

Association of the -160 C>A Polymorphism in the *CDH1* Promoter with Gastric Cancer: A Case-control Study

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ARTICLE INFO

Article history:

Received 08 September 2018
Accepted 14 November 2018
Available online 01 March 2019

Keywords:

Gastric cancer
CDH1
E-cadherin protein
Promoter region

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p-ISSN 2423-4257

e-ISSN 2588-2589

ABSTRACT

E-cadherin is a tumor suppressor protein that plays a crucial role in cell-cell adhesion junction and tissue architecture and it is hypothesized to participate in carcinogenesis. It has been shown that a polymorphism in the upstream of the transcription start site of the *CDH1* gene affects E-cadherin transcriptional regulation and seems to be associated with a variety of cancers. For the first time, we investigated the association of the rs16260 in the 5'-untranslated region of the *CDH1* gene with gastric cancer in Iranian population. Seventy-eight patients with gastric cancer and 72 healthy individuals were included and genotyped for this SNP using PCR-RFLP method. Our results showed that the frequency of the AA genotype in gastric cancer patients (16 of 78, 20.5%) was higher than healthy individuals (9 of 72, 12.5%), the frequency of the A allele in the patients group was higher than controls (OR=1.231, 95% CI= 0.772-1.962, *p*-value= 0.383), but statistical analysis revealed the absence of association between AA genotype and gastric cancer risk (OR=1.719, 95% CI= 0.656-4.502, *p*-value= 0.268). In conclusion, our results suggest that this substitution and the AA genotype have not a major impact on the individual's susceptibility to gastric cancer, and therefore this SNP may be an ethnicity-dependent risk factor. Further works with a larger sample size and including other criteria such as *H. pylori* infection status are needed for more accuracy.

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Please cite this paper as: Tafrihi M, Kalantari S, Shokrzadeh M. 2019. Association of the -160 C>A polymorphism in the *CDH1* promoter with gastric cancer: A case-control study. *J Genet Resour* 5(1): xxx-xxx. doi: 10.22080/jgr.2019.15741.1123

Introduction

Gastric cancer is the fifth most common cancer and the third leading cause of cancer-related death worldwide (Bray *et al.*, 2018). The role of factors including age, sex, diet, alcohol, smoking, *Helicobacter pylori*, low socioeconomic status, genetic factors, and a positive family history in the incidence and development of gastric cancer have been established (Lu *et al.*, 2015).

According to morphological features, Lauren classified gastric carcinomas into intestinal and diffuse type (Carcas, 2014). In Japanese classification, they are corresponding to differentiated and undifferentiated types, respectively (Ushijima and Sasako, 2004). In

intestinal type tumors, which commonly occur in elderly men, tumor cells express adhesion molecules and therefore, cell adhesions are established, tumor cells arranged in tubular or glandular formations and exhibit better prognosis. In contrast, in diffuse type which occurs in younger females, cells lack adhesions, and invade as single cells or collectively (Qiu *et al.*, 2013; Ma *et al.*, 2016).

The *CDH1* gene is located on chromosome 16q22.1, encodes the transmembrane glycoprotein E-cadherin. This protein is a calcium-dependent cell-cell adhesion molecule that has crucial roles in epithelial cell behavior and maintenance of tissue architecture and

integrity (Wong *et al.*, 2018). In normal epithelial tissues, E-cadherin acts as a tumor suppressor, mainly by enhancing the membrane localization of β -catenin protein at cell adhesion contacts, and sequestering the β -catenin oncoprotein from binding to LEF/TCF factor and inhibits its transcriptional activity of Wnt signaling target genes (Tafrihi *et al.*, 2007; Petrova *et al.*, 2016; Tafrihi and Nakhaei Sistani 2017). Downregulation or loss of expression of E-cadherin in different epithelial tumors which is associated with poor prognosis and survival in patients of a variety of cancers leads to cancer cell invasion, epithelial-mesenchymal transition (EMT), and metastasis (Onder *et al.*, 2008; Tafrihi *et al.*, 2014).

So far, there are different single nucleotide polymorphisms (SNPs) have been identified in the regulatory and/or coding regions of the *CDH1* gene that affect the expression and ability of E-cadherin protein to mediate cell-cell adhesion (Suriano *et al.*, 2003; Corso *et al.*, 2007). Accordingly, a C>A transversion at -160 from the transcription start site of the *CDH1* promoter has been identified, repeatedly. *In vitro* studies using *luciferase* reporter gene showed that this substitution leads to lower luciferase activity in different cell lines (Cattaneo *et al.*, 2006). It is suggested that this polymorphism is associated with increased susceptibility to various epithelial malignancies including gastric, colorectal, prostate, and breast cancer (Wang *et al.*, 2008; Wang *et al.*, 2012), and the minor

allele is regarded as a cancer genetic marker (Cattaneo *et al.*, 2006). However, so far, no study has been performed on the association of the rs16260 polymorphism with gastric cancer among the Iranian population. In this hospital-based, case-control study we have investigated the association of the rs16260 polymorphism in the promoter region of the *CDH1* gene with the risk of gastric cancer among a population in the north of Iran.

Materials and Methods

Samples

In this study, the blood samples of the 78 clinically diagnosed gastric cancer patients including 48 males and 30 females and 72 age-matched healthy individuals consisting of 34 males and 38 females were recruited from Baghban medical center in Sari (Table 1). The blood samples were collected into EDTA_{Na2} containing complete blood count/CBC tubes and stored at -20°C before genomic DNA extraction. The study protocols were approved by the ethical review board of the University of Mazandaran. All patients and controls were from Mazandaran province, agreed to participate in this study and the written informed consent that is in accordance with the principles laid down in the Helsinki II declaration was obtained from all of them.

Table 1. Demographic characteristics of patients and healthy individual's groups contributing this study

Samples	Patients group (n= 78)	Healthy individuals group (n= 72)
Age (years) (Mean± SD)	67.9 ± 13.18	68.5 ± 13.2
Sex (Male/Female)	48/30	43/38
diffused / Intestinal type	52/26	-

PCR-RFLP and SNP genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the salting-out method (Shokrzadeh *et al.*, 2017). The polymerase chain reaction-restriction length polymorphism (PCR-RFLP) method was performed to detect the rs16260 polymorphism. The PCR primers that were used in this study to amplify a fragment with 326 bp covering the *CDH1* -160 (Fig. 1A),

were forward primer 5'-TGATCCCAGGTCTTAGTGAGC-3' and reverse primer 5'-TCTGAACTGACTTCCGCAAGC-3' (Bioneer, South Korea). PCR was performed by Techne thermal cycler (Techne Co., UK) in a 25 μ l reaction volume containing 20-80 ng of genomic DNA, 10 pM of each primer, and WizPure PCR 2X master mix (Wiz Bio Solutions, South Korea). The amplification process consisted of

an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation step at 94 °C for 50 sec, annealing at 61.4 °C for 30 sec, polymerization at 72 °C for 40 sec, and with a final extension at 72 °C for 10 min. As a negative control, for each panel of PCR, distilled water was used instead of DNA in the reaction tube. For the RFLP analysis, PCR products were digested in a 10- μ l reaction volume containing 2.5 U of *HincII* restriction enzyme (Thermo Fisher Scientific, USA) and 1X reaction buffer at 37 °C overnight. The C allele yielded an undigested fragment of 326 bp and the presence of the A allele produces fragments of 213 and 113 bp. The digestion products were separated on a 3% agarose gel with a 50 bp molecular weight marker (GeneDireX, Taiwan) and visualized under UV light using Red-Type AlphaImager system (Proteinsimple, USA) after staining with ethidium bromide.

***In silico* analysis**

HaploReg software which is available at <https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php> is a tool that is used to explore annotations of noncoding variations in the genomes including SNPs at regulatory regions of the disease-associated loci. For this purpose, the reference SNP ID number was entered at the query rsID to analyze the chromatin state, histone variants and DNase hypersensitivity state of the sequence carrying the SNP. The PROMO ver. 3.0.2 software (using TRANSFAC ver. 6.4) that is available at http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3, was used to predict transcription factor binding sites in DNA sequences among those already experimentally verified. For this purpose, the sequence carrying the intended SNP was loaded at the query sequence to looking for potential transcription factor binding sites.

Statistical analyses

For both cases and healthy individuals, the Hardy-Weinberg equilibrium was tested. Odd ratios (OR) and 95% confidence interval (95% CI) for various genotypes were calculated.

Differences between case and control groups were analyzed by chi-squared test. A probability of $p < 0.05$ was considered significant. Statistical analysis was carried out with the SPSS ver. 23 (SPSS Inc., USA).

Results

At first, the 226 bp fragment containing the rs16260 polymorphism was amplified using polymerase chain reaction (Fig. 1B). The agarose gel electrophoresis of some RFLP products is shown in figure 1C. The A allele that provides a restriction site for *HincII* is cut and produces 213 and 113 bp fragments, whereas the C allele was not cut by the restriction enzyme (Fig. 1C).

Genotypes and allele frequencies

Statistical analyzes showed that the genotype distribution of patients and controls were consistence with Hardy-Weinberg equilibrium. Genotype distribution and allele frequencies of the *CDH1* rs16260 polymorphism in patients and healthy controls were shown in Table 2. Of the gastric cancer patients, 30 of 78 (38.5%) were homozygous for the C allele, 16 of 78 (20.5%) were for homozygous for the A allele and 32 of 78 (41%) were heterozygous (AC). In the healthy individual group, 29 of 72 (40.3%) had CC genotype, 9 of 72 (12.5%) had AA genotype and 34 of 72 (47.2%) were heterozygous (AC). The frequency of AA genotype in gastric cancer patients was higher than controls. The frequency of the A allele in the patients group was higher than controls (OR=1.231, 95% CI= 0.772-1.962, p -value= 0.383), but statistical analysis revealed absence of association between AA genotype and gastric cancer risk (OR=1.719, 95% CI= 0.656-4.502, p -value= 0.268).

***In silico* analysis**

Using the HaploReg ver. 4.1 software we found that the rs16260 polymorphism is located in DNase hypersensitive site in different tissues and cell lines. Also, modified histones H3K4me1 and H3K27ac are found in this region

that labels enhancers and H3K4me3 and H3K9ac histones are located in the promoter region of various cell lines and tissues. Our

analysis using PROMO ver. 3.0.2 showed that this substitution changes the transcription factor binding sites (Fig. 2).

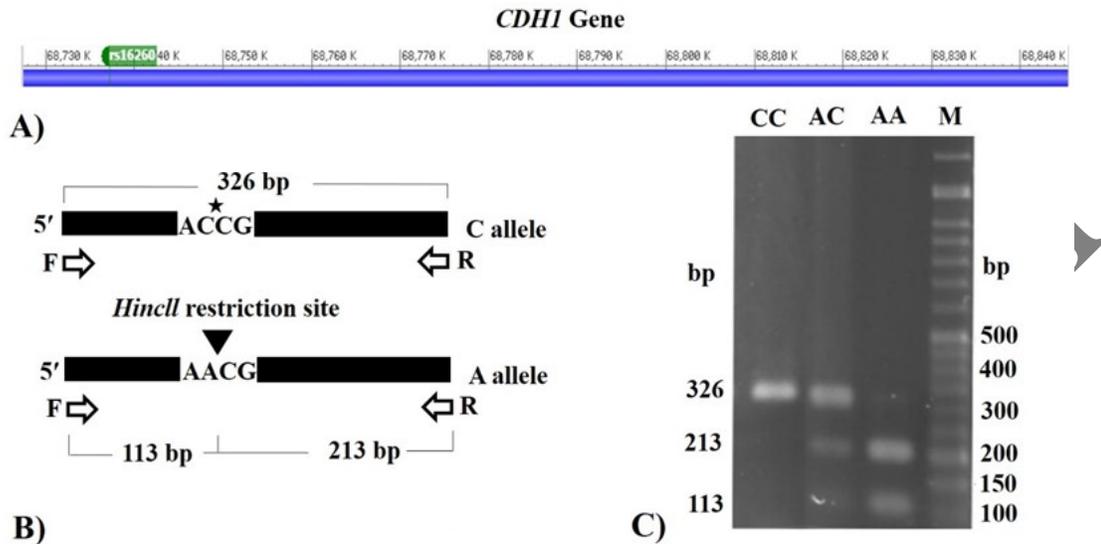


Fig.1. The *CDHI* gene map and PCR-RFLP results: Human *CDHI* gene map was obtained from NCBI, the rs16260 polymorphism is located on the promoter region of the gene (A); Schematic representation of the digestion of the PCR products with *HincII* restriction enzyme (B); The gel electrophoresis of 326 bp fragments containing rs16260 (C).

Table 2. Genotype and allele frequencies of the *CDHI* -160 C>A in cases and healthy individuals

Genotype	Patients (%)	Healthy individuals (%)	P-value	OR (95% CI)
CC	30 (38.5)	29 (40.3)	-	-
AC	32 (41.0)	34 (47.2)	0.792	0.910 (0.451 – 1.837)
AA	16 (20.5)	9 (12.5)	0.268	1.719 (0.656 – 4.502)
AC+AA	48 (61.5)	43 (59.7)	0.186	1.889 (0.731 – 4.878)
Allele	Patients (%)	Healthy individuals (%)	P-value	OR (95% CI)
C	92 (59.0)	92 (63.9)	-	-
A	64 (41.0)	52 (36.1)	0.383	1.231 (0.772 – 1.962)

Discussion

Single nucleotide polymorphisms are common DNA variations among people that may partly explain the individual's susceptibility to various diseases including cancer (Deng *et al.*, 2017). These variations, particularly in promoter or coding regions of a gene, may have serious

effects on its transcription or protein function (Li *et al.*, 2000).

Several studies have investigated the role of genetic variations in different genes in the etiology of gastric cancer (Wu *et al.*, 2002; Kiemeny *et al.*, 2006; Qiu *et al.*, 2009). It has been shown that several cancers are associated with rs16260 polymorphism of the *CDHI* gene (Forszt *et al.*, 2009; Memmi *et al.*, 2016).

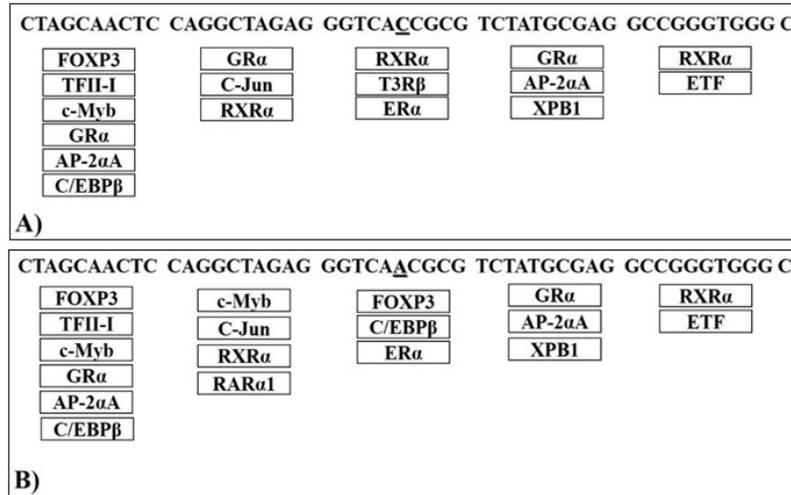


Fig. 2. A putative pattern of transcription factor binding site near the -160 polymorphic site of the *CDH1* promoter: The polymorphic nucleotide is shown as underline; **A)** The C allele; and **B)** the A allele.

The association of the rs16260 polymorphism with gastric cancer has been frequently investigated in different ethnic populations with conflicting results. For example, Humar *et al.* reported that in an Italian population, the -160 A allele is associated with an increased risk of sporadic diffused gastric cancer (Humar *et al.*, 2002). On the other hand, in Taiwanese gastric cancer patients of mixed histology, a reduced frequency of the AA genotype compared to control group has been shown (Wu *et al.*, 2002). It is suggested that, in diffused-type gastric cancer samples, the AA genotype has the protective role (Zhao *et al.*, 2015). Also, there are no statistically significant differences in genotype and allele frequencies between non-cardia gastric cancer patients and controls in Caucasian and Chinese populations (Pharoah *et al.*, 2001; Lu *et al.*, 2005). On the other hand, several studies have reported that the AA genotype is associated with an increase in the gastric cancer risk (Al-Moundhri *et al.*, 2010; Chu *et al.*, 2014). Several reasons may account for these discrepancies including modifying genes, environmental factors, and different ethnicities (Chen *et al.*, 2011; El-Husny *et al.*, 2016). Meta-analyses suggested that the rs16260 polymorphism is an ethnicity-dependent risk factor for gastric cancer (Gao *et al.*, 2008; Wang *et al.*, 2008; Jiang *et al.*, 2015) and also for other cancers (Geng *et al.*, 2012; Wang *et al.*, 2012). Due to controversial results from various ethnic

populations, we aimed to study the association of the rs16260 polymorphism in the promoter region of the *CDH1* gene and gastric cancer, for the first time among Iranian gastric cancer patients. In this study, we found that there is no statistically significant difference between cancer patients and control groups. Our analysis showed that patients with the CC genotype were more likely to contract gastric cancer than those with the AA genotype (OR= 0.582, 95% CI, 0.222-1.524, *p*-value = 0.268).

For more accurate analysis, linkage analysis of several tightly linked polymorphisms (Haplotypes) that modulate gastric cancer risk must be included. Depending on the combination of SNPs and genes, the influence of the rs16260 polymorphism may be varied (Zhang *et al.*, 2008).

The exact mechanism underlying the association of E-cadherin genotypes with gastric cancer remains to be well known, but this discrepancy may be related to differences in the affinity of DNA binding proteins to the two alleles of the E-cadherin promoter (Shin *et al.*, 2004; Li *et al.*, 2014).

The *in silico* analysis showed that this substitution eliminates the RXR α and T3R β binding sites and creates binding site for FOXP3 and C/EBP3 transcription factors. It has been shown that the rs16260 polymorphism in the promoter region of the *CDH1* gene changes the methylation status, and eliminates a CF-1

binding site and creates binding sites for RC2 and MCBF transcription factors (Borges *et al.*, 2010). Footprinting analyses showed that the C allele is protected from digestion by DNase I in the presence of the HeLa cell nuclear extracts. On the other hand, transfection of DU145 cells with plasmid carrying the A allele resulted in decreased transcriptional activity (Li *et al.*, 2000). Also, it has been shown that this SNP alters the secondary structure of paRNA and the risk allele facilitates the assembly of AGO1-iso miR-4534 complex that results in the *CDH1* gene silencing (Pisignano *et al.*, 2017).

In conclusion, the current study showed that the rs16260 polymorphism may not have an association with gastric cancer susceptibility in Mazandaran population. It is clear that this study have some limitations including low sample size and lack of information on smoking status, dietary habits and the prevalence of *H. pylori* infection in participants. Also analyzing other SNPs in the *CDH1* gene may fortify our results.

Research ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Acknowledgments

We thank Dr. Ali Tarayati for his statistical assistances and Dr. Abbas Mohammad pour for his collaboration in sample preparation. This study was supported by the University of Mazandaran Research Department.

Conflict of interest

The authors declare that they have no competing interests for this publication.

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