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ORIGINAL RESEARCH

Association of miRNA-499 rs3746444 A>G variants with adenocarcinoma of esophagogastric junction (AEG) risk and lymph node status

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Background: MicroRNAs (miRNAs) miRNA-499 rs3746444 A>G polymorphism may be complicated in the susceptibility to cancer. However, the correlation of this polymorphism with adenocarcinoma of esophagogastric junction (AEG) was unknown.

Patients and methods: A total of 1063 AEG patients and 1677 controls were included in this study to assess the association of miR-499 rs3746444 A>G with AEG risk. SNPscan™ genotyping assay was harnessed to obtain the genotypes of miRNA-499 rs3746444 A>G polymorphism.

Results: We identified that SNP miR-499 rs3746444 A>G increased the susceptibility of AEG (AG vs AA: adjusted OR=1.25, 95% CI=1.05–1.49, *P*=0.012 and AG/GG vs AA: adjusted OR=1.30, 95% CI=1.10–1.54, *P*=0.002). In a stratified analysis, we found that miR-499 rs3746444 A>G polymorphism had an increased susceptibility of AEG in several subgroups (male subgroup: AG vs AA: adjusted *P*=0.004 and AG/GG vs AA: adjusted *P*=0.002; female subgroup: GG vs AG/AA: adjusted *P*=0.046; <64 years subgroup: AG vs AA: adjusted *P*=0.006 and AG/GG vs AA: adjusted *P*=0.003; never smoking subgroup: AG vs AA: adjusted *P*=0.003 and AG/GG vs AA: adjusted *P*=0.001; and never drinking subgroup: AG vs AA: adjusted *P*=0.008 and AG/GG vs AA: adjusted *P*=0.002). The results of power calculation indicated that miR-499 rs3746444 A>G polymorphism increased the risk of AEG in overall comparison, male, <64 years, never smoking, and never drinking subgroups. Among the AEG cases, 625 patients accompanied by positive lymph node. However, the distribution of miRNA-499 rs3746444 A>G variants was no significant difference between different lymph node status.

Conclusion: Our findings indicate that miR-499 rs3746444 A>G polymorphism is significantly associated with AEG susceptibility. In the future, further exploration of this genetic factor in relation to AEG susceptibility with an adequate methodological quality is needed.

Keywords: miRNA-499, polymorphism, susceptibility, lymph node metastasis, adenocarcinoma of esophagogastric junction

Introduction

Recently, it is estimated that there will be 1,033,701 new gastric carcinoma (GC) cases and 782,685 GC-related deaths worldwide in 2018, ranking as the sixth most frequent malignancy and the second leading cause of cancer death.[1] The Siewert II and III adenocarcinoma of esophagogastric junction (AEG) is considered as a subtype of GC. However, AEG has a quite extraordinary clinicopathological characteristic and may be very different to common GC. The etiology of AEG is unclear. It is one of the human complex diseases, which may be caused by genetic predisposition and environmental factors.

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MicroRNAs (miRNAs) are a cohort of small non-coding RNA molecules, which contain about 22 nucleotides. It was found that miRNAs play important roles in RNA silencing and post-transcriptional regulation. MiRNAs take part in regulation of cell proliferation, differentiation, oncogenesis, apoptosis, and angiogenesis.[2–7] Alterations in the regulation of transcription may lead to the changes in miRNA expression in carcinogenesis.[8] Stojanovic et al reported that miRNAs expression profiles may consider as useful biomarkers of diagnosis in GC.[9]

Recently, a number of case–control studies were carried out to explore the relationship of miRNA-499 rs3746444 A>G variants with risk of GC.[10–15] Some previous studies reported that miRNA-499 rs3746444 A>G variants influenced the risk of GC and this polymorphism might be used as a potential biomarker for GC prediction.[11,14] The miRNA-499 rs3746444 A>G polymorphism was also associated with overall survival and progression-free survival among cases of neoadjuvant chemotherapy.[16] A meta-analysis indicated that miRNA-499 rs3746444 variants were risk factors for overall cancer development.[17,18] However, the correlation of miRNA-499 rs3746444 A>G variants with the susceptibility of AEG remains unclear. To shed some light on this issue, 2740 participants were included and analyzed the association between miRNA-499 rs3746444 A>G variants and the development of AEG.

Materials and methods

Subjects

The present case–control study was performed by cooperation among Fujian Medical University Union Hospital, the Affiliated Cancer Hospital of Fujian Medical University and the Affiliated People's Hospital of Jiangsu University. Two hundred and eighty AEG patients were consecutively enrolled between January 2014 and May 2016 from two Affiliated Hospitals of Fujian Medical University mentioned earlier. Seven hundred and eighty-three AEG cases were recruited from the Affiliated People's Hospital of Jiangsu University from January 2008 to November 2016 consecutively. In this study, all AEG cases were diagnosed as Siewert type II by gastroscope and operation. All AEG cases were pathologically confirmed. And 1677 healthy subjects without any cancer history served as controls, age, and gender matched. Before collecting blood samples, a written informed consent was obtained from each participant. This investigation protocol met with the Declaration of Helsinki and was approved by the ethics committee of Jiangsu University (No. K-20170050-Y). They were inquired by a questionnaire and face-to-face interview. The following information was collected: age, sex, alcohol consumption, and smoking history. The information of lymph node status was obtained from medical records. The TMN stage was determined by American Joint Committee on Cancer (AJCC, 7th edition). The related risk factors and clinical data are summarized in Table 1.

Selection of SNPs

MiRNA-499 rs3746444 A>G was selected according to some previous publications.[14,17,19,20] The corresponding information of miRNA-499 rs3746444 A>G SNP is presented in Table 2.

DNA extraction and genotyping

Blood was collected in EDTA test tube from 2740 participants. Details on blood draw, DNA extraction, and storage status were described in the previous study.[21] Genotyping was conducted at ABI 3730XL sequencer using a custom-designed SNPscan™ assay (Genesky Biotechologies Inc., Shanghai, China).[22] For quality control, the obtained variants of miRNA-499 rs3746444 A>G polymorphism were confirmed by genotyped in 4% randomly selected genomic DNA samples. And the results of the quality control were in accord with the first assays.

Statistical analysis

Age among cases and controls was expressed as the mean ±standard deviation (SD). Student's *t*-test was used to assess age difference between AEG patients and controls. Differences of the categorical variable (eg, age, gender, smoking, alcohol consumption, and the number of miR-499 rs3746444 A>G genotypes) between AEG patients and controls were determined by using χ^2-test. To calculate odds ratios (ORs) and their 95% confidence intervals (CIs), we used a multiple logistic regression model. In multivariate model, the confounding risk factors, including age (<64, ≥64), gender (male, female), smoking (ever, never), and alcohol consumption (ever, never) were used to adjust the risk to AEG. We also carried out stratified analyses to explore the relationship of miRNA-499 rs3746444 A>G polymorphism with AEG risk in different subgroups. In this study, the association of miRNA-499 rs3746444 A>G polymorphism with lymph node status in AEG patients was also assessed by a multiple logistic regression model. An online program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) was used to examine for deviation from Hardy–Weinberg equilibrium (HWE).[23] Statistical analyses were

Table 1 Distribution of selected demographic variables and risk factors in AEG cases and controls

Variable	AEG Cases (n=1063)		Controls (n=1677)		*P*[a]
	n	%	n	%	
Age (years)	64.19 (±8.63)		63.91 (±10.22)		0.451
Age (years)					0.165
<64	494	46.47	825	49.19	
≥64	569	53.53	852	50.81	
Sex					0.909
Male	759	71.40	1194	71.20	
Female	304	28.60	483	28.80	
Smoking status					**<0.001**
Never	773	72.72	1323	78.89	
Ever	290	27.28	354	21.11	
Alcohol use					**<0.001**
Never	908	85.42	1507	89.86	
Ever	155	14.58	170	10.14	
Lymph node status					
Positive	625	58.80			
Negative	438	41.20			
TMN stage					
I/II	305	28.69			
III/IV	758	71.31			

Notes: [a]Two-sided χ^2 test and Student *t*-test.
Abbreviation: TMN, tumor node metastasis.

Table 2 Primary information for miRNA-499 rs3746444 A>G polymorphism

Genotyped polymorphism	miR-499 rs3746444
Chromosome	20
NCBI Build 38	34990448
Region	nc transcript variant
MAF in our controls (n=1677)	0.150
P-value for HWE test in our controls	0.500
% Genotyping value	99.05

Abbreviations: MAF, minor allele frequency; HWE, hardy–weinberg equilibrium.

conducted with SAS 9.4 (SAS Institute, Cary, North Carolina), and all *P*-values are two-sided. In the present study, $P<0.05$ was considered as the level of significance. Power value ($\alpha=0.05$) was calculated by the online Power and Sample Size Calculator software (http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize).[24]

Results

Baseline characteristics

In total, 2740 age/gender-matched Chinese Han subjects (1063 AEG patients and 1677 non-cancer history controls) were included in this hospital-based case–control study to assess the correlation of miRNA-499 rs3746444 A>G polymorphism with risk of AEG. Clinicopathological features and confounding factors are listed in Table 1. There were no significant differences in age and gender (mean age of AEG patients vs controls: 64.19±8.63 years vs 63.91±10.22 years, $P=0.451$; number of cases vs controls (male/female): 759/304 vs 1194/483, $P=0.909$). There were statistically significant differences in smoking, alcohol consumption (smoking status of AEG patients vs controls (ever/never): 773/290 vs 1323/354, $P<0.001$; alcohol consumption of cases vs controls (male/female): 908/155 vs 1507/170, $P<0.001$). In AEG group, patients included 305 cases with stage I/II and 758 with stage III/IV of the disease. Tumor stage was determined according to AJCC (7th edition). Among the 1063 AEG cases, 625 patients accompanied by lymph node metastasis. The frequencies of the miRNA-499 rs3746444 polymorphism in controls did not deviate from the HWE ($P=0.500$, Table 2).

Association of miRNA-499 rs3746444 variants with AEG

Genotype frequency and percentage of the miRNA-499 rs3746444 A>G polymorphism are summarized in Table 3.

Table 3 The frequencies of miRNA-499 rs3746444 A>G polymorphisms in AEG patients and controls

Genotype	Overall cases (n=1063)		Stage I/II patients (n=305)		Stage III/IV patients (n=758)		Controls (n=1677)	
	n	%	n	%	n	%	n	%
AA	695	66.83	199	66.78	496	66.85	1214	72.52
AG	311	29.90	90	30.20	221	29.78	419	25.03
GG	34	3.27	9	3.02	25	3.37	41	2.45
AG+GG	345	33.17	99	33.22	246	33.15	460	27.48
AA+AG	1,006	96.73	289	96.98	717	96.63	1633	97.55
GG	34	3.27	9	3.02	25	3.37	41	2.45
G allele	379	18.22	108	18.12	271	18.26	501	14.96

We list the relationship between this SNP and AEG susceptibility in Table 4. SNP miRNA-499 rs3746444 A>G polymorphism had an increased susceptibility to AEG (AG vs AA: adjusted OR=1.29, 95% CI=1.08–1.53, *P*=0.005 and AG/GG vs AA: adjusted OR=1.30, 95% CI=1.10–1.54, *P*=0.002, Table 4).

Association of miRNA-499 rs3746444 A>G polymorphism with AEG in different subgroups

After adjustment by age, gender, smoking, and alcohol consumption, in stage III/IV patients, we also identified that miRNA-499 rs3746444 A>G polymorphism increased the risk of AEG (AG vs AA: adjusted OR=1.28, 95% CI=1.05–1.55, *P*=0.013 and AG/GG vs AA: adjusted OR=1.30, 95% CI=1.08–1.57, *P*=0.006, Table 4).

Table 5 summarizes the genotype frequencies of miRNA-499 rs3746444 A>G polymorphism in different subgroups. After adjustment by confounding factors (age, gender, smoking, and alcohol consumption), we found that miRNA-499 rs3746444 A>G polymorphism increased the risk of AEG in several subgroups (male subgroup: AG vs AA: adjusted OR=1.39, 95% CI=1.13–1.70, *P*=0.002 and AG/GG vs AA: adjusted OR=1.36, 95% CI=1.12–1.66, *P*=0.002; female subgroup: GG vs AA: adjusted OR=2.36, 95% CI=1.02–5.44, *P*=0.044 and GG vs AG/AA: adjusted OR=2.33, 95% CI=1.01–5.33, *P*=0.046; <64 years subgroup: AG vs AA: adjusted OR=1.47, 95% CI=1.14–1.89, *P*=0.003 and AG/GG vs AA: adjusted OR=1.45, 95% CI=1.13–1.85, *P*=0.003; never smoking subgroup: AG vs AA: adjusted OR=1.40, 95% CI=1.15–1.72, *P*=0.001 and AG/GG vs AA: adjusted OR=1.41, 95% CI=1.16–1.72, *P*=0.001; and never drinking subgroup: AG vs AA: adjusted OR=1.33, 95% CI=1.11–1.61, *P*=0.003 and AG/GG vs AA: adjusted OR=1.33, 95% CI=1.11–1.60, *P*=0.002, Table 5).

Power of this case–control study

For miRNA-499 rs3746444 A>G polymorphism, the power value (α=0.05) was 0.817 in AG vs AA genetic model and 0.882 in AG/GG vs AA genetic model among overall comparison, 0.729 in AG vs AA genetic model and 0.805 in AG/GG vs AA genetic model in III/IV patients subgroup, 0.883 in AG vs AA genetic model and 0.830 in AG/GG vs AA genetic model among male subgroup, 0.649 in GG vs AA genetic model and 0.536 in GG vs AG/AA genetic model among female subgroup, 0.854 in AG vs AA genetic model and 0.851 in AG/GG vs AA genetic model among <64 years subgroup, 0.902 in AG vs AA genetic model and 0.932 in AG/GG vs AA genetic model among never smoking subgroup, and 0.847 in AG vs AA genetic model and 0.871 in AG/GG vs AA genetic model among never drinking subgroup. The results of power calculation confirmed that miRNA-499 rs3746444 A>G polymorphism increased the risk of AEG in overall comparison, male, <64 years, never smoking and never drinking subgroups.

Association of miRNA-499 rs3746444 A>G polymorphism with lymph node status in AEG patients

In total, 1063 AEG patients were included in the present study to assess the relationship of the miRNA-499 rs3746444 A>G polymorphism with lymph node status of AEG. Among these AEG cases, 625 patients accompanied by positive lymph node. As listed in Table 6, the distribution of miRNA-499 rs3746444 A>G variants was no significant difference among different variants.

Discussion

Accumulating evidence suggests a vital role for heredity in determining potential risk for malignancy. Yang et al recently performed a meta-analysis and identified the

Table 4 Association of miRNA-499 rs3746444 A>G polymorphism with AEG

Genotype	Overall patients (n=1063) vs controls (n=1677)				Stage I/II patients (n=305) vs controls (n=1677)				Stage III/IV patients (n=758) vs controls (n=1677)			
	Crude OR (95%CI)	P	Adjusted OR[a] (95%CI)	P	Crude OR (95%CI)	P	Adjusted OR[a] (95%CI)	P	Crude OR (95%CI)	P	Adjusted OR[a] (95%CI)	P
GA vs AA	**1.30 (1.09–1.54)**	**0.003**	**1.29 (1.08–1.53)**	**0.005**	1.31 (1.00–1.72)	0.052	1.29 (0.98–1.70)	0.068	**1.29 (1.06–1.57)**	**0.010**	**1.28 (1.05–1.55)**	**0.013**
GG vs AA	1.45 (0.91–2.30)	0.118	1.46 (0.92–2.33)	0.112	1.34 (0.64–2.80)	0.437	1.41 (0.67–2.96)	0.362	1.49 (0.90–2.48)	0.123	1.50 (0.90–2.50)	0.118
GA/GG vs AA	**1.31 (1.11–1.55)**	**0.002**	**1.30 (1.10–1.54)**	**0.002**	**1.31 (1.01–1.71)**	**0.043**	1.30 (1.00–1.70)	0.052	**1.31 (1.09–1.58)**	**0.005**	**1.30 (1.08–1.57)**	**0.006**
GG vs GA/AA	1.35 (0.85–2.14)	0.207	1.36 (0.86–2.16)	0.194	1.24 (0.60–2.58)	0.564	1.31 (0.63–2.75)	0.467	1.39 (0.84–2.30)	0.202	1.40 (0.84–2.33)	0.192

Notes: [a]Adjusted for age, sex, smoking status, and alcohol use in a logistic regression model. Bold values are statistically significant ($P<0.05$).

relationship between miRNA-499 rs3746444 A>G variants and risk to overall cancer was significant involving 23,762 cases and 28,694 controls.[18] In addition, this study reported that miRNA-499 rs3746444 A>G variants also have been implicated in the risk of digestive system cancer. To the best of our knowledge, the current study is the first case–control study to explore the correlation of miRNA-499 rs3746444 A>G polymorphism with the susceptibility of AEG.

MiRNA-499 is a common miRNA which was implicated in posttranscriptional regulatory.[25] A previous study reported that miRNA-499 might facilitate the metastasis and cellular invasion of colorectal cancer by targeting PDCD4 and FOXO4.[26] Thus, miRNA-499 might be considered as a useful therapeutic target for patients with colorectal cancer. Li et al reported that miRNA-499-3p have been identified in GC tissues.[27] These findings indicated that miRNAs may contribute to the development of human GC. Recently, several case–control studies identified that miRNA-499 rs3746444 A>G polymorphism might alter the risk of GC.[11,14] Additionally, Tahara et al reported that the miRNA-499 rs3746444 G allele carrier was associated with a poorer prognosis in advanced GC performing chemotherapy.[16] However, some meta-analysis have reported negative signals of miRNA-499 rs3746444 A>G polymorphism with the risk of GC.[18,20,28,29] The possible interpretation for the lack of association could be the sufficient sample size in previous study. In this case–control study, we established that the relationship between miRNA-499 rs3746444 A>G variants and susceptibility to AEG was significant in overall comparison. In the subsequent stratified analyses, as summarized in Table 5, observation of this study also showed that miRNA-499 rs3746444 A>G polymorphism might increase the risk of AEG in male, <64 years, never smoking, and never drinking subgroups. Our results for the association between miRNA-499 rs3746444 A>G variants and AEG risk are based on related large sample sizes, which were analogous to the previous studies in Asians.[18,20,28,30] However, our findings might be deciphered with very caution. In the future, further case–control studies with larger sample sizes and rigorous matching criteria considering more gene-environment factors are needed to explore the potential relationships.

Several merits and limitations should be acknowledged when explaining these observed results. Merits of this case–control study including its large sample size and the matching confounding factors that it was enrolled to

Table 5 Stratified analyses between miRNA-499 rs3746444 A>G polymorphism and AEG risk by sex, age, smoking status, and alcohol consumption

Variable	miRNA-499 rs3746444 A>G (case/control)[a]			Adjusted OR[b] (95% CI); *P*				
	AA	AG	GG	AA	AG	GG	AG/GG	GG vs (AG/AA)
Sex								
Male	492/867	233/293	20/31	1.00	**1.39 (1.13–1.70); *P*: 0.002**	1.14 (0.64–2.04); *P*: 0.647	**1.36 (1.12–1.66); *P*: 0.002**	1.04 (0.59–1.85); *P*: 0.886
Female	203/347	78/126	14/10	1.00	1.05 (0.75–1.47); *P*: 0.761	**2.36 (1.02–5.44); *P*: 0.044**	1.15 (0.84–1.58); *P*: 0.391	**2.33 (1.01–5.33); *P*: 0.046**
Age								
<64	312/599	157/203	13/21	1.00	**1.47 (1.14–1.89); *P*: 0.003**	1.24 (0.61–2.52); *P*: 0.560	**1.45 (1.13–1.85); *P*: 0.003**	1.10 (0.54–2.24); *P*: 0.783
≥64	383/615	154/216	21/20	1.00	1.13 (0.89–1.45); *P*: 0.314	1.67 (0.89–3.14); *P*: 0.108	1.18 (0.93–1.49); *P*: 0.168	1.62 (0.87–3.02); *P*: 0.132
Smoking status								
Never	499/970	227/316	27/35	1.00	**1.40 (1.15–1.72); *P*: 0.001**	1.50 (0.90–2.51); *P*: 0.121	**1.41 (1.16–1.72); *P*: 0.001**	1.37 (0.82–2.28); *P*: 0.230
Ever	196/244	84/103	7/6	1.00	1.01 (0.71–1.43); *P*: 0.968	1.38 (0.45–4.24); *P*: 0.575	1.03 (0.73–1.45); *P*: 0.874	1.38 (0.45–4.21); *P*: 0.576
Alcohol consumption								
Never	593/1,097	266/370	27/38	1.00	**1.33 (1.11–1.61); *P*: 0.003**	1.32 (0.80–2.19); *P*: 0.278	**1.33 (1.11–1.60); *P*: 0.002**	1.22 (0.74–2.02); *P*: 0.437
Ever	102/117	45/49	7/3	1.00	0.98 (0.60–1.63); *P*: 0.949	3.60 (0.84–15.54); *P*: 0.086	1.11 (0.68–1.79); *P*: 0.687	3.62 (0.85–15.50); *P*: 0.083

Notes: [a]For miRNA-499 rs3746444 A>G, the genotyping was successful in 1040 (97.84%) AEG cases, and 1674 (99.82%) non-cancer controls; [b]Adjusted for multiple comparisons [age, sex, smoking status, and alcohol consumption (besides stratified factors accordingly)] in a logistic regression model; Bold values are statistically significant ($P<0.05$).

Table 6 Logistic regression analyses of association between miRNA-499 rs3746444 A>G polymorphism and lymph node status in AEG patients

Genotype	Positive (n=625)		Negative (n=438)		Crude OR (95%CI)	*P*	Adjusted OR[a] (95%CI)	*P*
	n	%	n	%				
miRNA-499 rs3746444 A > G								
AA	418	68.19	277	64.87	1.00		1.00	
AG	174	28.38	137	32.08	0.84(0.64–1.10)	0.212	0.83(0.63–1.09)	0.180
GG	21	3.43	13	3.04	1.07(0.53–2.17)	0.851	1.05(0.52–2.14)	0.891
AG+GG	195	31.81	150	35.13	0.86(0.66–1.12)	0.264	0.85(0.65–1.11)	0.224
AA+AG	592	96.57	414	96.96	1.00		1.00	
GG	21	3.43	13	3.04	1.13(0.56–2.28)	0.734	1.11(0.55–2.25)	0.768
G allele	216	17.62	163	19.09				

Note: [a]Adjusted for age, sex, alcohol use and smoking status.

analyze the relationship between miRNA-499 rs3746444 A>G variants in relation to susceptibility of AEG. Concerns relate to certain potential selections and risk factors. The participants among controls enrolled from two local hospitals, which might lead to some possible biases. If included controls were more likely to possess some benign diseases than those who did not participate in a physical examination, this could have led to a bias of the correlation we observed. Finally, obesity and overweight are an established cancer risk factor. AEG is regarded as an obesity-/overweight-related malignancy.[31–33] However, for lack of the body mass index (BMI) data, we did not match this confounding factor. If this bias existing in our study, then the increased susceptibility that we have observed may be an overestimate of the potential risk effects of miRNA-499 rs3746444 A>G polymorphism for AEG.

In summary, to our knowledge, this is the first case–control focusing on the possible correlation between miRNA-499 rs3746444 A>G polymorphism and risk of AEG. The significant association of AEG susceptibility with miRNA-499 rs3746444 A>G polymorphism observed suggests additional support for the vital role of miRNA in the development of AEG. In the future, further exploration of this genetic factor in relation to AEG susceptibility is needed.

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Disclosure

The authors have no potential conflicts of interest in this work.

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