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Epidermal growth factor *A61G* gene polymorphism, gastroesophageal reflux disease and esophageal adenocarcinoma risk

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Background: **Single-nucleotide polymorphisms of key cancer genes, such as *EGF A61G*, are associated with an elevated risk of esophageal adenocarcinoma (EAC). As gastroesophageal reflux disease (GERD) is an established risk factor for EAC, we evaluated whether the association between epidermal growth factor (*EGF*) polymorphism and EAC development is altered by the presence of GERD. *Methods: EGF* genotyping of DNA samples was performed and GERD history was collected for 309 EAC patients and 275 matched healthy controls. Associations between genotypes and EAC risk were evaluated using adjusted logistic regression. Genotype–GERD relationships were explored using analyses stratified by GERD history and joint effects models that considered severity and duration of GERD symptoms. *Results: EGF* variants (*A/G* or *G/G*) were more common ($P = 0.02$) and GERD was more prevalent ($P < 0.001$) in cases than in controls. When compared with the *EGF* wild-type *A/A* genotype, the *G/G* variant was associated with a substantial increase in EAC risk among individuals with GERD [Odds ratio 9.7; 95% confidence interval (CI), 3.8–25.0; $P < 0.001$] and a slight decrease in risk for GERD-free individuals (odds ratio 0.4; 95% CI = 0.22–0.90; $P = 0.02$). In the joint effects models, the odds of EAC was also highest for *G/G* patients (when compared with *A/A*) who either experienced frequent GERD of more than once per week (odds ratio 21.8; 95% CI = 5.1–94.0; $P < 0.001$) or suffered GERD for longer than 15 years (odds ratio 22.4; 95% CI = 6.5–77.6; $P < 0.001$). There was a highly significant interaction between the *G/G* genotype and the presence of GERD ($P < 0.001$). *Conclusions: EGF A61G* polymorphism may alter EAC susceptibility through an interaction with GERD.**

Introduction

The incidence of esophageal adenocarcinomas (EACs) continues to increase steadily and in some countries surpassing squamous cell carcinomas as the most common malignant histology affecting the esophagus (1). In North America, the annual rate of EAC has experienced a dramatic 3- to 4-fold increase in the last three decades alone (2,3). The prognosis of EAC remains relatively poor, however, with 5 year overall survival rates approximating 10–15% only (4). Our understanding of its etiology has been advanced by epidemiological studies that have identified certain clinical variables, such as male gender, smoking, obesity and possibly alcohol and other dietary and environmental factors, that are significantly associated with the development of EAC (5–8). Chronic gastroesophageal reflux disease (GERD) and Barrett's metaplasia are also noted to correlate with greater risk of EAC (9). Nonetheless, the precise relationship between these risk factors and EAC is purportedly more complex since only a small proportion of people with such risk factors ultimately develop EAC, suggesting that there are probably additional parameters and interactions that are important to esophageal carcinogenesis.

Genetic factors may be an important contributor to the risk of developing EAC. Alterations in certain key genes that govern DNA maintenance and repair have already been linked to elevated risks of developing various cancers. Epidermal growth factor (EGF) has been implicated in cell proliferation and differentiation, and overexpression of the *EGF* gene, as measured in tissue or in serum, has been shown to be associated with a higher risk of esophageal squamous and adenocarcinomas (10–13). In a recent case–control study conducted by our research group, it was demonstrated that the *G/G* genotype of a single-nucleotide $A \rightarrow G$ polymorphism at position +61 of the *EGF* gene was associated with an almost 2-fold greater risk of EAC and that *EGF A61G G/G* was also associated with higher EGF serum levels in GERD patients who were tumor free (13). Because a different research question was being answered in our previous study, patients who never had GERD symptoms were selected as controls. Therefore, it was not possible to determine whether *EGFA61G* exerts a direct effect on EAC risk or acts by an indirect mechanism through an interaction with GERD. In a preliminary study of animal models by Sui *et al.* (14), a potential gene–environmental interaction was suggested between EGF and GERD. To explore this observation further, we performed gene–GERD interaction and joint effects analyses in a case–control study to determine whether the association between *EGF* polymorphism and EAC risk is modified by the presence of GERD.

Abbreviations: AOR, adjusted odds ratio; BE, Barrett's esophagus; BMI, body mass index; CI, confidence intervals; EAC, esophageal adenocarcinoma; EGF, epidermal growth factor; GERD, gastroesophageal reflux disease.

Materials and methods

Characteristics of the study population

This study was conducted upon receiving full ethics approval from the institutional review boards at Massachusetts General Hospital (Boston, MA), Dana Farber Cancer Institute (Boston, MA), Harvard School of Public Health (Boston, MA) and Princess Margaret Hospital (Toronto, Canada). Cases and controls were all over the age of 18 years (adults). More than 85% of the eligible cases and controls were recruited. Written informed consent was obtained from all subjects prior to study participation. Cases consisted of histologically-confirmed EAC recruited from Massachusetts General Hospital/Dana Farber Cancer Institute between 1999 and 2006. Controls were recruited among healthy adults who were friends and non-blood-related family members of other cancer patients (mostly lung cancer) from Massachusetts General Hospital/Dana Farber Cancer Institute between 2002 and 2007 and did not have any prior history of personal cancers (with the exception of non-melanoma skin cancers); no controls were hospital patients. We performed age, gender and race frequency matching during the selection of controls. Detailed GERD data were collected from the study outset for cases and starting in 2002 for controls. Since >96% of cases and controls were Caucasians, we selected only Caucasian cases and controls who had detailed GERD symptoms (including data on GERD frequency and intensity of symptoms) for analyses. A total of 309 cases and 275 controls met these inclusion criteria. All 309 cases were originally recruited for the study by Lanuti *et al.* ($n = 312$; three cases were excluded from the current analyses because they did not have detailed GERD data). Among the 275 controls, 143 were GERD-free patients from the Lanuti *et al.* study (9), whereas the remainder consisted of consecutive patients who were excluded from the original Lanuti *et al.* analysis because they had GERD symptoms.

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 analyses. Variables of interest included: demographic information (age, gender, adult height and weight); information

regarding smoking and alcohol exposure and lifetime GERD symptoms up to 1 year prior to diagnosis (for cases) or 1 year prior to interview (for controls). Body mass index (BMI) was calculated using self-reported heights and weights when the patients were in their twenties. Smoking history was grouped into 'never smokers', 'ex-smokers' and 'current smokers', and the degree of smoking exposure was quantified in pack-years when applicable. To evaluate for the presence of GERD, participants were asked to indicate whether they experienced symptoms, such as heartburn, acid reflux or regurgitation (see Appendix I). To explore GERD further, we studied its severity and duration. Both severity and duration of GERD data were collected based on Likert scales. Severity was classified on the basis of GERD symptom frequency into 'more than once per month' (baseline), 'once or more per month and once or less per week' or 'more than once per week', whereas duration was categorized into '<1 year' (baseline), '≥1 and ≤15 years' or '>15 years'. Patients were considered GERD-free if they have less than one episode of GERD per month.

EGF genotyping

Using the Puregene DNA Isolation Kit (Gentra Symptoms, Minneapolis, MN), DNA was extracted from peripheral whole blood samples of study participants at the time of enrollment. Singlenucleotide polymorphisms at position +61 in the 5′-untranslated region of the *EGF* gene was detected using modified polymerase chain reactions and the Taqman approach (Applied Biosystems, Foster City, CA) with commercial primer sequences. Specific details of primers, probes and polymerase chain reactions methods and conditions are available from study investigators upon request. All genotyping were performed by laboratory technicians who were blinded to the case–control status and clinical data. As a quality control measure, a random selection of 15% of samples was repeated to validate accuracy and ensure reproducibility of the genotyping results. Two investigators independently reviewed all of the results, and disagreements were resolved by a third investigator.

Statistical analyses

Baseline demographics and clinical information were summarized with descriptive statistics and subsequently compared between cases and controls with Pearson's chi-square tests for categorical variables and Wilcoxon rank sum test for continuous variables. Using the wild-type genotype (*A/A*) as the reference, analyses of the other genotypes (e.g. heterozygous *A/G* and homozygous *G/G*) and their associations with EAC risk were examined with logistic regression models and subsequently adjusted for possible confounding factors such as age, gender, smoking history and adult BMI. Alcohol exposure was excluded from the models because of missing data from a number of cases and controls. Multiple strategies were implemented in the evaluation of gene–GERD interactions in order to ensure consistency and validity of the results, including the use of subset analyses stratified by GERD status (e.g. infrequent GERD of less than once per month versus frequent GERD of once or more per month) as well as genotype–GERD interactions and joint effects models that considered GERD severity and duration. Categories for the frequency and duration of GERD considered the number of individuals in each category; categories for which there were very few individuals (e.g. less than five subjects per cell) were collapsed together, if feasible. Crude and adjusted odds ratios (odds ratios and AORs, respectively) and corresponding 95% confidence intervals (CIs) for the risk of EAC were derived from these models. SAS version 9.1.3 (SAS Institute, Cary, NC) was used to perform all statistical analyses. All *P*-values were two sided where a value <0.05 was considered statistically significant.

Results

General characteristics of the study population

The distribution of baseline demographic, clinical and genotypic characteristics among cases and controls are summarized in Table I. Although only 143 of 275 controls overlapped with the controls used in the Lanuti *et al.* (9) study, demographic and genotypic characteristics were similar between these two control groups. In comparison with controls, cases had a higher median adult BMI ($P = 0.01$) and they were more probably to be former or current smokers ($P = 0.01$). Patients in the case group were also more probably to have experienced significant lifetime GERD, as measured by symptom frequency and duration (both $P < 0.001$). Otherwise, there were no significant differences between groups for the remaining variables including age, gender, pack-years of smoking and alcohol history (all $P > 0.05$), although alcohol data were collected in only a small subset of cases and controls.

Table I. Baseline characteristics of cases with and controls without EAC

Clinical parameter	Cases ($N = 309$)	Controls ($N = 275$)	*P* value comparing cases versus controls
Gender			
Male	89%	88%	0.62[a]
Female	11%	12%	
Median age (range)	64.1 (21–91)	62.9 (30–96)	0.49[b]
Median BMI[c] (range)	23.3 (15–36)	22.6 (14–36)	0.01[b]
Smoking status			
Never smoker	20%	31%	0.01[a]
Ex-smoker	55%	50%	
Current smoker	25%	19%	
Median pack-years of ever-smokers (range)	33.7 (0.2–212)	30.0 (0.1–218)	0.21[b]
Caucasian race	98%	98%	0.54[a]
Tumor stage of esophageal cancer			
I	8%	—	
II	40%	—	
III	25%	—	
IV	27%	—	
EGF genotype			
A/A	33%	40%	0.02[a]
A/G	42%	44%	
G/G	25%	16%	
GERD frequency			
<1 Mo. (GERD free)	51%	77%	<0.001[a]
≥1/Mo. and ≤1/wk	27%	14%	
>1/Wk	22%	9%	
GERD duration			<0.001[a]
<1 Year	34%	78%	<0.001[a]
≥1 and ≤15 Years	32%	12%	
>15 Years	34%	10%	
Alcohol history[d]			
Yes	94%	91%	0.32[a]
No	6%	9%	

mo., month; wk, week.
[a]Fisher's exact test.
[b]*t*-test/Wilcoxon rank sum test.
[c]Median BMI when patients were in their twenties.
[d]Alcohol history was only available for 246 cases and 66 controls.

Distribution of EGF genotype and association with EAC risk

The frequency of the *G/G* genotype for the *EGF A61G* polymorphism was significantly higher for cases than controls ($P = 0.02$). Table II shows that the odds of EAC were significantly increased for patients with the *EGF A61G* homozygous *G/G* genotype as evidenced by the crude analyses (odds ratio 1.88, 95% CI = 1.2–3.0, $P = 0.007$). This finding persisted in the adjusted analyses after accounting for age, gender, smoking history and adult BMI (AOR 1.90, 95% CI = 1.2–3.0, $P = 0.007$). No clear relationships were found for the heterozygous *A/G* genotype.

EGF polymorphism, GERD symptoms and EAC risk

We performed stratified analyses based on GERD status. In the subset of patients with self-reported GERD symptoms of more than once per month, there continued to be a very strong association between *G/G* genotype and EAC risk that was statistically significant (AOR 9.71, 95% CI = 3.8–25.0, $P < 0.001$). However, this correlation was no longer evident for the group of patients with GERD of less than once per month. In fact, these patients had a lower risk of EAC (AOR 0.44, 95% CI = 0.22–0.90, $P = 0.024$). For frequent GERD patients, the *A/G* genotype in comparison with *A/A*

Table II. OR of the risk of EAC by *EGF A61G* polymorphism in the overall cohort and in GERD versus GERD-free subsets

Clinical parameter	Total number of cases/controls[a]	*A/G versus A/A*		*G/G versus A/A*	
		OR (95% CI)	*P* value	OR (95% CI)	*P* value
Crude analysis					
Overall	309/275	1.16 (0.8–1.7)	0.43	1.88 (1.2–3.0)	0.007
GERD subset	150/62	3.27 (1.6–6.8)	0.002	8.95 (3.6–22.3)	<0.001
GERD-free subset	159/213	0.68 (0.44–1.06)	0.087	0.44 (0.22–0.87)	0.017
Adjusted analysis[b]					
Overall	309/275	1.22 (0.8–1.8)	0.30	1.90 (1.2–3.0)	0.007
GERD subset	150/62	3.59 (1.7–7.7)	0.001	9.71 (3.8–25.0)	<0.001
GERD-free subset	159/213	0.70 (0.44–1.11)	0.13	0.44 (0.22–0.90)	0.024

OR, odds ratio.
[a][*N/n*] where N = number of cases and n = number of controls. Overall: A/A [77/44]; A/G [131/122] and G/G [101/109]; total [309/275]; GERD subset: A/A [62/9]; A/G [68/27] and G/G [20/26]; total [150/62] and GERD-free subset: A/A [15/35]; A/G [63/95] and G/G [81/83]; total [159/213].
[b]Adjusted for age, gender, smoking status, pack-years and adult BMI.

Table III. Exploratory adjusted[a] OR for the joint effects of GERD frequency (A) or duration (B) and EGF *A61G* polymorphism on EAC risk

A. GERD frequency				
EGF genotype	<1/Mo	≥1/Mo and ≤1/wk	>1/Wk	Marginal OR
A/A	1.00 (81/83)[b]	1.51 (0.7–3.3) (19/14)	0.07 (0.01–0.6) (1/12)	1.00
A/G	0.70 (0.4–1.1) (63/95)	2.78 (1.4–5.4) (41/17)	3.09 (1.4–6.9) (27/10)	1.02 (0.7–1.5)
G/G	0.45 (0.2–0.9) (15/35)	3.05 (1.2–7.7) (22/7)	21.80 (5.1–94) (40/2)	1.25 (0.8–2.1)
Marginal OR	1.00	2.97 (1.9–4.7)	3.71 (2.2–6.4)	—
B. GERD duration				
EGF genotype	<1 Year	≥ 1 and ≤15 Years	>15 Years	Marginal OR
A/A	1.00 (45/83)	3.99 (1.8–8.9) (27/12)	4.09 (1.9–8.7) (29/14)	1.00
A/G	0.94 (0.56–1.58) (46/95)	5.02 (2.5–10) 44/16	8.25 (3.7–18) (39/10)	1.14 (0.8–1.7)
G/G	0.69 (0.32–1.45) (13/35)	8.62 (3.3–23) (28/6)	22.39 (6.5–78) (36/3)	1.38 (0.8–2.3)
Marginal OR	1.00	5.57 (3.5–8.9)	8.11 (4.9–13)	—

mo, month; wk, week.
[a]Adjusted for age, gender, smoking status, pack-years and adult BMI.
[b][*N/n*] where N = number of cases and n = number of controls.

also appeared to contribute to a higher risk of EAC risk (AOR 3.59, 95% CI = 1.7–7.7, $P = 0.001$).

We also conducted exploratory analyses on the joint effects of *EGF* polymorphisms with the different degrees of GERD severity and duration. For severity, the combination of the *G/G* genotype and severe GERD frequency of more than once per week exhibited the highest odds of EAC (AOR 21.8, 95% CI = 5.1–94) when compared with the reference group of *A/A* genotype and less than once per month (Table IIIA). For duration, the *G/G* genotype combined with a long GERD duration of >15 years conferred the greatest risk of EAC (AOR 22.4, 95% CI = 6.5–78) in comparison with the reference group (Table IIIB). Some estimates of risk had wide CIs since, despite our efforts, every classification of our GERD symptoms resulted in at least one cell having fewer than five individuals.

In *EGF* polymorphism–GERD interaction analyses, the interaction between *EGF A61G* (*G/G* versus wild-type) and presence or absence of GERD was highly statistically significant ($P < 0.001$). The interaction between *EGF* heterozygote and GERD presence/absence was also significant but to a lesser extent ($P = 0.003$). In exploratory interaction analyses involving severity or duration of GERD, we combined *A/A* and *A/G* together to reduce the number of interaction terms in each model and to ensure that there were the fewest categories with very small numbers in each cell. The interaction term between *EGF A61G* and GERD frequency of more than once per week was statistically significant ($P < 0.001$), but the interaction term between *EGF A61G* and GERD frequencies of between once a month to once per week was not ($P = 0.11$). Similarly, there was a statistically significant interaction term between the *EGF A61G* polymorphism and GERD duration of >15 years ($P = 0.02$) but not for *EGF A61G* and GERD duration of between 1 and 15 years ($P = 0.12$).

Discussion

Both a personal history of GERD and the presence of the *EGF A61G* homozygous variant genotype have been shown in separate studies to correlate with an increased risk of developing EAC (9,13). Their evaluation together, however, has not been studied to date. In our previous study, elevated serum EGF levels were detected in cancer-free individuals with GERD. Specifically, elevated serum EGF levels were found only among GERD patients with the *G/G* genotype, but not in those without GERD, highlighting the possibility that the *EGF*-signaling pathway promotes esophageal carcinogenesis more in the presence of GERD (13). We tested this biological hypothesis by examining the relationships among *EGF* polymorphisms, severity and duration of GERD symptoms and the risk of EAC in a case–control design study. In the current analyses, we found a highly statistically significant interaction between cumulative GERD exposure and *EGF* polymorphism ($P < 0.001$) in its association with EAC risk. Importantly, patients with severe or long-standing GERD and the *G/G* genotype were found to have over a 20-fold greater risk of EAC when compared with GERD-free individuals and the wild-type genotype.

The finding of an *EGF*–GERD interaction is consistent with prior knowledge that the *EGF* pathway is implicated in tumorigenesis. EGF is involved in epithelial cell proliferation and differentiation (12). More specifically, reports suggest that EGF plays a role in the stepwise progression from esophageal metaplasia to dysplasia and the subsequent transformation to adenocarcinoma among patients affected with Barrett's esophagus (BE) (15). Furthermore, the single-nucleotide $A \rightarrow G$ polymorphism at position +61 in the 5′-untranslated region of the *EGF* gene is associated with higher EAC risk (13). Genetic variations of *EGF* are also reportedly linked with more extensive invasion and worse prognoses for many other tumors, including malignant melanoma, glioblastoma multiforme and gastric cancer (16–18). However, not all patients with polymorphisms, amplification or overexpression of *EGF* develop EAC, supporting the potential importance of gene–environmental interactions in EAC carcinogenesis. Gene–environmental interactions have been noted in other cancers. For instance, gene–smoking interactions between DNA repair gene (e.g. *ERCC1*, *ERCC2*, *XRCC1*) polymorphisms and cigarette exposure have been shown to be an important contributor to lung cancer development (19–21). This study is the first to report a strong interaction between frequency, duration or presence of GERD and the *EGFA61G* polymorphism for EAC risk.

The precise mechanisms underlying the observed gene–GERD interaction in EAC risk are probably complex. Preliminary studies indicate that the *EGF* pathway may serve as a protective factor under normal conditions or during early acid reflux by helping to repair and maintain the integrity of the esophageal mucosa (22,23). The salivary EGF concentration has been shown to contribute to the quality of the esophageal mucosal barrier whereby patients with lower than normal salivary EGF levels are more predisposed to developing severe esophageal damage and BE from acid reflux (24,25). Studies also demonstrate that exposure of the esophagus to damaging factors or toxins is associated with an increase in intraluminal release of EGF, providing additional support of its potential contribution to mucosal protection and its role in regeneration of the esophageal epithelium (26). Overactivation of signaling pathways or overexpression of genes that are actively involved in tumor suppression predominates in this setting. This may explain our observation that in the absence of chronic reflux damage, the allele is actually protective against EAC. In contrast, in the presence of chronic reflux damage (i.e. GERD individuals in this study), these subtle repair and protective mechanisms are overwhelmed. Therefore, the balance shifts in favor of carcinogenesis when the *G/G* variant genotype occurs in combination with GERD (14). Further biological studies are required to confirm this hypothesis.

This study has several limitations. First, we do not have available esophageal cancer tissues to correlate EGF amplification or expression levels with polymorphism results. We also do not have a large independent BE population to determine whether this gene–environmental interaction is present in the precursor lesion. This latter relationship will be difficult to delineate since occult BE exists more often than occult EAC, and GERD symptoms commonly trigger investigations leading to a diagnosis of BE. Second, we chose to study a known modifier of EAC risk rather than perform single-nucleotide polymorphism discovery analyses, but given the specific association sought, this approach is valid. Nonetheless, other polymorphisms of the *EGF* gene or polymorphisms of additional genes that can control epithelial cell proliferation and differentiation were not studied. Third, GERD symptoms were collected based on self-report only and were not validated by medical record review or with confirmatory studies such as 24 h-pH monitoring. In addition, second hand smoking history, amount of alcohol intake, dietary pattern and other environmental and occupational exposures were neither accounted for nor adjusted in the logistic regression models because of missing or uncollected data. Given the strong relationship between GERD and *EGF* polymorphism with EAC risk, these potential confounders would probably have had minor influence on the results. Finally, results from the joint effects models must be interpreted with caution and viewed primarily as hypothesis generating, considering that these were derived from exploratory analyses, where some cells contained small numbers (Table IIIA and B). Our patient sample also consisted primarily of Caucasians and cannot be extrapolated to other races.

In summary, this study represents the first report among humans that *EGF* polymorphism exerts its effect on EAC susceptibility through an interaction with GERD. Specifically, the homozygous *G/G* variant genotype is associated with the greatest risk of EAC, especially among those with either severe or long-standing GERD, whereas in the absence of GERD, it may actually be protective through a mucosal defense mechanism. If validated prospectively, *EGF* genotyping and GERD symptoms together may offer an effective approach of identifying a subset of individuals with greater risk of developing EAC, with relevant implications for GERD screening programs. Regardless, these results compel further study to delineate the precise molecular mechanisms of this *EGF*–GERD relationship with EAC development.

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Appendix I

Questions about heartburn:

Have you ever had symptoms of heartburn?

○ Yes ○ No

If yes, prior to the past 5 years, how often did you have heartburn when the symptoms were at its worst?

○ Never or less than once a month
○ At least once a month
○ At least once a week
○ Several times (3–4) a week
○ More than four times a week

If yes, prior to the past 5 years, how often did you have heartburn AT NIGHT when the symptoms were at its worst?

○ Never or less than once a month
○ At least once a month
○ At least once a week
○ Several times (3–4) a week
○ More than four times a week

How long have you had symptoms of heartburn?

_____ years

Questions about acid reflux or regurgitation:

Have you ever had symptoms of acid reflux or regurgitation?

○ Yes ○ No

If yes, prior to the past 5 years, how often did you have acid reflux or regurgitation when the symptoms were at its worst?

○ Never or less than once a month
○ At least once a month
○ At least once a week
○ Several times (3–4) a week
○ More than four times a week

If yes, prior to the past 5 years, how often did you have acid reflux or regurgitation AT NIGHT when the symptoms were at its worst?

○ Never or less than once a month
○ At least once a month
○ At least once a week
○ Several times (3–4) a week
○ More than four times a week

How long have you had symptoms of acid reflux or regurgitation?

_____ years