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miR-581-Related Single Nucleotide Polymorphism, rs2641726, Located in MUC4 Gene, is Associated with Gastric Cancer Incidence

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Abstract MUC4 is aberrantly expressed in several carcinomas including breast, colon, ovarian, lung, prostate, stomach and pancreatic cancers. MUC4 can regulate cell apoptosis negatively and facilitate stomach tumorigenesis. In this research, we aimed to evaluate the possible association between rs2641726 (C > A) polymorphism of MUC4 and gastric cancer risk in the Iranian population. In this case-control study, we collected blood samples from 168 gastric cancer patients and 66 healthy subjects. Allele-specific primer polymerase chain reaction method was applied to genotype rs2641726 in the obtained DNA samples. This study demonstrated that rs2641726 C allele was significantly associated with the incidence of gastric cancer, odds ratio = 3.382, 95% confidence interval: 1.840–6.217 (P < 0.001). Furthermore, the distribution of this risk allele was highly enriched in the samples with stage III. In silico studies revealed that the C allele of rs2641726, located within MUC4 3'UTR, is potential to attenuate the interaction between miR-581 and MUC4 mRNA. This disturbing effect, which might result in higher expression of MUC4 oncoprotein, was proposed for the mechanism of action of the rs2641726 risk allele. rs2641726 C allele is significantly enriched in gastric cancer specimens. The attenuating effect of this allele on miR-581 and MUC4 interaction might be a potential mechanism of action by which C allele imposes its oncogenic impact.

Keywords rs2641726 · miR-581 · MUC4 · Gastric cancer · Single nucleotide polymorphism

Introduction

Gastric cancer is considered as the fourth most common cancer worldwide [1]. Gastric cancer is often asymptomatic at early stages and is diagnosed in advanced stages. Hence in most cases, gastric cancer patients have an average survival rate of 7–9 months [2]. Although early stages gastric cancer is surgically curable, early detection with accurate diagnosis rarely occurs. Meanwhile, high rate of gastric cancer incidence has reported in Japan, China, Venezuela, and Chile [3].

Various genetic and environmental parameters are important in gastric cancer etiology, among which Helicobacter pylori (H. pylori) is one of the most important ones. H. pylori is a gram-negative bacillus, mainly responsible for the development of gastric cancer. H. pylori infection could affect cellular signaling pathways increasing the rate of cancer incidence in 65–80% of gastric cancer patients [4, 5].

Mucins are one of the substantial family of proteins playing role in stomach tumorigenesis. Mucins are glyco-proteins with high molecular weight, responsible for the
MUC4 is a membrane-bound member of mucin family, negatively regulating cell apoptosis via both ErbB2-dependent and independent mechanisms. Due to its signaling and anti-adhesive properties, MUC4 is aberrantly expressed in several carcinomas; therefore, it could be accounted as a tumor biomarker [6, 7]. Variations in different parts of MUC4 gene have not been studied in gastric cancer. It has been shown that rs863582, rs842226, rs2550236, rs842225, and rs2688515 single nucleotide polymorphisms (SNPs) have an adverse effect on lung cancer in the Chinese population [8]. Furthermore, rs882605 and rs1104760 have been reported to be important in endometriosis development [9]. However, no study has been conducted to investigate the role of MUC4 SNPs in gastric cancer. Among different polymorphisms, SNPs located at miRNA target sites may have an effect on mRNA stability and/or expression [10–14]; and therefore, they could be more functional.

In this research, we have evaluated the possible association between a miRNA-related rs2641726 polymorphism (C > A), located in 3’UTR region of MUC4, and gastric cancer risk in Iranian population, along with association studies with metastasis, H. pylori infection, stage and lymph nodes invasion.

Materials and Methods

Study Subjects

234 peripheral blood samples from patients with gastric cancer (168 samples) and healthy controls (66 samples) from August 2015 and August 2016 were collected. The mean ± standard deviation (SD) of controls and cases were 43.3 ± 11.3 and 51 ± 15.7, respectively. The inclusion criterion for recruiting the gastric cancer samples was patients diagnosed with gastric cancer. The exclusion criterion for both groups was having any acute or chronic inflammatory disease, as they can modify MUC4 expression independently. Moreover, the control samples with any history of gastric cancer were excluded. In this study, gastric cancer samples were definite after clinical examination. All the donors, including the patients and healthy participant, were informed clearly and provided with a written consent.

All procedures performed in studies involving human participants were in accordance with the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran, regarding the 1964 Helsinki declaration.

SNP Genotyping

DNA was isolated from whole peripheral blood samples by using PrimePrep Genomic DNA Isolation kit (GeNetBio, Chungnam, South Korea), according to the producer’s protocol. The extracted DNA was diluted in 0.5 M TE and then stored at −20 °C. Allele-specific primer polymerase chain reaction (ASP-PCR) assay was applied to genotype SNP. SNP genotyping was performed using common forward: 5’-CTAGGCTACCTCAAGAGTCACCTCATC-3’, specific reverse (SNP): 5’-GGCCATCACCACATTATGAACTTG-3’ and specific reverse (wild-type): 5’-GGCCATCACACCACATTATGAACTTG-3’ primers.

Standard cycling was performed under the following conditions: Initial denaturation at 95 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 55.6 °C for 30 s, and 72 °C for 50 s, and the final one-step extension of 72 °C for 10 min. PCR reactions with A allele-specific reverse mutant and C allele-specific reverse wild-type primers were performed separately in two vials.

The allele-specific PCR products were separated by 2% agarose gel electrophoresis in 1 x Tris–Borate-EDTA (TBE) buffer at 100 V and stained with RedSafe Nucleic Acid Staining solution (Boca Scientific, Inc., Boca Raton, FL, USA) for visualization. The amplicon size of SNP rs2641726 polymorphism (C > A) was 298 bp for both alleles.

Bioinformatics Analysis

miRNASNP V2.0 bioinformatics online tool [15] was used to predict the effect of the rs2641726 polymorphism (C > A) on 3’UTR of MUC4 gene. KM Plotter online tool was recruited to extract the effect of MUC4 expression on the overall survival rate of gastric cancer patients [16]. In this database, the overall survival rate was calculated in 1065 gastric carcinoma samples, over the course of 150 months.

Statistical Tests

To evaluate the association between the SNP and gastric cancer, patient and control samples were compared using the SNPstate and SPSS software (version 16, SPSS Inc., Chicago, IL, USA). Number (percentage) and odds ratio (OR) along with 95% confidence interval (95% CI) were used to describe the statistical analyses. P < 0.050 was considered as statistically significant.
Results

PCR Condition

The temperature gradient of 55–62.5 °C was carried out for each set of primers in separate vials. The optimized temperature was 55.6 °C for annealing step. Increased levels of MgCl₂, dNTP, and PCR buffer were incorporated in order to achieve the optimum condition of PCR, which was 150 ng genomic DNA, 2.5 μl 10 × solution buffer, 0.75 μl MgCl₂ (100 mM), 1 μl dNTPs (10 mM), 0.2 μl Taq DNA polymerase (Bioron, Germany) and 0.5 μl of each primer (10 μM). Performing ASP-PCR in these conditions led to genotype each sample (Fig. 1).

Frequency of rs2641726 and Gastric Cancer

The frequency of rs2641726 genotypes and alleles, in 168 stomach cancer patients and 66 healthy cases, is shown in Table 1.

The statistical analysis showed that the studied population was in Hardy–Weinberg Equilibrium (HWE) (null hypothesis: the population is in HWE; P > 0.050). Moreover, we observed a significant association between genetic polymorphism rs2641726 (C > A) and incidence of gastric cancer. According to the hereditary pattern, the dominant model was selected (A/A: recessive and A/C–C/C: dominant, P < 0.001). It means that carrying C allele was conceived as the risk allele. Statistical analyses showed the association between rs2641726 C allele and increased incidence of gastric cancer, OR = 3.382, 95% CI: 1.840–6.217 (P < 0.001), meaning that the samples carried C allele in rs2641726 position increased the risk of gastric cancer around 3.4 times greater than A allele carriers.

Furthermore, studying clinicopathological features of the gastric cancer patients with presence/absence of C allele showed that this allele enhanced the risk of having stage III of gastric cancer as compared to other stages, OD = 2.571, 95% CI: 1.204–5.491 (P = 0.015). However, other characteristics did not show to have any association with either A or C alleles (Table 2).

In Silico Analysis

Bioinformatics studies showed that rs2641726 located in the 3′UTR of MUC4 is potential to interact with miR-581. In silico investigations also suggested that rs2641726 A allele may increase MUC4 mRNA:miR-581 stability based on calculated free energy difference between C and A alleles. Free energy for A and C allele was −15.2 and −12.4 kcal/mol, respectively.

Analyzing the Kaplan–Meier plots revealed that higher expression of MUC4 is associated with a higher hazard ratio (HR) in gastric cancer patients. According to the results, high MUC4-expressing patients had a median survival rate of 23.6 months, while low MUC4-expressing patients had a higher survival rate of 35.2 months, HR = 1.350, 95% CI: 1.140–1.590 (P < 0.001) (Fig. 2). The analysis of this cohort-based study (1065 gastric cancer patients) strongly supports the oncogenic function of MUC4 in stomach tumorigenesis.

Table 1 The frequency of rs2641726 genotypes and alleles in the studied population

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Gastric cancer</th>
<th>Control</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/A</td>
<td>68 (40)</td>
<td>46 (70)</td>
<td>114 (49)</td>
</tr>
<tr>
<td>A/C</td>
<td>76 (45)</td>
<td>14 (21)</td>
<td>90 (38)</td>
</tr>
<tr>
<td>C/C</td>
<td>24 (14)</td>
<td>6 (9)</td>
<td>30 (13)</td>
</tr>
<tr>
<td>Total</td>
<td>168</td>
<td>66</td>
<td>234</td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>212 (63)</td>
<td>106 (80)</td>
<td>318 (68)</td>
</tr>
<tr>
<td>C</td>
<td>124 (37)</td>
<td>26 (20)</td>
<td>150 (32)</td>
</tr>
<tr>
<td>Total</td>
<td>336</td>
<td>132</td>
<td>468</td>
</tr>
</tbody>
</table>

Data are shown as number (%)

Fig. 1 Representative PCR amplicons of allele-specific primer PCR (ASP-PCR) resolved by 2% agarose gel electrophoresis to detect the rs2641726 genotypes in control (top) and gastric cancer (bottom) DNA samples. Common forward + specific reverse (C allele), and common forward + specific reverse (A allele) were added in the first and second lanes of each genotyping group, respectively, resulting in the production of 298 bp DNA fragment.
Recently, it has been revealed that miRNAs are certain members of regulatory systems [17]. miRNAs are small noncoding RNAs, constituting of 18–24 nucleotides. The association between miRNAs and cancer was firstly observed in 2006 [8]. miRNAs have a significant role in cancer due to their function in the regulation of growth, differentiation, and apoptosis in the cell [9]. Mutations or SNPs in the miRNA-interacting region may disturb the properties of miRNA target mRNA, which eventually alters the protein expression [18].

In this study, we found the association between increased risk of gastric cancer and presence of C allele in rs2641726 position. Our studies also revealed that C allele is significantly more enriched in the samples categorized in stage III of gastric cancer.

According to our bioinformatics studies, rs2641726 C allele (risk allele) decreases the stability of interaction between miR-581 and MUC4 mRNA. Little is known about the role of miR-581 in cancer. However, it has been reported that miR-581 is down-regulated in Hepatocellular carcinoma [19] and prostate cancer [20], implying that this non-coding RNA might play a role as a tumor suppressor. According to the oncogenic activity of MUC4 and tumor suppressive function of miR-581, any alteration leading to the strengthened interaction of these two RNAs would have tumor suppressor effect. Whereas attenuating this interaction facilitates the oncogenic effect of MUC4. Hence, rs2641726 A allele (protective allele) is potential to strengthen the interaction of miR-581:MUC4, which would result in decreased expression of MUC4 protein. On the other hand, rs2641726 C allele, as a risk factor in our findings, attenuates this interaction and leads to the elevated oncogenic function of MUC4. Bearing all the above in mind, in vitro reporter assay is highly-recommended to validate the role of rs2641726 different alleles in the expression of MUC4.

This study was carried out with some demerits, including limited sample size, lack of in vitro assay to validate miR-581:MUC4 interaction and also lack of demographics of studied patients, such as alcohol consumption, smoking and family history of gastric cancer.

**Table 2** The association between rs2641726 C allele carriers and gastric cancer characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Status</th>
<th>A/A</th>
<th>A/C–C/C</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. pylori</td>
<td>Positive</td>
<td>44</td>
<td>50</td>
<td>–</td>
<td>0.727</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>22</td>
<td>22</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Metastasis</td>
<td>Positive</td>
<td>28</td>
<td>36</td>
<td>–</td>
<td>0.297</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>42</td>
<td>38</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>Stage I</td>
<td>10</td>
<td>8</td>
<td>–</td>
<td>0.143</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>50</td>
<td>84</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stage II</td>
<td>12</td>
<td>14</td>
<td>–</td>
<td>0.445</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>48</td>
<td>78</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stage III</td>
<td>12</td>
<td>36</td>
<td>2.571 (1.204–5.491)</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>48</td>
<td>56</td>
<td>–</td>
<td>0.432</td>
</tr>
<tr>
<td></td>
<td>Stage VI</td>
<td>26</td>
<td>34</td>
<td>–</td>
<td>0.751</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>34</td>
<td>58</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Lymph node</td>
<td>Positive</td>
<td>38</td>
<td>56</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>8</td>
<td>10</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

*Obtained from Chi square test

OR Odds ratio, CI confidence interval

**Fig. 2** KM plot illustrates the inverse effect of high MUC4 expression on the overall survival rate of gastric cancer patients

**Discussion**

Recently, it has been revealed that miRNAs are certain members of regulatory systems [17]. miRNAs are small noncoding RNAs, constituting of 18–24 nucleotides. The association between miRNAs and cancer was firstly observed in 2006 [8]. miRNAs have a significant role in cancer due to their function in the regulation of growth, differentiation, and apoptosis in the cell [9]. Mutations or SNPs in the miRNA-interacting region may disturb the properties of miRNA target mRNA, which eventually alters the protein expression [18].
Conclusion

rs2641726 C allele, risk factor, is highly and significantly enriched in gastric cancer samples, especially in tumors with stage III. The proposed C allele mechanism of action might be attenuating the MUC4 and miR-581 interaction, which eventually results in higher expression of MUC4 oncoprotein.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

Research Involving Human Participants and/or Animals All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

References