Accumulation of aberrant CpG hypermethylation by *Helicobacter pylori* infection promotes development and progression of gastric MALT lymphoma

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Abstract. Aberrant DNA hypermethylation is an important mechanism for the inactivation of tumor-related genes in human tumors. Gastric mucosa-associated lymphoid tissue (MALT) lymphomas arise from *Helicobacter pylori*-associated chronic gastritis; most patients are *H. pylori*-positive and eradication therapy is highly effective. In the present study, we used methylation-specific PCR to analyze the DNA methylation status of 11 tumor-related genes (*Kip2*, *p16*, *hMLH-1*, *p15*, *p73*, *MGMT*, *DAPK*, *MINT1*, *MINT2*, *MINT31* and *HCAD*) in 21 specimens of MALT lymphoma, 5 specimens of MALT lymphoma with large cell component (high-grade MALT lymphoma), 15 specimens of diffuse large B-cell lymphoma (DLBCL), 8 specimens of complete remission of MALT lymphoma after eradication therapy, 5 specimens with no evidence of malignancy and PBMCs from 10 healthy donors. The average number of methylated genes was significantly greater in gastric lymphomas as compared to normal controls (P<0.001). The CpG island methylator phenotype (CIMP) was observed in 93.3% (14/15) of DLBCLs, 100% (5/5) of high-grade MALT lymphomas and 61.9% (13/21) of MALT lymphomas; in contrast, CIMP was not found in the control group (0%). The average number of methylated genes and the CIMP incidence significantly increased with *H. pylori* infection. Furthermore, aberrant CpG methylation of specific genes, such as *p16*, *MGMT* and *MINT31*, was consistently associated with *H. pylori* infection. These findings strongly suggest that *H. pylori* infection causes the aberrant DNA hypermethylation of specific genes and induces CIMP, which is an important epigenetic mechanism for the development and progression of gastric MALT lymphoma; additionally, our findings provide new epigenetic markers.

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Introduction

Mucosa-associated lymphoid tissue (MALT) lymphoma, a common low-grade B-cell lymphoma arising from a background of chronic inflammatory diseases at a number of mucosal sites, was first described by Isaacson and Wright in 1983 (1). MALT lymphomas originating in the stomach are causatively linked to *Helicobacter pylori* infection and eradication of the bacterium with antibiotics leads to long-term complete remission of the lymphoma in ~70% of patients (2). Additional evidence links *Campylobacter jejuni* (3), *Chlamydia psittaci* (4), *Borrelia burgdorferi* (5) and hepatitis C virus (6,7) infection with MALT lymphoma of the small intestine, ocular adnexa, skin and splenic marginal zone, respectively. These organs are normally devoid of organized lymphoid tissue and lymphoma. MALT lymphomas arise from these sites as a result of chronic inflammatory or autoimmune disorders, such as *H. pylori*-associated chronic gastritis, lymphoepithelial sialoadenitis, Sjögren syndrome and Hashimoto thyroiditis (8,9). The common karyotypic alterations that characterize MALT lymphoma include the trisomies of 3 and 18 (10,11) and the translocations t(11;18) (q21;q21) (12,13), t(1;14)(p22;q32) (14,15), t(14;18)(q32;q21) (10,16), and t(3;14)(p14.1;q32) (17,18), which commonly activate the NF-κB pathway. The most common translocation is t(11;18) (q21;q21), which results from the fusion of the *API2* (apoptosis inhibitor 2) and the *MALT1* (MALT lymphoma-associated translocation) genes (19,20). Clonal identities of the immunoglobulin heavy chains between low-grade MALT lymphomas and coexisting diffuse large B-cell lymphoma (DLBCL) have been found in a considerable number of

patients, indicating that low-grade MALT lymphoma progresses to high-grade malignancy composed of large-sized lymphoma (DLBCL) (21,22). The detailed molecular mechanism of the progression to high-grade lymphoma has not been elucidated.

Genetic abnormalities found in various cancers do not provide the complete picture of molecular mechanism of cancers and malignancies. Epigenetic changes, mainly DNA methylation and histone modification, are additional mechanisms that contribute to the malignant phenotype (23-25). DNA methylation is a normal process used by mammalian cells in maintaining a normal expression pattern; it is involved in the regulation of imprinted gene expression and X-chromosome inactivation and in the fine-tuning of specific differentiation of cells and the development from stem cells (26-29). However, aberrant promoter hypermethylation of the CpG islands leads to epigenetic silencing of multiple genes, including tumor suppressor genes and has been recognized as an important mechanism in carcinogenesis (30-32). Furthermore, concordant promoter hypermethylation of multiple genes, the CpG island methylator phenotype (CIMP), has been found in gastric and colorectal carcinomas (33-35).

Our goal in the current study was to clarify the contribution of epigenetic abnormalities to disease development and progression. We comparatively evaluated the methylation status of 11 genes in gastric lymphomas and related diseases (MALT lymphoma, high-grade MALT lymphoma and DLBCL) and a control group that included complete remission specimens of MALT lymphoma after eradication therapy, specimens of no evidence of malignancy in the stomach and healthy donor peripheral blood mononuclear cells (PBMCs). In addition, we addressed the questions of whether CIMP is associated with gastric lymphoma development and/or progression.

Materials and methods

Specimens. Specimens were pathologically diagnosed at Okayama University Medical School from 1989 to 2000; there were 21 specimens of primary gastric MALT lymphoma (low-grade MALT lymphoma; L-MALT), 5 specimens of gastric MALT lymphoma with large cell component (high-grade MALT lymphoma; H-MALT) and 15 specimens of gastric DLBCL. The control group contained specimens from: 8 patients of complete remission (CR) of gastric MALT lymphoma after eradication therapy; 5 patients of no evidence of malignancy (NEM) in the stomach with infiltration of mononuclear cells, including chronic gastritis and 10 healthy donors of PBMCs. Informed consent was obtained from all patients and healthy volunteers. All the patient-derived materials in the present study were used following the guideline of Japanese Society of Pathology. All tissue samples were obtained by endoscopic biopsy with surgical resection followed by immediate freezing and storing at -80˚C. High molecular weight DNA was extracted by the phenol/chloroform method.

Sodium bisulfite modification and methylation-specific PCR (MSP). Sodium bisulfite modification of DNA was performed, as previously described (31,32). Briefly, 1 μg aliquots of genomic DNA were denatured by NaOH and modified by sodium bisulfite, which converts all unmethylated cytosines to uracils while leaving methylated cytosines unaltered. Modified DNA was purified, desulfonated with NaOH, precipitated with ethanol and resuspended in TE buffer. The methylation-specific PCR was performed to examine the methylation status of 11 tumor-related genes: cell-cycle regulators (*Kip2*, *p16*, *p15*) (36,37), mismatch repair (*MGMT*) (38), DNA repair (*hMLH-1*) (39), tumor suppressor (*p73*) (40), apoptosis (*DAPK*) (41), cell adhesion [*HCAD* (*CDH13*)] (42) and methylated in tumor (*MINT1*, *MINT2*, *MINT31*) (33,34) genes. CpGenome universal methylated DNA (Chemicon International Inc., Temecula, CA) was used as the methylation-positive control and PBMCs from healthy volunteers were used as negative controls. PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and visualized with a UV illuminator.

Evaluation of H. pylori infection. *H. pylori* infection status was analyzed by PCR methods. The PCR conditions and primer sets have been described previously (43). The primer sequence is listed in Table I. *H. pylori* genomic DNA (ATCC43504) was used as a positive control.

Statistical analysis. Statistical analysis was performed by using the Welch's t-test and two-tailed Fisher's exact test. SPSS software (version 11.5J, Chicago, IL) was used to perform the analysis.

Results

Methylation status of gastric MALT lymphoma and related diseases. Initially, we examined whether the aberrant DNA methylation in gastric lymphomas could be detected by MSP. CpG island methylation was analyzed in the promoter region of 11 tumor-related genes (*p15*, *p16*, *kip2*, *p73*, *DAPK*, *hMLH-1*, *MGMT*, *HCAD*, *MINT1*, *MINT2* and *MINT31*) in gastric lymphoma specimens (15 DLBCLs, 21 L-MALT lymphomas and 5 H-MALT lymphomas) and also non-lymphoma controls (8 specimens of CR after eradication therapy; 5 specimens of NEM in the stomach, including chronic gastritis; and PBMCs from 10 healthy volunteers).

Representative results of MSP are shown in Fig. 1. CpGenome was used as the MSP-positive control. DNA methylation was categorized in 5 groups (-, ±, +, ++ and +++) according to the signal intensity; '-' is no methylation signal; '±', very faint signal; + to +++, evident signal with different strengths. Only the categories from + to +++ were regarded as methylation-positive. Table II shows a clear difference in the methylation frequencies between the lymphoma and control groups.

The number of methylated genes shifted from low to high values according to the disease progression from healthy controls to L-MALT lymphoma and H-MALT lymphoma and DLBCL (Fig. 2A). The average number of methylated genes in each group is: healthy PBMCs, 0.4; NEM, 0.4; CR after eradication therapy, 1.4; *H. pylori* (-) L-MALT lymphoma, 3.5; *H. pylori* (+) L-MALT lymphoma, 5.5; H-MALT lymphoma, 7.8; and DLBCL, 6.4 (Fig. 2B). The number of

Table I. Primer sequences and PCR conditions for MSP analysis.

Genes	Primer sequences (5'-3') forward	Primer sequences (5'-3') reverse	Annealing temperature in ˚C (no. of cycles)
KIP2	MF 5'-ACACAACACACTTAACCTATAA-3'	MR 5'-CGCGGTCGTTAATTAGTCGC-3'	56 (38)
	UF 5'-ACACAACGCACTTAACCTATAA-3'	UR 5'-TTTGTTTTGTGGTTGTTAATTAGTTGT-3'	56 (40)
p16	MF 5'-TTATTAGAGGGTGGGGCGGATCGC-3'	MR 5'-GACCCCGAACCGCGACCGTAA-3'	66 (35)
	UF 5'-TTATTAGAGGGTGGGGTGGATTGT-3'	UR 5'-CAACCCCAAACCACAACCATAA-3'	66 (35)
hMLH-1	MF 5'-TTAATAGGAGAGGCGGATAGC-3'	MR 5'-CTATAAATTACTAAATCTCTTCG-3'	54 (35)
	UF 5'-TTAATAGGAAGAGTGGATAGTG-3'	UR 5'-TCTATAAATTACTAAATCTCTTCA-3'	54 (35)
p15	MF 5'-GCGTTCGTATTTTGCGGTT-3'	MR 5'-CGTACAATAACCGAACGACCGA-3'	65 (35)
	UF 5'-TGTGATGTGTTTGTATTTTGTGGTT-3'	UR 5'-CCATACAATAACCAAACAACCAA-3'	60 (35)
p73	MF 5'-GGACGTAGCGAAATCGGGGTTC-3'	MR 5'-ACCCCGAACATCGACGTCCG-3'	63 (35)
	UF 5'-AGGGGATGTAGTGAAATTGGGGTTT-3'	UR 5'-ATCACAACCCCAAACATCAACATCCA-3'	66 (35)
DAPK	MF 5'-GGATAGTCGGATCGAGTTAACGTC-3'	MR 5'-CCCTCCCAAACGCCGA-3'	64 (35)
	UF 5'-GGAGGATAGTTGGATTGAGTTAATGTT-3'	UR 5'-CAAATCCCTCCCAAACACCAA-3'	64 (35)
MGMT	MF 5'-TTTCGACGTTCGTAGGTTTTCGC-3'	MR 5'-GCACTCTTCCGAAAACGAAACG-3'	66 (35)
	UF 5'-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3'	UR 5'-AACTCCACACTCTTCCAAAAACAAAACA-3'	66 (35)
MINT1	MF 5'-AATTTTTTTATATATATTTTCGAAGC-3'	MR 5'-AAAAACCTCAACCCCGCG-3'	57 (35)
	UF 5'-AACAAAAAACCTCAACCCCACA-3'	UR 5'-AATTTTTTTATATATATTTTTGAAGTGT-3'	57 (35)
MINT2	MF 5'-TTGTTAAAGTGTTGAGTTCGTC-3'	MR 5'-AATAACGACGATTCCGTACG-3'	61 (35)
	UF 5'-GATTTTGTTAAAGTGTTGAGTTTGTT-3'	UR 5'-CAAAATAATAACAACAATTCCATACA-3'	61 (35)
MINT31	MF 5'-TGTTGGGGAAGTGTTTTTCGGC-3'	MR 5'-CGAAAACGAAACGCCGCG-3'	64 (35)
	UF 5'-TAGATGTTGGGGAAGTGTTTTTTGGT-3'	UR 5'-TAAATACCCAAAAACAAAACACCACA-3'	64 (35)
HCAD	1st F 5'-TTGGAAAAGTGGAATTAGTTGG-3'	1st R 5'-CCTCTTCCCTACCTAAAACA-3'	54 (35)
	MF 5'-TCGCGGGGTTCGTTTTTCGC-3'	MR 5'-GACGTTTTCATTCATACACGCG-3'	69 (25)
	UF 5'-GTAAAATGAGGGAGTGTTAGG-3'	UR 5'-AAACACACCCAACAACCCCTCT-3'	50 (25)
H. pylori	F 5'-ATTACTGACGCTGATTGTGC-3'	R 5'-CTGGAGAGACTAAGCCCTCC-3'	60 (40)

U, unmethylated sequences and M, methylated sequences.

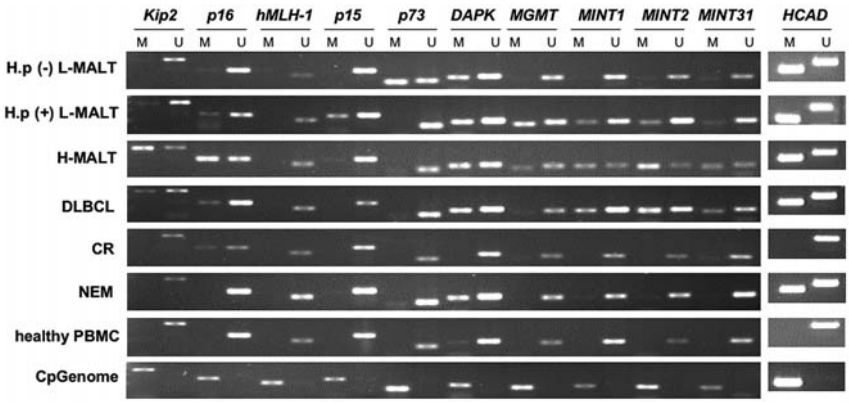

Figure 1. Representative results of methylation-specific PCR of 11 genes in low-grade MALT lymphoma (L-MALT), high-grade MALT lymphoma (H-MALT), diffuse large B-cell lymphoma (DLBCL), complete remission of MALT lymphoma (CR) after eradication therapy, no evidence of malignancy (NEM). Healthy PBMCs, used as negative control; CpGenom, used as positive control; M, methylated DNA and U, unmethylated DNA.

methylated genes was significantly greater in the lymphoma group compared to the control group (P<0.001, Kruskal-Wallis exact test). Interestingly, the frequency of methylated genes in CR patients after eradication therapy remarkably decreased, suggesting that aberrant DNA methylation was closely associated with the *H. pylori* infection.

Table II. Methylation profile of 11 genes in gastric lymphomas and related diseases.

			age/sex	H.p	KIP2	p16	hMLH-1	p15	p73	DAPK	MGMT	MINT1	MINT2	MINT31	HCAD	No of methylated genes
H-MALT		1	76/F	+	−/+	+	−	−/+	−	+	++	−	+	+	−	5
		2	49/M	+	+++	+++	−	−	−	++	+	++	+++	++	+++	8
		3	70/F	+	+	+++	−	−	+	++	++	++	+++	+	+++	9
		4	88/M	+	+	+	−	−	−	+	+	+	+	+	+++	8
		5	64/M	+	+++	+++	−	−	+++	++	++	+	+++	+	+++	9
		H-MALT total			80%(4/5)	100%(5/5)	0%(0/5)	0%(0/5)	40%(2/5)	100%(5/5)	100%(5/5)	80%(4/5)	100%(5/5)	100%(5/5)	80%(4/5)	100%(5/5)
DLBCL		1	58/F	+	+	−	−	−	−	−/+	−/+	−/+	+	+	++	4
		2	55/M	+	+++	−	−	−/+	+++	++	−/+	+	−	+	+++	6
		3	41/M	+	+	−/+	−	−	−	+	−	+	+++	+	+++	6
		4	61/F	+	+	+	+	−	+++	−/+	−	+	+++	+	++	8
		5	63M	+	−	−	+	++	−	+	−/+	+	+++	+	++	7
		6	66/M	+	+	++	+	−	−	−/+	+++	+	+++	−/+	−/+	6
		7	62/M	+	+	+++	−	+	+++	+	+	+	++	+	+++	10
		8	79/M	+	+	+++	−	+	−	+	+	+	+	+	−	8
		9	71/F	+	−/+	+++	−	−	+++	+	+	+	+	+	+++	8
		10	50/M	+	−	−	−	+++	−	+	+	−/+	+	−	+++	5
		11	73/F	+	+	+++	−	−	−	−	++	−/+	+	−/+	+++	5
		12	76/F	+	−/+	+++	−	−	−	−	−/+	−	+	−/+	−	2
		13	62/F	+	+	+	−	−	−	+++	−	+	+	+	+++	7
		14	71/M	+	++	−	−	−	+	+++	−	+	++	++	+++	7
		15	51/M	+	++	++	−	−	−	+++	−/+	+	++	++	+++	7
		DLBCL total			73.3%(11/15)	60%(9/15)	20%(3/15)	26.7%(4/15)	33.3%(5/15)	66.7%(10/15)	40%(6/15)	73.3%(11/15)	93.3%(14/15)	73.3%(11/15)	80%(12/15)	93.3%(14/15)

			age/sex	H.p	KIP2	p16	hMLH-1	p15	p73	DAPK	MGMT	MINT1	MINT2	MINT31	HCAD	No of methylated genes
L-MALT	MALT H.p(-)	1	34/F	−	−	−/+	−	−	−	−	−/+	−	−/+	−	+++	1
		2	47/F	−	+	−/+	−	−	−	−/+	−/+	−	−/+	−	+++	2
		3	48/F	−	++	−/+	−	−	+	+	−	−	−	−/+	+++	4
		4	47/F	−	−	+	−	−/+	−	−/+	−/+	−	−	−	+++	2
		5	54/F	−	+	+++	−	+	−	+	−/+	−/+	+++	+	+++	7
		6	62/M	−	+++	++	−	−/+	+++	++	+	−/+	+++	+	+++	8
		7	75/M	−	−	+	−	−	−	+	−	+	−	−/+	+	4
		8	69/M	−	−	−/+	−	−	−	+	+	−	−/+	−/+	−	2
		9	76/M	−	−	−	−	−	−	−/+	−/+	−	−	−/+	++	1
		10	80/M	−	−	−	−	−	−	−/+	−	−	−	−	−	0
		11	68/F	−	+	+	−	−	+	++	+	+	+	+	++	9
		12	67/F	−	−	−/+	−	−	−	++	−/+	−	−	−/+	++	2
		MALT H.p(-) total			41.7%(5/12)	41.7%(5/12)	0%(0/12)	8.3%(1/12)	16.7%(2/12)	58.3%(7/12)	25%(3/12)	16.7%(2/12)	25%(3/12)	25%(3/12)	83.3%(10/12)	41.7%(5/12)
	MALT H.p(+)	13	54/F	+	+++	+	−	−/+	−	+	+	−/+	+++	+	+++	7
		14	55/F	+	+	+	−	−/+	−	+	−/+	−/+	+	+	+++	6
		15	57/F	+	+++	+++	−	−	−	+	−/+	−/+	+	++	+++	6
		16	34/F	+	−	−/+	−	−	−	+++	+	+	−	−/+	+	4
		17	57/F	+	+++	+	−	−	−	+	+	−/+	+	+	+++	7
		18	70/F	+	+++	+++	−	−	++	+	+	−/+	+++	++	+++	8
		19	74/M	+	−/+	+	−	+	−	+	+	−/+	+	−	+++	6
		20	62/F	+	−	+	−	−	−	+	+	−/+	−	−	++	4
		21	63/M	+	−	−/+	−	−	−	+	+	−	−	−/+	−	2
		MALT H.p (+) total			55.6%(5/9)	77.8%(7/9)	0%(0/9)	11.1%(1/9)	11.1%(1/9)	100%(9/9)	77.8%(7/9)	11.1%(1/9)	66.7%(6/9)	55.6%(5/9)	88.9%(8/9)	88.9%(8/9)
		L-MALT total			47.6%(10/21)	57.1%(12/21)	0%(0/21)	9.5%(2/21)	14.3%(3/21)	76.2%(16/21)	47.6%(10/21)	14.3%(3/21)	42.9%(9/21)	38.1%(8/21)	85.7%(18/21)	61.9%(13/21)

		age/sex	H.p	KIP2	p16	hMLH-1	p15	p73	DAPK	MGMT	MINT1	MINT2	MINT31	HCAD	No of methylated genes
CR	1	53/F	−	−	−/+	−	−/+	−	+	−/+	−	++	−/+	++	3
	2	53/F	−	−	−	−	−	−	++	−	−	+	−	++	3
	3	69/M	−	−	−	−	−	−	+	−	−	−	−	−/+	1
	4	76/M	−	−	−	−	−	−	+	+	−	−	−	−	2
	5	64/M	−	−	−	−	−	−	−	−	−	−	−	−	0
	6	64/M	−	−	−/+	−	−	−	−	−	−	−	−/+	−	0
	7	81/M	−	−	+	−	−	−	−	−	−	−	−	−	1
	8	70/M	−	−	−/+	−	−	−	−/+	−	−	−	−	++	1
	CR total			0%(0/8)	12.5%(1/8)	0%(0/8)	0%(0/8)	0%(0/8)	50%(4/8)	12.5%(1/8)	0%(0/8)	25%(2/8)	0%(0/8)	37.5%(3/8)	0%(0/8)
NEM	1	53/F	−	−	−	−	−	−	−/+	−	−	−	−	−	0
	2	54/F	+	−	−	−	−	−	−/+	−	−/+	−	−	−	0
	3	55/F	−	−	−	−	−	−	−/+	−	−/+	−	−	+++	1
	4	55/M	−	−	−	−	−	−	−	−	−	−/+	−	++	1
	5	38/M	−	−	−	−	−	−	−	−	−	−	−	−/+	0
	NEM total			0%(0/5)	0%(0/5)	0%(0/5)	0%(0/5)	0%(0/5)	0%(0/5)	0%(0/5)	0%(0/5)	0%(0/5)	0%(0/5)	40%(2/5)	0%(0/5)
Healthy PBMC	1	48/M	nd	−	−	−	−	−	−/+	−	−	−	−	−	0
	2	29/M	nd	−	−	+	−	−	−/+	−	+	−	−	−/+	2
	3	46/M	nd	−	−	−	−	−	−	−	−	−	−	−/+	1
	4	27/F	nd	−	−	−	−	−	−	−	−	−	−	−	0
	5	24/F	nd	−	−	−	−	−	−	−	−	−	−	−	0
	6	27/F	nd	−	+	−	−	−/+	−	−	−	−	−	−	1
	7	20/M	nd	−	−	−	−	−	−/+	−	−	−	−	−	0
	8	41/M	nd	−	−	−	−	−/+	−	−	−	−	−	−/+	0
	9	41/M	nd	−	−/+	−	−	−/+	−	−	−	−	−	−	0
	10	50/M	nd	−	−	−	−	+	−	−	−	−	−	−	1
	PBMC total			0%(0/10)	10%(1/10)	10%(1/10)	0%(0/10)	10%(1/10)	0%(0/10)	0%(0/10)	10%(1/10)	0%(0/10)	0%(0/10)	0%(0/10)	0%(0/10)

Results of MSP were shown as -, -/+, +, ++, or +++ according to the signal intensity. DLBCL, diffuse large B-cell lymphoma; H-MALT, high-grade MALT lymphoma; MALT, MALT lymphoma; CR, complete remission of MALT lymphoma; NEM, no evidence of malignancy in stomach; PBMCs, healthy donor peripheral blood mononuclear cells. The number of methylated genes was shown in the right-end column. CIMP (+) was defined as more than or equal to 4 genes were methylated. Hp, *H. pylori* infection. nd: not done.

An analysis of the differences in methylation frequency of the 11 genes among various stages of gastric MALT lymphomas and related diseases revealed a statistically significant difference in the DNA methylation incidence between the lymphoma and control groups, especially in the *Kip2*, *p16*, *DAPK*, *MGMT*, *MINT1*, *MINT2* and *MINT31* genes

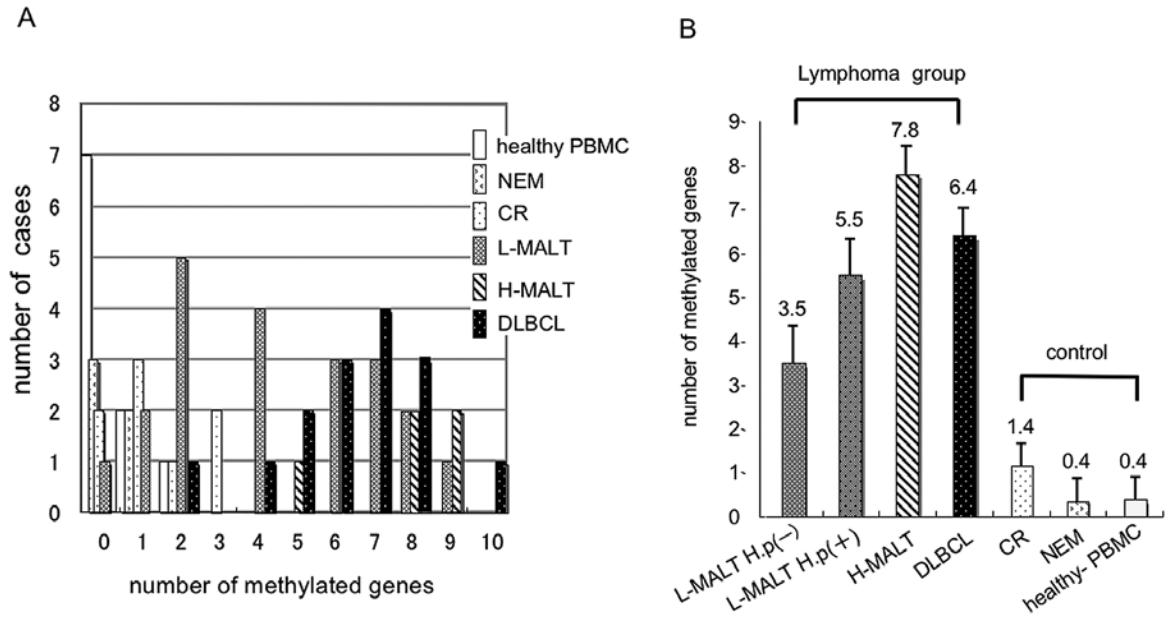

Figure 2. (A) The distribution of number of methylated genes in gastric lymphomas. The distribution profile of the methylated genes shifted from low to high according to the disease progression from healthy donor, NEM and CR to lymphoma groups from low-grade L-MALT to high-grade H-MALT and DLBCL. (B) The average number of methylated genes in lymphoma and control groups. Significant difference of average number of methylated genes was detected between lymphoma and control groups (P<0.001, Kruskal-Wallis exact test).

(Table III), suggesting that aberrant CpG methylation of particular genes was closely associated with the lymphomagenesis in the stage-specific manner.

CIMP in gastric lymphoma. An examination of the number of methylated genes among gastric lymphomas and controls revealed a clear boundary (between 3 and 4 methylated genes) in the distribution of the number of methylated genes (Fig. 3A). All specimens with ≥4 methylated genes belong to the lymphoma group. On the other hand, almost all of the specimens with <4 methylated genes were in the control group. Therefore, we defined the CIMP-positive specimens as those specimens with at least 4 methylated genes. Specimens with <4 methylated genes were CIMP-negative. The CIMP (+) or (-) status in the lymphoma and control groups is summarized in Fig. 3B, indicating that 93.3% (14 of 15) of DLBCL, 100% (5 of 5) of H-MALT lymphoma and 61.9% (13 of 21) of L-MALT lymphoma were CIMP-positive. Contrary, all specimens of CR (8 of 8), NEM (5 of 5) and healthy PBMCs (10 of 10) were CIMP-negative. The incidence of CIMP (+) specimens was significantly higher in the lymphoma group than in the control group (P<0.001, two-tailed Fisher's exact test). CIMP-positive specimens increased according to the disease progression from L-MALT lymphoma to H-MALT lymphoma and to DLBCL.

Next, we analyzed the correlation between CIMP status and the methylation of each gene (Table IV). Methylation of all genes except for the *hMLH1* gene was correlated with CIMP status (P<0.004, Fisher's exact test). There was a statistically significant difference in the incidence of CIMP between the two stages of MALT lymphoma and related diseases, such as between healthy PBMCs and L-MALT (P=0.001); healthy PBMCs and H-MALT (P=0.000); healthy PBMCs and DLBCL (P=0.000); and NEM and H-MALT

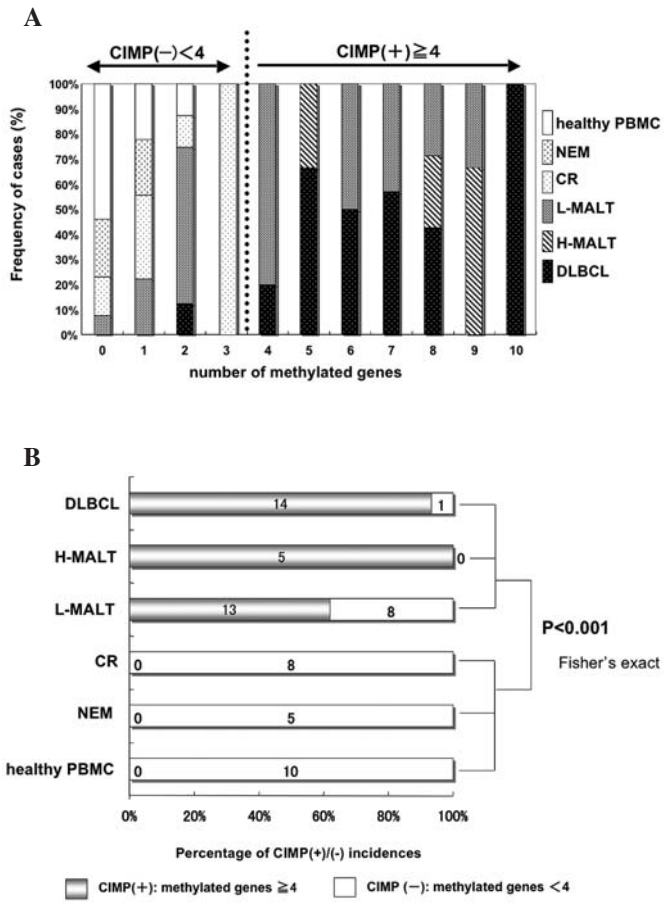

Figure 3. (A) Case frequency (%) of number of methylated genes among gastric lymphomas and controls including healthy donors, NEM and CR after eradication. (B) CIMP (+) or (-) status in lymphoma and control groups. The incidence of CIMP (+) specimens was significantly higher in lymphoma group than control group (P<0.001, two-tailed Fisher's exact test). The number in each column indicates the number of specimens.

Table III. Statistical differences in methylation frequency of 11 genes between various stages of gastric MALT lymphoma and related diseases (P-values[a]).

	Kip2	*p15*	*p16*	*p73*	*hMLH1*	*DAPK*	*MGMT*	*HCAD*	*MINT1*	*MINT2*	*MINT31*
Healthy PBMC NEM	-	-	1.000	1.000	1.000	-	-	0.095	1.000	-	-
Healthy PBMC Low MALT	**0.012**	1.000	**0.020**	1.000	0.323	**0.000**	**0.012**	**0.000**	1.000	**0.030**	**0.032**
Healthy PBMC High MALT	**0.004**	-	**0.002**	0.242	1.000	**0.000**	**0.000**	**0.004**	**0.017**	**0.000**	**0.000**
Healthy PBMC DLBCL	**0.001**	0.125	**0.018**	0.345	0.626	**0.001**	**0.051**	**0.000**	**0.004**	**0.000**	**0.001**
Healthy PBMC CR	-	-	1.000	1.000	1.000	**0.023**	0.444	0.069	1.000	0.183	-
Healthy PBMC Hp (+)	**0.000**	0.302	**0.001**	0.400	1.000	**0.000**	**0.001**	**0.000**	**0.024**	**0.000**	**0.000**
Healthy PBMC Hp (-)	**0.040**	1.000	0.162	0.594	0.455	**0.005**	0.221	**0.000**	1.000	0.221	0.221
NEM Low MALT	0.121	1.000	**0.042**	0.555	-	**0.004**	0.121	0.062	1.000	0.129	0.281
NEM High MALT	**0.048**	-	**0.008**	0.444	-	**0.008**	**0.008**	0.524	**0.048**	**0.008**	**0.008**
NEM DLBCL	**0.008**	0.530	**0.038**	0.266	0.539	**0.033**	0.260	0.131	**0.008**	**0.000**	**0.008**
NEM CR	-	-	1.000	-	-	0.105	1.000	1.000	-	0.487	-
NEM Hp (+)	**0.007**	1.000	**0.005**	0.309	1.000	**0.001**	**0.016**	**0.072**	**0.046**	**0.000**	**0.005**
NEM Hp (-)	0.245	1.000	0.245	0.515	-	**0.044**	0.515	0.117	1.000	0.515	0.515
Low MALT High MALT	0.330	1.000	0.129	0.558	-	0.545	**0.053**	1.000	**0.010**	**0.042**	**0.039**
Low MALT DLBCL	0.176	0.210	1.000	0.443	0.064	0.709	0.741	0.677	**0.001**	**0.004**	**0.049**
Low MALT CR	**0.027**	1.000	**0.044**	0.552	-	0.209	0.110	**0.019**	0.540	0.671	0.066
High MALT DLBCL	1.000	0.530	0.260	1.000	0.539	0.266	**0.038**	1.000	1.000	1.000	0.530
High MALT CR	**0.007**	-	**0.005**	0.128	-	0.105	**0.005**	0.266	**0.007**	**0.021**	**0.001**
DLBCL CR	**0.001**	0.257	0.074	0.122	0.526	0.657	0.345	0.071	**0.001**	**0.002**	**0.001**
CR Hp (+)	**0.001**	0.564	**0.004**	0.160	1.000	0.078	**0.019**	**0.021**	**0.006**	**0.002**	**0.000**
CR Hp (-)	**0.055**	1.000	0.325	0.242	-	1.000	0.619	0.062	0.495	1.000	0.242
Hp (+) Hp (-)	0.161	0.651	0.083	1.000	0.543	0.124	**0.043**	1.000	**0.038**	**0.000**	**0.013**
Low MALT Hp (+) Low MALT Hp (-)	0.670	1.000	0.184	0.603	-	**0.045**	**0.030**	1.000	1.000	0.087	0.203

[a]Fisher's exact test. Hp (+), *H. pylori* (+) MALT lymphoma with/without large cell compornent and DLBCL; Hp (-), *H. pylori* (-) MALT lymphoma with/without large cell compornent and DLBCL; NEM, no evidence for malignancy; low MALT, MALT lymphoma; high MALT, MALT lymphoma with large cell compornent. Bold numbers indicate significant differences of methylation frequency ($P<0.05$).

Table IV. Correlation between presence of CIMP and methylation of each gene.

		CIMP		
		Positive	Negative	P-value[a]
Kip2	Methylated	24	1	0.000
	Unmethylated	8	31	
p15	Methylated	6	0	0.002
	Unmethylated	26	32	
p16	Methylated	24	4	0.000
	Unmethylated	8	28	
p73	Methylated	11	1	0.003
	Unmethylated	21	31	
hMLH1	Methylated	3	1	0.613
	Unmethylated	29	31	
DAPK	Methylated	28	7	0.000
	Unmethylated	5	24	
MGMT	Methylated	19	3	0.000
	Unmethylated	13	29	
HCAD	Methylated	29	10	0.000
	Unmethylated	3	22	
MINT1	Methylated	18	1	0.000
	Unmethylated	14	31	
MINT2	Methylated	28	3	0.000
	Unmethylated	5	28	
MINT31	Methylated	24	0	0.000
	Unmethylated	8	32	

[a]Fisher's exact test.

(P=0.008) (Table V), indicating that the incidence of CIMP in each lymphoma group was significantly higher than that of the control group.

Effects of H. pylori infection on the number of methylated genes and CIMP. We analyzed the effects of *H. Pylori* infection on the number of methylated genes and CIMP status (Fig. 4). The distribution of the number of methylated genes with or without *H. pylori* infection in MALT lymphoma indicates that the number of methylated genes was shifted to a higher value in cases of *H. pylori* infection (Fig. 4A). The CIMP (+) incidence was significantly higher in *H. Pylori* (+) L-MALT (89%, 8/9) compared to *H. Pylori* (-) L-MALT (42%, 5/12) (P=0.037) (Fig. 4B). *H. Pylori* (+) gastric lymphoma also showed significantly higher CIMP (+) incidence (93%, 27/29) than *H. Pylori* (-) gastric lymphoma (42%, 5/12)(P<0.001) (Fig. 4C). Table VI shows a more detailed statistical analysis of the effects of *H. pylori* infection on the particular target gene methylation. The methylation frequency of specific genes, such as *p16*, *DAPK*, *MGMT*, *Kip2*, *MINT2*, *MINT31* and *HCAD*, significantly increased in *H. pylori* (+) L-MALT compared to healthy PMBCs (P<0.05). On the other hand, the methylation frequency of these genes (except for the *MINT2* gene) dramatically decreased in CR after eradication therapy compared to *H. pylori* (+) L-MALT

Table V. Statistical difference in the incidence of CIMP among various stages of MALT lymphoma and related diseases.

	CIMP		
	Positive	Negative	P-value[a]
Healthy PBMC	0	10	-
NEM	0	5	
Healthy PBMC	0	10	0.001
Low MALT	13	8	
Healthy PBMC	0	10	0.000
High MALT	5	0	
Healthy PBMC	0	10	0.000
DLBCL	14	1	
Healthy PBMC	0	10	-
CR	0	8	
Healthy PBMC	0	10	0.000
Hp (+)	27	2	
Healthy PBMC	0	10	0.040
Hp (-)	5	7	
NEM	0	5	0.039
Low MALT	8	13	
NEM	0	5	0.008
High MALT	5	0	
NEM	0	5	0.000
DLBCL	14	1	
NEM	0	5	-
CR	0	8	
NEM	0	5	0.000
Hp (+)	27	2	
NEM	0	5	0.515
Hp (-)	5	7	
Low MALT	13	8	0.281
High MALT	5	0	
Low MALT	13	8	0.051
DLBCL	14	1	
Low MALT	13	6	0.003
CR	0	8	
High MALT	5	0	1.000
DLBCL	14	1	
High MALT	5	0	0.001
CR	0	8	
DLBCL	14	1	0.000
CR	0	8	
CR	0	8	0.000
Hp (+)	27	2	
CR	0	8	0.055
Hp (-)	5	7	
Hp (+)	27	2	0.001
Hp (-)	5	7	

[a]Fisher's exact test. Hp (+), *H. pylori* (+) MALT lymphoma with/without large cell component and DLBCL; Hp (-), *H. pylori* (-) MALT lymphoma with/without large cell component and DLBCL; NEM, no evidence for malignancy; low MALT, MALT lymphoma; high MALT, MALT lymphoma with large cell component.

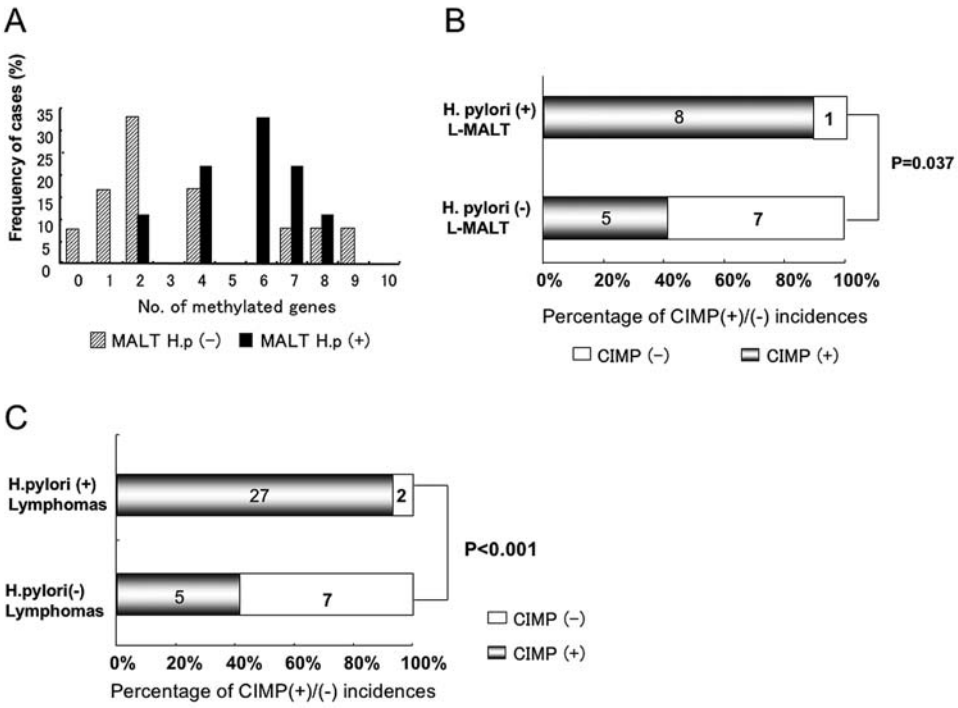

Figure 4. Association of *H. Pylori* infection with the number of methylated genes and CIMP status. (A) Distribution of number of methylated genes with/without *H. pylori* infection in MALT lymphoma. Profile of number of methylated genes was shifted to higher value of number of methylated genes according to the *H. pylori* infection. (B) Correlation between CIMP (+)/(-) status and *H. pylori* infection to low-grade MALT. The incidence of CIMP (+) specimens was significantly higher in *H. pylori* (+) L-MALT than *H. pylori* (-) L-MALT (P=0.037, two-tailed Fisher's exact test). (C) The incidence of CIMP (+) specimens was significantly higher in *H. pylori* (+) gastric lymphoma than *H. pylori* (-) gastric lymphoma, including MALT lymphoma, high-grade MALT lymphoma and DLBCL. (P<0.001, two-tailed Fisher's exact test).

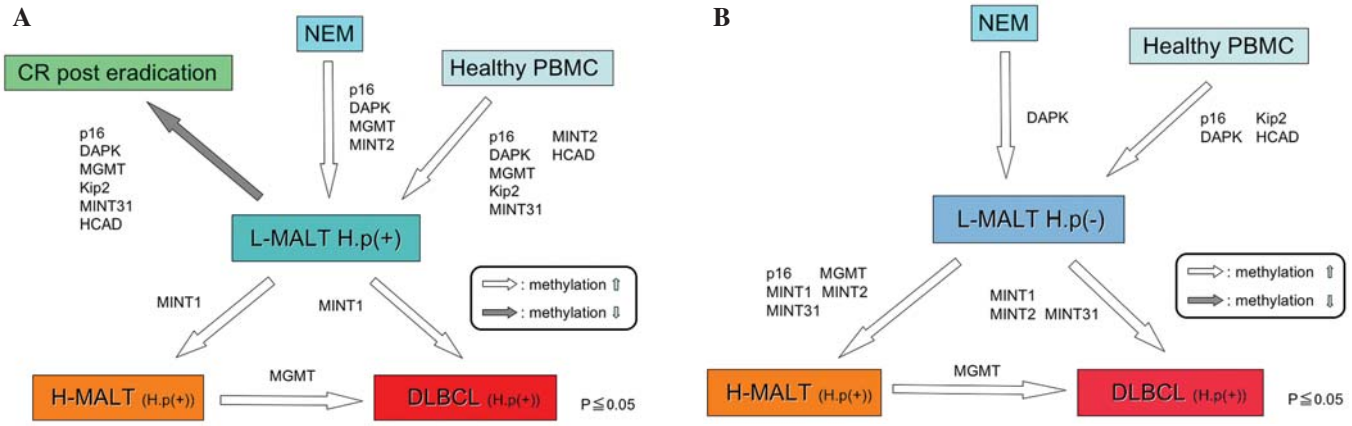

Figure 5. Schematic illustration of development and progression of gastric lymphoma in terms of specific gene methylation. Genes associated with white arrow indicate significant increase of methylation frequency from arrow start point status to end point status. On the other hand, genes associated with dark arrow show statistically significant decrease of methylation frequency from start-point to end-point status (P<0.05). (A) Schematic illustration of *H. pylori* (+) L-MALT related diseases in terms of CpG hypermethylation. (B) Schematic illustration of *H. pylori* (-) L-MALT-related diseases in terms of CpG hypermethylation. Statistical data was originated from Tables III and VI.

(P<0.05). These statistically significant alterations in the methylation frequency of the 11 genes among various clinical states, including MALT lymphoma with or without *H. pylori* infection and related diseases, are summarized in Fig. 5A and B.

Discussion

A close link between *H. pylori* infection and the development of gastric MALT lymphoma has been established by the detection of *H. pylori* infection in most patients of gastric MALT lymphoma (1,2,19,20). We previously reported that autoimmune reactions evoked by immunological cross-reactivity between *H. pylori* HSP60 and host HSP60 are important in the development of gastric MALT lymphoma because of the molecular mimicry between human HSP60 and *H. pylori* HSP60 (44-47).

In the present study, we investigated the aberrant DNA hypermethylation in gastric lymphoma, including L-MALT lymphoma, H-MALT lymphoma and DLBCL, showing that

Table VI. Statistical differences of methylation frequency in 11 genes between various clinical status and *H. pylori* infection (±) low grade MALT lymphoma (P-values[a]).

Low MALT HP (+)	Statistical differences of methylation frequency between two groups										
	Kip2	*p15*	*p16*	*p73*	*hMLH1*	*DAPK*	*MGMT*	*HCAD*	*MINT1*	*MINT2*	*MINT31*
Low MALT HP (+) Healthy PBMC	**0.011**	0.474	**0.005**	1.000	1.000	**0.000**	**0.001**	**0.000**	1.000	**0.003**	**0.011**
Low MALT HP (+) NEM	0.086	1.000	**0.021**	1.000	-	**0.000**	**0.021**	0.095	1.000	**0.031**	0.086
Low MALT HP (+) High MALT	0.580	1.000	0.505	0.505	-	-	0.505	1.000	**0.023**	0.258	0.221
Low MALT HP (+) DLBCL	0.412	0.615	0.657	0.351	0.266	0.118	0.105	1.000	**0.009**	0.130	0.412
Low MALT HP (+) CR	**0.029**	1.000	**0.015**	1.000	-	**0.029**	**0.015**	**0.050**	1.000	0.153	**0.029**
Low MALT HP (-)	Statistical differences of methylation frequency between two groups										
	Kip2	*p15*	*p16*	*p73*	*hMLH1*	*DAPK*	*MGMT*	*HCAD*	*MINT1*	*MINT2*	*MINT31*
Low MALT HP (-) Healthy PBMC	**0.040**	1.000	**0.040**	0.594	0.455	**0.005**	0.221	**0.000**	1.000	0.221	0.221
Low MALT HP (-) NEM	0.245	1.000	0.245	0.515	-	**0.044**	0.515	0.117	1.000	0.515	0.515
Low MALT HP (-) High MALT	0.294	1.000	**0.044**	0.600	-	0.245	**0.009**	1.000	**0.028**	**0.009**	**0.009**
Low MALT HP (-) DLBCL	0.130	0.342	0.449	0.698	0.231	0.706	0.683	1.000	**0.006**	**0.001**	**0.021**
Low MALT HP (-) CR	0.055	1.000	0.325	0.242	-	1.000	0.619	0.062	0.495	1.000	0.242

[a]Fisher's exact test. Hp (+), *H. pylori* (+) MALT lymphoma with/without large cell compornent and DLBCL; Hp (-), *H. pylori* (-) MALT lymphoma with/without large cell compornent and DLBCL; NEM, no evidence for malignancy; low MALT, MALT lymphoma; high MALT, MALT lymphoma with large cell compornent. Bold numbers indicate significant differences of methylation frequency ($P<0.05$).

the number of methylated genes is clearly greater in the lymphoma group as compared to the control group (Fig. 2A and B). Furthermore, according to the progression from L-MALT to H-MALT and to DLBCL, the number of methylated genes shifted from low to high values, indicating that the aberrant hypermethylated genes accumulated in the course of disease progression. There was a significant difference in the incidence of CIMP between the lymphoma group and the control group (Fig. 3B), suggesting that there are abnormalities in the regulatory and/or maintenance mechanism of DNA methylation status that cause aberrant hypermethylation to accumulate in specific genes in gastric lymphomas.

Interestingly, a specific group of genes was targeted for aberrant hypermethylation in a stage-specific manner in gastric lymphoma progression and related diseases (Tables III and VI and Fig. 5A and B). The group of genes, including *Kip2*, *p16*, *DAPK*, *HCAD*, *MGMT*, *MINT2* and *MINT31*, was significantly hypermethylated during the onset of *H. pylori* (+) L-MALT from healthy PBMCs (Table V, Fig. 5A). The same group of genes (except for the *MINT2*) was significantly de-methylated in the CR state post-eradication therapy from *H. pylori* (+) L-MALT (Table VI, Fig. 5A); these results clearly indicate that aberrant DNA hypermethylation of these set of genes is closely associated with *H. pylori* infection and also the onset of *H. pylori* (+) L-MALT ($P\leq0.05$). They suggest that abnormal epigenetic alterations, such as the aberrant DNA hypermethylation that was induced by *H. pylori* infection, are responsible for the triggering of *H. pylori* (+) L-MALT. Aberrant hypermethylation of *MINT1* is significantly associated with the progression from *H. pylori* (+) L-MALT to *H. pylori* (+) H-MALT or DLBCL ($P=0.023$ or $P=0.009$ respectively, Table VI, Fig. 5A). Aberrant hypermethylation of *MGMT* was also closely associated with the progression from *H. pylori* (+) H-MALT to DLBCL ($P=0.038$, Table III, Fig. 5A).

On the other hand, a small number of genes, including *p16*, *Kip2*, *DAPK* and *HCAD*, were associated with the onset of *H. pylori* (-) L-MALT from healthy PBMCs, suggesting that these genes contribute to the triggering of *H. pylori* (-) L-MALT independent of *H. pylori* infection (Table VI, Fig. 5B). In the progression from *H. pylori* (-) L-MALT to *H. pylori* (+) H-MALT, another group of genes, such as *p16*, *MGMT*,

MINT1, *MINT2* and *MINT31*, were significantly hypermethylated (Table VI, Fig. 5B). This process was associated with *H. pylori* infection, which was closely similar to the methylation profile alteration at the onset of *H. pylori* (+) L-MALT from healthy PBMC accompanied with the *H. pylori* infection. The aberrant hypermethylation of *MINT1* was significantly associated with the progression from L-MALT to H-MALT and also to DLBCL (Table III, Fig. 5B). Similarly, *MGMT* hypermethylation was significantly associated with the progression from H-MALT to DLBCL (Table III), comparing the *H. pylori* (+) L-MALT process (Fig. 5A) with the *H. pylori* (-) L-MALT process (Fig. 5B), it is clear that hypermethylation of specific target genes, such as p16, *MGMT*, *MINT2* and *MINT31*, is closely associated with *H. pylori* infection. It suggests the possibility that *H. pylori* infection perturbs or alters the epigenetic status, including the methylation profile, with the production of *H. pylori*-specific molecules, such as *CagA*, *VacA*, *OipA* and DNA methyltransferase (*hpy1M* or *hpyIIM*) (48,49), cytokines or inflammatory responses with the production of reactive oxygen species to cause aberrant hypermethylation of specific genes, followed by malignant transformation events including aberrant hypermethylation of other genes, alteration of miRNA expression profile, histone modification and/or chromatin organization changes. Contact of *H. pylori* with the epithelium induces transcription of the induced-by-contact-with-epithelium gene (*iceA1*); the presence of this gene product has been associated with duodenal ulceration and gastric adenocarcinoma (50). The expression of *H. pylori* methyltransferase *hpy1M* accompanies *iceA1* (48). *H. pylori* DNA methyltransferases may be one candidate to cause aberrant hypermethylation in addition to host DNA methyltransferases. The effectiveness of *H. pylori* eradication therapy in *H. pylori* (+) L-MALT is consistent with the reversibility of epigenetic modifications and alterations (including DNA methylation) in response to micro-environmental conditions, nutrition and drugs.

Our findings strongly suggest that *H. pylori* infection causes the aberrant DNA hypermethylation of specific genes and induces CIMP, an important epigenetic mechanism of the development and progression of gastric MALT lymphoma. The genes that were specifically hypermethylated by the *H. pylori* infection and disease progression could be used as highly sensitive epigenetic markers to detect *H. pylori* infection and the development and progression of gastric lymphoma, as well as to monitor the disease status.

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