DNA hypermethylation of the vimentin gene inversely correlates with vimentin expression in intestinal- and diffuse-type gastric cancer

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Abstract. The vimentin gene is a hallmark of epithelial-to-mesenchymal transition and has been observed to be overexpressed in various types of tumor cell line and tissue. Previous studies have reported correlations between vimentin DNA methylation levels and subsequent vimentin expression levels in solid tumors, including breast and colorectal cancer; however, to the best of our knowledge, such a correlation has not been reported for gastric cancer (GC) using Lauren classification. Therefore, the present study aimed to quantify DNA methylation levels of the vimentin gene using quantitative (q) methylation-specific polymerase chain reaction (PCR) in intestinal-type GC cell lines (MKN-28, AGS and MKN-1), diffuse-type GC cell lines (SGC-7901, SNU-5 and KATO III), the GES-1 immortalized human non-neoplastic gastric epithelial cell line, as well as in tumor and paratumor normal tissue samples. Furthermore, the present study analyzed the messenger RNA expression of the vimentin gene in these cell lines and tissues by reverse transcription-qPCR. A comparison of the clinicopathological features was conducted between patients, grouped according to the Lauren classification. The present study identified that the vimentin promoter region was hypermethylated in all GC cell lines and tumor tissue samples when compared with immortalized normal gastric epithelial cells and paratumor normal tissues. In addition, vimentin promoter methylation levels were observed to be higher in intestinal-type cell lines when compared with those of diffuse-type lines and tissues. Correspondingly, vimentin

expression levels were lower in intestinal-type gastric cell lines compared with those of diffuse-type cell lines and tissues, and were lowest in the non-neoplastic gastric cell line and paratumor normal tissues. Patients with diffuse-type GC were on average younger (P=0.023), and exhibited higher tumor (P=0.020), node (P=0.032) and TNM classification of malignant tumor stage (P=0.039) than those with intestinal-type GC. Following treatment of AGS cells (which demonstrated the highest methylation level of the vimentin gene) with 5-aza-2'-deoxy-cytidine, vimentin expression was restored significantly. Thus, the present study revealed that vimentin promoter methylation levels are inversely correlated with vimentin expression levels in GC (according to Lauren classification). High levels of methylation in the vimentin gene promoter region may be involved in carcinogenesis and the development of GC, and may provide a novel molecular classification for GC.

Introduction

Gastric cancer (GC) is a frequently occurring type of malignancy and the second most common cause of cancer-associated mortality worldwide (1). Gastric tumorigenesis is a complex, multi-step process involving alterations of numerous genes (2). Aberrant promoter methylation is an important mechanism for silencing certain tumor suppressor and tumor-associated genes, and is significant during the pathogenesis and progression of certain types of human cancer (3,4), including GC (5,6). Data suggests that DNA methylation may be a useful biomarker for cancer risk evaluation (3,5), early diagnosis (5), prognosis (4,5) and evaluation of sensitivity to chemotherapy (7).

The vimentin gene encodes an intermediate filament protein reported to be involved in cytoskeletal architecture (8-10), the immune response and stabilization of collagen messenger RNAs (mRNAs) (11). Given its multiple functions, vimentin is considered to be significant in the epithelial-to-mesenchymal transition (EMT), including upregulation of EMT-associated genes, adaptive responses to wound healing and pathological responses during cell invasion and metastasis (12). Lauren classification has been globally adopted and separates gastric carcinoma into intestinal and diffuse subtypes according to the morphological features of the tumor (13).

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GC is generally classified into two histological types, intestinal and diffuse, and each type develops through a distinct carcinogenic pathway (14). Phenotypically, the two types exhibit distinct macroscopic appearances, reflecting the differences in their microscopic growth patterns and molecular signaling pathways (15). In intestinal-type carcinoma, the macroscopic margins approximately correspond with microscopic spread, while the poor differentiation of diffuse-type carcinoma facilitates submucosal extension beyond the macroscopic borders (15). This difference in tumor extension is of clinical significance in the selection of a suitable treatment strategy.

Although upregulation of levels of vimentin expression during EMT have been well characterized in GC, to the best of our knowledge, no study has reported epigenetic regulation of the vimentin gene in GC using Lauren classification. In the present study, vimentin promoter DNA methylation, and vimentin expression in gastric cell lines and human tissue samples, grouped according to Lauren classification, were examined to determine whether a correlation between the two existed.

Materials and methods

GC cell lines and human tissue samples. Six gastric cancer cell lines (MKN-28, AGS, MKN-1, SGC-7901, SNU-5 and KATO III) and one normal gastric epithelial cell line (GES-1) (American Type Culture Collection, Manassas, VA, USA) were used in the present study. The MKN-28 cells were checked for contamination. All cell lines were cultured and maintained in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco Life Technologies, Carlsbad, CA, USA) and 1% penicillin/streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin; Invitrogen Life Technologies, Carlsbad, CA, USA) and incubated in 5% CO_2 at 37˚C.

Tumor and paratumor normal tissue samples of 64 patients with GC, who underwent radical gastrectomy at the Affiliated Hospital of Qingdao University (Qingdao, China), were collected between January 2008 and October 2011, and stored in liquid nitrogen. Written informed consent was obtained from all patients and the procedure of the present study was approved by the institutional review board of the Affiliated Hospital of Qingdao University. All samples were confirmed by histology and 36 diffuse-type and 28 intestinal-type samples, as well as their paired paratumor normal tissues, were selected for analysis. Clinicopathological features of patients enrolled in the present study are presented in Table I.

DNA and RNA isolation from cell lines and tissue samples. Genomic DNA extraction was conducted with the DNAeasy® Blood & Tissue kit (Qiagen GmbH, Hilden, Germany). RNAiso Plus Reagent (Takara Bio, Inc., Otsu, Japan) was used to extract total RNA, which was utilized in the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) prior to DNA isolation. For RT-qPCR, RNA was reverse transcribed using PrimeScript RT reagent (Takara Bio, Inc.) and the reaction product was treated with RNAse-free DNase I. Details of the RT-qPCR reaction are described below.

Sodium bisulfate modification and quantitative methylation-specific PCR (qMSP). A total of 1 μg genomic DNA was subjected to bisulfate treatment using an Epitect Bisulfate kit (Qiagen GmbH). The bisulfate-treated DNA was amplified by qMSP using a LightCycler480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland). Briefly, thermocycling was conducted in a final volume of 25 μl, containing 1.0 μl treated DNA sample, 100 nM of each primer and 12.5 μl SYBR Premix Ex *Taq II* (Takara Bio, Inc.). The PCR primer sequences for vimentin (GeneChem, Shanghai, China) were as follows: Methylated sense, 5'-TCGTTTCGAGGTTTTCGCGTTAGAGAC-3' and vimentin methylated antisense, 5'-CGACTAAAACTC GACCGACTCGCGA-3'. PCR amplification consisted of 40 cycles (95˚C for 5 sec and 55˚C for 30 sec) following an initial denaturation step (95˚C for 10 sec). Genomic DNA, methylated *in vitro* by CpG methyltransferase (Sss I; New England BioLabs, Inc., Ipswich, MA, USA), served as a positive control and a water blank served as a negative control. GAPDH served as an internal control for normalization. The percentage of vimentin promoter methylation in each sample was estimated using the following formula:

$$\text{Methylated vimentin (\%)} = \frac{M}{M+U} \times 100\% = \frac{1}{1 + 2^{(-\Delta Ct)}} \times 100\%$$

M, copy number of methylated vimentin; U, copy number of unmethylated vimentin; and $\Delta Ct = Ct_u - Ct_M$.

RT-qPCR gene expression analysis in gastric cell lines and tissue samples. Gene expression in gastric cell lines and tissue samples was quantified by RT-qPCR using a 7500 Real-Time PCR Platform (Applied Biosystems, Foster City, CA, USA). Vimentin and GAPDH expression levels were assessed using pre-designed TaqMAN probes VIM-Hs00185584_m1 and GAPDH-Hs02758991_g1, respectively (Applied Biosystems Life Technologies). Complementary DNA samples from three independent biological experiments were examined by RT-qPCR (50˚C for 2 min, denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C annealing for 1 min). For each experiment, the samples were analyzed in duplicate. Normalized vimentin expression was determined using the comparative Ct ($\Delta\Delta Ct$) method using Relative Quantification Study software (7300 Sequence Detection system, version 1.4; Applied Biosystems Life Technologies).

5-Aza-2'-deoxycytidine (5-Aza-dC) treatment. To further examine whether vimentin expression is regulated by DNA promoter methylation, the AGS cell line, which demonstrated the highest level of vimentin gene methylation among the six cell lines, was selected for further study. The AGS cell line was cultured in RPMI-1640 medium supplemented with 10% FBS. Cells in the logarithmic proliferative phase were seeded into a 96-well plate (Corning, New York, NY, USA) at a density of 5,000 cells/well and cultured in an incubator (Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2 saturated humidity for 24 h. Cells were subsequently treated with 1 μM 5-Aza-dC (Sigma-Aldrich, St. Louis, CA, USA) for 72 h, with replenishment of fresh medium containing 5-Aza-dC every 24 h. Cells in the treatment and control groups were then harvested. Demethylation of the vimentin promoter regions was detected using MSP, and vimentin mRNA expression

Table I. Clinicopathological features of patients exhibiting gastric cancer according to Lauren classification.

| Clinicopathological features | Cases, n | Lauren classification | | P-value |
		Diffuse-type (n=36)	Intestinal-type (n=28)	
Age, years	-	54.2±9.1[a]	69.7±9.6[a]	0.023
Gender				0.072
Male	41	15	18	
Female	23	21	10	
Maximal tumor size, mm	-	56.3±23.7[a]	51.4±22.6[a]	0.264
T stage (AJCC)				0.020
T1	6	1	5	
T2	10	4	6	
T3	39	28	11	
T4	9	7	2	
N stage (AJCC)				0.032
N0	18	6	12	
N1	9	4	5	
N2	16	11	5	
N3	21	16	5	
TNM stage (AJCC)				0.039
I	15	4	11	
II	22	13	9	
III	27	18	9	

[a]Data are expressed as the mean ± standard deviation. AJCC, American Joint Committee on Cancer; TNM, TNM classification of malignant tumors; T, tumor; N, (lymph) nodes; M, metastasis.
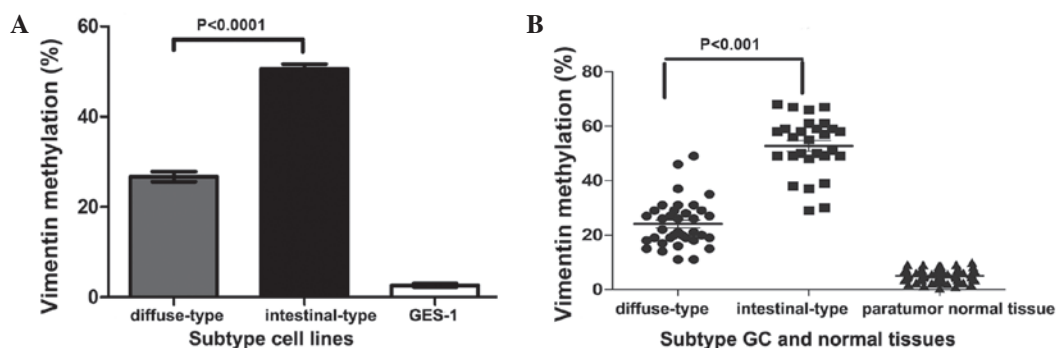

Figure 1. DNA methylation of the vimentin promoter in gastric cell lines and tissues. (A) DNA methylation of the vimentin promoter was analyzed using methylation specific-quantitative polymerase chain reaction technology in diffuse-type GC cell lines (SGC-7901, SNU-5, KATO III), intestinal-type GC cell lines (MKN-28, AGS, MKN-1) and immortalized human non-neoplastic gastric epithelial cells (GES-1), as well as in (B) tumor and paratumor normal tissues. GC, gastric cancer.

levels were measured using RT-qPCR. MSP and RT-qPCR were performed as described previously in the current study.

Statistical analysis. Statistical analyses were performed using SPSS (version 17.0; SPSS, Inc., Chicago, IL, USA). Vimentin DNA methylation was compared between diffuse-type and intestinal-type cell lines or between tumor and paratumor normal tissue samples by Student's t-test, and correlation of vimentin promoter methylation with vimentin expression was assessed by Pearson's correlation analysis. The χ^2 test or Student's t-test were

utilized to compare the clinicopathological data. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinicopathological features of patients with GC are associated with Lauren classification. Clinicopathological data were assessed for differences between diffuse- and intestinal-type GC cells. There were 36 (56.3%) patients with diffuse-type and 28 (43.7%) patients with intestinal-type GC.

Table II. Spearman's correlation analysis for vimentin methylation and expression.

Source	Vimentin Methylation, %	Expression	Pearson's r	P-value
Cell line				
Diffuse-type	26.71±1.04	0.285±0.014	-0.901	<0.001
Intestinal-type	50.60±1.03	0.184±0.006	-0.869	<0.001
GC tissue				
Diffuse-type	19.48±5.26	0.342±0.0521	-0.821	<0.001
Intestinal-type	61.02±9.19	0.158±0.055	-0.879	<0.001

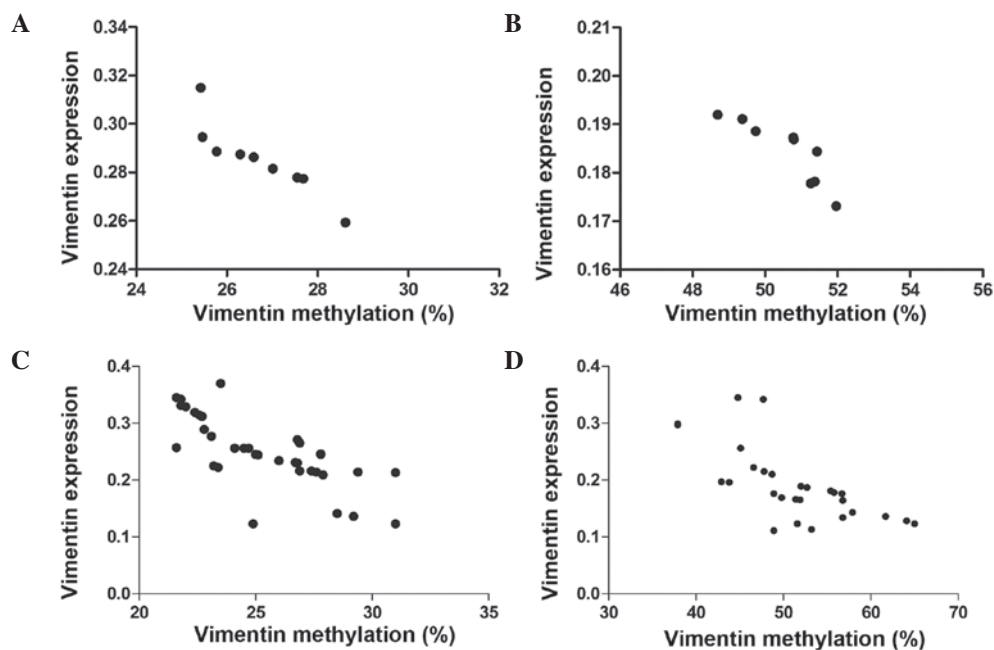Data are expressed as the mean ± standard deviation. GC, gastric cancer.

Figure 2. Correlations between vimentin methylation and relative expression levels in GC cell lines and tissues. Reverse transcription-quantitative polymerase chain reaction was used to analyze relative vimentin messenger RNA expression levels. A negative correlation was identified between vimentin DNA methylation and expression levels in (A) diffuse-type and (B) intestinal-type GC cell lines. (C and D) Comparable correlations were observed in diffuse- and intestinal-type of GC tissue. GC, gastric cancer.

As shown in Table I, a significant difference was observed between diffuse- and intestinal-type GC for age (P=0.023), tumor (T) stage (P=0.020), node (N) stage (P=0.020) and Tumor-Node-Metastasis (TNM) stage (P=0.039). There was no significant difference observed for gender (P=0.072) or maximal tumor size (P=0.264).

Vimentin promoter methylation and vimentin gene expression vary according to Lauren classification. In order to investigate epigenetic silencing mechanisms of the vimentin gene in GC, qMSP technology was applied to evaluate the DNA methylation status of the vimentin promoter. GES-1 immortalized human non-neoplastic gastric epithelial cells, intestinal-type GC cell lines (MKN-28, AGS, MKN-1) and diffuse-type GC cell lines (SGC-7901, SNU-5, KATO III), as well as tumor and paratumor normal tissue samples were analyzed. All GC cell lines and tissues that were assessed

demonstrated promoter hypermethylation (>10%), and methylation levels were significantly higher in intestinal-type compared with diffuse-type (P<0.0001; Fig. 1A and B). Conversely, GES-1 and paratumor normal tissues exhibited hypomethylation at the vimentin promoter (P<0.0001; Fig. 1A and B).

Subsequently, RT-qPCR was conducted to investigate the correlation between the vimentin promoter methylation and expression levels. Vimentin expression levels were strongly detected in diffuse-type cell lines and tissues, weakly detected in intestinal-type cell lines and tissues, and markedly reduced in GES-1 and paratumor normal tissues. Through comparison of vimentin promoter methylation and vimentin mRNA expression levels, an inverse correlation was identified (Table II) in the GC cell lines (Fig. 2A and B) and tissues (Fig. 2C and D). This may suggest that expression is negatively regulated by vimentin promoter DNA methylation.

Table III. Varying expression levels of vimentin messenger RNA following 5-Aza-dC treatment in AGS cells (n=3).

Group	Vimentin methylation, %	P-value	Vimentin expression	P-value
Control	51.53±0.31	0.002	0.1764±0.0023	0.014
5-Aza-dC	31.66±0.17		0.2251±0.0019	

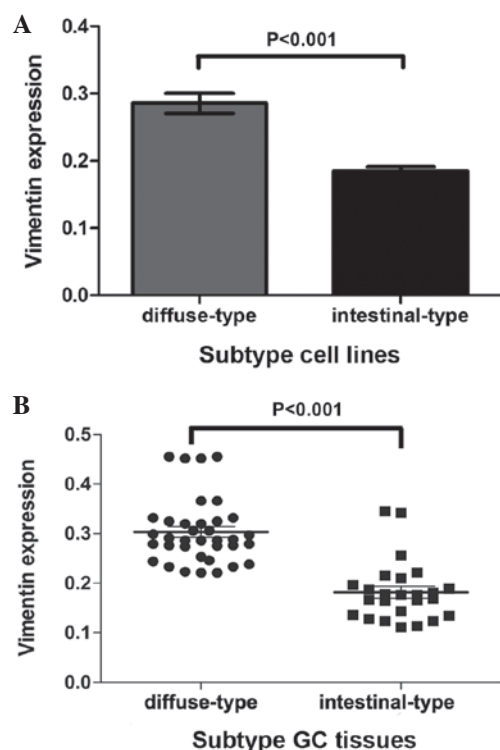Data are expressed as the mean ± standard deviation. 5-Aza-dC, 5-Aza-2'-deoxycytidine.

Figure 3. Diffuse-type GC exhibits higher vimentin expression levels compared with intestinal-type GC. (A) GC cell lines and (B) GC tissues. Statistical analysis was performed using Student's t test. GC, gastric cancer.

Vimentin expression levels are lower in intestinal- than diffuse-type GCs. To evaluate the expression of vimentin in the various Lauren histological types, vimentin expression levels in intestinal-type cell lines and GC tissues were compared with those in diffuse-type cell lines and GC tissues. The results revealed significantly lower vimentin expression levels in intestinal-type cells and GC tissues when compared with diffuse-type cells and GC tissues ($P<0.001$; Fig. 3).

5-Aza-dC treatment restores vimentin expression. Among the six cell lines examined, AGS cells demonstrated the highest levels of vimentin gene methylation and thus, this cell line was selected for further study. To further examine whether vimentin expression was regulated by vimentin promoter DNA methylation, AGS cells were treated with a DNA demethylating agent (5-Aza-dC) for 72 h. Following treatment, promoter DNA methylation was significantly reduced, which was accompanied by reactivation of vimentin expression ($P<0.01$; Table III). Although significantly increased, vimentin expression was still in the range for GC.

Discussion

Vimentin is a type III intermediate filament protein involved in cell attachment, migration and signaling (9,16). Vimentin regulates integrins, which are heterodimeric transmembrane cell adhesion receptors, and stimulates development and turnover of adhesive structures, particularly in endothelial cells (17,18). Vimentin also affects the activity of certain cell membrane-associated signaling pathways, including mitogen-activated protein kinase (19,20). Overexpression of vimentin in cancer cells strongly correlates with levels of invasiveness and poor prognosis (21). Increased vimentin expression levels have been observed in a number of types of epithelial cancer, including prostate, gastrointestinal, central nervous system, breast, malignant melanoma and lung (21). Consistent with previous findings, the present study demonstrated vimentin overexpression in GC cell lines and clinical GC tissues.

Over the past decade, knowledge of the importance of epigenetic events in the control of normal cellular processes, and aberrant events leading to tumor development and progression has increased (3-7,22). DNA methylation is a major epigenetic mechanism that has been intensively investigated in the context of gene regulation and abnormal gene silencing in cancer cells (3-6). DNA methylation-associated biomarkers are under investigation in various types of human cancer (3-5,7). Studies have previously investigated epigenetic alterations in the vimentin gene, with data demonstrating differential vimentin DNA methylation levels in solid tumors, including colorectal, cervical, bladder and pancreatic cancer (23-26). In patients with GC, frequent methylation of vimentin DNA in serum (27) and tissues (28,29) has been detected. The frequency of vimentin DNA methylation in GC cell lines and tissues observed in the present study is consistent with the findings of the above-mentioned studies. Additionally, the results of the present study demonstrated an inverse correlation between vimentin DNA methylation and vimentin expression in GC cell lines and tissues grouped according to Lauren classification. Furthermore, restoration of vimentin expression in AGS cells was achieved through DNA demethylation with 5-Aza-dC. Data from the present study indicates that the inverse correlation between vimentin DNA methylation level and vimentin expression level may be cancer-specific, as it was not observed in GES-1 cells (a non-neoplastic gastric epithelial cell line) or paratumor normal tissues. GES-1 cells and paratumor normal tissue demonstrated reduced levels of vimentin expression, while the vimentin promoter was observed to be hypomethylated. These results suggest the existence of an alternative mechanism for

the suppression of vimentin expression in normal cells, which requires further investigation.

The difference in tumor extension between intestinal and diffuse-type carcinoma is of clinical significance in the selection of a suitable treatment strategy (14,15). In the present study, the younger age (P=0.023), higher T (P=0.020), N (P=0.032) and TNM stage (P=0.039) of patients with diffuse-type GC indicated that diffuse-type was more invasive than intestinal-type GC, which may contribute to the poorer prognosis of patients with diffuse-type carcinoma (30). An improved understanding of the underlying pathogenesis and molecular events of the two histological subtypes may facilitate the development of novel diagnostic, therapeutic and preventive strategies for GC. The present study revealed differential hypermethylation at the vimentin gene promoter and an inverse correlation between vimentin DNA methylation and transcriptional expression levels among cancer cell lines and tissues, which were derived from the two types of GC. Intestinal-type GC exhibited higher promoter methylation levels and lower vimentin expression levels than those of diffuse-type. Considering the disparity in vimentin DNA methylation between the two types of GC and the marked correlation between vimentin expression, invasiveness and poor prognosis, vimentin DNA methylation may be involved in a mechanism that induces the distinct morphology and behavior of the two GC types by regulating vimentin mRNA expression. It has been reported that vimentin DNA methylation in colorectal cancer (CRC) tissues is significantly more frequent than that in normal and benign tissues and, therefore, vimentin promoter methylation may be involved in the carcinogenesis of CRC (31). The results of the present study demonstrated hypermethylation of the vimentin promoter in GC, which is comparable with CRC on a cytological and histological level. This suggests that the high level of methylation in the vimentin gene promoter region may be involved in carcinogenesis and the development of GC, and may serve as a novel molecular marker for GC.

In conclusion, the present study demonstrated that vimentin promoter methylation was inversely correlated with vimentin expression, and that the levels of vimentin expression were significantly different in the Lauren histological types of human GC. Vimentin gene hypermethylation may be associated with the occurrence and progression of GC, and detection of methylation of the vimentin gene may serve as a diagnostic marker for GC classification, which may provide guidance for the treatment and evaluation of GC.

Acknowledgements

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