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Single nucleotide polymorphisms in the matrix metalloproteinase gene family and the frequency and duration of gastroesophageal reflux disease influence the risk of esophageal adenocarcinoma

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Abstract

The matrix metalloproteinase (MMP) family of proteins mediates various cellular pathways, including apoptosis and angiogenesis. Polymorphisms of *MMP* genes are associated with increased esophageal adenocarcinoma (EAC) risk. Gastroesophageal reflux disease (GERD) is an established EAC risk factor. We examined whether *MMP* polymorphism-EAC risk is modified by GERD. In total, 309 EAC patients and 279 frequency-matched healthy controls underwent *MMP1 1G/2G*, *MMP3 6A/5A*, *MMP12 −82A/G* and *MMP12 1082A/G* genotyping. Questionnaires collected GERD history. EAC risk was analyzed using logistic regression, adjusted for key covariates and stratified by GERD. Joint effects models explored GERD severity and duration, whereas additional models explored genotype–GERD interactions in EAC risk. We determined that each *MMP1* and *MMP3* minor (variant) allele was independently associated with increased EAC risk (adjusted odds ratio (AOR) 3.2, 95% confidence interval (CI) 2.0–5.1, $p < 0.001$ and AOR 1.8, 95% CI 1.1–2.7, $p = 0.01$, respectively) only among those with GERD but not in GERD-free individuals (all p = nonsignificant). There were significant interactions between the *MMP1* variants and the presence of GERD ($p = 0.002$) and between *MMP3* variants and GERD ($p = 0.04$). There was an equally strong interaction between cumulative GERD severity and *MMP1* ($p = 0.002$). The AOR of each variant allele was 14.9 (95% CI 1.6–136) for individuals with severe GERD, 1.7 (95% CI 1.0–2.7) for mild-moderate GERD and 0.98 (95% CI 0.7–1.4) for those without GERD. This was further reflected in separate analyses of frequency and duration of GERD. In conclusion, *MMP1 1G/2G* (and possibly *MMP3 6A/5A*) polymorphisms alter EAC risk differentially for GERD and GERD-free individuals.

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Keywords

matrix metalloproteinase; gene polymorphism; gastroesophageal reflux disease; esophageal cancer

Although esophageal adenocarcinoma (EAC) is a relatively rare malignancy, its incidence is steadily increasing, especially among white males.[1,2] There has been a particularly sharp rise in new cases of EAC in several Western countries, in part due to increases in known risk factors such as overweight and obesity.[1-4] In the United States, the annual incidence of EAC has tripled over the last several decades.[3,4] Despite advances in diagnostic and therapeutic strategies, EAC has a poor prognosis with 5-year overall survival rates of under 15%.[5] Male gender, smoking, alcohol, chronic gastroesophageal reflux disease (GERD) and Barrett's metaplasia are among the many other established risk factors for the development of EAC.[6-10] As only a small minority of patients with such risk factors actually develops EAC, the precise causal relationship between these risk factors and EAC is probably very complex and points to additional variables that are crucial to esophageal carcinogenesis.

Genetic factors may be important to the development of EAC. Specifically, the matrix metalloproteinase (MMP) family of proteins is implicated in extracellular matrix and basement membrane degradation, which promote cellular invasion and metastatic spread of cancer through diverse pathways that govern apoptosis, angiogenesis, cellular adhesion and signaling and host immune response.[11] In particular, *MMP1*, *MMP3* and *MMP12* have been most significantly associated with various cancers.[12-16] Upregulated *MMP1* transcription is linked with an elevated risk of nasopharyngeal, thoracic and colorectal tumors,[12-14] whereas increased expression of *MMP3* is reportedly linked with a higher risk of lung malignancies.[15] Polymorphic variations in *MMP12* have been correlated with breast cancer outcomes,[16] and aberrant expression of several *MMP* genes has been associated with poorer esophageal cancer prognosis.[17,18] In Barrett's esophagus, *MMP* genes are overexpressed, suggesting a role for these genes in the stepwise progression from Barrett's metaplasia to EAC.[19,20]

Our recent case–control study of the *MMP* gene family found that the heterozygous (*1G/2G*) and homozygous (*2G/2G*) variant genotypes of *MMP1 1G/2G* (rs1799750) were independently associated with an approximately 1.5-fold to twofold greater risk of EAC.[21] Likewise, the homozygous (*5A/5A*) variant of *MMP3 6A/5A* (rs3025058) was associated with similar results (1.6 times greater risk).[21] The same variants were also associated with higher risk of Barrett's esophagus, a potential outcome of chronic GERD, among tumor-free patients. Polymorphisms in *MMP12* (*−82A/G* [rs2276109] and *1082A/G* [rs652438]) were not associated with EAC risk. Because this previous study mainly focused on a different research question, patients who never had GERD symptoms were chosen as controls. For this reason, it was not possible to evaluate whether GERD modified the association between *MMP1*, *MMP3* and *MMP12* polymorphisms and EAC risk. Ylitalo et al. described preliminary *in vitro* studies suggesting gene–environmental interactions between *MMPs* and reflux.[22,23] In our study, we extend this work into a human setting and hypothesize that the relationship between *MMP* polymorphisms and EAC risk is modified by a history, severity, frequency and duration of GERD.

Material and Methods

Study population

Our study was performed after receiving ethics approval from the Human Subjects Committee at Massachusetts General Hospital (MGH), Dana Farber Cancer Institute (DFCI) and Harvard School of Public Health (all in Boston, MA) and the Research Ethics Board at

Princess Margaret Hospital (Toronto, ON, Canada). Specific details of the case and control population have been reported previously.[24-26] Briefly, all cases and controls were adults (more than the age of 18 years). The recruitment rate for eligible cases and controls was more than 85%. Written informed consent was formally obtained from all participants before study enrollment. Incident cases of histologically confirmed or biopsy-proven EAC were recruited from either the MGH or the DFCI between 1999 and 2006. As this was an exploratory study, formal sample size calculations were not conducted; rather, we recruited as many eligible cases as possible from the study institutions during the study time period. Controls were recruited from the same hospitals between 2002 and 2007 among healthy adults who were friends or non-blood-related family members of cardiothoracic patients (most were lung cancer or cardiac patients and none had esophageal cancer). To be eligible, controls could not be hospital patients and all had no prior personal history of cancers (except for nonmelanoma skin cancers). Age, gender and race frequency matching were performed during the selection of controls.

Detailed GERD information was collected. Because the vast majority (more than 96%) of the case and control populations was Caucasian, we selected only Caucasian cases and controls who had GERD symptom information for analyses. A total of 309 cases and 279 controls met these inclusion criteria.

Clinical data collection

A trained research assistant conducted a personal interview with each participant at study entry to collect baseline demographic and clinical data, which served as covariates for our analyses. Demographic information included age, gender, adult height and weight, smoking and alcohol history (at 1 year before diagnosis for cases and at 1 year before interview for controls) and lifetime GERD symptoms (up to 1 year before diagnosis/interview for cases/controls, respectively). Body mass index (BMI) was determined using self-reported adult heights and weights when the patients were in their third decade of life because obesity-related EAC risk is associated with a latency period of 20–30 years. Smoking history was categorized into “never smokers,” “ex-smokers” and “current smokers.” Pack-years were used to characterize the extent of smoking exposure.

To evaluate whether cases and controls experienced GERD, participants were asked to indicate if they experienced typical GERD symptoms, such as heartburn, acid reflux or regurgitation (Appendix). To explore GERD in greater detail, we also characterized its severity and duration. Likert scales were used to collect information regarding GERD symptom severity and duration. GERD severity was classified by symptom frequency into “< once per month” (baseline), “≥ once per month and ≤ once per week” or “> once per week” while duration was categorized into “<1 year” (baseline), “ ≥1 and ≤15 years” or “>15 years.” If patients experienced less than one episode of GERD per month, they were considered GERD free in the analyses. The definition of GERD free was broader that the standard definition of GERD; we used this broader definition to study a wider distribution of symptoms. To allow for appropriate comparisons with other studies, we studied frequency, duration and cumulative severity variables separately. All data from the personal interviews were entered into an electronic database. Missing data or points requiring clarification were resolved through a follow-up telephone interview with participants.

DNA extraction and genotyping

All peripheral whole blood used for genotyping were collected from study participants at the time of enrollment. Using Qiagen DNA Purification Kits (Qiagen, Valencia, CA), DNA was extracted from these blood samples. Single nucleotide polymorphisms of the *MMP1*, *MMP3* and *MMP12* genes were detected using modified polymerase chain reactions and the

Taqman approach (Applied Biosystems, Foster City, CA) with commercial primer sequences. Specifically, *MMP1* and *MMP3* used ABI Assays-by-design and the following probes: *MMP1* probes, 5′ [6FAM]-GCCCTCTTGAACTCACATGTT ATG-3′ and 5′-ACTTTCCTCCCCTTATGGATTCC-3′; *MMP3* probes, 5′-GTCCTCATATCAATGTGG CCAAA-3′ and 5′ [6FAM]-CGGCACCTGGCCTAAAGAC-3′ (PMID: 15102660), whereas *MMP12* used ABI Assay-on Demand: ID: C_15880589_10. Laboratory personnel performing the genotyping were blinded to the case–control status and to all other clinical information. For quality control purposes and to validate accuracy and ensure reproducibility of the results, a random 15% sample underwent repeat genotyping. Failed, equivocal or discrepant results were also repeated before excluding these cases from the analysis. All results were independently reviewed by two investigators, and disagreements were resolved by consensus, blinded to clinical data.

Statistical considerations

Baseline demographics and clinical information were summarized with descriptive statistics. To compare between cases and controls, Pearson χ^2 and Wilcoxon rank-sum tests were conducted on categorical and continuous variables, respectively. The wild-type genotypes (*1G/1G* for *MMP1, 6A/6A* for *MMP3* and *A/A* for both *MMP12 −82A/G* and *MMP12 1082A/G*) served as the reference groups for comparing the variant genotypes. Associations between genotypes and EAC risk were first examined with unadjusted logistic regression. Models were subsequently adjusted for confounders such as age, gender, smoking history and adult BMI. Because data on alcohol exposure were missing from a number of cases and controls, this variable was excluded from the models. For the evaluation of gene–GERD interactions, we used subset analyses stratified by GERD status (*i.e.*, rare GERD of < once per month [also termed GERD free] *vs.* GERD of ≥ once per month) in addition to genotype–GERD interactions and joint effects models that explored GERD severity and duration.

Both frequency and duration of GERD symptoms were categorized such that the number of cells with too few individuals for any analysis would be minimized. In settings where there were less than five subjects per cell, for instance, these categories were collapsed together, if possible. We also performed analyses of cumulative severity of GERD that separated individuals with GERD into those with severe cumulative GERD (duration > 15 years and frequency of more than once per week) and those with mild-to-moderate cumulative GERD (all others) comparing them to individuals with rare or no cumulative GERD (duration < 1 year and frequency of less than once per month). For these analyses, an additive genetic model was used to simplify the number of interaction terms. Crude and adjusted odds ratios (ORs and AORs, respectively) and corresponding 95% confidence intervals (CIs) for the risk of EAC were derived from these various models. SAS version 9.1.3 (SAS Institute, Cary, NC) was used to perform all statistical analyses. *p*-values were two sided where a value <0.05 was considered to be statistically significant. We present data from the codominant or discrete genetic model of inheritance (primary analysis) and the additive genetic models of inheritance (a robust allelic dose model that fits well with biological rationale) as a secondary analysis.

Results

Characteristics of cases and controls

Table 1 summarizes the details of the baseline demographic, clinical and genotypic characteristics among cases and controls. In comparison to controls, cases had a slightly higher median adult BMI ($p = 0.01$), and they were more likely to have had a smoking history ($p = 0.01$). Cases were also more likely than controls to have experienced significant

lifetime GERD in terms of symptom frequency, duration and cumulative severity (all $p < 0.001$). Otherwise, there were no significant differences between the two groups with respect to age and race distribution, gender, pack-years of smoking and alcohol exposure (all $p > 0.05$).

Distribution of the MMP genotypes and their associations with EAC risk

All of the evaluated polymorphisms were in Hardy–Weinberg equilibrium. The homozygous *2G/2G* variant for the *MMP1* polymorphism was significantly more prevalent, whereas the wild-type *1G/1G* genotypes were less common among cases than for controls ($p = 0.01$). In contrast, the distribution of the *MMP3* and *MMP12* polymorphisms was not statistically different between cases and controls. Table 2 shows the ORs and AORs of EAC due to *MMP1* for the entire study cohort. In the crude analyses, the *MMP1* polymorphism was associated with EAC risk (global Wald $p = 0.009$). Specifically, the risk of EAC was significantly increased for patients carrying the *MMP1* homozygous *2G/2G* variant (OR 2.05, 95% CI 1.3–3.3, $p = 0.002$). After controlling for age, gender, smoking history and adult BMI, the adjusted analyses revealed a similar relationship (global Wald $p = 0.008$); the *2G/2G* genotype was associated with increased EAC risk (AOR 2.11, 95% CI 1.3–3.4, $p = 0.002$). Using an additive model of inheritance, the AOR was 1.45 (95% CI 1.2–1.8, $p = 0.002$) for each minor allele.

Similarly, when compared to the wild-type *MMP3 6A/6A* genotype, the homozygous *5A/5A* variant was marginally correlated with a greater EAC risk (AOR 1.70, 95% CI 1.0–2.8, $p = 0.04$), whereas the heterozygous *6A/5A* variant did not (AOR 1.19, 95% CI 0.8–1.8, $p = 0.42$); global Wald $p = 0.10$. Using an additive model of inheritance, the AOR was 1.31 (95% CI 1.0–1.7, $p = 0.04$) for each minor allele.

Of note, there were no associations between any of the *MMP12* genotypes and EAC risk; thus, all subsequent analyses focused only on *MMP1* and *MMP3*.

MMP gene polymorphisms, GERD and EAC risk

Subset analyses based on GERD status were subsequently performed. Table 2 shows the ORs and AORs of EAC due to *MMP1* for both GERD and GERD-free individuals. In the subset of patients with self-reported GERD symptoms of more than once per month, a very strong, statistically significant association between the *MMP1* polymorphism and EAC risk was observed, even after controlling for confounders (global Wald $p < 0.001$), which was driven by the increased risk of the *2G/2G* genotype compared to wild type (AOR 10.87, 95% CI 4.0–29.2, $p < 0.001$) and also by the *1G/2G* genotype (AOR 2.54, 95% CI 1.2–5.3, $p = 0.01$). This correlation, however, was absent in the group of patients with GERD of less than once per month (global Wald $p = 0.15$). In an additive model of inheritance, the AORs were 3.19 (95% CI 2.0–5.1, $p < 0.001$) for each minor allele in GERD patients and 0.79 (95% CI 0.6–1.1, $p = 0.14$) in GERD-free patients.

A similar, albeit weaker, relationship was also observed for *MMP3* where the *5A/5A* homozygous variant was associated with a higher EAC risk only in the subset of patients with self-reported GERD (AOR 3.05, 95% CI 1.2–7.5, $p = 0.01$; global Wald $p = 0.03$) but not in the group without GERD (AOR 0.87, 95% CI 0.4–1.7, $p = 0.69$; global Wald $p = 0.55$). The heterozygous *6A/5A* variant *MMP3* genotype was not associated with EAC risk regardless of GERD status. Using an additive model of inheritance, the AORs were 1.76 (95% CI 1.1–2.7, $p = 0.01$) for each minor allele in GERD patients and 0.95 (95% CI 0.7–1.3, $p = 0.75$) in GERD-free patients.

Our *MMP1* polymorphism–GERD interaction analyses revealed that there was a highly statistically significant interaction between *MMP1* and GERD (global Wald $p < 0.001$). This was due to a significant interaction term between the *2G/2G vs.* wild-type *1G/1G* and the presence or absence of GERD ($p < 0.001$) and a significant interaction term between the *MMP1* heterozygote *1G/2G* and GERD presence or absence ($p = 0.03$). The interaction term in the additive model of inheritance was equally significant ($p < 0.001$).

Interaction and joint analyses by GERD frequency and duration

We conducted exploratory interaction analyses that evaluated the frequency or duration of GERD. In our GERD frequency interaction models, we included two interaction terms: (*i*) *MMP1* and GERD of greater than once per week and (*ii*) *MMP1* and GERD between once a month and once per week. The interaction term between *MMP1* and GERD frequency was highly significant (global Wald $p < 0.001$). Both the interaction terms between *MMP1* and GERD frequency of greater than once per week ($p < 0.001$) and between *MMP1* and GERD frequencies of between once a month and once per week ($p = 0.03$) were significant. Similarly, for GERD duration (global Wald $p = 0.02$), there was also a statistically significant interaction term between the *MMP1* polymorphism and longstanding GERD of >15 years ($p = 0.009$), but not for the interaction term between *MMP1* and GERD duration of 1–15 years ($p = 0.13$). In contrast, our *MMP3*-GERD interaction analysis was entirely nonsignificant (global Wald $p = 0.14$) where only a trend in the interaction between the homozygous *5A/5A* variant and GERD presence/absence ($p = 0.08$) was observed.

Given the strong interaction between *MMP1* and GERD, further exploratory analyses on the joint effects of *MMP1* polymorphisms and the varying degrees of GERD frequency and duration were also conducted. For GERD frequency (Table 3), when compared to the reference group of the wildtype *1G/1G* genotype and mild GERD frequency of less than once per month, the combination of the variant *2G/2G* genotype and severe GERD frequency of more than once per week displayed the highest odds of EAC (AOR 55.3, 95% CI 7.3–417). For GERD duration (Table 4) where *1G/1G* and <1 year were used as the reference group for comparison, the *2G/2G* variant combined with a longstanding GERD duration of >15 years conferred the greatest risk of EAC (AOR 32.7, 95% CI 9.3–114). Of note, because every attempt at categorizing GERD frequency or duration resulted in at least one cell containing fewer than five individuals, this resulted in some estimates of EAC risk with wide CIs in the joint effects models.

Severity of cumulative GERD exposure

In light of small cell numbers in the joint effects models, we elected to analyze a composite definition for cumulative severity of GERD. For cases, 13% were classified as severe (frequency > once per week and duration >15 years), 53% were mild/moderate and 34% were rare/no GERD (frequency < once per month and duration <1 year). For controls, the corresponding proportions were 4, 18 and 78%, respectively. Interaction analyses found a strong interaction (global Wald $p = 0.002$) between cumulative GERD severity and *MMP1* polymorphism in an additive genetic model. In subset analyses, the AOR of each minor allele was 14.7 (95% CI 1.6–136) for individuals with severe GERD, whereas the AOR for mild/moderate GERD was 1.7 (95% CI 1.0–2.7). The AOR for rare/no GERD was 0.98 (95% CI 0.7–1.4).

Discussion

Chronic GERD and aberrant expression of *MMP* genes have been previously associated with an increased risk of developing EAC.[10,21] To our knowledge, however, the concurrent evaluation of GERD and polymorphisms of the *MMP* gene family together in their

association with EAC have not been conducted. In our previous investigation of *MMP* genes and EAC risk, the variant *1G/2G* and *2G/2G* genotypes of *MMP1* and the homozygous *5A/5A* variant of *MMP3* were individually found to be associated with an increased risk of EAC as well as Barrett's esophagus (a known outcome of chronic GERD) among cancer-free individuals.[21] A recent, separate analysis by our group showed that GERD can modify the associations between an epidermal growth factor polymorphism, *EGF A61G* and EAC risk through significant gene–GERD interactions.[26] To investigate the biological hypothesis that the *MMP* signaling pathway may also potentiate esophageal carcinogenesis more in the presence of GERD, we examined the relationships among *MMP1, MMP3* and *MMP12* polymorphisms, presence, frequency, duration and severity of GERD symptoms and EAC risk in a case–control analysis. We found statistically significant interactions between *MMP1 1G/2G* polymorphism and the presence, frequency, duration of GERD or cumulative GERD history. The borderline relationship between GERD and the *MMP3* homozygotes may be due to a minor association or may simply reflect linkage disequilibrium (LD) between *MMP1* and *MMP3* (LD $R^2 = 0.81$). We did not observe any interactions between *MMP12* polymorphisms and GERD. Of great potential clinical importance, patients with frequent, longstanding or cumulative GERD who carried the *MMP1* variant genotypes were found to have a much higher greater risk of EAC when compared to GERD-free individuals or those carrying the wild-type *1G/1G* genotype. Although the odds ratios were high, the CIs were wide as there were small numbers of patients fitting the most frequent, longstanding and severe GERD categories. Nonetheless, although a precise quantification of this risk is not possible, there was a clear synergistic effect of both longstanding and frequent GERD and *MMP1* polymorphism on EAC risk.

The finding of an *MMP1*–GERD interaction is consistent with our prior understanding of the role of the *MMP* family in carcinogenesis. As a group, *MMP* genes are involved in apoptosis, angiogenesis, cellular adhesion and signaling and extracellular matrix and basement membrane degradation, which are important contributors to the malignant process. Genetic alterations in the *MMP* genes have already been linked to an increased risk of developing various malignancies, including nasopharyngeal, lung and colorectal cancers.[12-14] *MMP* genes have been postulated to contribute to the histological transformation of esophageal metaplasia to dysplasia and adenocarcinoma among patients with Barrett's esophagus.[19,20] Polymorphisms within members of the *MMP* family, including *MMP1* and *MMP3*, are also associated with higher Barrett's esophagus and EAC risk.[21]

Because only a minor subset of patients with polymorphisms, amplification or overexpression of *MMP* genes actually develops EAC, the potential significance of other parameters, such as gene–environmental interactions, needs to be considered to fully understand EAC carcinogenesis. In fact, the importance of several gene–environmental interactions has already been proposed in other cancers. In lung cancer development, for instance, gene–smoking interactions between various DNA repair gene (e.g., *ERCC1, ERCC2* and *XRCC1*) polymorphisms and cigarette exposure are shown to be an important contributor to carcinogenesis.[27-29] Moreover, we recently demonstrated a significant gene–environmental interaction between the *EGF A61G* gene and GERD, which enhanced the risk of EAC by more than 20-fold.[26] A similar relationship was also observed between polymorphisms of the vascular endothelial growth factor gene and cigarette smoking with EAC.[30] To our knowledge, this report is the first to describe a strong and consistent interaction between frequency, duration, severity or presence of GERD and the *MMP1* polymorphism in their relationship with EAC risk.

The precise mechanisms underlying the observed gene–GERD interaction in EAC risk are likely complex. Our findings suggest that GERD frequency potentiates the gene–

environment interaction to a greater extent than GERD duration, leading to a relatively more dramatic increase in EAC risk. The reasons for this observed difference are unclear and may not even be a true finding. As the marginal OR for GERD duration is greater than GERD frequency (Tables 3 and 4), our power to detect interactions with GERD frequency is automatically greater. Although the joint effects models were based on small numbers of patients in some cells, categorization of individuals into severe *vs.* mild-to-moderate cumulative GERD exposure also confirmed such relationships. Further prospective studies are necessary to confirm these relationships with GERD frequency, duration or cumulative severity.

There are several limitations to the findings of our study. First, it was not possible to correlate *MMP* gene amplification or expression levels with the different polymorphism results because esophageal cancer tissues from the cases were not available for study. Second, GERD symptoms were collected based on patient self-report only and were not validated by either medical record review or objective confirmatory tests such as 24-hr pH monitoring. Information regarding the use of gastric acid suppression and antireflux medications, either prescribed or over the counter, was also unavailable but should be considered in future studies. Third, 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 these were either missing or not collected extensively during the interviews with study participants. However, these unmeasured confounding factors are unlikely to influence the results to any significant extent, considering the strong relationship observed between GERD and *MMP1* polymorphism with EAC risk.[31] Finally, results from the joint effects models should be viewed solely as hypothesis generating and must be interpreted cautiously because these estimates were based on small sample numbers (with resultant wide CIs). Our study cohort also consisted primarily of Caucasians from two affiliated hospitals in a single geographical area and therefore cannot be readily generalized to areas with very different demographic characteristics.

In summary, our study represents the first report in humans that the *MMP1 2G* variant allele is associated with EAC risk in individuals with a history of GERD but not in GERD-free individuals. Specifically, the homozygous *2G/2G* variant genotype is associated with the greatest risk of EAC, especially among those with either severe, frequency or longstanding GERD. Our findings suggest that the combination of *MMP1* genotyping and GERD symptoms may potentially be used in the future as an effective strategy for identifying a subset of individuals with a greater risk of developing EAC and who possibly may benefit more from aggressive GERD screening. These findings also raise the possibility of *MMP* as a potential therapeutic target in the management of EAC, but this hypothesis requires further investigation. Although our results carry the potential to inform the diagnosis and management of EAC, prospective studies are warranted to validate the results before we should modify our current practice. Such studies will also serve to enhance our understanding of the precise molecular mechanisms governing the *MMP1*–GERD interaction with EAC risk.

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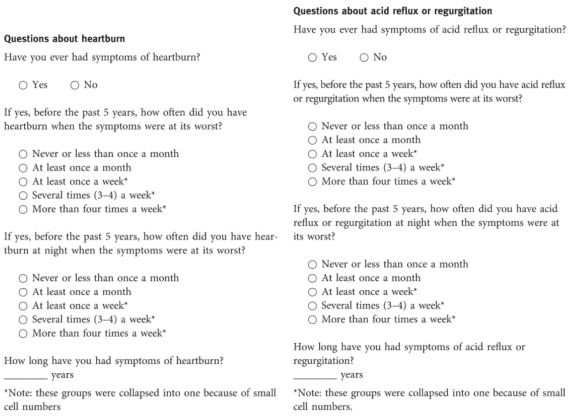
Appendix



Questions about heartburn

Have you ever had symptoms of heartburn?

○ Yes ○ No

If yes, before 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, before 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

*Note: these groups were collapsed into one because of small cell numbers

Questions about acid reflux or regurgitation

Have you ever had symptoms of acid reflux or regurgitation?

○ Yes ○ No

If yes, before 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, before 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

*Note: these groups were collapsed into one because of small cell numbers.

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Table 1

Clinical characteristics of esophageal adenocarcinoma cases and controls

Clinical parameter	Cases (total *n* = 309)	Controls (total *n* = 279)	*p* value comparing cases *vs.* controls
Gender			
Male	89%	87%	0.48**
Female	11%	13%	
Median age (range)	64.0 (55–70)	62.9 (55–72)	0.96***
Median BMI[1] (range)	23.1 (15–37)	22.6 (14–36)	0.01***
Smoking status			
Never smoker	20%	31%	0.01**
Ex-smoker	55%	50%	
Current smoker	25%	19%	
Median pack-years of ever smokers (range)	34.0 (15–55)	30.0 (13–52)	0.37***
Caucasian race	98%	98%	0.54**
Disease stage at diagnosis			
I	8%	–	
II	40%	–	
III	25%	–	
IV	27%	–	
MMP1 1G/2G			
1G/1G	25%	34%	0.01**
1G/2G	48%	48%	
2G/2G	27%	18%	
MMP3 6A/5A			
6A/6A	19%	23%	0.08**
6A/5A	54%	57%	
5A/5A	27%	20%	
MMP12–82A/G			
A/A	77%	81%	0.12**
A/G	21%	18%	
G/G	2%	1%	
MMP12 1082A/G			
A/A	88%	89%	0.88**
A/G	11%	10%	
G/G	1%	1%	
GERD frequency			
<1 month (GERD-free)	51%	78%	<0.001**

Clinical parameter	Cases (total *n* = 309)	Controls (total *n* = 279)	*p* value comparing cases *vs.* controls
≥1/month and ≤1/week	27%	14%	
>1/week	22%	8%	
GERD duration			
<1 year	34%	78%	<0.001**
≥1 and ≤15 years	32%	12%	
>15 years	34%	10%	
GERD cumulative severity [2]			
Rare to no GERD	34%	78%	<0.001**
Mild to moderate	53%	18%	
Severe	13%	4%	
Alcohol history [3]			
Yes	94%	91%	0.33**
No	6%	9%	

[1] Median BMI when patients were in their third decade of life.

[2] Severe GERD = duration > 15 years and frequency > once per week. Rare/no GERD = duration < 1 year and frequency < once per month. Mild–moderate GERD = all others.

[3] Alcohol history was only available for 246 cases and 67 controls.

** Fisher's exact test.

*** Wilcoxon rank sum test.

Table 2

Crude and adjusted odds ratios (ORs) for the risk of esophageal adenocarcinoma by *MMP1* polymorphism in the overall cohort and in GERD vs. GERD-free subsets

		Crude analysis			
		1G/2G vs. 1G/1G		*2G/2G vs. 1G/1G*	
Clinical parameter	**Total number of cases/controls**[1]	**OR (95% CI)**	***p* value**	**OR (95% CI)**	***p* value**
Overall	309/279	1.41 (0.9–2.1)	0.079	2.05 (1.3–3.3)	0.002
GERD subset	150/62	2.43 (1.2–5.0)	0.016	10.29 (3.9–27.3)	<0.001
GERD-free subset	159/217	1.06 (0.7–1.7)	0.804	0.57 (0.3–1.1)	0.085
		Adjusted analysis [2]			
		1G/2G vs. 1G/1G		*2G/2G vs. 1G/1G*	
		OR (95% CI)	***p* value**	**OR (95% CI)**	***p* value**
Overall	309/279	1.39 (0.9–2.1)	0.097	2.11 (1.3–3.4)	0.002
GERD subset	150/62	2.54 (1.2–5.3)	0.014	10.87 (4.0–29.2)	<0.001
GERD-free subset	159/217	0.99 (0.6–1.6)	0.982	0.55 (0.3-1.1)	0.078

[1] [*N*/*n*], where *N* = number of cases and *n* = number of controls. Overall: *2G/2G* [77/96]; *1G/2G* [150/133]; *1G/1G* [82/50]; total [309/279]. GERD subset: *2G/2G* [21/24]; *1G/2G* [66/31]; *1G/1G* [63/7]; total [150/62]. GERD-free subset: *2G/2G* [56/72]; *1G/2G* [84/102]; *1G/1G* [19/43]; total [159/217].

[2] Adjusted for age, gender, smoking status, pack-years and adult BMI.

Table 3

Exploratory adjusted[1] odds ratios (ORs) for the joint effects of GERD frequency and *MMP1* polymorphism on the risk of esophageal adenocarcinoma

	GERD frequency			
***MMP1* genotype**	**<1/month**	**≥1/month and ≤1/week**	**>1/week**	**Marginal OR**
1G/1G	1.00 [56/72][2]	1.56 (0.7–3.4) [20/15]	0.14 (0.0–1.2) [1/9]	1.00
1G/2G	1.01 (0.6–1.6) [84/102]	2.42 (1.1–5.1) [39/17]	3.21 (1.6–6.4) [27/14]	1.28 (0.9–1.9)
2G/2G	0.56 (0.3–1.1) [19/43]	5.12 (1.9–13.7) [22/6]	55.26 (7.3–417) [41/1]	1.58 (0.9–2.6)
Marginal OR	1.00	3.04 (1.9–4.8)	3.62 (2.1–6.2)	–

[1] Adjusted for age, gender, smoking status, pack-years and adult BMI.

[2] [*N*/*n*], where *N* = number of cases and *n* = number of controls.

Table 4

Exploratory adjusted[1] odds ratios (ORs) for the joint effects of GERD duration and *MMP1* polymorphism on the risk of esophageal adenocarcinoma

	GERD duration			
***MMP1* genotype**	**<1 year**	**≥1 and ≤15 years**	**>15 years**	**Marginal OR**
1G/1G	1.00 [33/72]	2.69 (1.2–6.2) [27/10]	5.21 (2.2–12.3) [17/14]	1.00
1G/2G	1.09 (0.6–1.9) [53/102]	5.18 (2.6–10.3) [49/20]	10.26 (4.5–23.2) [46/10]	1.35 (0.9–2.1)
2G/2G	0.90 (0.5–1.8) [18/43]	12.34 (3.9–39.2) [23/4]	32.65 (9.3–114) [41/3]	1.81 (1.1–3.1)
Marginal OR	1.00	5.86 (3.7–9.4)	8.15 (4.9–13.4)	–

[1] Adjusted for age, gender, smoking status, pack-years and adult BMI.