

New Variants in the *CDH1* Gene in Iranian Families with Hereditary Diffuse Gastric Cancer

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Abstract

Background: Hereditary diffuse gastric cancer (HDGC) is a heritable form of diffuse gastric cancer with very aggressive tumors, poor prognosis, and delayed clinical signs.

Method: We assessed 17 probands identified with HDGC upon gastrectomy according to the histopathological criteria confirmed by a pathologist and familial history. We extracted DNA from peripheral blood and formalin fixed paraffin-embedded tissues. DNA sequencing was done following PCR amplification of 16 exons and exon/intron boundaries of the *CDH1* gene and exon 2 of *CTNNA1* gene. The Multiplex Ligation-dependent Probe Amplification technique was performed on patients with no pathogenic variants in sequencing.

Results: Totally, 17 probands comprising seven males and 10 females were assessed. In three patients, we recognized the tumors in the early TNM stage (I, II), while in 14 cases, tumors were observed in the late stages (III, IV). Overall, DNA sequencing of the *CDH1* gene identified 16 variants (seven exonic including five new variants and nine intronic containing six new variants). Moreover, Multiplex Ligation-dependent Probe Amplification detected one deletion in exon 1 of two patients.

Conclusion: Our results showed that E-cadherin deficiency in HDGC was related to *CDH1* gene point mutations and large deletion with high heterogeneity, which should be considered in the diagnosis and treatment of HDGC patients.

Keywords: *CDH1* gene, Diffuse gastric cancer, Iranian families, Hereditary, Mutation

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Introduction

Gastric cancer (GC) is the fourth common cancer with 952,000 new cases and 723,000 deaths during 2012 and the second cause of mortality among all cancers.^{1,2} In addition, GC is estimated to be the eleventh cause of all deaths and account for 1.8% of all deaths by 2030.² GC is the most common cause of cancer-related mortality in Iran, the second prevalent cancer in males (14%), and the fourth in females (7%).^{2,3} Most of GC cases are sporadic, and familial aggregation could be observed in approximately 10% of the cases.^{4,5} Hereditary pattern is observed in a few cases (1-3%).⁶ According to Lauren histological classification, GC is divided into intestinal and diffuse type of adenocarcinoma.⁷ Hereditary diffuse gastric cancer (HDGC) is an autosomal dominant inherited form of DGC, a highly invasive tumor with a poor prognosis, high penetrance, and infiltrating pattern. This causes gastric wall thickening (Linitis plastica) without

forming a definite mass. Signet ring cell carcinoma (SRCC) or isolated cell type carcinoma are common histopathologic features of diffuse GC (DGC).⁸

Mutations in the *CDH1* gene is the most prevalent cause of HDGC and sporadic DGC.⁹ CTNNA1 (encoding the alpha-E-catenin) is the only identified gene involved in HDGC other than *CDH1* germline mutations. This gene has been reported in a large HDGC pedigree as a 2 bp germline deletion in exon 2.¹⁰ Germline mutations in CTNNA1, BRCA2, STK11, SDHB, PRSS1, ATM, MSR1, and PALB2 genes were reported in HDGC patients.¹¹ Furthermore, several genes including LMTK3, RHOA, PIK3CA, MED1, ARID1A, and MCTP22 were detected in the HDGC patients with somatic mutations.¹⁰ More than 80% of the HDGC carriers for *CDH1* mutations, whether male or female, might be inflicted with GC until the age of 80. Moreover, there is a 60% risk of lobular breast cancer until

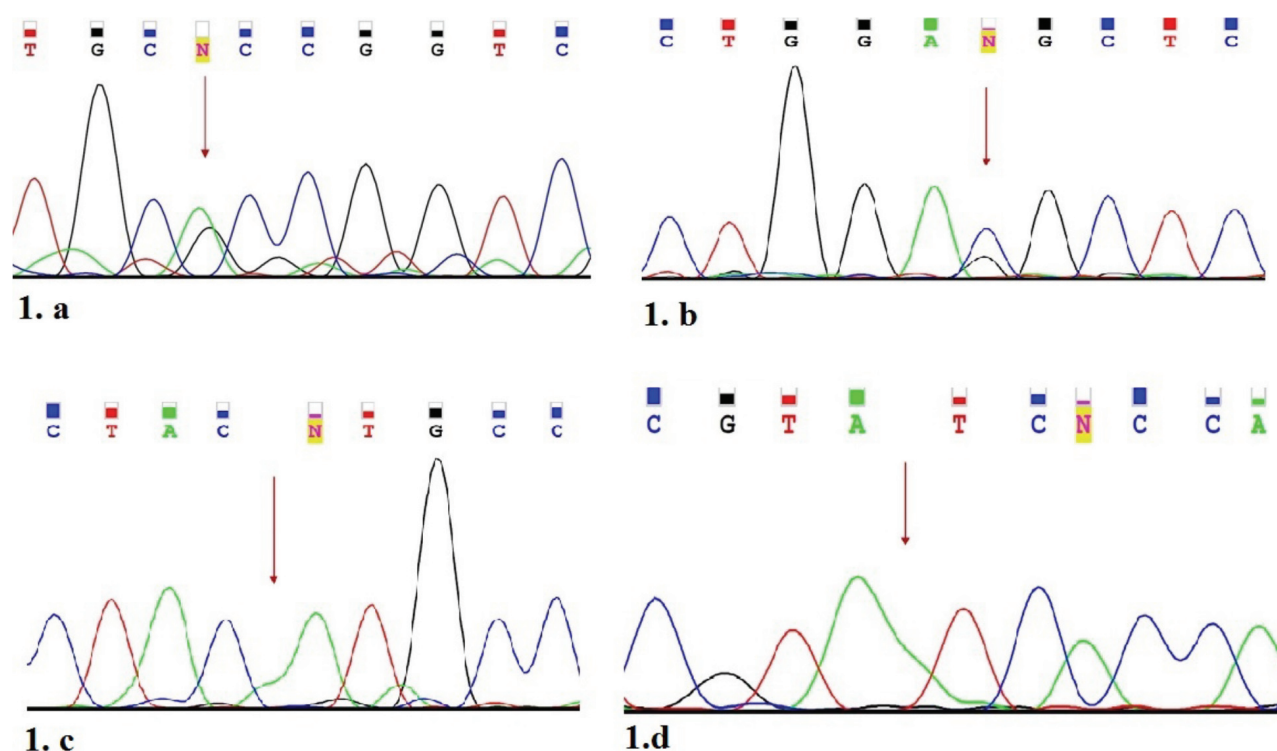


Figure 1 (a): Sequence electropherogram of exon 3 of the *CDH1* gene. Arrow indicates the location of the base substitution at c.181G>A (p.61T>A). (b): Sequence electropherogram of exon 15 of the *CDH1* gene. Arrow indicates the location of the base substitution at c.2331C>G (p.777D>E). 1 (c): Sequence electropherogram of exon 7 of the *CDH1* gene. Arrow indicates the location of the base deletion at c.889delA. (d): Sequence electropherogram of exon 9 of the *CDH1* gene. Arrow indicates the location of the base deletion at c.1177delA.

Table 1. Epidemiological and clinicopathological features of HDGC patients

Sample ID	Tissue type	Gender	Age of diagnosis	Stage	Histopathological type
A	Blood	Female	31	IV	Signet ring cell carcinoma
B	Blood	Female	34	IV	Signet ring cell carcinoma
4	FFPE	Male	49	IIIA	Signet ring cell carcinoma
14	FFPE	Male	36	IIIB	Signet ring cell carcinoma
15	FFPE	Male	61	IV	Signet ring cell carcinoma
18	FFPE	Female	37	IIIA	Signet ring cell carcinoma
20	FFPE	Male	78	IIIC	Signet ring cell carcinoma
22	FFPE	Male	31	IIIC	Signet ring cell carcinoma
29	FFPE	Female	70	IIIC	Poorly differentiated adenocarcinoma
37	FFPE	Female	30	IIIB	Poorly differentiated adenocarcinoma
40	FFPE	Male	51	IV	Signet ring cell carcinoma
41	FFPE	Female	57	IIIC	Poorly differentiated adenocarcinoma
42	FFPE	Female	27	IIIA	Poorly differentiated adenocarcinoma
43	Blood	Male	41	IIIB	Signet ring cell carcinoma
44	Blood	Female	29	II	Signet ring cell carcinoma
45	FFPE	Female	37	I	Signet ring cell carcinoma
48	FFPE	Female	72	II	Signet ring cell carcinoma

the age of 80 in women with *CDH1* germline mutation.¹²

CDH1 gene is located on 16q22.1, including 16 exons encoding E-cadherin.^{13, 14} E-cadherin is

a transmembrane protein that plays a pivotal role in cell adhesion and tumor suppression.¹⁵ The *CDH1* promoter hypermethylation is the most common epigenetic inactivation mechanism of

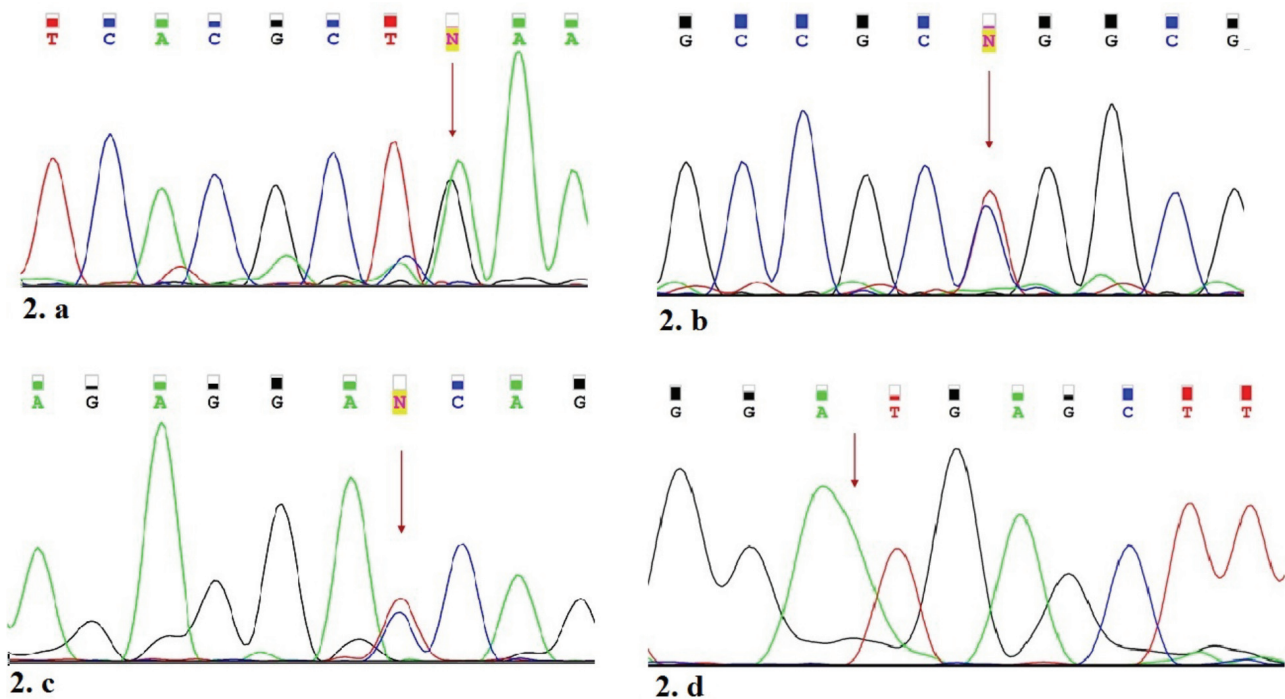


Figure 2 (a): Sequence electropherogram of the exon 3 of the *CDH1* gene. Arrow indicates the location of the base substitution at c.348G>A (p.116L>L). (b): Sequence electropherogram of exon 13 of the *CDH1* gene. Arrow indicates the location of the base substitution at c.2076T>C (p.692A>A). (c): Sequence electropherogram of exon 14 of the *CDH1* gene. Arrow indicates the location of the base substitution at c.2292C>T (p.764D>D). (d): Sequence electropherogram of exon 13 and the intron boundary of the *CDH1* gene. Arrow indicates the location of the base deletion at c.1937-58delA.

Table 2. Exonic and intronic variants in *CDHI* gene among HDGC patients

Sample ID	Type of tissue	Exon or exon/intron boundary	Substitution	Deletion	Heterozygosity	Amino acid	Chromosome location
14, 15, 20, 48.	FFPE	3	G>A	-	Heterozygote*	L116L	NM_004360.3:c.348G>A
20	FFPE	3	A>G	-	Heterozygote*	T61A	NM_004360.3:c.181A>G
A, B	Blood	7	-	A	Homozygote*	Exonic	NM_004360.4:c.889delA
B	Blood	9	-	A	Homozygote*	Exonic.	NM_004360.4:c.1177delA
14, 29, B, 15, 40, 41, 42, 46	FFPE/ Blood	13	T>C	-	Heterozygote/ Homozygote	A692A	NM_004360.:c.2076T>C rs:1801552
B	Blood	14	C>	-	Heterozygote	D764D	NM_004360:c.2292C>T rs: 61747636
29	FFPE	15	C>G	-	Heterozygote*	D777E	NM_004360:c.2331C>G
15	FFPE	4	G>C	-	Heterozygote	-	NM_004360:c.531+10G>C rs: 33963999
15, 22, A, B, 40, 41, 43, 46	FFPE/ Blood	13	-	A	Homozygote*	-	NM_004360.3:c.1937-58delA
22	FFPE	13	G>	-	Heterozygote*	-	NM_004360.4:c.2164+48G>A
22	FFPE	13	G>A	-	Heterozygote*	-	NM_004360.4:c.2164+51G>A
14, 15, 20, 22, 44,	FFPE	15	C>G	-	Heterozygote*	-	NM_004360.4:c.2296-48C>G
14, 15, 20, 22,	FFPE/ Blood	15	T>G	-	Heterozygote*	-	NM_004360.4:c.2296-44T>G
44, 48	FFPE/ Blood	15	C>G	-	Heterozygote	-	NM_004360:c.2296-22C>G rs:763184960
14, 15, 20, 22, 44, 48	FFPE/ Blood	15	C>G	-	Heterozygote	-	NM_004360.3:c.2439+105T>G
40	FFPE	15	T>G	-	Heterozygote*	-	NM_004360.4:c.2439+52G>A rs:33965115
43, 46	FFPE/ Blood	15	G>A	-	Heterozygote	-	

*New mutation, FFPE: Formalin fixed-paraffin embedded

the gene as the second hit in HDGC; however, other secondary substitution or insertion/deletion mutations have been reported with less frequency.¹⁴ To date, the majority of the germline mutations have been identified as single nucleotide substitutions.¹⁶ On average, 5% of the familial DGC cases are due to the large deletions up to several exons of the *CDHI* gene.^{17, 18} Large deletions in *CDHI* gene have been described as a reason for cancer susceptibility in Japanese,¹⁹ Canadian, and European familial GC patients.²⁰ In this study, we reported several new *CDHI* variants and a large deletion in Iranian patients with HDGC.

Materials and Methods

Patients and sampling

We identified 17 probands with HDGC using histological features and clinical criteria based on IGCLC 21. A pathologist confirmed DGC. We selected the samples from GC patients who, from January 2011 to April 2016, referred to Al-Zahra Hospital, a referral hospital in Isfahan

province, central Iran, and Alaa cancer control center, a charity-based foundation for cancer patients in Isfahan. Four samples belonged to blood's probands and 13 samples of probands were formalin fixed-paraffin embedded (FFPE) tumor tissues. We extracted the DNA of blood samples by Prime Prep Genomic DNA Isolation Kit (GeNet Bio, Korea). FFPE tumor samples were cut to 5-10µm thickness sections for DNA extraction using SDS-proteinase K digestion, phenol chloroform, and ethanol precipitation. All patients participating in the study or their families completed informed consent forms. The Review Board of Isfahan University of Medical Sciences approved this study with code number 394479.

DNA sequencing

We assessed the obtained DNA to sequence *CDHI* and *CTNNA1* genes in all the samples. Polymerase chain reaction (PCR) amplified the DNA. Specific primers amplified all coding exons and exon/intron boundary regions of the *CDHI* gene (NM_004360.4) and exon 2 of *CTNNA1*

Table 3. Effect of non-synonymous variants on protein function of *CDH1* gene

Amino acid substitution	PolyPhen 2	SIFT	I-Mutant v2.0	Mutation taster	PROVEAN	Mutation assessor	PhD-SNP	ConSurf
T61A	Benign: 0.005	Tolerated: 0.78	Decrease stability DDG: -0.99	Polymorphism.	Neutral	low- FI score: 1.085	Neutral	1
D777E.	Probably damaging: 0.985	Tolerated: 1	Decrease stability DDG: -0.48	Disease causing.	Neutral	low- FI score: 1.245.	Neutral	7

Ranges of score variation are depicted below the program names. Categorical predictions based on the following cut-off values: Polyphen-2: Benign= 0-0.2, Possibly damaging=0.2-0.85, Probably damaging=0.85-1; SIFT: Damaging if ≤ 0.05 , I-Mutant: Free Energy Change Value (DDG) <0 = Decrease stability, DDG >0 =Increase stability; Mutation Taster: disease causing= probably deleterious, disease causing automatic = known to be deleterious, polymorphism= probably harmless, polymorphism automatic= known to be harmless, PROVEAN: Deleterious if ≤ -2.5 , Mutation assessor: Functional impact of a variant (Func. Ipmact)= predicted functional (high, medium), predicted non-functional (low, neutral); PhD-SNP: Neutral: Neutral polymorphism, Disease: Disease-related polymorphism; ConSurf: The conservation scale: Variable=1-3, Average=4-6, Conserved=7-9

gene (NM_001903.4). We sequenced the PCR product of each reaction through the use of ABI 3130XL capillary sequencing platform (Applied Biosystems/Life Technologies, Carlsbad, CA, USA). The obtained sequences were analyzed using Chromas software, version 2.31.

In silico investigation of pathogenicity

We assessed the effect of non-synonymous variants on protein function using bioinformatics software tools, including Polyphen2, SIFT, I-Mutant, Mutation taster, Mutation assessor, PROVEAN, ConSurf, and PhD-SNP. Human Splice Finder (HSF) version 3 evaluated the effect of intronic variants on the splicing site.

Multiplex ligation-dependent probe amplification (MLPA)

We examined samples with no identified pathogenic point mutations to identify large deletions/duplications in *CDH1* and *CTNNA1* genes using SALSA P083-C2 *CDH1* MLPA kit (MRC-Holland, Amsterdam, Netherlands). The reactions were performed according to the manufacturer's instruction. Probe ratio (PR) described deletions or duplications. A PR of less than 0.7 presented a gene dosage reduction, and a PR of more than 1.3 indicated an increase in gene dosage.

Results

Clinicopathologic characteristics

Altogether, the mean age of the cases at diagnosis was 45.4 years (49.6 in men and 42.4 in women) and seven of the 17 (41%) patients were older than 45. In three patients (18%), we

recognized the tumors at an early TNM stage (I, II), and in 14 cases (82%), the tumors were detected at late stages (III, IV). SRCC and "poorly differentiated adenocarcinoma" were reported as the histopathological type of the tumor in 13 (76.5%) and 4 (23.5%) cases, respectively (Table 1).

Sequence analysis

DNA sequencing of the amplified PCR products exhibited eleven new variants (5 exonic and 6 intronic) in the *CDH1* gene of the samples (Table 2). Two exonic variants were non-synonymous (T61A and D777E), three were synonymous (L116L, A692A and D764D), and two were one-nucleotide deletion (c.889delA and c.1177delA). No single nucleotide changes occurred in exon 2 of *CTNNA1*. Using DNA sequencing, we further found one base pair deletion in *CDH1* gene at c.1937-58delA in eight samples. Interestingly, seven of these samples also carried exonic variants, two of which were predicted to be pathogenic. This could favor the benign effect of the intronic variant.

Amino acid substitutions

We detected two non-synonymous variants, including a single base pair substitution, A to G transition resulting in a single amino acid substitution at codon 61 as p.61T>A (Figure 1a), and a single base pair substitution C to G transversion leading to an amino acid substitution at codon 777 as p.777D>E (Figure 1b) (Table. 2). Moreover, we detected two deletions with one-nucleotide at c.889delA (Figure 1c) and c.1177delA (Figure 1d). The discovered synonymous variants were: a single base pair

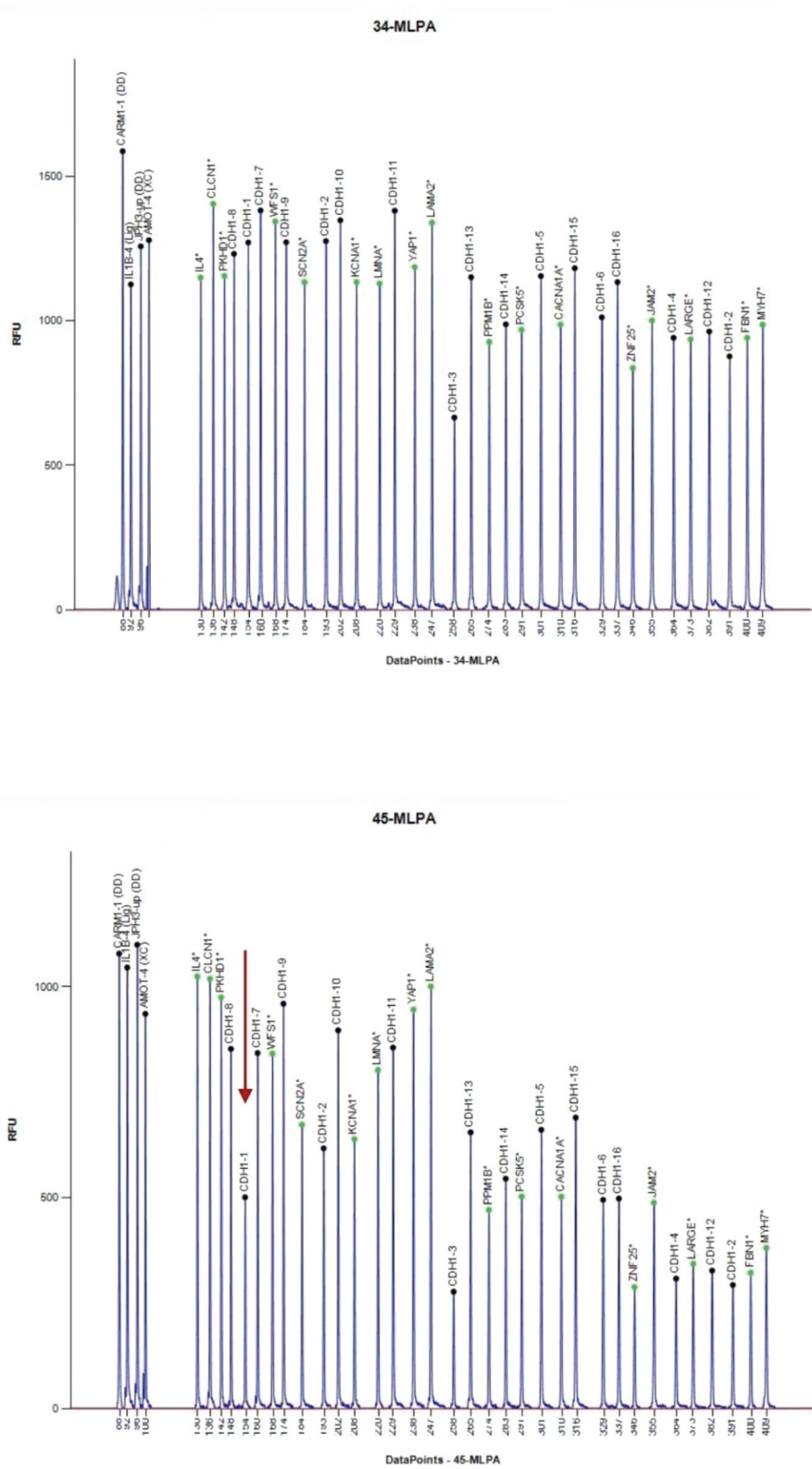


Figure 3. Coffalyser electropherogram of the *CDH1* gene. Above electropherogram relates to normal controls and the bottom belongs to the patients. Arrow indicates deletion in exon 1.

Table 4. Intronic variants effect on splicing site

Sample ID	Splice site type	Consensus value (0-100)
NM_004360.4:c.1937-58delA	Acceptor	70.07
NM_004360.4:c.2164+51G>A	Donor	72.25
NM_004360.4:c.2296-48C>G	Acceptor	69.78
NM_004360.4:c.2296-44T>G- c.2296-48C>G	Donor	73.78
NM_004360.4:c.2296-22C>G	Acceptor	82.33
NM_004360.4:c.2439+52G>A	Acceptor	66.32
NM_004360.3:c.2439+105G>T	Donor	66.12

Consensus value: splice site if >= 65

substitution, G to A nucleotide transition resulting in synonymous substitution at codon 116 (p.116L>L) (Figure 2a), a single base pair substitution, T to C nucleotide transition leading to a synonymous substitution at codon 692 (p.692A>A) (Figure 2b), and finally, a single base pair substitution, C to T transition leading to a synonymous substitution at codon 764 (p.764D>D) (Figure 2c) (Table. 2).

Effect of non-synonymous variants on protein function

Bioinformatics analysis by PolyPhen2, I-Mutant, Mutation taster, Mutation assessor, and ConSurf software tools suggested the p.777D>E substitution can have pathogenicity effect on the protein function (Table 3).

Intronic substitutions and splicing site-effects

Moreover, we identified certain likely benign variants in intronic sites including a single base pair deletion at c.1937-58delA (Figure 2d) and several single base pair substitutions containing the following: c.531+10G>C, (c.2164+48G>A, c.2164+51G>Ac.2296-48C>G, c.2296-44T>G c.2296-22C>G, c.2439+52G>A and finally (.2439+105T>G. The evaluation of intronic variants showed the potential effect of seven different variants on the splicing site (Table 4).

MLPA results

Using the MLPA technique, we found one large deletion in exon 1 (CDH1 probe 12651-L19938) in two patients (Figure 3). This change was confirmed by quantitative PCR by primers of exon 1 *CDH1* gene in patients. From the same

patient, we obtained the normal sample as the negative control for normalization. To analyze the results, we utilized the ABI Step One Plus (Applied Biosystems, Foster City, CA, USA) instrument and the $\Delta\Delta C_t$ method.

Discussion

Most GC cases are sporadic and in approximately 10% of cases, a familial aggregation is observed. Therefore, hereditary GC encompasses just a minority of cases.⁴⁻⁶ Almost 15 to 50% of families presenting with HDGC criteria (according to International Gastric Cancer Linkage Consortium) presented germline mutations in *CDH1* gene.²² Until 2010, the rate of *CDH1* gene mutations in DGC was reported 25 to 50%;¹⁶ however, using updated criteria, the rate of mutation was reduced down to 10-18% in countries with low GC incidence.^{22, 23} The HDGC incidence rate is unknown in Iran. In the present study, we identified somatic and germline variants in Iranian patients with HDGC in 15 of 17 (88.2%) cases. Regarding certain variants and single nucleotide polymorphisms in *CDH1* gene, there is a correspondence between our findings and other studies. For instance, substitutions at position c.2076T>C (A692A),²⁴ c.2292C>T (D764D), c.531+10G>C,²⁵ and c.2439+52G>A were previously described.²⁶ Based on table 2, other exonic and intronic variants marked with an asterisk were novel in our study.

Corso et al. detected mutations in two Italian patients with early onset DGC. In their study, one of the mutations was missense p.Arg224Cys and the other was a substitution -63C>A. Also, they found five out of 21 tumors in early TNM

stage (I, II), and 15 out of 21 in the late stages (III, IV).²⁷ Until now, only one study has evaluated *CDHI* gene mutations in a single family with HDGC in Iran, reporting a truncating mutation.²⁸ We found certain substitutions, small and large deletions in our samples located on exons 1, 3, 7, 9, 13, 14, and 15 of *CDHI* gene. Bioinformatics analysis suggested that the p.777D>E substitution in exon 15 showed that this mutation would negatively affect the protein function. Exon 1 of *CDHI* gene encodes the signal peptide domain and exon 3 of this gene encodes the propeptide of E-cadherin protein. The signal peptide domain is necessary for the import of the protein into the endoplasmic reticulum. Exons 7, 9, and 13 of *CDHI* gene encode the extracellular domain of E-cadherin, essential for cell-cell adhesion;²⁴ also, changes in this domain can destroy the cell adhesion. Exons 14 and 15 of *CDHI* encode the cytoplasmic domain of E-cadherin. Cytoplasmic domain binds to β -catenin and plays a fundamental role in tumor silencing.²⁸ Approximately 5% of *CDHI* mutations in HDGC patients were large deletions.^{17, 18} In the present study, we detected one large deletion in exon 1 in two cases by the MLPA technique. In 2009, Oliveira et al. identified some large deletions in exons 1, 2, 14, 15, and 16 of *CDHI* gene in HDGC families.¹⁸ In 2014, a large genomic deletion (c.1566-?_1711+?del) in exon 11 of the *CDHI* gene in a patient with no familial history of gastric cancer was reported;²⁹ moreover, Molinaro et al. reported a deletion with 1642 bp length, with breakpoints in introns 6 and 8 of *CDHI* gene (c.833-476_1138-463del).³⁰

We found several intronic variants in *CDHI* gene, including c.1937-58delA, c.2164+51G>A, c.2296-48C>G, c.2296-44T>G, c.2296-22C>G, c.2439+52G>A, and c.2439+105G>T. Bioinformatics analysis by HSF showed that they might generate new potential splice sites leading to incorrect splicing. Further functional data is required to reach a conclusion on the effects of these variants.

In conclusion, our results showed that E-cadherin deficiency in HDGC is associated with *CDHI* gene point mutations and large deletions with high heterogeneity. This should be considered

in the diagnosis and treatment of HDGC patients. These results highlight the important roles of *CDHI* gene, development of molecular and genetic testing by PCR sequencing, MLPA, and high throughput techniques such as whole exome sequencing and whole genome sequencing for an early diagnosis and prevention of the potentially lethal effects of HDGC in high risk individuals.

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Conflict of Interest

None declared.

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