Interactions between genetic polymorphisms in the apoptotic pathway and environmental factors on esophageal adenocarcinoma risk

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How genetic variations in apoptosis pathway interact with environmental factors to contribute to esophageal adenocarcinoma (EA) risk has not been comprehensively investigated. We conducted a case-only analysis in 335 Caucasian EA patients that were genotyped for 242 single nucleotide polymorphisms (SNPs) in 43 apoptotic genes. Gene–environment interactions were assessed using a two-step approach. First, random forest algorithm was used to screen for the potential interacting markers. Next, we used case-only logistic regression model to estimate the effects of gene–environment interactions on EA risk. Four SNPs (*PERP* rs648802; *PIK3CA* rs4855094, rs7644468 and *TNFRSF1A* rs4149579) had significant interaction with gastroesophageal reflux disease (GERD). The presence of variant alleles in *TP53BP1* rs560191, *CASP7* rs7907519 or *BCL2* rs12454712 enhanced the risk of smoking by 2.08–2.58 times [interaction odds ratio (ORi) = 2.08–2.58, adjusted P-value (P_{adj}) = 0.02–0.04]. Compared with patients carrying ≤1 risk genotype, the risk of GERD on EA was increased in persons with two (ORi = 1.89, P_{adj} = 0.016) or ≥3 (ORi = 4.30, P_{adj} < 0.0001) risk genotypes. Compared with cases with ≤1 risk genotype, smoking-associated EA risk increased by 3.15 times when ≥2 risk genotypes were present (ORi = 3.15, P_{adj} < 0.0001). In conclusion, interactions among apoptotic SNPs and GERD or smoking play an important role in EA development.

Introduction

Cumulative evidence has indicated that symptomatic gastroesophageal reflux disease (GERD), obesity, smoking and male gender are four major risk factors for developing esophageal adenocarcinoma (EA) (1,2). Although GERD is the strongest individual risk factor and subjects who have the most frequent reflux symptom carry >4-fold EA risk, most of them follow an indolent course for the entire life (3). On the other hand, nearly 50% of EA patients do not experience GERD-associated symptoms (2,4,5). Therefore, challenges arise

Abbreviations: BMI, body mass index; CI, confidence interval; EA, esophageal adenocarcinoma; GERD, gastroesophageal reflux disease; MAF, minor allele frequency; MDA, mean decrease of accuracy; ORi, interaction odds ratio; P_{adj}, adjusted P-value; SNP, single nucleotide polymorphism.

when considering the benefit and cost of endoscopic surveillance for EA among subjects with these risk factors, especially GERD (6). One of the explanations for this phenomenon stems from the complex interactions between lifestyle/environmental exposure and genetic factors. Since most EA cases are sporadic, genetic influences are more probably to be polymorphisms in multiple genes instead of single gene mutation. Evidence from pathway-based studies suggests that genetic variance may modulate the susceptibility of developing EA (7–9). Incorporating one's genetic background in the clinical setting could be a better strategy for risk assessment and cancer surveillance, and possibly provide a better understanding about how complex factors contribute to EA risk jointly. However, fewer studies have assessed interactions using a more comprehensive approach.

Apoptosis, or programmed cell death, is essential for normal tissues to regulate cell number and to eliminate unwanted or aging cells as an organism develops. Mutations and single nucleotide polymorphisms (SNPs) in apoptotic pathway genes that alter the ability of the cell to undergo apoptosis may induce cancers by allowing transformed cells to keep accumulating rather than dying (10). Our recent studies indicated that genetic polymorphisms in the apoptosis pathway, by themselves or through interaction with environmental factors, play an important role in the carcinogenesis of EA (7,11). In a case–control study covering 1330 functional/tagging SNPs categorized into14 cancer-related pathways, two apoptotic SNPs (*Caspase 7* rs312707 and *Caspase 9* rs4661636) were significantly associated with EA risk. Moreover, apoptosis pathway was found to be the most import one in pathway-based analysis (11). However, it is possible that other SNPs further down the significance list of individual effects exert their importance through gene–environment interaction. Here, we conducted a pathway-based case-only study to further explore the interactions between 242 apoptosis-related SNPs and those well-known EA risk factors. We applied a two-step approach to identify gene–environment interaction markers in EA cases. Our results showed that gene–environment interactions play important roles in the development of EA.

Materials and methods

Study population, interview and DNA preparation

Details of patient recruitment were described in our previous paper (11). In brief, they were Caucasian, >18 years old and histologically confirmed to have EA at Massachusetts General Hospital between 1999 and 2005 and at Dana Farber Cancer Institute between 2004 and 2005. All of them had a tumor center located at or above the gastroesophageal junction and had at least two-thirds of the bulk tumor located in the esophagus. Patients with secondary or recurrent cancers were excluded. The recruitment rate was 86%. Participants were interviewed using a modified questionnaire (12) immediately after enrollment to collect information of their demographic characteristics and smoking history. Smoking status was defined based on whether the patient smoked 1 year prior to diagnosis. Lifetime GERD-related symptoms were collected based on the questions described before (13). The presence of GERD was defined as having heartburn or regurgitation symptoms at least once per month for more than six continuous months in one's lifetime (7).

Blood samples were collected at the time of recruitment and DNA was extracted using the Puregene DNA Isolation Kit (Gentra Systems/Qiagen, Valencia, CA). This study was approved by the Human Subjects Committees of Massachusetts General Hospital, Dana Farber Cancer Institute and the Harvard School of Public Health (Boston, MA). All subjects signed the informed consent prior to study participation.

SNP selection, pathway categorization and genotyping

The criteria for SNP selection in the GoldenGate assay was described previously (11). The candidate SNPs selected in the apoptosis pathway were common SNPs with minor allele frequency (MAF) ≥ 5% in the HapMap Caucasian population (CEU). They were either missense/exonic SNPs, SNPs within untranslated regions and 2 kb 5′ of the gene or tagSNPs for genes with

little functional information available. The common non-synonymous SNPs were selected using SNP500Cancer Project and International HapMap Project. Potential functional non-synonymous SNPs were selected from the Predicted Impact of Coding SNPs database (14) and FASTSNP (15). SNPs on the Illumina Cancer Panel were chosen with priority. TagSNPs were selected using the r^2-based Tagger program (16) with pairwise $r^2 \geq 0.80$ and MAF $\geq 5\%$ among Caucasians. Apoptosis pathway genes were chosen based on the literature reports and information in the following database, the National Center for Biotechnology Information, Genetic Association Database (17), National Institute for Environmental Health Science GeneSNPs (18) and Kyoto Encyclopedia of Genes and Genomes (19). More frequently cited genes were given greater priority.

Genotyping was performed on the Illumina GoldenGate assay at the Broad Institute (Cambridge, MA) by laboratory personnel blinded to the subjects' clinical information. Forty-eight duplicate samples were randomly selected for quality control. The concordance rate of the replicate samples for all assays was >99%. As described previously (11), 1330 SNPs with MAF ≥ 0.01 and passed Hardy–Weinberg equilibrium ($P \geq 0.0001$) in the control subjects were successfully genotyped. Among them, 242 SNPs (43 genes) in apoptosis pathway (supplementary Table S1 is available at *Carcinogenesis* Online) were used for interaction analysis in the case-only model.

Statistical analysis

We applied a two-step approach to identify the SNPs, which may have interactions with environmental factors (20). First, we used a random forest algorithm (21), with one individual environmental factor (GERD, smoking or body mass index [(BMI) at age 18] as the outcome and apoptotic SNPs as the predictors, to impute the missing values (<4% in each variable) and then obtain the mean decrease of accuracy (MDA) for each SNP. The MDA measures the degree of loss if the corresponding SNP was 'removed' from the model and could be regarded as an 'importance' score for the SNP. The top important SNPs were selected based on the turning point of MDA in random forest importance plots. The turning points in the MDA plots were defined as the cut points to select important markers for subsequent interaction analyses.

In the second step, we used the 'important SNPs' to perform case-only logistic regression analysis using dominant genetic model (mutant carriers versus wild-type homozygotes). SNPs with adjusted P-value (P_{adj}) < 0.05 after adjustment for covariates and multiple comparison using false discovery rate were considered to be significant (22). To estimate cumulative risk effects of the significant SNPs, we combined the number of risk genotypes representing the degree of 'genetic risk score', which was included in the case-only logistic models to fit with individual environmental risk factors, respectively. Interaction odds ratios (ORi) and 95% confidence intervals (95% CIs) were calculated as an estimate of relative risk. All data analyses were performed using the SAS statistical package, version 9.l (SAS Institute, Cary, NC). Random forest analysis was performed using *rpart* package in R software, version 2.9.1.

Results

The mean age of the EA cases was 62.9 years and 295 (88%) were males. About half of them had history of GERD symptoms and 80.3% were ever smokers. Other demographic characteristics were shown in Table I. In random forest analysis, MDA scores were obtained by SNP–GERD, SNP-smoking and SNP–BMI models, respectively. To minimize random bias, 100 000 trees were constructed in each RF

model, allowing each marker to have ~100% of probability to be tested for 500 times. Based on MDA plot curves, the most important SNPs that had interaction with GERD (22 SNPs), smoking (17 SNPs) and BMI (15 SNPs) were selected for subsequent case-only logistic regression analysis (Figure 1).

In case-only logistic regression analysis, we found that five SNPs (rs651662, rs648802, rs4855094, rs7644468 and rs4149579) in three genes (*PERP*, *PIK3CA* and *TNFRSF1A*) had significant interaction with GERD; these interactions remained significant even after adjusting for covariates and false discovery rate (Table II). Two SNPs (rs651662 and rs648802) were in the gene *PERP* and were in high linkage disequilibrium ($r^2 = 0.99$). Another two significant SNPs in *PIK3CA* (rs4855094 and rs7644468) were not tagged to each other. Subjects carrying the variant allele of rs651662, rs648802, rs4855094 or rs7644468 significantly enhanced the risk of GERD to develop EA compared with subjects carrying homozygous wild genotypes. On the contrary, the risk of GERD was significantly reduced when one carries the wide-type allele of rs4149579 (ORi = 0.42, 95% CI = 0.22–0.81, P_{adj} = 0.04) (Table II). Since rs651662 and rs648802 were in linkage disequilibrium ($r^2 = 0.99$) and the later was a non-synonymous SNP, whereas the former located in intron, only rs648802 was used to combine with the other three SNPs to calculate the joint effect. There was a significant cumulative risk when those four SNPs were considered jointly. The risk of GERD on EA was increased when one has two (ORi = 1.89, 95% CI = 1.13–3.16) or ≥ 3 (ORi = 4.30, 95% CI = 2.25–8.21) risk genotypes comparing with those with ≤ 1 risk genotype (Table II).

For the SNP-smoking interaction, 4 of the top 17 SNPs were identified to be significant in logistic regression models (Table III). The presence of variant alleles in *TP53BP1* rs560191, rs2602141, *CASP7* rs7907519 or *BCL2* rs12454712 enhanced the risk of smoking by 2.08–2.58 times (ORi = 2.08–2.58, P_{adj} = 0.02–0.04). *TP53BP1* rs560191 and rs2602141 were tagged to each other ($r^2 = 0.99$) and the former was non-synonymous; thus, we dropped the later in the cumulative risk analysis. Taking those three SNPs together, persons with ≥ 2 risk genotypes had 3.15 times of interaction effect with smoking (ORi = 3.15, 95% CI = 1.80–5.52, P_{adj} < 0.0001) than those with ≤ 1 risk genotype (Table III). No SNPs were found to be significantly interacted with BMI in logistic regression analysis. The allele frequencies of the SNPs with significant interaction with environmental factors were shown in Supplementary Table S2, available at *Carcinogenesis* Online.

Discussion

This is a comprehensive study covering 242 apoptotic SNPs to evaluate the gene–environment interaction in EA. Case-only study with random forest analysis has been proven to be a reliable method to investigate gene–environment interaction in complex diseases (20). The EA patients in this study were incident cases; therefore, the ORi estimated the interaction under the assumption that the genetic and

Table I. Characteristics of 335 EA cases

Characteristics	EA cases
Age (years, mean ± SD)	62.9 ± 11.8
Gender, male (%)	295 (88.0%)
GERD[a], yes (%)	173 (50.6%)
Smoking, yes (%)	269 (80.3%)
Smoking (pack-years), mean ± SD, medium (minimum to maximum)	30.5 ± 31.0, 24.4 (0–212.0)
BMI at age 18, (kg/m^2, mean ± SD)	23.6 ± 3.8
BMI $\geq$ 25 at age 18 (%)	104 (31.0%)
Stage	
I–IIA	98 (29.3%)
IIB–IV	237 (70.7%)

[a]7 (2%) missing and imputed.
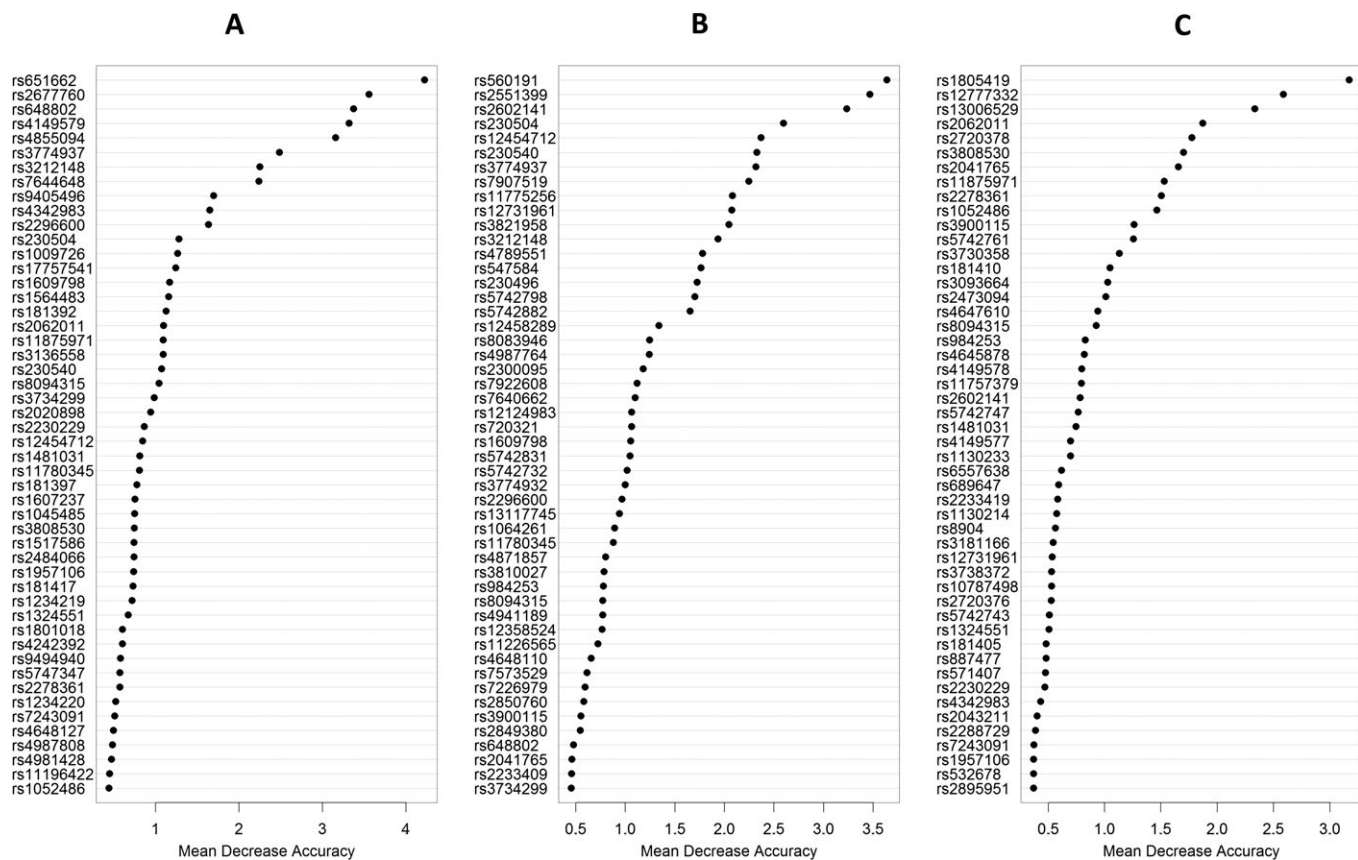
A   **B**   **C**

Fig. 1. Random forest importance plots for interaction between apoptotic SNPs and GERD (A), smoking (B) or body mass index (C).

environment factors were independent. In addition to identifying potential modifiers, we also demonstrated increased gene–GERD and gene–smoking interactions when those significant SNPs were considered jointly. Our results provide more evidence to how apoptotic genes modify the risk of GERD and smoking on EA risk. Dominant model of each SNP was used to calculate the ORi because it was more convenient and the sample size was limited in this study. We also used case–control model to test the interaction between smoking and the four significant SNPs found in this case-only analysis. The control subjects were described in our previous study (11). Three of them had very consistent results (rs560191, rs260214 and rs12454712; ORi = 2.11–2.28, *P*-value = 0.02–0.04). Although we did not find significant interaction between *CASP7* rs7907519 and smoking in case–control model, the trend was consistent (ORi = 1.20, 95% CI, 0.58–2.48, *P*-value = 0.61). Because GERD information was missing in >50% of our control subjects (11), we cannot perform case–control analysis for genotype–reflux interaction.

Evading apoptosis, a characteristic of transformed cells, has been well documented in Barrett's carcinogenesis (23,24). Gastric acid and bile acid are two principle components of refluxate that cause chronic esophageal inflammation and Barrett's transformation. Direct exposure of an EA cell line to acid leads to suppression of apoptotic genes, which may occur via p53-dependent mechanism (25). TP53 tumor suppressor induces the intrinsic apoptotic pathway and activates many downstream genes, including *PERP* (*TP53 apoptosis effecter related to PMP-22*) (26). Previous study also suggests that there is a reciprocal regulation between p53 and the PI3K–AKT pathway (27). The PI3K-AKT signaling pathway regulates many normal cell functions, including cell proliferation, survival and growth. It is also known to be anti-apoptotic in many different cell types (28). Amplification of *PIK3CA*, which encodes the 110 kDa subunit of PI3K, has been found in many human cancers (29,30). Treatment with a PI3K inhibitor decreases proliferation and increases apoptosis; therefore, it has be-

come a target for cancer therapy (28). Genetic polymorphisms of *PIK3CA* have been studied in the susceptibility of ovarian cancer (31). In addition to *PERP* and *PIK3CA*, we also found a significant interaction between the *TNFRSF1A* polymorphism and GERD. TNFRSF1A is one of the cognate receptors that binds to tumor necrosis factor superfamily ligands to activate the extrinsic apoptotic pathway (32). *TNFRSF1A* rs4149570 has been linked to the survival of early-stage non-small cell lung cancer (33). However, no studies investigated the association between *PERP*, *PIK3CA* or *TNFRSF1A* polymorphism and esophageal cancer risk.

Our data revealed that polymorphisms in *CASP7*, *TP53BP1* and *BCL2* modified the risk of smoking to develop EA. Long-term exposure to carcinogens in cigarette smoke such as polycyclic aromatic hydrocarbons leads to DNA damage, which accumulates if a cell evades cell cycle regulation and apoptotic mechanism. Caspase activation plays a primary role in the apoptotic cascade. A previous report has shown an inactivation mutation of *CASP7* in esophageal cancer cells (34). Polymorphisms in *CASP7* have been examined in several human cancers (35,36) but not in esophageal cancer until our recent report (11). In this study, we identified a significant interaction between *CASP7* rs7907519 and smoking in EA cases. Such information adds more evidence to the importance of genes in caspase cascade in the susceptibility of EA. TP53BP1 binds to TP53 and plays a role in responses to DNA damage. A study in Japan revealed that *TP53BP1 Asp353Glu* (rs560191) and *TP53 Arg72Pro* polymorphism had significant interaction in lung cancer risk. However, no gene–smoking interaction was found (37). BCL2 protein is one of the products of *BCL2* family and has anti-apoptotic function. It has been found to aberrantly expressed in Barrett's carcinogenesis (38). Polymorphisms in *BCL2* were reported in many human cancers such as ovarian cancer, leukemia and esophageal cancer (39–41). However, no study examined the association of *TP53BP1* or *BCL2* polymorphism and EA risk among Caucasians.

Table II. Case-only interaction between apoptotic SNPs and GERD on EA risk

SNPs	GERD		ORi[a] (95% CI)	P_{adj}[a]
	No, n (%)	Yes, n (%)		
PERP rs651662				
GG	68 (41.97)	43 (24.86)	reference	
GA + AA	94 (58.03)	130 (75.14)	2.13 (1.33–3.40)	0.02
PERP rs648802				
CC	67 (41.36)	43 (24.86)	reference	
CG + GG	95 (58.64)	130 (75.14)	2.09 (1.30–3.34)	0.02
PIK3CA rs4855094				
GG	151 (93.21)	145 (83.82)	reference	
GA + AA	11 (6.79)	28 (16.18)	2.76 (1.32–5.80)	0.04
PIK3CA rs7644648				
AA	123 (75.93)	109 (63.00)	reference	
GA + GG	39 (24.07)	64 (37.00)	1.89 (1.17–3.06)	0.04
TNFRSF1A rs4149579				
GG	130 (80.25)	157 (90.75)	reference	
GA + AA	32 (19.76)	16 (9.25)	0.42 (0.22–0.81)	0.04
[b]Cumulative risks of rs648802, rs4855094, rs76044648 and rs4149579				
≤1 risk genotype	65 (40.12)	37 (21.39)	reference	
2 risk genotypes	75 (46.30)	82 (47.40)	1.89 (1.13–3.16)	0.016
≥3 risk genotypes	22 (13.58)	54 (31.21)	4.30 (2.25–8.20)	<0.0001

[a]Adjusted for age, sex, BMI at age 18 (<25 versus ≥25) and smoking (+/−) (also false discovery rate for P_{adj}).
[b]Since rs651662 and rs648802 were in linkage disequilibrium, only the later was used to calculate the cumulative risks.

Table III. Case-only interaction between apoptotic SNPs and smoking on EA risk

SNPs	Smoking		ORi[a] (95% CI)	P_{adj}[a]
	No, n (%)	Yes, n (%)		
TP53BP1 rs560191				
CC	44 (66.67)	119 (44.24)	reference	
CG + GG	22 (33.33)	150 (45.76)	2.58 (1.45–4.59)	0.02
TP53BP1 rs2602141				
AA	43 (65.15)	118 (43.87)	reference	
AC + CC	23 (34.85)	151 (56.13)	2.38 (1.35–4.19)	0.02
CASP7 rs7907519				
CC	30 (45.45)	76 (28.25)	reference	
CA + AA	36 (54.55)	193 (71.75)	2.14 (1.23–3.76)	0.04
BCL2 rs12454712				
TT	36 (54.55)	99 (36.80)	reference	
TC + CC	30 (45.45)	170 (63.20)	2.08 (1.20–3.63)	0.04
[b]Cumulative risks of rs560191, rs7907519 and rs12454712				
≤1 risk genotype	39 (59.09)	85 (31.60)	reference	
≥2 risk genotypes	27 (40.91)	184 (68.40)	3.15 (1.80–5.52)	<0.0001

[a]Adjusted for age, sex, BMI at age 18 (<25 versus ≥25) and GERD (+/−) (also false discovery rate for P_{adj}).
[b]Since rs560191 and rs2602141 were in linkage disequilibrium, only the former was used to calculate the cumulative risks.

studies are needed to validate the results and elucidate how the genes interact with environmental factors to contribute to the cancer risk.

Obesity not only promotes reflux symptoms but also contributes to the progression of EA by inhibiting apoptosis through a reduced adiponectin (anti-inflammatory adipokine from adipose tissue) and ghrelin levels (42). It is possible that there is an interaction between increased BMI and apoptotic SNPs; however, our data does not support this hypothesis. This might suggest that the effect of obesity on EA involves in mechanisms other than polymorphisms in apoptotic genes. It is also possible that there is a high-dimensional interaction involving multiple SNPs and BMI, which is not easy to investigate. Because we only included 40 (12%) female cases, the interaction with gender is difficult to examine in the present study. *IL1B* rs1143634 has been shown to have significant interaction with GERD on EA risk in our previous case–control study (7). However, this association was not found in the current case-only model. This is possibly because case-only and case–control designs differ in estimating interaction parameters. In case-only analysis, the ORi tests whether the influence of a risk factor (e.g. GERD) is different among two groups of patients dichotomized by the presence or absence of a risk genotype. In case–control study, interaction was tested by measuring the departure of multiplicative joint effects of genetic and environmental factors on disease risk (43,44).

This study has several limitations. First, we did not validate our findings in another group, and these findings need to be replicated. Second, the case number in this study is relatively small because EA is an uncommon disease among Caucasians. However, we have >80% power to identify a significant pairwise interaction if the MAF, relative risk of interaction are ~20% and 2.5, respectively (45). Third, it is not clear how these polymorphisms affect the biological function of the genes. Finally, we did not provide data to prove that the gene and environmental factors in this study are independent although it was reasonable since EA is an uncommon disease. Violation of the independence assumption could make the multiplicative odds ratio of a case-only study very different from the ORi obtained from a case–control study (43,44).

In conclusion, our data suggest that the interaction among apoptotic SNPs and GERD or smoking play an important role in the carcinogenesis of EA. Replication in other populations and further mechanistic

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