Novel mechanism of a CDH1 splicing mutation in a Korean patient with signet ring cell carcinoma

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INTRODUCTION

Hereditary diffuse gastric cancer (HDGC) is defined by the International Gastric Cancer Linkage Consortium (IGCLC) as 2 or more cases of diffuse gastric cancer in first- or second-degree relatives, with at least one diagnosed before the age of 50 years, or 3 or more pathologically documented cases of diffuse gastric cancer in first- or second-degree relatives of any age (1). Although only a few gastric cancers are hereditary, the identification of such cases may provide valuable information for genetic counseling, genetic testing for gastric cancer susceptibility, and gastric cancer risk reduction strategies for as-yet unaffected family members (1). The vast majority of CDH1 gene mutation carriers (>90%) already exhibit microscopic intramucosal disease by prophylactic total gastrectomy (2).

One-third of all HDGC families harbor a germline mutation of the CDH1 gene, which encodes E-cadherin, a cell-to-cell adhesion protein that plays a fundamental role in the maintenance of cell differentiation and the normal architecture of epithelial tissues. E-cadherin deficiency provides an obvious explanation for the diffuse, scattered growth of HDGC tumors, as this protein is the central component of epithelial cell-to-cell adhesion (3, 4).

We report herein a novel mechanism of a CDH1 splicing mutation in a Korean patient with signet ring cell carcinoma of the stomach. This study reveals the disease-causing mechanism of this splicing mutation, and emphasizes the need for functional studies using RNA samples for the accurate interpretation of detected splicing variant. This is the first reported case of a CDH1 mutation in a Korean patient. [BMB reports 2011; 44(11): 725-729]

Subject

A 27-year-old Korean man had been complaining of dyspepsia for 7 years, and had symptoms of aggravated epigastric pain and indigestion for 2 months. Physical examination revealed no significant findings. He was an ex-smoker (had quit 8 years prior to examination) and had no underlying diseases such as diabetes or hypertension. A family history of stomach cancer was evident; his father and uncle died at the ages of 33 and 41, respectively, from the disease (Fig. 1).

A 2.5-cm hyperemic, friable, nodular lesion was observed on the angle of his stomach by gastroendoscopy, suggesting advanced gastric cancer. In addition, chronic active Helicobacter pylori (H. pylori) gastritis was found. An endoscopic biopsy was performed, and he was diagnosed with signet ring cell carcinoma. Computed tomography revealed no obvious stomach wall thickening and no evidence of lymph node or distant metastasis. The levels of tumor markers such as carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), and carbohydrate antigen 72-4 (CA72-4) were all within normal limits. The nodular lesion on the stomach angle...
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Fig. 1. Pedigree of the proband’s family, showing the autosomal dominant trait. The proband is indicated by an arrow. Shaded symbols indicate a diagnosis of cancer. Ages are shown directly below the symbol. Cancer diagnoses are followed by the age at diagnosis. Stomach: stomach cancer.

RESULTS

Sequence analysis
Direct DNA sequencing of the CDH1 gene revealed a heterozygous A to G transition at cDNA position 833-2 (c.833-2A > G), which is a splice site mutation (Fig. 3B). We performed further analysis using a sample of the patient’s RNA/cDNA to confirm the splicing defect. The reverse transcription-polymerase chain reaction (RT-PCR) products consisted of the expected band size and a larger band (Fig. 3A). Direct sequencing of the PCR products yielded dual peaks at the beginning of the exon 6/7 junction in both the forward and reverse reactions (Fig. 3B). The mutant sequence, which had a low peak height, was identical to the 79-bp sequence of the 3’ end of intron 6 when aligned with the reference sequence (NM_004360.3) of the CDH1 gene from genomic databases such as those at UCSC (University of California Santa Cruz) (http://genome.ucsc.edu/) and Ensemble (http://www.ensembl.org/) (Fig. 3B and C). We found that CDH1 c.833-2A > G generated a new acceptor site within intron 6, causing the insertion of a 79-bp intronic sequence between exon 6 and 7 (r.833-79_833-1ins), and resulting in a frame shift (Fig. 3C).

Bioinformatic analysis
The results of the bioinformatic analysis are summarized in Table 1. All 9 algorithms predicted the splice defect. In addition, the new potential predicted splice sites were consistent with the in vivo results obtained from all algorithms.

DISCUSSION

We report a first case of CDH1 mutation in a Korean patient with signet ring cell carcinoma of the stomach. Previously, Yoon et al. (6) reported 2 germline missense mutations in CDH1 in 2 of 5 Korean familial gastric cancer patients that were screened. However, subsequent repeated experiments...
Fig. 3. A splicing mutation confirmed by RNA/cDNA analysis. (A) A transcript abnormality in the CDH1 gene. Electrophoresis of cDNA from patient and normal control leukocytes after PCR. The forward primer is specific for exon 3 and the reverse primer is specific for the exon 8/9 junction. The expected product size is 838 bp. The open circle symbol indicates the abnormal transcript (917 bp) containing the new cryptic exon sequence. The square symbol indicates a normal transcript variant (682 bp) with the loss of exon 5. (B) Sequencing results of the CDH1 gene using RNA/cDNA. The results reveal an A to G transition at cDNA position 833-2 (arrow; c.833-2A>G) and dual peaks at the beginning of the exon 6/7 junction in both the forward and reverse reactions. The mutant sequence was aligned with the 3’-end of the IVS 6 region in the CDH1 gene reference sequence (NM_004360.3). (C) Schematic diagram of the pathogenic mechanism for this splicing mutation. A new acceptor splice site was generated at position c.833-81 within intron 6, marked by “ag”. The last 9-bp sequence (CTC TTC TAG) of exon 6 is identical to that of intron 6, yielding homozygous peaks on the chromatogram (bold font in Fig. 3B and 3C).

Using denaturing high-performance liquid chromatography (DHPLC) and automatic sequencing by authors from the same laboratory did not reveal any CDH1 germline mutation in these 2 families. They concluded that the previous incorrect results may have originated from experimental artifacts generated during the PCR single-strand conformation polymorphism (SSCP) or cloning–sequencing steps (7). Although gastric cancer is the second most common type of cancer worldwide, germline mutations in CDH1 are found in only 1-3% of all gastric cancers (8). In addition, inactivating germline CDH1 mutations occur less frequently in countries with high sporadic gastric cancer rates, such as Japan and Korea (4). This observation may be related to the obscured identification of families with inherited susceptibilities or population frequencies with low-penetrance alleles (4).

This study, based on sequencing of RNA/cDNA from the patient, demonstrates the pathogenetic mechanism of the CDH1 c.833-2A>G splicing mutation. We found that this mutation generated a new acceptor site at position c.833-81 within intron, causing the insertion of a 79-bp intronic sequence between exon 6 and 7 (r.833-79_833-1ins) and resulting in a frame shift. This result emphasizes the need for functional studies using RNA samples for the accurate interpretation of any splicing variant detected by DNA sequencing. Although the c.833-2A>G mutation was previously reported in a symptomatic patient with diffuse gastric cancer (9), the disease-causing mechanism of this splicing mutation was not described.

Analyses at the mRNA level are essential for detecting novel or unclassified variants that induce splicing alterations; however, such analyses are not always suitable as a routine laboratory procedure. The reliability and accuracy of predicting splicing alterations are valuable, making in silico splicing prediction useful. We compared 9 different algorithms for the novel CDH1 c.833-2A>G variant. All 9 algorithms predicted this variation as deleterious. The predicted new potential splice sites of all of the algorithms were consistent with the in vivo result (Table 1). Houdayer et al. (10) evaluated 6 in silico tools, including Splice Site Prediction by Neural Network (NNSplice), Splice-Site Finder, MaxEntScan, Automated Splice-Site Analyses, Exonic Splicing Enhancer (ESE) Finder, and Relative Enhancer and Silencer Classification by Unanimous Enrichment (RESCUE)-ESE. All 6 tools predicted 17 deleterious mutations that impacted canonical AG/GT dinucleotide splice sites. However, the sensitivity for unclassified intronic variants was only 38-85% in less-conserved intronic positions (±60 nucleotides from an AG/GT site). Thus, a combination of complementary in silico tools is necessary to overcome the limitations of individual in silico tools for less-conserved intronic
E-cadherin plays a central role in the maintenance of cell polarity, and its loss during tumorigenesis is associated with poorly differentiated cancers, often with signet ring cell morphology (4). HDGC initiation requires somatic downregulation of the second CDH1 allele, which in most cases, is caused by DNA promoter hypermethylation (4). Transient episodes of H. pylori infection or gastritis are sufficient to provoke stable DNA methylation (4). In the present proband, H. pylori gastritis may have promoted the downregulation of the second CDH1 allele.

The proband’s grandfather and grandmother were both healthy when they died in their 80s. Their lack of gastric cancer may be explained by partial penetrance. Although HDGC is inherited in an autosomal-dominant manner, the lifetime penetrance of CDH1 germline-mutation carriers is estimated at 70% (11). Unfortunately, we were unable to obtain specimens from the proband’s deceased father and uncle who died of gastric cancer 25-30 years prior to the proband’s diagnosis.

In conclusion, we report a novel mechanism of a CDH1 splicing mutation in a Korean patient with signet ring cell carcinoma of the stomach. This study demonstrates the disease-causing mechanism of this splicing mutation based on sequencing of RNA/DNA from the patient, and emphasizes the need for functional studies using RNA samples for the accurate interpretation of any splicing variant detected by DNA sequencing.

MATERIALS AND METHODS

Sequence analysis

Genetic analysis was performed on the patient after obtaining written informed consent. Genomic DNA was extracted from peripheral blood leukocytes using the Wizard Genomic DNA Purification kit according to the manufacturer’s instructions (Promega, Madison, WI, USA). All of the coding exons and flanking intronic regions of the CDH1 gene were amplified using primer sets designed by the authors (available upon request). PCR was performed using a thermal cycler (Model 9700; Applied Biosystems, Foster City, CA, USA). Cycle sequencing was performed on an ABI Prism 3100 Genetic Analyzer using the BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems). For RNA/DNA analysis, fresh peripheral blood samples containing EDTA from the patient were collected. RT-PCR was performed in 2 steps. First, cDNA was produced from total RNA using the RevertAid First Strand cDNA Synthesis kit (Fermentas, Hanover, MD, USA) with random hexamer primers according to the manufacturer’s protocol. In the second step, PCR was performed using sequence-specific primers designed by the authors that annealed to the beginning of exon 3 and to the end of the exon 8/9 junction. For RT-PCR analysis, the following primers were used: exon 3 forward, 5’-CCT GGG ACT CCA CCT ACA GCC TGT TTG CTG TTG TGC TTG-3’; exon 6 reverse, 5’-CTG GG ACT CCA CCT ACA GA-3’; and exon 8/9 reverse, 5’-TTG TAC GTG CTG CTG CGA TTG AA-3’. For RT-sequencing analysis, the following primers were used: exon 3 forward, above; exon 6 reverse, 5’-GGA TGA CAC AGC GTG AGA GA-3’; exon 5 forward, TGA CCC TGT TGG TGT CTT TA-3’; and exon 8 reverse, 5’-CAG CTG TTG CTG CTG TGC TTG TTG-3’.

We sequenced the mutation site in genomic DNA samples isolated from 96 normal healthy volunteers to show it was not a polymorphism.

Bioinformatic analysis

The CDH1 c.833-2A>G variant was analyzed in silico for 3’ splice sites using NNSplice (www.fruitfly.org/seq_tools/splice.html), GeneSplicer (http://cbcb.umd.edu/software/GeneSplicer), Human Splicing Finder (http://www.umd.be/HSF1/), MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), NetGene2 (www.cbs.dtu.dk/services/NetGene2), and Alamut version 1.5 (Interactive Biosoftware, Rouen, France). In each case, the software’s default thresholds were used.

**Table 1. In silico analysis of the CDH1 c.833-2A>G variant using various splicing prediction programs**

<table>
<thead>
<tr>
<th>Algorithm</th>
<th>Normal score</th>
<th>Mutant score</th>
<th>Results</th>
<th>New potential splice site (normal score, mutant score, % variation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNSplice</td>
<td>0.88</td>
<td>-</td>
<td>WT site broken</td>
<td>c.833-79 (0.95, 0.95, 0%)</td>
</tr>
<tr>
<td>GeneSplicer</td>
<td>10.68</td>
<td>-</td>
<td>WT site broken</td>
<td>c.833-79 (-, 7.00, new site generation)</td>
</tr>
<tr>
<td>Human Splicing Finder</td>
<td>86.51</td>
<td>57.56</td>
<td>WT site broken</td>
<td>c.833-79 (94.02, 94.02, 0%)</td>
</tr>
<tr>
<td>MaxEntScan</td>
<td>8.10</td>
<td>0.15</td>
<td>-84.15% variation</td>
<td>c.833-79 (12.15, 12.15, 0%)</td>
</tr>
<tr>
<td>Alamut-NNSplice</td>
<td>0.88</td>
<td>-</td>
<td>WT site broken</td>
<td>c.833-79 (0.95, 0.95, 0%)</td>
</tr>
<tr>
<td>Alamut-GeneSplicer</td>
<td>10.69</td>
<td>-</td>
<td>WT site broken</td>
<td>c.833-79 (6.89, 7.01, +1.7%)</td>
</tr>
<tr>
<td>Alamut-SiteFinder-like</td>
<td>87.55</td>
<td>-</td>
<td>WT site broken</td>
<td>c.833-79 (88.95, 88.95, 0%)</td>
</tr>
<tr>
<td>Alamut-MaxEntScan</td>
<td>8.10</td>
<td>-</td>
<td>WT site broken</td>
<td>c.833-79 (12.15, 12.15, 0%)</td>
</tr>
</tbody>
</table>

WT: wild-type; - below the threshold.
Acknowledgements
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REFERENCES


