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ORIGINAL ARTICLE

Case Control Study

Esophageal squamous cell cancer in a highly endemic region

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Abstract

AIM: To identify risk factors associated with esophageal cancer in Zambia and association between dietary intake and urinary 8-iso prostaglandin F2α (8-isoPGF2α).

METHODS: We conducted a prospective, case control study at the University Teaching Hospital. Subjects included both individuals admitted to the hospital and those presenting for an outpatient upper endoscopy. Esophageal cancer cases were compared to age and sex-matched controls. Cases were defined as patients with biopsy proven esophageal cancer; controls were defined as subjects without endoscopic evidence of

esophageal cancer. Clinical and dietary data were collected using a standard questionnaire, developed *a priori*. Blood was collected for human immunodeficiency virus (HIV) serology. Urine was collected, and 8-isoPGF2α was measured primarily by enzyme-linked immunosorbent assay and expressed as a ratio to creatinine.

RESULTS: Forty five controls (mean age 54.2 ± 15.3, 31 male) and 27 cases (mean age 54.6 ± 16.4, 17 males) were studied. Body mass index was lower in cases (median 16.8) than controls (median 23.2), P = 0.01. Histopathologically, 25/27 (93%) were squamous cell carcinoma and 2/27 (7%) adenocarcinoma. More cases smoked cigarettes (OR = 11.24, 95%CI: 1.37-92.4, P = 0.02) but alcohol consumption and HIV seropositivity did not differ significantly (P = 0.14 for both). Fruit, vegetables and fish consumption did not differ significantly between groups (P = 0.11, 0.12, and 0.10, respectively). Mean isoprostane level was significantly higher in cases (0.03 ng/mg creatinine) than controls (0.01 ng/mg creatinine) (OR = 2.35, 95%CI: 1.19-4.65, P = 0.014).

CONCLUSION: Smoking and isoprostane levels were significantly associated with esophageal cancer in Zambians, but diet, HIV status, and alcohol consumption were not.

Key words: Gastrointestinal cancer; Non-communicable diseases; Zambia

Core tip: The most common type of esophageal cancer in developing countries, including Sub-Saharan Africa, is squamous cell carcinoma, in contrast to the United States and United Kingdom, in which adenocarcinoma predominates. Yet, there are few studies evaluating risk factors, antioxidant status and the role of oxidative stress of esophageal cancer in Africa. This study explores the association of a non-invasive marker for oxidative stress in esophageal cancer.

INTRODUCTION

Esophageal cancer is the eight most common cancer worldwide and the sixth most common cause of cancer death[1-3]. Esophageal adenocarcinoma (EA) and esophageal squamous cell carcinoma (ESCC) are the two most common esophageal cancers worldwide. EA is most common in the western world (North America, Europe), whilst ESCC is most common in Africa, South America and China[4]. Data suggests an increasing trend (500% increase) of EA over the past 30 decades in Western world[5] and decreasing rates of ESCC[4]. In Sub-Saharan Africa, there is an increasing trend of ESCC, attributed to several factors including improved diagnostic capabilities and changing behaviour (smoking, obesity)[6]. In a systematic review evaluating the epidemiology of esophageal cancer in sub-Saharan Africa, Kachala reports an increase in esophageal cancer, predominantly ESCC, with varying incidence rates within the continent ranging from, 0.6 to 76.6/100000 and 0.6 to 36.5/100000, in males and females respectively[6]. In a retrospective study at the University Teaching hospital (UTH), Lusaka, Zambia, Kelly *et al*[7] found 96% of esophageal cancers were ESCC. The overall incidence of esophageal cancer in Zambia is estimated to be 9.1/100000[1], more than in United States (3-4/100000) or United Kingdom (6.5/100000).

Information regarding esophageal cancer within most countries in Africa is poor, primarily due to under-reporting because of lack of diagnostic resources and limited manpower[7]. Zambians with esophageal cancer often present with disease at a late stage, probably due to financial constraints and access to health care facilities[2]. According to GloboCan 2012, esophageal cancer is the 5th leading cause of cancer mortality in Zambia[1]. However, the age of onset of esophageal cancer is younger in the Zambian population compared to the United States and United Kingdom. Kelly *et al*[7] found that 28% of patients with esophageal cancer were younger than 45 years of age, compared to the United States or United Kingdom in which new cases occurring in individuals younger than 50 years of age were 7.6% and less than 10% respectively[8,9]. The phenomenon of esophageal cancer in young people has also been observed in Kenya. Of 2643 newly diagnosed cancers, 914 (34.6%) were esophageal cancer; of which 58 (6.3%) patients were under 30 years, 9 (1%) under 20 years, with the youngest patient diagnosed at age 14 years[2]. We propose that elucidation of the basis for this age difference may reveal important clues to the etiology and pathogenesis of esophageal cancer.

A review of the literature reveals that risk factors for esophageal cancer include environmental, lifestyle (diet, smoking and alcohol consumption), infectious, and genetic factors[3,10-14]. Alcohol consumption has been primarily associated with ESCC whilst smoking is a risk factor for both squamous cell and adenocarcinoma[3]. The specific dietary factors include vitamin/trace metal deficiencies, and increased nitrites and nitrosamines[15].

Dietary antioxidants, such as those found in fresh fruits and raw vegetables, particularly green leafy vegetables, are thought to play a protective role in

the development of cancer[16-20]. Oxidative stress is a result of increase production of reactive oxygen species which may play a role in carcinogenesis by causing damage to DNA, cell structure and increase proliferation rate[17-21]. Antioxidants such as those found in diet are thought to play a role in preventing and repairing damage induced by the reactive oxygen species[5]. This study aims to test the hypothesis that poor antioxidant status predisposes to esophageal cancer in Zambia using isoprostanes measured in urine as a surrogate marker of oxidative stress. Isoprostanes are prostaglandin like substances formed by free radical peroxidation of arachidonic acid[21]. The increase isoprostane level is a marker of free radical induced damage that leads to DNA damage and has a role in carcinogenesis. *In vivo* formation of isoprostanes in humans was not discovered until the 1990s[21,22], and this measurement of isoprostanes is considered one of the most reliable methods to assess *in vivo* status of oxidative stress[21], with urinary status being least invasive. Oxidative stress plays a role in carcinogenesis by increasing DNA damage, cell proliferation and apoptosis.

MATERIALS AND METHODS

Institutional Review Board approval was obtained from the University of Zambia School of Medicine research ethics committee and Washington University School of Medicine in St Louis, United States. Informed consent was obtained from all patients prior to enrolling patients in the study.

This was a prospective, case control study at the largest tertiary hospital, University Teaching Hospital (UTH) in Lusaka, Zambia, Southern Africa between November 2010 and January 2012. UTH is a national referral hospital based in Lusaka, the capital city of Zambia, and sees cases from all over the country, a catchment area of approximately 1.3 million[23].

Study design
We employed a case control study design. Cases and controls were matched by sex, and age (within age groups $\leq$ 30, 31-45, 46-60, or > 60 years). Our inclusion criteria for cases were: (1) age $\geq$ 18 years; and (2) histopathologically proven esophageal cancer; for controls: (1) age $\geq$ 18 years; and (2) upper gastrointestinal (GI) symptoms but no pathology seen at endoscopy. The exclusion criteria: (1) active chemotherapy or radiation therapy; and (2) inability to consent.

Data collection
For patients who met inclusion criteria, a questionnaire was used to collect demographic data (age, gender, occupation, socioeconomic status and education level); a medical history including family history, alcohol consumption history and smoking history); A food frequency questionnaire (FFQ), developed *a priori* by the research team was used to obtain dietary history. The FFQ was specific to the Zambian diet and 51 food items were classified into 7 categories: Fruits, vegetables, fish, meat, insects, cereals and starches. This food questionnaire assessed habitual food consumption of each food item by asking frequency in form of never, once per month, once per week, 2-3 times per week, 4-6 times per week, daily, 2-3 times per day, 4-6 times per day and 7 times per day. The analysis was performed of the 7 categories. A physical examination was performed on all subjects at initial enrollment. An upper endoscopy was performed using the Pentax 2990 series endoscopes. Cold biopsy forceps were used to obtain routine biopsies from all malignant appearing lesions for histopathology. Urine samples were collected to measure levels of isoprostanes using immunoassays. Blood samples were collected for human immunodeficiency virus (HIV).

Lab technique for immunoassay
Urine samples were collected to measure isoprostanes (8-iso prostaglandin F2α) using enzyme-linked immunosorbent assay (ELISA). In our prior study the use of ELISA was validated by comparing results against the gas chromatography/mass spectrometry[24]. Butylated hydroxytoluene (BHT) preservative stock solution was prepared with 50 mg/mL methanol and stored in a refrigerator at +4 degrees Celsius. Fasting spot urine samples were collected from all subjects; aliquots of 0.9 mL was added to 0.1 mL BHT in a cryopreservation tube then stored at -80 ℃. Urinary isoprostane and creatinine concentrations were measured using Isoprostane and Creatinine Microplate Assays (Oxford Biomedical, Oxford, MI) according to the manufacturer's instructions.

Statistical analysis
Data was analyzed using SAS. The cases and control were matched by age, so they are not independent cohorts of patients. Conditional logistic regression was used to take into account the matching while estimating the overall risk of esophageal cancer associated with smoking, alcohol consumption, HIV positive status, isoprostane and creatinine levels and standardized food consumption frequencies. The following variables were categorized and thus we were able to analyze them as dichotomous or polytomous independent variables using conditional logistic regression: age (< 30, 31-45, 46-60, > 60), education (none, primary, secondary, tertiary) and income (high *vs* low). The following were not categorized, but analyzed as continuous variables: BMI and MUAC. Mean isoprostane level was analyzed on a log scale to improve the fit of the logistic model. The odds of esophageal cancer were calculated per one unit increase in log isoprostane level. The results

Table 1 Baseline characteristics *n* (%)

Variable	Case (*n* = 27)	Control (*n* = 45)	*P* value
Age group			
< 30 yr	2 (7.41)	2 (4.4)	(reference)
31–45 yr	7 (26.0)	14 (31.1)	0.99
46–60 yr	6 (22.2)	11 (24.4)	0.99
> 60 yr	12 (44.4)	18 (40.0)	0.99
Gender			
Male	17 (63)	31 (69)	0.99
Female	10 (37)	14 (31)	(reference)
Education			
None	2 (8.3)	4 (8.9)	(reference)
Primary	15 (62.5)	18 (40.0)	0.76
Secondary	6 (25)	11 (24.4)	0.66
Tertiary	1 (4.1)	12 (26.7)	0.99
Income			
Low	21 (77.8)	15 (33.3)	(reference)
High	2 (7.4)	14 (31.1)	0.025
Irregular/unsure	4 (14.8)	16 (35.6)	0.019
Smoking status			
Never	19 (70)	42 (93)	(reference)
Ever	8 (30)	3 (7)	0.024
Alcohol intake			
Never	18 (67)	37 (82)	(reference)
Ever	9 (33)	8 (18)	0.140
Median BMI (kg/m^2)	16.8	24.5	0.014
HIV			
Positive	6 (22.2)	3 (6.7)	0.140
Negative	20 (74.1)	38 (84.4)	(reference)
MUAC (cm)	21.5 (19-25)	28 (26-31)	0.0024

Low income: less than $200 per month; Ever smoked or drank alcohol includes both current and former. A *P* value of < 0.05 was statistical significant. BMI: Body mass index; MUAC: Mid upper arm circumference; HIV: Human immunodeficiency virus.

are expressed as odds ratio with a 95%CI. *P* values test the null hypothesis that each of the following risk factors is equally likely to be present in those with esophageal cancer and in the controls with no esophageal cancer. A *P* value of less than 0.05 was considered to be statistically significant. Sample size calculations were based on a matched case-control study design. Given that there are few baseline data are available, the assumptions made were plausible but not necessarily accurate. We assumed that 20% of the controls have high isoprostane levels (indicating high levels of oxidative stress), and 50% of cases have high isoprostane levels, with 95% confidence and 90% power, we thus required 58 cases and 58 controls.

RESULTS

Patient characteristics
During the study period, we enrolled patients from across the country, 27 with esophageal carcinoma and 45 controls. We analyzed 27 case-control sets, 11 with one control and 16 with 2 controls per case. Of the 45 controls, 31 were males with a mean age of 54.2 years (SD ± 15.3) and of the 27 cases, 17 were males with a mean age of 54.67 years (SD ± 16.4) (Table 1). The median BMI for cases (16.8 kg/m^2)

Table 2 Logistic regression models of association of risk factors with esophageal cancer

Risk factor	Odds ratio	95%CI	*P* value
Isoprostane excretion (log)	2.35	1.19-4.65	0.014
Current/former smoker *vs* never	11.24	1.37-92.40	0.024
Current/former alcohol use *vs* never	2.49	0.744-8.34	0.140
HIV seropositive	3.450	0.656-18.14	0.140
Total fruits	0.761	0.542-1.07	0.110
Total vegetables	0.917	0.822-1.02	0.120
Total fish	0.364	0.108-1.23	0.100
Total animal products	0.555	0.162-1.90	0.350
Total insects	4.220	0.463-38.42	0.200

Associations between risk factors and cancer are presented as OR with 95%CI, and a *P* value of < 0.05 was required for statistical significance. HIV: Human immunodeficiency virus.

and controls (23.2) differed, and was statistically significant, *P* = 0.014. Social history revealed more cases than controls smoked, OR = 11.24, 95%CI: 1.37-92.4, *P* = 0.02. Neither alcohol consumption nor HIV seropositivity were statistically significant, *P* = 0.14 for both. Histopathologically, 25/27 (93%) cases were squamous cell carcinoma and 2/27 (7%) were adenocarcinoma. The location of the malignant lesions in the esophagus was as follows: 4/27 in the upper third, 6/27 in the middle and 4 in the lower third. Lesion location documentation was missing for 13 of the 27 cases.

Smoking and alcohol status
Ever having smoked (that is, current or former smoker) was more common among cases than among controls (OR = 11.2, *P* = 0.024). Being a current smoker was not demonstrably more common in either group (*P* = 0.21) (Table 2). Ever having smoked and ever having consumed alcohol (that is, having been both a smoker and a drinker, *vs* having done only one or neither of these things) was also more common among cases than controls (OR = 9.3, *P* = 0.040). This effect may be mostly due to smoking.

Food frequency questionnaire estimation of dietary intake
Standardized food frequencies did not differ in cases and controls, although total fruits, total vegetables and total fish may have higher values in controls (*P* = 0.11, 0.12 and 0.10, respectively). After correction for multiple testing none of the individual foods are clearly more commonly consumed with either cases or controls (individual food results not shown here).

Urinary isoprostane excretion
The mean isoprostane excretion into urine was significantly higher in cases (0.03 ng/mg creatinine) than controls (0.01 ng/mg creatinine), OR = 2.35, 95%CI: 1.19-4.65, *P* = 0.014. We found the higher isoprostane levels are associated with esophageal

cancer compared to the controls, $P = 0.014$. The risk increases by about 2.4 times with each unit increase on a log scale. There is no evidence that creatinine levels are associated with esophageal cancer ($P = 0.11$).

DISCUSSION

Esophageal cancer poses a significant global health burden, with majority of cases and deaths occurring in highly endemic areas, Asia and Southern Africa[1]. Late presentation especially in cases of ESCC has been identified as one of the risks for poor outcome and death[25]. Given the poor morbidity and mortality, it is important to understand the etiopathogenesis of cancer and develop tools for early detection. An increase in isoprostane levels has been found in association with other disorders such as cardiovascular (heart failure), pulmonary (asthma), neurologic (multiple sclerosis)[26], hepatic (cirrhosis) and cancers (prostate, breast)[21,22]. Oxidative stress has been implicated in various disorders including cancer of the prostate[27,28], lung[29], and breast[30]. An imbalance between pro-oxidant and antioxidants results in oxidative stress, and this may promote carcinogenesis. The advantage of measuring urinary isoprostanes as a marker of oxidative stress is that it is postulated to be chemically stable, detectable in all tissues hence allowing for normal range definition and evidence from animal models showing an increase in the setting of oxidative injury[21]. The prognostic aspect of F2 isoprostanes shows a direct relationship between isoprostane and disease state, specifically a higher level with increasing severity in conditions such as asthma explained by worsening inflammation[21]. However, there is not sufficient evidence to apply this direct relationship in the development and progression of esophageal squamous cell cancer.

In this study, we report risk factors associated with esophageal cancer in Zambians. This study contributes to what is currently known about the role of diet, oxidative stress and antioxidants in carcinogenesis. We found that smoking and elevated urinary isoprostane levels were associated with esophageal cancer in the Zambian patients, but diet composition, HIV status, and alcohol consumption were not. To our knowledge this is the first prospective study evaluating dietary factors and urinary isoprostane as a marker of oxidative stress as risk factors for esophageal cancer in Zambia. This is also one of a handful of publications related to esophageal cancer risk factors in Zambia. Our hypothesis was that poor antioxidant status as evidenced by poor dietary antioxidants predisposes to esophageal cancer in Zambia and the use of isoprostanes measured in urine was as surrogate non-invasive marker of oxidative stress. However, our analysis revealed that the consumption of total fruit, vegetables and fish consumption did not differ significantly between esophageal cancer cases and controls. The mean isoprostane level was significantly higher in esophageal cases than controls, which cannot be explained by dietary factors alone. A factor to consider is the smoking status of subjects and role in carcinogenesis. There is some evidence suggesting that cigarette smoking results in increase in oxidative stress given the oxidant content[31-33], thus would play a role in carcinogenesis. However, there is also data supporting the fact that many in the esophageal squamous cell carcinoma high risk areas do not smoke or consume significant amount of alcohol to explain the increase incidence and prevalence of ESCC. This is an area requiring further research.

The role of dietary factors, such as meat in esophageal carcinogenesis is inconsistent, attributing to the method by which the meat was prepared - boiled, fried or grilled[5]. There are also data supporting the role of carbohydrates in esophageal carcinogenesis, however this data is inconclusive with some studies showing an inverse association[5]. The Zambian diet is rich in carbohydrates with the main daily meal consisting of Nshima (a maize flour based food); however we do not believe this confounded our results given that Nshima is a staple part of the diet and consumed by all participants. The International Agency for Research on Cancer (IARC) working group has identified alcohol as a human carcinogen and implicated its role in ESCC; however in regards to red/processed meat the conclusion is "limited suggestive increase". There are several studies evaluating the role of diet in esophageal carcinogenesis with fewer differentiating the histologic subtype[34]. As mentioned, meat has been one of the most widely studied food items with inconsistent results. Most published studies are from Western nations such as United States, Europe, Asia and Australia. In a systematic review and dose response meta-analysis evaluating meat, fish and esophageal cancer risk, Salehi *et al*[34] analyzed 35 articles, of which 14 focused on ESCC and 13 did not differentiate ESCC from EAC. There was an overall positive association between red meat and those with ESCC[34]. The mechanism of red meat in carcinogenesis is believed to be related to pro-oxidant property of heme iron. The difference of meat consumption in Western countries *vs* Asian countries has to do with the meat being more processed in Western nations; however both processed and red meat contains N-nitroso compounds and heme iron[34]. Red meat is consumed more than processed meat in the Zambian population, with 20%-30% of households from most major cities[35]. We found no significant difference in meat consumption between controls and cases.

There are several limitations to our study. First patients with esophageal cancer may change their diet due to dysphagia or other clinical features related to cancer. However, the use of FFQ has the advantage of capturing diet overtime including prior to being symptomatic. Secondly, we cannot ascertain if the poor antioxidant status in patients with esophageal cancer is a cause or consequence of the cancer.

Thirdly, we did not measure the antioxidant content of the food items, proportion or mode of preparation. Finally, we did not achieve our calculated sample size due to limitations in research funding; however our data contributes significantly to current knowledge about esophageal cancer. There are also numerous strengths to this study. First this was a prospective, case control study that included subjects both hospital and community based setting. Secondly, the FFQ was developed *a priori* specific to the Zambian diet, but can also be applied to other countries within the region. Third, this study contributes significantly to the understanding of esophageal cancer and role of urinary isoprostanes as a non-invasive marker of oxidative stress. This knowledge can be used to guide further studies evaluating risk factors as it relatives to oxidative injury and carcinogenesis.

In conclusion, identifying risk factors for esophageal cancer is important, so as to characterize modifiable risks that can be altered with behavioural changes and thus contribute to decreasing overall disease incidence or set-up screening protocols for individuals at risk. To our knowledge there are no global guidelines for ESCC screening, however several techniques have been studied to identify precursor lesions. Screening and surveillance guidelines for Barrett's esophagus have been developed by the American Society for Gastrointestinal Endoscopy (ASGE), but no concrete guidelines exist for ESCC. Further studies are encouraged to understand the role of urinary isoprostanes in screening and progression of esophageal squamous cell cancer.

COMMENTS

Background
Globally, esophageal cancer poses significant morbidity and mortality with an increasing trend in sub-Saharan Africa. Broadly there are two main subtypes: adenocarcinoma and squamous cell carcinoma. The squamous cell carcinoma predominates in the developing countries, including sub-Saharan Africa, yet there are limited published studies from affected regions such as Zambia, Southern Africa. Interestingly, the age of presentation for esophageal cancer in the Zambian population is younger (less than 45 years) when compared to developed nations such as United States or United Kingdom. Recognizing risk factors (*i.e.*, genetic, dietary, environmental) is important in understanding carcinogenesis and developing tools for early detection and prevention of esophageal cancer. Oxidative stress plays a role is carcinogenesis *via* tumour cell proliferation and DNA damage. The ability of measuring oxidative stress could play a role in early detection of esophageal cancer and prevention of progression as a monitoring tool.

Research frontiers
One of the most reliable methods to assess *in vivo* status of oxidative stress is the measurement of isoprostanes. Urinary isoprostanes have been studied in other cancers (prostate, breast) as a marker of oxidative stress. To the best of the authors' knowledge, there are no published studies from Sub-Saharan Africa exploring the role of isoprostanes as a marker of oxidative stress in esophageal cancer.

Innovations and breakthroughs
The aim of this study was to identify risk factors associated with esophageal cancer in Zambia and association between dietary intake and urinary 8-iso prostaglandin F2α. There are no studies from Zambia or other sub-Saharan countries exploring this association. Oxidative stress has been implicated in various disorders including cancer of the prostate, lung, and breast. An imbalance between pro-oxidant and antioxidants results in oxidative stress, and this may promote carcinogenesis. This study contributes to what is currently known about the role of diet, oxidative stress and antioxidants in carcinogenesis.

Applications
Urinary isoprostanes could potentially serve as a biomarker for oxidative stress in individuals with esophageal cancer. Further studies are needed to explore role of isoprostanes in early detection of esophageal cancer and monitoring of treatment in those with diagnosed esophageal cancer.

Terminology
Oxidative stress plays a role in carcinogenesis by increasing DNA damage, cell proliferation and apoptosis. Oxidative stress is an imbalance between free radicals (unstable, reactive) and antioxidants, with resulting damage induced by free radicals. Urinary isoprostanes are prostaglandin like substances that can be measured in urine as markers of oxidative stress. Oxidative stress has been recognized in the development of cancer; hence urinary isoprostanes can be used as a non-invasive marker of oxidative stress.

Peer-review
This prospective, case control study provides more data about the association of isoprostanes with carcinogenesis and to our knowledge this is one of the first few studies exploring the role in esophageal cancer in Sub-Saharan Africa. This study contributes to current knowledge of esophageal cancer and risk factors. Information from this study could be used for further studies, development of screening protocols and management of esophageal cancer.

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