Original Article

Single nucleotide polymorphism of rs28416520 in *Piwil1* gene promoter region is associated with an increased risk of gastric cancer

LI Zhenzhen[1], ZHOU Lanting[2], ZHAI Lihong[2], XIAO Juan[2], CHENG Zhengjiang[1]

[1]*Clinical Laboratory Medicine Department, Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science, Xiangyang 441053, China;* [2]*Medical College, Hubei University of Arts and Science, Xiangyang 441053, China*

Abstract: **Objective** To analyze the correlation between the single nucleotide polymorphisms (SNPs) in the promoter of *Piwil1* gene and gastric cancer. **Methods** The expression of *Piwil1* mRNA in the tumor tissues of 3 patients with gastric cancer was detected by RT-qPCR, and RNA-Sequencing data from the Cancer RNA-Seq Nexus were analyzed for *Piwil1* mRNA expression in gastric patients. Blood samples were collected from 24 gastric cancer patients and 29 healthy control subjects for PCR amplification of *Piwil1* gene promoter region. The SNP loci in the promoter region of *Piwil1* gene were determined by direct sequencing, and the results were analyzed by SnapGene software. **Results** Analysis of the data from Cancer RNA-Seq Nexus and the results of RT-qPCR in 3 gastric cancer patients all showed significantly increased *Piwil1* expression in gastric cancer tissues compared with the adjacent tissues. Seven SNP loci in two CpG regions of the *Piwil1* gene promoter were genotyped, and only one SNP locus was found to be related to gastric cancer. The frequencies of GG, GA, and AA genotypes at the rs28416520 locus in CpG 67 region were 79.2%, 16.7%, and 4.1% in the gastric cancer group, and were 37.9%, 55.2%, and 6.9% in the control group, respectively, showing a significantly higher frequency of the GG genotype in gastric cancer group (OR=0.144, 95%*CI*: 0.045-0.564, χ^2=9.071, P<0.01). The frequency of allele G of the rs28416520 locus was significantly higher in gastric cancer group than in the control group (87.5% *vs* 65.5%; OR=0.271, 95%*CI*: 0.099-0.766, χ^2=6.856, P<0.01). The genotype or allele frequencies of the other 6 SNPs locus did not differ significantly between gastric cancer group and control group. **Conclusions** The expression of *Piwil1* is increased in gastric cancer tissues as compared with the adjacent tissues. The GG genotype and G allele of rs28416520 within CpG 67 region are associated with an increased risk of gastric cancer.

Keywords: gastric cancer; *Piwil1* gene; single nucleotide polymorphisms; CpG

INTRODUCTION

Gastric cancer is the fifth most common cancer worldwide and the third leading cause of cancer-related mortality, causing 723 000 deaths each year [1, 2]. Gastric cancer is often asymptomatic in the early stages, and roughly 80% to 90% of the patients are diagnosed at advanced stages. Some of the patients can experience relapses soon after surgery and distant metastasis, which is the leading cause of death in gastric cancer patients[3]. It is generally believed that the occurrence of gastric cancer is due to the dysfunction or epigenetic changes in the oncogenes and tumor suppressor genes, and exploration of molecular mechanism underlying gastric carcinogenesis and metastasis has become a hotspot in gastric cancer studies[4].

Tumor cells are characterized by their self-renewal and infinite proliferation capacities and strong migration ability that promotes tumor metastasis[5]. PIWI proteins are a subclass of the Argonaute family, expressed mainly in germline cells. PIWI binds with PIWI-interacting RNA (piRNA) to maintain the genomic integrity by regulating the transposons, thus playing a key role in sperm development and embryogenesis [6]. A recent report suggests that *Piwil1* plays a vital role in tumor cell proliferation, growth, and differentiation, and its expression increases progressively during the development of gastric cancer[7]. Studies have shown that the expression of *Piwil1* is significantly higher in tumor tissues than in adjacent tissues, and a low *Piwil1* expression is associated with a significantly improved overall survival of the patients [8-10]. Currently the mechanism of *Piwil1* overexpression in gastric cancer has not been clarified. Gene promoters play an indispensable role in gene transcription initiation, and their mutations often lead to abnormal gene expression[11]. In this study, we aimed to examine the single nucleotide polymorphism (SNP) in the promoter of *Piwil1* gene and explore how the SNPs correlate with carcinogenesis of gastric cancer.

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Corresponding author: CHENG Zhengjiang, PhD, E-mail: zjcheng11@163.com

METHODS

Blood and tissue sample collection

This study was performed under approval by the Ethics Committee of Xiangyang Central Hospital, and informed consents to participate in this study were obtained from all the participants. Anticoagulated blood samples with sodium citrate were collected from 24 patients with gastric cancer and 29 healthy individuals in Xiangyang Central Hospital between February, 2019 and March, 2020, and were stored at −80 ℃ before analysis. The mean age of the patients with gastric cancer and the normal control subjects were comparable (65.5±2.49 *vs* 65.9 ± 1.65 years, P>0.05). The gastric cancer group consisted of 17 male and 7 female patients, and the healthy control group included 19 male and 10 female subjects; the gender distribution was similar between the two groups (χ^2=0.170, P>0.05). Gastric cancer tissue and adjacent tissue specimens were collected during surgery from 3 of the patients diagnosed in 2019, frozen in liquid nitrogen and stored at −80 ℃. For all the patients with gastric cancer, the diagnoses were established histopathologically by the Department of Pathology of Xiangyang Central Hospital.

Reagents

The reagents for tissue lysis and genomic DNA extraction (including 10 mmol/L Tris, 10 mmol/L EDTA, 0.5% SDS, saturated NaCl, and proteinase K) were all routine laboratory reagents. Taq enzymes used for PCR amplification were purchased from Nanjing Novizan (2× Phanta Max Master Mix kit, P515-01). First-strand cDNA synthesis kit was from TOYOBO Biotech (Japan). The real-time quantitative detection reagent TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) was from Takara Biomedical Technology (Japan).

Extraction of genomic DNA

After thawing at 37 ℃, 200 μL of the blood samples was lysed in a centrifuge tube by adding 300 μL of the lysis reagent and digested at 55 ℃ for 3 h, followed by addition of 30% saturated NaCl and centrifugation at 12 000 r/min for 20 min at 4 ℃. The supernatant (150 μL) was transferred to a 1.5-mL centrifuge tube, in which 1 mL absolute ethanol was added and thoroughly mixed. After centrifugation at 12 000 r/min for 10 min at 4 ℃, the supernatant was discarded and 1 mL of 70% ethanol was added. After washing once, the tube was centrifuged at 12 000 r/min for 5 min at 4 ℃ and the supernatant was removed. The tube was inverted and allowed to dry at room temperature. An appropriate amount of double-distilled water was added into the centrifuge tube to dissolve DNA, and the concentration of the DNA sample was determined using Epoch™ microplate spectrophotometer (BioTek Instruments, Inc.). The samples were stored at −20 ℃ until use.

PCR and genotyping

The PCR primers for amplification of CpG 43 and CpG 67 in *Piwil1* promoter region were designed using the software Primer 5.0. The sequences of the primers are listed below:

CpG-43: 5'-CGGCCACATCAGAAACCGC-3'(forward),
5'-CTCGCCCTAGTCCTGGTCCTTG-3'(reverse);
CpG-67: 5'-GAGGCTGAGGCCTGAGTTGC-3' (forward),
5'-CACTCGCTGGAAATCACCTCT-3' (reverse).

All the primers were synthesized by TingKe Biological Technology. The PCR reaction system for amplification of CpG 43 and CpG 67 alleles consisted of 25 μL DNA polymerase mixture, 2 μL forward primer, 2 μL reverse primer, 1 μL genomic template, and 20 μL double-distilled water. PCR amplification was performed with initial pre-denaturation at 95 ℃ for 3 min followed by 35 thermal cycles of denaturation at 95 ℃ for 15 s, annealing at 60 ℃ for 15 s and extension at 72 ℃ for 30 s, with a final extension at 72 ℃ for 5 min. The PCR products were subjected to gel electrophoresis and sent to TingKe Company for sequencing.

Gastric cancer RNA-seq and dbSNP databases

The expression of *Piwil1* was analyzed based on gastric cancer RNA-seq data retrieved from Cancer RNA-Seq Nexus (http://syslab4.nchu.edu.tw/index.jsp). The alleles of rs28416520 in dbSNP database were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/snp/rs28416520#frequency_tab).

RNA extraction and reverse transcription reaction

Tissue homogenate was prepared on ice using a homogenizer with an appropriate amount of lysate. The total RNA was extracted using RNAsimple Kit (TianGen Biotech, Beijing, China) following the manufacturer's instructions. The concentration of the extracted RNA was measured with a spectrophotometer. Reverse transcription was performed using first-strand cDNA synthesis kit (Toyobo, Japan) in the following system: 1 μg total RNA, 4 μL reaction buffer (5×), 2 μL dNTP mixture, 1 μL RNase inhibitor, and 1 μL Oligo dT, and 1 μL reverse transcriptase in a total volume of 20 μL. All the samples were heated to 42 ℃ for 20 min, and the reaction was terminated by heating at 85 ℃ for 5 min. The obtained cDNA was diluted for use or stored at −20 ℃.

Real-time quantitative PCR for detecting Piwil mRNA expression

The standard two-step PCR was performed on ABI StepOne plus real-time quantitative PCR instrument using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus). The reaction was performed with pre-denaturation at 95 ℃ for 30 s followed by 40 cycles of denaturation at 95 ℃ for 30 s and annealing at 60 ℃ for 30 s. The primers used for real-time quantitative PCR detection of *Piwil1* were:

5'-CATGCTCTGCACTGACGTT-3' (forward),
5'-CTTGGTTGTATTGCTTCCTGT-3' (reverse).

Statistical analysis

GraphPad Prism 7.0 was used for statistical analysis of the data. Hardy-Weinberg equilibrium (HWE) was calculated using Chi-square (χ^2) test. Logistic regression analysis was used to calculate the odd ratio (OR) and 95% confidence interval (*CI*) of the genetic polymorphism in healthy and gastric cancer groups. The difference in gene expression was tested by a two-tailed, unpaired Student's *t*-test, and *P*<0.05 was considered to indicate a statistically significant difference.

RESULTS

Expression of Piwil1 in gastric cancer

We analyzed gastric cancer RNA-seq data from Cancer Transcriptome Analysis Website (Cancer RNA-Seq Nexus). The results showed that the expression of *Piwil1* increased significantly in gastric cancer tissues at all clinical stages as compared with normal tissues (Fig.1). We detected *Piwil* mRNA expression in gastric cancer tissues from 3 patients using real-time quantitative PCR, and the results showed a significantly increased *Piwil* mRNA expression in the tumor tissues as compared with the adjacent tissues (Fig.1B).

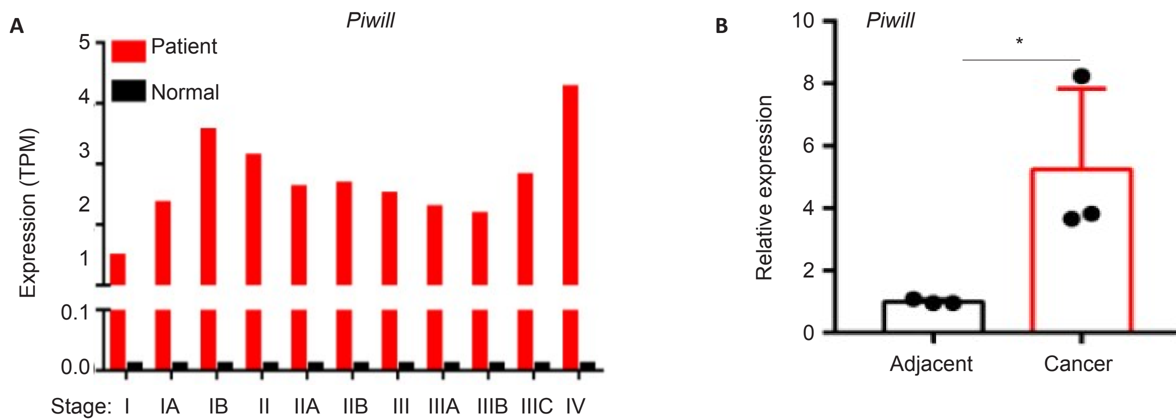

Fig.1 Expression of *Piwil1* in gastric cancer database and in gastric cancer tissues from 3 clinical patients. **A**: *Piwil1* expression in gastric cancer RNA-seq database; **B**: Real-time quantitative PCR for detecting *Piwil1* expression in tumor tissues and adjacent tissues from 3 gastric cancer patients. **P*<0.05.

PCR amplification of CpG 43 and CpG 67 alleles in Piwil1 gene promoter

The CpG 43 and CpG 67 alleles in the *Piwil1* promoter region were amplified by PCR using specific primers. The PCR product of CpG 43 region was 312 bp in length, and that of CpG 67 was 647 bp in length (Fig.2). Gel electrophoresis of the PCR product showed a single target band of the expected size. The product was sent directly for Sanger sequencing.

SNP loci in CpG 43 and CPG 67 and their correlation with gastric cancer

We analyzed SNP loci in CpG 43 and CPG 67 alleles in the *Piwil1* promoter region in 24 patients with gastric

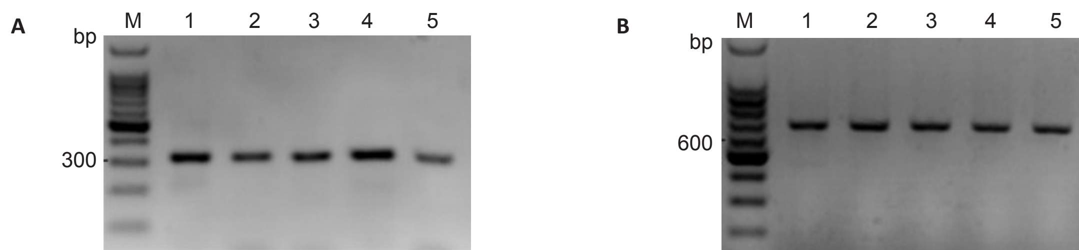

Fig.2 PCR products of CpG 43 and CpG67 in *Piwil1* promoter amplified from gastric cancer tissue samples. **A**: Gel electrophoresis of PCR products of CpG 43 allele. M: Marker (100 bp DNA Ladder); Lanes 1 to 5 are PCR products of CpG 43 region from different samples. **B**: Gel electrophoresis of PCR products of CpG 67 allele. M: Marker (100 bp DNA Ladder); Lanes 1 to 5 are PCR products of CpG 67 region from different samples.

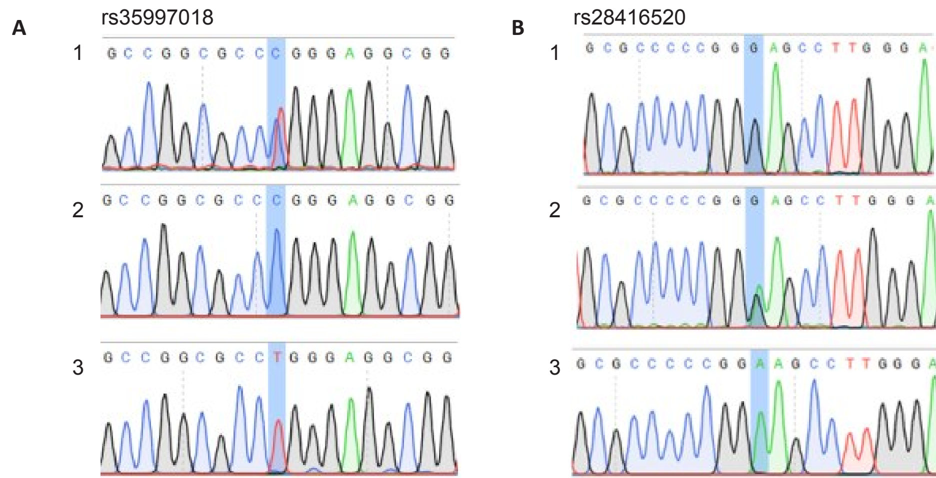

Fig.3 SNP of rs34990411 and rs28416520. **A**: Genotypes of rs35997018 (1: Genotype CT; 2: Genotype CC; 3: Genotype TT); **B**: Genotypes of rs28416520 (1: Genotype GG; 2: Genotype GA; 3: Genotype AA).

cancer and 29 healthy control subjects. We detected two CpG island regions in the *Piwil1* promoter region, and the distributions of the genotypes and alleles in the two groups are shown in Tab.1 and Tab.2.

Tab.1 Genotypes and alleles of the CpG 43 SNP locus in gastric cancer and control groups [*n* (%)]

CpG43 SNP	Model	Genotype	Gastric cancer (*n*=24)	Control (*n*=29)	OR (95%*CI*)	*P* value	χ^2
rs34990411	Co-dominant	GG	8(33.3)	8(27.6)	1		
		GT	11(45.9)	15(51.7)	0.733 (0.207-2.535)	0.626	0.236
		TT	5(20.8)	6(20.7)	0.833 (0.156-3.953)	0.816	0.053
		GT+TT	16(66.7)	21(72.4)	0.761 (0.251-2.299)	0.65	0.205
	Allele	G	27(56.2)	31(53.4)	1		
		T	21(43.8)	27(46.6)	0.893 (0.408-1.925)	0.773	0.083
rs79528159	Co-dominant	GG	22(91.7)	28(96.6)	1		
		GT	2(8.3)	1(3.4)	2.545 (0.277-38.02)	0.443	0.586
	Allele	G	46(95.8)	57(98.3)	1		
		T	2(4.2)	1(1.7)	2.478 (0.279-36.46)	0.45	0.569
rs182955466	Co-dominant	GG	21(87.5)	27(93.1)	1		
		GT	3(12.5)	2(6.9)	1.929 (0.362-11.49)	0.487	0.482
	Allele	G	45(93.8)	56(96.6)	1		
		T	3(6.2)	2(3.4)	1.867 (0.366-10.81)	0.498	0.458
rs147087823	Co-dominant	CC	23(95.8)	29(100)	1		
		CT	1(4.2)	0(0)	Infinity (0.134-Infinity)	0.267	1.232
	Allele	G	47(97.9)	58(100)	1		
		T	1(2.1)	0(0)	Infinity (0.134-Infinity)	0.269	1.22
rs35997018	Co-dominant	CC	17(70.8)	13(44.8)	1		
		CT	6(25)	13(44.8)	0.352 (0.112-1.218)	0.086	2.94
		TT	1(4.2)	3(10.4)	0.254 (0.018-1.953)	0.233	1.421
		CT+TT	7(29.2)	16(55.2)	0.334 (0.108-1.026)	0.057	3.616
	Allele	C	40(83.3)	39(67.2)	1		
		T	8(16.7)	19(32.8)	0.410 (0.162-1.055)	0.058	3.583

Tab.2 Genotypes and alleles of the CpG 67 SNP locus in gastric cancer and control groups [*n* (%)]

CpG67 SNP	Genotype/Allele	Gastric cancer (*n*=24)	Control (*n*=29)	OR (95%*CI*)	*P* value	χ^2
rs28416520	Genotype					
	GG	19 (79.2)	11 (37.9)	1		
	GA	4 (16.7)	16 (55.2)	0.144 (0.045-0.564)	0.002	9.071
	AA	1 (4.1)	2 (6.9)	0.289 (0.019-2.813)	0.310	1.028
	GA+AA	5 (20.8)	18 (62.1)	0.160 (0.050-0.552)	0.002	9.090
	Allele					
	G	42 (87.5)	38 (65.5)	1		
	A	6 (12.5)	20 (34.5)	0.271 (0.099-0.766)	0.008	6.856
rs375886343	Genotype					
	CC	23 (95.8)	29 (100.0)	1		
	CT	1 (4.12)	0 (0.0)	Infinity (0.134-Infinity)	0.267	1.232
	Allele					
	C	47 (97.9)	58 (100.0)	1		
	T	1 (2.1)	0 (0.0)	Infinity (0.134-Infinity)	0.269	1.220

The results of sequencing revealed 5 SNPs in the CpG 43 region. The frequency of CT + TT genotype of rs35997018 locus differed significantly between the gastric cancer group and the control group (29.2% *vs* 55.2%; OR=0.334, 95% *CI*: 0.108-1.026; χ^2=3.616, *P*=0.057). The frequencies of rs35997018 locus alleles C and T were 83.3% and 16.7% in gastric cancer group, similar to those of 67.2% and 32.8% in the control group, respectively (OR=0.41, 95% *CI*: 0.162-1.055; χ^2=3.583, *P*=0.058). The sequence of SNP rs35997018 is shown in Fig.3A. There were no significant differences in the genotype or allele frequencies of the other 4 SNP loci in the CpG 43 region, indicating that these SNP loci were not associated with gastric cancer (Tab.1).

There were two SNPs in the CpG 67 region. The frequency of rs28416520 genotype GA + AA was significantly lower in the gastric cancer group than in the normal control group (20.8% *vs* 62.1%; χ^2=9.090, *P*<0.01), and the frequency of rs28416520 allele G was significantly higher in gastric cancer group than in the control group (87.5% *vs* 65.5% ; χ^2=6.856, *P*<0.01), indicating that rs28416520 mutation may increase the risk of gastric cancer. We then compared the frequency of rs28416520 allele G with the data in dbSNP, and found that the mean frequencies of the alleles G were 87.5% in gastric cancer patients and 65.5% in control groups, respectively, similar to those found in Vietnamese and Korean cohorts (Tab.3). The sequence of SNP rs28416520 was shown in Fig.3B. The genotype or allele distributions of the SNP locus rs375886343 in the CpG 67 region were not significantly different between the 24 patients with gastric cancer and 29 healthy control subjects analyzed in this study (Tab.2).

DISCUSSION

As the third leading cause of cancer-related death worldwide, gastric cancer has a high incidence and often a poor prognosis of the patients[13-15]. Currently the role of *Piwil1* in the pathogenesis of gastric cancer remains unclear. *Piwil1* is a member of the PIWI subfamily of human AGO proteins, which regulates gene expressions by binding to piRNAs through the regulatory mechanisms including transposon silencing [16], gene silencing[17], DNA methylation [18], translation inhibition or activation [19, 20]. Recent studies suggested the involvement of the PIWI subfamily in the occurrence, progression, and prognosis of many malignancies[21]. Our analysis of the transcriptome data of Cancer RNA-Seq Nexus and the results of *Piwil1* mRNA detection in gastric cancer patients by real-time quantitative PCR all confirm that the expression of *Piwil1* is increased in gastric cancer, as is consistent with the results of previous studies[22].

We analyzed the SNP loci of CpG 43 and CpG 67 in *Piwil1* gene promoter region, and found that only the rs28416520 site was associated with the risk for gastric cancer. The SNPs of CpG 43 region were not found to be a risk factor for gastric cancer, but the frequencies of rs35997018 genotype CC + CT and the allele T were significantly lower in gastric cancer group than in the control group, indicating a possible association of the rs35997018 locus with the risk for gastric cancer. The other SNP loci of CpG 43, including rs79528159, rs182955466, rs147087823 and rs34990411, were not significantly different between the two groups. We identified two SNP loci in CpG 67 in the promoter region of *Piwil1* gene. Among them, the frequencies of rs28416520 genotype GG and allele G were significantly higher in gastric cancer patients than in the control group, suggesting a high likelihood that both rs28416520 genotype GG and allele G are high-risk factors for gastric cancer. The other SNP locus in CpG 67, rs375886343, was not found to correlate with the risk of

Tab.3 Frequencies of alleles of rs28416520 in dbSNP database

Study	Sample size (n)	G allele frequency (%)	A allele frequency (%)
Our data (gastric cancer)	24	0.875	0.125
Our data (control)	29	0.655	0.345
Vietnamese	216	0.722	0.278
KOREAN	2910	0.711	0.289
1000 Genomes	5008	0.680	0.320
TOPMED	125568	0.646	0.354
GnomAD	31184	0.629	0.371
ALFA	17772	0.614	0.386
Northern Sweden	600	0.565	0.435
ALSPAC	3854	0.559	0.441
TWINSUK	3708	0.554	0.446
Qatari	216	0.523	0.477
Estonian	4480	0.503	0.497
GENOME_DK	40	0.50	0.50
Siberian	32	0.47	0.53
SGDP_PRJ	280	0.368	0.632

gastric cancer. But given the small sample size in this study, further validation of these findings in studies with a larger sample size is needed.

The methylation of the CpG region may also regulate the expression of *Piwil1* [23, 24]. Our results indicate that the G mutation at the rs28416520 locus is a high-risk mutation in gastric cancer. G mutation at this site is associated with a greater chance of DNA methylation modification, but the relationship between the SNP mutation and DNA methylation in this region awaits further investigation. In addition, SNP loci can also occur in the coding region of the *Piwil1* gene, which is closely related to the occurrence of diseases [25] and needs to be examined in future studies. Given the heterogeneity of tumor [26], the correlation between the type of SNP and the expression level of *Piwil1* should be explored to determine whether a high-risk SNP mutation of gastric cancer is associated with an increased *Piwil1* expression.

Conclusion

We found significantly increased expression of *Piwil1* gene in gastric cancer tissues. Our results suggest that 5 SNPs of CpG 43 region within *Piwil1* gene promoter are not associated with the risk of gastric cancer, but the genotype GG and allele G mutations of rs28416520 at CpG 67 are associated with an increased risk of gastric cancer.

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胃癌中*Piwil1*基因启动子区rs28416520位点单核苷酸多态性及其临床意义

李珍珍[1],周兰庭[2],翟立红[2],肖 娟[2],程正江[1]
[1]湖北文理学院附属医院//襄阳市中心医院检验科,湖北 襄阳 441053;[2]湖北文理学院医学院,湖北 襄阳 441053

摘要:目的 分析*Piwil1*基因启动子区域的单核苷酸多态性(SNP)和胃癌的相关性。**方法** 利用实时定量PCR检测3例胃癌患者肿瘤组织中*Piwil1* mRNA的表达,并通过肿瘤转录组测序数据库分析胃癌组织中*Piwil1* mRNA的表达水平。采用PCR克隆出*Piwil1*基因启动子区的序列,直接测序法测定24例胃癌患者和29例健康对照者的基因多态性,并使用SnapGene软件对测序结果进行分析。**结果** 对Cancer RNA-Seq Nexus数据库查询结果和3例胃癌患者肿瘤组织和癌旁组织的实时定量PCR检测结果进行分析,发现胃癌组织中*Piwil1*基因表达量较癌旁组织高。检测到*Piwil1*基因启动子两个CpG区域的7个SNP位点,发现仅有一个SNP位点和胃癌有相关性。CpG 67区域rs28416520位点基因型GG、GA、AA在胃癌组中的频率分别为79.2%、16.7%、4.1%,在健康对照组中分别为37.9%、55.2%、6.9%,胃癌组GG基因型频率显著高于对照组(OR=0.144,95%*CI*:0.045~0564,$\chi^2=9.071$,$P<0.01$)。胃癌组rs28416520等位基因G的频率显著高于对照组(87.5% *vs* 65.5%;OR=0.271,95%*CI*:0.099~0.766, $\chi^2=6.856$,$P<0.01$)。其它6个SNP位点在胃癌组和正常组之间没有显著差异。**结论** *Piwil1*基因启动子CpG 67区域rs28416520位点基因型GG和等位基因G与胃癌发病风险升高相关,且*Piwil1*基因在胃癌肿瘤组织中表达升高。

关键词:胃癌;*Piwil1*基因;单核苷酸多态性;CpG

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作者简介:李珍珍,初级检验师,E-mail: 15123853369@163.com
通信作者:程正江,博士,教授,主任技师,E-mail: zjcheng11@163.com