Frequent p16 CpG island hypermethylation in primary remnant gastric cancer suggesting an independent carcinogenic pathway

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Abstract. Cancer found in the post-operative remnant stomach includes both newly developed cancer after surgery for benign-disease (PRC) and metachronous multiple cancer (MRC). Differences in the carcinogenic pathway between PRC and MRC have been suspected from clinical studies. However, no study has demonstrated the difference in molecular alteration between these diseases. P16 is inactivated predominantly by epigenetic change, rather than genetic alteration. We analyzed the methylation status and protein expression of the p16 gene in cancers of the remnant stomach. Eleven lesions of PRC, 24 lesions of MRC and corresponding non-cancerous tissue, as well as 13 primary gastric cancer (PC) lesions were examined. DNA was extracted by the micro-dissection method from paraffin-embedded surgical specimens. The methylation status of the promoter CpG island of the p16 gene was examined by using a methylation-specific polymerase chain reaction technique. To detect protein expression, immunohistochemical staining was employed. p16 promoter hypermethylation was observed more often in remnant gastric cancer than in PC. A significantly more frequent hypermethylation in the p16 gene was found in PRC (64%) than in MRC (21%) or PC (23%). Moreover, a significant correlation was found between p16 promoter hypermethylation and diminishment of protein expression in cancers of the remnant stomach. Silencing of the p16 gene by methylation of its promoter CpG island was suggested as a unique molecular mechanism in the carcinogenesis of PRC compared with MRC or PC.

Introduction

Cancer found in the post-operative remnant stomach includes both newly developed cancer after surgery for benign disease, and metachronous multiple cancer after primary gastric cancer surgery. Many former studies have demonstrated the clinical characteristics of these cancers found in the remnant stomach. Newly developed 'primary' remnant gastric cancer after surgery for benign disease (PRC) is often found in the stump of the remnant stomach after a long interval from the initial surgery (more than 20 years). The frequency of PRC has been reported to be around 1-3% (1) and the remnant stomach after distal gastrectomy is thought to be an organ at high risk of cancer development. Reflux of intestinal juices (2-4), de-nervation (5), and changes in bacterial inhabitation (6) have been reported as causative factors of cancer development in the remnant stomach. In contrast, cancer in the remnant stomach after primary gastric cancer surgery (or MRC, metachronous multiple remnant gastric cancer) is often found in the non-stump area after a shorter interval from the initial surgery than PRC (<10 years) (7). The involvement of *Helicobacter pylori* infection and chronic atrophic gastritis (8,9) on the carcinogenesis of MRC has been suggested, just as in primary gastric cancer (PC). Therefore, PRC and MRC have been suggested to have different characteristics and carcinogenic pathways, though both cancers develop in the remnant stomach.

Although several differences in the clinical features or the possible etiologies between PRC and MRC have been demonstrated as above, no molecular background has been able to display the difference of the two different types of cancer in the remnant stomach. Furthermore, several important genes, including p53 (10), bcl-2 (11), APC (12), or k-ras (13), have been found to demonstrate no unique alterations in remnant gastric cancer compared with PC. Our recent study (14) has suggested that genetic instability is more commonly found in remnant gastric cancer than PC. Moreover, the most recent report has indicated an accumulation of RUNX3 hypermethylation at the anastomotic mucosa of the remnant stomach (15). These results indicate that an abnormality in gene maintenance or epigenetic change rather than the alteration of a key gene might play a critical role in the mechanism of the carcinogenic pathway in the remnant stomach.

P16 is a tumor suppressor gene which maps to 9p21 and functions as an inhibitor of cyclin D-dependent protein kinases. P16 protein binds to cyclin-dependent kinase 4 (CDK4) and CDK6 (CDK6), and induces G1-phase arrest in the molecular machinery of the cell cycle by interfering with

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Table I. Clinicopathological characteristics of the patients.

Clinical features	PRC (n=11)	MRC (n=24)	p-value
Age (mean)	69.8	67.0	n.s.
Sex (male/female)	9/2	21/3	n.s.
Location (stump/other)[a]	7/4	16/ 8	n.s.
pN (0/1-4)	7/4	5/16[b]	n.s.
PStage (0,1/2-4)	6/5	12/12	n.s.
Interval from the initial surgery (mean; years)	(27.3)	(7.1)	
(-10/<10 years)	0/11	19/5	<0.001
Histological type (well/poor)	8/3	12/12	n.s.
Reconstruction method (BI/BII)	6/5	15/9	n.s.

[a]Stump included both anastomosis and suture line; [b]pN status at surgery was unknown in 3 cases. Well, well-differentiated adenocarcinoma included papillary and tubular carcinoma; Poor, poorly differentiated adenocarcinoma included poorly differentiated, mucinous and signet-ring cell carcinoma.

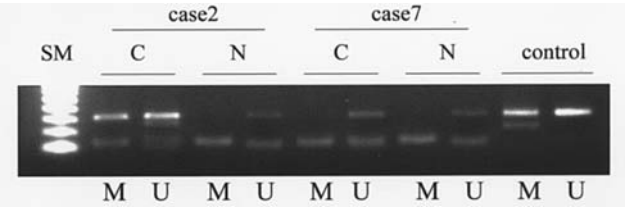

Figure 1. Representative results of the methylation-specific polymerase chain reaction. C and N indicate cancerous and non-cancerous tissue, respectively. M and U below the PCR products indicate the primers used for amplification; M represents primers for methylated and U for unmethylated p16 gene promoter CpG island, respectively. Both the 151-kb band indicating unmethylated p16 promoter CpG island and a 150-kb PCR product of methylated p16 products were found in the positive control lanes. At least one of the methylated or unmethylated products was clearly found in all samples examined. Bisulfite modified CpGenome™ Universal methylated DNA (Chemicon, Temecula, CA) was used as a DNA template for control of the methylated p16 gene. SM, size marker (50-bp DNA ladder).

Table II. Frequencies of p16 gene methylation.

	No.	Hyper-methylation	(%)	p-value
Non-cancerous tissue	48	3	(6.3)	<0.01[a]
Primary gastric cancer	13	1	(7.7)	n.s[b]
Remnant gastric cancer	35	2	(5.7)	
Cancerous tissue	48	15	(31.3)	
Primary gastric cancer	13	3	(23.1)	n.s[b]
Remnant gastric cancer	35	12	(34.3)	

[a]Significantly less frequent compared with cancerous tissue; [b]No significant difference was found compared with remnant gastric cancer.

binary cyclin D-CDK4 complexes (16). Loss of p16 or its transcripts breaks down the regulatory mechanism of the cell cycle; thus, p16 plays a role as a tumor suppressor gene. Inactivation of p16 is one of the most commonly observed abnormalities in human cancers (17). P16 is inactivated by a combination of genetic or epigenetic alterations. Rather than genetic alterations, epigenetic change may be the predominant mechanism associated with the loss of p16 function in sporadic carcinoma (18).

This study was undertaken to determine the involvement of epigenetic change in the carcinogenic pathway of the remnant stomach by investigating p16 promoter CpG island hypermethylation in remnant gastric cancer and non-cancerous tissue, and to analyze the relationship between methylation status, p16 protein expression, and the clinicopathological features of remnant gastric cancer, including both PRC and MRC.

Materials and methods

Patients. Surgically resected specimens from 35 cases with cancer in the remnant stomach were analyzed. Eleven of these patients had undergone initial distal gastrectomy because of benign disease (PRC: primary remnant gastric cancer). Twenty-four had undergone curative surgery because of primary gastric cancer (MRC: metachronous multiple gastric cancer). The curability at initial surgery for the MRC cases was confirmed by pathological review of the specimens at the initial surgery. Total remnant gastrectomy was performed for these remnant gastric cancers from 1986 to 2004 at Osaka City University Hospital. The clinical and pathological features of the patients are summarized in Table I. The interval between surgeries was significantly longer in cases with PRC than with MRC. Otherwise, no difference in the clinicopathological features was found between PRC and MRC. Thirteen cases of PC were also analyzed as a control. For each tumor, non-cancerous gastric mucosa without severe metaplasia, apart from the cancerous lesion, was available as an internal control.

Methylation-specific polymerase chain reaction (MSP). Samples for DNA extraction were obtained by the micro dissection method from formalin-embedded samples of both cancerous and non-cancerous tissues. Using hematoxylin-stained sections of 10 μm thick, the tissue was scraped by a scalpel. Digestion was performed in proteinase K buffer (Gibco, Gaithersburg, MD), pH 8.1 at 55˚C for 7 days. Proteinase K was inactivated by boiling for 8 min. The samples were rapidly cooled, and the DNA was stored at 4˚C.

Extracted DNA was modified by sodium bisulfite to determine the methylation status by MSP (16). Polymerase chain reactions were performed in 10 μl of reaction buffer containing 1 μl 10X buffer (Applied Biosystems, Warrington, UK), 0.5 μM deoxynucleotide triphosphates (Applied Biosystems), 5 μM each primer, 0.5 μl (20 ng/l) of extracted DNA, and 0.5 U Ampli Taq polymerase (Applied Biosystems). PCR amplification was carried out in a thermal cycler using the following conditions: 95˚C for 5 min for a hot start; then 45

Table III. p16 methylation status and clinicopathological features.

Factors	PRC		MRC	
	No.	Methylated (%)	No.	Methylated (%)
Sex				
Male	9	6 (66.7)	21	4 (19.0)
Female	2	1 (50.0)	3	1 (33.3)
Age				
<70	5	2 (40.0)	14	4 (28.6)
<71	6	5 (83.3)	10	1 (10.0)
Location				
Stump	7	4 (57.2)	16	3 (18.8)
Other	4	3 (75.0)	8	2 (25.0)
Interval				
1-10	-	-	19	5 (26.3)
<10	11	7 (63.6)	5	0 (0)
Histological type				
Well diff.	8	4 (50.0)	12	4 (33.3)
Poorly diff.	3	3 (100)	12	1 (8.3)
Stage				
Early	6	4 (66.7)	12	3 (25.0)
Advanced	5	3 (60.0)	12	2 (16.6)
Lymph node metastasis				
Negative	7	4 (57.1)	14	4 (28.6)
Positive	4	3 (75.0)	7	1 (14.3)
Reconstruction				
B-I	6	5 (83.3)	15	2 (13.3)
B-II	5	2 (40.0)	9	3 (33.3)
Total	11	7 (63.6)	24	5 (20.8)

cycles of 95˚C for 30 sec, 60˚C for 60 sec, and 72˚C for 50 sec; and finally, 72˚C for 10 min for extension. The primers used were as follows: unmethylated $p16^{INK4a}$ (5'-TTA TTA GAG GGT GGG GTG GAT TGT-3', 5'-CAA CCC CAA ACC ACA ACC ATA A-3') and methylated $p16^{INK4a}$ (5'-TTA TTA GAG GGT GGG GCG GAT CGC-3', 5'-GAC CCC GAA CCG CGA CCG TAA-3') (19). The PCR products were electrophoresed on a 2.5% agarose gel and visualized using ethidium bromide staining to determine the methylation status.

Immunohistochemistry. The protein expression of p16 was determined using the immunohistochemical staining technique, as described previously (20). Briefly, 4-μm sliced sections of formalin-fixed paraffin-embedded tissue were deparaffinized and incubated with 0.3% hydrogen peroxide in methanol for 30 min. Slides were autoclaved at 105˚C for 10 min in X10 target retrieval solution (Dako, Carpinteria, CA) for antigen retrieval. After blocking to reduce non-specific antibody binding with normal rabbit serum, a 1:100 dilution of a mouse monoclonal antibody to human p16 (sc1661; Santa Cruz Biochemicals, Santa Cruz, CA) was applied at 4˚C overnight, followed by three washes with PBS. The sections were incubated with secondary antibody and reacted with streptoavidin-biotin peroxidase reagent (Histofine SAB-PO (M) kit. Nichirei Co., Tokyo, Japan). Finally, diamino-benzidine and 1% hydrogen peroxidase were applied as chromogen and counterstained with hematoxylin and eosin. Normal tissue without severe atrophy adjacent to the tumor was used as a positive control for p16 immunoreactivity. Positive nuclear stains in less than 50% of the cancer cells was considered to be a diminishment in expression.

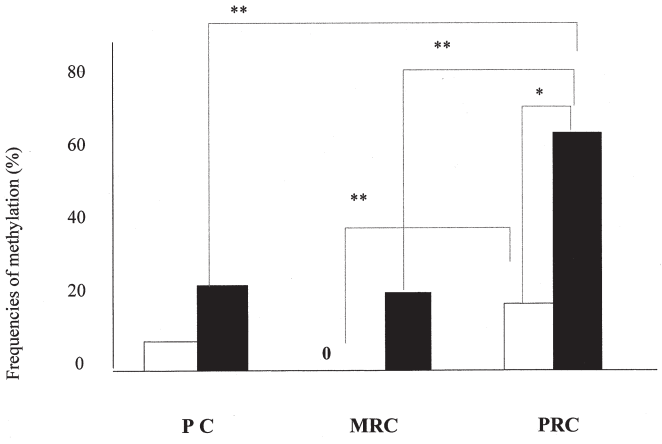

Figure 2. The frequency of p16 methylation found in cancerous (closed bars) and non-cancerous (open bars) tissues according to the patients' background. Remarkably, more frequent hypermethylation in the p16 gene promoter CpG island was found in cancerous tissue of PRC than of MRC (p=0.01) or PC (p=0.04). *p<0.01; **p<0.05.

Statistics. Statistical analyses were performed using Mann-Whitney's U-test and the Kruskal-Wallis test. A p-value <0.05 was considered significant.

Results

We were able to demonstrate a good reproducible result from MSP, as shown in Fig. 1. At least one of the methylated or unmethylated products was clearly found in all of the samples examined. Samples with a detectable methylated product were determined as positive for hypermethylation. Methylation of the p16 gene was found in only 2 of 35 (5.7%) non-cancerous tissues. In contrast, methylation was found in 12 of 35 (34.3%) cancerous tissues, and was significantly more frequent than that of non-cancerous tissues (Table II). P16 promoter hypermethylation was more commonly found in cancer of the remnant stomach (including PRC and MRC) than PC, although the difference was not significant. When the clinicopathological backgrounds of the cancer in the remnant stomach were compared, no significant correlation was found between p16 promoter methylation status and age, gender, pathological stage, histological type, reconstruction method, or tumor location (Table III). However, remarkably more frequent hypermethylation in the p16 gene was found in PRC (7/11; 63.6%) than in MRC (5/24; 20.8%, p=0.01) or PC (3/13; 23.1%, p=0.04) (Fig. 2).

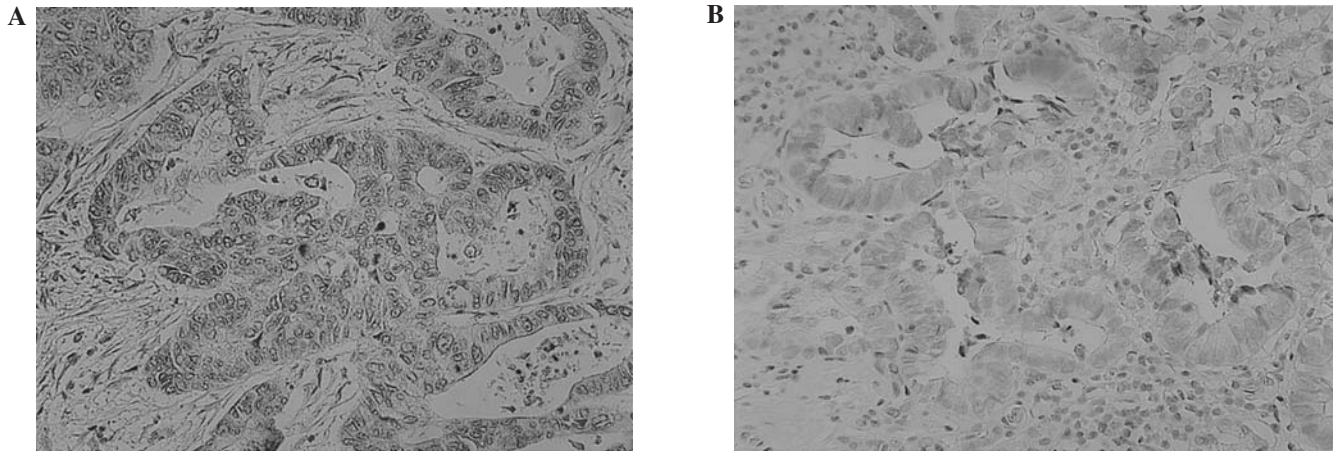

Figure 3. Representative results of the immunohistochemical analysis of P16 expression in remnant gastric carcinomas (original magnification, x40). P16 immunoreactivity localized in the nucleus was universally observed in p16 unmethylated cancerous tissue (A, case 3). Markedly diminished p16 immunoreactivity was observed in methylation-positive cancer cells in solid arrangement (B, case 7).

Table IV. Correlation between p16 protein expression and p16 hypermethylation in cases with RGC.

p16 Methylation	p16 Immunostaining		
	Positive (%)	Diminished (%)	Total
Negative	19 (82.6)	4 (17.4)	23
Positive	1 (8.3)	11 (91.7)	12
Total	20 (57.1)	15 (42.9)	35

In non-tumoral mucosa, every specimen was positive for P16 immunoreactivity. No loss in p16 immunoreactivity was found in any of the cases. Diminished immunoreactivity was found in 15 of 35 (42.9%) cancerous tissues. P16 expression was diminished in 7 of 11 (63.6%) cancerous lesions in PRC, 8 of 24 (33.3%) in MRC and 4 of 13 (30.8%) in PC. The diminishment in P16 expression was more often found in cases with PRC than MRC and PC, although the difference was not statistically significant (Fig. 3). Eleven of 12 (91.7%) cancerous tissues of the remnant stomach with hypermethylation showed diminished p16 protein expression. In contrast, 19 of 23 (82.6%) cancerous tissues without p16 methylation expressed the p16 protein. Thus, a significant correlation between p16 promoter methylation and diminished protein expression was found ($p<0.004$) (Table IV).

Discussion

The remnant stomach after partial gastrectomy is known as one of the critical organs at high risk of cancer development. Former epidemiological reports from European countries have suggested that the risk for developing cancer is statistically higher in the remnant stomach than that in the non-operative stomach (1,21,22). Although some studies carried out in places with high contraction rates for gastric cancer, such as Japan, have found no elevation in the cancer risk in the remnant stomach (23), typical PRC at the stump of the remnant stomach was often encountered similar to in European countries. Moreover, many experimental and clinical studies have suggested the existence of unique carcinogenic mechanisms at the stump area of the remnant stomach after distal gastrectomy. The influence of reflux of the duodenal juice on cancer development at anastomosis of the remnant stomach in animal models is well known (4). We have also observed the acceleration of glandular hypertrophy and cellular proliferation at anastomosis in clinical samples more predominantly in Billroth II type reconstruction, where reflux is more commonly found than in Billroth I type reconstruction (24-26). Kaminishi *et al* have reported that surgical denervation impairs the defense mechanism in the mucosa at the stump of the remnant stomach, leading to tumor development in experimental models (5). Therefore, PRC has been suggested to form a more independent entity than PC of the non-operated stomach. Still, little is known about the molecular backgrounds of PRC and no unique genetic alterations have been identified. In the present study, we investigated the p16 promoter methylation status in cancers of the remnant stomach and found a statistically higher rate of hypermethylation in PRC than in MRC or PC. This epigenetic change clearly led to a down-regulation of P16 protein expression in cancer and may be a unique molecular background of cancer development in the remnant stomach after surgery for benign disease.

The influences of epigenetic change in cancer development have been demonstrated well in inflammation-related cancers, including ulcerative colitis (27) and Barrett's esophagus (28). Chronic inflammation has also been reported to be involved in the process of gastric carcinogenesis (29). Although we found no remarkable accumulation of p16 methylation in the non-cancerous mucosa of the remnant stomach, the influences of chronic inflammation on the aberrant methylation of p16 in the stomach mucosa have been suggested (30). Former studies have found promoter CpG island hypermethylation, including that of p16, in precancerous tissues (31-34) and have suggested that methylation tends to accumulate throughout multi-step gastric carcinogenesis. In the present study, we did not use mucosa with severe inflammation or with obvious metaplasia as ‘non-cancerous tissue’, which may explain why we could

not detect frequent p16 methylation in the 'non-cancerous' mucosa in the present study. The involvement of p16 gene silencing in remnant gastritis, such as stomal gastritis and intestinal metaplasia, should be investigated to clarify the involvement of this epigenetic change in possible precursors of PRC.

Hypermethylation of p16 has been reported in PC (32,35). The frequency in p16 promoter methylation in former studies has varied from 25 to 43%. We compared PRC, MRC, and PC in the same series and confirmed a diminishment of p16 expression in methylated samples, and found that down regulation of p16 by methylation is a common molecular abnormality in PRC. The P16 gene regulates the cell cycle, and its dysfunction causes an acceleration of cell growth (36). Thus, a silencing of p16 function by promoter methylation might be a unique molecular mechanism in PRC that could trigger cancer development and/or progression. Furthermore, our results indicate that MRC and PC have a similar molecular background and are different from PRC. Similarities in MRC and PC have been suggested by many clinical investigations. We can therefore conclude that PRC and MRC are different entities, based not only on their clinical features but also on their molecular background.

The conditions of the stomal portion of the remnant stomach may be regarded as suitable for frequent p16 methylation. Regurgitation of the duodenal juice, including bile and pancreatic juices with high pH, is known as the main causative factor of stomal gastritis. Chronic mucosal inflammation with stomal gastritis and subsequent induction of superoxide and nitric oxide are known as strong inducers of DNA methyltransferase (37,38). Moreover, p16 methylation is frequently reported in cholangiocarcinoma (39-42), suggesting a strong involvement of bile in aberrant methylation. We have also found that PRC has a higher rate of methylation in genes other than p16 compared with PC (preliminary data, not shown). Furthermore, in a recent report, Nakase *et al* demonstrated that the involvement of hypermethylation in RUNX3 correlates well with the existence of stomal gastritis in the remnant stomach (15). Although they found a clear tendency toward RUNX3 methylation in stomal gastritis, no difference was found in the frequency of methylation between PRC, MRC, and PC. In contrast, p16 methylation in non-cancerous tissue was not frequent in our series, but it differed in cancer in the remnant stomach. Methylation in DNA is known to occur in a gene-specific manner, and the sensitivity to methylation differs between genes (43). It therefore appears that epigenetic change may occur in genes step-by-step and independently to develop cancer. Furthermore, aberrant methylation is known to induce DNA instability (44). It is therefore worth investigating the correlation between methylation and genetic instability in stomal gastritis and PRC.

Based on the present results we suggest that there are different molecular pathways in carcinogenesis of the remnant stomach after surgery for benign disease compared with that after cancer surgery, as has been demonstrated in many clinical investigations (7,26). Furthermore, as Klump has suggested (39), the promoter methylation status may be a candidate marker for diagnosis and for clinically unapparent neoplastic alterations in patients with remnant stomach. Further study is necessary to investigate the status of aberrant methylation in stomal gastritis in the remnant stomach to clarify the clinical significance of the correlation between mucosal inflammation and carcinogenesis in the remnant stomach.

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