Carcinogenesis vol.31 no.9 pp.1685–1693, 2010
doi:10.1093/carcin/bgq144
Advance Access publication July 12, 2010

Frequent silencing of *popeye domain-containing* genes, *BVES* and *POPDC3*, is associated with promoter hypermethylation in gastric cancer

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The *Popeye domain-containing* (*POPDC*) genes *BVES*, *POPDC2* and *POPDC3* encode proteins that regulate cell–cell adhesion and cell migration during development. Herein, we report the frequent downregulation of *BVES* and *POPDC3* by promoter hypermethylation in gastric cancer. *POPDC* expression in 11 gastric cancer cell lines and 96 paired gastric tumor and normal adjacent tissues was analyzed with quantitative reverse transcription–polymerase chain reaction. The methylation status of *BVES* and *POPDC3* was analyzed with methylated DNA immunoprecipitation sequencing, bisulfite sequencing and pyrosequencing. Expression of *BVES* and *POPDC3* was downregulated in 73% of the gastric cancer cell lines and in 69% (*BVES*) and 87% (*POPDC3*) of the gastric cancer tissues. The *BVES* and *POPDC3* promoter regions were hypermethylated in the gastric cancer cell lines in which they were silenced. Combined treatment with a DNA methylation inhibitor and a histone deacetylase inhibitor strongly induced *BVES* and *POPDC3* expression. *BVES* and *POPDC3* were hypermethylated in 69% (*BVES*) and 64% (*POPDC3*) of the gastric cancer tissues. We knocked down *POPDC3* expression with short hairpin RNAs and examined the consequences on cell migration and invasion. Knockdown of POPDC3 in SNU-216 cells caused increased cell migration and invasion. Thus, epigenetic inactivation of *BVES* and *POPDC3* occurs frequently in gastric tumors and may promote gastric cancer cell migration and invasion.

Introduction

The *Popeye domain-containing* (*POPDC*) gene family includes *BVES* (*blood vessel epicardial substance*, also known as *POPDC1*), *POPDC2* and *POPDC3*. *BVES* and *POPDC3* are organized in tandem on human chromosome 6q21, whereas *POPDC2* is on chromosome 3 (1). POPDC proteins are highly evolutionarily conserved transmembrane proteins expressed in embryonic epithelium, heart and muscle (1–4). BVES is the most-studied member of the POPDC protein family. It plays a role in cell–cell adhesion and cell migration (5). BVES accumulates at cell–cell contacts and regulates epithelial integrity by interacting with tight junctions (6,7). Studies have proposed roles for the POPDC family in epithelial morphogenesis and development, but its potential function in cancer is unknown.

Gastric cancer is the second-most frequent cause of cancer death in the world, but the precise mechanism of gastric carcinogenesis is not fully understood (8,9). DNA methylation is an important epigenetic change associated with cancer and may lead to transcriptional silencing of tumor suppressor genes and prodifferentiation factors (10–12). DNA methylation has been studied as a possible mechanism in carcinogenesis and used to find tumor suppressor genes and other cancer-related genes. Moreover, DNA methylation markers have been used for cancer diagnosis, tumor classification, prognosis and response to chemotherapy (13). To identify DNA methylation markers in gastric cancer, we previously studied global methylation patterns in gastric cancer cell lines and tissues with restriction landmark genomic scanning (RLGS), which involves two-dimensional gel electrophoresis of genomic DNA that had been digested with the methylation-sensitive enzyme *Not*I, identified aberrant methylation of several genes and suggested roles for these genes in gastric carcinogenesis (14–18). RLGS was also used to identify *POPDC3* as an aberrantly methylated gene in gastric cancer.

In our current study, we examined the expression patterns of *BVES*, *POPDC2* and *POPDC3* in gastric cancer tissues and found the frequent downregulation of *BVES* and *POPDC3*, which was associated with promoter hypermethylation. We also examined the effects of *POPDC3* knockdown on the malignant phenotype of gastric cancer cells and suggest that the frequent loss of *POPDC3* in gastric cancer probably promotes gastric cancer cell migration and invasion.

Abbreviations: 5-aza-dC, 5-aza-2'-deoxycytidine; ChIP, chromatin immunoprecipitation; EGF, epidermal growth factor; EMT, epithelial–mesenchymal transition; mRNA, messenger RNA; qRT–PCR, quantitative reverse transcription–polymerase chain reaction; RLGS, restriction landmark genomic scanning; shRNA, short hairpin RNA; TSA, trichostatin A.

Materials and methods

Cell lines and tissue samples

Eleven established gastric cancer cell lines from patients with gastric cancer were obtained from the Korean Cell Line Bank and were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic solution (all from Invitrogen, Carlsbad, CA). Frozen gastric cancer tissues and normal adjacent tissues (96 pairs) were collected from the Stomach Cancer Bank at Chungnam National University Hospital, Daejeon, Korea, from 2001 to 2002. The tissues had been obtained immediately after resection of the tumors. For each cancer tissue sample, the corresponding normal mucosa specimen was at least 3 cm away from the cancerous edge. Written informed consent was obtained from all patients, and the study was approved by the institutional review board (14).

Antibodies

Anti-histone H3 was purchased from Abcam (Cambridge, UK). Anti-acetyl-histone H3, anti-trimethyl-histone H3 (Lys27) and anti-trimethyl-histone H3 (Lys4) were purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal anti-POPDC3 was purchased from Protein Tech Group (Chicago, IL). Anti-ZO-1 and anti-occludin were purchased from ZYMED Laboratories (Invitrogen). Anti-E-cadherin was purchased from BD Biosciences (San Jose, CA).

Methylated DNA immunoprecipitation sequencing

Genomic DNA (5 μg) was fragmented at 44 psi for 1 min with a nebulizer (Illumina, San Diego, CA) and then subjected to methylated DNA immunoprecipitation using the MethylMiner methylated DNA enrichment kit (Invitrogen). Briefly, methylated DNA was precipitated from the fragmented genomic DNA (1 μg) via binding to the methyl-CpG-binding domain of human MBD2 protein that was coupled to magnetic Dynabeads. The methylated fragments were then eluted with 2 M NaCl elution buffer (Invitrogen) and purified with the Qiagen miniElute polymerase chain reaction (PCR) purification kit. The methylated DNA was processed to generate a library for sequencing with an Illumina Genome Analyzer. The sequencing libraries were generated using the ChIP-Seq Sample prep kit (Illumina). The sequences were mapped to the

human genome using ELAND from the Genome Analyzer data analysis pipeline. The sequencing data were visualized as a custom track in the University of California, Santa Cruz Genome Browser.

Promoter reporter assay

The DNA fragments comprising the *BVES* or *POPDC3* transcription start site were obtained with PCR using the primer sets listed in supplementary Table 1 (available at *Carcinogenesis* Online). Each DNA fragment was inserted into a pGEM-T easy vector (Promega, Madison, WI) and their was presence confirmed by sequencing. The HindIII/*Nco*I (for *BVES*) or *Sac*I/*Nco*I (for *POPDC3*) fragment was then subcloned into a pGL3-Basic vector (Promega). The luciferase assay was performed as described (17).

Quantitative reverse transcription–PCR

Quantitative reverse transcription (qRT)–PCR was performed as described (14–18). T_m and oligonucleotide sequences are listed in supplementary Table 1 (available at *Carcinogenesis* Online). β-Actin was amplified as the control. Relative quantification of target messenger RNA (mRNA) was analyzed with comparative threshold cycle (C_t) methods (19).

Bisulfite sequencing analysis

Bisulfite sequencing was performed as described (14–18). Bisulfite-modified DNA was amplified using primer sets designed to amplify sites −330 to 127 (for *BVES*) or −276 to 176 (for *POPDC3*) (supplementary Table 1 is available at *Carcinogenesis* Online).

5-Aza-2′-deoxycytidine and trichostatin A treatment

Gastric cancer cells (SNU-601, -620 and -638) were seeded in 10 cm dishes at a density of 1×10^6 cells 1 day before drug treatment. The cells were treated with 10 μM 5-aza-2′-deoxycytidine (5-aza-dC) (Sigma, St Louis, MO) every 24 h for 3 days and then harvested. Another set of cells was treated with 0.5 μM trichostatin A (TSA) (Sigma) for 1 day. To characterize the combined effect of 5-aza-dC and TSA, cells were treated with 10 μM 5-aza-dC every 24 h for 3 days and then with 0.5 μM TSA for 1 day. Total RNA was prepared and tested for restoration of *BVES* or *POPDC* expression with qRT–PCR.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed with an EZ ChIP assay kit (Upstate Biotechnology) with modifications of the manufacturer's protocol (14). Immunoprecipitated DNA was recovered using the QIAquick PCR Purification kit (Qiagen) and analyzed with quantitative polymerase chain reaction. The amount of immunoprecipitated DNA was normalized to that of the input DNA.

Pyrosequencing analysis

The promoter region of *BVES* (sites −1202 to −999) or *POPDC3* (−182 to 181) was amplified using primers designed by PSQ Assay Design (Biotage AB, Kungsgatan, Sweden) (supplementary Table 1 is available at *Carcinogenesis* Online). Pyrosequencing was performed as described (14–18).

Short hairpin RNA and generation of stable cell lines by lentiviral infection

A MISSION TRC short hairpin RNA (shRNA) target set that contained five short hairpin sequences in glycerol [TRCN0000129942 (POPDC sh#1), TRCN0000129690 (POPDC sh#2), TRCN0000129803 (POPDC sh#3), TRCN0000131042 (POPDC sh#4) and TRCN0000129618 (POPDC sh#5)], which targeted human *POPDC3* mRNA, was purchased from Sigma. The control shRNA vector contained a non-specific sequence that lacked a complementary sequence in the human genome (Sigma SHC005).

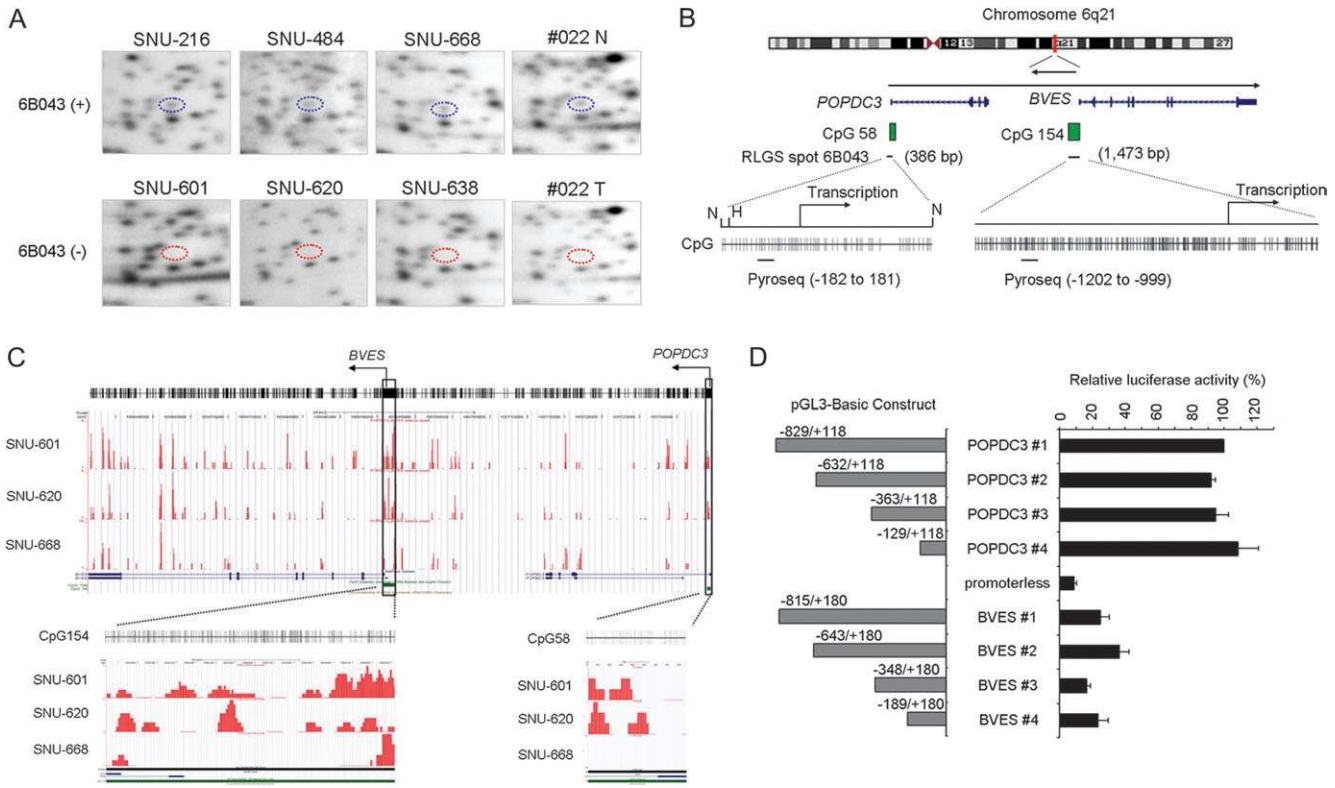

Fig. 1. RLGS profiles of *POPDC3* in gastric cancer. (**A**) Representative examples of decreased 6B043 spot intensity in RLGS gels. Blue ovals indicate the position of spot 6B043 from gastric cancer cell lines (SNU-216, -484 and -668) and from normal gastric tissue (#022N) and red ovals indicate decreased intensity of spot 6B043 from gastric cancer cell lines (SNU-601, -620 and -638) and gastric tumor tissue (#022T). (**B**) Schematic representation of the structures of *POPDC3* and *BVES* on human chromosome 6q21. CpG islands were predicted using the University of California, Santa Cruz genome browser (http://genome.ucsc.edu/). The location of spot 6B043 (386 bp) is indicated. N, *Not*I; H, *Hin*fI. CpG sites of spot 6B043 and CpG154 are indicated. Bars below the CpG sites indicate the regions subjected to pyrosequencing. (**C**) Methylated DNA immunoprecipitation sequencing analysis of cell lines SNU-601, -620 and -668. Methylated DNA immunoprecipitation sequencing data were visualized as a custom track in the University of California, Santa Cruz Genome Browser. CpG sites throughout *BVES* and *POPDC3* are shown. Red bars represent the methylation frequency of each 10 bp. (**D**) Luciferase assay of the regions surrounding the transcription start sites of *BVES* and *POPDC3*. SNU-216 cells were cotransfected with a pGL3-Basic empty vector (promoterless) or with a construct and a pRL-CMV vector (internal control). The luciferase activity of each construct was first normalized to that of its internal control and then to that of POPDC3#1 (100%). Each value is the mean ± SD of three independent experiments.

For lentivirus production, the shRNA vector was cotransfected with lentiviral packaging mix (Sigma) into 293T cells. SNU-216 cells were infected and selected with 0.8 μg/ml puromycin (Sigma). *POPDC3* mRNA reduction was assessed by RT–PCR, and POPDC3 protein reduction was assessed by western blotting.

Cell migration and invasion assay

Cells that stably expressed shRNA (5×10^4 cells per well) were plated in the upper compartment of a 24-well transwell tray (Corning, Corning, NY) in serum-free RPMI without or with epidermal growth factor (EGF; 20 ng/ml). The lower compartment contained RPMI with 10% fetal bovine serum. The cells were allowed to migrate through the intervening nitrocellulose membrane (8 μm pore size) (18). The invasion assay was performed as described (16).

Statistical analysis

The paired *t*-test was used to test differences in mRNA expression or promoter methylation between the set of primary gastric tumor tissues and their adjacent normal tissues. Each value was expressed as the mean ± the standard deviation of the mean. A value of $P < 0.05$ was considered significant. Sensitivity and specificity were calculated using receiver operating characteristic curves and the areas under the curves (20).

Results

Decreased spot intensity of the POPDC3 CpG island in the RLGS profile of gastric cancer

We previously performed RLGS analysis on gastric cancer cell lines and gastric cancer tissues to look for aberrantly methylated genes (14). The spot that corresponded to number 6B043 from our Master RLGS profile (21) and to number 5C25 from the Standard RLGS profile of the Costello Laboratory (22) showed a decreased intensity in 8 of the 11 (73%) gastric cell lines examined and in samples from primary gastric tumors when compared with those from adjacent normal tissues (Figure 1A). The 386 bp DNA fragment that corresponded to spot 6B043 partially overlapped the CpG island of

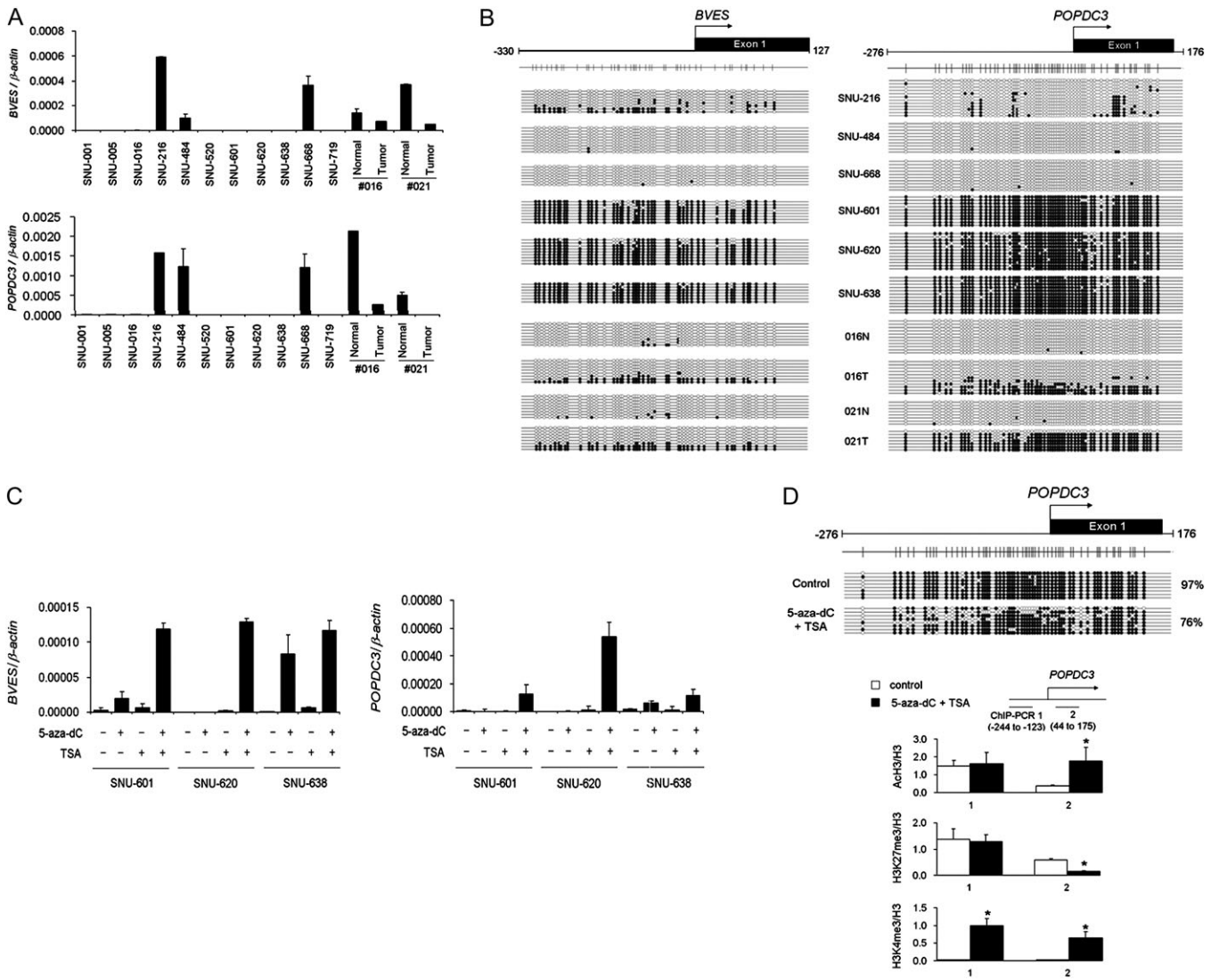

Fig. 2. Aberrant methylation of *BVES* and *POPDC3* in gastric cancer cell lines. (**A**) *BVES* and *POPDC3* mRNA expression in 11 gastric cancer cell lines and two pairs of gastric tumor tissues and adjacent normal tissues. Each value was normalized to the expression of *β-actin* and is the mean ± SD of three independent experiments. (**B**) Bisulfite sequencing analysis of the *BVES* and *POPDC3* promoters in gastric cancer cell lines and two pairs of gastric normal (N) and tumor (T) tissues. Bisulfite sequencing covered 46 CpG sites in the *BVES* promoter and 58 CpG sites in the *POPDC3* promoter. Open circles, unmethylated CpG sites; filled circles, methylated CpG sites. Each row represents the results for a single clone. (**C**) Effects of 5-aza-dC and TSA on *BVES* and *POPDC3* expression. qRT–PCR of *BVES* and *POPDC3* in SNU-601, -620 and -638 cells treated with 10 μM 5-aza-dC, 0.5 μM TSA or both. Each value is the mean ± SD of three independent experiments. (**D**) Bisulfite sequencing of *POPDC3* in untreated SNU-620 cells and in cells treated with 5-aza-dC and TSA (upper panel). ChIP analysis indicated a change in the histone marks associated with *POPDC3* loci in untreated SNU-620 cells or in cells treated with 5-aza-dC and TSA. Each value is the mean ± SD of six PCR samples in each of two independent experiments (lower panel). $^*P < 0.05$.

POPDC3 on chromosome 6q21 (Figure 1B). *POPDC3* and *BVES* are tandem genes on human chromosome 6, and each gene has a CpG island near its transcription start site. Methylated DNA immunoprecipitation sequencing of SNU-601, -620 and -668 showed that the methylation patterns throughout the two genes were similar in all three cell lines; however, the CpG islands of *BVES* and *POPDC3* were methylated only in SNU-601 and -620—the cell lines for which the RLGS spot of the *POPDC3* CpG island had decreased (Figure 1C). We then used the luciferase assay to examine the promoter activity of the CpG islands around the transcription start sites of *BVES* and *POPDC3* in SNU-216 cells. The sites −189 to 180 (*BVES*) and −129 to 118 (*POPDC3*) showed core promoter activity (Figure 1D).

BVES and POPDC3 are aberrantly methylated and silenced in gastric cancer cell lines

To examine the expression patterns of *BVES* and *POPDC3* in gastric cancer, we analyzed the mRNA expression levels of both genes in 11 gastric cancer cell lines and in two pairs of gastric normal and tumor tissues with qRT–PCR. *BVES* and *POPDC3* expression was silenced in 8 of the 11 (73%) gastric cancer cell lines and downregulated in both tumors (Figure 2A). We assessed the methylation status of each CpG site around the core promoter of *BVES* and *POPDC3* with bisulfite sequencing. SNU-216, -484 and -668 cells (with normal expression of *BVES* and *POPDC3*) had preserved hypomethylated CpG sites, but SNU-601, -620 and -638 cells (with reduced expression of *BVES* and *POPDC3*) showed heavily methylated CpG sites. In addition, these CpG sites were hypomethylated in the normal tissue section and moderately methylated in the tumor section of two paired tissues (Figure 2B), suggesting that hypermethylation of the *BVES* and *POPDC3* promoters correlates with decreased expression of these genes in gastric cancer.

DNA methylation is usually associated with histone modification (23). To examine whether silencing of *BVES* or *POPDC3* in gastric cancer cells could be reversed by treatment with the DNA methylation inhibitor 5-aza-dC (24), the histone deacetylase inhibitor TSA (25) or both, we treated the gastric cancer cell lines SNU-601, -620 and -638 with these agents. Treatment with a combination of 5-aza-dC and TSA induced the expression of the two genes more effectively than did treatment with either inhibitor alone (Figure 2C), suggesting that both DNA methylation and histone deacetylation play a causal role in *BVES* and *POPDC3* silencing in gastric cancer cells.

To examine whether reduced methylation of the *POPDC3* promoter is associated with restoration of *POPDC3* expression, we performed bisulfite sequencing. We observed a slight reduction in *POPDC3* promoter methylation in SNU-620 cells treated with both 5-aza-dC and TSA (Figure 2D). To determine whether open chromatin status was also associated with restoration of *POPDC3* expression, we performed ChIP assays. We designed two primer pairs with sequences close to the transcription start site of *POPDC3* and searched for the histone marks, AcH3 (histone H3 acetylation), H3K27me3 (histone H3 Lys27 trimethylation) and H3K4me3 (histone H3 Lys4 trimethylation). Treatment with both 5-aza-dC and TSA reduced the incidence of the repressive mark, H3K27me3, and increased the activating histone marks, AcH3 and H3K4me3 (Figure 2D).

Frequent methylation of BVES and POPDC3 in primary gastric cancer tissues

We performed pyrosequencing to measure the extent of CpG island methylation in *BVES* or *POPDC3* from 76-paired normal and tumor tissues for which genomic DNA was available. The mean *BVES* CpG island methylation level for the normal tissues was 9.4 ± 7.9% compared with 18.0 ± 9.6% for the tumor tissues (Figure 3A). Of the 76 tumors, 53 (69.7%) were hypermethylated at the six CpG sites ($P < 0.0001$). The mean *POPDC3* CpG island methylation level in normal tissues was 13.2 ± 8.9% compared with 26.9 ± 21.6% in tumor tissues (Figure 3B). Of the 76 tumors, 49 (64.5%) showed hypermethylation in the seven *POPDC3* CpG sites ($P < 0.0001$). Using the receiver operating characteristic curve and the methylation levels of

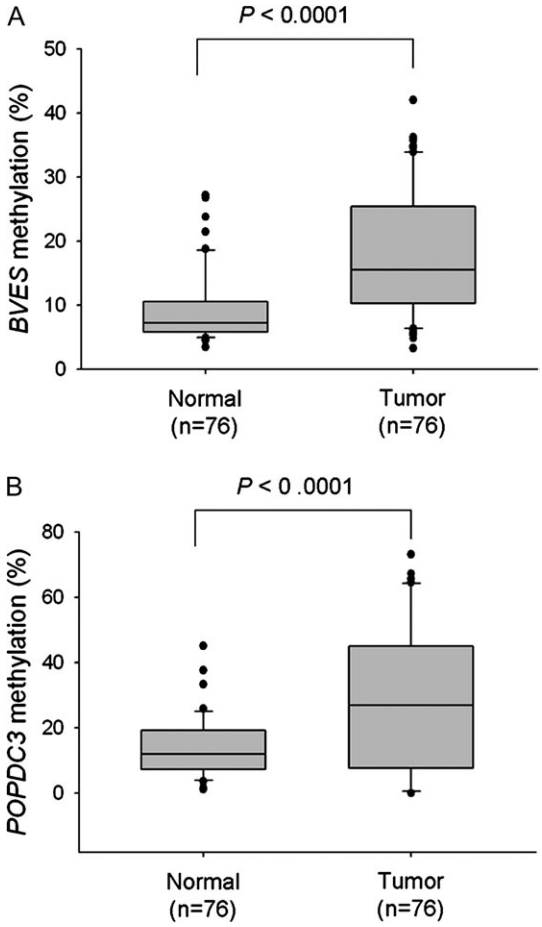

Fig. 3. Frequent methylation of *BVES* and *POPDC3* in gastric cancer tissues. (**A**) *BVES* methylation for 76-paired gastric normal and tumor tissues. Pyrosequencing analysis was performed at the six CpG sites of the *BVES* CpG island. The box plot shows the median, 25th and 75th percentiles, and the dots represent outliers. (**B**) *POPDC3* methylation in 76-paired gastric normal and tumor tissues. Pyrosequencing analysis was performed at the seven CpG sites of the *POPDC3* CpG island.

the normal and tumor tissues, we determined methylation cutoff values of 10.6% for *BVES* (0.73 sensitivity and 0.76 specificity) and 14.0% for *POPDC3* (0.64 sensitivity and 0.63 specificity). Supplementary Table 2 (available at *Carcinogenesis* Online) summarizes the *BVES* and *POPDC3* methylation results for gastric tumor tissues with respect to clinicopathologic characteristics. Neither *BVES* nor *POPDC3* methylation was associated with age, gender, histology or tumor stage.

BVES and POPDC3 expression is downregulated in gastric cancer

POPDC family genes are expressed in heart and muscle, but little is known about their expression in cancerous tissues. To elucidate the roles of *POPDC* in gastric carcinogenesis, we performed qRT–PCR in the 96-paired gastric tumor and adjacent normal tissues. Expression of *BVES* and *POPDC3* was significantly reduced in tumors ($P = 0.00012$ for *BVES* and $P = 0.00002$ for *POPDC3*), whereas *POPDC2* expression was not reduced ($P = 0.77210$) (Figure 4A). *BVES* expression was downregulated at least 2-fold in 69% (66 of 96) of the gastric tumors compared with their corresponding normal tissues, and *POPDC3* expression was also downregulated at least 2-fold in 87% (83 of 96) of the gastric tumors. In contrast, *POPDC2* expression was downregulated at least 2-fold in only 24% (23 of 96) of the gastric tumors. Figure 4B illustrates the relationship between specificity and sensitivity for *BVES*, *POPDC2* and *POPDC3* expression (receiver operating characteristic curves) in gastric cancer. *BVES* and

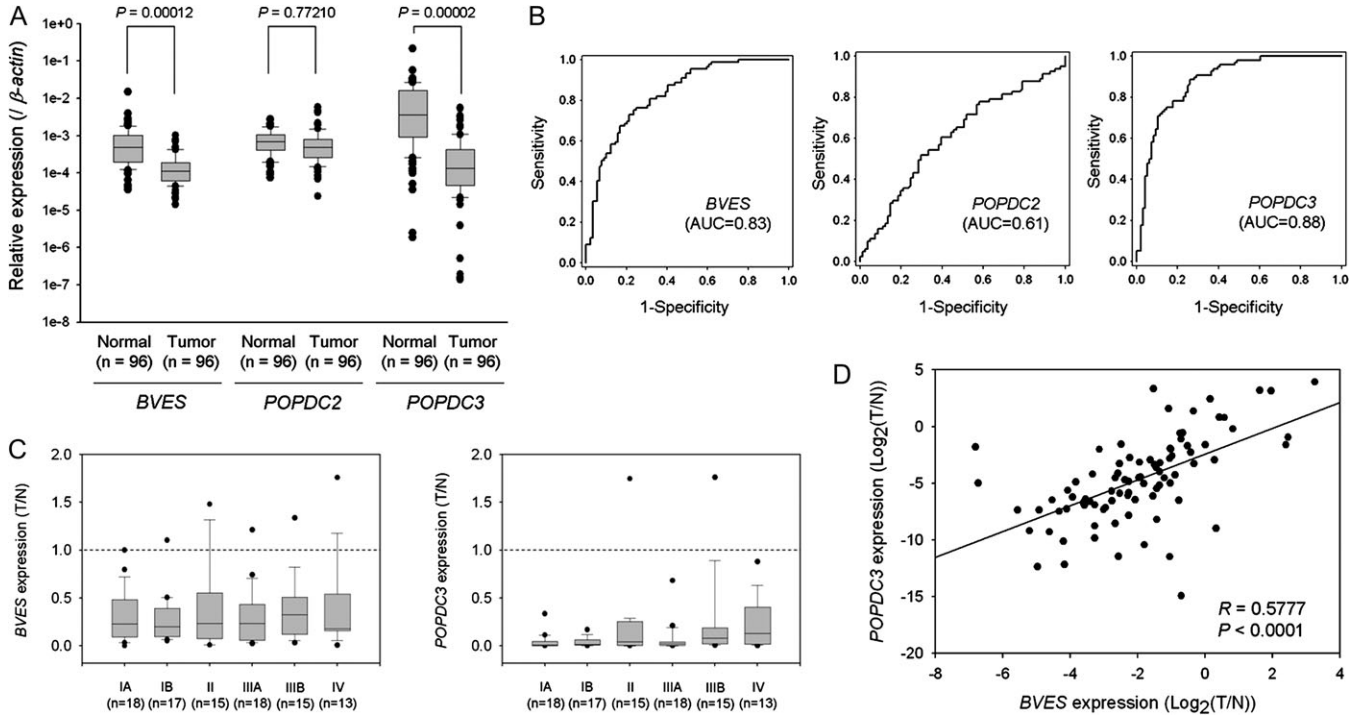

Fig. 4. qRT–PCR analysis of *POPDC* in gastric cancer cell lines and gastric cancer tissues. (**A**) *BVES*, *POPDC2* and *POPDC3* mRNA expression, relative to that of β-actin, in the 96-paired gastric normal and tumor tissues. Each box plot shows the median, 25th and 75th percentiles, and the dots represent outliers. (**B**) Receiver operating characteristic curves for *BVES*, *POPDC2* and *POPDC3* mRNA expression in the 96-paired gastric normal and tumor tissues. (**C**) Box plots of *BVES* and *POPDC3* expression with respect to tumor stage. Relative expression values are expressed as the ratio of tumor (T) to normal (N). (**D**) The relationship between *BVES* expression and *POPDC3* expression in the 96-paired gastric normal and tumor tissues. Relative expression values are the $\log_2$ ratio of tumor to normal.

POPDC3 showed large areas under their curves (0.83 for *BVES* and 0.88 for *POPDC3*), whereas that for *POPDC2* was only 0.61, suggesting that downregulation of *BVES* and *POPDC3* may be specific indicators of gastric cancer. Inactivation of *BVES* and *POPDC3* occurred in early stage as well as in advanced-stage tumors (Figure 4C). Interestingly, the expression of *BVES* was highly correlated with *POPDC3* expression in the gastric cancer tissues ($R = 0.5777$, $P < 0.0001$) (Figure 4D). This result is consistent with the highly correlated expression pattern of the two genes in gastric cancer cell lines (Figure 2A).

Immunohistochemistry of POPDC3 in primary gastric tumor tissues

To examine POPDC3 expression in normal gastric and tumor tissues, we performed immunohistochemistry on paraffin-embedded sections from nine matched normal and tumor tissues that showed *POPDC3* mRNA downregulation and promoter hypermethylation in the tumor sections. POPDC3 staining was detected in the normal cells, especially in chief cells. We also found POPDC3 expression in intestinal metaplasia, but the tumor cells showed decreased POPDC3 expression (Figure 5). Strong POPDC3 expression was detected in the cytoplasm, and weak expression was associated with cell membranes.

Repression of POPDC transcription during EGF-induced epithelial–mesenchymal transition

POPDC transcription has been suggested to be negatively regulated by EGF (4,26). Increased expression of EGF and EGF receptors has been reported as a potent stimulator of cancer cell migration and invasion (27,28). To examine the effect of EGF on *POPDC* expression, we treated SNU-216 cells, which showed normal levels of *POPDC* expression, with EGF (20 ng/ml). We observed epithelial–mesenchymal transition (EMT)-like morphological changes in the EGF-treated SNU-216 cells (Figure 6A). qRT–PCR showed that *BVES* and *POPDC3* mRNAs were rapidly downregulated following EGF treatment, but *POPDC2* mRNA was downregulated more slowly. As expected for EMT, *E-cadherin* mRNA was downregulated, whereas *Snail* expression was upregulated (Figure 6A). POPDC proteins may be involved in cell–cell adhesion through interactions with tight junctions (7). Thus, we next performed western blotting to explore whether the observed morphological changes in EGF-treated SNU-216 cells were accompanied by reduced expression of POPDC3 and the cell adhesion molecules, ZO-1, occludin and E-cadherin. The levels of POPDC3 and the tight junction proteins ZO-1 and occludin decreased following EGF treatment (Figure 6B). The steady-state level of E-cadherin was not significantly altered within 72 h after EGF treatment, but staining of intracellular membranes was diffuse (data not shown). EMT is an important event in cancer cell migration, invasion and metastasis (29–31). Repression of *BVES* and *POPDC3* during EMT implicates roles for these molecules in cancer cell migration and invasion.

POPDC3 silencing promotes gastric cancer cell migration and invasion

To investigate whether *POPDC3* inactivation is involved in gastric cancer cell migration, we examined the effects of shRNA-mediated silencing of *POPDC3* expression in SNU-216 cells. A non-targeting RNA was used as the control. Among the five lentiviral shRNAs used for POPDC3 knockdown (POPDC3 sh#1–sh#5), POPDC3 sh#1 and sh#2 were selected for their high knockdown efficiencies (data not shown). POPDC3 sh#1 and sh#2 inhibited *POPDC3* mRNA expression and protein expression (Figure 6C). Although POPDC3-targeted shRNAs should not, in theory, target any *BVES* mRNA sequences, they repressed *BVES* expression. Conversely, they had little effect on *POPDC2* expression. Interestingly, we found that knockdown of

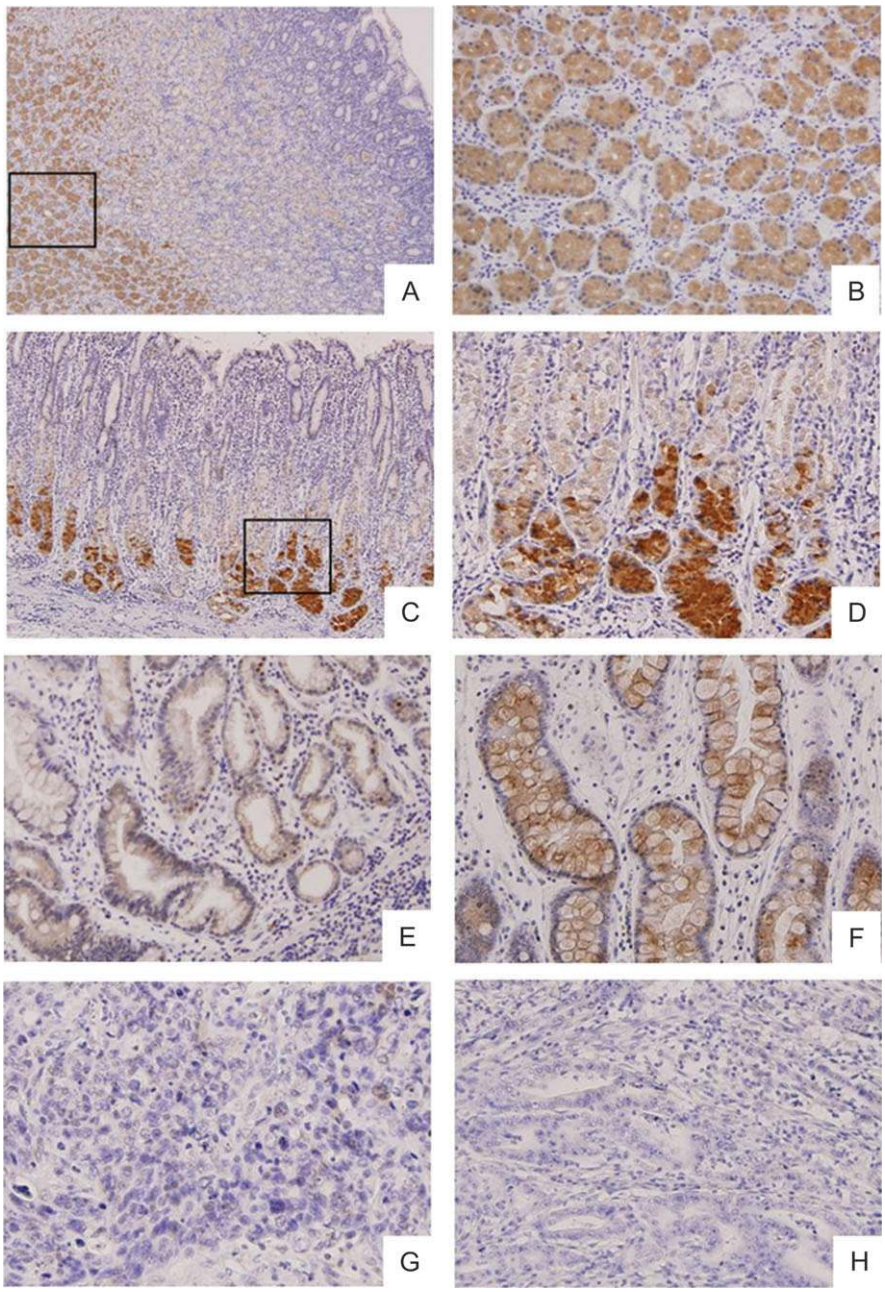

Fig. 5. Immunohistochemistry for POPDC3 in gastric normal and tumor tissues. Paraffin-embedded sections of matched normal and tumor samples were examined for POPDC3 expression using rabbit anti-POPDC3. (**A** and **B**) Staining in normal chief cells. (**C** and **D**) Staining in the basal portion of normal glands of the body-fundic region. (**E**) Staining in normal and intestinal metaplasia cells. (**F**) Staining in intestinal metaplasia cells. (**G**) Reduced expression in tumor tissue, stage III and diffuse type. (**H**) Reduced expression in tumor tissue, stage II and intestinal type. The boxed regions in (A) and (C) are shown at a higher magnification in (B) and (D), respectively.

BVES also repressed *POPDC3* expression but had little effect on *POPDC2* expression (data not shown), suggesting that *POPDC3* expression may affect *BVES* expression and vice versa. In confluent cultures, control cells showed a tightly packed cuboidal/epithelial appearance, which is characteristic of the parental SNU-216 cells, but cells that expressed POPDC3 sh#1 or sh#2 showed reduced cell–cell adhesion. To establish whether POPDC3 downregulation affects the migration and invasiveness of gastric cancer cells, we examined cell migration in transwell assays. POPDC3 sh#1 or sh#2 induced increased cell migration relative to that of control cells. Control cells treated with EGF showed increased migration relative to untreated control cells, and cells that expressed POPDC3 sh#1 or sh#2 showed dramatically increased cell migration. We also observed that knockdown of *POPDC3* promoted gastric cancer cell invasion throughout the Matrigel plugs (Figure 6D), suggesting that downregulation of POPDC3 induces gastric cancer cell migration and invasion.

Discussion

Herein, we report the frequent downregulation of *BVES* and *POPDC3* in human gastric cancer. Among the three *POPDC* genes, the expression of *BVES* and *POPDC3* was significantly reduced in gastric cancer cell lines and tumor tissues. We found that promoter hypermethylation is a major mechanism for inactivation of both genes in gastric cancer. *BVES* and *POPDC3* promoters were heavily

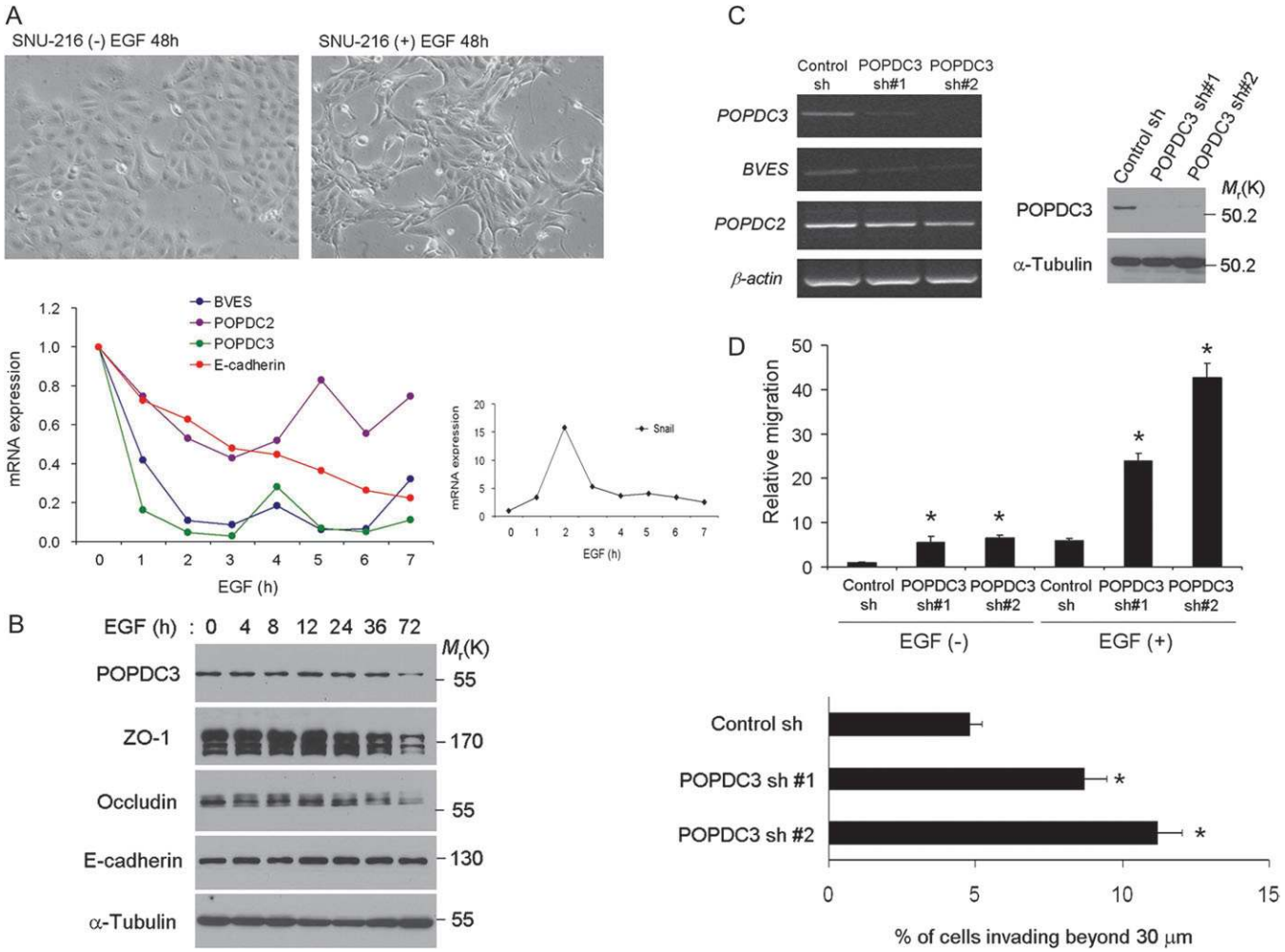

Fig. 6. Effects of *POPDC3* silencing on gastric cancer cell migration and invasion. (**A**) EMT-like morphological changes in SNU-216 cells following treatment with EGF (20 ng/ml) (upper panel). Downregulation of *POPDC* and *E-cadherin* and upregulation of *Snail* after treatment with EGF. Serum-starved SNU-216 cells were treated with EGF (20 ng/ml) for the indicated intervals, and mRNA expression was analyzed with qRT–PCR. Each datum represents three independent experiments (lower panel). (**B**) Western blotting to detect POPDC3 and cell adhesion molecules in EGF-treated SNU-216 cells. Serum-starved SNU-216 cells were treated with EGF (20 ng/ml) for the indicated intervals, and their cell lysates were subjected to western blotting. (**C**) Knockdown of *POPDC3* by shRNA. RT–PCR (left panel) and western blotting (right panel) of SNU-216 cells that stably expressed POPDC3 shRNA (POPDC3sh#1 or sh#2) or non-targeting control shRNA (Control sh). (**D**) Upper panel, cell migration assay. Cells that stably expressed shRNA were plated in the upper compartment of a transwell tray in serum-free RPMI with or without EGF (20 ng/ml). The lower compartment contained RPMI with 10% fetal bovine serum. The cells were allowed to migrate through the intervening nitrocellulose membrane for 16 h at 37°C. Data were obtained from three independent experiments performed in triplicate. The mean migration distances of the experimental cells were normalized with respect to that of the control cells (set to one). Lower panel, invasion assay. Cells that stably expressed shRNA were attached to the bottom of a Matrigel plug and were allowed to migrate through the Matrigel for 4 days at 37°C. Data were obtained from three independent experiments performed in triplicate. $^{*}P < 0.05$.

methylated in gastric cancer cell lines in which the two genes were silenced. Furthermore, *BVES* and *POPDC3* promoters were significantly hypermethylated in gastric tumor tissues compared with adjacent normal tissues, indicating that methylation of both genes is an important event in gastric carcinogenesis. While this work was in progress, Feng *et al.* (32) analyzed the methylation status of 27 genes including *BVES* in tumors and adjacent normal tissues from 49 patients with non-small cell lung cancer and showed that *BVES* was significantly methylated in the lung cancer samples. Thus, methylation of *BVES* appears frequently in gastric and lung cancer.

DNA methylation and histone modifications cause gene repression and can be dependent on one another (23). Treatment with both 5-aza-dC and TSA induced the expression of *BVES* and *POPDC3* more effectively than did treatment with either inhibitor alone. Furthermore, treatment with both inhibitors reduced DNA methylation and the repressive histone mark, H3K27me3, and increased the activating histone marks, AcH3 and H3K4me3, associated with the *POPDC3* promoter. Thus, *BVES* and *POPDC3* are repressed in gastric cancer by epigenetic mechanisms including DNA methylation and histone modifications, and *BVES* and *POPDC3* repression in gastric cancer can be effectively reversed with a pharmacological approach involving simultaneous inhibition of DNA methylation and histone deacetylation.

We suggest that promoter hypermethylation is a causal event for long-term repression of *BVES* and *POPDC3*, whereas EGF stimulation is an immediate repression mechanism for both genes in gastric cancer. *BVES*, *POPDC3* and *E-cadherin* mRNAs were downregulated, and *Snail* mRNA expression was upregulated in EGF-induced EMT in SNU-216 cells (Figure 6A). The EGF receptor signaling pathway leads to activation of Snail through the inactivation of glycogen synthase kinase-3β (27). Snail is a transcriptional repressor that binds to consensus E-box sequences (5′-CA(G/C)(G/C)TG-3′) in the promoters of its target genes (33). Cell adhesion molecule genes, e.g.

E-cadherin, *occludin* and some *claudin* genes are known targets of Snail. Interestingly, *BVES* and *POPDC3* have E-box sequences in their promoter regions. Further studies are needed to elucidate whether *BVES* and *POPDC3* are also target genes of *Snail* family transcription factors.

EGF is an important molecular factor in gastric cancer. Increased expression of EGF and EGF receptors correlates with deep invasion and poor prognosis of gastric cancer (34). EGF stimulates the Rho-family GTPases Rac1 and Cdc42 through the activation of guanine nucleotide exchange factors, e.g. Tiam1 (35), Sos1 (36), Vav2 (37) and Asef (38), which leads to membrane ruffling, lamellipodial protrusion and cell migration. Smith *et al.* (39) showed that BVES interacts with guanine nucleotide exchange factor GEFT and negatively regulates cell movement by regulating Rac1 and Cdc42. We suggest that EGF-induced downregulation of BVES and POPDC3 may result in increased cell migration through the activation of guanine nucleotide exchange factors and Rho-family GTPases. Further studies are necessary to clarify whether BVES and/or POPDC3 activity is related to EGF-induced cell migration signaling. We also suggest that epigenetic inactivation of *BVES* and *POPDC3* may accelerate EGF-induced cell migration signaling.

Although loss of *BVES and POPDC3* expression is associated with EMT, cell migration and invasion in SNU-216 cells, we did not find any significant correlation between *BVES* or *POPDC3* expression and clinical outcomes i.e. tumor grade and nodal or distant metastasis (supplementary Table 2 is available at *Carcinogenesis* Online). Promoter hypermethylation and downregulation of both genes is a frequent event in early stage as well as advanced-stage tumors. These data are consistent with the idea that critical changes in metastatic potential may be determined early during the development of cancer (40). van't Veer *et al.* (41) showed that certain prognostic indicators for breast cancer are found in primary tumors before the appearance of metastases. Moreover, micrometastases are often found in early-stage cancers (42). Thus, we suggest that frequent methylation and inactivation of *BVES* and *POPDC3* in early-stage gastric cancer may predispose cells to other critical changes that cause cancer metastasis.

BVES and *POPDC3* are located on the same chromosome, and the genomic region spanning *BVES* and *POPDC3* is evolutionarily conserved from chicken to human (1). Moreover, we found that the expression of *BVES* and *POPDC3* was highly correlated in our gastric cancer cell lines and tissues, suggesting that these two genes may have evolved via gene duplication and may be regulated in a similar manner. *BVES* and *POPDC3* were downregulated similarly within 7 h after EGF stimulation, which is in accordance with the highly correlated expression pattern of the two genes in the gastric cancer cell lines and tissues.

In summary, we showed that epigenetic inactivation of *BVES* and *POPDC3* occurred frequently in gastric cancer tissues and cell lines and may contribute to gastric cancer cell migration and invasion. We suggest that *BVES* and *POPDC3* may be important targets for gastric cancer therapies and are candidate biomarkers for gastric cancer detection and prognosis.

Supplementary material

Supplementary Tables 1 and 2 can be found at http://carcin.oxfordjournals.org/

Funding

21C Frontier Functional Human Genome Project from the Ministry of Science and Technology (FG09-11-01); Korea Health 21 R&D Project (A062254) from the Ministry of Health & Welfare of Korea.

Acknowledgements

Conflict of Interest Statement: None declared.

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Received February 25, 2010; revised June 3, 2010; accepted July 3, 2010