RESEARCH

A putative microRNA binding site polymorphism rs61882771 in the CD44 3'-UTR is associated with the risk of gastric cancer

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ABSTRACT

Objectives: Single nucleotide polymorphisms (SNPs) in potential microRNA (miRNA) binding sites in the 3'-UTR of target genes may dysregulate the target gene expression and subsequently contribute to the susceptibility to cancers. The cluster of differentiation 44 (CD44) is a surface gastric cancer stem cell marker and its 3'-UTR variants have been the subject of previous cancer risk association studies. However, this research provides the first evidence on the relationship between the rs61882771 (c.*2046T>C), located on microRNA-binding sites in the 3′-UTR of CD44 and gastric cancer.

Materials and methods: 252 gastric cancer patients and 216 non-cancer controls were included in the present work. High-resolution melting curve (HRM) analysis and sequencing were used to screen genotyping of the SNP rs61882771.

Results: This case–control study indicated that individuals carrying GC were at a higher risk compared with subjects carrying TT genotype (CT vs TT, OR=2.66, 95% CI=1.74-4.07, P<0.001; CC vs TT, OR=3.03, 95% CI=1.84-5.00, P<0.001; CT + CC vs TT, OR=2.79, 95% CI=1.91-4.09, P<0.001). Similarly, a significant difference in the allelic frequencies was found between GC patient and control groups (C vs T, OR=2.08, 95% CI=1.59-2.72, P<0.001). Furthermore, bioinformatics analysis using an online tool indicated that the rs61882771 variant may alter miRNAs capability to bind the 3'-UTR of CD44.

Conclusion: Consistent with the in silico analysis, our data suggest that polymorphism in the miRNA binding site in the CD44 3'-UTR (rs61882771) modify gastric cancer susceptibility.

KEYWORDS: CD44; MicroRNA; SNP; rs61882771; Gastric cancer susceptibility

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INTRODUCTION

Gastric cancer (GC) is the third most common cause of cancer leading deaths and the fifth most common malignancy throughout the world (1). In Iran, gastric cancer with the incidence rate of 26.1 and mortality rate of 19.9 per 100000 is a serious public health issue (2). Most of Iranian gastric patients who are diagnosed at an advanced stage experience relatively poorer survival rate as a result (3). Generally, Helicobacter pylori (HP) infection, salted and nitrated food, heavy alcohol drinking, and smoking are major risk factors associated with gastric cancer (4). Moreover, gene polymorphisms may cause significant risk variance in the development of various complex diseases (5-8), including GC.

CD44, a multifunctional glycoprotein adhesion molecule of the extracellular matrix, functions as a receptor for hyaluronic acid and osteopontin and also a co-receptor for growth factors and cytokines (9). CD44 is involved in many crucial cellular processes, including cell survival, proliferation, differentiation, adhesion, and migration (9, 10). Being a marker of cancer stem-like cells (CSCs) in many tumors (11), contributing to tumor cell proliferation, differentiation, invasion, migration (11), having a critical role in epithelial-mesenchymal transition (EMT) (12), and involving in resistance to chemotherapy (13) suggest that overexpression of CD44 causes poor prognosis in cancer patients (14). Particularly, in GC, CD44 has been reported to play remarkable roles in lymph node and peritoneal metastases and in resistance to chemotherapy (15-17). Therefore, CD44 may be useful in predicting early recurrence and poor overall survival (18-21).

Based on the critical roles of CD44 in cancer, several studies have examined the possible association of the genetic single nucleotide polymorphisms (SNPs) of CD44 gene and cancer risk. MicroRNAs (miRNAs), small non-coding RNA molecules of 19-25 nucleotides, negatively regulate gene expression during the translation process of the specific messenger RNA (mRNAs) by targeting the 3′ untranslated region (3′-UTR) at the post-transcriptional level (22). Therefore, SNPs located in the 3′-UTR of CD44 may affect its expression. For example, the rs13347 C>T polymorphism, located in the 3′-UTR of CD44 gene might influence the CD44 gene expression, since it has been reported in a number of cancers that subjects carrying TT andCT genotypes had remarkably higher levels of CD44 protein than those carrying CC genotype (23-25).

To date, no studies have tested the possible association between rs61882771 (c.*2046T>C), located in microRNA-binding sites in the 3′-UTR of CD44 and gastric cancer risk. Thus, this study was performed to evaluate the relationship between CD44 rs61882771 SNP and gastric cancer susceptibility as well as its other clinical characteristics among Iranian population for the first time. In addition, we looked further into the connected molecular function of the CD44 SNP alleles by performing bioinformatics assessment.

MATERIALS AND METHODS

Study subjects

This case-control study comprised 252 gastric cancer patients selected from Iranian population who were diagnosed with cancer in Shahid Sadooghi Hospital, Isfahan, Iran (Sept. 2013 to Nov. 2016). The diagnosis of gastric cancer was confirmed by clinical criteria and histopathology verification. Moreover, a total of 216 unrelated cancer-free Iranian controls were randomly chosen from the usual physical examinations for early diagnosis of gastric cancer in the same hospital. They were matched with the gastric cancer patients in terms of age, sex, and smoking status. The individuals with any hematological disorders, relationship, or previous history of cancers were excluded from the current study. The demographic characteristics and clinic-pathological variables' information were collected from the hospital records and are listed in Table 1.

Each subject signed a written consent, and this study was approved by the Ethical Committee of the Nourdanesh Institute of Higher Education.

Table 1. Demographic data of participants and clinical characteristics of GC patients

Parameters	GC case *n* (%)	Cancer-free control *n* (%)	*P*
Age (year, mean ± SD)	55.3 ± 11.6	54.7 ± 10.9	0.18
Gender Male Female	 170 (67.4) 82 (32.6)	 141 (65.3) 75 (34.7)	 0.62
Smoking status Smokers Nonsmokers	 191 (75.8) 61 (24.2)	 151 (69.9) 65 (30.1)	 0.15
HP infection Yes No N.A.	 57 (22.6) 54 (21.4) 141 (56)		
Tumor stages 1 2 3 4 N.A.	 27 (10.7) 39 (15.5) 72 (28.6) 90 (35.7) 24 (9.5)		
Lymphatic metastasis 0 1 2 3 N.A.	 27 (10.7) 63 (25) 42 (16.7) 36 (14.3) 84 (33.3)		
Distant metastasis Yes No N.A.	 96 (38.1) 120 (46.9) 36 (14.3)		

Prediction of miRNAs binding to the SNP

The PolymiRTS Database 3.0 (http://compbio.uthsc.edu/miRSNP/) (26) was utilized to predict the functional consequence of rs61882771 T>C substitution located in the 3'-UTR of CD44 which could alter binding energy of miRNAs-mRNA.

Genotyping

Five ml of venous blood samples from all participants were collected and stored in EDTA containing tube. Genomic DNA was extracted from the peripheral whole blood using PrimePrep Genomic DNA Isolation kit (GeNetBio, Chungnam, South Korea) based on the manufacturer's instructions. The purity and concentration of the isolated DNA were examined by a BioPhotometer (Eppendorf, Hamburg, Germany). The isolated DNA was stored at -20 °C before genotyping.

Genotypes of the rs61882771 SNP was screened using high-resolution melting curve (HRM) method. Polymerase chain reaction (PCR) amplifications were carried out in the Rotor-Gene 6000 instrument (Corbett Life Science, Valencia, CA, USA). The 3'-UTR of the CD44 gene was amplified by the primers, (forward: 5'- ATG GTT ACA GCC TCT ACA TGT C -3' and reverse: 5'- CAT CTC TGG CTA TGA GAC TTC TAT CAC -3'), producing a product of 72 bp. Briefly, the HRM-PCR reaction was initially performed in 10 µL volumes containing 5 µL H2O, 1X EVA-GREEN master mix, 0.5 µM forward primer, 0.5 µM reverse primer, and 0.5 ng genomic DNA. Real-time PCR was performed in Mic qPCR Hardware (Bio Molecular Systems, Upper Coomera, Queensland, Australia) with the following conditions: an initial denaturation at 95 °C for 15 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, extension at 72 °C for 20 s. Following the completion of the cycle program, PCR products were denatured at 95 °C for 1 min and then cooled to 40 °C for one minuteto form double-strand DNA. Next, the HRM analysis assessment was performed by gradually increasing the temperature from 60 to 95 °C at the rate of 0.1 °C/s. The data were analyzed by the Mic qPCR Software V1.4.1 (Bio Molecular Systems, Upper Coomera, Queensland, Australia). Variant alleles of rs61882771 of the CD44 gene recognized by HRM were randomly validated using direct sequencing technology.

Statistical analysis

To evaluate the association between the SNP and phenotypes, statistical analysis was performed by comparing two groups using the DeFinetti program (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Association tests were examined by Pearson's Chi-square test. Univariate and multivariate logistic regression

models were implemented to show odds ratios (ORs) and related 95% confidence intervals (95% CI). In this study, P<0.05 was considered statistically significant.

Table 2. Potential miRNAs with binding site of the CD44 3' UTR harbored the rs61882771 polymorphism

miRNA	miRNA sequence	miR site on CD44 3'-UTR with rs61882771
hsa-miR-1273a	GGGCGACAAAGCAAGACUCUUUCUU	TGTCG[T/C]C
hsa-miR-3180-3p	UGGGGCGGAGCUUCCGGAGGCC	CG[T/C]CCCA
hsa-miR-3196	CGGGGCGGCAGGGGCCUC	CG[T/C]CCCA
hsa-miR-6816-5p	UGGGGCGGGGCAGGUCCCUGC	CG[T/C]CCCA
hsa-miR-6852-5p	CCCUGGGGUUCUGAGGACAUG	[T/C]CCCAGG
hsa-miR-92b-5p	AGGGACGGGACGCGGUGCAGUG	CG[T/C]CCCA

Table 3- Association test of the rs61882771 polymorphism between GC case and control groups.

SNP (rs61882771)	GC case *n* (%)	Cancer-free control *n* (%)	OR (95 % CI)	*P*
Genotype				
TT	72 (28.6)	114 (52.8)	1.00 (reference)	
CT	111 (44)	66 (30.6)	2.66 (1.74-4.07)	<0.001
CC	69 (27.4)	36 (16.7)	3.03 (1.84-5.00)	<0.001
CT + CC	180 (71.4)	102 (47.3)	2.79 (1.91-4.09)	<0.001
Allele				
T	255 (50.6)	294 (68.1)	1.00 (reference)	
C	249 (49.4)	138 (31.9)	2.08 (1.59-2.72)	<0.001

RESULTS

Basic and clinical characteristics of subjects

The known characteristics of the 468 Iranian subjects, including 252 gastric cancer cases and 216 healthy controls are summarized in Table 1. There were no statistically significant differences between distribution of sex, age, and smoking status between gastric cancer and cancer-free groups. This validates that cases and controls are adequately matched.

The association between the rs61882771 (c.*2046T>C) SNP of the microRNA-binding site in CD44 and GC susceptibility

First of all, to select a miRNA correlated SNP in the 3'-UTR of CD44, we chose the rs61882771 due to its proper minor allele frequency. We then bioinformatically predicted miRNAs which can bind to the region of the rs61882771 CD44 3'-UTR polymorphism location using PolymiRTS Database 3.0 online software (Table 2). Therefore, we hypothesized that the expression regulation of CD44 might be impacted by the genotype at the

rs61882771 location. To investigate a possible relationship between the rs61882771 SNP and the occurrence of gastric cancer, a case–control study was conducted.

All patient and control subjects were successfully genotyped and its distributions are presented in Table 3. Interestingly, the Chi-square test results indicated that individuals carrying an allele of C at rs61882771 showed a significant association with a higher risk for GC comparing subjects carrying TT genotype (CT vs TT, OR=2.66, 95% CI=1.74-4.07, P<0.001; CC vs TT, OR=3.03, 95% CI=1.84-5.00, P<0.001; CT + CC vs TT, OR=2.79, 95% CI=1.91-4.09, P<0.001). Evidently, a significant difference in the allelic frequencies was found between gastric cancer patient and control groups (C vs T, OR=2.08, 95% CI=1.59-2.72, P<0.001).

Furthermore, to perform stratified association analysis between the SNP and clinical characteristics of gastric cancer, the included case group was divided into three subgroups based on tumor stages, lymphatic metastasis, and distant metastasis. However, genotype distributions or allelic frequencies of the rs61882771 (c.*2046T>C) SNP did not show any significant association in the stratified analysis (data are not shown).

DISCUSSION

Genetic and environmental factors, as well as their interactions, play a critical role in the development of cancers, including gastric cancer. It has confirmed that miRNAs are important regulators of biological processes (22, 27). Notably, increasing evidence has established that miRNA associated SNPs can have influence on the gene expression; therefore, are associated with cancer risk (6, 28). CD44 gene, a critical surface marker in cancer cells, is an ideal target gene for association studies and has been recognized as a poor prognostic molecule in several cancers (9, 29). It has been demonstrated that although the CD44 has multiple transcriptional variants, its 3'-UTR has a conserved sequence (10). As a result, polymorphisms located in the 3'-UTR of CD44, such as the rs13347, have been evaluated in several association studies (14). However, there is no reported investigation for CD44 rs61882771 polymorphism in any cancer type.

In the present study, we assumed that the rs61882771 (c.*2046T>C) SNP is located in the miRNAs target site in the 3'-UTR of the CD44 gene; hence, may modulate the CD44 protein expression and finally lead to alterations in the susceptibility to gastric cancer. On the basis of our case-control study with 252 histologically/clinically confirmed gastric cancer patients and 216 cancer-free controls, subjects carrying the rs61882771 C allele had an increased risk for gastric cancer incidence comparing to subjects with TT genotype (CT vs TT, OR=2.66, 95% CI=1.74-4.07, P<0.001; CC vs TT, OR=3.03, 95% CI=1.84-5.00, P<0.001; CT + CC vs TT, OR=2.79, 95% CI=1.91-4.09, P<0.001).
However, this SNP was not significantly related to the clinicopathological features of gastric cancer, including tumor stages, lymphatic metastasis, and distant metastasis (P≥0.05).

Furthermore, the bioinformatics assessment indicated that the C allele of the investigated SNP rs61882771 in the CD44 gene may cause alterations in the binding site for miR-1273a, miR-3180-3p, miR-3196, miR-6816-5p, miR-6852-5p, and miR-92b-5p. This finding may demonstrate that the SNP rs61882771 may change expression level of CD44 through alteration of microRNA-mediated regulatory function and as a result, contribute to gastric cancer development. This may explain the observed association between the SNP and the risk of gastric cancer.

There were some limitations in the present study. To begin with, various risk factors have been reported to contribute in gastric cancer carcinogenesis, such as HP infection, tobacco smoking, diet, and genetic elements (30). In this study, the information of other involved factors, such as gastroesophageal disease and obesity were not available to be considered. Secondly, without

survival information, prognostic value of the rs61882771 SNP in gastric cancer remains unclear.

Despite the limitations, our findings provide the first evidence that suggests an association between the CD44 rs61882771 polymorphism and gastric cancer risk. This variant may potentially change the expression level of CD44 and increase gastric cancer susceptibility. Our results may further reinforce the hypothesis that the miRNA related SNPs are important risk factors for cancers. Because of the important role of SNPs in the microRNA target genes, further studies can aim to investigate the impact of CD44 rs61882771 polymorphisms in other ethnic populations and also other cancer types. Moreover, further functional evidence is required to experimentally verify our in silico result.

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Conflict of interest statement

The author has declared that no competing or conflict of interests exist. The funders had no role in study design, writing of the manuscript and decision to publish.

Authors' contributions

Will be added in proof read version.

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