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Methylation of CpG islands of *p16* associated with progression of primary gastric carcinomas

Daya Luo*, Baozhen Zhang*, Lingbo Lv, Shengyan Xiang, Yahang Liu, Jiafu Ji and Dajun Deng

Peking University School of Oncology, Beijing Cancer Hospital and Beijing Institute for Cancer Research, Beijing, China

Inactivation of *p16* by methylation of CpG islands is a frequent early event in gastric carcinogenesis. The positive relationship between *p16* methylation and the clinical characteristics of gastric carcinomas (GC) has not been reported to date. In the present study, a DHPLC assay to quantify *p16* methylation was established (detection limit by fluorescence detector: 1:255 (Methlyated *vs* Unmethylated)). The proportion of methylated *p16* in the representative samples was confirmed and standardized by clone sequencing. Then, the DHPLC and two regular methylation-specific PCR (MSP) assays were used to detect *p16* methylation in 82 paired, resected GCs and their adjacent normal tissues. Results showed that the average proportion of methylated *p16* in GCs was significantly higher than that in their adjacent samples (12.90 *vs* 0.63%; *t*-test $P=0.005$). A much higher proportion of methylated *p16* was detected in GCs with metastases (local or distant) than without metastases (14.76 *vs* 2.61%; *t*-test $P=0.014$). A proportional relationship was observed between clinical stages and positive rates of *p16* methylation in GCs and/or adjacent tissues: 27.3, 37.5, and 58.8% (by DHPLC) for stage-I, -II, -III–IV of GCs, respectively (two-sided Fisher's exact test $P=0.016$). To confirm the data obtained by DHPLC, two MSP primer sets (*p16*-M and *p16*-M2) were also used to analyze *p16* methylation in the same set of samples simultaneously. Data of MSP assay using the primer set *p16*-M2, but not *p16*-M, correlated with that of DHPLC. These results imply that the primer set *p16*-M2 might be more suitable than *p16*-M to detect *p16* methylation in gastric tissues. In conclusion, the present data indicates that *p16* methylation correlates with progression of GCs significantly.
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Methylation of CpG islands is one of the crucial epigenetic pathways used to regulate gene transcription, which is involved in cell differentiation, parasite DNA defending, X-chromosome inactivation, and gene imprinting.[1,2] Aberrant methylation of CpG islands is tumor-specific, which may result in inactivation of tumor suppressor genes.[3–6]

$P16^{INK4A}$ (CDKN2A/MTS1) is a cell cycle regulator involved in the inhibition of G1 phase progression.[7] Methylation of *p16* CpG islands silences transcription of this gene.[8] It was reported that the *p16*-methylated cells could have an advantage in progression and metastasis in non-small-cell lung cancers.[9] Aberrant *p16* methylation was reported to occur frequently in multiple human cancers and relate to TNM stages of non-small-cell lung cancer and esophageal adenocarcinoma.[8–13]

In primary gastric carcinoma (GC), the frequency of *p16* inactivation by homozygous deletions ranged from 0–9%, by mutation from 0–2%, whereas by methylation from 32–42%,[14–20] which suggests that methylation is a major mechanism for *p16* inactivation in GC. It was reported that methylated *p16* was also observed in pre-malignant stages of GC.[21] We previously reported a positive association between aberrant *p16* methylation and the severity of glandular stomach pathology of Wistar rats induced by chemical carcinogen.[22] Recently, we observed that *p16* methylation was also associated with malignant transformation of human low-grade gastric dysplasia in a nested case–control study based

Correspondence: Dr D Deng, MD, Department of Etiology, Peking University School of Oncology and Beijing Institute for Cancer Research, Fu-Cheng-Lu No.52, Hai-Dian District, Beijing 100036, China.
E-mails: dengdajun@hotmail.com or dengdajun@bjmu.edu.cn
*These two authors contributed equally to this work.
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on a long-term population follow-up screening.[23] These studies indicated that *p16* methylation might play an important role in gastric carcinogenesis. However, relationship between *p16* methylation and clinical characteristics of GCs was not observed.[24,25] Gastric carcinomas is still the leading cause of cancer death in China and the second leading cause in the world.[26] It is necessary to clarify whether clinical characteristics of GC are associated with the proportion of methylated *p16*.

Denatured high-performance liquid chromatography (DHPLC) was successfully used to detect methylation of various CpG islands.[27–30] In the present study, a quantitative DHPLC assay was developed to detect *p16* methylation in 82 paired GCs and adjacent tissue samples. We found that the proportion of methylated *p16* in GCs was significantly higher than that in their adjacent nonmalignant tissues, and that *p16* methylation correlated with the observed clinical stages of GCs.

Materials and methods

Gastric Carcinoma Samples and Cell Lines

Eight-two paired surgical primary gastric carcinoma and adjacent mucosa samples (fresh-frozen at −70°C) were collected from patients at Beijing Cancer Hospital (63 males and 19 females, 35–81 years old, the average age 58 years). All clinical samples and histopathological information for each case were obtained according to approved institutional guidelines. The 1997 UICC-TNM criteria was used for classification of GCs. Human colon cancer cell line RKO and gastric cancer cell line MGC803 were cultured in RPMI 1640 medium (Gibco) supplemented with 10% of fetal bovine serum at 37°C with 5% CO_2.

DNA Preparation and Bisulfite Modification

Genomic DNA (2 μg) of cell lines and tissue samples was isolated with phenol/chloroform extraction as previously described.[31] The unmethylated-cytosines of the genomic DNA were converted to uridines by the addition of 5 M sodium bisulfite.[32] The Wizard® DNA Clean-Up System Kit (Promega) was used before PCR amplification.

Design of Primers and PCR Conditions

The universal primers used to amplify the antisense strand of methylated and unmethylated *p16* CpG island (GenBank accession number 527803) after bisulfite modification are 5′-**TT**T**TT** AGAGG ATTTG AGGGA **T**AGG-3′ (sense) and 5′-CTACC T**A**ATT CCAAT TCCCC T**A**CAA ACTTC-3′ (antisense) as previously described.[33] 35 CpG sites are located in the 392 bp amplicon (Figure 2d). The PCR products of *p16* CpG islands were amplified by hot-start PCR. HotStarTaq DNA polymerase (QIAGEN GmbH, Hilden, Germany) was used. A touchdown PCR protocol was used for amplification of *p16*: 95°C for 15 min → (95°C for 40 s → 70°C for 60 s, −1°C/cycle → 72°C for 60 s) × 10 cycles → (95°C for 40 s → 60°C for 60 s → 72°C for 60 s) × 30 cycles → 72°C for 10 min.

The genomic DNA samples of cell line RKO with methylated *p16* and MGC803 with unmethylated *p16* (determined by sequencing) were used as positive and negative controls. These DNA samples were also used to optimize the partial denaturing temperature needed for separation of the methylated and unmethylated *p16* amplicons for analysis with DHPLC and to calculate the standardization constant *K* for quantitative analysis of CpG methylation by DHPLC, as described in the next section.

To determine the detection limit for the methylated amplicons in the presence of the unmethylated amplicons for *p16*, different ratios of the *p16*-methylated genomic DNA from RKO cells *vs* the *p16*-unmethylated genomic DNA from MGC803 cells were prepared (1:1, 1:3, 1:7, 1:15, 1:31, 1:63, 1:127, 1:255), injected onto the WAVE System (Transgenomic, Inc., Omaha, USA), and analyzed at 57.6°C using both UV and fluorescence detection.

Amplification of the Methylated *p16* CpG Islands

The methylation status of the bisulfite-modified *p16* CpG islands was analyzed with methylation-specific polymerase chain reactions (MSP) as previously described.[34] Briefly, both the *p16*-M, *p16*-U, and *p16*-M2 primer sets for the methylated *p16* CpG islands were 5′-T**T**A**TT** AGAGG GTGGG GCGGA **T**CGC-3′ (sense one of *p16*-M and *p16*-M2), 5′-GACCC CG**AA**C CGCGA CCGT**A** **A**-3′ (antisense one of *p16*-M), 5′-CCACC T**AA**AT CG**A**CC TCCGA CCG-3′ (antisense one of *p16*-M2), 5′-T**T**A**TT**AGA GGGTGGGGTGGA**TT**G**T**-3′ (sense one of *p16*-U), and 5′-C**A**ACCCC**AA**ACC**A**C**A**ACC**A**T**AA**-3′ (antisense one of *p16*-U). Hot-start MSP was used for amplification of the methylated *p16* CpG islands. Thermal cycler conditions were denaturing at 95°C for 15 min, amplification for 35 cycles (95°C for 40 s, 64°C for 40 s, 72°C for 40 s), and extension at 72°C for 10 min. The reaction mixture (20 μl) contained about 10 ng of templates, 4 pmol of each primer, 4 nmol of dNTP, 1 unit of HotStarTaq DNA polymerase (QIAGEN GmbH, Hilden, Germany), and 2 μl of 10 × GC-buffer. Distilled water and genomic DNA of the *p16* unmethylated MGC803 cells were used as template for negative control. Genomic DNA of *p16*-methylated RKO was used as positive control. These controls were used in every individual MSP experiment. The methylated *p16* MSP products could be amplified with either the *p16*-M or *p16*-M2 primer set only in the positive control, but not in the negative controls. The MSP products were run on 8% PAGE gel.

Quantification Analysis for Methylation by DHPLC

The PCR products amplified by universal primers were analyzed by DHPLC on the WAVE™ DNA Fragment Analysis System coupled to the post-column HSX-3500 Accessory (Transgenomic, Inc., Omaha, USA) and the high-sensitivity fluorescence (FL) detector (excitation at 450 nm, emission at 520 nm) as previously described.[35] The PCR products were separated by a DNASep® analytical column (Transgenomic, Inc.) at 57.6°C, the partially denaturing temperature of the amplicon of the unmethylated *p16* as predicted by Transgenomic WAVEMaker software. The WAVE-HS1 FL-dye buffer (Transgenomic, Inc.) was used to enhance the FL-intensity of the PCR products (universal post-column labeling). Classical 3 × signal/noise (3S/N) criteria was used to determine the detection limit. Because the peaks for both the methylated and unmethylated *p16* CpG islands can be separated in the same DHPLC injection under partially denaturing conditions (57.6°C), the proportion of the methylated *p16* CpG island in the sample was calculated based upon the peak height of methylated *p16* divided by the sum of the peak heights of the methylated and unmethylated *p16* peaks from the DHPLC chromatogram. The proportion of methylated *p16* was calculated by the following formula:

$$\begin{aligned}&\text{Methylated\_CpG\_Islands\_(\%)}\\&= K\times(\text{peak\_height\_for\_methylated\_CpG\_islands})\\&\quad/(\text{total\_peak\_heights\_for\_methylated\_}\\&\quad\&\text{\_unmethylated\_CpG\_islands})\times 100\end{aligned}$$

where K is the standardization constant which is determined by the ratio of the exact proportion of the methylated CpG islands detected by clone sequencing to the non-adjusted proportion of methylated CpG islands. For *p16*, the *K*-value is 1.4996.

Clone Sequencing

Fresh PCR products of *p16* for each sample were amplified with the same universal primers, cloned with the AT clone kit (Tianwei Time Company, Beijing, China), and sequenced on ABI PRISM 3730 DNA Analyzer. About 15–20 clones with information of the target sequence were obtained for each sample. Two kinds of *p16* MSP products for the representative samples were also confirmed by direct sequencing as described previously.[23]

Results and discussions

The Proportion of Methylated *p16* CpG Islands can be Detected by DHPLC Quantitatively

We first developed a DHPLC assay to detect methylation of *hMLH1* CpG islands and subsequently used it to quantify the proportion of methylated *MT-3* in tissue samples previously.[27–29] DHPLC was also used to analyze *p16* methylation.[33] However, quantification of the proportion of methylated *p16* in tissue samples was not reported. To quantify the proportion of methylated *p16* in tissue samples with DHPLC, we performed a series of experiments. After bisulfite modification, a pair of universal primers was used to amplify both methylated and unmethylated *p16* in a same PCR reaction. Amplicons for methylated and unmethylated *p16* were separated at 57.6°C within a single DHPLC injection (Figure 1). The retention times (t_R) for the methylated and unmethylated *p16* peaks were 4.9 and 5.3 min, respectively, as determined by individual injections of the methylated and unmethylated products. A linear relationship ($y = 0.95x$, $R^2 = 9.99$; for fluorescence (FL)-Detector) was observed between peak heights of methylated *p16 vs* unmethylated *p16* PCR products at various dilution ratios (1:1, 1:3, 1:7, 1:15, 1:31, 1:63, 1:127, 1:255). Such linearity was also obtained among the PCR products amplified from the *p16* methylated genomic DNA of RKO cells diluted by the *p16* unmethylated genomic DNA of MGC803 cells (data not shown). Methylated *p16* was still detected by FL-Detector when it was diluted with unmethylated *p16* at ratio 1/256 (0.4%) (Figure 1a); by comparison, the detection limit for the UV-Detector was 1/32 (3.2%) (data not shown). Similar results were observed in two independent repeat experiments (data not shown). These results indicate that DHPLC with FL-Detector may be a very sensitive quantitative assay for the detection of *p16* methylation. As the proportion of methylated *p16* was directly calculated based on the peak heights of methylated and unmethylated *p16* in a single DHPLC injection, a DHPLC assay has an inherent advantage over other quantitative assays such as MSP (MethyLight), in which a separate reference PCR and a labeled probe are generally required to standardize the amount of template used for each sample.[36]

Validation and Standardization of the Proportion of Methylated *p16* by Clone Sequencing

To confirm the result of quantification of CpG methylation by DHPLC, the methylation status of *p16* CpG islands in four representative samples of GCs were quantified by DHPLC and clone sequencing simultaneously (Figures 1b and 2b). In order to determine the ratio of methylated *p16* in the sample in the DHPLC assay, the peak height for methylated *p16* (t_R 5.3 min) product was divided by the sum of the peak heights for methylated and unmethylated *p16* (t_R 4.9 min) products. For comparison using clone sequencing, the non-adjusted proportion of methylated *p16* and the ratio of the methylated clones to the total number of sequenced clones were used to calculate an exact proportion of methylated *p16*. The results showed that the non-adjusted

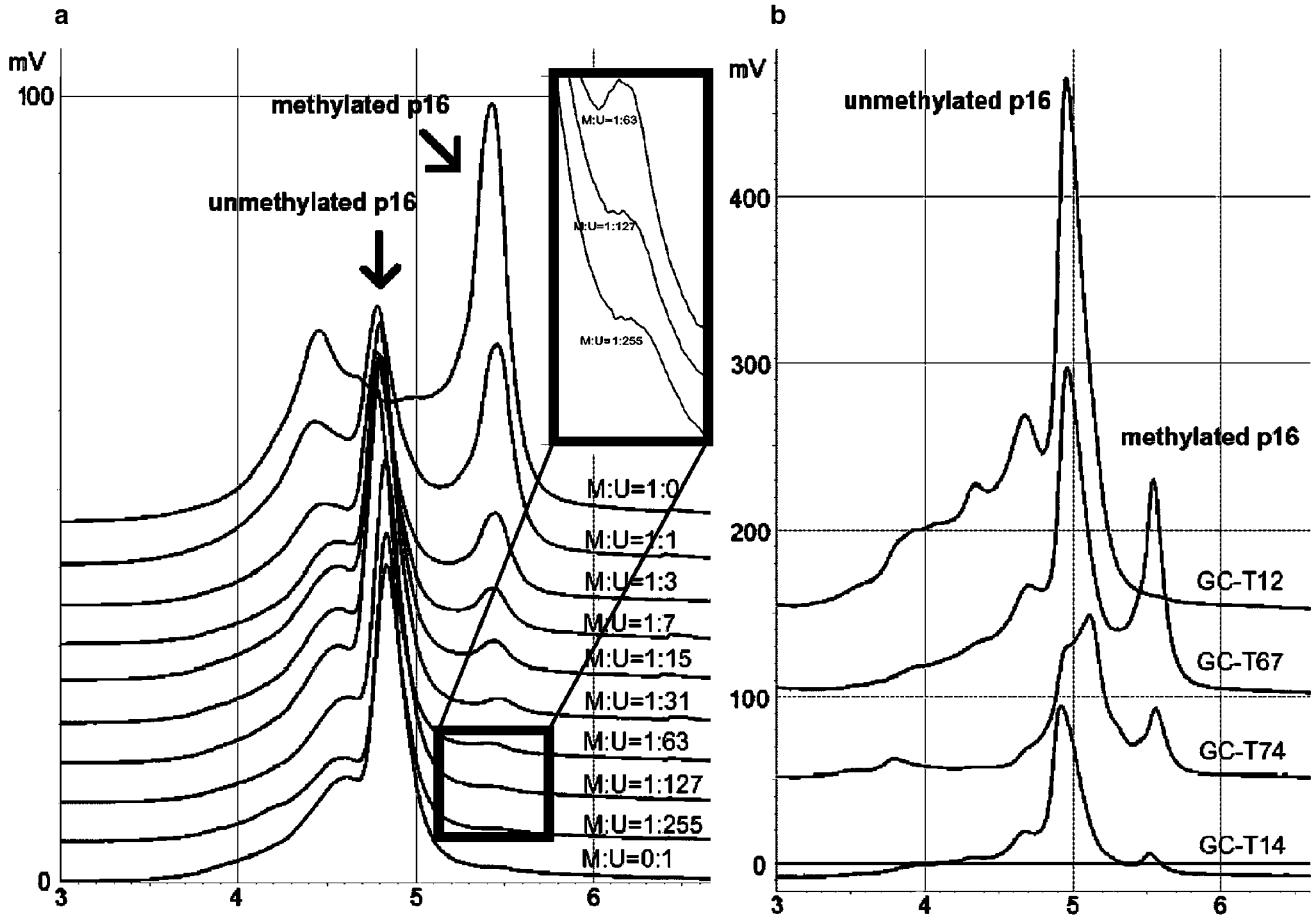

Figure 1 Analysis of methylation of CpG island of *p16* by DHPLC quantitatively. (**a**) Detection of the methylated *p16* amplicons diluted with the unmethylated amplicons at various ratios by the fluorescence detector at the partial denaturing temperature 57.6°C. (**b**) DHPLC chromatograms (fluorescence detection) of PCR products of methylated (if any) and unmethylated *p16* CpG islands amplified with universal primers from the bisulfite modified genomic DNA of four representative gastric carcinoma samples.

proportion of methylated *p16* in GCs by DHPLC with FL-Detector correlated with ratio of the methylated clones linearly ($R^2 = 0.977$, $y = 1.4996x$; Figure 2a and c). In addition, the non-adjusted proportion of methylated *p16* by DHPLC was lower than the exact proportion detected by clone sequencing. Therefore, the non-adjusted proportion of methylated *p16* was standardized with the slope constant (*K*) of 1.4996 during calculation of the proportion of each tested sample (as described in the Materials and methods section).

p16 Methylation is Associated with the Clinical Stages of GCs

p16 methylation is an early, frequent event in gastric carcinogenesis.[21–23] To investigate whether *p16* methylation correlates with clinical characteristics of GCs, the methylation status of *p16* CpG islands in 82 paired GCs and adjacent tissue samples from the operative cutting margin were quantified with DHPLC and analyzed with MSP as described in the Materials and methods section. Methylated *p16* was detected in GCs and/or adjacent tissues from 31 of 82 patients (17 GCs and 20 adjacent tissues) by DHPLC. The adjusted average proportion of methylated *p16* in GCs was >20-fold higher than that in their adjacent samples (12.90 *vs* 0.63%; *t*-test $P = 0.005$). Among six paired samples, in which methylated *p16* was detected both in tumors and normals, the proportion of methylated *p16* in tumor was always higher than that in normal (the adjusted average proportion, 14.71 *vs* 0.76%). Gastric carcinogenesis could be a multi-center process. Precancerous lesions are often observed in different sites within a stomach, especially in the cases with gastric carcinomas. Hence, that aberrant *p16* methylation might present in these lesions but not in the sampling site of tumor may account for the detection of *p16* methylation only in the adjacent normals.

A much higher positive rate and average proportion of methylated *p16* were detected in GCs ($n = 47$) with metastases (local or distant) than GCs ($n = 35$) without metastases (Table 1). The positive rate and adjusted average proportion of *p16* methylation in GCs were also associated with their TNM-based clinical stages (Table 2). When the normal and

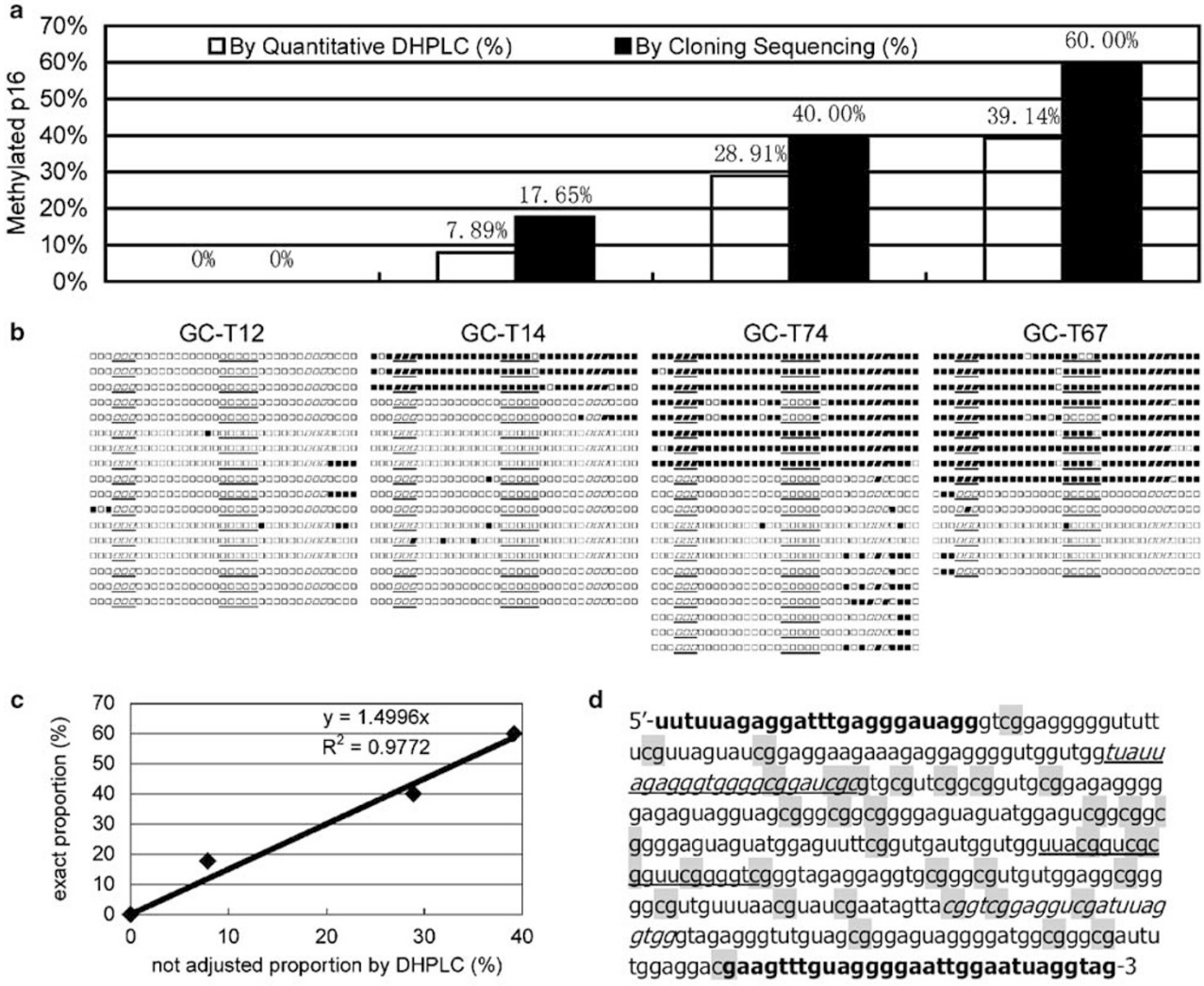

Figure 2 Validation of the proportion of methylated *p16* by clone sequencing. (**a**) Comparison of the exact proportion of methylated *p16* by clone sequencing with the non-adjusted proportion of methylated *p16* by DHPLC in PCR products from the bisulfite modified genomic DNA of four representative gastric carcinoma samples. (**b**) Results of clone sequencing of PCR products of four representative gastric carcinoma samples; each dot represents one CpG site; ■, methylated cytosines; □, unmethylated cytosines; each row of dots represents one clone; each clone block represents one sample; each sample's name is listed on the top of its corresponding clone block; 35 CpG sites in the fragment are highlighted; the underlined, and italic nucleotides are the primer set sequences for *p16*-M MSP, and *p16*-M2 MSP assay, respectively. (**c**) The linearity of the exact proportions and the non-adjusted proportions of methylated *p16* presented in (**a**). The *K-value* (1.4996) for methylated *p16* is calculated as described in method section. (**d**) The bisulfite modification sequence of the fragment of the methylated *p16* CpG island used for DHPLC analysis and clone sequencing; 35 CpG sites in the fragment are highlighted as described in (**b**) section; the bold sequences are the primer set for DHPLC.

tumors were taken together, there was a significant correlation ($P = 0.0328$, linear trend test by Epi Info 6.0). Thus, detection of methylated *p16* in adjacent tissues might also imply the presence of invasive tumor cells and poor prognosis. Correlation between *p16* methylation and age, sex, differentiation of GCs was not observed. To the best of our knowledge this is the first report, that correlated *p16* methylation with the progression of GCs both qualitatively and quantitatively.

It was reported that *p16* methylation by MSP was not associated with pathological characteristics of GCs.[24,25] To determine whether different assays result in different results, the *p16* methylation status in 82 above DHPLC-analyzed samples was also determined by MSP as previously described.[34] The primer set *p16*-M and *p16*-U, the most frequently used primers for detection of *p16* methylation, was used to amplify the methylated *p16* (150-bp) and the unmethylated *p16* (151-bp) MSP products. Methylated *p16* was detected in total 37 of the 82 tested cases with GC (22 GCs and 25 adjacent tissues) by MSP. Unmethylated *p16* was detected in all tested tissue samples. However, the relationship between *p16* methylation by MSP and clinical characteristics of GCs was not observed in the present study. Furthermore, the results by MSP did not correlate with those by DHPLC (Figure 3 bottom block);

Table 1 Results of the methylated *p16* CpG islands in gastric carcinomas with and without metastases by denatured high-performance liquid chromatography

Tissue samples containing the methylated p16	*GC cases with metastases* (n = *47*)	*GC cases without metastases* (n = *35*)	P-*value (two-sided)*
Tumor			
Case number (positive rate)	14 (29.79%)	3 (8.57%)	<0.02[a]
Methylated *p16* proportion (%)[b]	22.77±20.96	7.17±7.92	0.053[c]
Normal			
Case number (positive rate)	13 (27.66%)	7 (20.00%)	>0.05
Methylated *p16* proportion (%)	0.81±0.71	0.30±0.23	0.031
Tumor and/or normal			
Case number (positive rate)	22 (46.81%)	9 (25.71%)	0.051
Methylated *p16* proportion (%)	14.76±19.73	2.61±5.24	0.013

[a]Fisher's exact test.
[b]Adjusted percentage of copies of the methylated *p16* to both the methylated and unmethylated *p16* in samples containing methylated *p16*, average±s.d.
[c]Student's *t*-test.

Table 2 The methylated *p16* CpG islands was correlated with the clinical stages of gastric carcinomas

Tissue samples containing the methylated p16	*Stage I* (n = *33*)	*Stage II* (n = *32*)	*Stage III or IV* (n = *17*)
Tumor			
Case number (positive rate)	3 (9.09%)	9 (28.13%)	5 (29.41%)
Methylated *p16* proportion (%)[a]	7.17±7.92	23.19±21.45	22.01±22.49
Normal			
Case number (positive rate)	7 (21.21%)	7 (21.88%)	6 (35.29%)
Methylated *p16* proportion (%)	0.30±0.23	0.68±0.69	0.96±0.75
Tumor and/or normal			
Case number (positive rate)[b]	9 (27.27%)	12 (37.50%)	10 (58.82%)
Methylated *p16* proportion (%)	2.61±5.24	17.57±20.93	11.39±18.72

[a]Adjusted percentage of copies of the methylated *p16* to both the methylated and unmethylated *p16* in samples containing methylated *p16*, average±s.d.
[b]Analysis for linear trend in proportions by Epi Info 6.0 software, $P = 0.0328$.

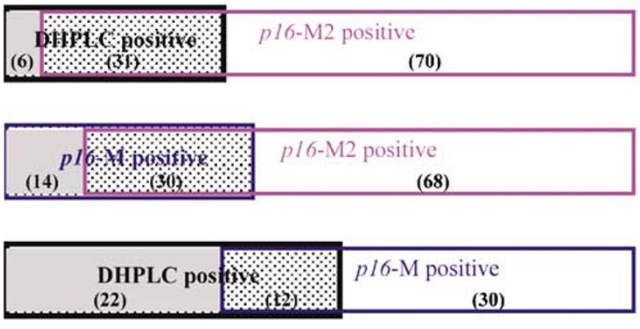

Figure 3 Comparison of detection of methylated *p16* in 164 gastric tissue samples with various assays. Blocks within the black frame represent the area of samples containing methylated *p16* by DHPLC; blocks within the purple frame represent the area of samples containing methylated *p16* by MSP using *p16*-M2 primer set; blocks within the blue frame represent the area of samples containing methylated *p16* by MSP using *p16*-M primer set; overlapped blocks represent the area of samples in which methylated *p16* was detected by two assays simultaneously. The exact positive sample number for each assay was also listed within each block.

p16 methylation was detected in only 35.3% (12/34) of DHPLC-positive samples by MSP using the primer set *p16*-M. A potential reason why the correlation was poor between the DHPLC results and the MSP results using the *p16*-M primer set is that MSP is a CpG-site specific assay that is used to detect methylation of a few CpG sites complementary to the primers. The MSP assay may work well when these complementary CpG sites are fully methylated or unmethylated such as in the case of *in vitro* cell lines. However, in the case of tissue samples, fully methylation of target CpG sites may be present in only a small number of copies. For example, full methylation of the complementary CpG sites was observed in some of clones within the antisense *p16*-M primer (Figure 2b, underlined blocks). This implies that the used primer set for 150-bp fragment of methylated *p16* might not be suitable for analysis of *p16* methylation in some kinds of tissues, such as stomach tissue. This might contribute to the observation that *p16* methylation

determination by MSP did not correlate with the clinical characteristics of GCs in the present study as well as previous investigations.[24,25]

Another set of MSP primers (*p16*-M2, 234-bp amplicon) were used to determine the amount of methylation of *p16* CpG islands.[34] In the present study, we observed that all CpG sites complementary to the primer set *p16*-M2 were methylated in all of the methylated clones of three representative GC samples (Figure 2b). To investigate whether *p16*-M2 is better than *p16*-M, we used the *p16*-M2 primer set to detect *p16* methylation in the same set of the bisulfite-modified GC and adjacent samples. The result indicated that the positive rate of *p16* methylation by *p16*-M2 was correlated with that determined by DHPLC ($P = 0.0025$). A total of 83.8% (31/37) of DHPLC-positive samples were also *p16*-M2 MSP positive (Figure 3 Top block). Such a correlation was not observed between determinations by the *p16*-M and the *p16*-M2 (Figure 3 middle block). In addition, the positive rate of *p16* methylation determined by *p16*-M2 in GCs was higher than that in their adjacent tissue samples (71.25 *vs* 54.32%, 2-sided Fisher's exact test, $P = 0.026$). A relationship between *p16* methylation (by *p16*-M2) and clinical stages of GCs was observed but not significant ($P = 0.065$, two-sides). These results indicated that the *p16*-M2 primer set correlates with clone sequencing and DHPLC well, and might be more suitable than the *p16*-M primer set for detection of *p16* methylation in gastric tissues. Whether the *p16*-M2 primer set is more suitable for other kinds of tissues is unknown.

The present data suggests that DHPLC may be a rapid and convenient quantitative assay for the detection of *p16* methylation in human tissue samples and that the progression of GCs is associated with *p16* methylation, both qualitatively and quantitatively.

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