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Risk assessment of esophageal adenocarcinoma using γ -H2AX assay

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Abstract

Background—Mutagen-induced DNA damage as measured in peripheral blood lymphocytes (PBLs) has been associated with increased risks of cancers. The formation of γ -H2AX is an early cellular response to DNA double-strand breaks (DSBs). We hypothesize that higher level of radiation-induced γ -H2AX in PBLs may be associated with an increased risk of esophageal adenocarcinoma (EAC).

Methods—Laser scanning cytometer-based immunocytochemical method was used to measure baseline and irradiation-induced γ -H2AX levels in PBLs from 211 EAC patients and 211 healthy controls. The ratio of induced γ -H2AX level to baseline level was used to evaluate individual susceptibility to DSBs. Relative risks for EAC associated with γ -H2AX were assessed by multivariable logistic regression analysis.

Results—Radiation-induced γ -H2AX level and the γ -H2AX ratio were significantly higher in cases than in controls. Dichotomized at the median in controls, a significantly increased risk for EAC was observed in association with high γ -H2AX ratio (odds ratio=2.94, 95% confidence interval=1.83–4.72). Quartile analyses showed significant dose-response associations between higher γ -H2AX ratio and increased risk of EAC (P for trend, 1.64E-06). In addition, joint effect between γ -H2AX ratio and smoking was observed: smokers who had high γ -H2AX ratio exhibited the highest risk of EAC (OR =5.53, 95% CI: 2.71–11.25) compared to never-smokers with low γ -H2AX ratio.

Conclusion—Radiation-induced DNA damage assessed by γ -H2AX ratio is associated with an increased risk of EAC.

Impact— γ -H2AX assay is a new and robust method to measure DSB damage in PBLs, which can be used to assess mutagen sensitivity and EAC risk.

Keywords

Double strand break; γ -H2AX; mutagen sensitivity assay; esophageal cancer risk

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No potential conflicts of interest were disclosed.

Introduction

Esophageal cancer is diagnosed in approximately 300,000 individuals around the world each year. In the United States, an estimated 17,990 new cases (14,440 men and 3,550 women) and 15,210 deaths (12,220 men and 2,990 women) are expected in 2013 (1). There are two major histologic types of esophageal cancer, esophageal squamous cell carcinoma and esophageal adenocarcinoma (EAC). In the Western world, EAC has the most rapidly increasing incidence among all solid tumors, and EAC currently accounts for more than 80% of new esophageal cancer cases in the U.S. (2). EAC frequently arises from Barrett's esophagus, a precursor lesion, and multiple modifiable risk factors have been identified for EAC, including smoking and obesity (3–7). The fact that only a small fraction of exposed individuals develop EAC suggests genetic predisposition to EAC.

It is widely recognized that DNA repair is of fundamental importance in maintaining genomic integrity and protecting against cancer. DNA damage/repair capacity can be quantified by phenotypic assays such as the mutagen sensitivity assay, which quantifies chromatid breaks induced by mutagens in short-term cultures of peripheral blood lymphocytes (PBLs) (8). Numerous studies have shown that individuals with higher mutagen sensitivity phenotype are at increased risk of various cancers (9–12). DNA double-strand breaks (DSBs) is one of the major forms of DNA damage. When DSB repair fails, the resulting chromosomal instability may lead to tumor formation or progression (13, 14).

One of the most well-established molecular events during DSB repair process is the phosphorylation of histone H2AX, referred to as γ -H2AX (15, 16). In mammalian genome, this phosphorylation event on Ser-139 at the C-terminus of the H2AX molecule is one of the earliest responses to DSBs, playing a critical role in the recognition, signaling, and repair of DSBs (17, 18). γ -H2AX foci can be easily detected in cell nuclei by various techniques, such as flow cytometry, immunoblotting, and immunofluorescence microscopy, which allows γ -H2AX to be used as a quantitative marker of DSBs in a wide range of applications. The potential applications of γ -H2AX as a marker of aging and aging-related diseases, predominantly cancer, are emerging (19, 20). However, to our knowledge, no molecular epidemiologic studies have been performed to evaluate γ -H2AX level with EAC risk.

In this case control study, we applied a new laser scanning cytometer-based quantitative immunofluorescence assay to measure baseline γ -H2AX level and the ratio of γ -H2AX before and after irradiation in PBLs. We hypothesized that higher level of radiation-induced DSBs, as represented by γ -H2AX ratio, is associated with an elevated risk of EAC.

Materials and Methods

Study population and epidemiologic data

In this study, 211 patients with EAC were identified from participants of an ongoing case-control study of EAC. All cases were newly diagnosed, histologically confirmed EAC patients who had not received chemotherapy or radiotherapy prior to recruitment. There were no age, gender, ethnicity, or clinical stage restrictions on recruitment. The 211 healthy controls, who had no prior history of cancer, except non-melanoma skin cancer, were identified and recruited using the random digit dialing method. In random digit dialing, randomly selected phone numbers from household were used to contact potential control volunteers in the same residency of cases accordingly to the telephone directory listings. Controls must have lived for at least 1 year in the same county or socioeconomically matched surrounding counties in Texas as the case resides and have no prior history of cancer. After a case was identified, a control was selected to match the case patient in age

(± 5 years), gender, and ethnicity. To minimize effects of population admixture, only non-Hispanic white individuals were included in the analysis.

Epidemiologic data were collected from all cases and controls in a 45-minute in-person interview using a structured questionnaire; data collected included demographic characteristics, family history of cancer, medical history, recent and prior tobacco use, alcohol consumption, and occupational exposures. An individual who had never smoked or had smoked no more than 100 cigarettes in his or her lifetime was considered a never smoker. An individual who had smoked at least 100 cigarettes in his or her lifetime was defined as an ever smoker. Ever smokers included former smokers and current smokers. A former smoker was a person who had quit at least 12 months before diagnosis (for cases) or 12 months before the interview (for controls). A current smoker was a person who was currently smoking or had quit less than 12 months before diagnosis (for cases) or less than 12 months before the interview (for controls). At the completion of the interview, a 40-ml sample of peripheral blood was drawn into coded and heparinized tubes and delivered to the laboratory for analyses. All study participants signed a written informed consent, and the study was approved by the institutional review boards of The University of Texas MD Anderson Cancer Center and the Kelsey–Seybold Clinics.

Whole-blood culture and γ -H2AX assay

Blood cultures were set up immediately after the samples were delivered to the laboratory. A total of 0.4 ml of whole blood was divided into two 0.2-ml aliquots, which were cultured in 1.8 ml of RPMI 1640 (JRM Biosciences, Lenexa, KS) supplemented with 15% fetal calf serum (Life Technologies, Grand Island, NY) and 1.25% phytohemagglutinin (Remel, Lenexa, KS) at 37°C for 72 hr. These two identical cultures for each study subject were prepared for detection of DSBs at baseline (untreated) and after irradiation (treated).

For the treatment set, cells were exposed to γ -irradiation at a dose rate of 1.64 Gy/min from a cesium-137 source (cesium irradiator Mark 1, Model 30; J.L. Shepherd and Associates, Glendale, CA) at room temperature. After irradiation, the blood cultures were quickly returned to a 37°C incubator for 1 hr. γ -H2AX phosphorylation was assessed in both untreated and irradiated cells using a modification of a previously described protocol (21). Briefly, the cells were fixed in 4% formaldehyde (Sigma Chemical Co., St. Louis, MO) for 10 min, after which Triton X-100 (0.12% in phosphate-buffered saline [PBS]) was added. The cells were then washed with cold wash buffer (PBS with 4% fetal bovine serum) and fixed again in cold 50% methanol in PBS for 10 min. After centrifugation, the cells were spotted onto slides and blocked in PBS with 4% bovine serum albumin for 30 min and incubated with 1:500 diluted (1 μ g/ml) mouse monoclonal γ -H2AX antibody (Biolegend, San Diego, CA) for 30 min. The cells were then washed and re-incubated with fluorescein isothiocyanate–conjugated horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA) for 30 min. Finally, the slides were mounted with propidium iodide counterstain (Abbott Molecular, Inc., Des Plaines, IL) and covered with a cover slip.

The fluorescence signals were measured by using a Cys laser scanning cytometer (CompuCyte, Cambridge, MA). Slides were scanned automatically by using a 488-nm laser, and cell counting continued until 5,000 dispersed and contoured cells were registered. For each subject, the average readings of fluorescence intensity per cell represented γ -H2AX level for baseline (untreated) and irradiated cells. The γ -H2AX ratio was defined as the ratio of γ -H2AX level in irradiated cells to that at baseline (in untreated cells) from the same individual. All of the experiments were done by lab personnel who had no knowledge of the case-control status of samples.

Statistical analysis

We performed the Pearson χ^2 to compare the distributions of categorical variables between cases and controls. For continuous variables, the Student t-test was used to assess the differences. The Wilcoxon rank sum test was performed to test the difference between cases and controls for pack-years, baseline and radiation-induced γ -H2AX levels, and γ -H2AX ratio. γ -H2AX ratio was dichotomized by median, tertiles, and quartiles on the basis of its distribution in the control group. Unconditional multivariable logistic regression was performed to estimate odds ratios (ORs), while adjusting for age, sex, smoking status, and presence of Barrett's esophagus where appropriate. All statistical tests were two-sided. Statistical analyses were performed using the STATA software (Version 10, College Station, TX). Differences with *P*-values <0.05 were considered statistically significant.

Results

Table 1 summarizes the demographic characteristics and γ -H2AX levels for the 211 EAC patients and 211 healthy controls. By study design, the cases and controls were well matched on age and gender. Approximately 88% of study participants were male. Never smokers were significantly over-represented among control participants (53.55%) compared with EAC cases (36.49%). Among ever smokers, the self-reported median pack-years smoked was significantly higher in cases than in controls (35.8 for cases and 24.8 for controls, *P*=0.02). Barrett's esophagus was significantly over-represented in EAC cases compared with controls (29.86% for cases and 2.34% for controls, *P*<0.001). There was no significant difference in baseline γ -H2AX level between cases and controls (860.9 vs. 791.7, *P*=0.62). However, the radiation-induced γ -H2AX level was significantly higher in EAC cases than in controls (1270.5 vs. 1105.9, *P*=0.04). Likewise, the γ -H2AX ratio was significantly higher in cases than in controls (1.48 vs. 1.40, *P*<0.001). In stratified analyses, there were no significant differences of baseline and radiation-induced γ -H2AX level, and the γ -H2AX ratio between different age and smoking groups in controls (Supplemental Table 1, Table 2). Female controls had significantly higher baseline level than male controls (*P*=0.030) (Supplemental Table 1), which resulted in significantly lower γ -H2AX ratio in female than in male controls (*P*=0.009) (Table 2). In all stratified subgroups, except the current smokers, cases had a significantly higher γ -H2AX ratio than controls (Table 2).

We next analyzed the association between γ -H2AX ratio and the risk of EAC (Table 3). Using the median γ -H2AX ratio in controls as the cutoff point, we found that higher γ -H2AX ratio was associated with a significantly increased risk of EAC, with an OR of 2.94 (95% confidence interval [CI], 1.83–4.72). Analysis of the data by the quartile distribution of γ -H2AX ratio in controls revealed a dose-response association between γ -H2AX ratio and EAC risk. Compared with individuals within the lowest quartile of γ -H2AX ratio, those in the lower mid, higher mid and highest quartiles had higher ORs, 0.91 (95% CI, 0.43–1.92), 1.59 (95% CI, 0.79–3.21) and 3.98 (95% CI, 2.11–7.84), respectively, with a significant trend (*P* for trend, 7.84E-07).

We further estimated the relative risk by age, gender, and smoking status, again using the median γ -H2AX ratio in controls as the cutoff point (Table 4). The risk appeared to be stronger in older (62 years or older) individuals than in younger (<62 years old) individuals, in women than in men, and highest in former smokers (OR 3.96, 95% CI, 1.96–7.99), intermediate in never smokers (OR=2.54, 95% CI, 1.20–5.37), but not significant in current smokers (OR=1.49, 95% CI, 0.32–6.99).

Table 5 shows the joint effect of γ -H2AX ratio and smoking on elevating the risk of EAC. Compared with never smokers with low γ -H2AX ratio, the ORs for ever-smokers with low γ -H2AX ratio, never smokers with high γ -H2AX ratio, and ever-smokers with high γ -H2AX

ratio were 1.66 (95% CI, 0.77–3.62), 2.61 (95% CI, 1.27–5.35), and 5.53 (95% CI, 2.71–11.25), respectively (P for trend =3.33E-07), although the interaction analysis between γ -H2AX ratio and smoking was not significant (P=0.70).

Discussion

In this case-control study, we used the γ -H2AX assay to measure baseline and irradiation-induced DNA damage and found that PBLs from EAC cases exhibited a significantly higher level of DNA damage after irradiation than PBLs from controls. To the best of our knowledge, no earlier studies have used the γ -H2AX assay to assess susceptibility to DNA damage and EAC risk, and our findings confirm the importance of genetic susceptibility in EAC etiology.

Evidence is accumulating that latent genetic instability plays an important role in cancer initiation, and DNA repair is of fundamental importance in maintaining genomic integrity. DNA damage in response to environmental carcinogens accumulates more rapidly in people with suboptimal DNA repair capacity than in individuals with normal DNA repair capacity. Therefore, inter-individual differences in DNA repair capacity have been suggested to have a significant impact on cancer susceptibility in the general population. In this study, we did not find a significant difference in baseline γ -H2AX levels between cases and controls; however, upon radiation challenge, cases exhibited significantly higher level of γ -H2AX accumulation, translating to an over 2-fold increased risk of EAC for those with high γ -H2AX ratio (radiation-induced to baseline γ -H2AX) with a significant dose-response association. Previous studies using other assays measuring mutagen sensitivity have demonstrated similar case-control differences in induced DNA damage. Wang et al. noted that the average number of chromatid breaks per cell induced by radiation exposure was significantly higher in young breast cancer patients than in controls [19]. Shao et al. used the comet assay to demonstrate that BPDE and radiation induced significantly higher levels of DNA damage in PBLs from EAC patients than those from controls [20]. Schabath et al. also used the comet assay to find that PBLs from bladder cancer patients showed greater DNA damage after BPDE and radiation exposure than PBLs from control subjects [21]. Our findings, using a more specific DSB assay than other assays measuring general DNA damage, are consistent with the notion that latent genetic instability plays an important role in cancer predisposition.

Our data showed that the association between high radiation-induced γ -H2AX and increased EAC risk was significant in never and former smokers, but not in current smokers. This may be due to elevated DNA repair capability in current smokers due to consistent smoking stimulation. Previous studies have suggested that current smokers tend to have greater DNA repair capacity than former or never smokers, and the expression levels of several DNA repair genes in PBLs were higher in smokers than in nonsmokers, suggesting that current smokers' DNA repair capacity may include an adaptive response to DNA damage induced by chronic tobacco carcinogen exposure (22). Alternatively, the small sample size of current smokers may result in this spurious observation. When we analyzed joint effect of γ -H2AX ratio and smoking on elevating the risk of EAC, we found a more than 5 fold increased risk of EAC cancer for smokers who had high γ -H2AX ratio, which suggested the combination of mutagen sensitivity with other risk factors could increase predictive power.

DNA DSBs result from exposure to ionizing radiation, radiomimetic drugs, or as a result of replication fork stalling (23). DSBs are considered the most deleterious damage to DNA and most difficult to repair and the faithful repair of DSBs is crucial for maintain genome integrity (24–26). Imaging DSBs in vivo is particularly informative, as their number and persistence would predict cell fate. Various techniques are being used to analyze DSBs, such

as pulsed-field electrophoresis, comet assay, and neutral filter elution (27, 28). However, none has been widely adopted in epidemiologic studies, and assays with higher throughput are being sought. Various techniques are being used to detect DSBs, such as pulsed-field electrophoresis, comet assay, and neutral filter elution (27, 28). However, none of these techniques has been widely adopted in epidemiologic studies due to assorted limitations including time-consuming and labor-intensive procedure and/or subjective interpretation. Robust assays for DSBs with high throughput potential are desired.

-H2AX is an early and almost universal feature of the eukaryotic response to DNA DSBs. Many studies have shown that the kinetics of -H2AX induction and its release from chromatin correlate with the rate of DSB rejoining, making -H2AX a sensitive marker of DSB damage and repair. -H2AX can be detected by various techniques, such as flow cytometry, immunoblotting, and immunofluorescence microscopy. The laser scanning cytometer-based immunocytochemical method we used in this study can be applied to analyze -H2AX automatically in thousands of lymphocytes in just a few minutes. This assay is rapid, simple, and objective compared to other traditional methods such as comet assay, which uses microscopy to quantify the -H2AX foci per cell and is time-consuming, laborious, and subjective. It is important to point out, that similar to most DNA repair assay, there is probably an overlap between DNA damage and repair in -H2AX assay(29). The -H2AX level may reflect susceptibility to DSBs, variation in repair capacity, or the summary results of both, depending on when the detection occurs. Many studies reported that -H2AX level reached a peak 1 or 2 hr after radiation exposure and maintained for approximately 1 hr before starting to decrease with time.(21, 30). In this study, the -H2AX level was detected at 1 hr post-irradiation, therefore, the -H2AX ratio represented the summary effect of DSB formation and repair, but reflected individual DSB susceptibility more than DSB repair capacity because the detection was conducted during the early phase of -H2AX kinetics, when DSBs repair mechanism had just been initiated.

In this study, the -H2AX ratio was applied to assess DNA damage after mutagen exposure. Some studies showed cancer cells and their analogous premalignant lesions generally display elevated endogenous -H2AX expression compared to normal cells, and the most plausible explanation for this elevation is the presence of higher numbers of endogenous DSBs(19, 31–33). In the current study, the slightly higher -H2AX level at base line was found in the cases compared to the controls, The use of induced -H2AX ratio should lessen confounding on baseline activity, and therefore allowing a more accurate comparison of susceptibility to DNA damage. Our results suggest that -H2AX ratio is robust for assessing EAC risk.

Our study had a few limitations. Firstly, reverse causation is an inherent limitation in any retrospective case-control studies, since the results might be affected by disease status or associated factors such as treatment. However, we only included newly diagnosed EAC cases and collected blood samples before any treatment, which should reduce the impact of disease status and treatment on the results. In addition, we did not find significant association of -H2AX ratio with stage or grade ($P=0.268$ for stage and $P=0.464$ for grade). Several previous studies have shown that response to mutagen challenges has high heritability and consistency (34–36). Nevertheless, future prospective validation is warranted. Secondly, we only detected IR-induced DSBs levels at a single time point (1hr) post-irradiation, which may not reflect DNA damage and repair capacity during an entire time course. Finally, although our sample size is sufficient for an overall association analysis, it is limited for stratified and interaction analyses. Large scale validation is necessary to confirm and extend our observations.

In conclusion, we have applied a new, robust, and relatively high-throughput γ -H2AX assay to measure DSB damage in PBLs. Our data suggest that susceptibility to DNA damage is associated with elevated risk of EAC. This is the first epidemiologic study using the γ -H2AX assay to assess mutagen sensitivity and EAC risk. Future prospective studies are needed to further validate our findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Characteristics of cases and controls

Variable	Cases, N (%)	Controls, N (%)	P
Age in years, mean (SD)	61.39(10.99)	61.71(10.18)	0.761 ^a
Sex			
Male	185(87.68)	185(87.68)	1 ^b
Female	26(12.32)	26(12.32)	
Smoker			
Never	77(36.49)	113(53.55)	<0.001 ^b
Former	99(46.92)	81(38.39)	
Current	34(16.11)	17(8.06)	
Pack-years, mean (SD)	35.85(33.73)	24.86(26.33)	0.022 ^c
Barrett's esophagus			
Yes	63(29.86)	5(2.34)	1.14E-15 ^b
No	132(62.56)	203(96.20)	
-H2AX level in PBLs			
Baseline ^d	860.97(394.56)	791.73(244.12)	0.620 ^c
-radiation-induced ^d	1270.54(588.00)	1105.92(337.00)	0.041 ^c
-H2AX ratio ^e	1.48(0.13)	1.40(0.09)	1.82E-10 ^c

^aStudent *t*-test.

^bPearson χ^2 test.

^cWilcoxon rank sum test.

^dOriginal readings of the fluorescence signals by cytometer.

^eThe ratio of radiation-induced -H2AX level to the baseline level of the same sample.

Table 2

-H2AX ratio by host characteristics in cases and controls

Variable	Cases			Controls		
	N	Mean (SD)	<i>P</i> ^a	N	Mean (SD)	<i>P</i> ^a
Overall	211	1.48(0.13)		211	1.40(0.09)	<0.001
Age, years						
<62 ^c	102	1.46(0.13)		102	1.41(0.10)	<0.001
62	109	1.49(0.13)	0.154	109	1.40(0.09)	0.272
Sex						
Male	185	1.48(0.12)		185	1.41(0.09)	<0.001
Female	26	1.46(0.16)	0.504	26	1.36(0.10)	0.008
Smoking status						
Never	77	1.48(0.14)	Ref.	113	1.40(0.09)	Ref.
Former	99	1.48(0.13)	0.825	81	1.39(0.10)	0.407
Current	34	1.47(0.12)	0.803	17	1.42(0.10)	0.822
Ever	133	1.48(0.12)	0.790	98	1.40(0.10)	0.516
Barrett's esophagus						
No	132	1.48 (0.12)		203	1.40 (0.09)	<0.001
Yes	63	1.48 (0.14)	0.495	5	1.36 (0.10)	0.294

^a *P*-values for the differences between subgroups were determined by the Wilcoxon rank sum test.

^b *P*-values for the difference between cases and controls.

^c Median age of the controls.

Table 3

Risk estimates of EAC by -H2AX ratio

Analysis method	Cases, N (%)	Controls, N (%)	Adjusted OR ^a	P
<i>By median^b</i>				
<1.40	59(27.96)	106(50.24)	1 (ref.)	
1.40	152(72.04)	105(49.76)	2.94(1.83–4.72)	<0.001
<i>By quartile^b</i>				
<1.362	30(14.22)	54(25.59)	1 (ref.)	
1.362–1.404	29(13.74)	52(24.64)	0.91(0.43–1.92)	0.81
1.404–1.445	41(19.43)	53(25.12)	1.59 (0.79–3.21)	0.19
1.445	111(52.61)	52(24.64)	4.98 (2.11–7.52)	<0.001
P for trend			7.84E-07	

^a Adjusted for age, sex, and smoking status and BE.

^b Median and quartiles determined by the distribution of -H2AX ratio in the controls.

Table 4
Risk estimates for EAC based on -H2AX ratio stratified by sex, age, and smoking status

Variable	Cases N (%)	Controls N (%)	Adjusted OR (95% CI) ^d	P
Age, years				
<62 ^c				
	<1.40	39(30.71)	60(47.24)	1.00 (ref.)
	1.40	88(69.29)	67(52.76)	1.91 (1.06–3.44) 0.031
62				
	<1.40	20(23.81)	46(54.76)	1.00 (ref.)
	1.40	64(76.19)	38(45.24)	6.24(2.65–14.69) <0.001
Sex				
Male				
	<1.40	49(26.49)	87(47.03)	1.00 (ref.)
	1.40	136(73.51)	98(52.97)	2.65 (1.61–4.37) <0.001
Female				
	<1.40	10(38.46)	19(73.08)	1.00 (ref.)
	1.40	16(61.54)	7(26.92)	11.50(1.87–70.50) 0.008
Smoking status				
Never				
	<1.40	22(28.57)	55(48.67)	1.00 (ref.)
	1.40	55(71.43)	58(51.33)	2.54 (1.20–5.37) 0.014
Former				
	<1.40	28(28.28)	45(55.56)	1.00 (ref.)
	1.40	71(71.72)	36(44.44)	3.96 (1.96–7.99) <0.001
Current				
	<1.40	9(26.47)	6(35.29)	1.00 (ref.)
	1.40	25(73.53)	11(64.71)	1.49 (0.32–6.99) 0.611
Ever				
	<1.40	37(27.82)	51(52.04)	1.00 (ref.)
	1.40	96(72.18)	47(47.96)	3.38 (1.80–6.38) <0.001

^a Adjusted for age, sex, and smoking status and BE when appropriate.

Table 5

Joint effect of -H2AX ratio and smoking on risk of EAC

-H2AX ratio	Smoking	Case, N(%)	Control, N(%)	Adjusted OR (95%CI) ^a	P value
<1.40	Never	20(10.70)	55(26.07)	1(reference)	
<1.40	Ever	31(16.58)	51(24.17)	1.66(0.77–3.62)	0.199
1.40	Never	51(27.27)	58(27.49)	2.61(1.27–5.35)	0.009
1.40	Ever	85(45.45)	47(22.27)	5.53(2.71–11.25)	2.50E-06
P for trend					3.33E-07
P for interaction					0.700

^a Adjusted for age, sex, smoking and BE