

# Short Communication

## API2-MALT1 Chimeric Transcripts Involved in Mucosa-Associated Lymphoid Tissue Type Lymphoma Predict Heterogeneous Products

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**Recent progress in molecular analysis of low-grade B cell lymphoma has revealed that API2 at 11q21 and a novel gene, MALT1 at 18q21, are involved in t(11;18)(q21;q21), a characteristic chromosome aberration for mucosa-associated lymphoid tissue (MALT) type lymphoma. We describe here the establishment of a reverse transcription-polymerase chain reaction (RT-PCR) assay that we used to analyze 22 cases of MALT lymphoma. All five cases that were shown to possess t(11;18)(q21;q21) showed the specific amplification of API2-MALT1 chimeric transcripts. Of the remaining 17 cases for which cytogenetic data were not available, three cases demonstrated the presence of fusion transcripts, indicating that a significant percentage of MALT lymphoma cases of the present series appeared to possess t(11;18). A single fragment was observed in each of these cases, but the size varied from case to case. Sequencing analysis revealed that there are two breakpoints in API2 and three in MALT1, and that all of the fusion transcripts are in-frame. On the basis of these results, four kinds of chimeric proteins can be predicted for the present series. Thus, the RT-PCR assay used here should serve as an effective molecular tool for understanding molecular pathogenesis and the clinical significance of API2-MALT1 for MALT lymphomas. (*Am J Pathol* 2000, 156:807–812)**

In B cell lymphoma, various oncogenes have been identified to be transcriptionally deregulated as a result of translocation with immunoglobulin genes,<sup>1</sup> including the *c-myc* gene in Burkitt's lymphoma,<sup>2</sup> the cyclin D1 gene in mantle cell lymphoma,<sup>3,4</sup> the BCL2 gene in follicular lymphoma,<sup>5</sup> and the BCL6 gene in diffuse large cell lymphoma.<sup>6</sup> Recent advances in the research of these specific gene alterations have enabled us to investigate the pathogenesis of hematolymphoid malignancies, as well as to use these genetic techniques for clinical applications, for example, as an aid for diagnosis and for monitoring of minimal residual diseases.

Malignant lymphoma of mucosa-associated lymphoid tissue (MALT) was first described by Isaacson and Wright.<sup>7</sup> Later, this type of lymphoma was characterized by a representative histological appearance with lympho-epithelial lesions and follicular colonization, an indolent clinical course, and frequent multicentric and extranodal involvement including the gastrointestinal tract, lung, thyroid, and mammary, salivary, and lachrymal glands.<sup>8</sup> Now MALT lymphoma is recognized as constituting a distinct clinicopathological disease entity. On the basis of its supposed cell origin, this lymphoma has been categorized in extranodal marginal zone B cell lymphoma for the revised European-American lymphoma (REAL) classification.<sup>9</sup> MALT lymphomas are sometimes associated with chronic inflammation triggered by chronic infection or autoimmune disorders, such as *Helicobacter pylori* gastritis, Sjögren's syndrome, and Hashimoto's thyroiditis.<sup>10–12</sup> This suggests that the proliferation of the lymphoma cells may depend on the presence of activated,

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**Table 1.** Clinicopathological Features of MALT Lymphoma Patients

Case	Age (years)	Sex	Origin	Histology	Stage	Other sites of involvement	Karyotype	RT-PCR product
1*	45	F	Lung	LG	IVA	Hilar LN, Pleural effusion	46,XX,t(11;18)(q21;q21)	+
2*	51	M	Lung	LG	IA	—	46,XY,t(11;18)(q21;q21)	+
3*	63	F	Lung	LG	IA	—	46,XX,t(11;18)(q21;q21), inv(16)(p13q22)	+
4*	45	M	Colon	LG+H	IIA	Mesenteric LN	46,XY,t(11;18)(q21;q21)	+
5*	56	M	Colon	LG	IA	—	46,XY,t(11;18)(q21;q21)	+
6	61	M	Lung	LG	IIA	Orbit	N.A.	—
7	76	M	Lung	LG	IA	—	N.A.	+
8	63	F	Lung	LG	IA	—	N.A.	+
9	56	M	Lung	LG	IIA	Salivary gland	N.A.	—
10	74	F	Lung	LG	IA	—	N.A.	—
11	57	F	Lung	LG	IA	—	N.A.	—
12	72	F	Stomach	LG+H	IIA	Gastric LN	N.A.	—
13	39	M	Stomach	LG	IA	—	N.A.	+
14	38	M	Stomach	LG	IA	—	N.A.	—
15	67	M	Stomach	LG+H	IIIA	Paratracheal LN Retroperitoneal LN	N.A.	—
16	54	F	Stomach	LG	IA	—	N.A.	—
17	46	M	Stomach	LG	IA	—	N.A.	—
18	60	F	Stomach	LG	IA	—	N.A.	—
19	45	F	Stomach	LG	IA	—	N.A.	—
20	27	M	Stomach	LG+H	IA	—	N.A.	—
21	54	F	Stomach	LG+H	IIA	Gastric LN	N.A.	—
22	63	M	Stomach	HG+L	IVA	Testis, Orbit Retroperitoneal LN	N.A.	—

\*Cases 1 through 5 are identical to the cases previously studied.<sup>18</sup>

LG, low grade; LG+H, low grade with partially high grade component; HG+L, high grade with residual low grade component; LN, lymph node; N.A., not available.

antigen-driven T cells.<sup>13</sup> The effectiveness of antibacterial therapy for gastric MALT lymphoma poses problems regarding oncogenesis of this type of lymphoma and whether MALT lymphoma is really neoplastic.<sup>14</sup>

Despite its well-recognized clinical and pathological characteristics, the cytogenetic features of MALT lymphoma have not been thoroughly studied. This is probably because of difficulties caused by the low mitotic activity of the lymphoma cells and the relatively low percentage of tumor cells in the tissue specimens of MALT lymphoma, which include heterogeneous reactive small lymphocytes and plasma cells as well as epithelial cells. However, in two studies of relatively large series, the recurrent chromosomal translocation t(11;18)(q21;q21) was identified as characteristic of MALT lymphoma.<sup>15,16</sup> Recently, we and others have shown that c-IAP2/HIAP1/MIHC/API2 gene on chromosome 11 and a novel gene, MALT1/MLT, on chromosome 18 were fused as a result of this specific translocation.<sup>17-19</sup> (MALT1 is used for the gene name of MALT1/MLT in the present report, as it was so designated by the Genome Nomenclature Committee.<sup>18</sup>) These analyses showed the presence of a chimeric API2-MALT1 transcript consisting of the N-terminal region of the API2 gene and the C-terminal region of the MALT1 gene in cases with t(11;18). The presence of different breakpoints on MALT1 cDNA was also demonstrated by these analyses. This prompted us to examine variations of the chimeric transcripts and the incidence of their involvement in a large number of MALT lymphoma cases. Detection of this chimeric product is important for exploration of the pathogenesis of MALT lymphoma as

well as for clinical application, because it represents direct evidence of the clonal expansion of lymphoma cells.

Here we report the establishment of a reverse transcription-polymerase chain reaction (RT-PCR) assay that can detect a chimeric transcript in all of our five MALT lymphoma cases with t(11;18) translocation. Furthermore, the chimeric API2-MALT1 transcript was found in three cases for which karyotype data were not available, indicating that the RT-PCR analysis is useful for detecting clonally aberrant cells in MALT lymphoma cases.

## Patients and Methods

### Patient Samples

We retrieved cases with available mRNA that met the criteria of MALT lymphoma from those entered in the surgical pathology files of the Aichi Cancer Center Hospital between January 1990 and December 1998. A total of 22 cases with MALT lymphoma arising in the lung and gastrointestinal tract were enrolled in the present study. The clinicopathological profiles of the samples used for the present analysis are summarized in Table 1. These cases included 16 cases of pure low-grade MALT lymphoma, five cases of low-grade with partially high-grade component, and one case of high-grade MALT lymphoma with residual low-grade component. The pathological diagnosis was established according to the REAL classification<sup>9</sup> in agreement with two independent pathol-

ogists (S. N. and T. S). The five cases with t(11;18)(q21;q21) were identical to those used for our previous analysis, which demonstrated aberrant transcripts in Northern blot analysis.<sup>18</sup> The karyotype data of the remaining 17 cases were not available.

### *RT-PCR Analysis*

The representative RT-PCR results shown in this report are those conducted with sense primer API2/S897-916(5'-CTGGTGTGAATGACAAGGTC-3') and antisense primer MALT1/AS1030-1049 (5'-CAAAGGCTGGT-CAGTTGTTT-3'), and with sense primer API2/S1203-1222 (5'-GTTCTACTACTGTGCAATG-3') and antisense primer MALT1/AS1030-1049. The nucleotide numbers are based on the sequences registered in GenBank under the accession numbers NM 001165 for API2 and AB026118 for MALT1. To detect any possible breakpoints in the coding region of API2 and MALT1, various primers were used. These include API2/S (sense) 674-693, API2/S897-916, API2/S1203-1222, API2/S1505-1524, API2/S1810-1829, API2/S2110-2129, API2/S2403-2422, API2/S2758-2777, and MALT1/AS (antisense) 1030-1049, MALT1/AS1124-1143, MALT1/AS1363-1382, MALT1/AS1506-1525, MALT1/AS1713-1732 for detecting API2-MALT1 chimeric transcripts. Primers for MALT1-API2 detection are MALT1/S60-79, MALT1/S383-402, MALT1/S569-588, MALT1/S921-940, MALT1/S1189-1208, MALT1/S1403-1422, MALT1/S1644-1663, MALT1/S1981-2000, MALT1/S2269-2288, and API2/AS1430-1449, API2/AS2017-2036, API2/AS2603-2622, API2/AS3146-3165. All combinations were examined against lymphoma cases with t(11;18) (cases 1 to 5).

Five micrograms of total RNA prepared with the guanidium-isothiocyanate method were converted to cDNA by means of reverse transcriptase (Superscript II, GIBCO-BRL, Bethesda, MD) in a total volume of 40  $\mu$ l, according to the manufacturer's protocol. To test various primer combinations, 0.5  $\mu$ l of the cDNA solution produced as described above was used for PCR amplification. The PCR was conducted in a total volume of 100  $\mu$ l containing 2.5 units of TaKaRaTaq (Takara, Otsu, Japan), 10 mmol/L Tris (pH 8.3 at 25°C), 1.5 mmol/L MgCl<sub>2</sub>, 50 mmol/L KCl, 0.2 mmol/L of each of the dNTPs and 100 pmoles of each primer. The touchdown PCR protocol from 63°C to 58°C was used.<sup>20</sup> The PCR regimen was as follows: 95°C for 3 minutes followed by 10 cycles of 1.5 minutes at 95°C, 1.5 minutes at 63°C (-1°C per two cycles), 2.5 minutes at 72°C, and 25 cycles for which the annealing temperature was held constant at 58°C, followed by a final extension at 72°C for 10 minutes.

After the reaction, 10  $\mu$ l of the PCR products was electrophoresed on a 1.2% gel and stained with ethidium bromide. For RNA quality control, a primer pair of 5'-GACTACCTCATGAAGATC-3' and 5'-GATCCACATCT-GCTGGAA-3' based on  $\beta$ -actin was used under PCR conditions of 35 cycles of 1.5 minutes at 95°C, 1.5 minutes at 53°C, and 2.5 minutes at 72°C, followed by a final extension for 10 minutes at 72°C.

### *Nucleotide Sequence Analysis*

The amplified fragments were separated on a low-melting-point gel electrophoresis and purified, after which 50 ng of the fragments were used for sequencing with the dideoxy chain termination method using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Foster City, CA).

## **Results**

### *Amplified Fragments Vary in Size*

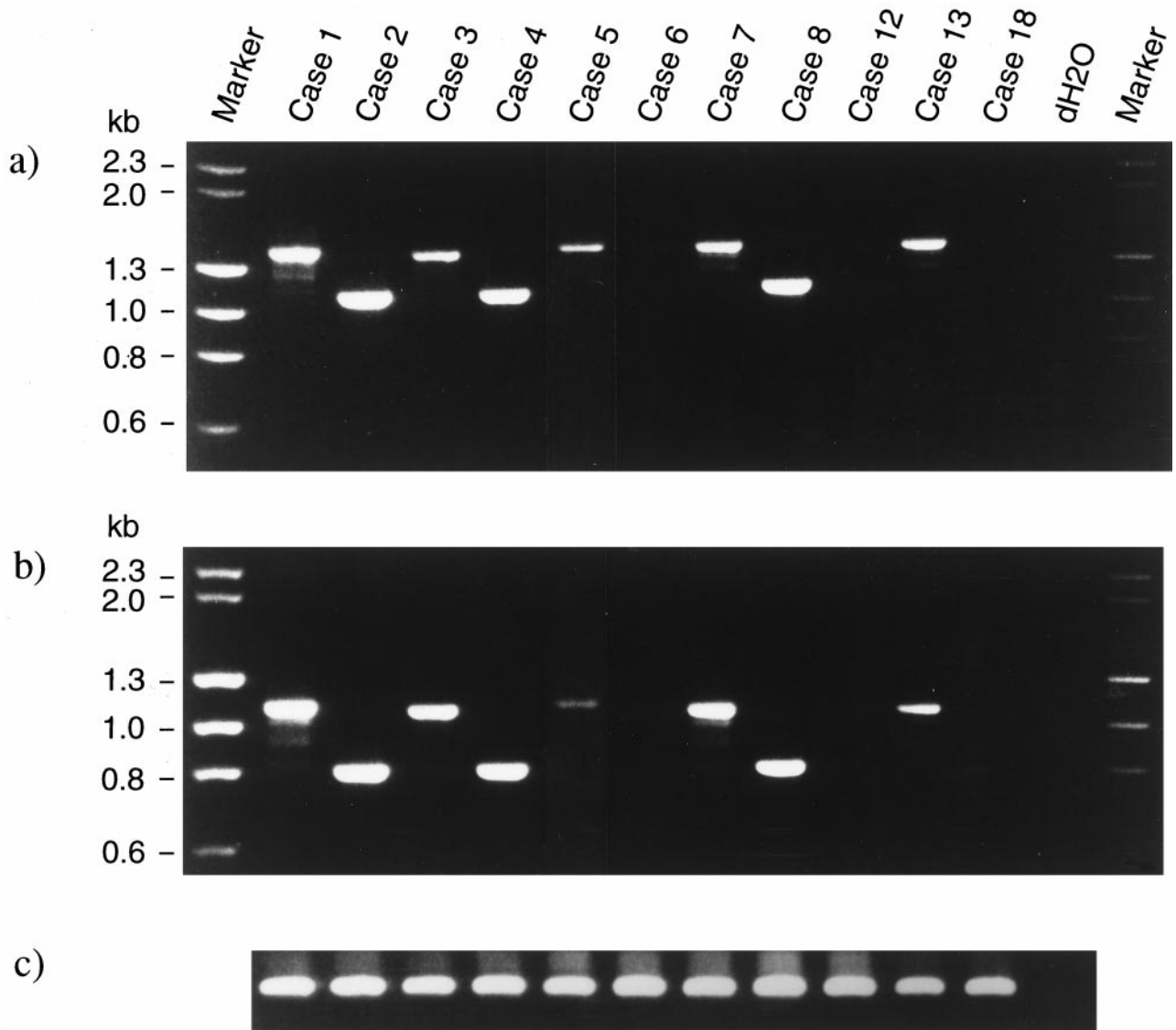
A previous study of ours revealed that the 5' region of the MALT1 gene is frequently deleted,<sup>18</sup> indicating that the API2-MALT1 chimeric transcripts are more important for pathogenesis of MALT lymphoma than the reciprocal transcript, MALT1-API2. It was also shown by Northern blot analysis that the API2-MALT1 chimeric transcripts were heterogeneous.<sup>18</sup> Therefore, to detect API2-MALT1 transcripts, we first analyzed samples with various sense primers of API2 and antisense primers of MALT1. The primers were designed to cover any of the possible breakpoints in the coding region of each of the genes. Specific amplification was found in eight out of the 22 cases examined (Table 1), and representative results obtained with the primer pair of API2/S897-916 and MALT1/AS1030-1049, and with that of API2/S1203-1222 and MALT1/AS1030-1049 are presented in Figure 1, a and b.

Figure 1a shows that amplified fragments varied in size from case to case ranging from 1487 to 1184 bp. When the primer pair of API2/S1203-1222 and MALT1/AS1030-1049 was used, the amplified fragments were reduced by 306 bp (Figure 1b). It should be noted that all of the cases with t(11;18)(q21;q21) were found to produce these specific products (Figure 1, a and b, and Table 1).

For the reciprocal transcripts, MALT1-API2 chimeric products were examined for amplification with the sense primers of MALT1 and the antisense primers of API2, but none of the samples in the present series produced any specific products (data not shown), which is in accordance with our previously reported FISH analysis showing a deletion at the 18q21 breakpoint region<sup>18,21</sup> and with the Northern blot analysis result that a probe 5' to the MALT1 breakpoints did not show any aberrant transcripts.<sup>18</sup>

### *Nucleotide Sequence Analysis*

The nucleotide sequence revealed that all of the eight amplified fragments were fusion products of API2 and MALT1. An important finding was that all of the eight fragments were fused in-frame (Figure 2b) and were predicted to produce chimeric proteins between API2 and MALT1, as shown schematically in Figure 2a. It was demonstrated that there are two breakpoints (A and B) in API2, and three breakpoints (a, b, and c) in MALT1 cDNA (Figure 2, a and b). The major breakpoint for API2 was



**Figure 1.** Reverse transcription-polymerase chain reaction assay. **a:** RT-PCR with sense primer API2/S897-916 and antisense primer MALT1/AS1030-1049 demonstrated specific fragments in cases 1-5, 7, 8, and 13. The remaining cases did not show any amplified fragments. The size of the amplified fragments is 1487 bp for cases 1, 5, 7 and 13, 1184 bp for cases 2 and 4, 1466 bp for case 3, and 1211 bp for case 8. **b:** The same cases also showed amplification when analyzed with sense primer API2/S1203-1222 and antisense primer MALT1/AS1030-1049. The amplified fragments are 306 bp smaller than those shown in **a**. The size of the fragments is 1181 bp for cases 1, 5, 7, and 13, 878 bp for cases 2 and 4, 1160 bp for case 3, and 905 bp for case 8. **c:** RT-PCR with a  $\beta$ -actin primer pair that amplifies 512 bp is shown for RNA quality control. dH<sub>2</sub>O represents PCR without any first-strand cDNA. Markers are  $\lambda$ HindIII and  $\phi$ HaeIII.

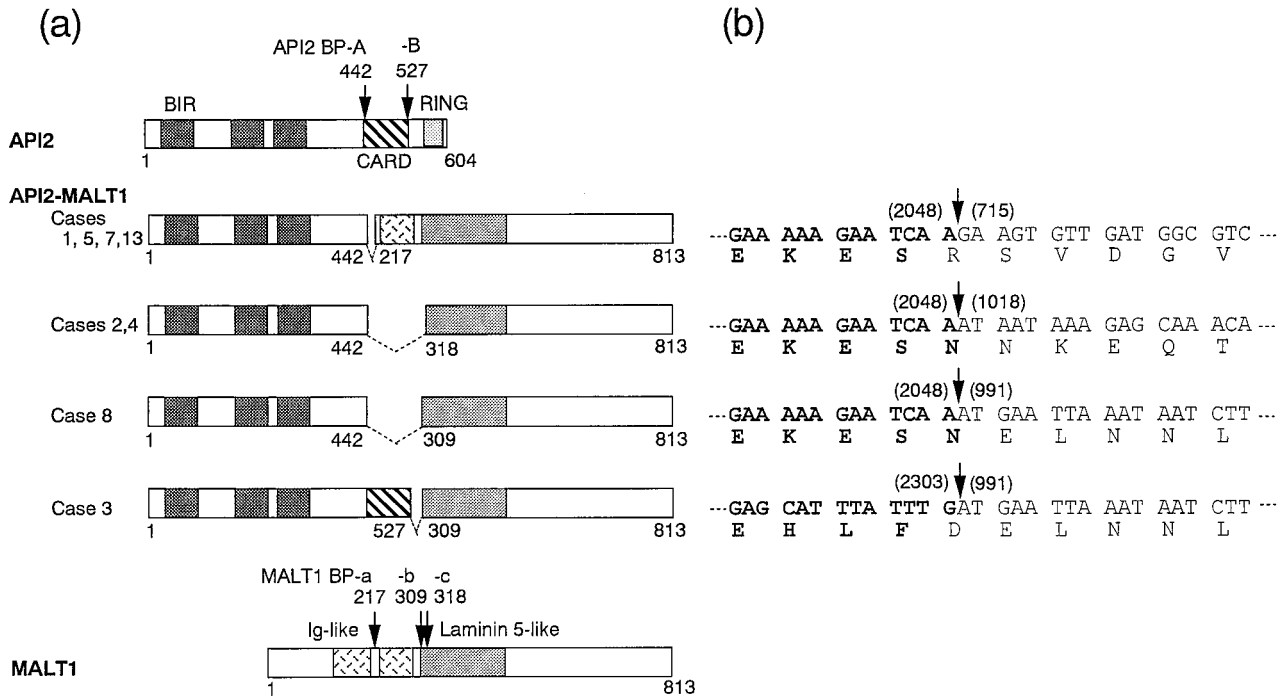
breakpoint A, because seven out of the eight cases had their breakpoints at A. The MALT1 breakpoints, on the other hand, were dispersed: four of the eight cases had their breakpoint at MALT1 breakpoint a, two at b, and two at c. The resulting chimeric proteins were predicted to comprise four kinds as shown in Figure 2a, and the major chimeric protein is the one fused at API2 breakpoint A and MALT1 breakpoint a. It should be noted that all of these chimeric proteins retain three baculovirus inhibitor of apoptosis repeat (BIR) domains.

### Discussion

Recent molecular analyses by us and others of MALT lymphomas with t(11;18)(q21;q21) revealed that distinct

regions of MALT1 gene were involved.<sup>17,18</sup> Indeed, the presence of various kinds of mRNA was demonstrated by our previous Northern blot analysis with MALT1, the size of which was found to range from 6.4 to 9.7 kb.<sup>18</sup> In the present study, we have demonstrated that there are three breakpoints in MALT1, although the difference in size observed in the Northern blot analysis cannot be explained simply by the difference in the fusion point. It was also found that there are two breakpoints in API2, a translocation partner gene of MALT1. Especially noteworthy is that all of the chimeric mRNAs were fused in-frame, leading to prediction of the chimeric API2-MALT1 products.

API2 was first identified as a molecule interacting with TRAF1 and TRAF2 and involved in the signal transduction of the anti-apoptotic pathway mediated by tumor necro-



**Figure 2.** Schematic representation of predicted chimeric products and nucleotide and amino acid sequences around the fusion points. **a:** API2 at the top is fused to MALT1 at the bottom in MALT lymphoma with t(11;18)(q21;q21). Numbers below the figures are based on the numbers of the predicted amino acid sequence of API2 (GenBank accession NM\_001165) and MALT1 (GenBank accession AB026118). The figure shows BIR (■) amino acid numbers 29–95, 169–234 and 255–322), CARD (▨) amino acid numbers 439–529) and RING finger (□) amino acid numbers 546–592) domains for API2 and immunoglobulin-like (Ig-like) domains homologous to those of CD22β (▤) amino acid numbers 141–194 and 241–294) and a domain homologous to the laminin 5 α3b subunit (▥) amino acid numbers 310–477). The four kinds of chimeric products identified in the present study are shown between the representations of API2 and MALT1. API2 breakpoints (API2 BP-A and -B) and MALT1 breakpoints (MALT1 BP-a, -b, and -c) are indicated by arrows together with the respective amino acid sequence numbers of API2 and MALT1. **b:** The nucleotide and amino acid sequences around the fusion sites of the chimeric product are shown. All of the products are fused in-frame. Bold type, API2 sequence; regular type, MALT1 sequence. Numbers in parentheses represent the nucleotide sequence numbers of either API2 or MALT1.

sis factor receptor II.<sup>22</sup> API2 contains three BIR domains, one caspase recruitment domain (CARD), and one RING finger domain.<sup>23</sup> The common domain of the inhibitor of apoptosis family is the BIR motif, which has been shown to play an essential role for anti-apoptotic function.<sup>24</sup> It is therefore strongly suggested that the BIR motifs maintained in the API2-MALT1 chimeric protein may well be a key domain for the development of MALT lymphoma.

The CARD domain has also been demonstrated to be an important domain interacting with molecules involved in apoptosis.<sup>25</sup> The API2-MALT1 chimeric transcripts demonstrated by Dierlamm et al suggested that the truncation of the CARD domain from API2 is one of the characteristics of the API2-MALT/MLT fusion products.<sup>17</sup> Although most of the chimeric products identified in the present study have been shown to have lost the CARD domain, one case proved to have retained the CARD domain (case 3, Figure 2a), suggesting that the removal of the CARD domain from the chimeric product is not essential.

MALT1, a novel gene at 18q21 and found in t(11;18)(q21;q21), shows homologies with the immunoglobulin-like domain of CD22β, the laminin-5 α3b subunit, and F2D3.6 of *Caenorhabditis elegans*.<sup>18</sup> The present study found the breakpoints of MALT1 to be relatively dispersed. Indeed, another breakpoint different from the three breakpoints of the present series has been reported by Dierlamm et al,<sup>17</sup> suggesting that there are at least four

different breakpoints in MALT1. Although the present analysis failed to identify important domain of MALT1 for the pathogenesis of MALT lymphoma, it should be noted that the C-terminal region may be important because of its consistent involvement in t(11;18) translocation. It is also conceivable that fusion with API2 might alter the function of API2 involved in the apoptotic pathway. Future studies focusing on API2, MALT1 and their chimeric protein API2-MALT1 are expected to shed further light on these issues.

In our current analysis, 8 of 22 (36%) MALT lymphoma cases were demonstrated, by means of the RT-PCR established in the present study, to possess chimeric products. Although the number of cases examined is limited, this assay is expected to be highly useful for clinical applications for diagnostic purposes and for the monitoring of clonal cells in MALT lymphoma patients. Recurrent detection of the API2-MALT1 chimeric transcripts suggests that low-grade lymphoma is in fact neoplastic (Table 1). There are still some difficulties in defining the classification of low-grade B cell lymphomas. The identification of clonal cells containing these chimeric transcripts is especially important for MALT lymphoma because the heterogenous cell populations in the lesion sometimes pose diagnostic problems. The present study suggested that MALT lymphomas with and without API2-MALT1 translocation might be subdivided into two

groups. The pathogenesis of the latter can be expected to become the subject of intense investigation.

The incidence of the genetic alterations of API2-MALT1 may be somewhat related to their anatomical sites of origin. The 56% (5/9) detection of this genetic alteration in pulmonary lymphomas is much higher than the 9% (1/11) in gastric lymphoma. This point deserves further investigation with a larger-scale study.

In conclusion, we have established an RT-PCR assay for the detection of API2-MALT1 and found that a significant percentage of MALT lymphomas involves the API2-MALT1 gene alteration. This RT-PCR assay for detecting chimeric transcripts is expected to become an important tool for a better understanding of marginal zone lymphoma. The function of API2-MALT1 remains to be elucidated, and further studies, including those of the clinicopathological significance of the presence or absence of API2-MALT1, should provide new insights into the molecular pathogenesis of MALT lymphoma.

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