BRITISH JOURNAL OF BIOMEDICAL SCIENCE, 2016
VOL. 73, NO. 4, 201–203
http://dx.doi.org/10.1080/09674845.2016.1233790

BIOMEDICAL SCIENCE IN BRIEF

miR-146a gene polymorphism and susceptibility to gastric cancer

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Article History Received 18 April 2016; Accepted 9 August 2016

Keywords Gastric cancer; SNP; rs2910164; miR-146a

Gastric cancer is the second most frequent malignancy in the world with approximately 989,600 newly diagnosed patients and 738,000 annual deaths.[1,2] The gastrointestinal (GI) cancers are the most common cancers in Iranian men and second-most common cancer in women, and are responsible for approximately half of all cancer-related deaths with 7300 new cases diagnosed annually.[3] Genetic variations in the genome, such as single nucleotide polymorphisms (SNPs) are important in susceptibility to cancer.[4] Furthermore, with the growth of the emerging field of post-transcriptional regulation in recent years, attention to biogenesis and function of microRNA (miRNA) has increased. miRNAs are endogenous, non-small coding RNAs, 18–23 nucleotides in length that bind to 3′-UTR of target mRNA and inhibit gene expression.[5]

Previous investigations on *miR-146a*, an important cancer-related miRNA, showed that expression alters in breast, colorectal and prostate cancers.[6–8] The rs2910164 polymorphism is associated with gastric cancer risk.[9,10] *miR-146a* is a key regulator of NF-κB pathway that exerts its effect by binding to IRAK1 and TRAF6 mRNAs.[11] *Pre-miR-146a* C/G SNP (rs2910164) is located in the stem region of *pre-miR-146a* and may potentially affect the expression level of *miR-146a*.[12]

To test the hypothesis of a link between *miR-146a* and gastric cancer, we determined the genotype of 120 patients with this disease, using 120 healthy individuals as controls. The control group included 90 men and 30 women with an average age of 60.5. The patients were 88 men and 32 women with an average age of 61.5. Subjects were recruited from the public hospitals affiliated to Shiraz University of Medical Sciences, Iran. The patients were confirmed to have gastric cancer by standard histological techniques. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences. The healthy control subjects had no family history of cancer or autoimmune diseases, and were matched for age, gender and ethnicity with gastric cancer patients. Tissue samples were collected from participants after obtaining informed consent. Patients' clinicopathological information including differentiation grade, vascular, lymphatic and perineural invasion were obtained from 40 clinical and pathological records. In connection with the vascular, lymphatic and perineural invasion we had access to information from 69, 75 and 55 individuals, respectively.

DNA from tissue sample was extracted using salting out method and was kept at 4 °C.[13] Genotyping was performed using polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) assay. The 147bp DNA fragments containing the target polymorphic site in gene (rs2910164) were amplified using the following primers: forward primer 5′-CATGGGTTGTGTCAGTGTCAGAGCT-3′ and 5′-TGCCTTCTGTCTCCAGTCTTCCAA-3′ reverse primer.[14] Briefly, 20µl PCR mixture containing 300 ng of genomic DNA, with 0.5pmol of both primers, 2µl of 10X PCR buffer, 3.5 mM $MgCl_2$, 0.5 mM of 10 µmol dNTP mix and 1UTaq DNA polymerase. PCR was performed with initial denaturation of 95 °C for 5 min followed by 35 cycles of 95 °C for 30 S, 57 °C for 30 S and 72 °C for 30 S with a final extension at 72 °C for 5 min. PCR product was digested with 3U of restriction enzyme *Sac* I, at 37 °C overnight and then separated on a 3% agarose gel. The intact PCR product with the size of 147 bp indicated that the wild type homozygous CC genotype is present. Digestion of PCR product by *Sac* I yielded two bands with the size of 122 bp and 25 bp. However, the 25 bp bands was not strong to enough to detect on agarose gel, so we detected the mutant homozygous GG genotype as a 122 bp fragment and heterozygote CG genotypes as a two bands with the fragment sizes of 147 bp and 122 bp (Figure 1).

Differences in frequency of rs2910164 genotypes between patients and control subjects were compared

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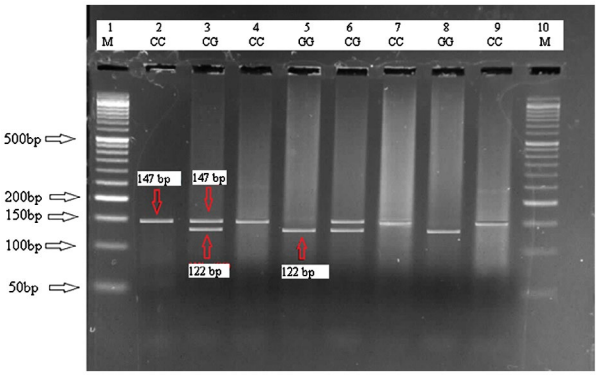

Figure 1. The PCR-RFLP results of 8 samples with rs2910164 polymorphism of miR-146a gene.

by χ^2*test*. Deviation of the observed genotype distribution from Hardy–Weinberg equilibrium was analyzed by a chi-square goodness of fit test. The odd ratio (OR) and 95% confidence interval (CI) were used to survey the level of strength of variant genotypes and risk of gastric cancer. To test the dominant model of inheritance, we improved our analysis by combining the GG and CG vs. CC genotype groups. The wild type genotype CC was used as reference and *P-value* < 0.05 considered as significant limit in all tests. All calculation statistics were performed by SPSS version 18.00 (SPSS Inc, Chicago IL, USA).

No statistically significant differences were observed in the age and sex between case and control groups based on the calculated *p-value* $p = 0.51$, $p = 0.77$, respectively. The distributions of *miR-146a* polymorphism genotype in patient and control groups are listed in Table 1. Genotype frequency is according to Hardy-Weinberg in both groups (Table 1). In relation to the distribution of genotypes according to $p = 0.91$ and $\chi^2 = 0.18$, have no different genotype distribution between two groups. We compared G allele frequency between two groups, finding *P-values* and χ^2 of 0.18 and 1.36, respectively. Due to large *P*-value ($p > 0.05$), the G allele frequencies did not differ between two groups.

The correlation of the genetic change effect with gastric cancer risk was calculated using *P-value* and OR. GG, GC and CC genotypes (The wild-type homozygote CC genotype were being used as a reference genotype) are listed in Table 1. There is no link between genotype and gastric cancer risk.

To study the effect of age on genotype, we divided the population into two groups, which include over 60 years and less than 60 years. The results of *P-value* and OR for individuals aged ≥ 60 years are 0.67 and 1.19, for individuals aged <60 years are 0.4 and 1.4, respectively. In the control group, 16 women and 65 men were CC genotype and in the cancer group, 12 women and 61 men were CC genotype. *P-value* and OR in men were 0.67 and 1.15, in women are 0.2 and, 0.39, respectively. Thus there is no association between the rs2910164 polymorphism and gender. The effect of rs2910164 polymorphism in

Table 1. Genotype distribution of cancer and control population and estimate risk of cancer with variant genotype.

Genotype	Case (%)	Control (%)	OR (95%)	*P-value*
CC	73 (60.83)	81 (67.5)	1.00	–
CG	38 (31.67)	34 (28.33)	1.90 (0.64–6.2)	0.23
GG	9 (7.5)	5 (4.17)	1.6 (0.49–5.27)	0.43
CG+GG	47 (39.17)	39 (32.5)	1.00 (0.2–50.39)	0.90
C-allele	184 (76.67)	196 (81.67)	–	–
G-allele	56 (23.33)	44 (18.33)	–	–

Table 2. Association between variant miR-146a genotypes and clinicopathological characteristics.

Variable	CG+GG	CC	OR (95%CI)	*P-value*
Tumour differentiation				
Well	15	38	1.00	–
Moderate	8	30	0.60 (0.20–1.90)	0.64
Poor	6	23	0.90 (0.29–3.20)	0.45
Vascular invasion				0.93
Positive	4	15	0.94 (0.26–3.40)	
Negative	11	39	1.00	
Lymphatic invasion				0.73
Positive	16	30	1.18 (0.5–3.20)	
Negative	9	20	1.00	
Perineural invasion				0.66
Positive	7	20	1.20 (0.36–4.47)	
Negative	6	22	1.00	

miR-146a was also evaluated with tumour stage and progressions consist of tumour differentiation, lymphovascular and perineural invasion. SNP rs2910164 genotype polymorphism in *miR-146a* has no effect on tumour progression stage (Table 2). It is noteworthy that in relation to the characteristics of the tumour, we did not have access to all patients' information, and the analysis was done on the basis of available information.

Many human disorders are associated with changes in the genome containing SNPs. The roles of SNPs have been investigated in development and progression of many diseases including various types of cancers.[15]

In this study, we investigated the effect of rs2910164 polymorphism in *pre-miR-146a* and risk of gastric cancer in a population. The rs2910164 polymorphism in *miR-146a* can alter the C: U to G: U mismatch and this change can influence the processing stages of *pre-miR-146a* to mature *miR-146a* and level expression (8). The relationship between the rs2910164 polymorphisms and cancer risk is under-explored. Chae et al. observed that C > G polymorphism increased colorectal cancer risk in a Korean population [7] whilst Shen et al. [6] in studied the effect of C > G rs2910164 SNP polymorphism in the breast and ovarian cancers and found patients with mutant allele G predispose to earlier age of breast and ovarian cancers. However, Hu et al. and Catucci et al. [16,17] found no link between rs2910164 polymorphism and risk of breast and ovarian cancers. Our data found no significant differences between allele distribution in patients with gastric cancer and control subjects. A similar result was also

derived from the study of breast cancer.[18] The diversity of results in C > G rs2910164 genotype distribution between patients and control groups in different populations might be due to differences in race, sample sizes and environmental conditions.

We studied the relationship between genotype and cancer. Finding no relationship between the rs2910164 polymorphism of *miR-146a* gene and risk of gastric cancer. This is similar to the result of C/G polymorphism in *miR-146a* gene and risk of cancer in a Chinese population.[19] However, investigations performed on gastric cancer [9,20] showed that the rs2910164 polymorphism increased the risk of gastric cancer in Japanese and Chinese populations. Our analysis on the rs2910164 polymorphism of *miR-146a* mutation did not show any association between gastric cancer risk with age and gender. We also found that the rs2910164 polymorphism of *miR-146a* gene mutation did not have any association with sex and high risk of gastric cancer in men. We also studied the effect of *miR*-146a mutation on tumour progression and so examined relationships between rs2910164 polymorphism and tumour differentiation, lymphatic and vascular invasion. Stratified analysis showed no significant association between variant genotypes and clinicopathological parameters. Similar results [20] reported no significant relationship between rs2910164 polymorphism and clinicopathological characteristics of gastric cancer.

This work represents an advance in biomedical science because it studies the effect of genetic changes in the risk of gastric cancer, and so this SNP cannot be used as a marker to identify individuals at high risk for stomach cancer.

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