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ORIGINAL PAPER

Association analysis of genetic variants in microRNA networks and gastric cancer risk in a Chinese Han population

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Abstract
Purpose To investigate associations between genetic variants involved in microRNA networks (microRNA biogenesis, microRNA and microRNA binding sites) and risk of gastric cancer.
Methods We genotyped 19 SNPs of the microRNA-related genes in a case–control study of 311 gastric cancers and 425 cancer-free controls in a Chinese Han population.
Results We found that two of the SNPs were significantly associated with gastric cancer. Inhibitory effect of minor allele T of rs2071504 SNP within the exon of POLR2A gene was significantly associated with gastric carcinogenesis ($p = 0.033$, aOR = 0.742, 95%CI = 0.564–0.977) and the SNP rs895819 in the miR-27a gene with the minor allele C presented significantly reduced risk to gastric cancer ($p = 0.037$, aOR = 0.771, 95%CI = 0.604–0.985). Further stratified analysis with regard to clinical pathological parameters of the patients indicated that the SNP rs2071504 was associated with lymph node metastasis ($p = 0.021$, aOR = 0.529, 95%CI = 0.307–0.910) and TMN stage ($p = 0.021$, aOR = 0.532, 95%CI = 0.311–0.908), respectively.
Conclusions Our findings provided evidence that the SNP rs2071504 in the exon of POLR2A gene would not only confer a decreased risk of gastric cancer, but also influence lymph node metastasis and TMN stage of gastric cancer in the Chinese population.

Keywords MicroRNA · Gastric cancer · Single-nucleotide polymorphism

Introduction

Gastric cancer was the second leading cause of cancer death worldwide and accounted for approximately 10% of newly diagnosed cancers (Crew and Neugut 2006). Widespread metastasis was a major reason for the dismal outcome of gastric cancer patients (Fidler 2003). Recent studies suggested that microRNAs played important roles in gastric cancer development, infiltration and metastasis. MicroRNAs were differentially regulated in gastric cancer (Tseng et al. 2011; Xu et al. 2011).

MicroRNAs were a family of 21- to 25-nucleotide-long, noncoding small RNA that bound to the target transcript in the 3′-UTR. They inhibited the translation of proteins and destabilized their target mRNA (Baek et al. 2008). MicroRNAs were predicted to regulate about 30% of the human genome including genes in stress resistance, fat metabolism, and cell proliferation and apoptosis pathways (Ambros 2003; Lewis et al. 2005). Genetic variants involved in microRNA biogenesis genes, in microRNA genes as well as in microRNA binding sites genes might affect microRNA-mediated cell regulation (Clague et al.

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2010; Mishra and Bertino 2009; Landi et al. 2008; Peng et al. 2010).

MicroRNA biogenesis included generation of large primary transcripts (pri-microRNA) encoded by the RNA polymerase II (POLR2A), excision of a stem-loop structure by the nuclear RNaseIII enzyme (Drosha) to generate precursor (pre) transcripts (pre-microRNA), transportation of the pre-microRNA to the cytoplasm by Exportin-5 and processing by another RNaseIII enzyme (Dicer) into an about 22-base mature duplex RNA till they reach their mature and functional form (Bartel 2004; Liang et al. 2011). Any alteration in each step during the maturation process could affect microRNA production. It was demonstrated that impaired microRNA processing and maturation enhanced cellular transformation and carcinogenesis (Kumar et al. 2008).

It was reported that point mutations in microRNA genes had a functional effect on the development of chronic lymphocytic leukemia (CLL). A germline mutation in pri-miR-16-1 might confer kindred with familial CLL cancer susceptibility and result in low levels of miR-16-1 expression (Calin et al. 2005). Associations of SNPs in microRNA genes with cancer risk were proved by case–control studies (Tian et al. 2009; Hoffman et al. 2009; Peng et al. 2010). The variants in the genes affected microRNA functions in the following ways: (1) through the transcription of the primary transcript, (2) through pri-microRNA and pre-microRNA processing and (3) through effects on microRNA–mRNA interactions (Ryan et al. 2010).

On the other hand, disruption of microRNA-dependent regulation by single-nucleotide polymorphisms (SNPs) in the microRNA binding site of target mRNAs was a mechanism for altered gene expression in cancer (Ryan et al. 2010). Rs1434536, a SNP in miR-125b target site in the BMPR1B, was differentially regulated by microRNA-125b due to the C or T alleles (Saetrom et al. 2009). The variant rs3783553 in the 3′UTR of IL1A led to a 38% decrease in the risk of developing HCC. The TTCA insertion allele for rs3783553 disrupted a binding site for miR-122 and miR-378, thereby increasing the transcription of IL1A in vitro and in vivo (Landi et al. 2008). Increasing evidence implicated genetic variants in microRNA networks attributed to progression of cancer; thus, we investigated the genetic role of SNPs in microRNA networks and gastric cancer risk.

Materials and methods

Study population

This study was a hospital-based case–control study. Patients with gastric cancers ($n = 311$) were collected from the First Affiliated Hospital of Anhui Medical University of China between March 2008 and July 2009 and received no preoperative chemotherapy or radiotherapy before surgical gastrectomy. The patients were comprised of 246 men and 65 women with an average age of 60.4 ± 10.4 years (ranging from 24 to 83 years). In addition, a total of 336 men and 89 women control subjects were matched with an average age of 60.6 ± 8.4 years (ranging from 30 to 86 years). These control subjects without a history of cancers were recruited from patients who visited the hospital for a conventional cancer screening program. Information on demographic characteristics such as gender, age, smoking habits, alcohol consumption and family history of cancer were obtained from a personal interview administered by professional doctors. Smoking habit was defined as non-smoker and smoker. Individuals who smoked one cigarette per day for over one year were defined as smokers. Alcohol consumption was defined as non-drinker and drinker. Individuals who consumed more than 200 mL alcohol per day were defined as drinker. All the subjects tested were Chinese Han population from the same geographic regions of China. Informed consents were obtained from all the participants. This study was approved by the ethics committee for genome research of the Anhui Medical University and was conducted according to the Declaration of Helsinki Principles.

Methods

Extraction of peripheral blood DNA

Blood sample was collected from each subject in EDTA and stored at −80°C. Genomic DNA was extracted from peripheral blood by using QIAamp DNA Blood Midi Kit (Qiagen Inc., Germany) according to the manufacturer's protocol. The concentration of DNA was measured by the Nanodrop Spectrophotometer (ND-1000, USA) of full wavelength and standardized to 50 ng/μl.

SNP selection and Sequenom assay

A panel of 19 SNPs either in the exon region that may affect the protein expression or already showed association with cancers based on previous studies relevant to microRNA networks were selected based on a criteria of the MAFs > 0.05. They included (1) microRNA-related machinery SNPs: rs2071504, rs2228128, rs2228133 and rs6761 (POLR2A), rs1106841 (Expotin-5) and rs10139161 (Dicer1); (2) SNPs in microRNAs relevant to cancer genesis: rs895819 (miR-27a), rs2289030 (miR-492), rs6505162 (miR-423), rs2910164 (miR-146a), rs7372209 (miR-26a) and rs531564 (miR-124); and (3) SNPs in microRNA binding sites: rs1044129 (PYR3) rs1071738 (PALLD), rs4351800

(SYT9), rs9332 (MTRR), rs998754 (GABRA1), rs3737336 (CDON) and rs3210967 (ZDHHC7). The candidate SNPs were in Hardy–Weinberg Equilibrium and in consistence with the database in the Han Chinese from International HapMap Project website (http://hapmap.ncbi.nlm.nih.gov/).

Fifteen ng of genomic DNA was used. Locus-specific PCR and detection primers were designed using the MassARRAY Assay Design 3.0 software (Sequenom, San Diego, California, USA) at the Key Laboratory of Dermatology at Anhui Medical University, Ministry of Education, China, following the manufacturer's instructions. The DNA samples were amplified by multiplex PCR reactions, and the PCR products were then used for locus-specific single-base extension reactions. The resulting products were desalted and transferred to a 384-element SpectroCHIP array. Genotyping analysis of the SNPs for fast-track validation analysis was performed using the Sequenom mass array system with MALDI-TOF MS for allele detection. Genotyping quality was examined by a detailed QC procedure consisting of >95% successful call rate, duplicate calling of genotypes, internal positive control samples and Hardy–Weinberg Equilibrium (HWE) testing. The mass spectrograms were analyzed by the MassARRAY Typer software (Sequenom).

SNP function analysis and MicroRNA target site prediction

SNP function analyses were performed by F-SNP database (http://compbio.cs.queensu.ca/F-SNP/). MicroRNA target sites prediction were performed by TargetScan (http://www.targetscan.org/) and dbSMR (http://miracle.igib.res.in/polyreg/). TargetScan was used to predict biological targets of miRNAs, which were conserved 8mer and 7mer sites that matched the seed region of each miRNA and annotated human UTRs and their orthologs (Bartel 2009). dbSMR was used to select miRNA binding sites within 200 nt of SNPs that may affect miRNA accessibility to the target site.

Data analysis

We used a logistic regression model to analyze association of the SNPs with the risk of gastric cancer. Hardy–Weinberg Equilibrium in each SNP was determined according to control samples. The association between variants and gastric cancer risk was analyzed by calculating the adjusted odds ratios (aOR) and 95% confidence intervals (95% CI). The p values reported in the study were based on two-sided probability test with a significance level of $p < 0.05$. The statistical software plink v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) and Stata/SE version 10 were used for statistical analyses (StataCorp LP, College Station, TX).

Results

We searched for potential associations of 19 SNPs from microRNA networks with gastric cancer. We found that two of the 19 SNPs used in this study were associated with gastric cancer (Tables 1–3). The SNP rs2071504 in the exon of POLR2A gene presented a significantly reduced susceptibility to gastric cancer with the minor allele T ($p = 0.033$, aOR = 0.742, 95%CI = 0.564–0.977, Table 1). The minor allele C of the SNP rs895819 in the miR-27a gene showed a reduced risk to gastric cancer ($p = 0.037$, aOR = 0.771, 95%CI = 0.604–0.985, Table 2). Further genetic model analysis revealed that the homozygous genotype TT of rs2071504 accounted for lower proportion in cases (2.32%) than in controls (5.30%) in the codominant model and significantly reduced the risk to gastric cancer ($p = 0.040$, aOR = 0.403, 95%CI = 0.169–0.961, Table 4). The homozygous genotype CC of the variant rs895819 appeared lower predominant in cases than in controls in the codominant (2.34 vs. 7.75%) and recessive (2.43 vs. 8.40%) models and significantly reduced the risk of gastric cancer ($p = 0.003$, aOR = 0.282, 95%CI = 0.121–0.655 and $p = 0.003$, aOR = 0.289, 95%CI = 0.126–0.665, respectively, Table 4). We further performed the stratified case-only logistic regression association analysis with regard to the two SNPs in

Table 1 Association between SNPs in MicroRNA-related machinery and gastric cancer risk

Genes	SNP ID	Locations	Alleles	HWE		MAF		p Value	OR (95% CI)
				Controls	Cases	Controls	Cases		
POLR2A	rs2071504	Exon	C → T	0.178	0.834	0.205	0.161	**0.033**	**0.742 (0.564,0.977)**
	rs2228128	Exon	T → C	1.000	0.071	0.036	0.039	0.724	1.103 (0.638,1.907)
	rs2228133	Exon	T → C	0.892	0.750	0.234	0.236	0.916	1.013 (0.791,1.297)
	rs6761	3′UTR	T → C	0.902	1.000	0.437	0.430	0.793	0.972 (0.786,1.201)
Expotin-5	rs1106841	Exon	A → C	0.713	0.277	0.073	0.089	0.260	1.245 (0.849,1.825)
Dicer1	rs10139161	Intron	C → G	0.259	1.000	0.394	0.381	0.613	0.946 (0.762,1.173)

The ORs (95%CI) and p values were derived from an age- and gender-adjusted logistic regression. p values under 0.05 were indicated in bold font

Table 2 Association between SNPs in MicroRNAs and gastric cancer risk

MicroRNA	SNP ID	Alleles	HWE		MAF		*p* Value	OR (95% CI)
			Controls	Cases	Controls	Cases		
miR-27a	rs895819	T → C	1.000	0.004	0.279	0.230	**0.037**	**0.771 (0.604,0.985)**
miR-492	rs2289030	C → G	0.232	0.406	0.243	0.221	0.342	0.886 (0.691,1.137)
miR-423	rs6505162	C → A	0.425	0.286	0.191	0.201	0.632	1.067 (0.819,1.389)
miR-146a	rs2910164	C → G	0.320	0.809	0.436	0.398	0.148	0.854 (0.689,1.057)
miR-26a	rs7372209	C → T	0.084	1.000	0.275	0.277	0.950	1.008 (0.796,1.274)
miR-124	rs531564	C → G	1.000	0.290	0.147	0.163	0.406	1.130 (0.847,1.507)

The ORs (95%CI) and *p* values were derived from an age- and gender-adjusted logistic regression. *p* values under 0.05 were indicated in bold font

Table 3 Associations between SNPs in microRNA binding sites and gastric cancer risk

Genes	SNP ID	Alleles	HWE		MAF		*p* Value	OR (95% CI)
			Controls	Cases	Controls	Cases		
PYR3	rs1044129	A → G	0.367	0.233	0.421	0.407	0.603	0.945 (0.763,1.169)
PALLD	rs1071738	G → C	0.334	0.066	0.113	0.109	0.813	0.960 (0.687,1.341)
SYT9	rs4351800	A → C	0.081	0.229	0.340	0.315	0.330	0.894 (0.715,1.119)
MTRR	rs9332	C → T	0.129	0.214	0.178	0.167	0.584	0.925 (0.700,1.222)
GABRA1	rs998754	T → G	0.319	0.419	0.446	0.455	0.751	1.035 (0.838,1.277)
CDON	rs3737336	T → C	0.643	0.176	0.305	0.307	0.935	1.010 (0.804,1.267)
ZDHHC7	rs3210967	G → A	0.695	0.168	0.495	0.495	0.995	0.999 (0.810,1.232)

The ORs (95%CI) and *p* values were derived from an age- and gender-adjusted logistic regression

Table 4 Genotype distributions in gastric cancer and controls

SNP ID	Genotype	Frequency		Codominant model		Dominant model		Recessive model	
		Controls	Cases	OR (95% CI)	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*
rs2071504	CC	267	211	1		1		1	
	TC	127	83	0.827 (0.594,1.151)	0.260	0.764 (0.556,1.050)	0.098		
	TT	22	7	**0.403 (0.169,0.961)**	**0.040**			0.426 (0.180,1.012)	0.053
rs895819	TT	214	166	1		1		1	
	CT	167	122	0.942 (0.691,1.283)	0.704	0.836 (0.619,1.128)	0.241		
	CC	32	7	**0.282 (0.121,0.655)**	**0.003**			**0.289 (0.126,0.665)**	**0.003**

The ORs (95%CI) and *p* values were derived from an age- and gender-adjusted logistic regression. *p* values under 0.05 were indicated in bold font. Codominant model meant DD versus Dd versus dd. Dominant model meant (DD, Dd) versus dd. Recessive model meant DD versus (Dd, dd). D was the minor allele, and d was the major allele

different clinical pathological parameters, including tumor location, tumor size, histologic subtype, depth of invasion, lymph node metastasis and TNM stage. We found that the SNP rs2071504 showed significant associations with lymph node metastasis ($p = 0.021$, aOR = 0.529, 95%CI = 0.307–0.910) and TMN stage ($p = 0.021$, aOR = 0.532, 95%CI = 0.311–0.908, Table 5).

In this study, different databases produced various outcomes about the SNP rs2071504 in the exon of POLR2A gene. F-SNP database provided integrated information about the functional or pathological effects of SNPs collected from 16 bioinformatics tools and databases at the splicing, transcriptional, translational and posttranslational levels. It identified that the C to T variation of rs2071504 did not lead to change the protein coding, but the spicing regulation predication tool, ESEfinder, indicated that this variation affected the gene splicing. Targetscan database sorted over 900 microRNA-27a relevant conserved binding targets. dbSMR database demonstrated that microRNA-27a obviously affected expression of IRF8 mRNA (Fig. 1).

Table 5 Analysis of the polymorphisms and clinical pathological characteristics of gastric cancer

		rs2071504				rs895819		
Parameter	CC	TC + TT	OR (95% CI)	*P*	TT	CT + CC	OR (95% CI)	*P*
Tumor location								
Cardia	91	32	1		69	50	1	
Antrum	50	19	1.081 (0.556,2.100)	0.819	39	29	1.026 (0.562,1.875)	0.933
Corpus	33	19	1.637 (0.818,3.276)	0.163	29	22	1.047 (0.539,2.032)	0.892
Tumor Size								
≥5 cm	98	33	1		75	53	1	
<5 cm	79	42	1.579 (0.917,2.720)	0.100	67	51	1.077 (0.649,1.787)	0.774
Histologic type								
Tubular or Papillary	165	71	1		133	97	1	
Others	23	9	0.909 (0.401,2.063)	0.820	19	13	0.938 (0.442,1.991)	0.868
Depth of invasion								
T1	16	12	1		18	10	1	
T2	32	16	0.667 (0.255,1.740)	0.407	23	23	1.800 (0.686,4.726)	0.233
T3	137	48	0.467 (0.206,1.058)	0.068	109	72	1.189 (0.519,2.722)	0.682
Lymph node metastasis								
N0	67	39	1		61	43	1	
N1 + N2	117	36	**0.529 (0.307,0.910)**	**0.021**	84	65	0.911 (0.549,1.513)	0.719
TNM stage								
I + II	81	46	1		71	53	1	
III + IV	106	32	**0.532 (0.311,0.908)**	**0.021**	79	56	0.950 (0.580,1.556)	0.837

The ORs (95%CI) and *p* values were derived from an age- and gender-adjusted logistic regression. *p* values under 0.05 were indicated in bold font

Fig. 1 dbSMR showed that mir-27a targeted IRF8 at location 946–964 and the intramolecular structure at the target site. The variant miR-27a rs895819 affected the miRNA-binding with IRF8

Has-miR-27a targets IRF8 at location 946 to 964

miRNA:	3'	CGCCUUGAAUCGGUGACACUu
Binding Pattern:		|:|||--||-|:-|||||||-
Target site:	5'	GTGGA--TTTGTGACTGTGAg

Intramolecular Structure at Target Site

Intramolecular Structure with Ancestral Allele	..XXXXXXXXXX.XXXXX
Intramolecular Structure with Polymorphic Allele	.XXXX........XXX.X

The 'X' represents bound bases at the target site

Discussion

In this study, we performed a genetic association analysis of the variants in microRNA networks with gastric cancer. We demonstrated that rs2071504 in microRNA-related machinery genes and rs895819 in microRNA genes were significantly associated with susceptibility to the entity. None of the selected SNPs in microRNA binding sites showed associations with gastric cancer risk. To the best of our knowledge, this was the first study showing that SNPs in microRNA networks might be novel susceptibility markers linked to gastric cancer.

MicroRNAs were posttranscriptional regulators. Differentiated microRNA expression was associated with gastric carcinogenesis. Let-7f was downregulated in highly metastatic potential gastric cancer cell lines GC9811-P and SGC7901-M. Overexpression of let-7f in gastric cancer could inhibit invasion and migration of gastric cancer (Liang et al. 2011). Tseng et al. found that elevated miR-148a level in gastric cancer tissues was strongly correlated

with tumor distant metastasis, organ and peritoneal invasion and reduced survival rate (Tseng et al. 2011). POLR2A genes encoded the largest subunit of RNA polymerase II that was responsible for synthesizing microRNAs in eukaryotes. MicroRNAs bound to complementary sequences on target POLR2A mRNAs and resulted in translational repression or target degradation and gene silencing in a reverse modulatory way (Barrera et al. 2008). Previous investigations proved that antisense oligonucleotides targeted at the SNPs in POLR2A inhibited the tumor genotype-specific growth in vivo (Langley and Fidler 2007). In this study, the minor allele T of the synonymous coding SNP rs2071504 in POL2A gene conferred a decreased susceptibility to gastric cancer development. Analysis by the ESEfinder, a splicing regulation prediction tool (Smith et al. 2006), indicated that the C to T change of rs2071504 might affect the gene splicing and interrupt microRNA pairing to the 3′-untranslated regions (UTRs) of POLR2A genes and specified mRNA cleavage or repression of protein synthesis. This variation would be associated with decreased cancer risk (Reshmi et al. 2011; Brennecke et al. 2003). This protective effect of the C to T of rs2071504 polymorphism was obvious. Our clinical data provided the evidence that genotype TC + TT of rs2071504 revealed significant associations with lymph node metastasis ($p = 0.021$) and TMN stage ($p = 0.021$), suggesting that the POLR2A gene variant played an inhibitory role in gastric cancer progression. Further analysis of POLR2A gene rs2071504 would be a valuable indicator for predicting progress and development of gastric cancer, and relevant functional study needs to be performed to test this mechanism.

MiR-27a gene was located on the chromosome 19p13.13. It acted as an oncogene in the development of cancers (Ma et al. 2010). MiR-27a was upregulated in human gastric adenocarcinoma. Suppressing microRNA-27a inhibited gastric cancer cell growth (Liu et al. 2009). MiR-27a was identified as a key regulator of p44 mRNA by destabilizing the p44 subunit of the TFIIH complex during the G2-M phase, thereby modulating the transcriptional shutdown (Portal 2011). Previous studies demonstrated that mutations in microRNAs could influence microRNAs expression and function (Calin et al. 2005; Raveche et al. 2007; Duan et al. 2007). A common polymorphism (rs895819) in Mir-27a acted as an important factor of the gastric cancer susceptibility by modulating miR-27a and ZBTB10 level (Sun et al. 2010). In this study, we found that the SNP rs895819 in miR-27a showed a protective effect. The T to C change of the variant rs895819 might affect the structure of miR-27a; therefore, it would alter pre-microRNA maturation and reduce microRNA-mediated posttranscriptional suppression (Portal 2011). By means of the databases of Targetscan and dbSMR, we found that a number of conserved targets motifs were highly miR-27a dependent, of which, the variant rs895819 of miR-27a obviously affected gene splicing and expression of IRF8 mRNA. Downregulation of IRF8 expression contributed to resistance to apoptosis and to the metastatic phenotype in metastatic tumor cells (Yang et al. 2009). We supposed that genetic alternation of the SNP rs895819 in miR-27a would affect progress of gastric cancer via the IRF-8 pathway.

The limitations how to interpret the results took place in this study. Although the sample size collected was acceptable, it attributed to lower power of significance in the association analysis by the case–control study and in the stratified association analysis of the SNPs by clinical subtypes. And contribution of a genetic risk variant to disease development was often influenced by the genetic effect and the population frequency of the risk variant that often vary from population to population. This study was only dedicated to a genetic analysis in a Chinese Han population; therefore, it would be highly advisable to deeply analyze integrated effects of genetic and environment factors, such as smoking, drinking and *H. pylori* infection, and to further validate the data in a larger panel of samples from distinct populations.

In conclusion, this was the first study providing evidence that the SNP rs2071504 in the exon region of POLR2A gene conferred not only a decreased risk of gastric cancer, but also influenced lymph node metastasis and TMN stage of gastric cancer in the Chinese population. Our findings would provide novel insights into the molecular mechanisms of tumor pathogenesis and could eventually contribute to the development of early diagnosis for gastric cancer.

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