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# Methylation of $p16^{INK4A}$ and $p57^{KIP2}$ are involved in the development and progression of gastric MALT lymphomas

Ki Ouk Min, Eun Joo Seo, Hi Jeong Kwon, Eui Jin Lee, Won Il Kim, Chang Suk Kang and Kyoung-Mee Kim

*Department of Clinical Pathology, The Catholic University of Korea, College of Medicine, Inchon, Korea*

**$p16^{INK4A}$ and $p57^{KIP2}$ are inhibitors of cyclin-dependent kinases and their inactivation by methylation has been reported as a major tumorigenic mechanism in tumors. To examine whether methylation of $p16^{INK4A}$ and $p57^{KIP2}$ is involved in the development and progression of gastric MALT lymphomas, 24 gastric low-grade lymphomas of MALT, 11 diffuse large B-cell lymphomas, and 10 each case of gastric lymphoid follicles with and without *H*elicobacter *pylori* infection were studied. *H. pylori* infection was positive in 85.7% of the gastric lymphomas. In the gastric lymphoid follicles positive for *H. pylori*, methylation of $p16^{INK4A}$ was detected in 10% of cases, while methylation of $p57^{KIP2}$ was not detected. In low-grade MALT lymphomas, $p16^{INK4A}$ and $p57^{KIP2}$ were methylated in 41.7 and 29.2% of the cases, respectively. In diffuse large B-cell lymphomas, methylation of $p16^{INK4A}$ and $p57^{KIP2}$ was found in 72.7 and 36.4% of the cases, respectively. All but one case with $p16^{INK4A}$ and $p57^{KIP2}$ methylation was *H. pylori* positive and most of them were stage I. Our results indicate that methylation of $p16^{INK4A}$ followed by $p57^{KIP2}$ methylation involves during the tumorigenesis of gastric MALT lymphomas associated with *H. pylori* infection. As methylation of these two genes was more frequent in the higher grade ($P<0.05$), it may contribute to the malignant progression of gastric MALT lymphomas.**
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Hypermethylation of CpG islands within promoter regions of genes is associated with transcriptional inactivation and represents an important mechanism of gene silencing in the pathogenesis of malignancies.[1] This epigenetic phenomenon acts as an alternative to mutations and deletions for disrupting the tumor suppressor gene function.[2] A large number of genes that involve several fundamental cellular pathways may be affected in virtually all types of human cancers by aberrant CpG island methylation. $p16^{INK4A}$ and $p57^{KIP2}$ genes are the suppressors of cyclin-dependent kinase (CDK), a member of the tumor suppressor gene, and their inactivation in various tumor cells has been reported.[3–5] $p16^{INK4A}$ is a member of INK4A/ARF family and is inactivated by a loss, deletion, or hypermethylation of the promoter region of the gene. In non-Hodgkin's lymphomas, inactivation of $p16^{INK4A}$ is primarily due to hypermethylation rather than to genetic alterations. The frequencies of $p16^{INK4A}$ methylation in non-Hodgkin's B-cell lymphoma have been reported to be 15–46%.[6–8] As the incidence of $p16^{INK4A}$ methylation in diffuse large B-cell lymphomas is higher than in the low-grade lymphomas, this is thought to be involved in the malignant transformation.[6,9,10] In gastric MALT lymphomas, methylation of $p16^{INK4A}$ is between 58 and 67%, which is higher than other lymphomas. So, inactivation of $p16^{INK4A}$ by methylation is thought to play an important role in the development of MALT lymphomas.[7,10–12]

$p57^{KIP2}$ is a member of the Cip/Kip family of CDK inhibitors that intervenes in the formation of the cyclin-CDK complex and is considered a putative tumor suppressor gene.[5,13–15] In mouse, $p57^{KIP2}$ gene is expressed from the maternal allele only, and the paternal allele is methylated and repressed in all tissues examined.[16] In humans, ~90% of the expression comes from the maternal allele, but no differential methylation between the two parental

Correspondence: Dr K-M Kim, MD, Department of Clinical Pathology, Our Lady of Mercy Hospital, The Catholic University of Korea, 665 Bupyung-dong, Bupyung-gu, Inchon 403-720, Korea.
E-mail: kkmkys@catholic.ac.kr
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alleles has been reported.[17–20] Inactivation of $p57^{KIP2}$ is known to be involved in the development of human malignancies such as hepatocellular carcinoma,[21,22] urinary bladder carcinoma,[23] and gastric carcinoma,[24] as well as Beckwith–Wiedemann Syndrome.[25] Inactivation of $p57^{KIP2}$ gene expression other than mutation was suggested due to rare occurrence of *p*57 gene mutations in these cancers. Recently, it has been reported that methylation of $p57^{KIP2}$ occurs frequently in malignant lymphomas of B-cell type[26] and adult acute lymphocytic leukemia.[27] However, studies on $p57^{KIP2}$ methylation in gastric MALT lymphomas are not reported and its clinical significance is not established yet.

Infection with *Helicobactor pylori* is one of the most important causes of gastric MALT lymphoma.[28,29] In Korea, the infection rate of *H. pylori* is around 60% in adults.[30] Here, we investigated two major inhibitors of CDK in gastric MALT lymphomas to evaluate whether methylation of $p16^{INK4A}$ and $p57^{KIP2}$ is involved in the development and progression of the gastric MALT lymphomas associated with *H. pylori* infection.

## Materials and methods

### Study Population

In total, 35 paraffin-embedded tissue sections of patients from 1996 to 2003 who were diagnosed as having gastric MALT lymphomas were used: 29 cases were biopsy specimens and six cases were surgical samples. The patients' age ranged from 22 to 80 years with a mean age of 56.3 years. The male to female ratio was 3:2. As a control group, 10 of each case of benign gastric lymphoid hyperplasia negative and positive for *H. pylori* were used.

### Pathologic Examinations

After review of all hematoxylin and eosin-stained sections used for diagnosis, the cases were classified as low-grade lymphoma of MALT (extranodal marginal zone B-cell lymphoma of MALT type) when there are dense diffuse infiltrates of centrocyte-like cells with monocytoid differentiation and typical lymphoepithelial lesion, which eventually leads to eosinophilic degeneration and disintegra-

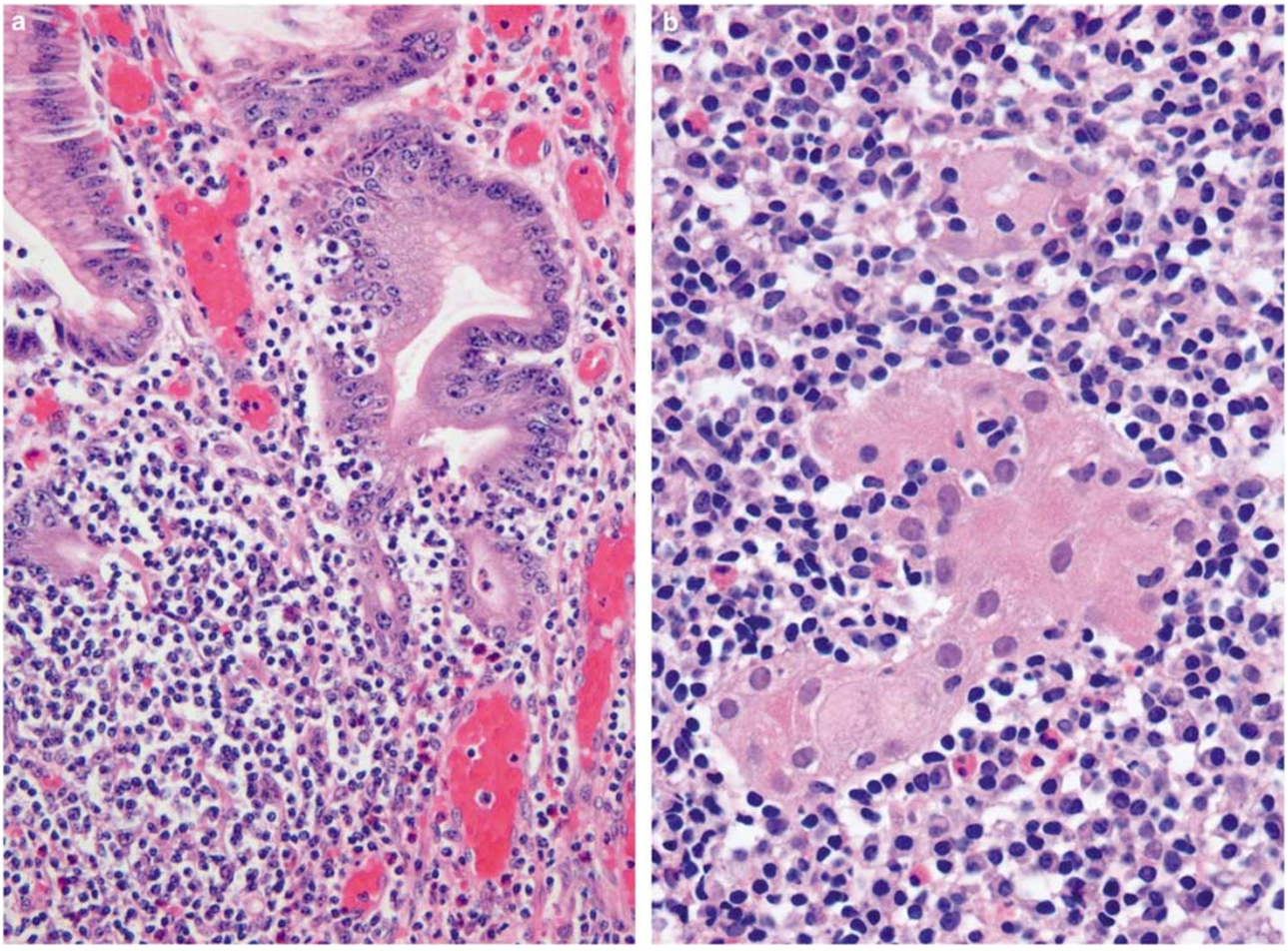


**Figure 1** Photomicrograph of representative examples of low-grade lymphomas of MALT with typical lymphoepithelial lesions (**a**) and damaged, eosinophilic epithelial cell nests surrounded by lymphoid tumor cells (**b**).

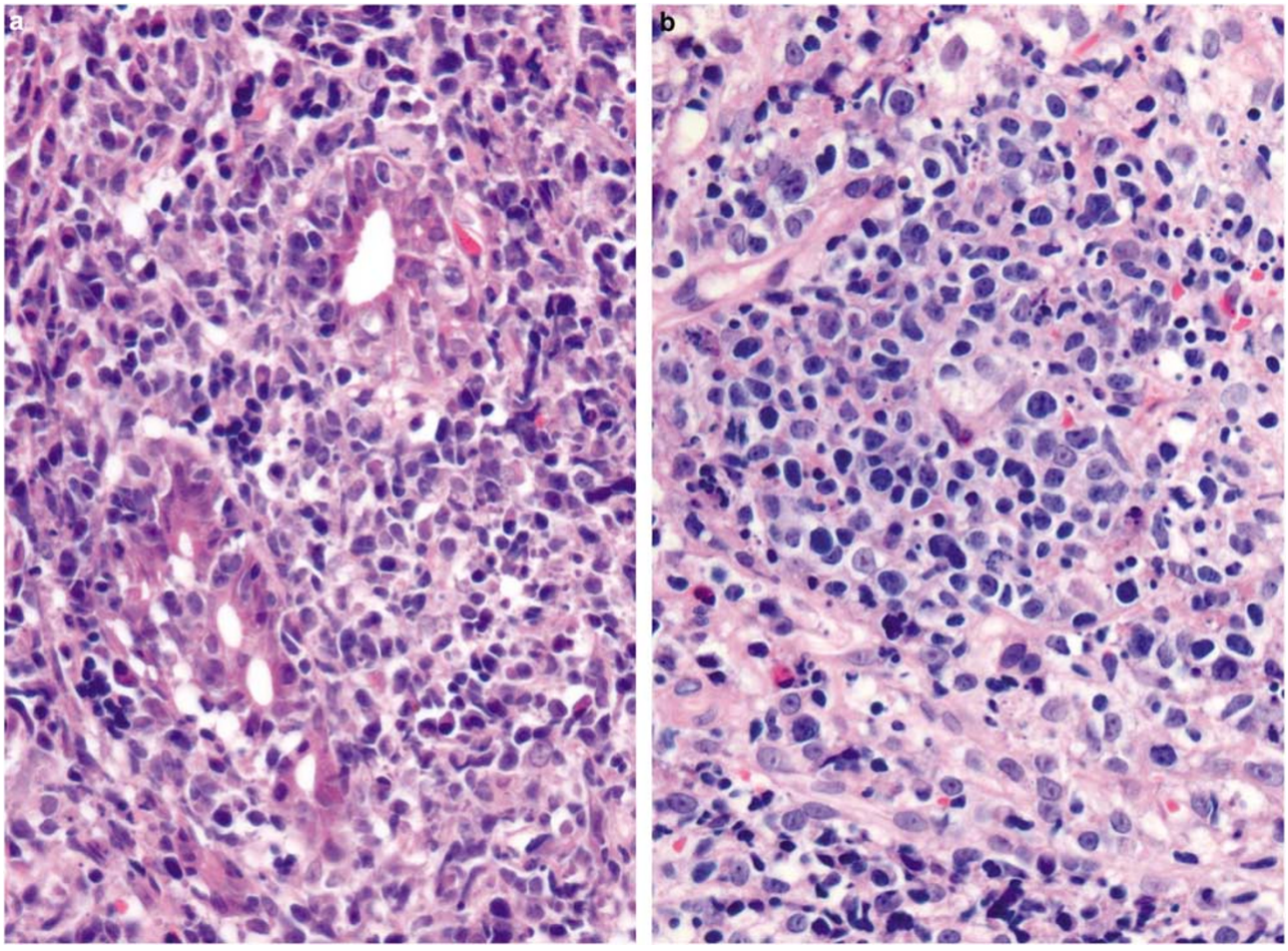


**Figure 2** Photomicrograph of representative examples of diffuse large B-cell lymphomas with lymphoepithelial lesions (**a**) and infiltration of large, atypical centroblast-like tumor cells (**b**).

tion of glandular epithelium (Figure 1). Diffuse large B-cell lymphoma was diagnosed if compact clusters, confluent aggregates, or sheets of transformed large centroblast- or immunoblast-like cells with B-cell immunophenotype were found (Figure 2).[31,32]

To detect *H. pylori*, Warthin–Starry silver staining was performed and the grades of infection were defined by the visual analog scales of the updated Sydney system.[33]

### Methylation-Specific PCR for *p*16 and *p*57

To assess the methylation status, methylation-specific polymerase chain reaction (MS-PCR) on the $p16^{INK4A}$ and $p57^{KIP2}$ genes was performed. The paraffin-embedded tissues were sectioned to 10 $\mu$m thickness. After they were stained with hematoxylin and eosin, the selected areas were collected with a 26-gauge needle by manual microdissection. The cells attached to the tip of the needle were transferred into a 1.5 ml eppendorf tube containing 20 $\mu$l proteinase K buffer solution (0.5% Tween 20, 1 mM EDTA pH 8.0, 50 mM Tris pH 8.5). They were incubated in a 55°C water bath for 12–15 h and next they were incubated at 100°C for 5 min to inactivate proteinase K, and stored at 5°C until further use. DNA (1 $\mu$g) was diluted with 50 $\mu$l distilled water and 5 $\mu$l 0.3 N NaOH was added. The mixture was kept in a 50°C water bath for 10 min; for treating this mix with bisulfite, 550 ml 1 mM hydroquinone (Sigma, St Louis, USA) and 550 ml 3.5 M sodium bisulfite (Sigma, St Louis, USA) were mixed well at pH 5.0 and reacted at 50°C for 16 h. The primer sequences, annealing temperatures, and the sizes of products are summarized in Table 1. The reaction mixture was as follows: 50 ng DNA modified by bisulfite, 5 pmol primers, 4 mM dNTP, 10 × PCR reaction Buffer (20 mM Tris-HCl, 100 mM KCl, 0.1 mM EDTA, and 1 mM DTT), and 1.25 U of *Taq* polymerase (EX taq, TAKARA, Japan) in a total volume of 20 $\mu$l. After utilizing a Thermal cycler (MJ Research PTC-100, Watertown, USA), the PCR products were subjected to 6% polyacrylamide gel electrophoresis, stained with ethidium bromide and assessed under a UV-transilluminator. If only the unmethylated bands were detected, the samples were evaluated as negative for methylation. If

**Table 1** Primer sequences and conditions for methylation-specific PCR

| *Primer names* | *Primer sequences (5′–3′)* | *Product sizes (bp)* | *Annealing temperature (°C)* |
|---|---|---|---|
| p*16*$^{INK4A}$ | | 151 | 60 |
| uF | TTATTAGAGCTGTGGGGTGGATTGT | | |
| uR | CAACCCCAAACCACAACCATAA | | |
| mF | TTATTAGAGGGTGGGGCGGATCGC | 150 | 65 |
| mR | GACCCCGAACCGCGACCGTAA | | |
| p*57*$^{KIP2}$ | | 270 | 62 |
| uF | TTTGTTTTGTGGTTGTTAATTAGTTGT | | |
| uR | ACACAACACACTTAACCTATAA | | |
| mF | CGCGGTCGTTAATTAGTCGC | 263 | 64 |
| mR | ACACAACGCACTTAACCTATAA | | |

unmethylated bands were detected with the methylated bands or if only the methylated bands were detected, the samples were evaluated as positive for methylation.

### Statisctical Analyses

Statistical analysis was performed using the SPSS software (SPSS Inc., Chicago, IL, USA) using $\chi^2$ test and probability values less than 0.05 were considered statistically significant.

## Results

### Clinical and Histopathologic Findings

The endoscopic findings included ulcer in 26 cases and erosion in seven cases. Nodular lesion and atrophy were detected in one case each. The location of the lesion was in the antrum in 20 cases, and this was followed by the lower body in eight cases and the midbody in seven cases. The microscopic examination of the gastric tissues revealed infiltration of atypical lymphocytes in the lamina propria and prominent lymphoepithelial lesions. On the histological classification, 24 cases were low-grade MALT lymphomas and 11 cases were diffuse large B-cell lymphomas. Among 11 diffuse large B-cell lymphomas, coexistent low-grade MLAT lymphomas, lymphoepithelial lesion and/or evidence of follicular colonization suggesting MALT origin were observed in eight cases. In the remaining three cases of large B-cell lymphomas, the patients showed no evidences of immunodeficiency.

The disease stage, as classified by the modified Ann Arbor system,[34] was stage I in 29 cases, stage II in two cases, stage III in three cases, and stage IV in one case. Bone marrow involvement was detected in the stage IV case. In our study population, four patients died and the remaining patients were still alive for a period of 9–80 months (mean time: 37 months). Four patients with poor outcome consisted of three high-grade and one low-grade lymphomas of MALT type with a mean age of 72.8 years.

With regard to *H. pylori* infection, 30 cases (85.7%) were *H. pylori* positive and five cases were *H. pylori* negative. The degree of *H. pylori* infection was mild in 19 cases, moderate in four cases, and severe in seven cases. The clinicopathologic characteristics of the patients are summarized in Table 2.

### Methylation of *p16* and *p57* in Gastric MALT Lymphomas

In the control gastric mucosa with lymphoid follicles that was negative for *H. pylori*, methylation of $p16^{INK4A}$ and $p57^{KIP2}$ was not detected. In the group positive for *H. pylori* and containing lymphoid follicles, methylation of $p16^{INK4A}$ gene was found in one case, while $p57^{KIP2}$ was not methylated. Methylation of $p16^{INK4A}$ was found in 18 of 35 cases (51.4%) and methylation of $p57^{KIP2}$ was detected in 11 of 35 cases (31.4%) (Figure 3). Methylation of either $p16^{INK4A}$ or $p57^{KIP2}$ was found in 25 of 35 cases (71.4%) and methylation of both genes was found in four of 35 cases (11.4%). For the grades of lymphomas, in the low-grade lymphomas of MALT, methylation of $p16^{INK4A}$ gene was found in 10 of 24 cases (41.7%) and methylation of $p57^{KIP2}$ was found in seven cases (29.2%). In the large B-cell lymphomas, methylation of $p16^{INK4A}$ gene was detected in eight of 11 cases (72.7%) and methylation of $p57^{KIP2}$ was detected in four cases (36.4%) (Table 3). Overall, methylation of $p16^{INK4A}$ and $p57^{KIP2}$ in the diffuse large B-cell lymphomas was higher than in the low-grade MALT lymphomas ($P<0.05$).

When comparing methylation status with the modified Ann Arbor stages, 18 cases with $p16^{INK4A}$ methylation consisted 12/29 stage I, 2/2 stage II, 3/3 stage III, and 1/1 stage IV. For $p57^{KIP2}$ methylation, 11 cases included 9/29 stage I, 1/2 stage II, and 1/3 stage III. Although the number of cases with advanced disease stages was small, most methylation of both genes started to be detected in the early stages. Among four patients who died of gastric lymphomas, three large B-cell lymphomas were methylated in both genes and their clinical stages

**Table 2** Clinicopathologic characteristics of the patients

| *Cases no.* | *Sex* | *Age (years)* | *Endoscopic findings* | *Topography* | *Histologic grade* | *Grades of* H. pylori | *Clinical stage* | *Follow-up* |
|---|---|---|---|---|---|---|---|---|
| 1 | M | 41 | Ulcer | Antrum | L | +++ | I | Alive |
| 2 | F | 70 | Ulcer | Antrum | L | + | I | Alive |
| 3 | F | 46 | Ulcer | Lower body | L | + | I | Alive |
| 4 | M | 38 | Ulcer | Antrum | DLBL[a] | +++ | I | Alive |
| 5 | M | 41 | Ulcer | Antrum | DLBL[a] | + | I | Alive |
| 6 | M | 66 | Ulcer | Antrum | DLBL[a] | + | II | DOD |
| 7 | F | 55 | Ulcer | Antrum | L | +++ | I | Alive |
| 8 | F | 58 | Erosion | Midbody | L | ++ | I | Alive |
| 9 | F | 58 | Ulcer | Midbody | L | ++ | I | Alive |
| 10 | M | 54 | Ulcer | Antrum | L | + | IV | Alive |
| 11 | F | 55 | Ulcer | Lower body | L | + | I | Alive |
| 12 | F | 72 | Nodular lesion | Antrum | L | + | I | Alive |
| 13 | F | 55 | Ulcer | Lower body | L | + | I | Alive |
| 14 | M | 42 | Erosion | Antrum | L | – | I | Alive |
| 15 | M | 39 | Atrophy | Antrum | L | +++ | I | Alive |
| 16 | F | 32 | Ulcer | Antrum | L | + | I | Alive |
| 17 | M | 64 | Ulcer | Lower body | L | +++ | I | Alive |
| 18 | M | 66 | Ulcer | Lower body | L | – | I | DOD |
| 19 | M | 72 | Erosion | Antrum | L | + | I | Alive |
| 20 | M | 46 | Ulcer | Lower body | L | + | I | Alive |
| 21 | M | 79 | Ulcer | Antrum | DLBL[a] | +++ | I | DOD |
| 22 | F | 22 | Ulcer | Midbody | DLBL[a] | +++ | II | Alive |
| 23 | M | 76 | Erosion | Lower body | DLBL | – | I | Alive |
| 24 | F | 61 | Ulcer | Midbody | L | + | III | Alive |
| 25 | F | 67 | Ulcer | Antrum | L | + | III | Alive |
| 26 | M | 55 | Erosion | Body | L | + | I | Alive |
| 27 | M | 50 | Ulcer | Antrum | L | ++ | I | Alive |
| 28 | M | 65 | Erosion | Antrum | L | + | I | Alive |
| 29 | M | 50 | Ulcer | Midbody | DLBL | – | I | Alive |
| 30 | M | 74 | Ulcer | Antrum | L | + | I | Alive |
| 31 | M | 62 | Erosion | Antrum | DLBL[a] | ++ | I | Alive |
| 32 | F | 80 | Ulcer | Midbody | DLBL[a] | + | III | DOD |
| 33 | M | 64 | Ulcer | Antrum | DLBL | + | I | Alive |
| 34 | M | 41 | Ulcer | Antrum | DLBL[a] | + | I | Alive |
| 35 | F | 55 | Ulcer | Lower body | L | – | I | Alive |

L, low-grade MALT lymphoma; DLBL, diffuse large B-cell lymphoma; DOD, dead of disease.
[a]Diffuse large B-cell lymphoma of MALT origin.

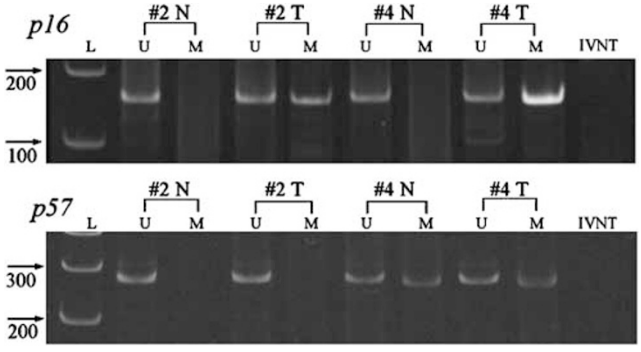


**Figure 3** Photograph of methylation-specific PCR. The sizes of PCR products are 151 bp for unmethylated $p16^{INK4A}$, 150 bp for methylated $p16^{INK4A}$, 270 bp for unmethylated $p57^{KIP2}$, and 263 bp for methylated $p57^{KIP2}$ (L, ladder; N, control gastric mucosa; T, tumor; IVNT, *in vitro* negative control).

were I, II, and III in each case. However, methylation of $p57^{KIP2}$ was not detected in the stage IV patients.

Out of 18 cases with $p16^{INK4A}$ methylation, *H. pylori* infection was positive in 83.3% of the cases and negative in 16.7% of the cases. In the 11 cases with $p57^{KIP2}$ methylation, *H. pylori* infection was found in 90.9% of the cases and it was negative in 9.1% of the cases (Table 4).

## Discussion

Altered methylation patterns can be used as biomarkers for cancer detection, for the assessment of prognosis, and for the prediction of response to antitumor treatment. Furthermore, clinical trials using epigenetically targeted therapies have yielded promising results for acute and chronic leukemias as well as for myelodysplastic syndromes. The exploration of our growing knowledge about epigenetic aberrations may help to develop novel strategies for the diagnosis and treatment of hematopoietic malignancies in the future.[2] In the non-Hodgkin's B-cell lymphomas and MALT lymphomas, $p16^{INK4A}$ gene has been reported to be methylated in 15–67% of tumors.[7,10–12] Martinez-Delgado[7] have reported that in MALT lymphomas, $p16^{INK4A}$ gene is methylated in 44% of the low-grade MALT lymphomas and in all the diffuse large B-cell lymphomas; thus, it is associated with the malig-

**Table 3** Correlation between the methylation status of $p16^{INK4A}$ and $p57^{KIP2}$ genes and the histologic types of gastric lymphomas

| | p*16INK4A* | p*57KIP2* | p*16INK4A* or p*57KIP2* | p*16INK4A* and p*57KIP2* |
|---|---|---|---|---|
| Low-grade MALT ($n=24$) | 10 (41.7%) | 7 (29.2%) | 16 (66.7%) | 1 (4.2%) |
| Large B-cell ($n=11$) | 8 (72.7%) | 4 (36.4%) | 9 (81.8%) | 3 (27.3%) |
| Total ($n=35$) | 18 (51.4%) | 11 (31.4%) | 25 (71.4%) | 4 (11.4%) |

**Table 4** Correlation between the methylation status and *H. pylori* infection

| H. pylori *infection* | p*16INK4A methylation* (n = *18*) | p*57KIP2 methylation* (n = *11*) |
|---|---|---|
| Negative | 3 (16.7%) | 1 (9.1%) |
| Positive | 15 (83.3%) | 10 (90.9%) |

nant transformation of lymphomas. For our study on 35 cases of gastric MALT lymphomas, methylation of $p16^{INK4A}$ was detected in 51.4% of the cases, which is in agreement with previous reports. In addition, methylation of $p16^{INK4A}$ was detected in 41.7% of the low-grade MALT lymphomas and in 72.7% of the diffuse large B-cell lymphomas. This supports the previous findings that $p16^{INK4A}$ methylation is involved in the development of MALT lymphoma as well as being involved in the malignant progression to a higher grade.[7,8,10]

Although methylation study on $p57^{KIP2}$ gene has only rarely been investigated, 54.9% of the non-Hodgkin's lymphomas and 50% of the acute lymphocytic leukemias showed methylation of $p57^{KIP2}$.[26,27] In our experiment on $p57^{KIP2}$ gene, the primers described in previous paper[26] were modified after checking the sequences from the Genebank (http://www.ncbi.nlm.nih.gov). In this first study of $p57^{KIP2}$ methylation in the gastric MALT lymphomas, 31.4% of the cases were methylated and the diffuse large B-cell lymphomas showed higher frequency of methylation than the low-grade MALT lymphomas. Similar to $p16^{INK4A}$, methylation of $p57^{KIP2}$ may be involved in the development and the malignant transformation of gastric MALT lymphoma.

When comparing methylation status and the disease stages, methylation of $p16^{INK4A}$ and $p57^{KIP2}$ was detected in the cases with early stage. However, there was no methylation of $p57^{KIP2}$ in the gastric mucosa with or without *H. pylori* infection and only rare methylation of $p16^{INK4A}$ in the *H. pylori*-positive gastric mucosa with lymphoid follicles. The abrupt and frequent occurrence of methylation of those two genes in the stage I MALT lymphomas suggests that this may be involved early in the development of gastric MALT Lymphoma.[35]

In our study, three out of four patients with poor prognosis showed diffuse large B-cell lymphomas with methylation of both of $p16^{INK4A}$ and $p57^{KIP2}$ genes although their clinical stages were I in two cases and II in one case. Although limited numbers of cases prohibit further clinical interpretation, methylation of these genes as a predicting factor needs to be evaluated.

For the correlation of methylation and *H. pylori* infection, 83.3% of the cases with $p16^{INK4A}$ methylation and 90.9% of the cases with $p57^{KIP2}$ methylation were infected with *H. pylori*, and this suggests that *H. pylori* infection may play an important role in the methylation of these genes. It has been reported that in MALT lymphoma, methylation of $p16^{INK4A}$ gene was not detected after treating *H. pylori* with antibiotics.[12,36,37] In this study, the control gastric mucosa with lymphoid follicles showed methylation of $p16^{INK4A}$ in 10% of the cases. These findings suggest that infection with *H. pylori* itself may play an important role in methylation. DNA damage with the considerable production of reactive oxygen species and inducible nitric oxide synthase caused by *H. pylori* infection will be a potential mechanism,[38] but more research is required to elucidate this mechanism. Based on the observation that methylation is involved in the initial stage of the development of MALT lymphomas, efforts have been made to apply methylation of $p16^{INK4A}$ and $p57^{KIP2}$ genes in clinics. It has been reported that the assessment of methylation of $p16^{INK4A}$ gene may be used as an early diagnostic tool for lung cancer.[39] In addition, by using it as a follow-up marker in non-Hodgkin's lymphoma patients, it may be used for evaluating the outcome of therapy and for the early detection of recurrence.[11] As MS-PCR is a sensitive, simple, rapid, and inexpensive method to determine the methylation status of CpG islands from very small amounts of DNA, the assessment of methylation may reflect the status of residual cancer cells with more sensitivity than the histological findings and so facilitate detection of recurrence. From such an aspect, our research data also suggest the possible application of $p16^{INK4A}$ and $p57^{KIP2}$ methylation as a marker for the early diagnosis and detection of residual tumor of gastric MALT lymphomas.

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