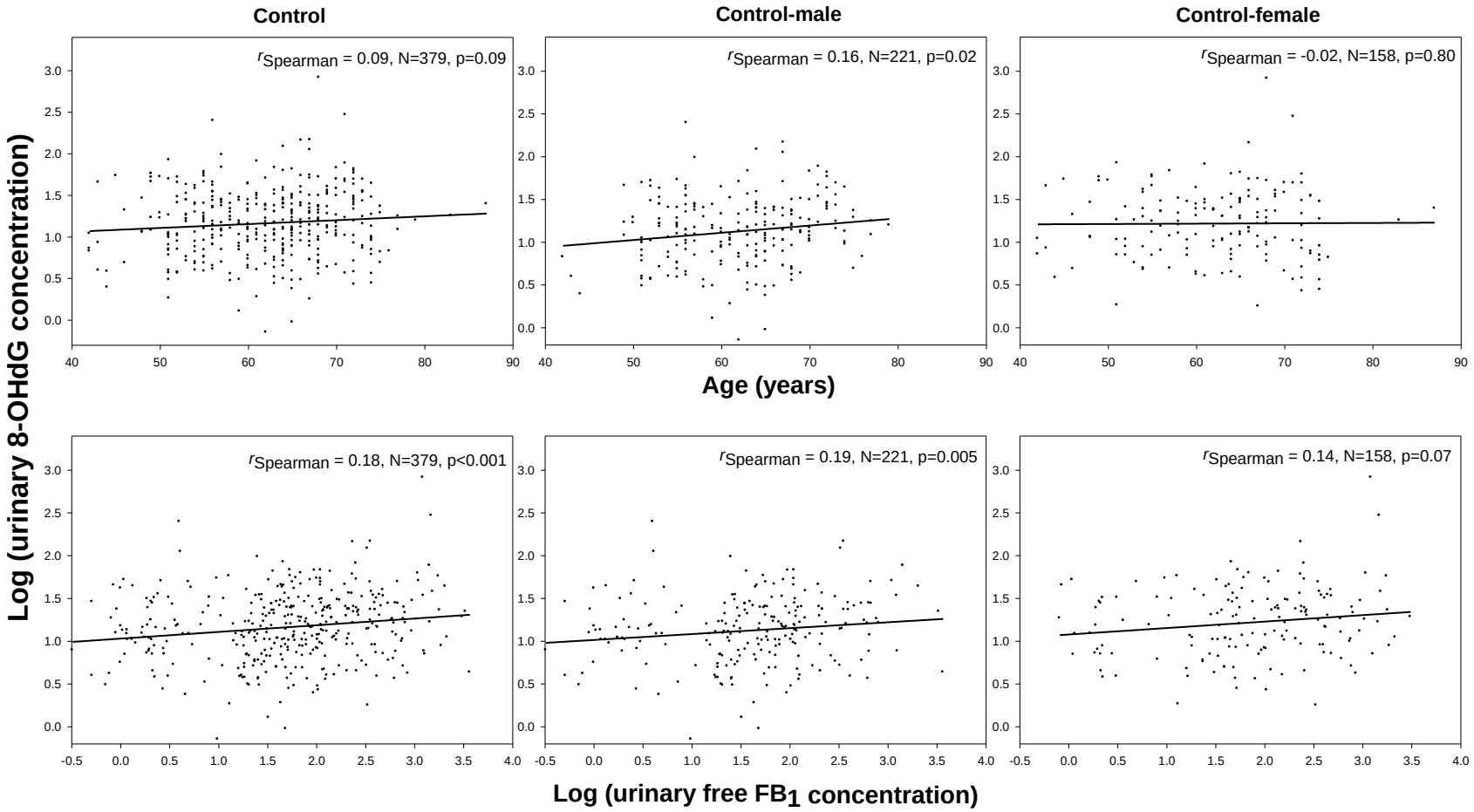

Figure 4.7 Correlation of logarithm of urinary 8-OHdG concentration [Log(ng/mg creatinine)] and age, as well as logarithm of urinary free FB_1 concentration [Log(pg/mg creatinine)] in controls, male controls, and female controls. P values were obtained using Spearman rank correlation test.

4.4 Genetic polymorphisms and ESCC risk

4.4.1 Genetic polymorphisms of xenobiotic metabolizing enzymes and the risk of ESCC

Table 4.19 and 4.20 present the ESCC risks associated with GSTM1 and GSTT1 genotypes. No significant differences were found in GSTM1 with or without stratification of gender. For GSTT1, the null type (mutant type) was associated with an increased risk of ESCC (OR= 1.47, 95% CI: 1.04 – 2.09). After stratified by gender, the significance was observed in males.

Table 4.19 GSTM1 genotype and ESCC risk

	Genotype	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]
All	Wild type	93 (49.2) [c]	180 (47.5)	0.70	1.0
	Null type	96 (50.8)	199 (52.5)		0.93 (0.66 – 1.32)
Male	Wild type	59 (53.2)	108 (48.9)	0.46	1.0
	Null type	52 (46.9)	113 (51.1)		0.84 (0.53 – 1.33)
Female	Wild type	34 (43.6)	72 (45.6)	0.77	1.0
	Null type	44 (56.4)	86 (54.4)		1.09 (0.63 – 1.87)

[a,] p values were calculated using chi-square test or fisher exact test, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression or unconditional logistical regression adjusted with age.
[c] percentage may not add to 100 because of rounding.

Table 4.20 GSTT1 genotype and ESCC risk

	Genotype	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]
All	Wild type	89 (47.1) [c]	215 (56.7)	0.03	1.0
	Null type	100 (52.9)	164 (43.3)		1.47 (1.04 – 2.09)
Male	Wild type	48 (43.2)	125 (56.6)	0.02	1.0
	Null type	63 (56.8)	96 (43.4)		1.71 (1.08 – 2.71)
Female	Wild type	41 (52.6)	90 (57.0)	0.52	1.0
	Null type	37 (47.4)	68 (43.0)		1.20 (0.63 – 2.07)

[a,] p values were calculated using chi-square test or fisher exact test, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression or unconditional logistical regression adjusted with age.
[c] percentage may not add to 100 because of rounding.

4.4.2 Genetic polymorphisms of inflammation-related genes and the risk of ESCC

Tables 4.21–4.24 present the ESCC risks associated with genetic polymorphisms, as well as allele frequencies of inflammation-related genes, COX-2, NF-κB, TNF-α and iNOS. No significant associations were observed between ESCC risk and these genetic polymorphisms, except in female subjects, an increased risk of ESCC was associated with iNOS Ser608Leu heterozygous type (Ser/Leu) (OR=1.97, 95% CI: 1.04 – 3.74). However, after combined with mutant type, the significance disappeared.

Table 4.21 COX-2 G765C genotype and ESCC risk

	Genotype	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]
All	Wild type	178 (93.7)	335 (91.5)	0.37	1.0
	Heterozygote	12 (6.3)	31 (8.5)		0.75 (0.38 – 1.40)
	Mutant type	0 (0.0)	0 (0.0)		-
	Allele frequency (%)				
	G	96.8	95.8	0.42	
	C	3.2	4.2		
Male	Wild type	104 (93.7)	197 (92.9)	0.79	1.0
	Heterozygote	7 (6.3)	15 (7.1)		0.88 (0.35 – 2.22)
	Allele frequency (%)				
	G	96.8	96.5	1.00	
	C	3.2	3.5		
Female	Wild type	74 (93.7)	138 (89.6)	0.31	1.0
	Heterozygote	5 (6.3)	16 (10.4)		0.58 (0.20 – 1.64)
	Allele frequency (%)				
	G	96.8	94.8	0.36	
	C	3.2	5.2		

[a,] p values were calculated using chi-square test or fisher exact test, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression or unconditional logistical regression adjusted with age.
[c] percentage may not add to 100 because of rounding.

Table 4.22 NF-κB -94 ins/delATTG genotype and ESCC risk

	Genotype	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]
All	D/D	46 (24.5) [c]	81 (21.9)	0.73	1.0
	D/I	130 (69.1)	261 (70.5)		0.84 (0.56 – 1.28)
	I/I	12 (6.4)	28 (7.6)		0.74 (0.34 – 1.60)
	D/D	46 (24.5)	81 (21.9)	0.46	1.0
	D/I+I/I	142 (75.5)	289 (78.1)		0.84 (0.55 – 1.26)
	Allele frequency (%)				
	D	59.0	57.2	0.56	
	I	41.0	42.8		
Male	D/D	27 (24.6)	52 (23.7)	0.98	1.0
	D/I	76 (69.1)	152 (69.4)		0.96 (0.56 – 1.65)
	I/I	7 (6.4)	15 (6.9)		0.90 (0.33 – 2.49)
	D/D	27 (24.6)	52 (23.7)	0.87	1.0
	D/I+I/I	83 (75.5)	167 (76.3)		0.96 (0.56 – 1.63)
	Allele frequency (%)				
	D	59.1	58.4	0.93	
	I	40.9	41.6		
Female	D/D	19 (24.4)	29 (19.2)	0.60	1.0
	D/I	54 (69.2)	109 (72.2)		0.75 (0.39 – 1.46)
	I/I	5 (6.4)	13 (8.6)		0.58 (0.18 – 1.91)
	D/D	19 (24.4)	29 (19.2)	0.36	1.0
	D/I+I/I	59 (75.6)	122 (80.8)		0.73 (0.38 – 1.42)
	Allele frequency (%)				
	D	59.0	55.3	0.49	
	I	41.0	44.7		

[a,] p values were calculated using chi-square test or fisher exact test, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression or unconditional logistical regression adjusted with age.
[c] percentage may not add to 100 because of rounding.

Table 4.23 TNF-α G308A genotype and ESCC risk

	Genotype	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]
All	Wild type	166 (87.4)	322 (85.0)	0.008	1.0
	Heterozygote	22 (11.6)	32 (8.4)		1.44 (0.80 – 2.57)
	Mutant type	2 (1.05)	25 (6.6)		0.15 (0.03 – 0.63)
	Wild type	166 (87.4)	322 (85.0)	0.52	1.0
	Heterozygote+mutant	24 (12.6)	57 (15.0)		0.83 (0.50 – 1.36)
	Allele frequency (%)				
	G	93.2	89.2	0.03	
	A	6.8	10.8		
Male	Wild type	97 (87.4)	188 (85.1)	0.06	1.0
	Heterozygote	12 (10.8)	16 (7.2)		1.45 (0.66 – 3.19)
	Mutant type	2 (1.8)	17 (7.7)		0.23 (0.05 – 1.00)
	Wild type	97 (87.4)	188 (85.1)	0.57	1.0
	Heterozygote+mutant	14 (12.6)	33 (14.9)		0.82 (0.42 – 1.61)
	Allele frequency (%)				
	G	92.8	88.7	0.10	
	A	7.2	11.3		
Female	Wild type	69 (87.3)	134 (84.8)	0.17	1.0
	Heterozygote	10 (12.7)	16 (10.1)		-
	Mutant type	0 (0.0)	8 (5.1)		-
	Wild type	69 (87.3)	134 (84.8)	0.60	1.0
	Heterozygote+mutant	10 (12.7)	24 (15.2)		0.81 (0.37 – 1.79)
	Allele frequency (%)				
	G	93.7	89.9	0.23	
	A	6.3	10.1		

[a,] p values were calculated using chi-square test or fisher exact test, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression or unconditional logistical regression adjusted with age.
[c] percentage may not add to 100 because of rounding.

Table 4.24 iNOS Ser608Leu genotype and ESCC risk

	Genotype	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]
All	Wild type	140 (73.7)	282 (74.6)	0.04	1.0
	Heterozygote	50 (26.3)	83 (22.0)		1.22 (0.81 – 1.84)
	Mutant type	0 (0.0)	13 (3.4)		-
	Wild	140 (73.7)	282 (74.6)	0.81	1.0
	Heterozygote+mutant	50 (26.3)	96 (25.4)		1.05 (0.70 – 1.57)
	Allele frequency (%)				
	C	86.8	85.6	0.59	
	T	13.2	14.4		
Male	Wild	84 (75.7)	156 (70.6)	0.18	1.0
	Heterozygote	27 (24.3)	57 (25.8)		0.87 (0.50 – 1.50)
	Mutant type	0 (0.0)	8 (3.6)		-
	Wild	84 (75.7)	156 (70.6)	0.33	1.0
	Heterozygote+mutant	27 (24.3)	65 (29.4)		0.76 (0.44 – 1.31)
	Allele frequency (%)				
	C	87.8	83.5	0.17	
	T	12.2	16.5		
Female	Wild	56 (70.9)	126 (80.3)	0.04	1.0
	Heterozygote	23 (29.1)	26 (16.6)		1.97 (1.04 – 3.74)*
	Mutant type	0 (0.0)	5 (3.2)		
	Wild	56 (70.9)	126 (80.3)	0.11	1.0
	Heterozygote+mutant	23 (29.1)	31 (19.8)		1.61 (0.88 – 2.95)
	Allele frequency (%)				
	C	85.4	88.5	0.78	
	T	14.6	11.5		

[a,] p values were calculated using chi-square test or fisher exact test, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression or unconditional logistical regression adjusted with age.
[c] percentage may not add to 100 because of rounding.
* $p < 0.05$ using conditional logistic regression or unconditional logistical regression adjusted with age where appropriate.

4.4.3 Genetic polymorphisms of DNA repair genes and the risk of ESCC

Table 4.25 presents the ESCC risks associated with hOGG1 Ser326Cys polymorphism. The heterozygote (Ser/Cys) was associated with a decreased risk of ESCC (OR = 0.60, 95% CI=0.38 – 0.94), as well as in female subjects (OR = 0.47, 95% CI=0.23 – 0.99). After combing heterozygote with mutant type, the association was marginally significant (OR = 0.65, 95% CI=0.42 – 1.00) in all subjects and disappeared in female subjects.

Table 4.26 presents the ESCC risks associated with XPD Lys751Gln polymorphism. Significant increased risks of ESCC were observed in subjects with heterozygous type (Lys/Gln) (OR = 1.71, 95% CI=1.09 – 2.67), as well as in female subjects with heterozygous type (OR = 2.66, 95% CI=1.29 – 5.48). After combining heterozygote and mutant type, significant increased risks of ESCC were still observed in all subjects (OR = 1.72, 95% CI=1.11 – 2.69) and female subjects (OR = 2.65, 95% CI=1.30 – 5.36). No significant difference was observed in male subjects.

Table 4.25 hOGG1 Ser326Cys genotype and ESCC risk

	Genotype	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]
All	Wild type	41 (21.8) [c]	57 (15.0)	0.07	1.0
	Heterozygote	80 (42.6)	193 (50.9)		0.60 (0.38 – 0.94)*
	Mutant type	67 (35.6)	129 (34.0)		0.74 (0.45 – 1.20)
	Wild type	41 (21.8)	57 (15.0)	0.05	1.0
	Heterozygote+mutant	147 (78.2)	321 (85.0)		0.65 (0.42 – 1.00)
	Allele frequency (%)				
	C	43.1	40.5	0.41	
	G	56.9	59.5		
Male	Wild type	22 (20.0)	32 (14.5)	0.42	1.0
	Heterozygote	52 (47.3)	115 (52.0)		0.65 (0.35 – 1.23)
	Mutant type	36 (32.7)	74 (33.5)		0.71 (0.36 – 1.39)
	Wild type	22 (20.0)	32 (14.5)	0.20	1.0
	Heterozygote+mutant	88 (80.0)	189 (85.5)		0.67 (0.37 – 1.23)
	Allele frequency (%)				
	C	43.6	40.5	0.45	
	G	56.4	59.5		
Female	Wild type	19 (24.4)	25 (15.8)	0.11	1.0
	Heterozygote	28 (35.9)	78 (49.4)		0.47 (0.23 – 0.99)*
	Mutant type	31 (39.7)	55 (34.8)		0.74 (0.35 – 1.56)
	Wild type	19 (24.4)	25 (15.8)	0.12	1.0
	Heterozygote+mutant	59 (75.6)	133 (84.2)		0.58 (0.30 – 1.14)
	Allele frequency (%)				
	C	42.3	40.5	0.77	
	G	57.7	59.5		

[a,] p values were calculated using chi-square test or fisher exact test, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression or unconditional logistical regression adjusted with age.
[c] percentage may not add to 100 because of rounding.
* $p < 0.05$ using conditional logistic regression or unconditional logistical regression adjusted with age where appropriate.

Table 4.26 XPD Lys751Gln genotype and ESCC risk

	Genotype	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]
All	Wild type	148 (77.9)[c]	327 (86.1)	0.04	1.0
	Heterozygote	40 (21.1)	51 (13.4)		1.71 (1.09 – 2.67)*
	Mutant type	2 (1.0)	2 (0.5)		2.83 (0.32 – 16.7)
	Wild type	148 (77.9)	327 (86.1)	0.01	1.0
	Heterozygote+mutant	42 (22.1)	53 (14.0)		1.72 (1.11 – 2.69)*
	Allele frequency (%)				
	A	88.4	92.8	0.02	
	C	11.6	7.2		
Male	Wild type	89 (80.2)	187 (84.2)	0.46	1.0
	Heterozygote	21 (18.9)	34 (15.3)		1.31 (0.72 – 2.39)
	Mutant type	1 (0.9)	1 (0.5)		2.09 (0.13 – 33.78)
	Wild type	89 (80.2)	187 (84.2)	0.35	1.0
	Heterozygote+mutant	22 (19.8)	35 (15.8)		1.33 (0.74 – 2.41)
	Allele frequency (%)				
	A	89.6	91.9	0.39	
	C	10.4	8.1		
Female	Wild type	59 (74.7)	140 (88.6)	0.01	1.0
	Heterozygote	19 (24.1)	17 (10.8)		2.66 (1.29 – 5.48)*
	Mutant type	1 (1.3)	1 (0.6)		2.38 (0.15 – 38.74)
	Wild type	59 (74.7)	140 (88.6)	0.006	1.0
	Heterozygote+mutant	20 (25.3)	18 (11.4)		2.65 (1.30 – 5.36)*
	Allele frequency (%)				
	A	86.7	94.0	0.01	
	C	13.3	6.0		

[a,] p values were calculated using chi-square test or fisher exact test, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression or unconditional logistical regression adjusted with age.
[c] percentage may not add to 100 because of rounding.
* $p < 0.05$ using conditional logistic regression or unconditional logistical regression adjusted with age where appropriate.

4.5 Gene-gene interaction and gene-environment interaction

4.5.1 Combinative effect of genetic polymorphisms for ESCC risk

Joint effects of measured genetic polymorphisms in our study on the risk of ESCC were investigated. Only genes showing significant results are presented in tables 4.27–4.30. Subjects harboring GSTT1 null type and GSTM1 wild type were at increased risk of ESCC compared with those harboring wild types of GSTT1 and GSTM1. Subjects harboring XPD Lys/Gln + Gln/Gln genotypes and COX-2 wild type, TNF-α wild type, and/or GSTT1 null type were at increased risk of ESCC in comparison with subjects harboring wild type of these polymorphisms.

Table 4.27 GSTM1, GSTT1 and ESCC risk

	Genotype	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]
All	GSTM1-GSTT1				
	Wild-wild	37 (19.6)	100 (26.4)	0.08	1.0
	Wild-null	56 (29.6)	80 (21.1)		1.86 (1.12 – 3.08)*
	Null-wild	52 (27.5)	115 (30.3)		1.23 (0.74 – 2.03)
	Null-null	44 (23.3)	84 (22.2)		1.46 (0.86 – 2.50)
Male	GSTM1-GSTT1				
	Wild-wild	23 (20.7)	59 (26.7)	0.12	1.0
	Wild-null	36 (32.4)	49 (22.2)		1.88 (0.99 – 3.59)*
	Null-wild	25 (22.5)	66 (29.9)		0.97 (0.50 – 1.89)
	Null-null	27 (24.3)	47 (21.3)		1.47 (0.75 – 2.89)
Female	GSTM1-GSTT1			0.46	
	Wild-wild	14 (18.0)	41 (30.0)		1.0
	Wild-null	20 (25.6)	31 (19.6)		1.89 (0.83 – 4.32)
	Null-wild	27 (34.6)	49 (31.0)		1.61 (0.75 – 3.47)
	Null-null	17 (21.8)	37 (23.4)		1.35 (0.58 – 3.11)

[a,] p values were calculated using chi-square test or fisher exact test, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression or unconditional logistical regression adjusted with age.
[c] percentage may not add to 100 because of rounding.
* p <0.05 using conditional logistic regression or unconditional logistical regression adjusted with age where appropriate.

Table 4.28 GSTT1, XPD Lys751Gln genotype and ESCC risk

	Genotype	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]
All	GSTT1-XPD			0.007	
	Wild-Lys/Lys	64 (33.9)	178 (47.0)		1.0
	Wild- Lys/Gln+Gln/Gln	25 (13.2)	37 (9.8)		1.85 (1.09 – 3.31)*
	Null-Lys/Lys	83 (43.9)	148 (39.1)		1.55 (1.04 – 2.30)*
	Null- Lys/Gln+Gln/Gln	17 (9.0)	16 (4.2)		2.65 (1.30 – 5.39)*
Male	GSTT1-XPD			0.07	
	Wild-Lys/Lys	36 (32.4)	100 (45.3)		1.0
	Wild- Lys/Gln+Gln/Gln	12 (10.8)	25 (11.3)		1.33 (0.61 – 2.93)
	Null-Lys/Lys	53 (47.8)	86 (38.9)		1.70 (1.02 – 2.85)*
	Null- Lys/Gln+Gln/Gln	10 (9.0)	10 (4.5)		2.80 (1.07 – 7.30)*
Female	GSTT1-XPD			0.03	
	Wild-Lys/Lys	28 (35.9)	78 (49.4)		1.0
	Wild- Lys/Gln+Gln/Gln	13 (16.7)	12 (7.6)		3.02 (1.23 – 7.41)*
	Null-Lys/Lys	30 (38.5)	62 (39.2)		1.35 (0.73 – 2.49)
	Null- Lys/Gln+Gln/Gln	7 (9.0)	6 (3.8)		3.25 (1.01 – 10.50)*

[a,] p values were calculated using chi-square test or fisher exact test, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression or unconditional logistical regression adjusted with age.
[c] percentage may not add to 100 because of rounding.
* p <0.05 using conditional logistic regression or unconditional logistical regression adjusted with age where appropriate.

Table 4.29 XPD Lys751Gln, COX-2 G765C genotype and ESCC risk

	Genotype	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]
All	COX2-XPD			0.08	
	G/G-Lys/Lys	139 (73.2)	289 (79.0)		1.0
	G/G-Lys/Gln+Gln/Gln	39 (20.5)	46 (12.6)		1.70 (1.06 – 2.69)*
	G/C-Lys/Lys	9 (4.7)	25 (6.8)		0.74 (0.34 – 1.63)
	G/C-Lys/Gln+Gln/Gln	3 (1.6)	6 (1.6)		1.13 (0.28 – 4.54)
Male	GSTT1-XPD			0.77	
	Wild-Lys/Lys	83 (74.8)	166 (78.3)		1.0
	Wild-Lys/Gln+Gln/Gln	21 (18.9)	31 (14.6)		1.37 (0.74 – 2.54)
	Null-Lys/Lys	6 (5.4)	12 (5.7)		1.00 (0.36 – 2.75)
	Null- Lys/Gln+Gln/Gln	1 (0.9)	3 (1.42)		0.66 (0.07 – 6.45)
Female	GSTT1-XPD			0.03	
	Wild-Lys/Lys	56 (70.9)	123 (79.9)		1.0
	Wild-Lys/Gln+Gln/Gln	18 (22.8)	15 (9.7)		2.66 (1.25 – 5.67)*
	Null-Lys/Lys	3 (3.8)	13 (8.4)		0.50 (0.14 – 1.83)
	Null- Lys/Gln+Gln/Gln	2 (2.5)	3 (2.0)		1.45 (0.24 – 8.92)

[a,] p values were calculated using chi-square test or fisher exact test, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression or unconditional logistical regression adjusted with age.
[c] percentage may not add to 100 because of rounding.
* $p < 0.05$ using conditional logistic regression or unconditional logistical regression adjusted with age where appropriate.

Table 4.30 XPD Lys751Gln, TNF-α G308A genotype and ESCC risk

	Genotype	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]
All	TNF-α-XPD			0.04	
	G/G-Lys/Lys	128 (67.4)	280 (73.9)		1.0
	G/G-Lys/Gln+Gln/Gln	38 (20.0)	42 (11.1)		1.98 (1.22 – 3.22)*
	G/A+A/A-Lys/Lys	20 (10.5)	46 (12.1)		0.97 (0.56 – 1.68)
	G/A+A/A-Lys/Gln+Gln/Gln	4 (2.1)	11 (2.9)		0.78 (0.25 – 2.47)
Male	TNF-α-XPD			0.22	
	G/G-Lys/Lys	76 (68.5)	161 (72.9)		1.0
	G/G-Lys/Gln+Gln/Gln	21 (18.9)	27 (12.2)		1.66 (0.88 – 3.13)
	G/A+A/A-Lys/Lys	13 (11.7)	25 (11.3)		1.10 (0.53 – 2.27)
	G/A+A/A-Lys/Gln+Gln/Gln	1 (0.9)	8 (3.62)		0.27 (0.03 – 2.17)
Female	TNF-α-XPD			0.045	
	G/G-Lys/Lys	52 (65.8)	119 (75.3)		1.0
	G/G-Lys/Gln+Gln/Gln	17 (21.5)	15 (9.5)		2.61 (1.21 – 5.63)*
	G/A+A/A-Lys/Lys	7 (8.9)	21 (13.3)		0.76 (0.31 – 1.91)
	G/A+A/A-Lys/Gln+Gln/Gln	3 (3.8)	3 (1.9)		2.27 (0.44 – 11.65)

[a,] p values were calculated using chi-square test or fisher exact test, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression or unconditional logistical regression adjusted with age.
[c] percentage may not add to 100 because of rounding.
* $p < 0.05$ using conditional logistic regression or unconditional logistical regression adjusted with age where appropriate.

4.5.2 Inflammation pathway and ESCC risk

As shown in table 4.31, to evaluate the possible additive effect of multiple genetic polymorphisms in the inflammation pathway on ESCC risk, we estimated the combined effect of these genetic polymorphisms (COX-2 G765C, NF-κB -94 ins/delATTG, TNF-α G308A and iNOS Ser608Leu) and then stratified by host characteristics. Compared with the reference group (subjects carrying no mutant allele), no significant differences were found in subjects with one or more mutant alleles, with or without stratification of host characteristics. A borderline significance of interaction between gender and mutant alleles of inflammation pathway on ESCC risk was detected (p=0.052).

Table 4.31 Inflammation pathway and ESCC risk

Number of adverse alleles	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]	P interaction
Overall					N/A
0	29 (15.4) [c]	41 (11.6)	0.31	1.0	
1	94 (50.0)	172 (48.6)		0.77 (0.45 – 1.32)	
≥2	65 (34.6)	141 (39.8)		0.65 (0.37 – 1.14)	
				0.15	
Gender					0.052
Male					
0	14 (12.7)	25 (12.0)	0.12	1.0	
1	61 (55.5)	93 (44.7)		1.17 (0.56 – 2.42)	
≥2	35 (31.8)	90 (43.3)		0.70 (0.32 – 1.49)	
				0.08	
Female					
0	15 (19.2)	16 (11.0)	0.13	1.0	
1	33 (42.3)	79 (54.1)		0.44 (0.20 – 1.00)	
≥2	30 (38.5)	51 (34.9)		0.62 (0.27 – 1.44)	
Smoking status					0.37
Smoker					
0	13 (14.9)	19 (11.2)	0.19	1.0	
1	48 (55.2)	81 (47.7)		0.86 (0.39 – 1.89)	
≥2	26 (29.9)	70 (41.2)		0.53 (0.23 – 1.24)	
				0.07	

Table 4.26 Continued

Number of adverse alleles	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]	P interaction
Non-smoker					
0	16 (15.8)	22 (12.0)	0.62	1.0	
1	46 (45.5)	91 (49.5)		0.68 (0.33 – 1.42)	
≥2	39 (38.6)	71 (38.6)		0.75 (0.35 – 1.60)	
				0.80	
Drinking status					0.85
Drinker					
0	17 (14.8)	26 (12.2)	0.64	1.0	
1	56 (48.7)	99 (46.8)		0.86 (0.43 – 1.72)	
≥2	42 (36.5)	88 (41.3)		0.73 (0.36 – 1.50)	
				0.38	
Non-drinker					
0	12 (16.4)	15 (10.7)	0.41	1.0	
1	38 (52.1)	72 (51.4)		0.66 (0.28 – 1.56)	
≥2	23 (31.5)	53 (37.9)		0.54 (0.22 – 1.34)	
				0.24	
Urinary FB_1 level					0.48
$FB_1 \leq 56.92$					
0	4 (10.26)	22 (12.2)	0.70	1.0	
1	21 (53.9)	83 (46.1)		1.44 (0.45 – 4.65)	
≥2	14 (35.9)	75 (41.7)		1.06 (0.31 – 3.55)	
				0.65	
$FB_1 > 56.92$					
0	25 (16.8)	19 (10.9)	0.31	1.0	
1	73 (49.0)	89 (51.2)		0.61 (0.31 – 1.20)	
≥2	51 (34.2)	66 (37.9)		0.58 (0.29 – 1.17)	
				0.29	

[a,] p values were calculated using chi-square test or fisher exact test, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression or unconditional logistical regression adjusted with age.
[c] percentage may not add to 100 because of rounding.

4.5.3 Combinative effects of family history and genetic polymorphisms on ESCC risk

Table 4.32 presents the joint effect of family history of cancer and GSTT1 and XPD on ESCC risk. Subjects having family history of cancer in first-degree relatives and carrying GSTT null genotype had a significant increased risk of ESCC compared to those without family history of cancer and carrying GSTT1 wild type (OR=2.51, 95% CI: 1.28-4.89). Similarly, subjects having family history of cancer and harboring XPD Gln genotype had the highest risk of ESCC (OR=3.50, 95% CI: 1.42- 8.62), comparing to those only has family history of cancer or XPD Gln genotype. No additive effects of family history and GSTT1 and XPD genotype were found.

Table 4.32 Family history and genetic polymorphisms on ESCC risk

Family history of cancer	Genotype	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]
	GSTT1			0.002	
No	Wild type	50 (26.5) [c]	148 (39.1)		1.0
	Null type	44 (23.3)	97 (25.6)		1.03 (0.54 – 1.94)
Yes	Wild type	39 (20.6)	67 (17.7)		1.64 (0.85 – 3.17)
	Null type	56 (29.6)	67 (17.7)		2.51 (1.28 – 4.89)*
	XPD			<0.001	
No	Lys/Lys	74 (39.0)	215 (56.6)		1.0
	Lys/Gln+Gln/Gln	21 (11.1)	31 (8.2)		3.09 (1.28 – 7.46)*
Yes	Lys/Lys	74 (39.0)	112 (29.5)		2.25 (1.30 – 3.90)*
	Lys/Gln+Gln/Gln	21 (11.1)	22 (5.8)		3.50 (1.42 – 8.62)*

[a,] p values were calculated using chi-square test or fisher exact test, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression or unconditional logistical regression adjusted with age.
[c] percentage may not add to 100 because of rounding.
* p <0.05 using conditional logistic regression adjusted with possible confounding factors.

4.5.4 Combinative effects of genetic polymorphisms and environmental exposure on ESCC risk

Table 4.33 presents the joint effect of GSTT1 genetic polymorphism and possible environmental exposure risk factor of ESCC on ESCC risk. Significant increased risks of ESCC were found in subjects with high urinary FB_1 regardless GSTT1 genotype, and subjects who intake pickled/salty food frequently regardless GSTT1 genotype.

Table 4.34 presents the joint effect of XPD genetic polymorphism and possible environmental exposure risk factor of ESCC on ESCC risk. Significant increased risks of ESCC were found in smoker carrying XPD Gln genotype, non-drinker carrying XPD Gln genotype, subjects with high urinary FB_1 regardless XPD genotype, and subjects who intake pickled/salty food frequently regardless XPD genotype. Additive effects of urinary FB_1 and XPD genotype, as well as pickled/salty food intake and XPD genotype, were detected.

Table 4.33 Combinative effect of GSTT1 genotype and environmental exposure on ESCC risk

	Genotype	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]
Smoking status				0.17	
Non-smoker	Wild type	36 (19.1) [c]	86 (22.8)		1.0
	Null type	38 (20.1)	69 (18.3)		1.07 (0.52 – 2.23)
Smoker	Wild type	53 (28.0)	128 (33.9)		1.13 (0.58 – 2.22)
	Null type	62 (32.8)	95 (25.1)		1.71 (0.85 – 3.43)
Alcohol drinking				0.11	
Non-drinker	Wild type	44 (23.3)	113 (29.8)		1.0
	Null type	57 (30.2)	83 (21.9)		1.33 (0.69 – 2.57)
Drinker	Wild type	45 (23.8)	102 (26.9)		1.16 (0.55 – 2.43)
	Null type	43 (22.8)	81 (21.4)		1.49 (0.68 – 3.28)
Urinary FB_1				<0.001	
Low	Wild type	18 (9.5)	111 (29.3)		1.0
	Null type	22 (11.6)	78 (20.6)		1.56 (0.66 – 3.67)
High	Wild type	71 (37.6)	104 (27.4)		5.08 (2.36 – 10.96)*
	Null type	78 (41.3)	86 (22.7)		6.22 (2.85 – 13.58)*
Pickled/salty food intake				<0.001	
Infrequent	Wild type	53 (28.0)	158 (41.7)		1.0
	Null type	43 (22.8)	108 (28.5)		1.28 (0.69 – 2.37)
Frequent	Wild type	36 (19.1)	57 (15.0)		2.56 (1.28 – 5.11)*
	Null type	57 (30.2)	56 (14.8)		2.98 (1.54 – 5.74)*

[a,] p values were calculated using chi-square test or fisher exact test, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression adjusted with possible confounding factors.
[c] percentage may not add to 100 because of rounding.

Table 4.34 Combinative effect of XPD genotype and environmental exposure on ESCC risk

	Genotype	Cases (%)	Controls (%)	*P*-value[a]	OR (95%CI) [b]
Smoking status				0.06	
Non-smoker	Lys/Lys	63 (33.2) [c]	134 (35.4)		1.0
	Lys/Gln+Gln/Gln	12 (6.3)	21 (5.5)		1.86 (0.70 – 4.94)
Smoker	Lys/Lys	85 (44.7)	192 (50.7)		1.23 (0.73 – 2.07)
	Lys/Gln+Gln/Gln	30 (15.8)	32 (8.4)		2.76 (1.29 – 5.89)*
Alcohol drinking				0.07	
Non-drinker	Lys/Lys	83 (43.7)	169 (44.5)		1.0
	Lys/Gln+Gln/Gln	19 (10.0)	27 (7.1)		2.26 (1.01 – 5.06)*
Drinker	Lys/Lys	65 (34.2)	158 (41.6)		1.13 (0.60 – 2.11)
	Lys/Gln+Gln/Gln	23 (12.1)	26 (6.8)		2.30 (0.94 – 5.63)
Urinary FB_1				<0.001	
Low	Lys/Lys	34 (17.9)	164 (43.2)		1.0
	Lys/Gln+Gln/Gln	6 (3.2)	26 (6.8)		1.56 (0.46 – 5.35)
High	Lys/Lys	114 (60.0)	163 (42.9)		4.64 (2.58 – 8.36)*
	Lys/Gln+Gln/Gln	36 (19.0)	27 (7.1)		9.35 (4.16 – 21.04)*
Pickled/salty food intake				<0.001	
Infrequent	Lys/Lys	80 (42.1)	227 (59.7)		1.0
	Lys/Gln+Gln/Gln	17 (9.0)	39 (10.3)		1.80 (0.82 – 3.97)
Frequent	Lys/Lys	68 (35.8)	100 (26.3)		2.11 (1.23 – 3.61)*
	Lys/Gln+Gln/Gln	25 (13.2)	14 (3.7)		5.70 (2.42 – 13.43)*

[a,] p values were calculated using chi-square test or fisher exact test, as appropriate.
[b] odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression adjusted with possible confounding factors.
[c] percentage may not add to 100 because of rounding.

4.6 Multivariate analysis of environmental exposure and individual susceptibility factors in ESCC risk

Table 4.35 presents the multivariate analysis result for ESCC risk and investigated environmental and genetic factors. After adjustment of potential confounding factors, significant increased risks of ESCC were found in subjects having medical history of esophageal lesion (OR=5.93, 95% CI: 2.16 – 16.28), favoring salty food 10 years ago (OR=2.38, 95% CI: 1.46 – 3.87), favoring high temperature food 10 years ago (OR=2.66, 95% CI: 1.63 – 4.35), consuming pickled/salty food frequently (OR=2.31, 95% CI: 1.40 – 3.82), consuming mildewed food frequently 10 years ago (OR=2.41, 95% CI: 1.43 – 5.50), having family history of cancer in first-degree relative(s) (OR=1.97, 95% CI: 1.19 – 3.26), having high urinary 8-OHdG level (high vs low: OR=2.56, 95% CI: 1.38 – 4.73), having high urinary FB_1 level (p=0.01), and harboring XPD Lys/Gln heterozygous or Gln/Gln mutant type (OR= 2.25, 95% CI: 1.20 – 4.25). Protective effects on ESCC risk were found in subjects consuming garlic frequently (OR=0.39, 95% CI: 0.22 – 0.70), drinking tea regularly (OR=0.41, 95% CI: 0.22 – 0.77), and having high BMI level (OR=0.86, 95% CI: 0.80 – 0.92).

Table 4.35 Multivariate analysis of environmental and genetic factors on ESCC risk

Characteristics	*P*-value[a]	OR_{ad} (95%CI) [b]
Esophageal lesion	<0.001	5.93 (2.16 – 16.28)
Preference for salty food (10-years-ago)	<0.001	2.38 (1.46 – 3.87)
Preference for high temperature food (10-years-ago)	<0.001	2.66 (1.63 – 4.35)
Frequent pickled/Salty food intake	0.001	2.31 (1.40 – 3.82)
Mildewed food intake (10-years-ago)	0.003	2.41 (1.29 – 4.50)
First-degree family history of cancer	0.008	1.97 (1.19 – 3.26)
Frequent garlic intake	0.002	0.39 (0.22 – 0.70)
Tea drinking	0.006	0.41 (0.22 – 0.77)
BMI	<0.001	0.86 (0.80 – 0.92)
Urinary 8-OHdG level (tertile)	0.007	-
Moderate		1.48 (0.79 – 2.79)
High		2.56 (1.38 – 4.73)
Urinary free FB_1 level	0.01	1.00 (1.00 – 1.001)
XPD Lys751Gln	0.01	2.25 (1.20 – 4.25)

[a, b] p value and adjusted odds ratio (OR) and 95% confidence interval (CI) were calculated using conditional logistic regression.

Chapter 5

ESOPHAGEAL CANCER CASE-CONTROL STUY – DISCUSSION

5.1 Questionnaire data

In the present case-control study, esophageal lesion, preference for salty food (10-years-agoe), preference for high temperature of food (10-years-agoe), frequent pickled/salty food intake, mildewed food intake (10-years-agoe), and first-degree family history of cancer were all associated with increased risk for ESCC in Huaian, while cigarette smoking and alcohol drinking were not significantly associated with ESCC risk. Frequent garlic intake, tea drinking, and BMI were protective factors for ESCC development. Our study confirmed that multiple environmental factors, especially dietary exposure factors, play important role in ESCC developments in Huaian high risk area.

Consumption of pickled vegetables has long been suspected as a risk factor of ESCC. Early investigation in Linxian high risk population found daily consumption of pickled vegetables as an integral part of the diet in local population [91]. The mutagencity of pickled vegetables were demonstrated by the presence of cancer induced by pickled vegetable extract in rodents [91, 288]. In addition, in pickled food collected from ESCC high risk areas, high levels of carcinogenic N-nitrosamines and/or fungal toxins have been found, suggesting a possible mechanism in ESCC development [289, 290]. These observations initiated population studies in etiology of pickled vegetable in ESCC. As described in the recent meta-analysis using 34 human epidemiological studies in Asian

populations, an approximately two-fold increased risk of ESCC was associated with pickled vegetables in the overall analysis as well as in the majority of individual studies [291]. In agreement with the meta-analysis, our study found a significant increased ESCC risk for frequent pickled vegetable intake, with an adjusted odds ratio of 2.31 (95% CI, 1.40–3.82). Since most significant results were from case-control studies (reviewed in [291]), further studies in prospective populations are warranted to confirm this finding.

Consumption of mildewed food was also commonly observed in ESCC high risk areas, especially in low socioeconomic population in developing countries [91]. In addition to facilitating formation of N-nitroso compounds, multiple mycotoxins have been isolated in moldy staples. In corn samples collected from Cixian and Linxian ESCC high risk areas, FB_1, type A (T-2 toxin, HT-2 toxin, isoneosolaniol, etc) and type B trichothecenes (dexoynivalenol [DON]-related) were widely detected [42]. The levels of FB_1, type A and type B trichothecene were higher in moldy corn than apparently fine corn samples [42]. A comparative study in China detected trichothecenes and zearalenone (ZEA) in corn and wheat samples from high- and low-incidence areas, and the detectable rate and levels of DON were significantly higher in samples from high incidence areas [292]. Fumonisins are the most investigated and detected mycotoxin in staples in ESCC high risk areas. Although not a confirmed human carcinogen, accumulating evidence has linked FNs or *Fusarium*-contaminated food with esophageal cancer in high incidence areas in China, Iran and South Africa [5-8]. In the present study, mildewed food intake frequency was significantly higher in cases than controls both now (14.2% vs. 8.7%) and 10 years ago (17.9% vs. 8.4%), supporting the contributing role of mildewed food consumption in ESCC risk. However, it is notable that in our study, mildewed food

intake is a crude concept obtained through interview to reflect the overall status and attitude toward moldy food in study population. Thus, it may only be used as a possible indictor, which has been used and shown inconsistent results in human epidemiological studies [293-295]. Considering the variation in occurrence of fungi species and types of mycotoxins, it is important to determine the level and nature of the mycotoxins present in the food consumed by study subjects before confirming the linkage between consumption of mildewed food and esophageal cancer.

We found that preference to high temperature of food significantly increased the risk of ESCC, showing an odds ratio and 95% CI of 2.66 and 1.63–4.35, respectively. Consistently, in tea drinkers, those favoring hot tea had an increased risk of ESCC, supporting the etiology role of hot food and beverage in ESCC risk. This is consistent with a previous case-control study in Yangzhong County, China, showing that consumption of hot food was associated with increased ESCC risk in both males and females, with odds ratio (95% CI) of 2.13 (1.39–3.25) and 3.05 (1.73–5.36), respectively [296]. In the case-control study conducted by Castellsagué and colleagues in populations from Argentina, Brazil, Uruguay and Paraguay, consumption of very hot beverage excluding mate and including mate was possible risk factor of ESCC with odds ratio (95% CI) of 2.45 (1.72–3.49) and 2.07 (1.55–2.76), respectively [297]. Since exact measurement of the temperature of consumed food in a relative long period is complicated and nonpractical, obtaining clear evidence of food or beverage temperature in ESCC formation is problematic. Based on current evidence (reviewed in [108]) and our findings, although inconclusive, high-temperature food or beverage is highly suspected as a possible risk factor of ESCC.

Accumulating studies showed that BMI was associated positively the risk of EAC, but inversely with the risk of ESCC [27, 298]. A prospective study conducted in 45 areas in China measured height and weight and calculated BMI in male subjects and followed up for 10 years [298]. After adjustment of both smoking and alcohol drinking, higher BMI in baseline appeared to be a protective effect against esophageal cancer. Among males with BMI $\geq$ 18.5 kg/m^2, each 5 kg/m^2 higher BMI was associated with a 30% (95%: 18–41%) lower risk of death from esophageal cancer [298]. Similarly, Another cohort of 29,584 adults in Linxian high risk area with 1958 ESCC cases after 15 years of follow-up also found that higher BMI was associated with decreased ESCC risk (RR: 0.71, 95% CI: 0.62–0.82) [27]. In additional, in a meta-analysis of 9 case-control studies and 4 cohort studies, each 5 kg/m^2 higher BMI was associated with 51% (95% CI, 30–79%) and 31% ((95% CI, 25–37%) lower risk of esophageal cancer, respectively [298]. In the present study, we calculated BMI based on self-reported weight and height at 1 year prior to recruitment to avoid/reduce the bias related to weight loss due to disease development. In agreement with previous studies [27, 298], BMI was strongly associated with decreased ESCC risk, with an odds ratio of 0.86 (95% CI, 0.80–0.92) after adjustment of potential confounding factors. Although the inverse association between BMI and ESCC was strong, the relationship may not be necessarily causal. Deficiencies of nutrients, including total calorie, proteins, vitamin A, vitamin C and several micronutrients, have been were associated with increased ESCC risk, especially in low socioeconomic high-risk population [91, 97-101]. In the present study, majority of study subjects were within the normal weight (BMI: 18.5–24.9 kg/m^2) (70.5% in cases and 67.9% in controls). Also, in regarding of local economic status over the past decades, the

low BMI in study subjects was possibly related to long-term poor nutrition status in development of ESCC in this population. Further analysis fully adjusting nutrients intake factors in this population is valuable in estimate the causal relationship of BMI in ESCC formation.

Garlic is widely consumed as a spice especially in East Asia populations. It contains several organosulfur compounds and flavoniods, which may exhibit anticarcinogenic effects through modulation of xenobiotic-metabolizing enzymes [299], inhibition of cell proliferation and induction of apoptosis [300]. Although garlic intake has been associated with decreased risk of several cancer sites, such as colorectal cancer, breast cancer, stomach cancer and prostate cancer, overall, the evidence in esophageal cancer was rare and limited to case-control studies (reviewed in [301]). In the case-control studies conducted by Gao and colleagues in high- and low- incidence areas of esophageal cancer in Jiangsu Province, China, Frequent intake of garlic (≥1 time/week) were associated with decreased esophageal cancer, with the adjusted odds ratio of 0.30 (95% CI, 0.19–0.47) compared to infrequent intake of garlic (<1 time/month) in the high incidence area [302, 303]. Similarly, in the low incidence area, garlic intake was also associated with decrease of esophageal cancer risk [302]. A series of case-control studies conducted by Galeone and colleagues found that high use of garlic was positive association with decreased risk of esophageal cancer, with the odds ratio of 0.43 (95% CI, 0.28–0.67). [304]. In agreement with these studies, the present study found a reverse association of frequent garlic intake and ESCC risk (adjusted OR=0.39, 95% CI: 0.22–0.70).

Cigarette smoking and alcohol consumption are two identified risk factors for ESCC [80-87]. They were generally believed to be the major causes of ESCC in Europe and US

population, while their contributing role to ESCC risk in high incidence areas in developing countries was inconsistent and less conclusive [27, 88-90]. In a study conducted in Jiangsu Province, China, the prevalence of smoking and alcohol drinking were similar between high- and low- incidence areas despite the ESCC incidence showed a significantly geographic variation [305]. In our study, no significant difference was found in both smoking and alcohol consumption status between cases and controls. About 70% of males were smokers or alcohol drinkers, while only 38% of females were smokers and 10% were alcohol drinkers. The gender difference in these factors seems not in parallel with the gender difference in incidence of esophageal cancer, that is, about half of cases occurred in females [31]. This is consistent with previous cohort study in Linxian which had comparable esophageal cancer incidence in males and females, reporting smoking in <1% of females and 60% of males [27]. In our study, no association of ESCC risk was found with smoking exposure measures (duration, intensity, cumulative dose), passive smoking, and alcohol drinking exposure measures (duration, intensity, cumulative consumption) with or without stratified by gender. Our result, together with previous finding in high-risk populations in China and Iran [27, 88-90], supports the hypothesis that smoking and alcohol drinking are not the major contributing factors for the extremely high incidence in these areas; other environmental carcinogen exposure may overwhelm the effect of smoking and alcohol drinking in ESCC formation in these populations.

Tea is the most commonly consumed beverages worldwide after water. Tea and tea polyphenols have been shown to inhibit tumor formation and growth through decreasing cell proliferation, increasing apoptosis and suppressing angiogenesis in different animal

models, whereas its health benefit in humans, especially cancer prevention effect, remains inconclusive [306]. As reviewed by Islami and colleagues [108], the results of association between tea consumption and esophageal cancer was mixed. The majority of significant inverse associations were from East-Asian countries, especially in China, where green tea is mostly used [108]. In the present study, regular tea drinking is an important protective factor of ESCC, with an odds ratio of 0.41 (95% CI, 0.22–0.77) after adjustment of possible confounding factors. And green tea was used by more than 90% of subjects. This is consistent with previous studies conducted in other Chinese populations [126, 296, 303, 307], while inconsistent results exist [308-310]. In a case-control study of esophageal cancer (83% of ESCC) conducted in Shanghai by Gao and colleagues [307], a protective effect of green tea consumption was observed in females, with an odds ratio of 0.50 (95% CI, 0.30–0.83), while no effect was found in males. The protective effect was more significant in female subjects consuming more tea (≥150 gram/month: OR=0.34, 95% CI: 0.17–0.69), and for a long time (≥3500 gram/month×year: OR=0.29, 95%: 0.13–0.65) [307]. In our study, no significant difference between cases and controls was observed regarding tea drinking start age, duration and intensity (gram/month) in subjects who consumed tea on a regular basis. The lack of significant difference may be due to the relatively low percentage of subjects who consumed tea. In tea drinkers, those favoring hot tea than cold/warm tea had an increased risk of ESCC, with an odds ratio of 2.58 (95% CI, 1.21 – 5.50). This factor could weaken the protective effect of tea consumption in ESCC risk, while related studies were rare. In another esophageal cancer case-control study in high risk area in China, although not statistically significant, the elevated odds ratio in ever drinking green tea (OR=1.2, 95% CI: 0.9–1.5) decreased after adjusted for

tea temperature (OR=1.0, 95% CI: 0.7–1.3). [308]. Therefore, further study in evaluation of these tea consumption-related paramters is valuable in assess the exact effect of tea component in cancer risk.

Identification of clinically relevant precursor lesions is of great importance in early detection and prevention in esophageal cancer. Several studies in high risk Asian populations have described possible ESCC precursor lesions, while the results were inconsistent [311-317]. Crespi, Muňoz and colleagues found an extremely high prevalence of histological esophagitis, atrophy and dysphasia in two endoscopic surveys conducted in Iranian and Chinese high risk populations and little to no evidence of present of oesphageal reflux [311-313]. In Linxian, 84% of subjects had a chronic esphagitis, which was accompanied by atrophy in 10% of these subjects and by dysplasia in 8% [311]. The prevalence was significantly higher than the endoscopic survey in a low-risk Chinese population, where 28% of subjects had esophagitis, accompanied by 0.4% atrophy and no dysplasia [314]. Re-examination of 20 Linxian subjects 1 year later showed progression of the esphagitis to atrophy and dysplasia in 4 subjects and to cancers in 4 others [313]. Furthermore, these lesions were assoicated with alcohol drinking [318], tobacco smoking [318-320], and consumption of burning hot beverages [320]. However, Yang and Qiu reported no difference in the prevalence of esophagitis or atrophy in high versus low risk Chinese populations [315, 316]. Instead, they found a high prevalence of dysplasia in the high risk population and an association with progression to esophageal cancer. A prospective study following up an endoscoped cohort in Linxian for 13.5 years found that dysplasia and carcinoma in situ were associated with a increased risk of ESCC, while no evidence in associating esophagitis with ESCC risk [317]. In our study,

12.2% of cases and 2.4% of controls reported a medical history of esophageal lesion. Since most mild esophagitis patients reported no symptom or non-specific symptom, and considering the relative low socio-economic status in this population, the low rate of esophageal lesion in cases and controls is acceptable. Consistent with previous studies [311-314], chronic esophagitis was the most common lesion, diagnosed in more than 70% cases and controls, supporting esophagitis as a possible precursor lesion in ESCC. Further studies using endoscopic screening in Huaian high risk population are warranted to identify and validate possible precursor lesions in this population.

5.2 Urinary fumonisin exposure biomarkers and ESCC risk

In the present study, we found a significant association between urinary free FB_1 level and esophageal cancer risk. The urinary free FB_1 level was significantly correlated with corn meal intake level in study subjects. Urinary free FB_1 level was significantly higher in females than males, which is consistent with the results of urinary Sa, So, and the Sa/So ratio. Also, a significant increased risk of ESCC was observed in subjects with high urinary Sa/So ratio level. These findings support dietary exposure of FB_1 as a risk factor for high incidence of ESCC in this area, and suggest application of urinary free FB_1 as a potential FB_1 exposure biomarker in human epidemiology study. To our knowledge, there has been no previously published data investigating urinary free FB_1 level and esophageal cancer risk in a case-control study in literature.

Several studies have evaluated the risk of esophageal cancer associated with dietary FB_1 exposure using cross-sectional design. An initial cross-sectional study conducted in esophageal cancer high- and low-incidence areas of Transkei, South Africa, found a

correlation between the proportion of kernels in both moldy and healthy maize samples infected by *F. verticilloides* and esophageal cancer [321]. Consistently, a recent study in Transkei region reported a significantly higher estimated daily intake of FB_1 in esophageal cancer high-incidence area than in low-incidence area [8]. Similar results were also observed in studies in Chinese high-risk populations. A study in Cixian County, one of the highest ESCC incidence area in China, found that the FB_1 level in corn samples from high-incidence areas was approximately 3-fold of the level in low-incidence areas[61]. In Henan Province, the contamination rate of fumonisins was about twice higher in corn samples from an esophageal cancer high-risk county (detectable rate: 48%) than from a low-risk county (detectable rate: 25%) [43], and the estimated daily intake of FB_1 in the high-risk county 1.6–1.9 times higher than in the low-risk county [322]. Our previous study found that the FB_1 level in corn samples collected from Huaian was significantly higher than those from Huaitai, an ESCC low-incidence area [62]. These cross-sectional studies, through comparing FB_1 contamination in corn samples from different areas, strongly suggested a possible link between FB_1 exposure and esophageal cancer risk. Unlike these, in the present study, we used a case-control design that recruited ESCC cases and health controls from the same area and matched by age, gender and residency. Furthermore, we calculated odds ratio after adjustment of other possible environmental confounding factors. These provide a direct and more accurate estimation of association between dietary FB_1 exposure and ESCC development. Also, we estimated FB_1 exposure on an individual basis by measuring several FB_1 exposure biomarkers which was validated in animal models. Our results show that high urinary FB_1 level was significantly associated with increased ESCC risk, which provide a strong

supporting evidence in associating dietary FB_1 exposure and ESCC development.

Toxicokenetics of FB_1 was characterized by rapid distribution and elimination, negligible absorption, and lack of metabolism in animal models *in vitro* or *in vivo* [9]. These characteristics suggest urinary FB_1 as a short-term biomarker of FB_1 exposure, which has been validated in animal studies [54]. However, it is still possible serving as a FB_1 exposure biomarker in human cancer risk estimation if the study subjects have relatively stable corn and corn products intake frequency/habit and FB_1 contamination in food has not changed dramatically over a relative long period. Our previous cross-sectional study in China reported a higher urinary FB_1 level in a population with high estimated daily FB_1 intake than that with lower daily FB_1 intake [79]. Consistently, a study in Mexican females found those with high Tortilla consumption had a 3-fold higher mean urinary FB_1 level compared to low Tortilla consumers [78]. In the present study, we not only observed a significant association between ESCC risk and urinary free FB_1 level in a dose-response manner, but also found a significant correlation between urinary free FB_1 level and corn meal intake level, which was estimated based on food frequency intake for the previous 12-month periods through in-person interviews. In general, these data support application of urinary free FB_1 in human cancer epidemiology study.

Inhibition of *de novo* sphingolipid biosynthesis and disruption of sphingolipid metabolism are known biological effects of FB_1, and therefore, Sa, So, and the Sa/So ratio was proposed as biomarkers for fumonisin exposure and testified in animal studies (reviewed in [9]). However, no significant difference was found in these biomarkers in cross-sectional studies conducted in Africa [73], Argentina, Brazil and Italy [72], Portugal [71], and an esophageal cancer case-control study in Linxian, China [70]. In

agreement with these studies, we did not observe an association between ESCC risk and urinary Sa, So, and the Sa/So ratio. But, after categorized urinary Sa/So ratio using tertile of controls, we did find an increased risk in high Sa/So ratio group (urinary Sa/So ratio≥0.26) than low Sa/So ratio group (urinary Sa/So ratio<0.14), while the increased risk was not statistically significant in moderate Sa/So ratio group (urinary Sa/So ratio: 0.14–0.26). Regarding urinary Sa and So level, no significant elevated odds ratios were found after categorized to high-, moderate-, and low- level groups. Also, we did not observe a correlation between urinary Sa/So ratio with either urinary free FB_1 level, or corn meal intake. Overall, the association between these urinary sphingolipids metabolites biomarkers and ESCC risk was not significant, or as significant as the association between urinary free FB_1 and ESCC risk. Therefore, it is reasonable to conclude that using urinary sphingolipids biomarkers, including Sa, So and the Sa/So ratio, may not be as valid and sensitive as urinary free FB_1 serving as FB_1 exposure biomarker in human study.

Although studies have suggested the potential role of FB_1 exposure in esophageal cancer development, confirmation of FB_1 exposure in human disease risks seems very difficult, partly due to fewer validated FB_1 biomarkers available for epidemiological study and for quantitative evaluation of human exposure and disease risk. Ideally, putative biomarkers are validated in pilot animal studies where sensitivity, specificity, accuracy, and reliability parameters can be established. Then, epidemiological studies are required to demonstrate the role of biomarkers in human pathogenesis and validate its application in human populations [323]. In previous animal study, we have established and validated a gradient immunoaffinity-HPLC-fluorescence method for the analysis of

urinary free FB_1 as a exposure biomarker in F344 rats [54]. Given the significant results in animal studies, we extended our research to human population by cross-sectional investigation of urinary free FB_1 in two high-risk populations exposed to different dietary FB_1 levels. Results of the cross-sectional study confirmed the association between urinary free FB_1 and estimated daily FB_1 intake [79]. In the present study, we detected a significant association between urinary free FB_1 and ESCC risk. In summary, our results support urinary free FB_1 as a potential exposure biomarker of FB_1 and its application to human epidemiological study. Further validation of this biomarker through cohort study, and intervention study should be highly anticipated.

5.3 Oxidative DNA damage and ESCC risk

In the present study, we found an association between urinary 8-OHdG level and ESCC risk, showing a dose-response relationship. The association was significant in subjects with high 8-OHdG level (≥22.83 ng/mg creatinine) compared to low 8-OHdG level (<10.16 ng/mg creatinine). The increased ESCC risk was more obvious in males, subjects consuming pickled food infrequently, alcohol drinkers, non-smokers, and tea drinkers. The association remains significant regardless age, tobacco smoking status, and tea drinking status after stratified by these factors. Also, urinary 8-OHdG level was significantly correlated with urinary free FB_1 level in control group, as well as age in controls. These results provide supporting evidence of the etiology role of oxidative stress in ESCC carcinogenesis and confirmed using of urinary 8-OHdG as biomarker in cancer risk study. To our knowledge, there has been no previously publication in investigating urinary 8-OHdG level and ESCC risk in a case-control study in literature.

As the most common oxidative stress biomarker, 8-OHdG reflects the general status of oxidative DNA damage caused by imbalance between production of ROS, detoxification and DNA repair ability of host system, Therefore, as a non-specific biomarkers, the levels of 8-OHdG are determined or affected by multiple exogenous factors, especially carcinogen exposures, which has been demonstrated in numerous studies (reviewed in [10, 113]). In our study, urinary 8-OHdG was positively correlated with urinary free FB_1 level, which in turn, was an obvious risk factor of ESCC in this case-control study after adjustment of all possible confounding factors. Based on these results, we postulate that oxidative stress may be one mechanism responsible for FB_1-induce carcinogenesis in human, which was evidenced by previous studies [120, 324, 325]. In human and rat glioblastoma cells and mouse hypothalamic cells as well, production of ROS increased after treatment of 10–100 μM FB_1 for 48–144 hours [120, 324]. FB_1 was also able to enhance oxidative stress and cell damage by increasing the rate of oxidation and promoting production of free radicals in membranes [325].

Besides, in the present study, urinary 8-OHdG was positive correlated with age in control group, especially in male subjects. There is accumulative evidence that aging might be caused by cumulative effects of ROS generated throughout the lifespan, which is also called the free radical theory of aging proposed by Haman (reviewed in [326, 327]). In agreement with the present findings, several studies reported a correlation between age and 8-OHdG level in human colorectal tissue [328, 329], leukocytes [330], and urine [331]. In case group, no correlation was found between urinary 8-OHdG level and age, as well as urinary free FB_1 level. The impact of health status in ESCC patients might explain the non-significance. Also, since elevation of 8-OHdG level cannot

entirely be attributed to age and FB_1 exposure, especially in cases, other environmental exposure and lifestyle factors might be heavily involved. In our study, the association between urinary 8-OHdG level and ESCC risk was more obvious in subjects consuming pickled food infrequently, alcohol drinkers, non-smokers, and tea drinkers, suggesting a possible role of these factors in 8-OHdG level.

In the present study, after adjustment of potential confounding factors, high urinary 8-OHdG level was associated with increased ESCC risk. This is consistent with a previous study in 18 ESCC patients and 12 health controls [122], which found significant higher concentration of serum 8-OHdG in cases than controls. They also found the level of 8-OHdG changed with disease status: in most patients the 8-OHdG level decreased after esophagectomy treatment and were comparable to the level in healthy controls, while in patients who had disease progression during the whole time of treatment, the 8-OHdG level remained high [122]. Thus, it's reasonable to anticipate that the 8-OHdG levels in ESCC patients are elevated with cancer development. In our study, urine samples of subjects were collected at time of recruitment. The urinary 8-OHdG levels reflect the oxidative stress status in cases after diagnosis of cancer. Therefore, there is a possibility that our significant result in associating urinary 8-OHdG with increased ESCC risk might be biased by the collection time of biological samples. Further study in cohort population is of great value in confirming the role of alternation of 8-OHdG levels in ESCC risk.

5.4 Genetic polymorphisms and ESCC risk

ESCC is a complex disease attributed mainly to the incorporation of multiply environmental risk factors. However, even in high risk areas, only a small portion of

people developed ESCC, suggesting that individual susceptibility plays an important role in ESCC development. Studies suggest that several host factors, including metabolic enzyme activity, DNA repair, and inflammation reactions contribute to individual susceptibility to cancer [164, 332]. Polymorphisms of genes involved in these host factors may confer different risks of susceptibility to cancers, which have been demonstrated in numerous epidemiological studies in different cancer site. In this study, we investigated genetic polymorphisms in xenobiotic-metabolic enzymes, inflammation-regulating proteins, and DNA repair enzymes in exploring their association with ESCC risk.

Numerous studies have investigated the relation of polymorphisms of phase II enzyme GSTM1 and GSTT1 to esophageal cancer, most of which were conducted in Asian populations. Individuals who carried null GSTM1 or GSTT1 genes lack the respective enzyme functions which are responsible for the detoxification of xenobiotics and can thereby modulate individual risk for cancer [123, 332]. The null GSTM1 genotype appears to be common in Asian populations, presenting in about 30–50% of individuals [333], whereas the frequency of the null GSTT1 genotype varies significantly among ethnicities. In this study, both GSTM1 and GSTT1 null genotype commonly existed, reporting in 52.5% and 43.3% of healthy controls, respectively. No association between GSTM1 null genotype and ESCC risk was observed, whereas GSTT1 null genotype was associated with increased ESCC risk, especially in male subjects. The significant association between GSTT1 and ESCC risk disappeared after adjustment of other environmental and genetic risk factors. This suggests that the contributing role of GSTT1 null genotype in ESCC risk in this high-risk population is weak or none. This is consistent with previous findings [126, 281, 334-336]. Although conflicting results exist

[128, 129], most studies did not find a significant association between GSTT1 and esophageal cancer risk, especially in esophageal cancer high incidence area [126, 281, 334-336]. GSTM1 is the most studied genotype in relation to esophageal cancer risk. Although several studies found a significant increased risk of esophageal cancer in subjects who carried GSTM1 null genotype (reviewed in [332]), two meta analysis failed to detect the association between GSTM1, as well as GSTT1 genotype and esophageal cancer risk [337, 338].

5.4.2 Genetic polymorphisms of COX-2, NF-κB, TNF-α and iNOS

Chronic inflammation has been proven to be a key event in carcinogenesis. Although the exact mechanism is complex and not well known, multiple approaches have been made to identify its contribution to cancer risk. Our approach is through investigation of inflammation genes polymorphisms in relation to cancer development. In general, no significant association was found between genetic polymorphisms of genes, COX-2, NF-κB, TNF-α and iNOS, and ESCC risk.

In our study, TNF-α G308A genotype was not assicated with ESCC risk. This is consistent with the case-control study in 291 ESCC cases and 437 healthy controls from North China that did not find a significant association between TNF-α G308A genotype and ESCC risk [140]. In this north Chinese population, the frequencies of TNF-α -308 G/G, G/A, and A/A genotype were 89.4%, 9.2% and 1.4%, respectively, which were comparable with our results indicating the genotype frequencies of 85.0%, 8.4% and 6.6%, respectively, healthy controls.

The promoter polymorphism of NF-κB -94 ins/del ATTG has been reported to be

associated with increased risk of several cancer sites in a few Chinese populations, including head and neck cancer [152, 153], gastric cancer [154], liver cancer [151], prostate cancer [155], bladder cancer[156], and cervical cancer [150]. However, one previous study found null association between NF-κB -94 ATTG polymorphisms and risk of colorectal cancer, renal cell carcinoma and leukemia [339]. Since only one case-control study in each cancer site has been published and most of these studies had a relative small sample size, more studies are highly warranty to confirm such an association. No previous study has been done regarding the NF-κB -94 ins/del ATTG polymorphism and esophageal cancer risk. In the present study, the genotype frequencies of NF-κB -94 del/del, del/ins and ins/ins in controls were 21.9%, 70.5% and 7.6%, respectively, which did not significantly different from the genetic frequencies in cases.

iNOS Ser608Leu is a novel genetic polymorphism which has been associated with increased gastric cancer risk recently [161, 284]. In a previous case-control study conducted in Caucasian population, iNOS Ser608Leu polymorphism was failed to be associated with risk of reflux esophagitis, Barrett's esophagus, and esophageal adenocarcinoma [340]. Other than that, no similar study has been done to investigation the role of iNOS genetic polymorphism in esophageal cancer risk. In the present study, the genetic frequencies of iNOS 608 Ser/Ser, Ser/Leu and Leu/Leu were 74.6%, 22.0% and 3.4%, respectively, in controls, which is coincident with the result in the previous study conducted in Chinese population [284].

In our study, 93.7% of cases and 91.5% of controls had COX-2 G765C wide type (G/G), showing no significant difference. The high frequency of wild type was also reported in other Chinese populations. Tan and colleagues in a Chinese population

reported COX-2 G765C wide type in 95.2% of health controls [341]. Similarly, in an ESCC case-control study in China, COX-2 G765C wide type was found in 95.7% of controls, which did not differ significantly from the frequency in ESCC patients [342].

5.4.3 Genetic polymorphisms of hOGG1 and XPD

DNA damage that is not repaired leads to apoptosis or mutation, which in turn is an important mechanism of carcinogenesis. Numerous studies have demonstrated that the defects in DNA repair system can lead increase of individual susceptibility to cancer [164]. In the present study, we investigated genetic polymorphisms of hOGG1 Ser326Cys and XPD Lys751Gln in cases and controls.

hOGG1 gene is an important DNA repair gene involved in basic excision repair pathway, which is the primary defense against oxidative DNA damage [165]. Although reduced capacity to repair 8-OHdG was observed for variant hOGG1 326Cys protein [343], controversial evidence exists showing no difference in 8-OHdG activity by hOGG1 polymorphisms (reviewed in [344]). In the present study, we found that compared to hOGG1 Ser/Ser wild type, Ser/Cys genotype significantly decreased ESCC risk, but no significance was found for Cys/Cys genotype or combined Ser/Cys and Cys/Cys genotype. The significant association between hOGG1 Ser/Cys genotype and ESCC risk disappeared after adjustment of other environmental and genetic risk factors. This is consistent with a previous study recruited 419 ESCC patients and 480 controls in China that found no significant association between hOGG1 Ser326Cys polymorphism and ESCC risk [345].

XPD gene in nucleotide excision repair pathway is among the most extensively

studied DNA repair genes for its potential implication in cancer risk [172]. Numerous studies have investigated the association between XPD Lys751Gln polymorphism and risk of several cancer sites, however, results remain controversial [346, 347]. With respect to esophageal cancer, variant XPD Gln/Gln genotype was significant associated with increased risk of ESCC in a Chinese population, with an odds ratio of 6.17 (95%CI, 1.76–25.63) [348]. In the present study, XPD Gln/Gln genotype was rarely observed in both cases (1.0%) and controls (0.5%). The frequency of XPD Gln/Gln genotype in our controls was lower than but still comparable to the frequency (1.3%) in another Chinese population [348]. In our study, XPD Lys/Gln genotype was significantly associated with increased ESCC risk. Also, Compared to XPD Lys/Lys wild type, combination of XPD Lys/Gln and Gln/Gln genotypes significantly increased ESCC risk after adjustment of potential environmental exposure factors and other genetic factors if applicable, with an adjusted odds ratio of 2.25 (95% CI, 1.20 – 4.25). These data strongly support contributing role of XPD Lys751Gln polymorphism to ESCC in this high risk population. This is consistent with a previous study in a Sweden population, which found an increased risk of ESCC for XPD Lys/Gln genotype (OR=2.0, 95% CI: 1.1–3.9) [349]. Although the exact effect in unclear, the result is biologically plausible. A previous study conducted by Spitz and colleagues found that individuals carried mutant XPD 751 Gln allele had lower nucleotide excision repair capacity than those with wild genotype [286]. Our finding is also in agreement with a previous case-control study conducted in Huaian high risk population [350], which measured the gene expression level of XPD from peripheral blood mononuclear cells of study subjects and found significant association between reduced expression of XPD and increased ESCC risk.

5.4.4 gene-gene interaction

Esophageal cancer is a complex disease attributed to integrated outcome of environmental factors and individual susceptibility factors. The interplay of these factors is hypothesized to play an important role in cancer development. Although a possible contribution of gene-gene interaction to cancer risk has been found [351], the genetic polymorphisms involved were not comparable and most studies have provided inconsistent results [351, 352]. With respect to esophageal cancer, most of the studies in investigating the role of genetic polymorphisms in ESCC risk did not find significant gene-gene interaction [332]. In the present study, we did not find a combinative effect in ESCC risk for double null genotype of GSTM1 and GSTT1, and for mutant genotype of both hOGG1 and XPD. After grouping the examined SNPs (COX-2, NF-κB, TNF-α and iNOS) in the inflammation pathway, no combinative effect in ESCC risk was observed either. However, the combined GSTT1 null and XPD heterozygous or homozygous mutant genotype was assoicated with increased risk of ESCC, with an odds ratio of 2.65 (95% CI, 1.30–5.39), which is higher than GSTT1 null genotype only or XPD heterozygous or homozygous mutant genotype only, while no additive effect was found.

Overall, in our study, except XPD Lys751Gln polymorphism, no supporting evidence was provided in associating genetic polymorphic xenobiotic metabolizing genes, inflammation genes, and DNA repair genes with ESCC risk. These non-significant results may be due to several reasons. First of all, because cancer development is a long-term process involved multiple environmental and host factors, a single genetic polymorphism or gene-gene interaction alone may not contribute significantly to cancer risk. Secondly, the size of population required to determine a risk of a polymorphism is depending on the

allele frequency of the polymorphism. The relative low frequency of minor allele in the genetic polymorphism requires an extremely large sample size. For example, using 80% power, more than 300 cases and 600 controls are necessary for odds ratio of more than 1.5 and a minor allele frequency of 0.4. In our case, the minor allele frequencies in several genes were even lower than 0.1, which require a much larger sample size. For gene-gene interaction, larger sample size is anticipated. In our case-control study of 190 cases and 380 matched controls, we 1:2 matched cases and controls to increase the power and efficiency to detect possible risk factors in this case-control study. However, we cannot rule out the possibility of association between these genetic polymorphisms with ESCC risk given the relatively limited sample size. Besides, in our study, we matched cases with controls from the same village. These controls may share same or close related ancestors with cases, which may results in small genetic difference in-between.

5.5 Gene-environment interactions

Comparing with gene-gene interaction, gene-environment interaction has been more extensively studied and suggested as an important mechanism in esophageal carcinogenesis. Most of these studies focused on interactions between genetic polymorphisms and cigarette smoking or alcohol drinking. For instance, synergistic effect has been reported between cigarette smoking and GSTT1 null or GSTM1 null genotype in terms of increased esophageal cancer risk [126, 127, 353]. Our results did not support such an additive effect between genetic polymorphisms and smoking in ESCC risk. However, our results showed that in smokers, XPD heterozygous or homozygous mutant genotype was associated with an increased risk of cancer in comparing with non-smokers

with XPD wild type (OR=2.96, 95% CI: 1.29–5.89). The increased risk of ESCC was not significant in non-smokers with XPD heterozygous or homozygous mutant genotype ((OR=1.86, 95% CI: 0.70–4.94). These results hint a possible role of XPD genetic polymorphism and smoking in ESCC risk.

In the present study, combinative effects (additive or multiplicative) were observed for XPD 751 heterozygous or homozygous mutant genotype and high-than-average urinary free FB_1 level (OR=9.35, 95% CI: 4.16–21.04), as well as XPD 751 heterozygous or homozygous mutant genotype and frequent consumption of pickled/salty food (OR=5.70, 95%CI: 2.42–13.43). The gene-environment interaction in our study supports that XPD Lys751Gln polymorphism may contribute to ESCC risk through modification of risk attributable to environment exposures. Although no reports are available for these specific environmental exposures with XPD genetic mutations in ESCC development, several previous studies implied that XPD Lys751Gln genotypes might interact with environmental exposure in cancer developments. Terry and colleagues found that XPD Lys751Gln polymorphism may interact with PAH-DNA adduct and cigarette smoking in breast cancer development [354]. XPD Lys751Gln polymorphism was also been found interacted with duration of aflatoxin exposure and level of AFB_1-DNA adducts in HCC development in a high-risk Chinese population [355]. Therefore, based on our findings and previous studies [354, 355], XPD Lys751Gln polymorphism might interact with environmental carcinogen exposure in intensifying cancer risk, strongly supporting the role of gene-environment interaction in cancer development.

5.6 Multivariate analysis of genetic and environmental factors in ESCC risk.

After multivariate analysis with adjustment of all possible confounding factors, esophageal lesion, preference for salty food (10-years-ago), preference for high temperature of food (10-years-ago), frequent pickled/salty food intake, mildewed food intake (10-years-ago), first-degree family history of cancer, high level of urinary 8-OHdG, free FB_1 level, and XPD 751 Lys/Gln or Gln/Gln genotypes were all associated with increased risk for ESCC. Increased BMI, frequent garlic intake and tea drinking were protective factors for ESCC development in this population. To our knowledge, no previous study has been published for investigating multiple environmental and genetic factors in ESCC development in Huaian high-risk population. Our results provided supporting evidence in regarding the contribution of environment exposure, especially dietary exposure, genetic polymorphisms, as well as gene-environment interactions in ESCC development.

Our findings should be interpreted with caution in light of limitations. The inherited weakness of case-control design makes it vulnerable to bias. In the present study, all ESCC cases were recruited after they were clinically diagnosed, who may have undergone a major dietary change after diagnosed. Although we carefully asked their dietary behaviors before disease occurrence, the possibility of recall bias exists. Also, we collected biology samples from subjects at the time of recruitment. The dietary changes may have an effect on biomarker measurement which may not evenly reflect the long-term dietary exposure status in cases compared to controls. Another limitation is the sample size. As described before, the relative small sample size did not allow sufficient number of subjects in subgroups, especially for genetic polymorphism analysis, which reduce the statistical power and increase the possibility of random error. Further

epidemiology studies based on large-scale prospective design is anticipated to clarify the association between these environmental and genetic factors, as well as their interactions and ESCC risk as we observed in the present study.

Chapter 6

HEPATOCELLULAR CARCINOMA CASE-CONTROL STUY

Titled

Genetic Variations of Hepatitis B Virus and Serum Aflatoxin-Lysine Adduct on High Risk of Hepatocellular Carcinoma in Southern Guangxi, China[1]

[1]Xu L, Qian G, Tang L, Su J, Wang J-S. 2010. Accepted by Journal of Hepatology. Reprinted here with permission of publisher.

6.1 Abstract

Background/Aims: Southern Guangxi area is one of the endemic areas for hepatocellular carcinoma (HCC) in China. This study is to evaluate roles of genetic variations of hepatitis B virus (HBV) and aflatoxin B_1 (AFB_1) exposure in formation of HCC in this high-risk area.

Methods: The study recruited 60 HCC patients and 120 age-, gender-, residency- matched controls. HBV genotype and basic core promoter (BCP) mutations were determined by nested-PCR/direct sequencing. Serum AFB_1-lysine adduct was measured by high performance liquid chromatography-fluorescence detection.

Results: HBV Genotype C was predominant in 75.0% of cases and 84.2% of controls. $1762^T/1764^A$ double mutations, 1753^V mutations, and 1752^V mutations were associated with HCC risk evidenced by the adjusted odds ratio (OR) [95% confidence interval (95% CI)] of 3.89 (1.40-10.77), 2.87 (1.49-5.49), and 5.96 (1.75-20.25), respectively. The adjusted OR (95% CI) was 6.94 (1.68-27.78) for subjects having $1762^T/1764^A$ double mutations with high AFB_1-lysine adduct level; 2.01 (0.24-14.29), for those only having $1762^T/1764^A$ double mutations, and 4.26 (1.16-15.38) for those only having high AFB_1-lysine adduct level, respectively. The adjusted OR was 5.13 (1.79-14.71) for subjects having 1753^V mutations with high AFB_1-lysine adduct level; 1.20 (0.47-3.08) for those only having 1753^V mutations, and 2.28 (1.01-5.31) for those having high AFB_1-lysine adduct level, respectively.

Conclusions**:** These data confirmed association of BCP mutations with HCC risk and the additive effects of $1762^T/1764^A$ double mutations and 1753^V mutations with dietary

AFB_1 exposure on this high-risk area for HCC.

6.2 Introduction

Chronic hepatitis B virus (HBV) infection is the major cause of hepatocellular carcinoma (HCC) worldwide [356]. Although the mechanism is not well defined, variations in the viral genome, including specific genotypes and mutations, were thought contributed to the formation of HCC [357, 358]. To date, eight HBV genotypes are classified (A-H) based on an inter-group divergence of equal or greater than 8% in the complete nucleotide sequence [21]. Infection with the genotype C was associated with greater severity of liver diseases compared to genotype B, though controversy exists [357, 359]. Basic core promoter (BCP) resides in the overlapping HBV functional X gene domain and controls the transcription activity of procore RNA [360]. Mutations in this region were anticipated to influence HBV-induced chronic infection and hepatocarcinogenesis [262, 263]. $1762^{T}/1764^{A}$ double mutations (1762A-to-T and 1764G-to-A) in BCP region were found commonly existed in HCC patients in some high-risk populations and were suggested as a potential biomarker for hepatocarcinogenesis [256-258, 358, 361, 362]. Also, 1753^{V} mutations (1753T-to-C/A/G) were associated with progression of liver disease [260, 261]; nevertheless, the role in HCC development, especially in high risk populations, remains controversy [256, 261, 264].

Southern Guangxi area is one of the HCC endemic areas in China, reporting more than 50/100,000 people per year of incidence and mortality [16]. Chronic HBV infection and dietary aflatoxin (AF) exposure are the two major risk factors for HCC in this area

[17], while hepatitis C virus (HCV) infection is extremely uncommon [280]. AFB_1-lysine adduct in serum has been considered the most reliable biomarker in monitoring long-term human AFB_1 exposure [19]. Association of this adduct with HCC has been well established in high-risk populations in China [227], while its application in assessing HCC risk in southern Guangxi high-risk population has not yet reported.

To these ends, a case-control study was conducted to assess the role of HBV genetic variations, including HBV genotypes and BCP region mutations, dietary AFB_1 exposure measured by serum AFB_1-lysine adduct level, and their interactions in HCC risk in the Southern Guangxi population.

6. 3 Materials and Methods

6.3.1 Study subjects, data collection and sample collection

A total of 60 HCC patients were recruited at Guangxi Medical University Cancer Hospital from August 2004 to August 2005. The eligibility criteria for cases were: 1) permanent resident in southern Guangxi area; 2) confirmed diagnosis of HCC as primary tumor by histological (surgical biopsy) or non-histological criteria (positive imaging test results, serum α-fetoprotein greater than 400ng/ml, and clinical features); 3) No previous diagnosis of other cancers; 4) positive HBV surface antigen (HBsAg) test result when admitted into the hospital, using a enzyme-linked immunosorbent assay (ELISA) kit (Kehua Bio-engineering, Shanghai, China) and 5) negative HCV infection demonstrated by anti-HCV test using a ELISA kit (Kehua Bio-engineering, Shanghai, China) . The 120 population-based controls were recruited from an on-going cohort study conducted in Southern Guangxi area and were frequency-matched to cases on gender, age (in 10-year

age groups) and place of residence (township). The inclusion criteria for these controls were: 1) normal liver and renal function tests as well as electrocardiograms; 2) negative for serum α-fetoprotein; 3) no cancer history; 4) no use of prescribed medications; 5) no pregnancy or lactation for female volunteers; 6) positive HBsAg test, and 7) negative HCV infection by anti-HCV test. Demographic information of cases was obtained from the case history in the hospital archive. Demographic information of controls was collected through in-person interviews by trained interviewers who administered structured questionnaires. Additionally, whole blood samples (7ml) were reserved from cases at time of admission to hospital and collected from controls at time of recruitment. Serum samples were separated and stored frozen (-20℃) for shipping to the US laboratory for further analysis. The study protocol was approved by the Institutional Review Board for human subject protections at Texas Tech University and Guangxi Cancer Institute.

6.3.2 Determination of HBV genotypes and mutations

HBV DNA was extracted from 100 μl serum using a commercial viral extraction kit (Qiagen, Valencia, CA). A nested polymerase chain reaction (nPCR) was designed to amplify the major part of HBV X gene including BCP region. First round amplification was carried out using the sense primer H1 5' - GCT TTC ACT TTC TCG CCA AC -3' and antisense primer RH1 5' - TGG AGG CTT GAA CAG TAG GAC -3' with 767bp of PCR product. Second round amplification used first round PCR product as a template and was processed using the sense primer HBX1 5' - GCC AAG TGT TTG CTG ACG C -3' and antisense primer RHBX1 5' - AAA GTT GCA TGG GGC TGG TG -3' with 648 bp

of PCR product. HBV amplicons were cleaned-up by a commercial gel purification kit (Qiagen, Valencia, CA) and sequenced subsequently in the ABI 3730XL sequencers (MCLAB, South San Francisco, CA). HBV genotypes were determined by the web based program-Viral Genotyping Tool [363]. For mutation analysis, the sequences generated were compared with non-mutational reference sequence (AY641563) using the BLAST program.

6.3.3 Measurement of serum AFB_1-lysine adduct

Levels of serum AFB_1-lysine adduct were measured by a high performance liquid chromatography (HPLC)-fluorescence detection [364] with modifications. In brief, 150 µL of the serum sample was digested by Pronase (Calbiochem, San Diego, CA) for 3 hours at 37 ℃ and purified with Oasis Max cartridge (Water Co., Milford, MA). After elution with 2% formic acid in methanol, the eluents containing AFB_1-lysine adduct were evaporated until dry and reconstituted with 10% methanol in PBS.

HPLC analysis was carried out on an 1100 liquid chromatography system (Agilent, Wilmington, DE). Chromatographic separation was performed on an Agilent C18 column (5 µM, 250 X 4.6 mm). The mobile phase consisted of 20 nm ammonium phosphate monobasic (pH 7.2) and methanol in a linear gradient profile. The concentration of AFB_1-lysine adduct was monitored at the wavelength 405 nm (excitation) and 470 nm (emission). Typical mean retention time was 12.7 minutes for AFB_1-lysine adduct. The results of AFB_1-lysine adduct concentration was adjusted by serum albumin level. The limit of detection is 0.5 pg/mg albumin.

6.3.4 Statistical Analysis

Fisher's exact test, Chi-square test, t-test, or Wilcoxon test were used as appropriate to examine difference between cases and controls. Odds ratio (OR) and 95 percent confidence intervals (95% CI) for HCC risk were calculated using unconditional logistic regression, and a full assessment of potential confounding factors was conducted. First-degree family history of cancer was added as a confounding factor into the final logistic regression model since it was unevenly distributed in cases and controls (Table 6.1). Age and gender were fit into the final model too. Other variables were included in final model if they changed the target odds ratio by 10 percent or more when added to the unadjusted model [365]. Stratified analyses were performed on HBV genotypes, BCP mutations, and AFB_1-lysine adduct levels to evaluate whether HCC risk differed on these factors. Considering the distribution frequency in each subgroup, the mean level of AFB_1-lysine adduct in the control group was used as cut point in stratification of low or high AFB_1-lysine levels. A p-value of less than 0.05 (two-tailed) was considered statistically significant. All data were analyzed by using SAS software version 9.1.5 (SAS Institute Inc., Cary, NC, USA).

6.4 Results

As shown in Table 6.1, the cases and controls, in addition to frequency-matched age, gender and residency, had similar demographic characteristics except for first-degree of family history of cancer. Among 14 cases with first-degree family history of cancer, there were 12 liver cancers (85.7%), one esophageal cancer (7.1%), and one rectum cancer (7.1%). Among 13 controls with first-degree family history of cancer, there were 10 liver

cancers (76.9%), one esophageal cancer (7.7%), one lung cancer (7.7%), and one gastric cancer (7.7%), respectively.

Genotypes B, C, A+C and B+C were detected with genotype C predominant in both cases and controls (75% and 84.2%) (Table 6.2). No significant difference was found in the distribution of genotypes between the cases and controls (p=0.280).

The distribution of BCP mutations was presented in Table 6.3. Detectable $1762^{T}/1764^{A}$ double mutations was found in 91.7% (55/60) cases and 72.5% (88/120) controls with significant difference (p=0.007). 1762^{T} single mutation was rare and only found in one genotype C-control. When stratified by HBV genotypes, $1762^{T}/1764^{A}$ double mutations/1762^{T} single mutation still showed significant difference between cases and controls. In genotype B-cases, the detectable rate of $1762^{T}/1764^{A}$ double mutations was 69.2% (9/13), which was significantly higher than the rate of 23.5% (4/17) in genotype B-controls after adjustment of age, gender, first-degree family history of cancer (p=0.017). The detectable rate of 97.8% (44/45) in genotype C-cases was also significantly higher than the rate in genotype C-controls (83.2%, 84/101) after adjustment of age, gender, first-degree family history of cancer (p=0.044). Of particular interest, a significantly higher rate of $1762^{T}/1764^{A}$ double mutations was found in genotype C-subjects compared to genotype B-subjects (case group: 97.8% vs. 69.2%, p=0.007; control group: 82.2% vs. 23.5%, p<0.001).

The rate of 1753^{V} mutations occurs more in cases than controls (50.0% vs. 25.8%, p=0.001) (Table 6.3). Subjects having 1753^{V} mutations had an increased risk for HCC (adjusted OR=2.78, 95% CI: 1.43-5.40). After stratifying by HBV genotype, rates of 1753^{V} mutations were significantly higher in genotype C-cases compared to genotype C-

controls (60.0%, 27/45 vs. 29.7%, 30/101, p<0.001), whereas these types of mutations were rarely detected in genotype B-cases (2/13) and -controls (1/17), showing no significant difference (p=0.565). In all 1753^{V} mutations, 1753^{C} mutation was the major type detected in both cases (83.3%) and controls (90.3%). There were other 5 1753^{G} mutations (4 cases and 1 control) and 3 1753^{A} mutations (1 case and 2 controls).

Moreover, co-existence of 1753^{V} mutations and $1762^{T}/1764^{A}$ double mutations was usually detected. Among 30 cases with 1753^{V} mutations, 29 (96.7%) had $1762^{T}/1764^{A}$ double mutations; and of the 31 controls with 1753^{V} mutations, 29 (93.5%) had the double mutations. In addition, combinations of 1753^{C} mutation and $1762^{T}/1764^{A}$ double mutations were detected in 40.0% (24/60) of cases and 22.5% (27/120) of controls (age-, gender-, family history of cancer- adjusted OR: 2.35, 95% CI: 1.18-4.70, p=0.016).

Besides, several other mutations in BCP region were detected in study subjects at low frequencies. 1752^{V} mutation (A-to-T/C/G) was found in 10 (16.7%) cases and 4 (3.3%) controls, showing a significant increase of HCC risk (p=0.004) (Table 6.3). Mutation at position 1751 (G-to-A) was detected in 1 (1.7%) case. Mutation at position 1757 (G-to-A) was detected in 3 (5.0%) cases and 5 (4.2%) controls. Also, mutation at position 1768 (T-to-A) was found in 3 (5%) cases, and 1 of them was accompanied by a point mutation at position 1766 (C-to-T).

Distribution of serum AFB_1-lysine adduct in HCC cases and controls was illustrated in Figure 6.1. The average level of serum AFB_1-lysine adduct in cases was 11.10 ± 30.13 (mean ± SD) pg/mg albumin, with the median level of 4.84 pg/mg albumin (range: 0.54-227.13). The average level of AFB_1-lysine adduct in matched control group was 6.46±8.34 (mean ± SD) pg/mg albumin with a median level of 3.77 pg/mg albumin

(range: 0.79-51.93). Although levels of AFB_1-lysine adduct were higher in cases than controls, no statistically significant difference was found (p=0.115).

Data for both AFB_1-lysine adduct levels and HBV BCP mutations on HCC risk was presented in Table 6.4. The additive effects of $1762^T/1764^A$ double mutations and 1753^V mutations and dietary AFB_1 exposure were found: the adjusted OR (95% CI) was 6.94 (1.68-27.78) for subjects having $1762^T/1764^A$ double mutations with high AFB_1-lysine adduct level; 2.01 (0.24-14.29) for those only having high AFB_1-lysine adduct level, and 4.26 (1.16-15.38) for those only having $1762^T/1764^A$ double mutations, respectively. The adjusted OR was 5.13 (1.79-14.71) for subjects having 1753^V mutations with high AFB_1-lysine adduct level; 1.20 (0.47-3.08) for those only having high AFB_1-lysine adduct level, and 2.28 (1.01-5.31) for those only having 1753^V mutations, respectively.

6.5 Discussion

Our study showed a significant association of increased HCC risk with HBV $1762^T/1764^A$ double mutations, 1753^V mutations, and 1752^V mutations in the Southern Guangxi area, a high-risk area for HCC in China. In addition, we found additive effects of HBV BCP mutations (both $1762^T/1764^A$ double mutations and 1753^V mutations) and high serum AFB_1-lysine adduct level on HCC risk. To our knowledge, there has been no previously published data on exploring interactions of HBV genomic variations (HBV genotypes, BCP mutations) and AFB_1 exposure biomarkers on HCC risks in literature.

HBV genotype B and C are the two common genotypes in China [252, 366, 367] and Vietnam [368, 369], a neighboring country to Southern Guangxi, where more than 80% of subjects had genotypes B or C. A previous study in Guangxi area reported 100% of

genotype C in 20 asymptomatic HBV carriers [370], which was higher but still comparable to 84.2% of genotype C in our controls.

Several studies reported that genotype C was associated with higher HCC risk as compared to genotype B [357, 359]. However, the lack of such association was also reported by several other studies. A cohort study following up HBV infected children for 15 years in Taiwan found no increased HCC risk in genotype C-children, and instead, genotype B was the predominate type (74% in cases) in HCC patients [251]. Also, genotype C was found predominant without difference in both HCC patients and controls in Qidong, another HCC high-risk area in China [253]. Our study did not show such association, too. Additionally, we found a significantly higher frequency of $1762^{T}/1764^{A}$ double mutations in genotype C-subjects than genotype B-subjects, which is consistent with previous studies [253, 371] suggesting a possible linkage between $1762^{T}/1764^{A}$ double mutations and genotype C.

$1762^{T}/1764^{A}$ double mutations and 1753^{V} mutations are the two most common mutations in HBV BCP region. Although pathogenic mechanism remains unclear, *in vitro* study found that $1762^{T}/1764^{A}$ double mutations and 1753^{V} mutations could suppress the expression of HBeAg and induce a modest increase in viral replication rate [262, 263]. The association of $1762^{T}/1764^{A}$ double mutations with increased HCC risk was well established in high-risk populations in China and South Africa [250, 256, 257, 372]. The relationship between 1753^{V} mutations and HCC development is less studied [261], and remains controversial, especially in high risk populations [256, 261, 264]. Our study supported the association of both $1762^{T}/1764^{A}$ double mutations and 1753^{V} mutations with HCC risk. The noteworthy fact is that 1753^{V} mutations presented at a

lower frequency in both cases (50.0%) and controls (25.8%) than $1762^{T}/1764^{A}$ double mutations (91.7% and 74.1%, respectively), and always occurred along with the double mutations. Therefore, it's reasonable to hypothesize that mutations in BCP region may appear in a time-dependent manner: $1762^{T}/1764^{A}$ double mutations may be involved in the early stage of hepatocarcinogenesis and 1753^{V} mutations may occur in a later stage. Previous study found that $1762^{T}/1764^{A}$ double mutations were persistently detected in patients over a period of four years before diagnosis of HCC [358]. Although no such longitudinal study on 1753^{V} mutations, a study reported that the prevalence rate of 1753^{V} mutations dramatically increased with severity of liver diseases: 0% in asymptomatic HBV carriers, 9% in chronic hepatitis patients, and 47% in liver cirrhosis or HCC patients [260]. Therefore, $1762^{T}/1764^{A}$ double mutations and 1753^{V} mutations may serve as valuable biomarkers for HCC risk among individuals with HBV chronic infection, especially in high risk areas.

Besides, our study found that 1752^{V} mutations in BCP region were also associated to HCC risk. There is no previous published data indicated such an association. Based on low frequency of 1752^{V} mutations was detected in study subjects (3.3%-16.7%), it seems that these mutations appear later after 1753^{v} mutations, which suggests these mutations as a possible biomarker in monitoring high-risk population. In a previous study conducted in Qidong, China, 1766^{T} or 1768^{A} mutation accompanying $1762^{T}/1764^{A}$ double mutations was associated with increased HCC risk [253]. However, the point mutation at position 1766 and 1768 was rarely found in our study subjects.

The contributing role of AFB_1 to HCC risk is confirmative in many populations[373]. Combined dietary AF exposure and chronic HBV infection were correlated with HCC

risk in high-risk areas in China. In a nested case-control study from a Shanghai cohort, the relative risk for HCC in HBsAg and urinary AFB_1 metabolites positive subjects was found 59 times higher than those negative in HBsAg and urinary AFB_1 metabolites [220]. Two other studies in Taiwan confirmed the combinative effect of HBV infection and AFB_1 exposure in HCC risk [224, 225]. As proposed [374], it is possible that chronic HBV infection could fix and enhance DNA damage and oxidative stress induced by AFB_1, and vice versa. Since BCP mutations could alter HBV function/toxicity [262, 263], we anticipated that interaction between HBV and AF on HCC could be aggravated by BCP mutations. Our study indicated the additive effects of serum AFB_1-lysine adduct level with both $1762^T/1764^A$ double mutations and 1753^V mutation on risk for HCC.

Exposure to AF-contaminated food in Southern Guangxi residents was repeatedly reported over the past several decades [15-17, 375]. This study confirmed the high AF-exposure in both cases and controls, which were matched with residence to ensure similar dietary intake background. Also, to avoid temporal variation as a modifying factor, the blood samples were collected within the same season of the year in case and control groups. Thus, overwhelming dietary exposure to AF of may result in no significant difference of AFB_1-lysine adduct levels between cases and controls in this study. Another possibility is the limitation of AFB_1-lysine adduct. Although AFB_1-lysine adduct is considered as a reliable long-term exposure biomarker (2-3 months) [19], it does not necessarily reflect the lifetime exposure status in study subjects.

In summary, this study confirmed the association of BCP mutations with HCC development; based on data from this study and available evidence, accumulation of $1762^T/1764^A$ double mutations, 1753^V mutations, and 1752^V mutations in sequence may

occur in different stage of hepatocarcinogenesis. Limitations of this study include the nature of the case-control design and the limited sample size. Further evaluation and confirmation will be valuable in cohort studies with a larger sample size.

Table 6.1 Demographic characteristics of HCC cases and controls

Characteristics	Cases	Controls	p-value
Age (years) (mean ± SD*)	41 ± 9	42 ±7	0.300
Gender [n (%)]			
Male	54 (90.0)	108 (90.0)	
Female	6 (10.0)	12 (10.0)	1.000
Current cigarette smoker [n (%)]			
Yes	26 (43.3)	63 (52.5)	
No	34 (56.7)	57 (47.5)	0.246
Total pack-years of smoking (mean ± SD)	16.6 ± 9.0	23.2 ± 14.1	0.058
Alcohol drinker [n (%)]			
Yes	20 (33.3)	36 (30.0)	
No	40 (66.7)	84 (70.0)	0.649
First-degree family history of cancer [n (%)]			
Yes	14 (23.3)	13 (10.8)	
No	46 (76.7)	107 (89.2)**	0.027

* SD, standard deviation
** $p < 0.05$ from chi-square test

Table 6.2 HBV genotypes in HCC cases and controls

Groups	N	HBV genotypes [n (%)]			
		B	C	B+C	A+C
HCC cases	60	13 (21.7)	45 (75.0)	0 (0.0)	2 (3.3)
Controls	120	17 (14.2)	101 (84.2)	2 (1.7)	0 (0.0)

Table 6.3 HBV BCP mutations in HCC cases and controls

BCP Mutations		Controls (%)	Cases (%)	OR (95% CI)*	Adjusted OR (95%CI)
$1762^{T}/1764^{A}$ mutations	No ($1762^{A}/1764^{G}$)	31 (25.8)	5 (8.3)	1.00	1.00
	Yes ($1762^{T}/1764^{G}$ or $1762^{T}/1764^{A}$)**	89 (74.1)	55 (91.7)***	3.83 (1.41-10.41)	3.89 (1.40-10.77)$^{\#}$
1753^{V} mutations	No (1753^{T})	89 (74.2)	30 (50.0)	1.00	1.00
	Yes ($1753^{C/A/G}$)	31 (25.8)	30 (50.0)***	2.87 (1.50-5.50)	2.78 (1.43-5.40)$^{\#\#}$
1752^{V} mutations	No (1752^{A})	116 (96.7)	50 (83.3)	1.00	1.00
	Yes ($1752^{T/C/G}$)	4 (3.3)	10 (16.7)***	5.80 (1.74-19.37)	5.96 (1.75-20.25)$^{\#}$

* OR, odds ratio; CI, confidence interval
** 1 single mutation ($1762^{T}/1764^{G}$) was found in control group, all others were $1762^{T}/1764^{A}$ double mutations.
*** $p<0.05$ from unconditional logistic regression
$^{\#}$ adjusted for age, gender, first-degree family history of cancer; $^{\#\#}$ adjusted for age, gender, alcohol drinking status, first-degree family history of cancer.

Table 6.4 HCC risks in relation to HBV mutations and AFB_1 exposure in cases and controls

BCP mutations	AFB_1-lysine adduct	Control (%)	Case (%)	OR (95% CI)	Adjusted OR (95%CI)*
$1762^T/1764^A$ double mutations or 1762^T single mutations	*Concentration (pg/mg alb.)*				
No	< 6.46	22 (18.3)	3 (5.0)	1.00	1.00
	≥ 6.46	9 (7.5)	2 (3.3)	1.63 (0.23-11.45)	2.01 (0.24-14.49)
Yes	< 6.46	66 (55.0)	36 (60.0)**	4.00 (1.12-14.27)	4.26 (1.16-15.38)
	≥ 6.46	23 (19.2)	19 (31.7)**	6.06 (1.57-23.37)	6.94 (1.68-27.78)
1753^V mutations	*Concentration (pg/mg alb.)*				
No	< 6.46	65 (54.2)	21 (35.0)	1.00	1.00
	≥ 6.46	24 (20.0)	9 (15.0)	1.16 (0.47-2.89)	1.20 (0.47-3.08)
Yes	< 6.46	23 (19.2)	18 (30.0)**	2.42 (1.10-5.33)	2.28 (1.01-5.31)
	≥ 6.46	8 (6.7)	12 (20.0)**	4.65 (1.67-12.89)	5.13 (1.79-14.71)

* adjusted for age, gender, first-degree family history of cancer
** $p<0.05$ from unconditional logistic regression

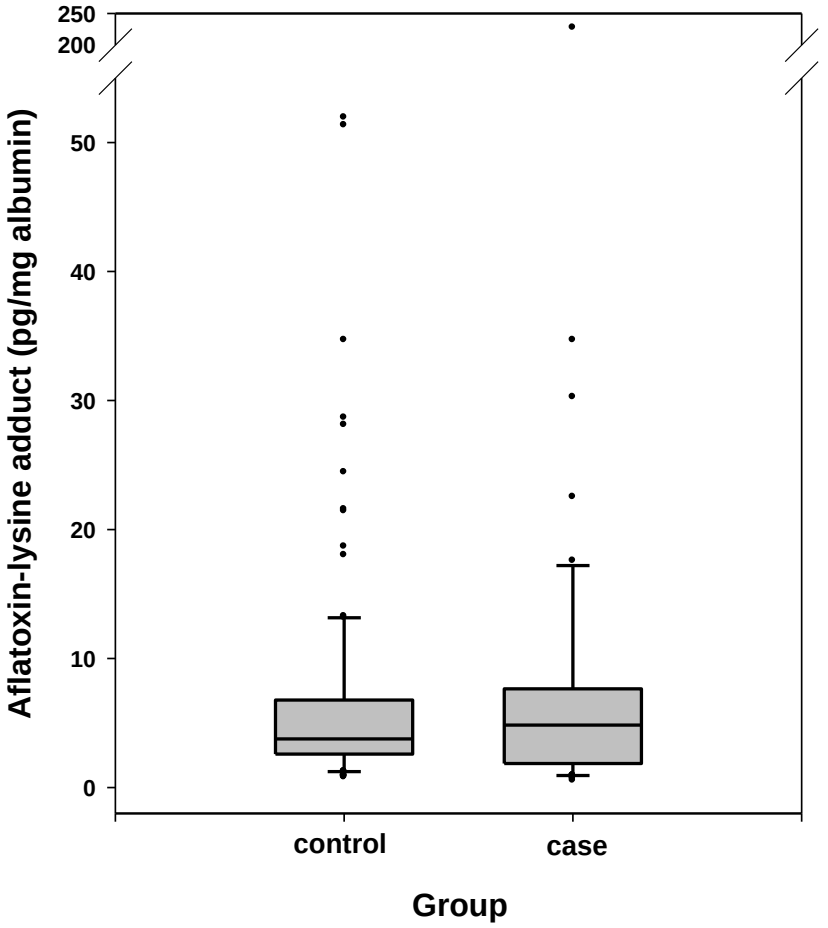

Figure 6.1 Serum AFB_1-lysine adducts level in cases and controls. The median and interquartile range of percentages in cases and controls were 4.84 (1.88-7.65) pg/mg albumin and 3.77 (2.62-6.78) pg/mg albumin, respectively. The dots, error bars and upper and lower ends of the box represent outliers, spread, and first and third quartiles, respectively.

Chapter 7

CONCLUSIONS

In this dissertation research, two population-based case-control studies were designed and performed to investigate possible etiological factors of ESCC and HCC in Huaian and South Guangxi, China, respectively. Several conclusions can be drawn from this study:

1. The ESCC case-control study reveals that medical history of esophageal lesion was associated with increased ESCC risk. Majority of the esophageal lesion clinically diagnosed was chronic esophagitis. These findings support chronic esophagitis as a possible precursor lesion for ESCC.
2. In the ESCC case-control study, preference for salty food ten years ago, preference for consuming food with high temperature tne years ago, frequent intake of pickled/salty food and frequent intake of mildewed food ten years ago, are risk factors for ESCC in Huaian high-risk population.
3. Regular tea drinking and frequent garlic intake are protective factors for ESCC, together with the risk factors shown above, suggesting the contributing role of dietary behavior/habit in ESCC development in this high-risk area.
4. Increased BMI was associated with reduced risk of ESCC in this high risk population, reflecting the etiological role of nutritional status in ESCC development.

5. Cigarette smoking and alcohol drinking were not associated with ESCC risk, suggesting that these factors are not major risk factors for ESCC in this high risk area.
6. Urinary free FB_1 was associated with increased risk for ESCC, supporting the contribution of dietary FB_1 exposure to ESCC risk in this high risk area. Furthermore, the urinary free FB_1 level was significantly positive correlated with corn meal intake level in study subjects. These provide strong supporting evidence in application of urinary free FB_1 as exposure biomarker in human epidemiology study in future.
7. Urinary Sa and So was not associated with ESCC risk in the present study. Only high level of urinary Sa/So ratio was assoicated with increased ESCC risk. In general, the Sa/So ratio is not as valid and sensitive as urinary free FB_1 serving as dietary FB_1 biomarker in this ESCC high risk population.
8. The present study shows that urinary 8-OHdG level was associated with increased risk of ESCC and positive correlated with urinary free FB_1 level, confirming that oxidative DNA damage caused by environmental toxin exposure play an important role in ESCC development.
9. XPD Lys751Gln polymorphism was associated with ESCC risk, suggesting its role in modifying the risk of ESCC. Moreover, XPD Lys751Gln polymorphism interacted with urinary free FB_1 level as well as frequent pickled/salty food intake in relation to ESCC risk, further confirming its contribution to ESCC risk and supporting the role of gene-environment interaction in etiology of ESCC in this high risk population.

10. The HCC case-control study reveals that HBV genotype C is the predominant genotype in South Guangxi HCC high risk population.
11. HBV $1762^{T}/1764^{A}$ double mutations, 1753^{V} mutations, and 1752^{V} mutations in BCP domain are risk factors for HCC in South Guangxi high risk area.
12. HBV $1762^{T}/1764^{A}$ double mutations and 1753^{V} mutations can interact with serum AFB_1-lysin adduct level resulting in combinative effects on increased HCC risk. This indicates the interaction between etiological role of HBV mutations and dietary aflatoxin exposure in HCC development in this high risk population.

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