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Promoter hypermethylation and protein expression of the *p16* gene: analysis of 43 cases of B-cell primary gastric lymphomas from China

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The *p16 (CDKN2a/INK4a)* gene is an important tumor-suppressor gene, involved in the *p16*/cyclin-dependent kinase/retinoblastoma gene pathway of cell cycle control. The p16 protein is considered to be a negative regulator of the pathway. Promoter hypermethylation resulting in inactivation of the *p16* gene has been found in various hematopoietic malignancies, including non-Hodgkin's lymphoma, and may play a role in transformation/clinical aggressiveness of those tumors. However, the p16 protein expression in primary gastric lymphoma has not been studied. In this study, we characterize protein expression and promoter hypermethylation of the *p16* gene in B-cell primary gastric lymphomas from China. In all, 43 cases of B-cell primary gastric lymphoma were investigated. They consisted of 24 (56%) cases of diffuse large-cell lymphoma, 12 (28%) cases of extranodal marginal zone lymphoma, six (14%) cases of extranodal marginal zone lymphoma with large-cell transformation and one (2%) case of follicular lymphoma. Loss of p16 protein expression was found in 34 (79%) out of 43 cases, while the remaining nine (21%) cases showed positivities for the p16 protein. All 43 cases were further characterized by methylation-specific polymerase chain reaction (PCR) to analyze *p16* promoter hypermethylation status. In total, 11 (26%) of 43 cases were positive for *p16* promoter hypermethylation. Among those, 10 (30%) out of the 33 cases negative for the p16 immunostaining showed promoter hypermethylation, whereas only one (10%) out of the 10 cases that were positive for the p16 immunostaining displayed promoter hypermethylation. Of the 43 cases, 30 had limited pathologic data at the time of resection. Primary gastric lymphoma involved extragastric sites (lymph node or liver) in 17 (57%) of 30 cases, while the remaining 13 (43%) cases were only limited to the stomach. Loss of p16 protein expression was found in 14 (82%) of 17 cases with extragastric involvement and in 11 (85%) of 13 cases without such involvement. In conclusion, loss of p16 protein expression is frequent in those B-cell primary gastric lymphomas and approximately one-third of such loss correlated with promoter hypermethylation. Despite limited pathologic data, loss of p16 protein expression appears not to be correlated with extragastric involvements.
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The *p16 (CDKN2a/INK4a)* gene is an important tumor-suppressor gene, involved in the *p16*/cyclin-dependent kinase/retinoblastoma gene pathway of cell cycle control, in which the p16 protein is considered to be a negative regulator involved in the inhibition of G1 phase progression.[1] The human p16 protein contains 156 amino acids and was first discovered in a yeast two-hybrid system to detect proteins that interact with human cyclin-dependent kinase.[2] The tumor-suppressor function of *p16* is attributed to its ability to inhibit the catalytic activity of the cyclin-dependent kinase 4-6/cyclin D complex that is required for phosphorylation of retinoblastoma protein.[3,4] Inactivation of *p16* gene plays an important role not only in tumorigenesis but is also considered to be a poor prognostic event in certain malignancies.[1–4] DNA methylation is a frequent mechanism of transcriptional silencing in human cancer.[5] Recently, aberrant methylation of multiple promoter-associated CpG islands of

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tumor-suppressor genes has been found in a variety of human malignancies. Promoter hypermethylation, in addition to gene deletion and point mutation of *p16* locus, has been found to be one of the main mechanisms of *p16* inactivation.[6–9] Hypermethylation of the 5′CpG islands of *p16* gene promotor region has been reported in colon, bladder, breast, head and neck, and lung carcinoma as well as in glioma, melanoma, leukemia and lymphoma.[10–15] In a previous study, we analyzed the histologic and immunologic features of primary gastric lymphomas from China and suggested that nuclear BCL10 expression may predict a poor prognosis with early extragastric involvement.[16] However, the status of *p16* expression in these lymphomas has not been studied. In this study, p16 protein expression patterns were analyzed and promoter methylation of *p16* gene in these primary gastric lymphomas was characterized.

Materials and methods

Tissue Acquisition

Paraffin-embedded tissues of resection ($n = 39$) and biopsy ($n = 4$) specimens of 43 primary gastric lymphomas were obtained from the files between 1996 and 2000 in Cancer Hospital, Chinese Academy of Medical Sciences and Qindao University Hospital. Diagnosis was verified by City of Hope pathologists using the recent WHO (World Health Organization) classification.[17] The available pathologic data concerning the status of extragastric involvement (such as lymph node and liver) at the time of surgical resection were also reviewed.

Immunohistochemical Staining for the p16 Protein

Immunohistochemical staining was performed using a monoclonal antibody against the p16 protein (Ab-4, clone 16P04 or JC2; NeoMarkers, Fremont, CA, USA). Positive p16 expression was defined as clear, nuclear and cytoplastic staining. The p16 immunohistochemical staining results were interpreted as follows: positive (+), if positive immunohistochemical staining in both nuclei and cytoplasm is present in more than 50% tumor cells; and negative (−), if positive p16 immunohistochemical staining is present in less than 10% tumor cells. The positive p16 immunohistochemical staining case typically consists of more than 50% of strongly stained tumor cells, whereas the negative p16 immunohistochemical staining case contains either no positive cells or in rare cases, less than 10% weakly stained tumor cells. In our study, cases are fairly easily separated into the positive or negative group because they either showed well-recognizable strong and diffuse stains (positive) or faint, scattered staining that is difficult to spot (negative). We did not encounter cases with staining intensity and percentage that fell between the criteria outlined above for positive and negative cases. Known positive and negative cases from our previous studies on head and neck carcinoma were used as control.[18] Two pathologists (QH and CYF) evaluated the immunohistochemical staining results independently.

Sample Collection and DNA Extraction

DNA samples were collected using the EX-WAX™ DNA Extraction Kit (Intergen Co.,. New York, NY, USA) from one 5-μm-thick tissue section, scratched from a glass slide. Human placental DNA (Sigma) was used as a negative control and CpGenome™ universal methylated human DNA (Intergen Co., New York, NY, USA) served as a positive control.

Bisulfite Modification of DNA for Methylation-specific PCR

DNA samples from the primary gastric lymphomas, negative and positive controls, respectively, were subjected to bisulfite modification prior to methylation-specific PCR using a CpGenome™ DNA modification Kit (Intergen Co., New York, NY, USA).

PCR Amplification and Primers

Amplification of the promoter region of the *p16* gene was carried out in a Touchgene Gradient Thermal Cycler (Techne Inc., Princeton, NJ, USA) in a 50 μl PCR reaction mixture containing 2μl of bisulfite-treated genomic DNA, dinucleotide triphospates (dNTPs) (each at 200 μM), primers (50 pmol each per reaction), 2.5 mM $MgCl_2$ and 1.25 U Hotstar Taq (Qiagen, Valencia, CA, USA) in 1 × PCR buffer. All reagents were supplied with the Qiagen Hotstar Taq Kit (Qiagen, Valencia, CA, USA). The only exception was the dNTP mix (Roche Molecular Biochemicals, Indianapolis, IN, USA).

Primers for *p16* gene were designed as follows: 5′-TTA TTA GAG GGT GGG GTG GAT TGT-3′ (sense) and 5′-CAA CCC CAA ACC ACA ACC ATA A-3′ (antisense) for the unmethylated reactions; 5′-TTA TTA GAG GGT GGG GCG GAT CGC-3′ (sense) and 5′-GAC CCC CGA ACC GCG ACC GTA A-3′ (antisense) for the methylated reactions as described previously.[8,15] Both primers were purchased from Operon Technologies Inc. (Alameda, CA, USA). The PCR conditions were as follows: initial denaturation and hot start at 95°C for 15 min, then 40 cycles consisting of 30 s at 95°C, 30 s at 60°C (unmethylated reactions) or 65°C (methylated reactions) and 1 min at 72°C followed by a final 5-min extension at 72°C. Positive and negative control DNA samples and controls without DNA were used for each set of PCRs.

Subcloning and DNA Sequencing of PCR Products

Amplified PCR products were ligated into a pCR4-TOPO vector and transformed into Escherichia coli (*E. coli*) using TOPO TA cloning Kit for sequencing (Invitrogen Life Technologies, Carlsbad, CA, USA). Plasmid DNA isolated from *E. coli* colonies was sequenced using an Applied Biosystems (Foster City, CA, USA) Model 377 DNA Sequencer as previously described.[19] The templates used for the sequencing were at 100 ng/μl. A T3 universal primer was used in all reactions at 1.6 μM. The Applied Biosystems Dye Terminator Kit version 2.0 was used according to the manufacturer's instructions.

Results

Histopathology of the Primary Gastric Lymphomas

The mean age for the patients was 51 years with a range from 15 to 77 years. The male/female ratio was 26:17. All 43 primary gastric lymphomas were of B-cell lineage demonstrated by immunohistochemistry analysis. Histologically, they consisted of 24 (56%) cases of diffuse large-cell lymphoma, 12 (28%) cases of extranodal marginal zone lymphoma, six (14%) cases of extranodal marginal zone lymphoma with large-cell transformation and one (2%) case of follicular lymphoma (Figure 1). The extranodal marginal zone lymphoma with large-cell transformation is defined by the presence of large aggregates or sheets of large lymphoid cells adjacent to the otherwise typical extranodal marginal zone lymphoma. The lymphomas expressed various B-cell-associated antigens, but all were positive for CD20. Some cases expressed CD23 (2/43), BCL-2 (31/43) and BCL-6 (16/43), and a subset also showed coexpression of T-cell-related antigen CD43 (13/43). *Helicobacter pylori* (*H. pylori*) organisms were detected in 88% of all cases by histologic examination on hematoxylin and eosin (H & E)-stained sections.

p16 Protein Expression by Immunohistochemistry

The expression of p16 protein was analyzed by immunohistochemistry using a specific antibody reacting against p16 protein. Loss of p16 protein expression (negative) was found in 34 (79%) out of 43 cases, while the remaining nine cases (21%) showed p16 protein expression (positive) in lymphoma cells (Figure 2). Among these 34 p16 immunostaining negative cases, there were 18 cases of diffuse large-cell-lymphoma, five cases of marginal zone lymphoma/diffuse large-cell lymphoma and 11 cases of marginal zone lymphoma.

p16 Gene Promoter Hypermethylation by Methylation-specific PCR Analysis

All 43 cases were further characterized by methylation-specific PCR analysis to analyze *p16* promoter hypermethylation status. In all, 11 (26%) of 43 cases were positive for *p16* promoter hypermethylation. Among these, 10 out of the 33 cases negative for the p16 protein immunostaining showed promoter hypermethylation, whereas only one (10%) out of

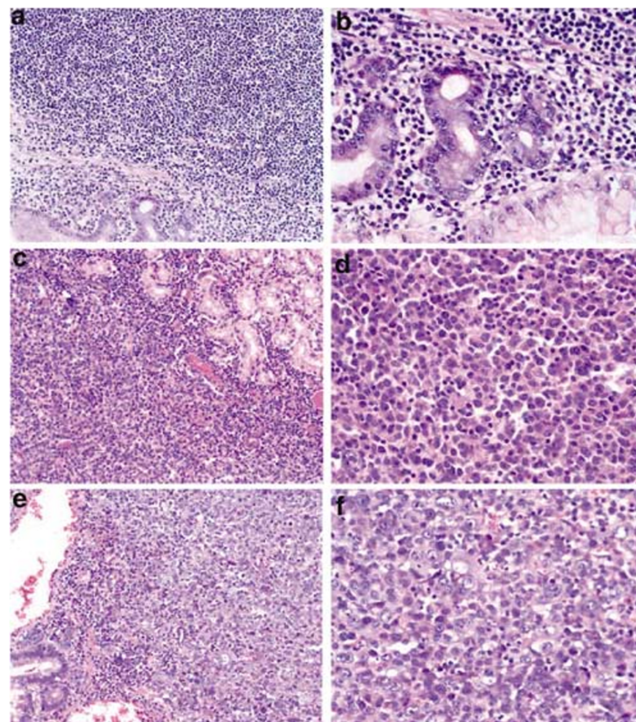

Figure 1 Histologic analysis of primary gastric lymphomas from China. Marginal zone lymphoma (MZL) of case #39 at low magnification (× 200) (**a**) and high magnification (× 400) (**b**); marginal zone lymphoma with large-cell transformation (MZL/DLCL) of case #36 at low magnification (× 200) (**c**) and high magnification (× 400) (**d**); diffuse large-cell lymphoma (DLCL) of case #25 at low magnification (× 200) (**e**) and high magnification (× 400) (**f**).

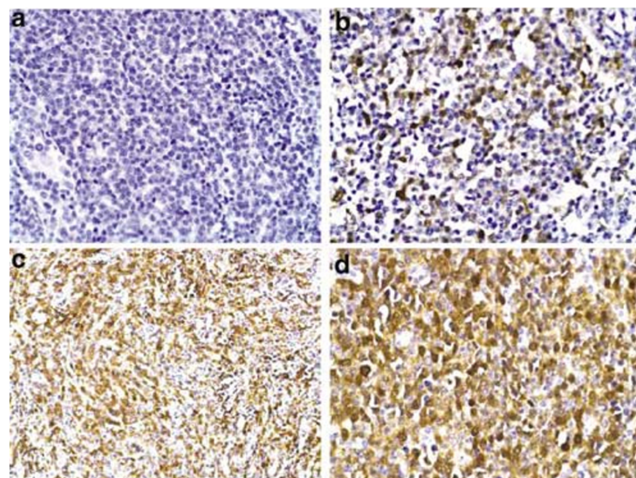

Figure 2 Immunohistochemical analysis for *p16* expression in primary gastric lymphomas. (**a**) Negative staining of case #30 (× 400); (**b**) Focal positivity for p16 expression of case #40 (× 400); (**c**) Diffuse positivity of p16 expression of case #5 (× 200) and (**d**) Diffuse positivity of p16 expression of case #3 (× 400).

the 10 cases that were positive for the p16 protein immunostaining displayed promoter hypermethylation (Figure 3; Table 1). The methylation status of the methylation-specific products was confirmed by direct DNA sequencing (Figure 4)

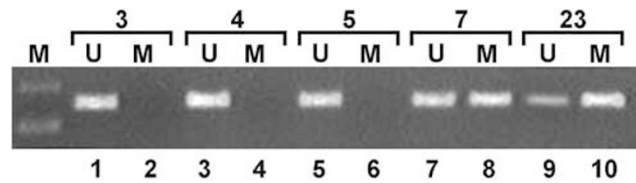

Figure 3 Bisulfite-modified genomic DNA harvested from cases #3 (lanes 1, 2), #4 (lanes 3, 4), #5 (lanes 5, 6), #7 (lanes 7, 8) and #23 (lanes 9, 10) was used in PCR reactions with the unmethylated-(U) or methylated-specific (M) primer sets.

Correlation of p16 Protein Expression with Available Pathologic Data

Of the 43 cases, 30 resection specimens had available pathologic data. Primary gastric lymphomas involved extragastric sites (lymph node or liver) in 17 (57%) of 30 cases, while the remaining 13 cases (43%) were limited to the stomach only at the time of resection. Absence of p16 protein expression was found in 14 (82%) of 17 cases with extragastric involvement and in 11 (85%) of 13 cases without such involvement (Table 1).

Table 1 Summary of pathologic and experimental data in 43 PGL patients

Case no.	*Sex*	*Age*	*Diagnosis*	*LN*	*p16 IHC*	*p16 MSP*
Case 1	F	15	DLCL	2/9	–	–
Case 2	F	42	DLCL	NA	–	–
Case 3	F	40	DLCL	3/12	+	–
Case 4	M	31	DLCL	3/20	+	–
Case 5	F	35	DLCL/MZL	NA	+	–
Case 6	M	65	DLCL	NA	–	–
Case 7	M	66	DLCL	0/13	–	+
Case 8	M	77	DLCL	NA	+	–
Case 9	F	36	MZL	NA	–	+
Case 10	F	52	DLCL	NA	–	–
Case 11	M	38	MZL	0/4	+	–
Case 12	M	70	DLCL	NA	–	–
Case 13	M	71	DLCL	10/23	–	–
Case 14	F	62	DLCL/MZL	15/23	–	+
Case 15	F	48	DLCL	NA	–	–
Case 16	F	49	DLCL	NA	+	–
Case 17	M	64	DLCL/MZL	0/15	–	–
Case 18	M	55	DLCL	Liver[a]	–	–
Case 19	M	80	MZL	NA	+	+
Case 20	M	75	MZL	2/4	–	+
Case 21	M	39	MZL	NA	–	–
Case 22	M	69	DLCL	4/25	–	–
Csae 23	F	54	DLCL	20/40	–	+
Case 24	M	51	MZL	0/37	–	+
Case 25	M	63	DLCL	NA	–	–
Case 26	M	57	FL	3/25	+	–
Case 27	M	65	MZL	0/37	–	–
Case 28	F	49	DLCL	0/12	–	–
Case 29	F	40	MZL	14/37	–	–
Case 30	M	51	DLCL	18/37	–	+
Case 31	M	64	DLCL	17/34	–	–
Case 32	F	45	MZL	0/16	–	–
Case 33	M	74	MZL	2/24	–	–
Case 34	F	69	DLCL	0/8	+	–
Case 35	M	50	MZL	0/17	–	–
Case 36	F	38	DLCL/MZL	26/26	–	–
Case 37	F	52	DLCL/MZL	0/25	–	–
Case 38	M	49	DLCL	7/10	–	–
Case 39	F	65	MZL	15/42	–	–
Case 40	M	45	DLCL	0/24	+	–
Case 41	M	43	DLCL	0/23	–	+
Case 42	M	43	DLCL/MZL	0/16	–	+
Case 43	M	60	DLCL	NA	–	+

[a]liver involvement.
LN: lymph node status ('*X*/*Y*'; *X* = number of nodes positive for lymphoma involvement; *Y* = total number of lymph nodes); IHC: immunohistochemical staining; MSP: methylation-specific PCR; DLCL: diffuse large-cell lymphoma; MZL: marginal zone lymphoma; FL: follicular lymphoma; NA: data not available.

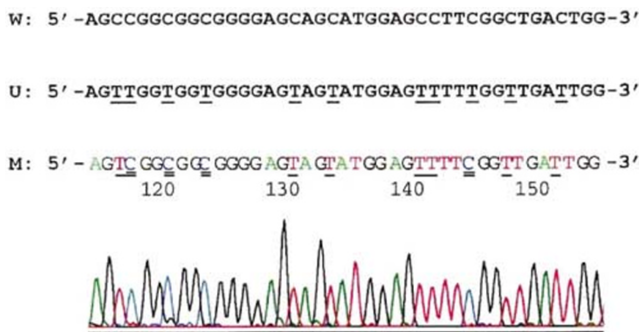

Figure 4 DNA sequence of methylation-specific PCR products: W, a wild-type DNA sequence; U, bisulfite-modified unmethylated DNA sequence. All cytosines (C) are converted to thymines (T) following bisulfite modification (single underlined). M, bisulfite-modified methylated DNA sequence (chromatogram from a cloned DNA sample from case 7 in Figure 3 and Table 1). All cytosines (C) at the non-CpG sites are converted to thymines (T) following bisulfite modification (single underlined). By contrast, all cytosines (C) at the CpG sites are methylated, and as a result, they remain as cytosines (C) following bisulfite modification (double underlined).

Discussion

The development of primary gastric lymphoma is believed to be a multistep process in which genetic and epigenetic events accumulate as a result of chronic exposure to antigen stimulation, leading eventually to clonal expansion, resulting from a loss of cell cycle control, selected cell growth and finally the development of clinically overt cancer.[20,21] Primary gastric lymphoma in Western countries is largely composed of marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT), which is closely related to *H. pylori* infection.[20,21] Primary gastric lymphoma development is considered to be a series of genomic aberrations that contribute, step by step, to increasing genomic instability and the ultimate establishment of a population of autonomously growing neoplastic cells.[20] The translocation t(11;18) (q21;q21) was identified in a fraction of low-grade MALT lymphoma cases, ranging from 21 to 60% of examined tumors.[21] The translocation t(1;14) involving *BCL10* oncogene was described as another characteristic abnormality in low-grade MALT lymphoma, albeit at a much less frequency.[22,23] Recent studies suggested that t(11;18) translocation, closely associated with nuclear BCL10 expression, was correlated with advanced MALT lymphoma, which might not respond to anti-*H. pylori* therapy.[22,23]

In present study, we found that the lack of p16 protein expression is quite frequent (79%) in primary gastric lymphoma from China. Although the p16 protein expression status of primary gastric lymphomas is not known in the literature, the frequency of *p16* inactivation in this study appears to be somewhat higher than that in previous studies on other site B-cell lymphomas/leukemias of Western population. Sanchez-Beato *et al*[24] detected inactivation of *p16* in 43.5% cases of aggressive diffuse large-cell lymphoma, Dalle *et al*[25] found loss of *p16* expression in 38.1% of acute lymphoblastic leukemia and Child *et al*[26] identified 43% *p16* inactivation in cutaneous B-cell lymphoma. Further studies would be needed to compare the frequency of p16 inactivation of primary gastric lymphomas in the Western population. The relationship of p16 inactivation or aberrant genetic or epigenetic changes of the *p16* gene with tumor progression or patient survival has been investigated in various forms of human tumor, such as breast cancer,[26,27] cutaneous melanoma,[28,29] colorectal carcinoma,[10] central nervous system malignancies,[29–31] lung cancer,[12] and head and neck carcinoma[32,33] with no general consensus. However, there appears to be evidence supporting a role for *p16* inactivation in the transformation of low-growth fraction lymphomas into their aggressive variants.[34,35] The histological progression of follicular lymphoma to aggressive large-cell lymphoma is frequently accompanied by 9p21 deletions that often result in reduction or loss of p16 expression. In mantle cell lymphoma, *p16* deletions are associated with aggressive forms, a finding that suggests the possibility that cyclin D1 overexpression and *p16* inactivation might not be completely redundant alterations.[36]

Mechanisms of *p16* silencing include loss of heterozygosity, homozygous deletion, point mutation and promoter region hypermethylation. The latter appears to be a major reason for *p16* inactivation in many previous studies. There appears to be good agreement of *p16* promoter methylation with lack of protein expression in B-cell lymphomas.[24] Sanchez-Beato *et al*[24] found that *p16* inactivation was seen in 27 (44%) out of 62 cases of aggressive B-cell lymphomas as a result of hypermethylation (20 of 62 cases), 9p21 deletion (seven of 44 cases) or *p16* mutation (two of 62 cases). In the current study, we found that absence of *p16* expression was identified in 10 (30%) of 33 primary gastric lymphoma cases with promoter hypermethylation of the gene. In contrast, only one (10%) of 10 cases with positive p16 immunostaining showed promoter hypermethylation. The lack of overall correlation between *p16* promoter hypermethylation and protein expression could be attributed to a large number of cases (70%) in which lack of p16 protein expression was not accompanied by the presence of *p16* promoter methylation. Therefore, other mechanisms must contribute to the inactivation of *p16* gene in those primary gastric lymphomas, such as point mutation and/or gene deletion. In one case, p16 protein expression is accompanied by positive *p16* promoter methylation (Table 1). This has been previously documented in another study[18] and could be due to intratumor heterogeneity of methylation pattern or the presence of minor component of tumor cells that showed epigenetic silencing of the *p16* gene. Nevertheless, these results indicate that approximately

one-third of *p16* inactivation correlated with promoter hypermethylation and suggest that epigenetic silence of tumor-suppressive gene may play a role in tumorigenesis of those primary gastric lymphomas.

BCL10, an apoptosis regulatory molecule containing an amino-terminal caspase-recruitment domain, is overexpressed as a result of t(1;14) (p22;q32), a translocation that is also recurrently detected in a subgroup of MALT lymphomas. Abnormal nuclear expression of BCL10 and the t(11;18) translocation tend to appear together, and their joint occurrence is associated with advanced MALT lymphoma.[21,37] The present study also suggests that the absence of *p16* expression appears to be correlated with nuclear expression of BCL10 in these lymphomas from our previous study (manuscript in preparation).[16] Six (67%) of nine cases that expressed the p16 protein were negative for BCL10 expression, whereas 22 (65%) of 34 case that were negative for the p16 protein expressed the BCL10 gene. However, no statistic significance can be obtained based on the relatively small number of case studies. Therefore, the exact relationship between *p16* gene inactivation and BCL10 expression remains unclear.

Although lack of p16 protein expression is present in the majority of the primary gastric lymphomas, there is no significant difference between p16 positive or negative primary gastric lymphomas with regard to the frequency of extragastric involvement at the time of resection. Inactivation of *p16* gene does not appear to be correlated with extragastric involvement in these patients. However, this view may even be of less significance due to not only the small number of cases analyzed but also limited pathologic data available for statistical analysis. More studies are needed to further establish the exact roles of *p16* inactivation in the development of primary gastric lymphomas.

In summary, we found that loss of p16 protein expression was seen in up to 79% of the primary gastric lymphomas and that approximately one-third of loss of p16 protein expression was associated with promoter hypermethylation. Despite limited pathologic data, loss of p16 protein expression appears not to be correlated with extragastric involvements.

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