

Genetic Variations in MicroRNA-Related Genes Are Novel Susceptibility Loci for Esophageal Cancer Risk

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Abstract

MicroRNAs (miRNA) can act as oncogenes or tumor suppressors and modulate the expression of approximately one third of all human genes. To test the hypothesis that adverse alleles in miRNA-related genes may increase the risk for esophageal cancer, we assessed the associations between esophageal cancer risk and 41 potentially functional single nucleotide polymorphisms (SNP) in 26 miRNA-related genes in a case-control study of 346 Caucasian esophageal cancer patients (85.5% with esophageal adenocarcinoma) and 346 frequency-matched (age, gender, and ethnicity) controls. Seven SNPs were significantly associated with esophageal cancer risk. The most notable finding was that the SNP rs6505162, which is located in the pre-*mir423* region, was associated with a per-allele odds ratio of 0.64 [95% confidence interval (95% CI), 0.51-0.80; *P* for trend < 0.0001]. This association remained significant after we corrected for multiple comparisons. A common haplotype of the *GEMIN4* gene was associated with a significantly reduced risk of esophageal cancer (odds ratio, 0.65; 95% CI, 0.42-0.99). We did a combined unfavorable genotype analysis to further evaluate the cumulative effects of the promising (risk associated) SNPs. In comparison with the low-risk group (fewer than three unfavorable genotypes), the medium-risk group (three unfavorable genotypes) had a 2.00-fold (95% CI, 1.31-3.08) increased risk and the high-risk group (more than three unfavorable genotypes) had a 3.14-fold (95% CI, 2.03-4.85) increased risk (*P* for trend < 0.0001). Results for the risk of esophageal adenocarcinoma were similar to the overall risk results. The present study provides the first evidence that miRNAs may affect esophageal cancer risk in general and that specific genetic variants in miRNA-related genes may affect esophageal cancer risk individually and jointly.

Esophageal cancer ranks sixth in cancer-related deaths worldwide with an increasing incidence rate (1, 2). It is estimated that there will be 16,470 new cases and 14,280 deaths in the United States in 2008 (3). The majority of the esophageal cancer patients are diagnosed at advanced stage with poor prognosis and the overall 5-year survival rate is 16% in the United States (3), highlighting the importance of targeted prevention and early detection in the control of this disease. Major risk factors for esophageal squamous cell cancer are tobacco smoking and alcohol consumption (3, 4), whereas reflux disease is the most common risk factor for esophageal adenocarcinoma. The distinct risks exhibited by individuals exposed to similar

known risk factors implied that genetic predisposition might play an important role in esophageal cancer etiology (2, 5).

MicroRNAs (miRNA) are a class of noncoding RNA molecules with ~22 nucleotides in length. A large number of miRNA genes (~1,000) were predicted to exist in the human genome, accounting for 1% to 5% of all predicted human genes (6). To date, there are 678 human miRNAs deposited in the miRBase miRNA registry (7). miRNAs play important roles in the etiology of many human diseases through post-transcriptionally regulating the expression of approximately one third of all human genes (8, 9). The biogenesis of miRNAs is a complex process involving multiple proteins and RNAs (10). Large primary precursors of miRNAs (pri-miRNA) are first transcribed mainly by RNA polymerase II. Nuclear cleavage of the pri-miRNAs by the microprocessor complex, which contains the DROSHA RNase, a member of the RNaseIII family, and DGCR8, a double-stranded RNA-binding protein, produces a stem-loop intermediate miRNA precursor (pre-miRNA). After the transportation from nucleus to cytoplasm via RAN GTPase and exportin 5 (XPO5), pre-miRNAs are further processed to produce the mature miRNAs through another round of RNase cleavage at both ends by DICER complex, including DICER, GEMIN3, GEMIN4, AGO1, and AGO2. The resultant mature miRNAs are capable of negatively regulating the expression level of multiple genes

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through binding to the 3' untranslated region of the target genes at the posttranscriptional level (10–12).

miRNA expression profiles have been frequently reported to be correlated with the etiology, classification, progression, and prognosis of multiple human cancers, including esophageal cancer (8, 13–17). However, whether genetic variants of miRNA-related genes have an influence on esophageal cancer risk has largely remained unknown. We recently published the first study showing that single nucleotide polymorphisms (SNP) in miRNA biogenesis genes and miRNA genes were associated with the risk of bladder cancer individually and jointly (18). In the current study, we hypothesized that these genetic variants might also modulate the risk of esophageal cancer. To test this hypothesis, we selected 41 potentially functional polymorphisms, including 24 SNPs in 11 miRNA processing genes and 7 SNPs in 7 pre-miRNA genes along with 10 SNPs in 8 pri-miRNA genes, and evaluated their individual and joint associations with esophageal cancer in a case-control study. To our knowledge, this is the first study that systematically evaluates the effects of genetic polymorphisms in miRNA-related genes on the risk of esophageal cancer.

Materials and Methods

Study population

Newly diagnosed and histologically confirmed esophageal cancer patients were recruited from The University of Texas M. D. Anderson Cancer Center (Houston, TX). There was no restriction on recruitment criteria for age, sex, and ethnicity. Controls were healthy individuals without a previous history of cancer (except nonmelanoma skin cancer). They were recruited from a large pool of individuals seeking routine health checkups or addressing health concerns at the Kelsey-Seybold Clinic, the largest private multispecialty physician group in the Houston metropolitan area. Control subjects were frequency matched to cases by age (± 5 years), sex, and ethnicity. In this article, we only reported data from the Caucasian population due to the insufficient number of minority populations.

Epidemiologic data

All study participants provided written informed consent and were interviewed by trained M. D. Anderson staff interviewers to gather comprehensive epidemiology data, including a 45-min standardized risk factor questionnaire and one 45-min food frequency questionnaire. After completion of the interview, a 40-mL blood sample was drawn from each individual and sent to the laboratory for immediate molecular analyses. This study was conducted in accordance with the institutional review boards of M. D. Anderson and Kelsey-Seybold Clinic.

SNP selection and genotyping

Genomic DNA was isolated from peripheral blood using QIAamp DNA extraction kit (Qiagen) according to the manufacturer's protocol. Detailed information for gene and SNP selection was described by Yang et al. (18). In short, we extensively searched the database of the International HapMap Project,⁴ dbSNP,⁵ and miRNA registry⁶ to identify potentially functional polymorphisms that had minor allele frequency of >0.01 in Caucasians for all the SNPs and were located in functional regions, including exons, promoters (within 2 kb of the gene), or untranslated regions for miRNA biogenesis pathway SNPs.

Only one SNP was selected in the same haplotype block (defined as $r^2 = 0.8$). Genotyping was done using the SNPlex Genotyping System (Applied Biosystems). The SNPlex assay provides high-throughput genotyping and robust SNP detection with a high concordance rate. Briefly, we submitted a list of candidate SNPs that met our selection criteria to Applied Biosystems for evaluation and design of a pool of SNP-specific probes. Fragmented genomic DNA (at 99°C for 10 min) was hybridized to the allele-specified oligonucleotide probes and locus-specific oligonucleotide probes for oligonucleotide ligation reaction. After purification to remove unligated probes, linkers, and genomic DNAs, purified ligation products were amplified by PCR using a pair of universal primers. The resulting amplicons were bound on streptavidin-coated plates and nonbiotinylated strands were removed. The hybridization of the single-stranded PCR amplicon and a set of fluorescently labeled, mobility-modified ZipChute probes enabled the analysis of genotype information by Applied Biosystems 3730 DNA Analyzer. Genotype calls were automatically made by GeneMapper software (19).

Statistical analysis

The differences in patient characteristics and genotypes from each SNP between cases and controls were assessed by Pearson's χ^2 or Fisher's exact tests. Student's *t* test was used to examine any significant differences between cases and controls for continuous variables, such as age and pack-years. The χ^2 test for Hardy-Weinberg equilibrium was applied to each SNP among controls. For each SNP, we did unconditional multivariate logistic regression to compute odds ratio (OR) and 95% confidence interval (95% CI) adjusting for age, sex, and smoking status, where appropriate. We tested three different genetic models, including dominant model (comparing homozygous wild-type genotype with variant allele-carrying genotypes), recessive model (comparing wild-type allele-carrying genotypes with homozygous variant genotype), and additive model (*P* for trend). The best-fitting model among the three models was the one with the smallest *P* value. If the genotype counts for the homozygous variant genotype were less than five in both cases and controls, we only considered the dominant model that had the highest statistical power. To examine whether the genetic effects of SNPs on esophageal cancer risk were modified by smoking and age, we did stratified analysis by smoking status and different age groups. An individual who smoked >100 cigarettes in his or her lifetime was defined to be an ever smoker. Ever smokers consisted of former smokers, current smokers, and recent quitters. Former smokers were those who had quit smoking at least 1 years before diagnosis (for cases) or enrollment into this study (for controls). Recent quitters were those who had quit within 1 years of diagnosis (for cases) or enrollment into this study (for controls). The median age in controls was used as the age cutoff point. We also tested interaction between stratification variables and genetic variants by adding a product term into the logistic regression model. Cumulative effects of SNPs that had a borderline significant effect (*P* for the best-fitting model < 0.1) on esophageal cancer risk were assessed by counting the number of unfavorable genotypes in each subject. We categorized each subject into low-, medium-, and high-risk groups based on the tertile distribution of the number of unfavorable genotypes in controls. Haplotypes for each individual were inferred using the PHASE program (20, 21) and were included in the analysis when the probabilities of certainty were at least 95%. ORs and 95% CI for each haplotype were estimated using unconditional logistic regression adjusting for age, sex, and smoking status. All *P* values reported were two sided. Stata 8.0 software package (Stata Co.) was used to conduct the above analyses. Given the number of SNPs investigated, we applied the Benjamini-Hochberg method to address the multiple comparison issue. The Benjamini-Hochberg method controlled the false discovery rate (FDR), which is defined as the expected proportion of erroneous rejections of the true null hypothesis to the total number of rejected. We controlled FDR at 5% level and calculated FDR-adjusted *P* value at this level to assess the statistical significance of each SNP after correction for multiple comparisons.

⁴ <http://www.hapmap.org>

⁵ <http://www.ncbi.nlm.nih.gov/projects/SNP/>

⁶ <http://microrna.sanger.ac.uk>

Results

Characteristics of study subjects

A total of 346 white esophageal cancer patients and 346 frequency-matched controls were included in this study. As shown in Table 1, no significant difference was observed for cases (63.30 ± 11.00 years) and controls (63.20 ± 10.63 years) on age ($P = 0.90$), gender ($P = 1.00$), and alcohol drinking ($P = 0.96$). Cases were more likely to be current smokers (21.39%) than controls (8.38%; $P < 0.001$) and had higher body mass index (BMI; 29.74 ± 5.51) than controls (28.83 ± 5.16; $P = 0.041$). Among ever smokers, cases reported heavier cigarette consumption (40.35 pack-years) than controls (32.78 pack-years; $P = 0.01$). The histology of esophageal cancer cases was 296 adenocarcinoma (85.5%), 42 squamous cell carcinoma (12.1%), 6 other types (1.7%), and 2 unspecified (0.6%). Similar to the overall analysis, no significant difference was observed for esophageal adenocarcinoma patients and controls on age ($P = 0.50$), gender ($P = 0.25$), and alcohol drinking ($P = 0.72$). Esophageal adenocarcinoma patients had significantly higher cigarette consumption ($P = 0.03$) and BMI ($P = 0.005$) than controls.

Risk associated with individual SNPs

Table 2 listed the associations of individual SNPs with esophageal cancer risk. Among the 41 SNPs, 2 SNPs (rs10719 in *DROSHA* and rs17276588 in *Let7f-2*) showed significant departure from Hardy-Weinberg equilibrium and were excluded from further analyses (data not shown). Due to missing data on BMI and alcohol information on 98 cases, we did all our

analyses with or without BMI and alcohol adjustment. We found that the ORs and 95% CI were similar with or without BMI and alcohol adjustment. Furthermore, the seven significant SNPs were also significant when adjusting for BMI and alcohol. We also assessed the association of SNPs with risk of esophageal adenocarcinoma patients, and the results were similar to the analysis of all cases (Table 2). Therefore, the results presented below were risk estimates without BMI and alcohol adjustment for all the esophageal cancer patients. Overall, there were seven SNPs significantly associated with esophageal cancer risk. Among them, additive model was the best-fitting model for two SNPs (rs6505162 in *mir423* and rs5745925 in *mir631*), dominant model was the best-fitting model for one SNP (rs213210 in *mir219-1*), and recessive model was the best-fitting model for four SNPs (rs11614913 in *mir196a-2*, rs14035 in *RAN*, rs531564 in *mir124-1*, and rs11077 in *XPO5*). The most significant association was a SNP (rs6505162) in the pre-miRNA region of *mir423* that showed a significantly reduced esophageal cancer risk in an additive genetic model (per-allele OR, 0.64; P for trend < 0.0001 ; Table 2). This association remained significant after adjusting for multiple comparisons using FDR at 5% level. Compared with the homozygous wild-type genotype of rs6505162, individuals with the heterozygous and homozygous variant genotype had a significantly reduced esophageal cancer risk with ORs of 0.58 (95% CI, 0.41-0.82) and 0.43 (95% CI, 0.27-0.68), respectively (data not shown).

To examine whether the effects of genetic variations were modified by epidemiologic factors, we did stratified analyses

Table 1. Characteristics of esophageal cancer cases and controls

Variables	Control	Case		Esophageal adenocarcinoma	
	n (%)	n (%)	P*	n (%)	P†
Age, mean (SD)	63.20 (10.63)	63.30 (11.00)	0.905	62.63 (10.41)	0.496
Pack-years, mean (SD)	32.78 (30.02)	40.35 (32.36)	0.014	39.49 (30.78)	0.030
BMI [‡] , mean (SD)	28.83 (5.16)	29.74 (5.51)	0.041	30.12 (5.53)	0.005
Sex					
Male	306 (88.44)	306 (88.44)		270 (91.22)	
Female	40 (11.56)	40 (11.56)	1.000	26 (8.78)	0.248
Total	346	346		296	
Smoking status					
Never	154 (44.51)	78 (22.54)		67 (22.64)	
Ever	192 (55.49)	268 (77.46)	<0.001	229 (77.36)	<0.001
Former	163 (47.11)	194 (56.07)		163 (55.07)	
Current and RQ	29 (8.38)	74 (21.39)	<0.001	66 (22.30)	<0.001
Total	346	346		296	
Drink alcohol [§]					
Yes	308 (90.86)	225 (90.73)		200 (91.74)	
No	31 (9.14)	23 (9.27)	0.957	18 (8.26)	0.718
Total	339	248		218	

Abbreviation: RQ, recent quitter.

* P value for testing differences between controls and all the esophageal cancer cases.

† P value for testing differences between controls and esophageal adenocarcinoma cases.

‡BMI is calculated using the usual weight over the last 3 years.

§Missing usual weight and alcohol for 98 cases.

Table 2. Genetic polymorphisms in miRNA-related SNPs and esophageal cancer risk

Gene	SNP	Alleles*		MAF		All cases				Esophageal adenocarcinoma			
						Best-fitting genetic model		OR (95% CI) [†]	P	OR (95% CI) [‡]	P	OR (95% CI) [‡]	P
						Case Control Model	OR (95% CI) [‡]						
Biogenesis pathway													
<i>DROSHA</i>	rs6877842	G/C	0.19	0.17	DOM	1.23 (0.88–1.71)	0.228	1.25 (0.87–1.80)	0.225	1.23 (0.87–1.74)	0.235	1.29 (0.88–1.88)	0.190
<i>DGCR8</i>	rs417309	G/A	0.09	0.07	DOM	1.24 (0.81–1.90)	0.316	0.89 (0.55–1.46)	0.647	1.30 (0.84–2.00)	0.242	0.99 (0.60–1.62)	0.964
	rs3757	G/A	0.26	0.24	DOM	1.22 (0.89–1.66)	0.217	1.32 (0.94–1.86)	0.115	1.19 (0.86–1.65)	0.289	1.22 (0.85–1.74)	0.273
	rs1640299	G/T	0.5	0.47	DOM	1.30 (0.90–1.87)	0.163	1.24 (0.83–1.84)	0.296	1.20 (0.82–1.75)	0.343	1.12 (0.74–1.68)	0.603
<i>XPO5</i>	rs11077	A/C	0.4	0.4	REC	1.58 (1.03–2.45)	0.038	1.84 (1.16–2.93)	0.010	1.47 (0.93–2.32)	0.101	1.61 (0.98–2.62)	0.059
<i>RAN</i>	rs14035	C/T	0.34	0.31	REC	1.99 (1.17–3.38)	0.011	1.93 (1.09–3.40)	0.023	2.14 (1.24–3.71)	0.006	1.97 (1.09–3.54)	0.024
<i>DICER</i>	rs3742330	A/G	0.1	0.08	DOM	1.25 (0.82–1.90)	0.302	1.43 (0.91–2.23)	0.117	1.29 (0.84–1.98)	0.251	1.46 (0.92–2.31)	0.108
	rs13078	T/A	0.19	0.21	REC	0.59 (0.27–1.31)	0.197	0.51 (0.20–1.29)	0.158	0.63 (0.28–1.43)	0.268	0.48 (0.18–1.26)	0.137
<i>TRBP</i>	rs784567	C/T	0.47	0.49	DOM	1.09 (0.76–1.56)	0.638	0.94 (0.63–1.38)	0.739	1.11 (0.76–1.61)	0.598	0.95 (0.64–1.43)	0.821
<i>AGO1</i>	rs636832	G/A	0.08	0.09	DOM	0.89 (0.58–1.37)	0.593	0.79 (0.49–1.28)	0.331	0.90 (0.57–1.42)	0.656	0.85 (0.51–1.40)	0.517
	rs595961	A/G	0.12	0.15	ADD	0.82 (0.60–1.14)	0.239	0.80 (0.56–1.15)	0.235	0.87 (0.62–1.21)	0.403	0.85 (0.58–1.24)	0.398
<i>AGO2</i>	rs4961280	C/A	0.21	0.18	DOM	1.30 (0.94–1.81)	0.119	1.21 (0.85–1.74)	0.294	1.18 (0.84–1.67)	0.343	1.12 (0.77–1.64)	0.548
<i>GEMIN4</i>	rs910924	C/T	0.29	0.29	REC	1.14 (0.64–2.06)	0.655	0.97 (0.49–1.92)	0.938	1.17 (0.63–2.15)	0.625	1.09 (0.54–2.17)	0.811
	rs2740348	G/C	0.17	0.17	REC	0.67 (0.24–1.86)	0.445	0.72 (0.23–2.20)	0.559	0.67 (0.23–1.95)	0.459	0.83 (0.27–2.56)	0.749
	rs7813	T/C	0.43	0.45	ADD	0.91 (0.73–1.13)	0.376	0.87 (0.68–1.11)	0.259	0.91 (0.72–1.15)	0.417	0.89 (0.69–1.15)	0.364
	rs910925	G/C	0.44	0.46	ADD	0.91 (0.73–1.14)	0.418	0.89 (0.70–1.13)	0.337	0.92 (0.73–1.16)	0.469	0.91 (0.71–1.17)	0.468
	rs3744741	C/T	0.12	0.15	DOM	0.78 (0.55–1.12)	0.18	0.81 (0.54–1.20)	0.286	0.81 (0.56–1.18)	0.268	0.85 (0.56–1.28)	0.442
	rs1062923	T/C	0.18	0.18	REC	0.59 (0.24–1.46)	0.256	0.86 (0.35–2.12)	0.741	0.61 (0.24–1.55)	0.295	0.85 (0.33–2.18)	0.738
	rs4968104	T/A	0.26	0.28	DOM	0.88 (0.64–1.20)	0.409	0.85 (0.61–1.20)	0.360	0.88 (0.64–1.22)	0.439	0.87 (0.61–1.24)	0.431
<i>GEMIN3</i>	rs197414	C/A	0.16	0.12	ADD	1.32 (0.95–1.83)	0.093	1.45 (1.02–2.06)	0.041	1.32 (0.94–1.85)	0.113	1.41 (0.97–2.05)	0.068
	rs197388	T/A	0.21	0.2	DOM	1.10 (0.80–1.52)	0.552	1.17 (0.83–1.67)	0.371	1.09 (0.78–1.53)	0.609	1.18 (0.82–1.71)	0.365

Abbreviations: MAF, minor allele frequencies; REC, recessive; DOM, dominant; ADD, additive.

*Major/minor alleles.

[†]OR adjusted for age, sex, smoking status, BMI, and alcohol drinking for the corresponding best-fitting model using subjects with BMI and alcohol information.

[‡]OR adjusted for age, sex, and smoking status.

[§]Significant after adjusting for multiple comparisons using FDR at 5% level.

Table 2. Genetic polymorphisms in miRNA-related SNPs and esophageal cancer risk (Cont'd)

Gene	SNP	Alleles*		MAF		All cases				Esophageal adenocarcinoma			
						Best-fitting genetic model		OR (95% CI)†	P	OR (95% CI)‡		P	
						Case Control	Model			OR (95% CI)‡	P	OR (95% CI)‡	P
	rs197412	T/C	0.41	0.41	REC	1.19 (0.78–1.83)	0.423	1.38 (0.87–2.18)	0.170	1.05 (0.67–1.65)	0.838	1.21 (0.75–1.97)	0.434
<i>HIWI</i>	rs1106042	G/A	0.06	0.05	DOM	1.03 (0.62–1.71)	0.912	0.91 (0.51–1.63)	0.760	1.10 (0.65–1.84)	0.728	0.98 (0.54–1.77)	0.946
Pre-miRNA													
<i>mir146a</i>	rs2910164	G/C	0.23	0.24	DOM	0.87 (0.63–1.21)	0.409	0.85 (0.60–1.23)	0.394	0.91 (0.65–1.28)	0.593	0.86 (0.59–1.25)	0.435
<i>mir196a-2</i>	rs11614913	C/T	0.5	0.43	REC	1.73 (1.16–2.56)	0.0066	1.76 (1.15–2.70)	0.0089	1.71 (1.13–2.57)	0.011	1.78 (1.14–2.78)	0.011
<i>mir423</i>	rs6505162	C/A	0.32	0.44	ADD	0.64 (0.51–0.80)	<0.0001 [§]	0.57 (0.44–0.73)	<0.0001	0.60 (0.48–0.77)	<0.0001	0.56 (0.43–0.73)	<0.0001
<i>mir492</i>	rs2289030	C/G	0.07	0.06	DOM	1.21 (0.75–1.95)	0.423	1.11 (0.66–1.89)	0.688	1.35 (0.83–2.21)	0.225	1.25 (0.73–2.13)	0.421
<i>mir604</i>	rs2368392	C/T	0.27	0.29	DOM	0.86 (0.63–1.19)	0.362	0.98 (0.69–1.39)	0.912	0.88 (0.63–1.23)	0.449	0.98 (0.68–1.41)	0.911
<i>mir608</i>	rs4919510	C/G	0.2	0.2	REC	1.41 (0.61–3.27)	0.428	1.52 (0.62–3.77)	0.362	1.38 (0.56–3.36)	0.483	1.47 (0.56–3.86)	0.428
<i>mir631</i>	rs5745925	CT/–	0.11	0.07	ADD	1.58 (1.07–2.34)	0.022	1.57 (1.03–2.41)	0.037	1.68 (1.12–2.52)	0.013	1.59 (1.02–2.49)	0.040
Pri-miRNA													
<i>mir26a-1</i>	rs7372209	C/T	0.31	0.28	ADD	1.25 (0.98–1.59)	0.075	1.35 (1.04–1.76)	0.025	1.28 (0.99–1.65)	0.056	1.33 (1.01–1.74)	0.045
<i>mir30a</i>	rs1358379	A/G	0.05	0.05	DOM	1.00 (0.58–1.73)	0.993	1.23 (0.70–2.18)	0.474	1.12 (0.64–1.95)	0.697	1.30 (0.72–2.33)	0.387
<i>mir30c-1</i>	rs16827546	C/T	0.04	0.03	DOM	1.80 (0.96–3.36)	0.066	1.43 (0.69–2.97)	0.332	1.94 (1.02–3.70)	0.044	1.53 (0.73–3.22)	0.262
<i>mir100</i>	rs1834306	C/T	0.44	0.43	DOM	1.14 (0.82–1.59)	0.445	1.14 (0.79–1.65)	0.484	1.12 (0.79–1.59)	0.526	1.07 (0.73–1.57)	0.718
<i>mir124-1</i>	rs531564	C/G	0.15	0.11	REC	11.27 (1.43–88.62)	0.021	8.79 (1.06–73.17)	0.044	13.29 (1.69–104.35)	0.014	9.57 (1.16–79.13)	0.036
<i>mir219-1</i>	rs107822	G/A	0.22	0.24	REC	0.48 (0.20–1.15)	0.0996	0.50 (0.19–1.32)	0.165	0.48 (0.19–1.21)	0.118	0.56 (0.21–1.48)	0.242
	rs213210	T/C	0.08	0.06	DOM	1.75 (1.10–2.80)	0.019	1.66 (1.00–2.74)	0.050	1.61 (0.98–2.64)	0.058	1.54 (0.90–2.61)	0.113
<i>mir373</i>	rs12983273	C/T	0.15	0.15	REC	1.61 (0.49–5.28)	0.431	1.69 (0.47–6.09)	0.422	1.98 (0.60–6.47)	0.260	1.99 (0.55–7.19)	0.291
	rs10425222	C/A	0.03	0.03	DOM	0.93 (0.46–1.88)	0.835	0.89 (0.41–1.92)	0.757	0.81 (0.38–1.73)	0.590	0.87 (0.39–1.94)	0.730

based on gender, smoking, and age. The sample size is too small to do separate analysis for female subjects. The protective effect of *mir423* rs6505162 remained significant among male (P for trend < 0.0001); for subjects ≤ 64 years old (P for trend < 0.001), but not for subjects >64 years old; and for both never smokers (P for trend = 0.001) and ever smokers (P for trend = 0.013; Table 3). The protective effect of *mir423* rs6505162 was over twice among subjects ≤ 64 years old than that among subjects >64 years old. *mir196a-2* rs11614913 showed approximately twice the risk of esophageal cancer among never smokers than ever smokers (OR, 2.52 and $P = 0.006$ for never

smokers; OR, 1.41 and $P = 0.166$ for ever smokers). The interactions between *mir423* rs6505162 and age group were significant ($P = 0.019$), whereas the interactions between *mir196a-2* rs11614913 and smoking did not reach statistical significance ($P = 0.18$).

Cumulative effect of selected SNPs on esophageal cancer risk

To further assess the cumulative effects of miRNA-related genetic variants on esophageal cancer risk, we did an unfavorable genotype analysis using the 11 SNPs that showed at least a

borderline significant association with esophageal cancer risk (P for best-fitting model < 0.10). They were rs11077 (MM), rs14035 (MM), rs197414 (WW+WM), rs11614913 (MM), rs6505162 (WW), rs5745925 (WW+WM), rs7372209 (WW+WM), rs16827546 (WW+WM), rs531564 (MM), rs107822 (WW+WM), and rs213210 (WW+WM), where WW is homozygous wild-types, WM is heterozygotes, and MM is homozygous variants. We found that, compared with the low-risk group with two or less unfavorable genotypes, the OR was 2.00 (95% CI, 1.31-3.08) for the median-risk group with three unfavorable genotypes and 3.14 (95% CI, 2.03-4.85) for the high-risk group with four or more unfavorable genotypes (Table 4). In addition, we also observed a significantly increased risk of esophageal cancer with increasing number of unfavorable genotypes (per-unfavorable genotype OR, 1.56; P for trend < 0.0001 ; Table 4). In histology-specific analysis, similar cumulative effects were obtained for esophageal adenocarcinoma.

Haplotype analysis

We conducted haplotype analyses for the miRNA-related genes with at least two SNPs in this study, including *DGCR8*, *DICER*, *AGO1*, *GEMIN4*, *GEMIN3*, *mir219-1*, and *mir373* (Table 5). The only common haplotype showing significant association with esophageal cancer risk was a haplotype of the *GEMIN4* gene (WWWWMMWW; W: wild-type allele, M: variant allele), in the order of rs910924, rs2740348, rs7813, rs910925, rs3744741, rs1062923, and rs4968104. Compared with the most common *GEMIN4* haplotype WWWWWWW, this haplotype was associated with a reduced esophageal cancer risk with an OR of 0.65 (95% CI, 0.42-0.99).

Discussion

In this study, we systematically evaluated the individual as well as joint effects of 41 genetic variants in 26 miRNA-related genes on esophageal cancer risk. The most notable finding was a SNP in the pre-*mir423* region that was associated with reduced esophageal cancer risk and with a significant gene-dosage effect. We further showed the haplotypic and cumulative effects of multiple genetic variants on risk prediction, highlighting the importance of using a pathway-based polygenic approach in genetic association studies of complex human diseases such as cancer.

The roles played by genetic variants of the miRNA-harboring regions in tumorigenesis have only been evaluated in a few studies with mixed results. It was reported that polymorphisms in pre-miRNA regions are scarce and unlikely to be physiologically functional (22–24). However, it has also been observed that sequence variations in both mature and precursor miRNAs may functionally affect the biogenesis of mature miRNAs (25, 26). Consistent with the latter observation, we recently reported that genetic variants in both miRNA processing pathway genes and miRNA genes might affect bladder cancer susceptibility both individually and jointly (18). In the current study, we found that these variants were also associated with the development of esophageal cancer. Among the 11 SNPs that showed at least borderline significance with esophageal cancer risk, 4 of them, including *GEMIN3*, *mir423*, *mir26a-1*, and *mir124-1*, were also associated with at least a borderline significant risk of bladder cancer. *GEMIN3* and *mir124-1* had the association in the same directions for both cancers, whereas *mir423* and *mir26a-1* exhibited

the association in the opposite directions. Several studies have reported that specific miRNA expression signatures could be used as predictors of esophageal cancer diagnosis and prognosis (15, 16). Moreover, miRNA processing genes have also been associated with the development and survival of multiple cancers, including esophageal cancer (27, 28). Nonetheless, it remained to be determined whether the genetic variants identified in our studies modulate esophageal cancer risk through their influences on the functions or expressions of their host genes with further functional assessment through *in vitro* and *in vivo* experiments.

Three of the seven SNPs identified in the scarce pre-miRNA region were associated with a significantly altered risk of esophageal cancer. The polymorphism in the pre-*mir423* remained significant after adjusting for multiple comparisons using FDR at 5% level, suggesting that this result was unlikely due to chance. We also observed significant interactions between *mir423* and age. *mir423* was reported to be expressed in human leukemia cell lines and was significantly up-regulated after the induction of a potent tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (29). Moreover, the expression of *mir423* has also been reported to be significantly altered in several other common human diseases, such as heart disease and Alzheimer's disease (30, 31). In addition, the *mir423* SNP was also associated with a borderline significantly increased risk of bladder cancer in the opposite direction to that of esophageal cancer, suggesting a potential role in cancer type-specific risk modulation by *mir423* or this variant (18). A polymorphism in the pre-*mir196a-2* gene was associated with a 1.7-fold increased risk in our study. Consistently, this SNP was recently found to confer a reduced survival rate of patients with non-small cell lung cancer, probably due to an influence on the expression level of mature *mir196a-2* (32). We also found a polymorphism in the pre-*mir631* gene that was associated with a significantly increased esophageal cancer risk. *mir631* was first identified from human colorectal tissues, and as yet, there has not been any study evaluating the cancer implications (33). Additionally, several SNPs in the pre-miRNA regions were also found to exhibit at least a borderline significance. Most of their mature miRNAs showed a significantly different expression patterns between tumor and normal tissues (34–36). However, whether these SNPs have any functional effect on the expression of the mature miRNAs needs to be further assessed.

In this study, we also found two significant SNPs in the miRNA processing pathway genes that were associated with an increased esophageal cancer risk (one in *XPO5* and the other in *RAN*). Both SNPs are located in the 3' untranslated region and therefore may potentially influence the mRNA stability of their host genes. The *XPO5* SNP was also associated with an increased risk of renal cell carcinoma (37). The direct interactions between *XPO5* and *RAN* proteins are essential to the transportation of pre-miRNAs from nucleus to cytoplasm through the nuclear pore complex in a GTP-dependent manner (38). Knocking down *XPO5* expression using RNA interference led to decreased miRNA levels (38). This result was consistent with the observation that global reduction in miRNA expression and enhanced tumorigenesis resulted from reduced expression of other essential miRNA processing genes (28). Therefore, functional analyses of the two SNPs in *XPO5* or *RAN* using *in vitro* or *in vivo* experiments, such as luciferase

Table 3. Genetic polymorphisms in selected miRNA-related SNPs and esophageal cancer risk stratified by host characteristics

Gene, SNP	Male		Never smoker		Ever smoker		Subjects ≤ 64 y*		Subjects > 64 y	
	Case/control	OR (95% CI) [†]	Case/control	OR (95% CI) [‡]	Case/control	OR (95% CI) [‡]	Case/control	OR (95% CI) [§]	Case/control	OR (95% CI) [§]
<i>XPO5</i> , rs11077	300/295		75/150		265/184		181/171		159/163	
0+1	247/255	1	58/126	1	221/162	1	153/147	1	131/141	1
2	53/40	1.58 (0.99–2.51)	17/24	1.55 (0.77–3.12)	44/22	1.61 (0.92–2.82)	33/24	1.56 (0.86–2.83)	28/22	1.65 (0.87–3.11)
<i>P</i>		0.0541		0.2155		0.0925		0.1437		0.1252
<i>P</i> trend		0.5910		0.6860		0.2350		0.5250		0.0710
<i>RAN</i> , rs14035	305/302		78/151		267/190		184/177		161/164	
0+1	267/282	1	66/138	1	234/177	1	167/165	1	133/150	1
2	38/20	2.05 (1.14–3.67)	12/13	2.08 (0.87–4.97)	33/13	2.03 (1.03–3.99)	17/12	1.38 (0.62–3.07)	28/14	2.77 (1.35–5.69)
<i>P</i>		0.0162		0.0994		0.0406		0.4269		0.0054
<i>P</i> trend		0.1950		0.5640		0.2290		0.6850		0.1580
<i>mir196a-2</i> , rs11614913	272/298		70/151		237/187		163/173		144/165	
0+1	198/246	1	47/126	1	177/153	1	125/144	1	99/135	1
2	74/52	1.74 (1.14–2.64)	23/25	2.52 (1.30–4.88)	60/34	1.41 (0.87–2.28)	38/29	1.45 (0.82–2.56)	45/30	2.01 (1.16–3.50)
<i>P</i>		0.0096		0.0062		0.1660		0.2013		0.0135
<i>P</i> trend		0.0360		0.0240		0.1940		0.1840		0.0530
<i>mir423</i> , rs6505162	291/299		76/151		253/188		172/173		157/166	
0	143/92	1	39/44	1	120/67	1	89/51	1	70/60	1
1+2	148/207	0.47 (0.33–0.67)	37/107	0.39 (0.22–0.69)	133/121	0.61 (0.41–0.91)	83/122	0.36 (0.23–0.58)	87/106	0.78 (0.49–1.25)
<i>P</i>		<0.0001		0.0012		0.0145		<0.0001		0.3022
<i>P</i> trend		<0.0001		0.0010		0.0130		<0.0001		0.4910
<i>mir631</i> , rs5745925	302/292		77/148		265/184		183/168		159/164	
0	239/255	1	64/129	1	211/159	1	146/151	1	129/137	1
1+2	63/37	1.87 (1.18–2.95)	13/19	1.42 (0.66–3.07)	54/25	1.69 (1.00–2.85)	37/17	2.18 (1.15–4.14)	30/27	1.27 (0.70–2.32)
<i>P</i>		0.0073		0.3742		0.0481		0.0167		0.4271
<i>P</i> trend		0.0030		0.2590		0.0440		0.0200		0.2690
<i>mir124-1</i> , rs531564	302/304		78/152		264/192		181/177		161/167	
0+1	290/303	1	77/151	1	252/192	1	177/177	1	152/166	1
2	12/1	10.90 (1.38–86.09)	1/1	1.94 (0.12–31.61)	12/0	NA	4/0	NA	9/1	7.38 (0.89–61.34)
<i>P</i>		0.0235		0.6415		NA		NA		0.0644
<i>P</i> trend		0.1870		0.5910		NA		NA		0.3860
<i>mir219-1</i> , rs213210	300/304		76/153		262/191		179/178		159/166	
0	251/270	1	61/129	1	223/177	1	152/157	1	132/149	1
1+2	49/34	1.75 (1.07–2.86)	15/24	1.35 (0.66–2.76)	39/14	2.18 (1.14–4.18)	27/21	1.50 (0.79–2.85)	27/17	2.01 (1.00–4.02)
<i>P</i>		0.0255		0.4162		0.0182		0.2145		0.0491

*Cutoff point was the median age in controls.

[†]Adjusted for age and smoking status (never, former, current).[‡]Adjusted for age and gender.[§]Adjusted for age, gender, and smoking status (never, former, current).

Table 4. Cumulative effect analysis by the number of unfavorable genotypes from miRNA-related SNPs and esophageal cancer risk

Unfavorable genotypes	Case	Control	OR* (95% CI)	P
Low risk (0–2)	69	142	1 (reference)	
Medium risk (3)	86	86	2.00 (1.31–3.08)	0.001
High risk (4–7)	102	71	3.14 (2.03–4.85)	0
P for trend				0

NOTE: Unfavorable genotypes: rs11077 (MM), rs14035 (MM), rs197414 (WW+WM), rs11614913 (MM), rs6505162 (WW), rs5745925 (WW+WM), rs7372209 (WW+WM), rs16827546 (WW+WM), rs531564 (MM), rs107822 (WW+WM), and rs213210 (WW+WM), where WW is homozygous wild-types, WM is heterozygotes, and MM is homozygous variants.

*OR adjusted for age, sex, and smoking status.

assay or site-directed mutagenesis, would be necessary to further characterize the biological mechanisms underlying these observed associations.

To further assess the potential implications of the physiologic roles played by the miRNAs identified in our study, we generated a list of candidate transcripts targeted by each of these miRNAs using TargetScan, a computational method that searches the potential miRNA regulation targets (data not shown; refs. 9, 39, 40). There were 11 predicted targets for *mir423*. Two of them, *PABPC1* (41) and *FGFR2* (42), were shown to be associated with the etiology or prognosis of esophageal cancer. Takashima et al. (41) showed that down-regulation of *PABPC1* was associated with tumor progression, including increased tumor size, locally invasive tumors, and poor overall survival. Yoshino et al. (42) showed that overexpression of *FGFR2* was highly correlated with well-differentiated esophageal cancer. Six HOX genes, including *HOXA5*, *HOXA7*, *HOXA9*, *HOXB6*, *HOXB7*, and *HOXC8*, were among the predicted targets of *mir196a-2*. *HOXA7* and *HOXC8* have been shown *in vitro* as the targets of *mir196* (43). Furthermore, Chen et al. (44) showed that the expression levels of *HOXC7* and

Table 5. Haplotypes in miRNA-related genes and esophageal cancer risk

Gene	Haplotype	Case	Control	OR* (95% CI)	P
<i>DGCR8</i>	WWW	274	306	1 (reference)	
	WMM	179	164	1.17 (0.88–1.56)	0.267
	WWM	162	153	1.12 (0.83–1.50)	0.463
	MWW	63	48	1.42 (0.94–2.16)	0.096
<i>DICER</i>	WW	487	477	1 (reference)	
	WM	113	126	0.87 (0.65–1.16)	0.342
	MW	46	43	1.01 (0.65–1.57)	0.964
<i>AGO1</i>	WW	598	584	1 (reference)	
	MM	52	60	0.95 (0.64–1.43)	0.815
	WM	26	42	0.63 (0.38–1.05)	0.075
<i>GEMIN4</i>	WWWWWWW	163	138	1 (reference)	
	WMMWWW	130	146	0.71 (0.50–1.01)	0.058
	WMMMWWW	98	103	0.79 (0.54–1.14)	0.21
	WWWWMW	57	62	0.74 (0.48–1.14)	0.17
	WWWMMW	58	74	0.65 (0.42–0.99)	0.044
	Other [†]	18	21	0.83 (0.44–1.60)	0.583
<i>GEMIN3</i>	WWW	402	397	1 (reference)	
	WWM	132	142	0.88 (0.66–1.17)	0.362
	MMM	98	78	1.16 (0.82–1.64)	0.398
	WMM	43	50	0.87 (0.55–1.38)	0.552
	Other [†]	11	5	2.82 (0.93–8.54)	0.067
<i>Pre-mir219-1</i>	WW	467	478	1 (reference)	
	MW	81	116	0.73 (0.53–1.02)	0.062
	MM	44	36	1.42 (0.87–2.34)	0.164
<i>Pre-mir373</i>	WW	557	562	1 (reference)	
	MW	90	93	0.99 (0.71–1.37)	0.953
	Other [†]	12	9	1.12 (0.45–2.80)	0.809

Abbreviations: M, mutant allele; W, wild-type allele.

*OR adjusted for age, sex, and smoking status.

[†]Haplotypes with frequency <1% were grouped together.

HOXA8 were associated with esophageal squamous cell carcinoma. *CDK6*, an oncogene in the cell cycle control pathway, was predicted as a potential target of *mir124-1*. In accordance, two microarray studies have confirmed the modulating effect of *mir124-1* on *CDK6* (45, 46). In addition, a borderline significantly increased risk of bladder cancer was also observed for the rare homozygous genotype of *mir124-1* (18). Taken together, these exploratory analyses highly suggested that the miRNAs identified in our study might possess physiologic significance in the regulation of esophageal cancer development. Our next step will be to both characterize the biological effect by the significant SNPs on the expression or functions of their miRNAs and experimentally validate the promising genes that are potential targets of these miRNAs.

Because esophageal cancer is a common human malignancy that involves multiple genes and SNPs, the candidate gene approach that considers one gene/SNP at a time may not be able to detect the modest effect associated with each SNP. In this study, we took a pathway-based approach that evaluated the cumulative effect of multiple unfavorable genotypes. We categorized the study subjects into different risk groups based on the number of unfavorable genotypes. There was a significantly increased trend of esophageal cancer risk with increasing number of unfavorable genotypes. These findings highlighted the importance of taking a multi-genic approach in pathway-based association studies to identify signatures of genetic variations as predictors of cancer risk.

We also assessed the effects of common haplotypes of those genes with at least two SNPs included in our study on esophageal cancer risk. Only one common haplotype of the *GEMIN4* gene showed significant risk association. The only difference between this haplotype and the most common *GEMIN4* haplotype (that contained the wild-type allele in all the SNPs) was that this haplotype contained the variant allele of rs3744741. Because rs3744741 did not show a significant finding in the single SNP analysis, there may be potential interaction effects

between the several *GEMIN4* SNPs in the modulation of esophageal cancer risk. *GEMIN4* belongs to a large protein complex that processes pre-miRNAs in the final step of miRNA maturation. Several studies have shown the involvement of *GEMIN4* in miRNA processing through its interaction with other protein factors in a 15S ribonucleoprotein complex (47, 48). The rs3744741 SNP is a nonsynonymous SNP that leads to an Arg to Gln amino acid change in the exon 2 of *GEMIN4*. However, it remained to be answered whether this SNP directly influences the interaction between *GEMIN4* and other proteins.

Our study has several strengths. We systematically evaluated a panel of pathway-based novel SNPs in miRNA and miRNA biogenesis pathway genes. We restricted our analysis to Caucasians to reduce the possible effects of population stratification. We matched our controls to cases so as to eliminate the potential confounding effects of age and gender. We addressed the multiple comparison issue by using the FDR approach to minimize the probabilities of chance findings. Last, our study was strengthened by the unfavorable genotype and haplotype analyses we conducted to further assess the joint and interaction effects of the informative genetic variants we identified. We also note, however, that the haplotypes were constructed using functional rather than tagging SNPs and therefore might not reflect the accurate linkage disequilibrium pattern of the genes. Furthermore, future large-scale studies are necessary to confirm our present findings.

In conclusion, we have provided the first evidence on the potential roles of miRNA in the modulation of esophageal cancer risk. Our results suggest that certain polymorphisms in miRNA-related genes may affect the etiology of esophageal cancer individually, interactively, and jointly.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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