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p16/INK4a gene methylation is a frequent finding in pulmonary MALT lymphomas at diagnosis

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***p16/INK4a* gene alterations have been associated with tumor progression in lymphoid malignancies. However, their significance in mucosa-associated lymphoid tissue (MALT) lymphoma is unclear. We investigated *p16* gene methylation and mutation in a large series of untreated cases of pulmonary MALT lymphoma and diffuse large B-cell lymphoma (DLBL), and correlated *p16* gene alterations with a MALT lymphoma-specific *API2–MALT1* fusion and the clinicopathologic features of MALT lymphoma. The *API2–MALT1* fusion was detected by multiplex reverse transcription polymerase chain reaction in 25/60 (42%) cases of MALT lymphoma, but none of 11 DLBLs. Methylation-sensitive single-strand conformation analysis showed that *p16* gene methylation was frequently detected in 36/60 (60%) cases of MALT lymphoma. The gene was similarly methylated in DLBL cases (6/11, 55%). A *p16* gene mutation was found in one (*p16* gene-methylation) of 44 MALT lymphomas and in none of six diffuse large B-cell lymphomas. Statistical analysis showed that the *p16* gene methylation status did not correlate with *API2–MALT1* fusion or any of the clinicopathologic factors including serum LDH, clinical stage, and increased large cells. These findings suggest that *p16* methylation is not associated with tumor progression, but may be an early event in MALT lymphomagenesis that might be maintained through the progression of the tumor.**
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Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) runs an indolent clinical course, involves extranodal sites, and exhibits a wide spectrum of histologic features consisting of centrocyte-like cells, lymphoepithelial lesions, and follicular colonization. Pre-existing chronic inflammation, for example, *Helicobacter pylori* gastritis, Hashimoto's thyroiditis, or Sjogren's syndrome, is considered to be important in the development of MALT lymphoma. Progression to diffuse large B-cell lymphoma (DLBL) may occur in some cases.[1] An *API2–MALT1* gene fusion was cloned from a recurring chromosomal translocation of t(11;18)(q21;q21).[2,3] Since this fusion is often the sole gene abnormality found in MALT lymphoma and has not been found in other types of lymphoma, *API2–MALT1* fusion has been considered as a specific MALT lymphoma-causing alteration.[4–6] *API2* is a member of the *IAP* (inhibitor of apoptosis) gene family, and is essential for the suppression of apoptosis.[7] MALT1 is identical to a novel caspase-like protein termed paracaspase,[8] although its biologic function remains unclear. It has been suggested that *API2–MALT1* fusion transcripts lead to an increased inhibition of apoptosis and thereby help MALT lymphoma cells to survive.[9]

Cell cycle progression is regulated by complexes formed between cyclins and cyclin-dependent kinases (CDKs). CDK4 and CDK6 bind to D-type cyclins in the G1 phase of the cell cycle and control G1/S transition through the phosphorylation of retinoblastoma protein. The activity of cyclinD–CDK4/6 complexes is subjected to additional levels

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of regulation, including the association with CDK inhibitors.[10] The *p16/INK4a* gene, mapped to 9q21, encodes for nuclear protein that can block cell cycle progression by effectively inhibiting the kinase activity of CDK4/6, thereby exerting a negative control on cell proliferation. *p16* gene has been considered as a tumor-suppressor gene, and among CDK inhibitors, only *p16* gene is frequently silenced in a variety of tumors by epigenetic or genetic abnormalities including promoter CpG methylation and less frequently, allelic loss and gene mutation.[10,11] In lymphoid malignancies, *p16* gene silencing, mainly induced by gene methylation, is frequently found in Hodgkin and non-Hodgkin lymphomas,[12–14] and has been associated with tumor progression.[15–19] However, it has also been suggested that the methylation of this gene is one of the early events in the development of lymphoid malignancies.[20–22]

MALT lymphoma constitutes more than 80% of all primary lung lymphomas.[23] It should be noted that the involvement of *API2–MALT1* fusion is particularly high (up to half of all cases) in pulmonary MALT lymphoma compared with MALT lymphomas at other sites,[24] and may define a distinctive clinicopathologic subgroup of pulmonary MALT lymphomas.[25] Therefore, pulmonary MALT lymphoma is a good model for study of lymphomagenesis. Like other lymphoid malignancies, the *p16* gene silencing has been associated with tumor progression in MALT lymphoma.[16] However, recent studies showed frequent *p16* gene methylation in gastric MALT lymphomas at diagnosis, suggesting that it might be an early event.[26,27] To clarify lymphomagenetic and clinicopathologic significances of *p16* gene alterations, we investigated the promoter hypermethylation and sequence mutation of this gene in a large series of pulmonary MALT lymphomas, and correlated these changes with *API2–MALT1* fusion.

Materials and methods

Case Selection

Specimens of MALT lymphoma ($n=60$) and diffuse large B-cell lymphoma ($n=11$) obtained at the initial presentation of the patients were fixed in formalin, and embedded in paraffin. The diagnosis of MALT lymphoma was made according to the criteria of the World Health Organization classification.[1] All of the cases were within the morphological boundaries of MALT lymphoma, and exhibited the following immunophenotype: CD20+, CD79a+, CD3−, CD45RO−, CD5−, CD10−, CD23−, and cyclin D1−. A precise microscopic observation revealed that 46 of 60 cases were 'typical' in histology.[1,25] However, seven cases showed an increased number of large cells (5% < large cells < 10%, without solid or sheet-like proliferations), and seven cases exhibited marked plasmacytoid differentiation (plasmacytoid tumor cells > 90%). The mean age of MALT lymphoma patients was 62.1 years with a range of 31-94 years. The male/female ratio was 22/38. The mean age of DLBL patients was 61 years with a range of 37-84 years. The male/female ratio was 7/4. Some of the tumors in this series were included in our previous study.[25]

Extraction of Genomic DNA and Total RNA

On examination of tissue sections under a dissecting microscope, selected areas were retrieved using a serial H&E section as a guide. Genomic DNA and total RNA were extracted in digestion buffer containing proteinase K as previously described.[25,28] For all cases included in this study, monoclonality was confirmed by a polymerase chain reaction (PCR) clonality assay of the immunoglobulin heavy chain gene and/or immunohistochemistry for immunoglobulin light chains as previously described.[25] We also confirmed that all cases had a sufficient quality of RNA by reverse transcription (RT)-PCR for beta-actin mRNA (190 base).[28] As normal controls, 12 lung and 14 lymph node specimens were used and these were obtained from carcinoma patients during surgery.

Multiplex RT-PCR for the *API2–MALT1* Fusion Transcript

The *API2–MALT1* fusion transcript was detected using archival paraffin sections according to the method we previously reported.[28] All eight variant fusion transcripts that have been reported to date can be detected with this assay (Figure 1a; accession number L49432 for *API2* and accession number AF130356 for *MALT1*). Briefly, total RNA was subjected to first-round multiplex one-tube RT-PCR, then to second-round nested multiplex PCRs (three in parallel: second PCR-A, second PCR-B, and second PCR-C). This RT-PCR assay using paraffin tissues is highly efficient and has 94% of the sensitivity and 100% of the specificity obtained with RT-PCR using frozen materials.[25]

DNA Bisulfite Modification and Methylation-Specific Single-Strand Conformation Analysis (MS-SSCA)

Genomic DNA extracted from lymphoma samples and a Raji lymphoma cell line (methylation-positive control) were examined for *p16* gene methylation by MS-SSCA.[29] In brief, DNA was first heated at 98°C for 10 min, and then for another 10 min at 37°C with the addition of freshly prepared 3 N NaOH. After addition of 10 nmol/l hydroquinone and 4.8 mol/l sodium bisulfite, the mixture was incubated at 55°C overnight. DNA modification was completed by adding 0.2 mol/l NaOH/90% ethanol. A 194 bp fragment of the *p16* gene promoter was amplified by

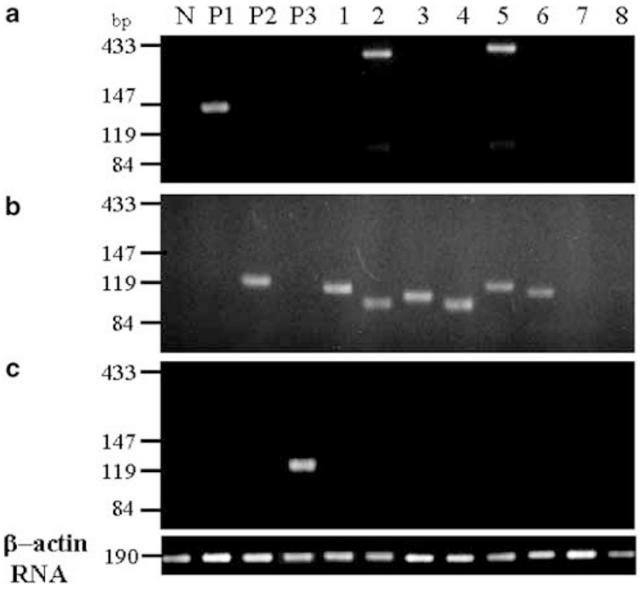

Figure 1 Detection of the *API2–MALT1* fusion transcript by multiplex RT-PCR. (**a**) Second PCR-A, (**b**) Second PCR-B, (**c**) Second PCR-C; N, negative control (normal lymph node); P1, P2, P3, positive controls for Second PCR-A, -B, and -C, respectively; Lanes 1 – 6, pulmonary MALT lymphomas positive for the *API2–MALT1* fusion transcript; lanes 7 and 8, pulmonary MALT lymphomas negative for the fusion transcript. Note that the fusion transcript was detected in both Second PCR-A and Second PCR-B (lanes 2 and 5). Beta-actin mRNA (190 base pairs) is amplified in all cases.

PCR using the following primers specific to the modified sequences: 5′-GGGGGAGATTTAATTTGG-3′ and 5′-CAACCCCTCCTCTTTCTT-3′. After denaturation, the PCR samples were immediately electrophoresed on a 30% MDE gel (Cambrex Bio Science Rockland, Rockland, ME, USA) with a constant voltage of 10 V/cm for 4 h at 20°C. Gels were then stained with a SYBR Green I (Molecular Probes, Eugene, OR, USA), and bands were visualized with UV light.

Mutation Analysis of the *p16* Gene by PCR-Single-Strand Conformation Polymorphism (SSCP)

PCR amplification of the *p16* gene was performed using a primer pair for exon 1 (GGGCGGCGGGGAG CAGC and GCTGCAGACCCTCTACCCAC) and two primer pairs for exon 2 (5′-ACCCTGGCTCTGAC CATTCTGT-3′ and 5′-GGCATCGCGCACGTCCAGC-3′ and 5′-GGGCTTCCTGGACACGCTGGT-3′ and 5′-GGAAGCTCTCAGGGTACAAATTCTCA-3′).[30] After denaturation, the PCR products were electrophoresed and separated using a GeneGel Exel 12.5/24 Kit (GenePhor System, Amersham Biosciences, Piscataway, NJ, USA) at two different temperatures, 15°C and 5°C, and the gels were subjected to silver staining. Mutational bands were excised from the gels and reamplified using the same PCR primers. The amplified DNA was directly sequenced by means of cycle sequencing with dye-labeled terminators (BigDye Terminators, Applied Biosystems, Foster City, CA, USA) and analyzed on an automated DNA sequencer.

Statistical Analysis

Statistical evaluation of data from two groups was performed using Fischer's exact test and Student's *t*-test. All analyses were two-tailed. A probability value of $P<0.05$ for each test was regarded as statistically significant.

Results

API2–MALT1 Fusion Transcript

The multiplex RT-PCR detected *API2–MALT1* fusion in 25/60 (42%) cases of pulmonary MALT lymphoma. The bands varied in size depending on the breakpoints and primer sets used (Figure 1). The *API2* breakpoints were restricted to 1446 (accession number. L49432) in all cases with one exception which had a breakpoint at 1701. In contrast, the *MALT1* breakpoint (accession number. L49432) varied with nine cases at 814, 10 cases at 1123, and six cases at 1150. *API2–MALT1* fusion transcript was negative for MALT lymphomas with increased large cells (0/7 cases) and DLBLs (0/11).

p16 Gene Methylation Analysis by MS-SSCA

The *p16* gene methylation was detected in 36/60 (60%) cases of MALT lymphoma: 32/53 (60%) and 4/7 (57%) cases of MALT lymphomas without and with an increased component of large cells, respectively (Figure 2). The gene methylation was detected in 6/11 (55%) cases of DLBL. Statistically significant difference was not obtained between any two of the above groups. *p16* gene methylation was detected in none of 12 normal lung tissues or 14 normal lymph node specimens.

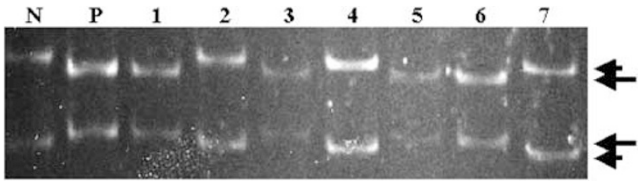

Figure 2 MS-SSCA of *p16* gene methylation. N, negative control; P, positive control (Raji lymphoma cell line); lanes 1, 3, 5, and 6, pulmonary MALT lymphomas showing *p16* gene methylation; lanes, 2, 4, and 7, pulmonary MALT lymphomas showing no methylation. In comparison with bands of normal controls (short arrows), those of *p16* gene methylation cases show a mobility shift (long arrows).

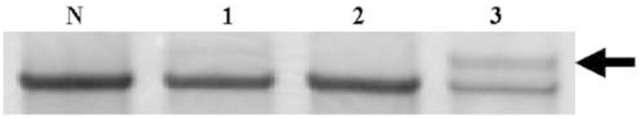

Figure 3 PCR-SSCP of *p16* gene mutation. An aberrant SSCP pattern for exon 2 (lane 3, arrow) resulted in a missense mutation. N, negative control.

p16 Gene Mutation Analysis by PCR-SSCP

The *p16* whole coding region was amplified in 44 cases of MALT lymphoma and eight cases of DLBL. One MALT lymphoma case (2%) showed a nucleotide substitution of G to A at codon 135 that changed GGG (glycine) to GAG (glutamic acid) (Figure 3). This case showed 'typical' MALT lymphoma histology and *p16* gene methylation by MS-SSCA. None of the DLBL cases possessed *p16* gene mutation.

Correlation of *p16* Gene Methylation with Clinicopathologic Factors and *API2–MALT1* Fusion

Available clinicopathologic factors included age, sex, chief complaints, autoimmune disease, serum LDH, clinical stage, B-symptoms, 'typical' histology, increased large cells, and marked plasmacytoid differentiation. *p16* gene methylation correlated with none of these factors, nor did it correlate with *API2–MALT1* fusion status (Table 1).

Table 1 Correlation of *p16* gene methylation with clinicopathologic and molecular factors

	p16 *gene*		
	Methylated (n = *36*)	*Unmethylated* (n = *24*)	P
Clinical factors			
Age			
Mean	60.3	64.8	NS
<60	17	6	
>60	19	18	NS
Sex			
Male	12	10	
Female	24	14	NS
Chief complaints			
Present	10	5	
Absent	26	19	NS
Autoimmune disease			
Positive	3	3	
Negative	33	21	NS
LDH (n = 55)			
Elevated	5	1	
Normal	27	22	NS
Stage (n = 55)			
I, II	24	21	
III, IV	8	2	NS
B-symptoms (n = 55)			
Present	4	2	
Absent	28	21	NS
Histopathologic factors			
Histology			
'Typical'	29	17	
'Atypical'	7	7	NS
Increased large cells			
Yes	4	3	
No	32	21	NS
Marked plasmacytoid differentiation			
Yes	3	4	
No	33	20	NS
API2–MALT1 *fusion*			
Positive	15	10	
Negative	21	14	NS

NS, not significant.

Discussion

p16/INK4a gene methylation was detected in diagnostic specimens of more than half of the cases of not only MALT lymphoma but also MALT lymphoma with increased large cells and DLBL. The MS-SSCA used in this study is a rapid and specific procedure for detecting gene methylation, especially in paraffin-embedded tissues, and gives fewer false-positive results.[29] The influence of *p16* gene methylation on background non-neoplastic cells is considered to be minimal because no methylation was detected in normal pulmonary tissues in the present and previous studies.[31] *p16* gene mutation was found in one MALT lymphoma case, which involved methylation of the *p16* gene. Consequently, gene methylation was a major occurrence among *p16* gene alterations in our study, which is in accord with previous reports.[10–16] Gene deletion is another means of *p16* gene silencing, but has been rare in MALT lymphomas.[32,33] We attempted to detect *p16* gene deletions using seven microsatellite markers,[16,34] however, most of the cases were not amplifiable or informative. We found no evidence of *p16* deletion in eight cases (six of MALT lymphoma and two of DLBL) that were suitable for evaluation (unpublished data).

The most important finding of our study is that *p16* gene was frequently methylated in pulmonary MALT lymphomas (60% of the cases) at diagnosis. It was methylated with similar frequencies in MALT lymphomas with increased large cells (57%) and DLBLs (55%), and its gene methylation did not correlate with any of the clinical factors such as serum LDH, stage, and B-symptoms. *p16* gene methylation has been considered to be a molecular event associated with the progression of MALT lymphoma to its high-grade counterpart.[15–19] However, our findings suggest that *p16* gene methylation is not associated with the tumor progression but rather may contribute to the

development of MALT lymphoma. Although the gene methylation was detected in DLBL as frequently as MALT lymphoma in our study, this observation may be explained by maintenance of the early phase gene abnormality through the history of the tumor. The hypothesis that *p16* gene methylation is an early event in MALT lymphomagenesis may be supported by the recent findings that *p16* gene repression is crucial for immortalization of several human cells[35,36] and by the frequent *p16* gene methylation found in monoclonal gammopathy of undetermined significance, in the plaque phase of mycosis fungoides, and in other premalignant lesions.[20–22,37]

Several MALT lymphoma-specific or -associated gene alterations have been reported, such as translocations between the *immunoglobulin heavy chain* gene and *BCL10* gene or *MALT1* gene, trisomy 3, and trisomy 18, as well as *API2–MALT1* fusion.[38] These seem to be mutually exclusive, and constitute up to 80% of the pulmonary MALT lymphoma cases.[38] A recent study of an *API2–MALT1* transgenic mouse model showed that the expression of the fusion protein did not induce spontaneous development of MALT lymphomas,[39] suggesting that the fusion gene alone would not be sufficient for MALT lymphoma development. This would be also the case for other gene alterations. In this study, while *p16* gene methylation was not directly associated with *API2–MALT1* fusion, we speculate that it is important as one of the early events of MALT lymphomagenesis and plays a role in tumor development in cooperation with several MALT lymphoma-specific or -associated gene alterations. To date, several studies have shown that methylation-associated silencing of tumor suppressor genes is one of the cancer-predisposing hits described in Knudson's two hit theory.[11]

Unlike genetic alterations, changes in DNA methylation are potentially reversible. This possibility has attracted considerable attention from a therapeutics standpoint. Nucleoside-analogue inhibitors of DNA methyltransferases, such as 5-aza-2′-deoxycytidine, are able to demethylate DNA and restore silenced gene expression.[40,41] Recently, some non-nucleoside inhibitors of DNA methyltransferases have also been reported as promising candidate agents for future cancer therapies based on epigenetics, including procainamide and procaine.[42] The clinical and therapeutic significance of these agents in pulmonary MALT lymphoma should be investigated.

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