Methylation of \textit{p16} CpG Islands Associated with Malignant Transformation of Gastric Dysplasia in a Population-Based Study

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ABSTRACT

\textbf{Purpose:} Inactivation of \textit{p16} by aberrant methylation of CpG islands is a frequent event in carcinomas and precancerous lesions of various organs, including the stomach. The aim of this study is to investigate the relationship between \textit{p16} methylation and malignant transformation of human gastric dysplasia (DYS) based on follow-up endoscopic screening in a high-risk population.

\textbf{Experimental Design:} Genomic DNA samples were extracted from paraffin blocks of gastric mucosal biopsies that were histopathologically diagnosed as low-grade DYS from patients who developed gastric carcinomas \textit{(GCs\ ((n = 21))} and those that did not do so \textit{(n = 21)} during 5 years of follow-up. The methylation status of \textit{p16} CpG islands of each sample was detected by methylation-specific PCR, denatured high-performance liquid chromatography, and sequencing.

\textbf{Results:} Aberrant \textit{p16} methylation was observed in 5 of 21 samples of DYS that progressed to GC but in 0 of 21 samples that did not progress to GC \textit{(P = 0.048, two-sided)}. Sequencing results confirmed that all CpG sites were methylated in the analyzed sequence from these five \textit{p16}-methylated cases. Furthermore, \textit{p16} methylation was also observed in the five subsequent GCs. Unmethylated \textit{p16} CpG islands were detected in all of the samples without \textit{p16} methylation.

\textbf{Conclusions:} Our findings suggest \textit{p16} methylation is correlated with the malignant transformation of gastric DYS, and \textit{p16} methylation might be a useful biomarker for prediction of malignant potential of gastric DYS.

INTRODUCTION

Gastric carcinoma (GC) is the second leading cancer death in China and in the world. Gastric dysplasia (DYS) \textit{(noninvasive neoplasia, the Padova International Classification]} is a premalignant lesion of intestinal-type GC. It was reported that about 90\% of low-grade DYS lesions do not progress to malignancy during long-term follow-up \textit{(1, 2)}.

Current diagnosis of DYS is based primarily on morphological criteria \textit{(3)}. It is virtually impossible to identify the malignant potential of low-grade DYS lesions on histopathological grounds alone. Thus, predicting the malignant potential of this lesion is eagerly awaited.

\textit{P16}\textsuperscript{INK4A} (CDKN2/MTS1), an inhibitor of the cyclin D-dependent protein kinase 4/6, is a cell cycle regulator involved in the inhibition of G\(_1\) phase progression \textit{(4)}. Loss of function of \textit{p16} results in higher cyclin D-dependent protein kinase activity and thus leads to aberrant phosphorylation of retinoblastoma, which accelerates cell growth. Inactivation of \textit{p16} by homozygous deletion or point mutation is one of the most commonly observed aberrations in tumors, indicating that \textit{p16} is a tumor suppressor gene \textit{(5)}. An alternative mechanism for inactivation of \textit{p16} is aberrant methylation of CpG island extending from the promoter region to exon 1, which silences transcription of this gene \textit{(6)}. Aberrant \textit{p16} methylation was reported to occur frequently in a variety of human cancers \textit{(6–8)}. In GC, the frequency of \textit{p16} inactivation by homozygous deletions ranged from 0\% to 9\%, the frequency of \textit{p16} inactivation by mutation ranged from 0\% to 2\%, and the frequency of \textit{p16} inactivation by methylation ranged from 32\% to 42\% \textit{(9–15)}, which suggests that methylation is a major mechanism for \textit{p16} inactivation in GC.

Aberrant \textit{p16} methylation was also observed frequently in premalignant stages of GC \textit{(16)}. We previously reported a positive association between aberrant \textit{p16} methylation and the severity of glandular stomach pathology of Wistar rats with cancer induced by chemical carcinogen \textit{(17)}. In the present study, aberrant \textit{p16} methylation in relation to malignant progression of DYS was investigated through a nested case-control study in a population with a high risk of GC.

MATERIALS AND METHODS

\textbf{Specimens.} In 1989, 1994, and 1999, the Beijing Institute for Cancer Research conducted three surveys of precancerous gastric lesions in Linqu County, a rural area in which residents had a high risk of GC, in Shandong Province, China \textit{(1, 18–20)}. The Institutional Review Boards of the Beijing Institute for Cancer Research approved these surveys, and all participants...
Methylation of p16 and Gastric Dysplasia Progression

gave written informed consent. Briefly, an endoscopic screening program for GC was launched among 3399 residents aged 35–64 years in 1989, and a repeat endoscopic screening was offered to 83% of eligible follow-up members in 1994 and 94% of eligible follow-up members in 1999 to determine the progression of precancerous gastric lesions at baseline. The endoscopic biopsy specimens were taken from seven standard sites in the stomach (four from the antrum, one from the angularis, and two from the body of the stomach). The biopsies were fixed immediately in buffered formalin and then embedded, sectioned, and stained with H&E. The microscopic slides were studied by a panel of three senior pathologists at the Department of Pathology, Beijing Institute for Cancer Research. The presence or absence of DYS, carcinoma, and other pathological changes was recorded for each biopsy, and a global diagnosis of each case represented the most advanced lesion. The three surveys used the same endoscopic and histopathological procedures, and histopathological diagnoses were made by the same pathologists blinded to the previous evaluations.

Biopsies with low-grade DYS (the global diagnosis) in 1989 and 1994 at baseline that either progressed to GC or persisted in DYS at the corresponding sites during the follow-up (in 1994 and 1999, respectively) were selected for detection of p16 promoter methylation. All biopsy samples (n = 21) of DYS that progressed to GC were used if sections were available from the paraffin block. An equal number of DYS samples (n = 21) that persisted in DYS were selected from the tissue block archive, according to pathological grade, sampling site, age, and sex.

Genomic DNA Extraction and Bisulfite Treatment.
All sections from the margin of the gastric tissue biopsies embedded in paraffin that were not eligible for preparation of diagnosis slides were collected into 1.5-ml microcentrifuge tubes. After the collected sections were dewaxed by xylene and rehydrated with graded ethanol, they were mixed with lysis buffer containing proteinase K and digested at 37°C overnight. Genomic DNA (about 10 ng) was extracted with a genomic DNA purification kit (Promega, Madison, WI) and modified with sodium bisulfite to convert the unmethylated cytosines to uridines (17, 21).

Amplification of the Methylated p16 CpG Island. The methylation status of the bisulfite-modified p16 CpG island (GenBank accession No. GI 16944057, antisense strand) was analyzed with methylation-specific PCR (MSP) as described previously (22). The primers for the methylated p16 CpG island were 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ (sense) and 5′-GACCCCCGAACCGGCAGCGCTGAA-3′ (antisense). Hot-start MSP was used for amplification of the methylated p16 CpG islands. Thermal cycles were as follows: denaturation at 97°C for 5 min, followed by addition of DNA polymerase; amplification for 35 cycles (95°C for 45 s, 64°C for 45 s, and 72°C for 45 s); and extension at 72°C for 10 min. The reaction mixture (20 μl) contained about 10 ng of templates, 10 pmol of each primer, 40 nmol of deoxynucleotide triphosphate, 1 unit of Taq DNA polymerase (Takara, Kyoto, Japan), and 10 μl of 2× GC buffer I (Takara). Distilled water and genomic DNA of human GC (p16 unmethylated by sequencing) or lymphocytes were used as a template for negative control. Genomic DNA of p16-methylated human GC (p16 methylated by sequencing) or colon cancer cell line RKO (23) was used as positive control. The control MSP products were run on the gel first. When no false positive or false negative methylated p16 MSP products were observed, the sample MSP products were analyzed further by denaturing high-performance liquid chromatography (DHPLC). In the pilot study, positive detection of p16 methylation by the above-mentioned MSP protocol correlated negatively with P16 expression in surgically obtained GCs by immunohistochemistry. Methylated p16 was detectable in 80% of P16-negative tissues and 32% of P16-positive samples (P = 0.003). Therefore, this protocol was used in the present nested case-control study.

Analysis by DHPLC. DHPLC is used frequently to size double-stranded DNA fragments and detect point mutation and CpG methylation (22, 24–26). In the present study, the MSP products (150 bp) of p16 CpG island were detected by DHPLC at 48°C, the nondenaturing temperature suitable for sizing of the amplicons of bisulfite-modified DNA samples (22). In brief, DHPLC was performed with the WAVE DNA Fragment Analysis System (Transgenomic, Inc.). MSP products were introduced into the mobile phase at an injection volume of 5 μl by the autosampler. A DNAsep analytical column was used as the solid phase. The products were eluted from the column with a binary gradient of 0.1 m triethylammonium acetate and 0.1 m triethylammonium acetate in 25% acetonitrile during mobile phase at a flow rate of 0.9 ml/min at 48°C. Elution gradients were predicted automatically by WAVEmaker 4.0 software (Transgenomic, Inc) according to the target size (150 bp). The eluted products were detected by UV analysis at 260 nm.

Confirmation of the Existence of the Modified Templates in MSP. If the methylated p16 CpG island was not observed by DHPLC, the unmethylated p16 CpG islands were further amplified by MSP to exclude the possibility of a false negative result of unsuccessful DNA preparation and bisulfite modification, which would result in the negative detection of methylated products by DHPLC. Because of the difficulty in obtaining additional sections from the invaluable biopsy paraffin archives, the above p16 methylation-negative MSPs were directly used in the confirmation assay.

The primers for unmethylated p16 CpG island and 1 unit of fresh DNA polymerase were added to each of the above MSPs, including negative control. The primers for the unmethylated island were 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ (sense) and 5′-CAACCCCCACAAACCACACCATA-3′ [antisense (21)]. Touchdown MSP was used. Thermal cycles were as follows: denaturation at 95°C for 5 min; followed by 30 cycles of amplification (95°C for 30 s→62°C (−0.3°C/cycle) for 30 s→72°C for 30 s); 10 additional cycles of annealing at 53°C; and a final extension at 72°C for 10 min. The reamplified MSPs were analyzed again by DHPLC as described above.

Sequencing of the MSP Products of Methylated p16 CpG Islands. To further confirm the specificity of MSP and the reliability of DHPLC analysis, the MSPs in which the methylated p16 fragments were observed by DHPLC analysis were subsequently purified and sequenced as described previously (25).

Statistical Analysis. Comparisons were made with the χ² test, t test, and Fisher’s exact test.
RESULTS

A total of 42 patients (21 cases progressed to GC, and 21 cases remained with DYS on follow-up) were studied. Information on the sex, age, lesion site, and DYS grade of each of the cases enrolled in the present study is presented in Table 1 and Fig. 1. The period ratio, sex ratio (M:F), 9:1; average age, 54.5 years; site ratio (body:antrum), 4:6.

A chromatographic peak corresponding to the 150-bp MSP product of all five methylated p16 CpG island was detected in dysplastic biopsy samples of 5 of 21 progressive DYS cases (23.8%; Fig. 2A), whereas none was found in any biopsy samples of 21 DYS cases without progression (Fig. 2B; two-sided Fisher’s exact test, P = 0.048). Furthermore, the methylated p16 MSP products were observed in all five GCs that originated from the p16-methylated DYS lesions on PAGE gel (Fig. 3) and DHPLC chromatograms (data not shown).

The MSP products of all five p16-methylated DYS samples were further analyzed by sequencing. Sequence information was obtained from four of five processed p16-methylated samples, Dys3245, Dys3504, Dys0335, and Dys3002 (Fig. 4). Results showed that all cytosines in the CpG sites in the testing sequence persisted as cytosines and that cytosines not in the CpG sites were converted to thymidines, indicating that all CpG sites analyzed were methylated.

To verify the existence of amplifiable templates in MSPs that did not exhibit methylated p16 products by DHPLC, the unmethylated p16 CpG islands in the same reaction were amplified with primer specific for nonmethylation and analyzed again by DHPLC. The unmethylated p16 products were observed in all of the samples examined (Fig. 2, C and D).

DISCUSSION

Aberrant methylation of the p16 promoter CpG islands is the major cause of inactivation of this tumor suppressor gene in GC (13–15) and various other human cancers (6–8). In the present study, we observed for the first time that aberrant methylation of p16 CpG island was correlated with malignant transformation of DYS. The methylated p16 CpG island was also present in all the GCs that progressed from the p16-methylated DYS lesions. Recent dynamic studies on a rat model for gastric carcinogenesis and on precursor lesions of human GC suggested that p16 methylation might be an early event in the development of GC (16, 17). Taken together, these results indicate that inactivation of p16 might play an important role in early-stage gastric carcinogenesis and thus may provide a specific biomarker for prediction of the malignant potential of DYS.

Aberrant p16 methylation has also been observed frequently in DYS of the cervix, esophagus, lung, and oral mucosa (27–31). It has been reported that aberrant p16 methylation could not predict the evolution of precancerous bronchial lesions within a 2-year follow-up (32). The follow-up time might be too short to display the effect of aberrant p16 methylation on carcinogenesis. More studies are needed to evaluate whether

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‡ Age when endoscopic biopsy was taken.

§ Lesion sites: A, antrum; B, body.

¶ Bold indicates samples with aberrant p16 methylation that progressed to gastric carcinoma.
Fig. 1 Photo of gastric dysplasia (DYS) lesions and carcinomas, stained with H&E. A–E (Dys4635, Dys4711, Dys1011, Dys2120, and Dys0222), gastric DYS lesions without progression (×200); F–J (Dys3504, Dys3245, Dys2003, Dys2184, and Dys0335), gastric DYS lesions that progressed to gastric carcinoma (×200); K–O, gastric carcinomas that originated from DYS lesions F–J (×100).
p16 methylation could predict malignant transformation of DYS and other precancerous lesions of these organs. Epigenetic alterations, in addition to multiple genetic abnormalities, are involved in the development of carcinoma (33). Hypermethylation or imprinting of several genes was used to predict susceptibility to human cancer development. For example, malignant progression of myelodysplastic syndromes was associated with methylation of p15INK4B (34, 35). The majority of microsatellite-unstable sporadic colon cancers were correlated with transcription silence of hMLH1 by CpG methylation (36). Disorders of imprinting of H19, SNRPN, Igf2, Igf2r, and others are involved in syndromes that are frequently accompanied by predisposition to childhood tumors (37).

Aberrant p16 methylation silences transcription of this
tumor suppressor gene and consequently promotes cell proliferation through the cyclin D-dependent protein kinase 4-retinoblastoma 1-transcription factor pathway (4, 6). In present study, we observed that aberrant p16 methylation correlated positively with the malignant transformation of DYS. However, such epigenetic alteration of p16 was detectable only in 5 of 21 progressive DYS cases. This indicates that aberrant p16 methylation may be one of the molecular pathways of gastric carcinogenesis. Thus, it is reasonable that aberrant p16 methylation may account for malignant transformation of a proportion of DYS cases (24% in present study). Other genetic and epigenetic abnormalities that occur in gastric DYS are currently under investigation.

The advantages of using DHPLC (as compared with agarose gel and PAGE) to size DNA fragments include its high sensitivity, high efficiency, and high reproducibility. It is easy to clarify chromatogram data by using the typical criteria of 3 × peak signal versus baseline noise (3S/N) as the minimum detection limit. Therefore, if possible, it might be better to use DHPLC to size the MSP products. Results obtained by DHPLC analysis were confirmed by sequencing.

MSP is a very sensitive assay for detection of CpG methylation (21). Methylation of CpG islands in a few cells will result in positive detection. MSP is useful in precancerous lesions such as DYS, in which genes might be inactivated only in a very limited number of cells. This is different from other gene expression assays, such as protein immunostaining and mRNA reverse transcription-PCR, which present the gene expression status in the majority of cells. The combination of MSP and DHPLC analyses demonstrates clear advantages for clinical applications.

In conclusion, methylation of p16 CpG island was positively correlated with progression of gastric DYS to GC in this high-risk population. Aberrant methylation of p16 promoter CpG islands therefore might be useful to predict the malignant potential of DYS identified specifically in gastric biopsies.

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