Original Article

Methylation and Polymorphism in CDH1 Gene Promoter Among Patients with Diffuse Gastric Cancer

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Abstract

Background: The promoter methylation and single nucleotide polymorphisms (SNPs) affect the transcription activity of cancer-related genes in several cancers including diffuse gastric cancer (DGC). Here we aimed to evaluate the promoter methylation status and the rs16260 at the promoter region of the CDH1 gene in DGC. **Methods:** This case-control study was performed of 48 formalin-fixed paraffin-embedded (FFPE) blocks of DGC patients and 41 fresh frozen tissue samples of healthy individuals. Methylation status was evaluated using methylation-specific polymerase chain reaction (PCR) and the rs16260 at the promoter region of the CDH1 gene was assessed using PCR and sequencing method. **Results:** The occurrence of methylation at the promoter region of the CDH1 gene in DGC patients was significantly higher than control samples ($P < 0.0001$). The methylated status was significantly associated with the poor differentiated histological type of DGC ($P = 0.0428$). The frequency of AC genotype and the A allele in DGC patients was significantly higher than the control subjects ($P = 0.006$ and 0.003, respectively). **Conclusions:** Here we showed that methylation at the CDH1 promoter may contribute to the DGC development, and also the AC genotype was associated with the risk of DGC.

Keywords: *Cadherin 1, methylation, polymerase chain reaction, sequence analysis, stomach neoplasms*

Introduction

Gastric cancer (GC) is the fourth most common cancer and the second cause of mortality among all cancers in the world, and although there is a decrease in the global incidence of GC in recent years, in some northern regions of Iran is the most common malignancy.[1,2] based on differential epidemiological, histological, clinicopathological, and molecular features, and also biological behavior GC is divided into two main subtypes including intestinal GC, and diffuse GC which is described as a poorly cohesive and infiltrative tumor that in some cases a signet ring cell morphology is obvious. Despite the decreasing occurrence of intestinal type over the world, the incidence of diffuse type is persistent or even increasing.[3-6]

Germline Cadherin 1 (CDH1) gene mutations confer a high lifetime risk of developing diffuse gastric (DGC).[7] CDH1 gene encodes, Cadherin, a tiny calcium-dependent protein expressed in the epithelial cell membrane. The function of E-cadherin is cell adhesion and intrusion suppression. The CDH1 gene encodes cadherin-E protein.[8] Reduced expression of CDH1 gene has been found to be involved in the dysfunction of cell–cell adhesion system, leading to cancer invasion and metastasis.[9] Epigenetic modifications and gene polymorphism are two types of alterations which may result in reduced expression of CDH1.[10,11]

As one of the major epigenetic mechanisms, abnormal methylation in the promoter of several genes including CASP8, hMLH1, CDH1, and MDR1 involved in the progression of GC. Hypermethylation at the promoter region of the CDH1 gene has been found in both sporadic and hereditary diffuse gastric cancer (SDGC and HDGC). In fact, hypermethylation of the CDH1 gene promoter is found in both subtypes (intestinal and diffuse) but its frequency is higher in the diffuse type.[12] It has been shown that methylation at the promoter of the CDH1 gene serves as the second hit in more than half of the SDGC cases harboring CDH1 mutations.[13,14]

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Besides, multiple CDH1 gene polymorphisms are associated with a decreased expression level. Among them, the well-known one is in the -160C > A (rs16260) single nucleotide polymorphism (SNP) at the promoter which has shown that A allele cause 68% decrease in the CDH1 gene transcription in comparison to the C allele.[10] The rs16260 SNP, located in the promoter region at the upstream of the transcriptional start site of the CDH1, and it has been revealed that involved in transcriptional activity and regulation of catenin-containing complexes formation. Actually as mentioned above, the transition from C to A nucleotide at the -160 leads to decreased transcriptional activity, which is associated with the increased risk of several cancers in different ethnicities.[15]

The purpose of this case-control study was to assess the promoter methylation status of the CDH1 gene, and also genotyping of rs16260 SNP in the CDH1 gene promoter in DGC patients and healthy controls and also understanding the association of the promoter methylation status with the clinicopathologic characteristics.

Methods

Study population

The study population of this case-control study comprises of 38 formalin-fixed paraffin-embedded (FFPE) blocks of SDGC patients and 10 blocks of FFPE from HDGC patients collected and diagnosed in the Taleqani Hospital, Shahid Beheshti University of Medical Sciences, Iran, and Al-Zahra hospital, Isfahan, Iran between 2007 and 2017. These cases were confirmed by a sophisticated pathologist based on histopathological features and absent criteria based on International Gastric Cancer Linkage Consortium (IGCLC). Forty-one fresh frozen tissue sample of individuals who referred to hospital for other diseases and the absence of DGC in them confirmed by a pathologist were collected and considered as healthy controls. Informed consent forms were signed by all the participants or their families. The study was approved by the Review Board of Isfahan University of Medical Sciences, according to the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration.

DNA extraction and Bisulfite treatment

All formalin-fixed paraffin-embedded (FFPE) samples were cut into 5–10 μm slices. DNA was extracted from FFPE sections and also fresh frozen tissue samples of healthy controls using the One-4-All Genomic DNA Miniprep Kit (Bio Basic Inc., Canada) according to the manufacturer's protocol and eluted in 50-μL of Tris- EDTA (TE) buffer. The quality and quantity of isolated were evaluated using NanoDrop™ 2000 (Eppendorf, Germany) instrument. The proper amount of extracted genomic DNA (about 1 μg) of both patients and healthy subjects were converted by sodium bisulfite treatment using Epitect Bisulfite kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol.

Methylation-specific polymerase chain reaction (MS-PCR)

In order to evaluate the methylation status of the CDH1 gene promoter the methylation-specific polymerase chain reaction (MS-PCR) method was used. Using the Methprimer2 software, two pairs of specific primers capable of distinguishing between methylated and unmethylated DNA sequences at the promoter of the CDH1 gene were designed whose sequence is shown in Table 1. Positive and negative control samples were purchased from Biolab England (New England). To perform the MS-PCR, a PCR mix containing 2 μl of bisulfite pre-treated DNA, 10 μl of Master Mix, 10 μl of d H2O, 1 μl of each primer (10 mol/μl), and 1 μl MgCl2 in a final volume of 25 μl was prepared. This mix was prepared in two parallel tubes for each sample, one with methylated primers and another with unmethylated primers. PCR cycling conditions were: initial denaturation at 96°C for 5 min, followed by 40 cycles of 97°C denaturation for 10 s, 62°C for 30 s, 72°C extensions for 30 s, and a final extension at 72°C for 7 min. The MSP products were separated by electrophoresis on 2.5% agarose gel.

Genotyping via PCR and sequencing

In order to assess the polymorphic substitution rs16260 SNP at the promoter of the CDH1 gene of the patients and control samples were amplified using the following primers: forward, 5′-TCCCAGGTCTTAGTGAGCCA-3′; reverse, 5′-GGCCACAGCCAATCAGCA-3′. PCR products were sequenced with the Sanger method using the ABI 3130XL capillary sequencing instrument (Applied Biosystems/ Life Technologies, Carlsbad, CA, USA). Chromatograms of the sequences were analyzed using chromas software (version 2.13).

Statistical analyses

In order to evaluate the significance of methylation status in DGC patients compared to healthy controls, the Chi-squared test was used. The association between clinicopathological data and the methylation status of the CDH1 gene promoter

Table 1: Primer sequences for MS-PCR of the CDH1 gene promoter

Primer ID	Sequence	Product size
Methylated		112 bp
Forward primer	TGTAGTTACGTATTTATTTTTAGTGGCGTC	
Reverse primer	CGAATACGTCGAATCGAACCG	
Unmethylated		120 bp
Forward primer	TGGTTGTAGTTATGTATTTATTTTTAGTGGTGTT	
Reverse primer	ACACCAATACAACAAATCAAACCAAA	

also was assessed using the Chi-squared test. For both cases and healthy individuals, the Hardy–Weinberg equilibrium was tested. Odds ratios (OR) and 95% confidence interval (95% CI) for various genotypes were calculated. Differences between case and control groups were examined using the Chi-squared test. Differences in genotypic distribution and allelic frequency between patients and controls were measured by the Chi-squared test. A probability of $P < 0.05$ was considered significant. All the statistical analysis were performed using GraphPad Prism 8 (GraphPad Software).

Results

Epidemiological and clinicopathologic data

Among 48 cases included in this study (32 males and 16 females), 38 patients diagnosed as SDGC and 10 as HDGC. The age range of patients was between 29 and 83 years with the mean age of 56.73 years. The healthy control subjects were age and gender matched. Fourteen out of 48 patients (29.1%) identified at the early TNM stages of the tumor (I, II), and in 26 cases (54.2%) tumor had been detected in advanced stages (III, IV). But for 8 patients (16.7%), the TNM stage of the cancer was unknown. "Signet ring cell carcinoma" and " poorly differentiated adenocarcinoma" known as two histopathological types were found in 30 (62.5%) and 18 (37.5%) of cases, respectively [Table 2].

MS-PCR analysis

To evaluate the methylation status of subjects the MS-PCR was performed. The prevalence of methylated, hemimethylated and unmethylated CpG dinucleotides within the CDH1 gene promoter of DGC patients was 20 (41.7%) and 10 (20.8%), and 18 (37/5%), respectively. Also, it has been revealed that non of control samples was methylated. Statistical analysis showed that the occurrence of methylation at the promoter region of the CDH1 gene in DGC patients was significantly higher than control samples ($P < 0.0001$) [Table 2]. Figure 1 showed samples of methylated, hemimethylated and unmethylated bands.

Association between methylation status and clinicopathological characteristics

As illustrated in Figure 2, methylation status of the promoter was compared between SDGC and HDGC and

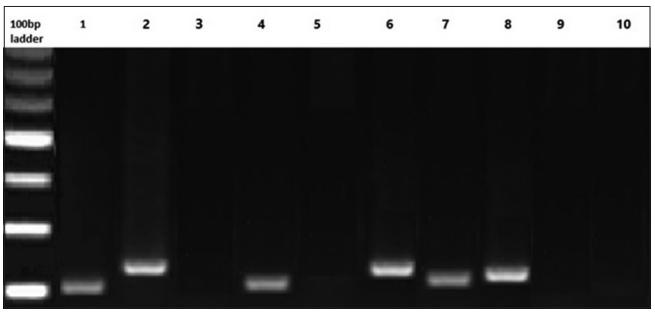

Figure 1: Visualization of PCR products using 2.5% gel electrophoresis. ladder 100bp; line1, 2: heterozygote (hemimethylated); line 3,4: homozygote for methylation (methylated); line 5,6: homozygote for unmethylation (unmethylated); 7: positive control for methylation; 8: positive control for unmethylation; 9: negative control for methylation; 10: negative control for unmethylation. Product size for methylated and unmethylated products was 112bp and 120bp, respectively

the result revealed that there is no significant difference between the groups ($P = 0.536$). The methylation status was not associated with various age groups and different genders of patients ($P = 0.5524$ and 0.5451, respectively), but there was a significant association between the methylated status in the promoter of CDH1 gene and the advanced stages of DGC ($P = 0.0312$). Statistical analysis showed that methylated status was significantly associated with the poor differentiated histological type of DGC ($P = 0.0428$) [Table 2].

Genotyping of rs16260 SNP in the CDH1 gene promoter

Genotyping of rs16260 SNP in the CDH1 gene promoter done for all patients with DGC and healthy controls using PCR and sequencing. The genotype distribution of both patient and control subjects were consistence with Hardy–Weinberg equilibrium. Table 3 showed the genotype distribution and allele frequencies of the rs16260 SNP at the promoter region of the CDH1 gene in patients and healthy controls. out of 48 patients with DGC, 19 (39.6%) were homozygous for the C allele, seven cases (14.6%) were for homozygous for the A allele and the genotype of 22 cases (45.8%) was heterozygous (AC). In the 41 healthy individuals, 30 (72%), 9 (22%), and 2 (6%) had CC, AA, and AC heterozygous genotypes, respectively. Statistical analysis showed that the frequency of AC genotype in DGC patients was significantly higher than the control subjects ($P = 0.006$). The frequency of the A allele in the patients was significantly higher than

Table 2: Association of methylation status with clinicopathologic characteristics

Characteristics	Status	Total	Proportion (%)	Methylated	Unmethylated	Hemimethylated	*P*
Age	≤55	25	52.08	11	8	6	0.5524
	>55	23	47.92	9	10	4	
Gender	M	32	66.7	13	11	8	0.5451
	F	16	33.3	7	7	2	
Differentiation	Poor	18	37.5	10	3	5	0.0312
	Signet	30	62.5	10	15	5	
TNM stage	I, II	14	29.1	3	8	3	0.0428
	III, IV	26	54.2	14	6	6	
	unknown	8	16.7	4	3	1	

Table 3: Allele, genotype frequencies of rs16260 (-160C>A) within the CDH1 gene in patients with DGC and healthy controls

Genotypes	Patients (%)	Controls (%)	OR* (95% CI**)	*P***
CC	19 (39.6)	30 (72)	Reference	-
AC	22 (45.8)	9 (22)	0.26 (0.1017 to 0.7123)	0.006
AA	7 (14.6)	2 (6)	0.18 (0.03611 to 0.8535)	0.063
AC and AA	-	-	0.24 (0.09495 to 0.5856)	0.0026
Alleles				
C	60 (62.5)	69 (84)	Reference	-
A	36 (37.5)	13 (16)	0.33 (0.1673 to 0.6702)	0.003

*OR, Odds-ratio, **CI, Confidence-interval, ***$P<0.05$

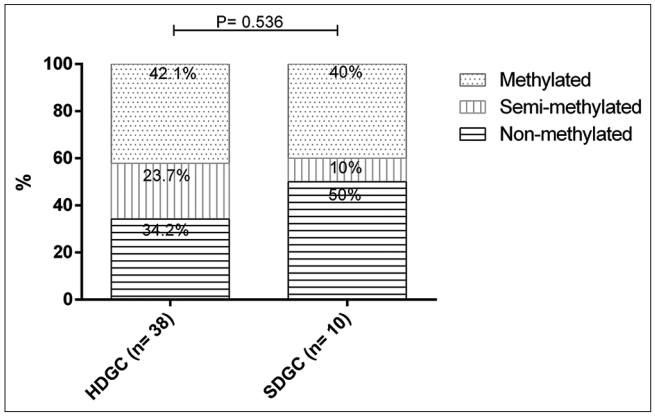

Figure 2: The comparison of methylation status of the promoter between SDGC and HDGC patients. SDGC: Sporadic Diffuse Gastric Cancer; HDGC: Hereditary Diffuse Gastric Cancer. Data analysis was done by Chi-Square

healthy controls (P = 0.003). Figure 3 showed a sample of chromatograms with CC, AA, and AC genotypes at the rs16260 SNP in the CDH1 gene promoter.

Discussion

E-Cadherin as the product of the CDH1 gene is a homophilic molecule that plays a role in cell–cell adhesion. This transmembrane glycoprotein has five extracellular domains and a cytoplasmic domain which acts in a complex three molecules including α-, β-, and γ-catenins.[16-18] Defects in E-cadherin leads to loss of contact inhibition, which is a critical initial step in the carcinogenesis. Besides, the dysfunction of E-cadherin contributes in metastasize process.[19,20] The critical role of

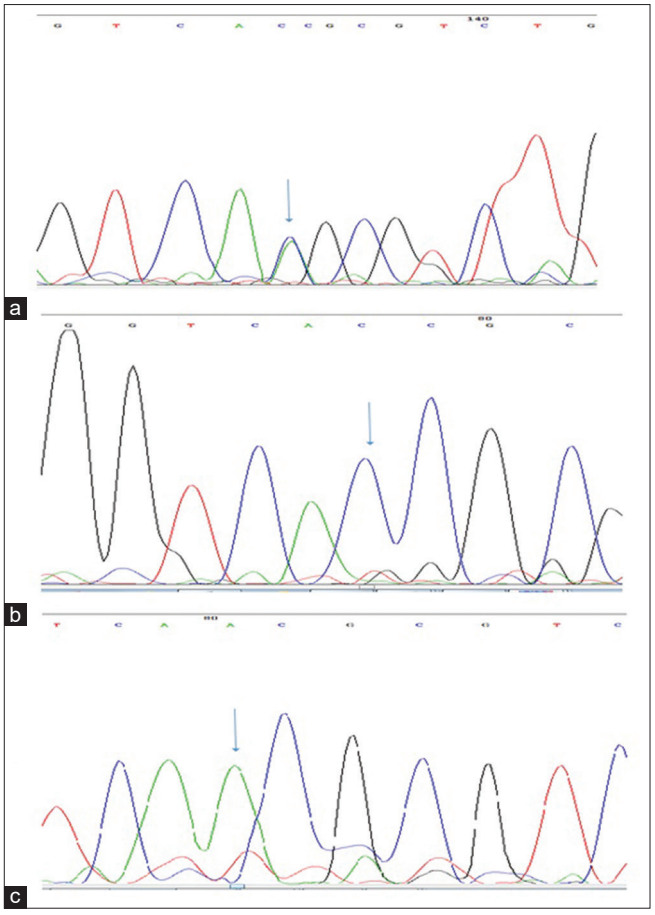

Figure 3: Chromatogram for PCR products of each genotype in rs16260 SNP at the promoter region of the CDH1 gene. (a). AC heterozygous genotype at the rs16260 SNP position (b) CC homozygous genotype at the rs16260 SNP position. (c) AA homozygous genotype at the rs16260 SNP position

E-cadherin in gastric carcinogenesis was established for a long time due to the relation of familial GC to germline mutations of the CDH1 gene.[21] To our knowledge, promoter methylation of CDH1 and also rs16260 polymorphism had been reported to be associated with the development of DGC cancer.[13,22]

In this case-control study, we investigated the associations among CDH1 gene promoter methylation and rs16260 polymorphism at the promoter region of this gene with GC risk. We found a significant difference between the methylation status of DGC patients compared to healthy controls. also, our data revealed that there was a significant association between the poor differentiated histological subtype and advances stages of cancer in DGC patients.

In a study by Lee *et al*. using MS-PCR, it had been revealed that methylation of the CDH1 promoter was observed in 73.6% of DGC cases, but this hypermethylation was not associated with age, gender, histological type, and TNM stages.[23] Although like their data, we found no association with age and gender but our data showed a significant association between CDH1 promoter methylation and tumor histological type and advanced stage, which may be because of different ethnical origins. Oliviera and *et al.* showed that 32.1% of evaluated neoplastic lesions were hypermethylated at the promoter region of the CDH gene. They stated that epigenetic changes act as the second hit in CDH1 in HDGC patients.[14] This research group in another study indicated that CDH1 promoter methylation has the same role in SDGC cases.[13] In concordance with these results, we could suggest the CDH1 promoter methylation as a possible step in the carcinogenesis process of DGC.

In the second part of the study, we evaluated the polymorphism of rs16260 in the promoter of the CDH1 gene and showed that the frequency of AC genotype in GC patients was significantly higher than the controls. Also, we showed that the frequency of the A allele in the patients group was significantly higher than controls.

To date, several studies performed on the association of the rs16260 polymorphism with the risk of GC in different ethnicities. In the study using European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, it had been revealed that the rs16260 SNP was not associated with GC risk.[10] A study in 2002 performed on an Italian population by Humar *et al*. revealed that the A allele at the -160 position is associated with an elevated risk of SDGC.[22] Another study suggested a protective role for the AA genotype in DGC patients.[24] Here in our study, there was no association between this genotype and the risk of DGC, instead, we found a significant association with the AC genotype. Two other studies did not find any significant differences in genotype and allele frequencies between GC patients and healthy controls in two different ethnicities.[8,25] The presence of modifier genes, environmental factors, and different ethnicities may explain differences between our results and other controversial results mentioned before. Another recent study in our country using PCR RFLP found no statistically significant association between genotype and allele frequencies of rs16260 and patients compared to controls.[26] We could mention the evaluation method that we used (PCR and sequencing) as the suggested reason to explain the difference observed between our data and previous Iranian study.

It has been stated that A allele decreased the transcriptional activity of the CDH1 gene about 70% and thereby increased susceptibility to GC.[27] A meta-analysis showed that the AA genotype of the rs16260 SNP was associated with a significantly elevated risk of GC among Asians, but not among Europeans.[28] Lik our data, a study performed by Zhan and *et al*. showed that AC genotype in rs16260 SNP at the promoter CDH1 gene was significantly increased the risk of DGC.[29]

Conclusions

In conclusion, we showed that methylation of the CDH1 gene promoter was significantly associated with the risk of DGC and also was associated with poorly differentiated subtype and advanced stage of DGC in patients. Therefore it may be involved in the DGC carcinogenesis process and even in progression and outcome. We also showed that variants of rs16260 in the CDH1 promoter may contribute to the DGC risk. However, like any research, this study had its limitations. The small sample size, the method used to evaluate the methylation status, and quality of samples may affect the significance of results. Further studies with larger sample size using more accurate methods like Methylation Sensitive High Resolution Melting (MS-HRM), evaluating the expression level of CDH1 gene may give a better insight into the risk of DGC development in our population.

Ethical approved

This study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.MUI.REC.1394.3.950).

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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