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Original Article

Hsa-miR-34b/c rs4938723 T>C, *pri-miR-124-1* rs531564 C>G, *pre-miR-125a* rs12975333 G>T and *hsa-miR-423* rs6505162 C>A polymorphisms and the risk of gastric cardia adenocarcinoma

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Abstract: Gastric cardia adenocarcinoma (GCA) is among the leading causes of cancer related deaths and its incidence is increasing recently. Along with environmental factors, genetic factors might also play an important role in the carcinogenesis of GCA. We conducted a hospital based case-control study to evaluate the effect of single nucleotide polymorphisms (SNPs) in *Hsa-miR-34b/c* rs4938723 T>C, *pri-miR-124-1* rs531564 C>G, *pre-miR-125a* rs12975333 G>T and *hsa-miR-423* rs6505162 C>A on the risk of GCA. A total of 330 cases and 608 controls were recruited for the study. Ligation detection reaction (LDR) method was used to determine individual genotypes. No significant association was observed between these SNPs and overall GCA risk in all genetic comparison models, respectively. However, in stratification analysis, SNP in *hsa-miR-423* rs6505162 C>A was associated with increased risk of GCA in female patients, whereas decreased risk of GCA in male patients. Further large scale studies in different ethnic groups are warranted to validate these findings.

Keywords: Gastric cardia adenocarcinoma, single nucleotide polymorphism, microRNA, miR-423,rs6505162, risk of cancer

Introduction

Gastric cardia adenocarcinoma (GCA) is one of the most common malignant tumors of stomach and is among the leading causes of cancer related deaths and disabilities [1]. Incidence and prevalence of GCAs have been found to increase sharply in recent years [2]. When GCA tissues were compared to matched normal tissues, expressions of the most of the genes were found to be altered, 199 genes out of 367 genes were up-regulated whereas 168 genes were down-regulated [3].

Micro RNAs (miRNAs) are non-coding RNAs made up of 20 to 25 nucleotides with the function of post transcriptional regulation of gene expression. miRNAs have very significant roles in the regulation of variety of biological processes at molecular level [4]. Any alteration in miRNAs affecting their functions make them very important players in carcinogenesis [5]. Functional single nucleotide polymorphisms (SNPs) in miRNAs may influence the miRNA-dependent gene expression regulation and contribute to the carcinogenesis [6].

miR-423 lies within the first gene nuclear speckle splicing regulated protein. It is involved in alternate splicing of mRNAs [7]. The pre-miRNAs of miR-423 contain two mature transcript at 3' and 5' ends. Several studies have found alteration in expression of matured miR-423 in various cancers including colorectal cancer, nasopharyngeal cancer, mesothelioma and breast cancer. Its expression was reduces in mesothelioma and breast cancer whereas increased in head and neck cancers suggesting miR-423 to be an independent prognostic indicator of disease [8-10].

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

Variable	Cases (n=330)		Controls (n=608)		p[a]
	n	%	n	%	
Age (years) mean ± SD	65.06 (±8.37)		64.19 (±6.66)		0.103
Age (years)					0.746
<60	89	26.97	170	27.96	
≥60	241	73.03	438	72.04	
Sex					0.965
Male	223	67.58	410	67.43	
Female	107	32.42	198	32.57	
Tobacco use					**0.006**
Never	209	63.33	438	72.04	
Ever	121	36.67	170	27.96	
Alcohol use					0.072
Never	233	70.61	462	75.99	
Ever	97	29.39	146	24.01	

[a]Two-sided χ^2 test and student t test; Bold values are statistically significant (P<0.05).

SNPs in Hsa-miR-34b/c rs4938723 occur within its CpG island which is a probable binding site of GATA-X transcription [11]. Polymorphisms in Hsa-miR-34b/c rs4938723 are associated with increased risk of hepatocellular carcinoma [12] and nasopharyngeal carcinoma [13]; whereas, decreased risk of colorectal cancer [14]. They are also associated with risk and survival of breast cancer [15]. Polymorphisms in pri-miR-124-1 rs531564 and pre-miR-125a rs12975333 are also found to be associated with different malignancies. SNP in pri-miR-124-1 rs531564 is associated with increased risk of esophageal cancer in males [16] and cervical cancer in females [17]. SNP in pre-miR-125a rs12975333 is found in Antwerp area and is associated with increased risk of breast cancer [18].

We conducted this hospital-based case-control study to evaluate the effect of functional SNPs in *Hsa-miR-34b/c* rs4938723 T>C, *pri-miR-124-1* rs531564 C>G, *pre-miR-125a* rs12975333 G>T and *hsa-miR-423* rs6505162 C>A on the risk of GCA. Genotyping analyses were performed for the four SNPs in 330 cases and 608 controls.

Materials and methods

Ethical approval of the study protocol

This hospital-based case-control study was approved by the Review Board of Jiangsu University (Zhenjiang, China). We have complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects and/or animals. All subjects provided written informed consent to be included in the study.

Study subjects

Three-hundred and thirty cases with GCA and 608 controls were consecutively recruited from the Affiliated People's Hospital of Jiangsu University and Affiliated Hospital of Jiangsu University (Zhenjiang, China) between October 2008 and December 2010. The selection criteria for cases recruitment were patients with diagnosis of GCA undergoing the surgical treatment for the first time; with confirmed diagnosis by biopsy and histopathological studies; and without history of any other kind of gastro-intestinal or other kinds of malignancy. The controls were patients without cancer frequency (usually trauma patients) recruited from the two hospitals mentioned above during the same time period. The controls were matched to the cases with regard to age (±5 years) and sex.

Each subject was personally questioned by trained interviewers using a pre-tested questionnaire to obtain information on demographic data (e.g., age, sex) and related risk factors (including tobacco smoking and alcohol consumption). Individuals who smoked one cigarette per day for >1 year were defined as "smokers". Subjects who consumed ≥3 alcoholic drinks a week for >6 months were considered to be "alcohol drinkers".

Isolation of DNA and genotyping by ligation detection reaction

Venous blood samples (2 ml) were collected from each subject in ethylene diaminetetraacetic acid (EDTA) tube. Buffy coat was extracted and stored at -80°C. QIAamp DNA Blood Mini Kit (Qiagen, Berlin, Germany) was used to isolate genomic DNA from the stored buffy coats

Table 2. Primary information for *hsa-miR-34b/*crs4938723 T>C, *pri-miR-124-1* rs531564 C>G, *pre-miR-125a* rs12975333 G>T and *hsa-miR-423* rs6505162 C>A polymorphisms

Genotyped SNPs	*hsa-miR-34b/* crs4938723 T>C	*pri-miR-124-1* rs531564 C>G	*pre-miR-125 a*rs12975333 G>T	*hsa-miR-423* rs6505162 C>A
Chromosome	11	8	19	17
Gene Official Symbol	MIR34B/C	MIR124-1	MIR125A	MIR423
Function	ncRNA	ncRNA	ncRNA	ncRNA
ChrPos (Genome Build 36.3)	110887775	9798109	56888340	25468309
Regulome DB Score[a]	5	5	5	1f
TFBS[b]	Y	Y	Y	Y
Splicing (ESE or ESS)	-	-	-	Y
MAF[c] for Chinese in database	0.400	0.178	Unknown	0.200
MAF in our controls (n=608)	0.329	0.148	0.000	0.187
p value for HWE[d] test in our controls	0.838	0.289	-	0.244
Genotyping method[e]	LDR	LDR	LDR	LDR
% Genotyping value	98.29%	96.59%	96.59%	95.31%

[a]http: //www.regulomedb.org/; [b]TFBS: Transcription Factor Binding Site (http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm); [c]MAF: minor allele frequency; [d]HWE: Hardy-Weinberg equilibrium; [e]LDR: Ligation detection reaction.

[19]. Sample DNA were amplified by PCR according to the manufacturer's recommendations. Genotyping work was performed using the ligation detection reaction (LDR) method with technical support from the Shanghai Biowing Applied Biotechnology Company [20]. For quality control, repeated analyses were done for randomly selected samples with high DNA quality.

Statistical analyses

SAS 9.1.3 (SAS Institute, Cary, NC, USA) was used for the statistical analysis. Demographic characteristics of the subjects and their selected variables between cases and controls were analyzed using chi square and student T test.

Odd Ratios (ORs) and their 95% Confidence Interval (CI) were used to estimate the association between the risk of GCA and the selected four SNPs using logistic regression analysis for crude ORs and adjusted ORs when adjusting for age, gender, smoking and alcohol drinking status. Observed genotypic frequency to the expected ones among control subjects were compared by using Hardy-Weinberg equilibrium (HWE) which was tested by goodness-of-fit chi square test.

Results

Demographic data

Demographic characteristics of cases and controls in the study are summarized in **Table 1**. Chi square test revealed that cases and controls were matched proportionally in regard to age (P=0.103) and gender (P=0.965). Smoking rates were significantly higher in case subjects than in control subjects (P=0.006).

Primary information for four genotyped SNPs

Table 2 lists the primary information of the selected four genotyped SNPs. The success rate of genotyping in all subjects ranged from 95.31% to 98.29%. The repeated analysis of all four selected SNPs revealed 100% concordance rates. The MAF in controls were in close similarity to MAF of Chinese people for all selected SNPs in database. The observed genotypic frequencies for hsa-miR-34b/crs4938723 T>C, pri-miR-124-1 rs531564 C>G andhsa-miR-423 rs6505162 C>A polymorphisms were in HWE (P=0.838, P=0.289 and P=0.244, respectively). The observed minor allele frequency for pre-miR-125a rs12975333 G>T was 0.000, making the HWE analysis not available.

Associations between hsa-miR-34b/c rs4938723 T>C, pri-miR-124-1 rs531564 C>G, pre-miR-125a rs12975333 G>T and hsa-miR-423 rs6505162 C>A polymorphisms and risk of GCA

Genotypic distributions of *hsa-miR-34b/c* rs4938723 T>C, *pri-miR-124-1* rs531564 C>G, *pre-miR-125a* rs12975333 G>T and *hsa-miR-423* rs6505162 C>A are shown in **Table 3**. Single locus, recessive model and dominant

Table 3. Logistic regression analyses of associations between *hsa-miR-34b/c*rs4938723 T>C, *pri-miR-124-1* rs531564 C>G, *pre-miR-125a* rs12975333 G>T and *hsa-miR-423* rs6505162 C>A polymorphisms and risk of GCA

Genotype	Cases (n=330)		Controls (n=608)		Crude OR (95% CI)	p	Adjusted OR[a] (95% CI)	P
	n	%	n	%				
hsa-miR-34b/c rs4938723T>C								
TT	132	40.74	270	45.15	1.00		1.00	
TC	148	45.68	262	43.81	1.16 (0.87-1.54)	0.328	1.18 (0.88-1.59)	0.257
CC	44	13.58	66	11.04	1.36 (0.88-2.11)	0.162	1.36 (0.88-2.10)	0.173
CC vs. TC vs. TT								0.326
TC+CC	192	59.26	328	54.85	1.20 (0.91-1.58)	0.198	1.22 (0.93-1.61)	0.160
TT+TC	280	86.42	532	88.96	1.00		1.00	
CC	44	13.58	66	11.04	1.27 (0.84-1.91)	0.256	1.24 (0.83-1.88)	0.298
C allele	236	36.42	394	32.94				
pri-miR-124-1 rs531564 C>G								
CC	225	70.31	429	73.21	1.00		1.00	
CG	90	28.13	141	24.06	1.22 (0.89-1.66)	0.214	1.24 (0.91-1.70)	0.176
GG	5	1.56	16	2.73	0.60 (0.22-1.65)	0.319	0.61 (0.22-1.71)	0.348
GG vs. CG vs. CC								0.247
CG+GG	95	29.69	157	26.79	1.15 (0.85-1.56)	0.353	1.18 (0.87-1.60)	0.295
CC+CG	315	98.44	570	97.27	1.00		1.00	
GG	5	1.56	16	2.73	0.57 (0.21-1.56)	0.271	0.58 (0.21-1.61)	0.294
Gallele	100	15.63	173	14.76				
pre-miR-125a rs12975333 G>T								
GG	320	100.00	586	100.00	1.00		1.00	
GT	0	0.00	0	0.00	-	-	-	-
TT	0	0.00	0	0.00	-	-	-	-
hsa-miR-423 rs6505162 C>A								
CC	218	69.65	380	65.40	1.00		1.00	
CA	82	26.20	185	31.84	0.77 (0.57-1.05)	0.102	0.77 (0.57-1.06)	0.105
AA	13	4.15	16	2.75	1.42 (0.67-3.00)	0.363	1.50 (0.70-3.20)	0.296
AA vs. CA vs. CC								0.139
CA+AA	95	30.35	201	34.60	0.82 (0.61-1.11)	0.199	0.83 (0.62-1.12)	0.216
CC+CA	300	95.85	565	97.25	1.00		1.00	
AA	13	4.15	16	2.75	1.53 (0.73-3.22)	0.263	1.62 (0.76-3.44)	0.210
A allele	108	17.25	217	18.67				

[a]Adjusted for age, sex, smoking status and alcohol consumption.

model analyses were done. No association was observed between *hsa-miR-34b/c* rs4938723 T>C, *pri-miR-124-1* rs531564 C>G and *hsa-miR-423* rs6505162 C>A polymorphisms and risk of GCA. All genotypes of *pre-miR-125a* rs12975333 G>T were GG homozygote.

Stratification analysis of hsa-miR-423 rs6505162 C>A polymorphism and risk of GCA

Stratification analysis was done to find out the effect of hsa-miR-423 rs6505162 C>A genotype on GCA risk in respect to selected demographic characteristics (**Table 4**). Analysis suggested that hsa-miR-423 rs6505162 CA genotype (adjusted OR=0.65, 95% CI=0.44-0.96, P=0.030) and CA+AA genotype (adjusted OR=0.68, 95% CI=0.47-0.99, P=0.042) were associated with lower risk of GCA in male patients compared to CC genotype, respectively. In female patients, however, hsa-miR-423 rs6505162 AA genotype was associated with significantly increased risk of GCA (adjusted OR=10.08, 95% CI=1.15-88.74, P=0.037) compared to CC genotype. In addition, hsa-miR-423 rs6505162 AA genotype was also associated with higher risk of GCA (adjusted OR=9.68, 95% CI=1.11-84.64, P=0.040) com-

Table 4. Stratified analyses between *hsa-miR-423* rs6505162 C>A polymorphism and GCA risk by sex, age, smoking status and alcohol consumption

Variable	*hsa-miR-423* rs6505162 C>A (case/control)[a]				Adjusted OR[b] (95% CI); p; p_h[c]				
	CC	CA	AA	CA+AA	CC	CA	AA	CA+AA	AA vs. (CA+CC)
Sex									
Male	151/249	50/127	8/15	58/142	1.00	0.65 (0.44-0.96); p: 0.030	0.96 (0.39-2.34); p: 0.928	0.68 (0.47-0.99); p: 0.042	1.09 (0.45-2.64); p: 0.851
Female	67/131	32/58	5/1	37/59	1.00	1.14 (0.67-1.94); p: 0.634	10.08 (1.15-88.74); p: 0.037	1.30 (0.77-2.18); p: 0.323	9.68 (1.11-84.60); p: 0.040
Age									
<63	62/107	18/51	4/3	22/54	1.00	0.75 (0.46-1.23); p: 0.251	1.10 (0.31-3.87); p: 0.883	0.78 (0.49-1.25); p: 0.298	1.20 (0.34-4.19); p: 0.778
≥63	156/273	64/134	9/13	73/147	1.00	0.85 (0.56-1.27); p: 0.422	2.10 (0.76-5.81); p: 0.151	0.93 (0.63-1.38); p: 0.719	2.21 (0.81-6.07); p: 0.122
Smoking status									
Never	139/270	51/133	8/13	59/146	1.00	0.75 (0.50-1.11); p: 0.147	1.24 (0.48-3.22); p: 0.660	0.79 (0.54-1.15); p: 0.217	1.35 (0.52-3.48); p: 0.534
Ever	79/110	31/52	5/3	36/55	1.00	0.90 (0.52-1.57); p: 0.720	2.95 (0.67-13.02); p: 0.152	1.01 (0.59-1.72); p: 0.972	3.05 (0.70-13.31); p: 0.138
Alcohol consumption									
Never	156/290	58/138	9/12	67/150	1.00	0.81 (0.56-1.18); p: 0.274	1.65 (0.66-4.13); p: 0.285	0.87 (0.61-1.25); p: 0.459	1.76 (0.71-4.36); p: 0.226
Ever	62/90	24/47	4/4	28/51	1.00	0.75 (0.40-1.42); p: 0.381	1.41 (0.31-6.35); p: 0.653	0.81 (0.44-1.48); p: 0.489	1.54 (0.35-6.85); p: 0.569

[a]The genotyping was successful in 313 (94.85%) GCA cases, and 581 (95.56%) controls for *hsa-miR-423* rs6505162 C>A; [b]Adjusted for age, sex, smoking status and alcohol consumption (besides stratified factors accordingly) in a logistic regression model; [c]p_h for heterogeneity.

pared to CA+CC genotype. No significant association was found in different age group or smoking and drinking condition in the study.

Discussion

We conducted this hospital-based case-control study to investigate whether there is any association between hsa-miR-34b/c rs4938723 T>C, pri-miR-124-1 rs531564 C>G, pre-miR-125a rs12975333 G>T and hsa-miR-423 rs6505162 C>A polymorphisms and risk of GCA. Significant differences were observed between smoking status of the cases and that of controls (P=0.006). In the stratification analysis when hsa-miR-423 rs6505162 CC homozygote genotype was taken as a reference, the CA genotype and CA+AA genotype showed significantly decreased risk of GCA in male patients (P=0.030 and P=0.042 respectively). In female patients, however, when the CC genotype was taken as a reference, the AA genotype showed significantly increased risk of GCA (P=0.037). The AA genotype was also associated with increased risk of GCA compared to CA+CC genotype in female patients (P=0.040).

miRNAs are small non-coding RNAs with very important functions of post translational regulations of gene expression. They play very important roles in regulating the expression of cellular proteins and pathways of cell proliferation [21]. SNPs in miRNAs usually occur in the step of biogenesis, sequence of miRNA or miRNA binding site. When it occurs in biosynthesis step, it results in abnormality either in pri-miRNA, pre-miRNA or mature miRNA. If SNP occurs at miRNA sequence, it may cause production of different pri-miRNA, pre-miRNA or mature miRNA, in turn causing production of miRNA with different target profile. When it occurs at non-pairing allele at miRNA binding site, it may affect the target gene at 3'UTR region which may result into deregulation of its function [22]. SNPs in the regulatory pathway of miRNA may disrupt their function which may cause carcinogenesis [23]. Their link to the cancer development was first described by Calin et al. in 2005 [24].

miRNAs regulate functions of many oncogenes and tumor suppressor genes. When their function are disrupted by SNPs in the regulatory pathway, it either functions as oncogene by inhibiting expression of tumor suppressor genes and enhancing oncogenes expression or functions as tumor suppressor by up-regulating expression of genes responsible for apoptosis and inhibition of cell proliferation [25-27].

SNP in hsa-miR-423 rs6505162 is linked with increased risk of esophageal and decreased risk of breast and colorectal cancer. SNP in hsa-miR-34b/c rs4938723 is associated with several types of malignancies including nasopharyngeal, hepatocellular, colorectal and breast cancer. SNP in pri-miR-124-1 rs531564 is associated with bladder cancer in male and breast cancer in female. Similarly, SNP in pre-miR-125a rs12975333 is linked with breast and nasopharyngeal carcinoma. In the present study, no significant association was found between hsa-miR-34b/c rs4938723 T>C, pre-miR-125a rs12975333 G>T and pri-miR-124-1rs531564 C>G polymorphisms and risk of GCA. In stratification analysis, however, miR-423rs6505162 C>A was associated with decreased risk of GCA in male patients and increased risk of GCA in females. The reasons behind these findings are unclear, however, it may result from very limited numbers of subjects were available for AA genotype, especially in female subgroup. It may also be due to the lifestyle and the hormonal differences between male and female patients. The different hormonal level when combined to certain SNPs may play catalytic role in the pathogenesis of GCA development.

There were some major limitations of this case control study. Patients were recruited from the two affiliated hospitals in a small area and numbers of subjects for the different genotypes were also limited which may not be sufficient to represent the general population. We were also not able to obtain detailed information about cancer metastasis, overall survival, viral infection and immune parameter of the subjects included in the study.

Conclusions

In conclusion our study showed that hsa-miR-423 rs6505162 C>A is associated with decreased risk of GCA in male patients whereas increased risk in female patients. Findings regarding polymorphisms in miRNAs and their possible roles may be very promising in determining the risks of GCA and further larger studies with functional analysis in various ethnic

groups and in different geographical regions are required.

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Disclosure of conflict of interest

None.

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