BARHL2 Methylation Using Gastric Wash DNA or Gastric Juice Exosomal DNA is a Useful Marker For Early Detection of Gastric Cancer in an H. pylori-Independent Manner

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OBJECTIVES: The main purpose of this study was to develop a methylation analysis pipeline by using gastric wash-derived DNA and/or gastric juice-derived exosomal DNA (exoDNA), and to evaluate its suitability for the early detection of gastric cancer (GC) in clinical settings.

METHODS: We analyzed alterations of BarH-like 2 homeobox protein (BARHL2) in GC cell lines and tissues, as well as in DNA obtained from 128 gastric washes and 30 gastric juice-derived exosomes. GC cell lines were transfected with plasmids encoding BARHL2 and subjected to proliferation, colony formation, and gene expression analyses.

RESULTS: High levels of BARHL2 methylation were detected in three of seven GC cell lines; consistent with this, these cell lines expressed low levels of BARHL2. Treatment of these cell lines with 5-aza-2′-deoxycytidine restored BARHL2 expression. Levels of BARHL2 methylation in 18 normal and 14 atrophic gastritis samples were low irrespective of Helicobacter pylori infection. High levels of BARHL2 methylation were observed in gastric wash-derived DNA obtained from early GC patients before endoscopic resection (ER), but methylation was significantly lower after curative ER. Analysis using gastric juice-derived exoDNA samples revealed that BARHL2 methylation yielded an area under the curve of 0.923 with 90% sensitivity and 100% specificity with respect to discriminating GC patients from non-GC controls. BARHL2 nuclear immunoreactivity was found in all normal gastric epithelial cells and in cells from patients with gastritis and adenoma. In contrast, loss of BARHL2 expression was observed in the vast majority of the GC tissues. Finally, transfection of BARHL2 into MKN7 and MKN45 cell lines significantly inhibited their proliferation and ability to form colonies.

CONCLUSIONS: Methylation analysis of BARHL2 using gastric wash-derived DNA and/or gastric juice-derived exoDNA could be useful for early detection of GC in clinical settings.

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Subject Category: Stomach

INTRODUCTION

Gastric cancer (GC) is the third highest cause of global cancer mortality.1 It is a heterogeneous disease with multiple environmental etiologies and alternative carcinogenic pathways.2–6 The development of noninvasive biomarkers to detect early GC (EGC) and/or reflect an individual’s cancer risk is essential to reduce GC mortality.7,8 Among the various methods for detection of genetic and epigenetic alterations,6 DNA methylation is more appropriate than mutations for molecular detection of GC.

Previous strategies have relied on the comparison of methylation levels between tumor and adjacent nontumor sites, to find genes that are specifically methylated in cancer. However, discovery of tumor-specific hypermethylated genes using this strategy is challenging, because chronic inflammation of the gastric mucosae (mainly due to H. pylori infection and aging) also induces aberrant methylation.9–14 Moreover, the process relies on endoscopic biopsy, which is a topical procedure that only samples a small portion of tissue.15 As a result of the uneven distribution of the atrophy or intestinal metaplasia, this restricted biopsy can lead to flawed evaluation of methylation status.16

In light of these problems, we have developed a method that uses gastric wash-derived DNA for EGC detection and have employed it to detect methylation of genes such as MINT25 (ref. 15). Gastric washes contain large amounts of DNA recovered from cells on the surface of the stomach, making it simple to collect DNAs from patients endoscopically, both before and after endoscopic resection (ER). Comparing DNA methylation levels in each of the patients before and after treatment is useful for the identification of genes that are specifically methylated in EGC; this process is not biased by
factors such as aging, chronic inflammation or H. pylori infection. On the other hand, it is possible that GC is subject to field effects, and therefore markers that are not altered by the ER could still play a role in its diagnosis. In this regard, several markers such as miR34b/c and miR-124a-3 (ref. 13) have been already reported.

As minimal invasive treatment is widely used for EGC patients, the identification of appropriate markers for detection of residual tumors after non-curative ER is critical.\(^{17,18}\) One of our main objectives is to develop markers that could be useful for detection of tumors that remain after non-curative ER and/or those that recur after curative ER in EGC patients. Therefore, in the present study we focused on markers that exhibit quantitative changes following ER.

We previously performed methylated CpG island amplification microarray (MCAM) analysis using 12 gastric washes (6 before and 6 after ER in each of the same patients).\(^{19}\) The 18 probes (total 36,579) corresponding to 11 unique genes (total 9,021) were selected as candidate tumor-specific methylated genes after calculations were made using Gene-Spring GX (Agilent Technologies, Santa Clara, CA) software based on the DNA methylation intensity measurements. Among these genes, we have shown that the silencing of sex determining region Y-Box 17 (SOX17) occurs frequently in EGC and may have a key role in the development and progression of the disease.

A number of genes are differentially methylated in GC and noncancer tissues following H. pylori infection. On the other hand, H. pylori eradication decreases aberrant DNA methylation in a gene-specific manner. These issues complicate the successful development of a methylation analysis pipeline in GC. Although there has been a striking decrease in the prevalence of H. pylori infection, especially in younger populations, it is important to identify biomarkers for early detection of GC that are not affected by either H. pylori infection or history of eradication. In this regard, SOX17 methylation is not ideal, because levels of SOX17 methylation were significantly affected by H. pylori infection in gastritis samples.

The BarH-like 2 homeobox protein (BARHL2) gene is a candidate H. pylori-independent biomarker. This is because 6 of the aforementioned 18 probes methylated in EGC analyzed by MCAM corresponded this gene and BARHL2 was one of the most significantly altered genes based on the Cy5/Cy3 (pre-ER/post-ER) signal in MCAM. A pilot study showed that BARHL2 methylation may not be affected by H. pylori infection. Although it is not known whether BARHL2

| Table 1 Clinical features of patients and controls |

| Test set | Validation set | Control set |
|---------|---------------|-------------|
| n = 6 | n = 64 Average | BARHL2 gene methylation (%) | P-value | n = 32 Average | BARHL2 gene methylation (%) | P-value |
| Age | 71.3 ± 8.4 | 28.4 ± 13.0 | 0.39 | 55.4 ± 17.5 | 8.1 ± 3.6 | 0.78 |
| Male | 3 47 | 70.2 ± 8.3 | 29.3 ± 13.4 | | |
| Female | 3 17 | 74.4 ± 8.0 | 25.9 ± 11.8 | | |
| Endoscopic appearance | | | | | | |
| Polypoid | 0 5 | 28.4 ± 14.6 | | | | |
| Slightly elevated | 6 29 | 27.3 ± 13.9 | | | | |
| Flat | 0 1 | 14.0 ± 0.0 | | | | |
| Slightly depressed | 0 29 | 29.9 ± 12.7 | | | | |
| Histology (adenocarcinoma) | | | | | | |
| Well differentiated | 6 44 | 28.1 ± 13.0 | | | | |
| Moderately differentiated | 0 20 | 29.1 ± 13.3 | | | | |
| Stage | | | | | | |
| I | 6 64 | 28.4 ± 13.0 | | | | |
| II/III/IV | 0 0 | 29.1 ± 13.0 | | | | |
| Helicobacter pylori infection | | | | | | |
| Positive | 3 43 | 28.9 ± 13.6 | 0.63 | 11 | 8.5 ± 2.9 | 0.73 |
| Negative | 3 21 | 27.2 ± 11.8 | | 21 | 8.0 ± 4.0 | |
| Locations (stomach) | | | | | | |
| Upper body | 0 11 | 22.6 ± 12.1 | 0.14 | 21 | 8.0 ± 4.0 | |
| Middle/lower body | 6 53 | 29.6 ± 13.0 | | | | |
| Atrophy | | | | | | |
| Non-atrophy | 0 0 | NA | | 18 | 9.0 ± 3.9 | 0.13 |
| Closed type | 0 18 | 25.4 ± 13.0 | | 6 | 6.2 ± 3.7 | |
| Open type | 6 46 | 29.5 ± 13.0 | | 8 | 7.6 ± 2.5 | |
| Intestinal metaplasia | | | | | | |
| Positive | 0 54 | 29.0 ± 13.4 | 0.38 | 4 | 8.7 ± 3.1 | 0.76 |
| Negative | 6 10 | 24.9 ± 10.5 | | 28 | 8.1 ± 3.7 | |

BARHL2, BarH-like 2 homeobox protein; NA, not applicable.
has a role in human malignancy, the function of BarH family of homeodomain proteins as transcriptional regulators has an impact on cell fate specification, cell differentiation, migration, and survival. Together, these data indicate that BARHL2 inactivation may play a role in GC. In this study, we analyzed epigenetic alterations of BARHL2, as well as the role of the BARHL2 protein in GC cell biology. We also determined whether analysis of BARHL2 methylation using gastric wash-derived DNA and/or gastric juice-derived exosomal DNA (exoDNA) could be applied for the detection of EGC.

MATERIALS AND METHODS

Patient characteristics and sample collection of gastric washes, biopsies, and gastric juices. DNA was extracted from 128 samples obtained after 140 gastric washes performed for patients who underwent ER for EGC at St Marianna University School of Medicine Hospital (Kanagawa, Japan), between March 2005 and February 2010. Gastric washes (70 before and 70 after ER for each patient) were obtained consecutively from patients who agreed to participate in this study. In addition to tumor samples, non-neoplastic gastric washes were collected consecutively from 32 non-GC controls who underwent endoscopic examination and were diagnosed with normal findings \( n = 15 \) or atrophic gastritis \( n = 18 \) endoscopically. Characteristics of the included patients and controls are described in Table 1. The study was conducted in accordance with all rules and regulations of the St Marianna University School of Medicine Institutional Review Board (1498 and #2470) and informed consent was obtained from each patient. Sample collection of gastric washes has been reported previously. Gastric washes were aspirated through the suction channel of the endoscope into specimen collection containers (No. 111219, Fortegrow Medical, Tochigi, Japan). The containers were directly connected to the endoscope modulator and the washes were vacuumed manually. The samples were then immediately centrifuged and the pellets were frozen at \(-80^\circ C\). After the collection of gastric washes, biopsy samples were obtained using biopsy forceps under endoscopic guidance for \( H. pylori \) analysis. Mucosal samples (~5 mm in diameter each) of the gastric body and antrum were collected by biopsy. Independently, gastric juices were obtained consecutively from 20 consenting GC patients. Non-neoplastic gastric juices were collected consecutively from 10 non-GC controls who underwent endoscopic examination and were diagnosed with normal findings \( n = 5 \) or atrophic gastritis \( n = 5 \) endoscopically. Characteristics of included patients and controls were described in Table 2. Exosomes were extracted from gastric juices using ExoQuick-TC Exosome Precipitation Solution (System Biosciences (SBI), Palo Alto, CA) with some modification as described previously.

Cell lines. Seven GC cell lines (MKN1, MKN7, MKN45, MKN74, NUGC3, KatoIII, and NUGC4) were obtained from the American Type Culture Collection (Manassas, VA) and the Japanese Collection of Research Bioresources (Tokyo, Japan). All cell lines were maintained in appropriate media containing 10% fetal bovine serum in plastic tissue culture plates.

DNA and RNA preparation. DNA was extracted from GC cell lines, gastric washes, microdissected formalin-fixed paraffin-embedded tissues after ER \( n = 30 \), and exosomes...
using the standard phenol–chloroform method. Total RNA was extracted from the collected cells and microdissected formalin-fixed paraffin-embedded tissues (n=8) using Trizol solution (Invitrogen, Carlsbad, CA).

DNA methylation analysis. Bisulfite PCR reaction was performed using an EpiTect Bisulfite Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. One microliter of bisulfite-treated DNA was used as a template. The primers used were 5'-AGTAAATGATAATGGAAGGGTTA-3' as a sense primer and 5'-TACACTCCRAAAACTCCATA-3' as an antisense primer. After PCR, the biotinylated strand was captured on streptavidin-coated beads (Amersham Bioscience, Uppsala, Sweden) and incubated with sequencing primer (5'-TYGTTYGTAGGTAGGAT-3'). Pyrosequencing quantitatively measures the methylation status of several CpG sites in a given promoter. These adjacent sites usually show highly concordant methylation. Therefore, the mean percentage of methylation at detected sites was used as a representative value for gene promoter.

5-Aza-2'-deoxycytidine and trichostatin A treatment of cells. To analyze restoration of gene expression, MNK7, MKN45, and NUGC3 were incubated for 96 h with 1 or 5 μM of 5-aza-2'-deoxycytidine (5-aza-dC) and/or 200 nM of trichostatin A (TSA) after which they were collected and RNA was extracted for further analysis.

Reverse transcription-PCR. First-strand cDNA was prepared by reverse transcription of 1 μg samples of total RNA using Superscript III Reverse Transcriptase (Invitrogen). Real-time quantitative reverse transcription-PCR was carried out using Taqman Gene Expression Assays (BARHL2, Hs00751752_s1, and glyceraldehyde-3-phosphate dehydrogenase, Hs_00266705_g1 (Applied Biosystems, Foster City, CA)) or SYBR green (CDX1, CDX2, PDX1, and SOX2) with an ABI 7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. SDS2.1 software (Applied Biosystems) was used to perform comparative delta-Ct analysis. Glyceraldehyde-3-phosphate dehydrogenase served as an endogenous control.

BARHL2 expression in gastric tissues by immunofluorescence. Immunofluorescence analysis of BARHL2 was performed on 4 μm sections of five gastritis and five adenoma tissue specimens and tissue microarray (SuperBChip Laboratories, Seoul, Korea). After deparaffinization, antigen retrieval was performed by incubation in 10 mM citrate buffer (pH 6.0) (DAKO, Carpinteria, CA) in a heated (97 °C) water bath for 40 min. Nonspecific binding was blocked by immersing the sections in a Tris-buffered saline/5% bovine serum albumin solution for 10 min. Sections were incubated with a mouse monoclonal antibody to BARHL2 diluted 1:20 for 60 min. Antibody to BARHL2 was detected using Alexa Fluor 568 goat anti-mouse IgG (Molecular Probes, Eugene, OR) diluted 1:700 for 30 min. Sections were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (Vysis, Downers Grove, IL). All incubations were performed at room temperature.

In vitro growth assay. For cell growth kinetics, 2 × 10^4 cells per well were seeded on the culture plates. The number of cells was counted at the indicated times in triplicate, excluding dead cells identified by trypan blue staining.

Colony formation assays. Cells (0.5 × 10^5) were plated in 2 cm² culture dishes for 24 h before transfection. The cells were then electroporated with a Myc-DDK-tagged pCMV6-BARHL2 expression vector or empty vector (RC217326 and PS100001, OriGene Technologies, Rockville, MD) using a Nucleofector II Device (Lanza, Basel, Switzerland) and the Nucleofector Kit V (Lanza) according to the manufacturer's recommended protocol. After transfection, cells were preserved for 14 days in a medium containing 0.2 mg/ml of G418 for MKN7 and 0.6 mg/ml of G418 for MKN45, and stained with Giemsa. The resultant colonies were then stained with crystal violet and cells were counted in triplicate cultures using NIH Image software. Western blotting was carried out using anti-BARHL2 antibody (AF1924, R&D Systems, Minneapolis, MN) and anti-tubulin-α monoclonal antibody (TA50011, OriGene Technologies).

Flow cytometry analysis. Cells were seeded in a 6-well plate at a density of 10,000 cells/well. The cells were incubated for 3 days at 37 °C in a CO₂ incubator, allowing for medium depletion and cell synchronization. The cells were then electroporated with Myc-DDK-tagged pCMV6-BARHL2 expression vector or empty vector using a Nucleofector II Device (Lanza) and the Nucleofector Kit V (Lanza) according to the manufacturer's recommended protocol. Cells were then washed and further incubated for 48 h followed by fixation, staining, and cytometric analysis using a Cell Cycle Phase Determination Kit (Cayman, Cayman Chemical, Ann Arbor, MI). Western blotting was carried out as described previously.

Statistical analysis. Methylation levels (percentage) were analyzed as a continuous variable for comparison. Statistical analysis was performed with Mann–Whitney’s U-test, Kruskal–Wallis test, or Spearman’s rank correlation coefficient. Difference with P<0.05 was considered significant. For data obtained by using exoDNA, sensitivity and specificity were analyzed using a receiver operating characteristic curve and the area under the curve was used to assess BARHL2 methylation levels, distinguishing GC patients from the non-GC controls. All statistical analyses were performed using PRISM software for Windows, version 4 (GraphPad Prism, San Diego, CA).

RESULTS

Selection of BARHL2 as a candidate gene for EGC detection by MCAM analysis. Human BLAT search sequence analysis of the 5’-regulatory region of BARHL2 showed that there is a CpG island encompassing its transcription start site (UCSC Genome Bioinformatics Group, Santa Cruz, CA). We designed primers for bisulfite-pyrosequencing analysis in a region downstream of the transcription start site (Figure 1a).
Figure 1  Methylation and expression of the BarH-like 2 homeobox protein (BARHL2) gene in GC cell lines. (a) Schema of the promoter region of the BARHL2 gene and its CpG island (black bar). Three arrows show the pyrosequencing primers used for methylation analysis. (b) Relative levels of expression and methylation of BARHL2 in seven gastric cancer (GC) cell lines. Levels of BARHL2 expression were normalized to GAPDH. (c) Restoration of BARHL2 expression in GC cell lines treated with 5-aza-2′-deoxycytidine (5-aza-dC) and trichostatin A (TSA). (d) Increase in BARHL2 expression in NUGC3 cells treated with TSA.

Figure 2  BarH-like 2 homeobox protein (BARHL2) silenced by DNA methylation in early gastric cancer (EGC). (a) Gastric wash-based pyrogram of the BARHL2 gene in EGC (left, unmethylated; right, methylated). (b) Correlation of methylation levels between gastric washes and formalin-fixed paraffin-embedded (FFPE) samples in the same patient. (c) Expression of BARHL2 in EGC with or without DNA methylation. Real-time PCR was carried out using RNA extracted from tumor tissues by laser capture microdissection after endoscopic resection (ER).
Silencing of BARHL2 is associated with promoter CpG island hypermethylation in GC cell lines. Three of the GC cell lines had low global methylation levels (MKN74, 34%; NUGC3, 13%; and KatoIII, 16%). However, hypermethylation was detected in three other lines (MKN7, 75%; MKN45, 96%; and NUGC4, 92%) (Figure 1b). To confirm the role of DNA methylation in transcriptional repression of BARHL2, we treated MKN7 and MKN45 cell lines, in which BARHL2 was methylated, with 5-aza-dC alone or in
combination with the histone deacetylases inhibitor, TSA. Treatment of these cell lines with 5-aza-dC restored BARHL2 expression and co-treatment with TSA elicited a synergistic effect (Figure 1c). On the other hand, treatment of NUGC3 cell line with TSA (but not 5-aza-dC) increased BARHL2 expression (Figure 1d).

**Gastric wash-based BARHL2 methylation analysis in patients with EGC before and after ER.** To evaluate gastric wash-based DNA methylation of the BARHL2 gene, we carried out quantitative bisulfite pyrosequencing analysis using 32 control samples (in which GC was not present) and a panel of 128 GC samples (example in Figure 2a). There was a significant correlation between BARHL2 methylation levels in gastric wash and formalin-fixed paraffin-embedded samples prepared from resected cancer tissues (Figure 2b). There was also a correlation between BARHL2 methylation levels and gene expression levels in eight EGC samples (Figure 2c). Methylation levels of BARHL2 in control samples were low irrespective of H. pylori infection (Figure 3a) or age (Figure 3b). In contrast, BARHL2 methylation levels were significantly higher in EGC samples before ER than they were in control samples. BARHL2 methylation levels did not significantly correlate with co-
variates (Table 1). There were no significant differences in \textit{BARHL2} methylation levels between \textit{H. pylori}-positive and -negative EGC samples (gastric washes in pre-ER). After ER, methylation levels significantly decreased to levels of controls (Figure 3c). When the methylation levels were compared between EGC samples before ER and those after ER in each patient, \textit{BARHL2} methylation levels significantly decreased to levels of controls in most patients after ER (Figure 3c).

\textit{BARHL2} methylation analysis in GC cell lines and in patients with GC. Analysis of \textit{BARHL2} methylation levels in exosomal and nuclear DNA from GC cell lines using quantitative bisulfite pyrosequencing yielded concordant results. Quantitative bisulfite pyrosequencing analysis of \textit{BARHL2} revealed varying levels of methylation in gastric juice-derived exoDNA. Concordant \textit{BARHL2} methylation levels were observed between exosomal and tissue nuclear DNA in 10 patients with GC (Figure 4a). We further analyzed 10 GC cases (a total of 10 EGCs and 10 advanced GCs) and 10 non-GC control samples. \textit{BARHL2} methylation levels did not significantly correlate with covariates (Table 2). Receiver operating characteristic curve analysis showed that the \textit{BARHL2} methylation level is a potential biomarker for differentiating GC patients from non-GC controls, with an area under the curve of 0.923 (\(p < 0.001\)) (Figure 4b). When the cutoff value for \textit{BARHL2} methylation was 20\%, sensitivity was 90\% and specificity was 100\%.

Loss of \textit{BARHL2} expression in GC tissues. The nuclei of all normal gastric epithelial cells and those from individuals with gastritis (\(n = 5\)) and adenoma (\(n = 5\)), were positive for \textit{BARHL2} staining (Figures 5a and b). In contrast, loss of \textit{BARHL2} expression was observed in 90\% (45/50) of GC tissues, irrespective of tumor grading or stage (Figure 5c and data not shown).

Expression of exogenous \textit{BARHL2} suppressed \textit{in vitro} growth and colony formation, but did not affect cell cycle distribution in GC cell lines. We next performed \textit{in vitro} growth and colony formation assays to determine whether \textit{BARHL2} had potential tumor suppressor activities. Overexpression of \textit{BARHL2} in MKN7 and MKN45 cells (which express low endogenous levels of \textit{BARHL2}) significantly

！ Figure 6 Suppression of \textit{in vitro} growth and colony formation of gastric cancer (GC) cell lines by \textit{BarH}-like 2 homeobox protein (\textit{BARHL2}). (a) Suppression of GC cell line growth by \textit{BARHL2}. The number of cells was counted at indicated times after stable transfection of MKN7 and MKN45 with pCMV6 (empty vector) or pCMV6-BARHL2 (\textit{BARHL2}). (b and c) Suppression of colony formation of GC cell lines by \textit{BARHL2}. Colony counts were obtained 14 days after stable transfection of MKN7 and MKN45 with pCMV6 or pCMV6-BARHL2.
reduced growth and colony formation in vitro (Figures 6a–c). However, fluorescence-activated cell sorting analysis did not reveal any perturbation of the cell cycle profile following exogenous BARHL2 expression (Figure 7).

Effects of BARHL2 expression on the expression of transcriptional factors involved in the differentiation of GC cell lines. We further analyzed the effects of BARHL2 on the expression of selected transcription factors involved in cellular differentiation (CDX1, CDX2, PDX1, and SOX2) in GC cell lines. Although the effects in MKN7 cells were marginal (Figure 8a), transfection of BARHL2 into MKN45 cells resulted in a significant induction of all the transcription factors analyzed (Figure 8b).

DISCUSSION

Here we demonstrate that silencing of BARHL2 was correlated with hypermethylation of its promoter in GC cell lines. Treatment of these cell lines with 5-aza-dC restored BARHL2 expression. Moreover, combined treatment of 5-aza-dC and TSA synergized to restore BARHL2 expression, indicating that cytosine methylation and histone deacetylation have a role in silencing of this gene in GC. The synergistic role was more evident in the MKN45 cell line than in MKN7 cells. This may be because patterns of methylation, histone acetylation, and the expression of other transcription factors involved in the regulation of BARHL2 transcription are different between the cell lines. In this regard, increased histone acetylation may be associated with increased BARHL2 expression in BARHL2 methylation-negative NUGC3 cells treated with TSA.

We examined BARHL2 methylation status in gastric wash-derived DNA samples obtained from control samples and EGC patients before and after ER. We found that levels of BARHL2 methylation as determined from gastric washes and formalin-fixed paraffin-embedded samples were well correlated, supporting the notion that gastric washes reflect biopsy results at the DNA methylation level.15 There was a correlation between BARHL2 methylation status and gene expression in EGC samples, suggesting that BARHL2 methylation is functionally significant.

Interestingly, BARHL2 methylation was low in 18 normal and 14 gastritis samples, irrespective of H. pylori infection. As for EGC samples before ER, there was no significant difference in BARHL2 methylation levels between EGC samples with and without H. pylori infection. We note that clinical tests for H. pylori infection detect only the current (culture and urease tests) or recent (serum antibody test) status of H. pylori infection and cannot detect past exposure to H. pylori.9,23,24 Nevertheless, these results suggest that
**BARHL2** methylation is a tumor-specific event that is not influenced by atrophy of the gastric mucosa or *H. pylori* infection that may accompany gastric carcinogenesis.

**BARHL2** methylation levels were significantly higher in EGC samples before ER than they were in control samples. Following curative ER, **BARHL2** methylation levels significantly decreased to levels of controls in most patients. Therefore, gastric wash-based **BARHL2** methylation analysis could be useful for early detection of remaining tumors after non-curative ER and/or recurrence after curative ER in EGC patients.

Nevertheless, an endoscopy is necessary to obtain gastric washes. Exosomes in the gastric juice may provide an alternative to gastric washes that can be used for GC molecular diagnostics. Using exoDNA derived from gastric juice, we were able to detect **BARHL2** methylation, which reflects the nuclear DNA methylation status of the corresponding tumor. When the cutoff value for **BARHL2** methylation was 20%, sensitivity was 90% and specificity was 100%. **BARHL2** methylation was detected in both early and advanced GC of intestinal and diffuse types. These findings suggest that **BARHL2** methylation analysis of exoDNA derived from gastric juice has utility as a biomarker for detection of both early and advanced GC. The high specificity of the approach is supported by the fact that **BARHL2** methylation is not influenced by atrophy of the gastric mucosa or *H. pylori* infection.

Combined with its high methylation frequency, these properties make **BARHL2** methylation an excellent candidate for future diagnostic applications for the early detection of GC.

The results of methylation analyses were supported by our results with immunohistochemistry. **BARHL2** expression was frequently downregulated in GC tissues, irrespective of tumor grading or stage, but was preserved in gastric epithelial cells, as well as those from patients with gastritis or adenoma. These results further support the notion that **BARHL2** silencing is a tumor-specific event in GC.

**BARHL2** methylation was detected in only three of seven GC cell lines. **BARHL2** was selected as a marker based on the MCAM data of six test set samples (well or moderately differentiated cancer). Importantly, **BARHL2** methylation was detected in not only well-differentiated but also poorly differentiated GC cell lines. Considering the data of clinical samples, therefore, the relatively low frequency of **BARHL2** methylation in GC cell lines does not necessarily discourage the role of **BARHL2** methylation as a diagnostic tool.

Given the silencing of **BARHL2** we observed, we hypothesized that restoration of expression might suppress GC cell growth. This was indeed the case in both MKN7 and MKN45 cell lines, although the cell cycle phases in these cells were not affected by **BARHL2**. We also observed that **BARHL2** induced expression of several transcription factors involved in cell differentiation in MKN45 cells but not in MKN7 cells. We suggest that this is because MKN7 is already a well-differentiated GC line, whereas MKN45 is poorly differentiated. The BarH family of homeodomain proteins has essential roles in cell fate specification, cell differentiation, migration, and survival through transcriptional regulation. Therefore, **BARHL2** may regulate gastric epithelial cell features through modulation of the network of transcriptional factors and its inactivation may have a role in GC.

As gastrointestinal endoscopy is costly and painful for patients, it is difficult to incorporate the technique into routine clinical settings, especially in developing countries. In contrast, gastric juice samples can be obtained easily and repeatedly, and exoDNA is not easily denatured by gastric acidity. Although our data require further validation, detection of **BARHL2** methylation in gastric washes and/or gastric juice-derived exosomes may be a novel and less invasive tool for EGC detection.

**CONFLICT OF INTEREST**

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