Endogastric capsule for *E-cadherin* gene (*CDH1*) promoter hypermethylation assessment in DNA from gastric juice of diffuse gastric cancer patients

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**Background:** We investigated whether an endogastric capsule (EC) may be a valuable tool for collecting DNA from exfoliated cells from the gastric mucosa and for carrying out an analysis of promoter methylation status of the *E-cadherin* (*CDH1*) gene in poorly differentiated, diffuse gastric cancer (DGC).

**Material and methods:** Consecutive patients with a confirmed diagnosis of poorly differentiated DGC underwent collection of gastric juice by EC. Subjects without cancer and premalignant lesions were also accrued as controls. The samples of gastric juice were processed for DNA isolation and amplification. Then they were used for analysis of *CDH1* promoter hypermethylation.

**Results:** The procedure successfully allowed the analysis of *CDH1* promoter hypermethylation in 20 patients and 14 controls. This pilot study showed feasibility of the procedure and a significantly different *CDH1* promoter hypermethylation status between DGC patients and controls was detected.

**Conclusions:** The EC may represent an innovative and noninvasive tool for the analysis of a specific epigenetic change in DGC patients. Our findings deserve additional studies as this method may represent a cost-effective tool for early detection of sporadic as well as hereditary DGC in *CDH1* germline mutations carriers.

**Key words:** *E-cadherin*, gastric adenocarcinoma, gastric neoplasms, hypermethylation

**introduction**

Over the past decades, a steady decline has occurred in gastric cancer incidence and mortality in many countries, but stomach cancer remains the second most frequent cancer worldwide, with about 12% of cancer deaths each year [1]. A major strategy for facing this health care problem is the identification of at risk individuals, for prevention and early detection of the disease. The knowledge on molecular alterations, which are involved in the carcinogenic process of gastric carcinoma [2], may lead to new, and hopefully more effective means for controlling this lethal disease.

Germline truncating mutations in the *E-cadherin* gene (*CDH1*) have been found in several families with hereditary diffuse gastric cancer (HDGC) [3]. In the majority of cases of HDGC, the wild-type allele was found to be inactivated by *CDH1* promoter hypermethylation [4]. Aberrant methylation of gene regulatory elements, a well-known epigenetic change, acts as an important alternative to genetic alteration for gene inactivation. Hypermethylation of normally unmethylated CpG rich areas, also known as CpG islands, which are located in or near the promoter region of many genes, has been associated with delayed replication, condensed chromatin and inhibition of transcription initiation. Loss of *E-cadherin* expression with *CDH1* promoter hypermethylation was also shown to occur frequently in sporadic cases of diffuse gastric carcinomas and it is now considered to play a relevant role in the development of the disease [5–7].

*CDH1* promoter hypermethylation was found to occur in the early stages of the disease and it may also be detected in preneoplastic lesions of the gastric mucosa [8]. According to these findings, *CDH1* promoter hypermethylation may represent a marker for identifying individuals who are at risk for the development of poorly differentiated, diffuse gastric cancer (DGC).

We previously demonstrated that an endogastric capsule (EC) is a simple noninvasive method for collecting gastric juice and exfoliated cells from the gastric mucosa [9]. We hypothesized that this sampling could be also effective for DNA isolation and amplification and the analysis of *CDH1* promoter hypermethylation. We tested this hypothesis in a pilot study, which included a series of patients with sporadic, poorly differentiated DGC and controls. The effectiveness of this
procedure may represent an innovative tool for detecting a specific epigenetic change to be used as a marker of the disease.

**Material and Methods**

**Patient Characteristics**

The study population consisted of consecutive subjects who were diagnosed with poorly differentiated, diffuse gastric carcinoma (pure diffuse subtype or mixed intestinal and diffuse). Diagnoses were made by upper endoscopy and biopsies of suspected lesions. After diagnosis, patients were asked to undergo EC for collecting gastric juice. A control group was also planned and it consisted of individuals who underwent upper endoscopy with negative findings or benign lesions. Subjects with precancerous lesions such as intestinal metaplasia and dysplasia were excluded.

**EC and Processing of Samples**

The EC was conceived by one of the investigators (PM) and was made by Quercetti, Turin, Italy [9]. It is composed of three main components, which are illustrated in Figure 1. The external envelope is made of gelatin material and it measures 14 mm in length and 5 mm in diameter. The inner capsule is made of plastic inert material and it measures 13 mm in length and 4 mm in diameter. The inner capsule is pierced with three holes and contains a rolled-up piece of absorbent paper. A thin nylon thread, 45–50 cm in length, is connected to the cover of the inner EC, and is fitted with a small button at the opposite extremity, to be kept between two adjacent teeth. Patients were asked to fast overnight and to swallow the capsule while drinking a small amount of water (generally half a glass). They fastened the small button of the nylon thread between two adjacent teeth and the capsule was left into the gastric cavity for 60 min to allow digestion of the outer envelope, penetration of the gastric juice into the inner capsule and saturation of the absorbent paper. Once removed from the inner capsule, the absorbent paper was unrolled. First, it was used for touch deposition on slides for microscopy analysis of cellularity after neutralization of the samples. Subsequently, each sample was subjected to DNA extraction, amplification and coamplification: a methylated set of primers and probe for the CDH1 promoter and an internal reference set of primers and probe, designed to avoid CpG nucleotides, for MYOD1 gene. MYOD1 was used as control for input DNA. The coamplification of CDH1 and MYOD1 genes was carried out with 2 μl of modified DNA sample added to 23 μl containing 1× Hot-Rescue Real-Time PCR Kit–Fluo Probe (Diatech srl, Fano, Italy), 400 nM of each CDH1 primer, 600 nM of each MYOD1 primer, 200 nM of each CDH1 and MYOD1 probe, 3 μl MgCl₂ and 0.2 μl Hot-Rescue DNA Polymerase (5 U/μl).

The sequences of primers and probe used to amplify and detect methylated CDH1 were 5’-AAATTTTACTGATTAGGGTTATGC CGGT (forward), 6FAM-5’-CGGCCACCGGACCTGCGAT-TAMRA (probe) and 5’-TCCCCAAGGAACTAAGGAC (reverse). The sequences of primers and probe used to amplify and detect MYOD1 were 5’-CCAACTGGAAATCCCTCCTCAT (forward), JOE-5’-TCCCTTCCATTCCACAACTTAATACCTC-TAMRA (probe) and 5’-TGATTAATTTAAGGTGGTTAGAAGGA (reverse). Real-time PCR amplification was carried out at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. Amplification of MYOD1 occurs independent of its methylation status, whereas the amplification of CDH1 is proportional to the degree of cytosine methylation within the amplicon.

CpGenome™ Universal Methylated DNA (Chemicon, Temecula, CA) was used as methylated positive control (C+) during sodium bisulfite treatment and MSP assay. The ratio between CDH1 and MYOD1 in C+ was used as measure of a 100% status and the ratio between the values obtained in sample and C+ multiplied by 100 were used as measure of methylation degree following the formula:

\[
\left(\frac{50 - C_{\text{sample}}}{50 - C_{\text{C+}}}\right) \times 100 = \%\text{sample’s CDH1 methylation}
\]

Investigators who carried out genetic analyses were not informed about the origin of each sample (patient or control). Enrolled patients gave their informed consent before study entry.

**Statistical Analysis**

Statistical analysis was carried out using the software SPSS 11.0 (SPSS Inc., Chicago, IL). Differences in CDH1 promoter hypermethylation values between patients and controls were studied by means of the t unpaired test and statistical significance was set at P < 0.05.

**Results**

In this prospective investigation, the study population consisted of 20 subjects with poorly differentiated, diffuse gastric carcinoma and 14 controls. Median age in the 20 patients was 59 years (48–81 years) and their characteristics are shown in Table 1. Among the 14 controls there were nine men and five women with a median age of 55 years (18–70 years). The EC did not cause any side-effects and all of the 34 subjects were fully compliant with the procedure. The presence of the EC in the stomach was confirmed by upper abdomen ultrasonography. In all 34 cases, microscopy of the gastric juice showed the presence of normal gastric cells (Figure 3A) and gastric cancer cells (Figure 3B).
As shown in Table 1, in the 20 patients, the level of CDH1 promoter hypermethylation from gastric juice analysis was comparable among stages. Also, it matched with CDH1 promoter hypermethylation analysis in the available tumor tissue from 13 cases. Median level of CDH1 promoter hypermethylation in the 20 patients was 65% (minimum value 0% and maximum value 83%) and it was 0% (minimum value 0% and maximum value 66%) in the 14 controls. The difference was significant with two-tailed $P < 0.0001$. When the 20 patients were dichotomized according to the observed median age (59 years) the median level of CDH1 promoter hypermethylation between patients aged <59 years (55%) and patients aged >59 years (66%) did not differ significantly.

Table 1. Characteristics of the 20 studied patients and results of methylation analysis

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age</th>
<th>Histology</th>
<th>Grading</th>
<th>Stage</th>
<th>CDH1 methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>63</td>
<td>Mixed</td>
<td>G2</td>
<td>EGC</td>
<td>66%</td>
</tr>
<tr>
<td>M</td>
<td>57</td>
<td>Mixed</td>
<td>G3</td>
<td>EGC</td>
<td>70%</td>
</tr>
<tr>
<td>F</td>
<td>50</td>
<td>DGC</td>
<td>G3</td>
<td>EGC</td>
<td>74%</td>
</tr>
<tr>
<td>M</td>
<td>80</td>
<td>Mixed</td>
<td>G2</td>
<td>EGC</td>
<td>60%</td>
</tr>
<tr>
<td>M</td>
<td>67</td>
<td>DGC</td>
<td>G2</td>
<td>EGC</td>
<td>61%</td>
</tr>
<tr>
<td>M</td>
<td>70</td>
<td>Mixed</td>
<td>G2</td>
<td>pT2N0M0</td>
<td>70%</td>
</tr>
<tr>
<td>M</td>
<td>58</td>
<td>Mixed</td>
<td>G2</td>
<td>pT2N0M0</td>
<td>negative</td>
</tr>
<tr>
<td>F</td>
<td>50</td>
<td>DGC</td>
<td>G3</td>
<td>pT2N0M0</td>
<td>73%</td>
</tr>
<tr>
<td>M</td>
<td>52</td>
<td>DGC</td>
<td>G2</td>
<td>pT2N0M0</td>
<td>60%</td>
</tr>
<tr>
<td>M</td>
<td>76</td>
<td>Mixed</td>
<td>G3</td>
<td>pT2N0M0</td>
<td>67%</td>
</tr>
<tr>
<td>M</td>
<td>49</td>
<td>DGC</td>
<td>G2</td>
<td>pT2N0M0</td>
<td>60%</td>
</tr>
<tr>
<td>F</td>
<td>60</td>
<td>DGC</td>
<td>G3</td>
<td>pT2N0M0</td>
<td>72%</td>
</tr>
<tr>
<td>M</td>
<td>51</td>
<td>Mixed</td>
<td>G3</td>
<td>pT3N0M0</td>
<td>51%</td>
</tr>
<tr>
<td>M</td>
<td>48</td>
<td>DGC</td>
<td>G3</td>
<td>pT1N1M0</td>
<td>Negative</td>
</tr>
<tr>
<td>M</td>
<td>80</td>
<td>Mixed</td>
<td>G3</td>
<td>pT2N1M0</td>
<td>81%</td>
</tr>
<tr>
<td>M</td>
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<td>DGC</td>
<td>G3</td>
<td>pT2N1M0</td>
<td>83%</td>
</tr>
<tr>
<td>M</td>
<td>60</td>
<td>DGC</td>
<td>G3</td>
<td>pT2N1M0</td>
<td>65%</td>
</tr>
<tr>
<td>F</td>
<td>81</td>
<td>Mixed</td>
<td>G3</td>
<td>pT3N1M0</td>
<td>79%</td>
</tr>
<tr>
<td>M</td>
<td>49</td>
<td>DGC</td>
<td>G3</td>
<td>pT3N1M0</td>
<td>61%</td>
</tr>
<tr>
<td>M</td>
<td>77</td>
<td>DGC</td>
<td>G3</td>
<td>pT3N2M0</td>
<td>51%</td>
</tr>
</tbody>
</table>

GJ, gastric juice; EGC, early gastric cancer; DGC, diffuse gastric cancer.

Figure 2. An ultrasonography image showing the antral location of the endogastric capsule.

As shown in Table 1, in the 20 patients, the level of CDH1 promoter hypermethylation from gastric juice analysis was comparable among stages. Also, it matched with CDH1 promoter hypermethylation analysis in the available tumor tissue from 13 cases. Median level of CDH1 promoter hypermethylation in the 20 patients was 65% (minimum value 0% and maximum value 83%) and it was 0% (minimum value 0% and maximum value 66%) in the 14 controls. The difference was significant with two-tailed $P < 0.0001$. When the 20 patients were dichotomized according to the observed median age (59 years) the median level of CDH1 promoter hypermethylation between patients aged <59 years (55%) and patients aged >59 years (66%) did not differ significantly.

Figure 3. Microscopy identification of populations of cells collected by the endogastric capsule. Normal gastric cells (A) and gastric cancer cells (B).
discussion

Our findings indicate that the EC is a promising simple, noninvasive tool, which allows for CDH1 promoter hypermethylation analysis using gastric juice from DGC patients. Patients and controls were fully compliant with the procedure and the EC did not cause any immediate or delayed side-effect. Its acceptability (swallowing and no evident external component) together with easiness of execution of the procedure may also favor the EC over collection of gastric juice with other possible devices, such as the nasogastric tube. Insertion of the nasogastric tube often causes major discomfort to patients and though rarely, it was associated with complications [10]. To our knowledge, the nasogastric tube has never been demonstrated to be effective for collecting and isolating gastric cells. To this purpose, the EC with its nontraumatic, 60-min persistence into the gastric cavity was effective in all studied subjects.

As shown in Figure 3, gastric cancer cells are detectable in gastric juice from EC samples. This phenomenon can be explained on the basis of the exfoliation of cancer cells into the gastric lumen and the physiological attempt of the stomach for digestion of the EC as a foreign body. We did not observe marked variability in CDH1 hypermethylation levels among different stages of gastric adenocarcinomas. In fact, this phenomenon tends to occur early in the development of the disease [7, 11]. Notably, marked levels of CDH1 hypermethylation were detected in patients with early gastric carcinoma in this study. The presence of CDH1 promoter hypermethylation has been described to be age-dependent [12]. Our study population did not include very young patients, however, when we dichotomized the sample according to the patients’ median age, CDH1 hypermethylation levels were not found to be significantly different between patients aged <59 years and patients aged ≥59 years. The isolated DNA from gastric juice is not tumor-derived only: it represents a mixture with DNA from normal cells. However, CDH1 promoter hypermethylation levels in samples from gastric juice were comparable to those determined in paired tumor tissue and this finding would contribute to support the reliability of the results of CDH1 hypermethylation status when determined by the EC.

CDH1 promoter hypermethylation is a key event for the loss of CDH1 expression and it was found to be the second hit in germline CDH1 mutation carriers, within the frame of the HDGC syndrome [3]. Also, it has been demonstrated that this event is common in the sporadic gastric carcinoma. Tamura et al. [5] found CDH1 promoter hypermethylation in the 83% of poorly differentiated diffuse gastric carcinomas. On this basis, we focused on the analysis of CDH1 promoter hypermethylation as a potential epigenetic marker for detection of the disease. Actually, it is not the sole epigenetic change occurring in gastric adenocarcinomas [13–15]. Therefore, the analysis of hypermethylation in additional genes (i.e. p16) might represent a step forward for a broader use of the EC. Indeed, a combined analysis of the hypermethylation status in multiple genes would allow detection of diffuse gastric carcinomas in the absence of CDH1 promoter hypermethylation and nondiffuse gastric adenocarcinomas [14].

The use of the EC for achieving information on the CDH1 promoter hypermethylation status may not only represent a strategic innovation for early detection of poorly differentiated DGC, but it might be used for prognostic purposes. In fact, the presence of CDH1 promoter hypermethylation was found to be associated with unfavorable survival of DGC patients treated with radical surgery [16]. Due to exploratory nature of this study, we cannot draw any firm conclusion for the widespread use of the EC in the early diagnosis of DGC. However, these data are encouraging and a large-scale study has been planned for confirming these findings.

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references