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Interactions between Environmental Factors and Polymorphisms in Angiogenesis Pathway Genes in Esophageal Adenocarcinoma Risk: A Case-Only Study

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Abstract

BACKGROUND—Gastroesophageal reflux symptoms (GERD), higher body mass index (BMI), smoking, and genetic variants in angiogenic pathway genes have been individually associated with increased risk of esophageal adenocarcinoma (EA). However, how angiogenic gene polymorphisms and environmental factors jointly affect EA development remains unclear.

METHODS—Using a case-only design (n = 335), we examined interaction between 141 functional/tagging angiogenic SNPs and environmental factors (GERD, BMI, smoking) in modulating EA risk. Gene-environment interactions were assessed by a two-step approach. First, we applied random forest (RF) to screen for important SNPs that had either main or interaction effects. Second, we used case-only logistic regression (LR) to assess the effects of gene-environment interactions on EA risk, adjusting for covariates and false-discovery rate (FDR).

RESULTS—RF analyses identified three sets of SNPs (17 SNPs-GERD, 26 SNPs-smoking, and 34 SNPs-BMI) that had the highest importance scores. In subsequent LR analyses, interactions between 3 SNPs (rs2295778 of *HIF1A*, rs133376 of *TSC2*, and rs2519757 of *TSC1*) and GERD, 2 SNPs (rs2295778 of *HIF1A*, rs2296188 (*VEGFR1*) and smoking, and 7 SNPs (rs2114039 of *PDGFRFA*, rs2296188 of *VEGFR1*, rs11941492 of *VEGFR1*, rs3756309 of *PDGFRB*, rs7324547 of *VEGFR1*, rs17619601 of *VEGFR1*, and rs17625898 of *VEGFR1*) and BMI were significantly associated with EA development (all FDR ≤ 0.10). Moreover, these interactions tended to have a

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None.

SNP dose-response effects for increased EA risk with increasing number of combined risk genotypes.

CONCLUSIONS—These findings suggest that genetic variations in angiogenic genes may modify EA susceptibility through interactions with environmental factors in a SNP dose-response manner.

Keywords

Esophageal adenocarcinoma; angiogenesis pathway genes; gene-environment interaction; case-only analysis

Esophageal adenocarcinoma (EA) has the fastest rising incidence of all solid tumors in the US and in Western Europe over the last four decades^{1, 2}. The rapidly increasing incidence and sporadically development feature of EA suggest that environmental influences dominate its etiology³. Over recent years, it has been established that gastroesophageal reflux disease (GERD), smoking, higher body mass index (BMI) or obesity, and certain genetic variations are individually associated with EA risk^{4, 5}. However, no single factor can fully explain the rising incidence of EA. It has been suggested that gene-environment interactions play more important role in cancer etiology than do individual factors alone⁶.

Angiogenesis is a process of new blood vessel formation from endothelial precursors. This process facilitates tumor growth by providing oxygenation and nutrition to tumor cells^{7–9}. Conversely, inhibition of angiogenesis results in suppression of tumor growth^{10, 11}. The importance of angiogenesis specifically in EA has been supported by a large body of research, including in vitro, in vivo, and clinical studies⁹. Overexpression of angiogenic mediators, such as vascular endothelial growth factor (*VEGF*), inducible nitric oxide synthase (*iNOS*), and cyclooxygenase (*COX*)-2 have been associated with EA development^{8, 9, 12} and prognosis^{9, 13}.

Previously, we and others have reported that functional genetic variations that modulate angiogenic mediator gene expression, protein production, and angiogenesis processes are associated with increased risk of developing malignancies, including EA^{5, 14–17}. However, these associations only partly explain the etiology of EA, suggesting that studying one or a few SNPs or genes may not be sufficient to explain complex diseases such as EA because they are unlikely to result from the effect of only one or a few genes. In the present study, we examine further whether interactions between multiple SNPs in the angiogenesis pathway genes and environmental factors affect EA susceptibility. We investigated 143 functional and tagging SNPs in major angiogenesis genes in 335 EA cases. We used a case-only design and a two-step analytical approach to improve statistical power for interaction estimations¹⁸. Our findings highlight the importance of complex gene-environment interactions in the development of EA.

MATERIALS AND METHODS

Patients

A total of 335 histologically confirmed EA patients were recruited at Massachusetts General Hospital (MGH) from 1999 to 2005 and at Dana Farber Cancer Institute (DFCI) between 2004 and 2005. This study was approved by the Human Subjects Committees of MGH, DFCI, and the Harvard School of Public Health (HSPH). Written informed consent was obtained from all subjects prior to study participation. Since 96% of cases were Caucasians, we selected only Caucasians for analysis¹⁹.

Upon enrollment, a trained research assistant conducted a personal interview with each participant to collect baseline clinical data that served as important covariates in the analysis. Variables of interest included: demographic information (age, gender, adult height and weight), information regarding smoking exposure and lifetime GERD symptoms up to 1 year prior to diagnosis. Body mass index (BMI) was calculated using self-reported height and weight when the patients were in their twenties. Smoking history was grouped into “never smokers” and “ever smokers”. To assess the presence of GERD, participants were asked to indicate whether they experienced symptoms, such as heartburn, acid reflux, or regurgitation¹⁷.

SNP Selection and Genotyping

DNA was extracted from whole blood using the Puregene DNA isolation Kit (Gentra System). Candidate genes were selected based on their roles in angiogenesis and on published evidence of their relationship to carcinogenesis¹⁹. Angiogenesis pathway was defined based on information in KEGG and GeneGO databases. SNPs were either functional SNPs with recorded functional information in the literature, or tagging SNPs with pairwise $r^2 \geq 0.8$ and minor allele frequency (MAF) $\geq 5\%$ in Caucasians. A total of 141 SNPs in 13 angiogenic genes (*FGFR4*, *HIF1*, *HIF1AN*, *MMP1*, *MMP3*, *PDGFRA*, *PDGFRB*, *THBD*, *TSC1*, *TSC2*, *VEGF*, *VEGFR1*, *VEGFR2*) were selected. Genotyping was determined using the Illumina Goldengate assay on BeadChip by laboratory personnels blinded to the subjects' clinical information. Ten percent of duplicate samples were randomly included in the testing plates for quality controls.

Statistical Analysis

We restricted our data analysis to Caucasians since most EA cases (>90%) in our study cohort were Caucasians. We applied a case-only study design which is considered a more powerful method for analyzing gene-environment interaction than a case-control study of the same size¹⁸. We used a two-step approach to investigate gene-environment interactions. In the first step, we applied random forest (RF) to screen for SNPs most likely to be associated with environmental factors. RF consists of a collection of classification or regression trees grown from a random selection of variables on boot-strapped samples (bagging). The importance of a variable (average importance, $AvImp$) can be estimated by the increase in misclassification for the left-out (out-of bag) samples when using a data vector containing the original variable values and a vector with randomly permuted values. It has been shown by simulation studies that RF importance measures are able to simultaneously detect single-locus heterogeneity models and multiplicative interaction models^{20, 21}. RF can also capture joint variable effects by a recently introduced joint-importance measure²², which extends the concept of single importance by jointly permutating the values of variables of interest. The aim of the second-step was to obtain valid parameter estimates for the selected variables. Case-only logistic regression model^{23, 24} was used to evaluate the associations of gene-environment interaction markers with EA development, adjusting for covariates, such as, age, gender. False-discovery rate (FDR) was assessed to account for multiple comparisons. Polymorphisms were categorized into homozygous wild-type, and variant allele-containing genotypes. GERD, smoking, and BMI were dichotomized into 0 and 1.

RESULTS

Patients

A total of 335 patients were included in the analyses. Demographic and tumor characteristics are listed in Table 1. The majority of EA cases (88.0%) were male. The mean age at the time of diagnosis for the entire cohort was 62.9 years ($\pm SD 11.8$). 52% of patients

reported to have GERD symptoms and 80% of EA cases were ever-smokers. About 31% cases were overweight ($BMI \geq 25$) at the age of 18. Twenty-nine percent were stage I–IIA and seventy-one percent were stage IIB–IV. All cases were without a prior EA diagnosis at enrollment.

Examination of the Independence between Environmental Exposures and Genetic Factors

In case-only analysis, the odds ratio (OR) can be interpreted as the multiplicative interaction between gene and environment under the assumption that the gene and environment are independent²⁵. To determine the independence assumption, the relationship between the gene and environment in a sample of controls from the same base population as the cases should be examined²⁶. A lack of association between the exposures and SNPs among the controls would indicate that the exposures and SNPs are independent²⁷. To determine whether environmental factors and SNPs were independent in our study, we examined their correlations in a sample of 313 controls from the same base population as the cases¹⁵. We found no significant correlations between any environmental factors and SNPs studied, suggesting that the independence assumption in the present study was not violated.

RF Analysis

SNPs-GERD, SNPs-smoking, and SNPs-BMI interactions were screened by RF, respectively. The RF algorithm generates an importance score for each variable that quantifies the relative contribution of that variable to the prediction accuracy (Figure 1). After ranking the importance score in each of three runs of RF using 100,000 trees, the classification errors of each RF run were estimated by sliding window sequential forward feature selection (SWSFS) algorithm²⁸. The SWSFS analysis identified three sets of SNPs (17 SNPs with GERD, 26 SNPs with smoking, and 34 SNPs with BMI, respectively) that had the lowest classification error rates (Figure 2). These three sets of SNPs were chosen for subsequent SNP-GERD, SNP-smoking, and SNP-BMI interaction analyses, respectively, in case-only logistic regression models,

Case-only Logistic Regression Analysis

Table 2 shows the odds ratios (ORs) of SNPs-GERD interaction for ARDS risk, as determined by the case-only design. Significant associations were found between 2 SNPs (rs2295778 of *HIF1N*, rs13337626 of *TSC2*, respectively) and GERD combinations and EA risk. Interactions between 2 SNPs (rs2295778 of *HIF1N*, rs2296188 of *VEGFR1*, respectively) and smoking were also significantly involved in EA development. When we assessed ORs effects for higher BMI and angiogenic SNPs, significant associations were detected between 7 SNPs (rs2114039 of *PDGFRFA*, rs2296188 of *VEGFR1*, rs17619601 of *VEGFR1*, rs7324547 of *VEGFR1*, rs11941492 of *VEGFR1*, rs1770857 of *PDGFRB*, rs1762598 of *VEGFR1*) and higher BMI, respectively.

To investigate the cumulative effects of multiple risk SNPs, we analyzed the interactions between combined risk genotypes and environmental risk factors (GERD, smoking, and BMI), respectively. We found that increased evidence for gene-environment interaction was associated with increasing number of risk genotypes, suggesting the existence of a SNP dose-response in the gene-environment interactions in EA development (Table 3).

DISCUSSION

Understanding the interplay between the genetic and environmental factors involved in the development of EA will help guide early detection and personalized treatment strategies for this devastating disease. One of the limitations that slows progress in this regard is the sample size requirement for statistical power in case-control studies²⁹. This is particular

difficult, if not impossible, for uncommon diseases such as EA. Recently, case-only design has been proved to be an effective and powerful approach in the assessment of interactions in the etiology of disease^{27, 30–32}. In the present study, we used case-only analysis to examine gene-environment interactions in EA carcinogenesis. Our results indicated that specific genetic variations in angiogenic pathway genes modify EA risk through interactions with GERD, BMI, and smoking. In addition, we found that gene-environment interaction may contribute to EA risk in a dose-response fashion.

In many candidate gene approach of polygenic diseases, the effect of each individual SNP is generally limited, highlighting the need for a more compressive approach for association studies³³. In this study, we grouped functional related SNPs and genes into pathway for analysis. The pathway-based multigenic approach combining multiple SNPs that interact in the same pathway may amplify the effect of individual SNP and enhance the predictive power. Analyzing SNP data at a pathway level may have several advantages over analysis of single SNPs or multiple SNPs within a single gene. Grouping the SNPs by biological pathway allows for a biologically meaningful interpretation of the results. In addition, it is often difficult to replicate the findings of individual SNPs or haplotypes due to different linkage disequilibrium patterns or different allele frequencies among different study populations. Thus, it may be more feasible and meaningful to perform replication at the level of biological pathways, though there have few studies using this type of pathway analyses.

It is biologically plausible that genetic variation in angiogenic pathway genes may modify the effect of GERD, BMI, and smoking on EA risk. GERD has been well documented to be able to cause esophageal inflammatory injuries³⁴. Such injuries may lead to cancer through the inflammation-metaplasia-dysplasia-adenocarcinoma sequence in the esophagus by triggering a cascade of activation of oncogenes and/or inactivation of tumor suppressor genes, and DNA mutations^{34, 35}. Inflammatory mediators can modulate angiogenesis directly, or indirectly through up-regulation of VEGF activities³⁶. Angiogenic mediators, such as VEGF, can also enhance inflammatory processes, resulting in more severe inflammation³⁷. Inflammatory inhibitors, such as corticosteroids have been shown to limit both inflammation and angiogenesis processes³⁸. Cigarette smoke can induce *5-LOX* expression which plays an important role in activation of MMP-2 and VEGF to induce angiogenic process and promotion of inflammation-associated adenoma formation in mice³⁹. Additionally, it has been shown that smoke-induced *VEGF* expression and release were mediated by inflammatory responses⁴⁰. Increased levels of angiogenic mediators (VEGF-C, VEGF-D, sVEGF-R2, Ang-2, HGF) as well as the angiogenesis inhibitor endostatin are present in overweight and obese subjects^{41, 42}. Conversely, serum VEGF levels significantly decreased after weight loss following Roux-en-Y gastric bypass or low-fat diet treatment⁴³.

In this study, stronger interactions were observed between rs2295778 (*HIF1*)-GERD and rs2295778 (*HIF1*)-smoking. HIF is a transcription factor that, in hypoxia, induces angiogenesis activating angiogenic factors, such as VEGF and VEGF receptor^{44, 45}. Increased tumor expression of *HIF-1* and *VEGF* was correlated with more aggressive lesions on histological studies of human cancers⁴⁶. Both GERD and smoking are known to be associated with inflammation³⁴. Inflammatory cytokines increased *HIF-1* expression through NF-kappaB pathway⁴⁷. HIF-1 can also induce inflammatory responses⁴⁸ by cell autonomous NF-kappaB activation⁴⁹. One important mechanism underlying the cross-talk between NF-kappaB and HIF-1 is that NF-kappaB binds at a distinct element in the proximal promoter of the *HIF-1* gene⁵⁰. Overexpression of *HIF1* has been seen in the Barrett's metaplasia-dysplasia-adenocarcinoma sequence⁵¹ and associated with inflammation in Barrett's metaplasia⁵². Cigarette smoke exposure impairs angiogenesis by

inhibiting VEGF through decreased expression of *HIF-1*alpha in hypoxic conditions⁵³. *HIF-1* alpha protein expression is associated with *VEGF* gene and protein expression in acetic acid-induced esophageal ulcers⁵⁴.

Our study had several limitations. First, we only considered functional SNPs and tagging SNPs, rather than a comprehensive SNP approach that would capture most of the genetic variation in each gene. Therefore, based on our findings, we can not exclude potential interaction roles of those SNPs for which we did not included in the present study in EA risk. Additionally, there is no gold standard or pathway definition and different databases have different guidelines for their pathway construction. Consequently, the gene content of pathways representing the same biological process may vary between different databases, and this may have a major impact on the sensitivity and specificity of this approach. We tried to minimize this impact by selecting pathways from three commonly used and manually curated resources. Third, we restricted our analyses to Caucasians, as most of the subjects in our cohort were white (96%). The results of this study may not be generalized to other ethnic populations.

In summary, our findings supported the hypothesis that genetic variations in the angiogenesis pathway genes can contribute to EA risk through interactions with GERD, smoking and BMI. Our results also provide further support for using pathway-based approach to identify the complex relationship between genetic polymorphisms and cancer susceptibility involving multiple factors.

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Figure 1.

Importance score plots of SNP-GERD (A), SNP-smoking (B), and SNP-BMI (C) interactions ranked by case-only random forest analysis. Mean decrease accuracy (MDA) was estimated from 100,000 tests (trees), respectively. Higher MDA means higher contribution of the interaction to EA risk.

Figure 2.

Prediction error rate of angiogenic SNPs using GERD (A), BMI (B), and smoking (C) as outcomes. X-axis denotes the number of SNPs in the model, where the SNPs are included one by one based on their MDA ranks ranging from the most (left) to the least (right) importance. Y-axis is the error rate given by random forest (out of bag error estimation from 10,000 trees). The SNP sets with the lowest error rates were selected for further case-only logistic regression analysis.

Table 1

Clinical and Demographic Characteristics of EA Cases

Characteristics	EA Cases (n = 335)
Age (years, mean $\pm$ SD)	62.9 $\pm$ 11.8
Gender	
Male (%)	295 (88%)
Female (%)	40 (12%)
GERD symptoms*	
Yes (%)	173 (52%)
No (%)	162(48%)
Smoking status	
Ever smoker (%)	269 (80%)
Never smoker (%)	66 (20%)
Pack-years, median (min ~ max)	24.4 (0 ~ 212.0)
BMI	
BMI at age 18, (kg/m ² , mean $\pm$ SD)	23.6 $\pm$ 3.8
BMI $\geq$ 25 at age 18 (%)	104 (31%)
BMI <25 at age 18 (%)	231 (69%)
Stage	
I–IIA	98 (29%)
IIB–IV	237 (71%)

* 7 (2%) missing and imputed

Table 2

Interaction Between Angiogenic SNPs and Environmental Factors (GERD, Smoking, and BMI) on EA Risk: Case-Only Analysis

Interaction Markers	ORi (95% CI)	P	FDR
SNP-GERD[#]			
rs2295778 (<i>HIF1N</i>)-GERD	2.23 (1.41–3.50)	0.0005	0.0080
rs13337626 (<i>TSC2</i>)-GERD	2.40 (1.27–4.51)	0.0067	0.0512
SNP-Smoking[*]			
rs2295778 (<i>HIF1AN</i>)-Smoking	0.44 (0.26–0.78)	0.0043	0.0630
rs2296188 (<i>VEGFR1</i>)-Smoking	0.50 (0.28–0.87)	0.0138	0.1035
SNP-BMI^{&}			
rs2114039 (<i>PDGRFA</i>)-BMI	2.06 (1.29–3.30)	0.0026	0.0286
rs2296188 (<i>VEGFR1</i>)-BMI	0.41 (0.24–0.73)	0.0023	0.0286
rs17619601 (<i>VEGFR1</i>)-BMI	0.26 (0.09–0.75)	0.0130	0.0572
rs7324547 (<i>VEGFR1</i>)-BMI	1.84 (1.14–2.98)	0.0129	0.0572
rs11941492 (<i>VEGFR1</i>)-BMI	0.52 (0.32–0.84)	0.0083	0.0572
rs1770857 (<i>PDGFRB</i>)-BMI	1.89 (1.12–3.18)	0.0164	0.0601
rs17625898 (<i>VEGFR1</i>)-BMI	0.38 (0.16–0.88)	0.0232	0.0742

ORi: interaction odds ratio; BMI: BMI at age 18 (< 25 vs. ≥ 25); FDR: False discovery rate; FDR < 0.10 was considered noteworthy.

[#] adjusted for age, sex, BMI at age 18 (< 25 vs. ≥ 25) and smoking (+/-).

^{*} adjusted for age, sex, BMI at age 18 (< 25 vs. ≥ 25) and GERD (+/-).

[&] adjusted for age, sex, GERD (+/-), and smoking (+/-).

Table 3

Cumulative Effects of Gene (Risk Genotypes^{*})-Environment (GERD, Smoking, and BMI) Interactions on EA Risk: Case-Only Analysis

Risk Genotypes of rs2295778 and rs13337626	GERD		Cumulative ORi (95% CI)	P
	No (n)	Yes (n)		
No risk genotype	95	63	1 (reference)	-
1 risk genotype	62	92	2.24 (1.42–3.52)	4.91E-4
2 risk genotype	6	17	4.27 (1.60–11.43)	3.81E-3
Trend			2.16 (1.49–3.13)	4.81E-5

Risk Genotypes of rs2295778 and rs2296188	Smoking		Cumulative ORi (95% CI)	P
	No (n)	Yes (n)		
No risk genotype	17	28	1 (reference)	-
1 risk genotype	34	122	2.18 (1.07–4.44)	3.22E-2
2 risk genotypes	15	119	4.82 (2.15–10.80)	1.35E-4
Trend			2.20 (1.47–3.28)	1.25E-4

Risk Genotypes of rs2114039, rs2296188, rs17619601, rs7324547, rs11941492, rs1770857, and rs17625898	BMI≥25		Cumulative ORi (95% CI)	P
	No (n)	Yes (n)		
Risk genotypes ≤3	88	12	1 (reference)	-
4 risk genotypes	80	32	2.93 (1.41–6.08)	3.83E-3
5 risk genotypes-	46	32	5.10 (2.40–10.83)	2.24E-5
>5 risk genotypes	17	28	12.07 (5.15–28.32)	1.02E-8
Trend			2.19 (1.70–2.82)	1.23E-9

* SNPs with FDR <0.10 were considered noteworthy.