API2-MALT1 Fusion Transcripts Involved in Mucosa-Associated Lymphoid Tissue Lymphoma

Multiplex RT-PCR Detection Using Formalin-Fixed Paraffin-Embedded Specimens

Hiroshi Inagaki,* Mitsukuni Okabe,* Masaru Seto,[†]
Shigeo Nakamura,[‡] Ryuzo Ueda,[§] and
Tadaaki Eimoto*

From the Departments of Pathology* and Medicine,[§] Nagoya City University Medical School, Nagoya; the Laboratory of Chemotherapy,[†] Aichi Cancer Center Research Institute, Nagoya; and the Department of Pathology and Clinical Laboratories,[‡] Aichi Cancer Center Hospital, Nagoya, Japan

Malignant lymphoma of mucosa-associated lymphoid tissue (MALT) type is a distinct clinicopathological disease entity in the category of extranodal marginal zone B-cell lymphoma. Recently, we and others have shown that the *API2* gene on chromosome 11 and the *MALT1* gene on chromosome 18 are fused as a result of t(11;18)(q21;q21) in MALT lymphomas. Here we report a detection assay that can be used for formalin-fixed, paraffin-embedded specimens. It consists of a multiplex one-tube reverse transcriptase-polymerase chain reaction (RT-PCR) followed by three parallel multiplex nested polymerase chain reactions. Eight variants of the fusion transcripts have been reported to date. When these variants were used as positive controls, all were successfully detected. The subsequent direct sequencing confirmed the results. Using this rapid and simple method, we could detect *API2-MALT1* fusion transcripts in 5 of 15 (33%) archival cases of MALT lymphoma for a frequency comparable with those of RT-PCR assays using frozen materials. The lung was the preferential anatomical site of origin of MALT lymphomas harboring *API2-MALT1* fusion. No fusion transcript was detected in any of 20 high-grade B-cell lymphomas. Our multiplex RT-PCR assay, which can be used for routinely-processed paraffin samples, should serve as a useful molecular tool for clarifying the clinicopathological significance of *API2-MALT1* fusion in MALT lymphoma. (*Am J Pathol* 2001, 158:699–706)

zone B-cell lymphoma (MZBL) according to the revised European-American lymphoma classification² and the forthcoming World Health Organization classification. This type of lymphoma is characterized by an indolent clinical course and a characteristic histological appearance including lymphoepithelial lesions and follicular colonization. Multicentric and extranodal involvement in the gastrointestinal tract, lung, thyroid, breast, and salivary and lacrimal glands is frequently observed in MALT lymphoma.³ Chronic inflammations, such as *Helicobacter pylori* gastritis, Hashimoto's thyroiditis, and Sjögren's syndrome, may make important contributions to its development.^{4–6} This association with inflammation suggests that proliferation of the lymphoma cells may depend on the presence of activated, antigen-driven T cells.⁷ Gastric MALT lymphoma sometimes shows regression as a result of anti-bacterial therapy against *H. pylori*, which makes the oncogenesis of this type of lymphoma and its neoplastic nature problematic.⁸

t(11;18)(q21;q21) has been identified as a recurring cytogenetic abnormality in MZBLs, particularly in those of MALT type.^{9–13} Because balanced translocations are thought to play a pathogenetic role in the development of B-cell lineage lymphomas such as Burkitt's lymphoma,¹⁴ follicular lymphoma,¹⁵ and mantle cell lymphoma,^{16,17} the presence of t(11;18)(q21;q21) may provide some clues to the pathogenesis of extranodal MZBLs. Recently, we and others showed that the *c-IAP2/HAIP1/MIHC/API2* gene on chromosome 11 and a novel gene, *MLT/MALT1*, on chromosome 18 were fused as a result of this specific translocation.^{18,19} These studies showed the presence of chimeric *API2-MALT1* transcripts, 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)(q21;q21). After the discovery of the *API2-MALT1* fusion in MZBLs of MALT type, ~100 MALT lymphomas were examined for this novel fusion, and one-third of the cases were found to be positive.^{18–22} This recurrent de-

Malignant lymphoma of mucosa-associated lymphoid tissue (MALT) type, first described by Isaacson and Wright,¹ is recognized as a distinct clinicopathological disease entity in the category of extranodal marginal

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Address reprint requests to Hiroshi Inagaki, M.D., Department of Pathology, Nagoya City University Medical School, 1-Kawasumi, Mizuho-ku, Nagoya, 467-0841, Japan. E-mail: hinagaki@med.nagoya-cu.ac.jp.

tection of the *API2-MALT1* fusion suggested that MALT lymphoma is in fact neoplastic.

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 necrosis factor receptor II.²³ *API2* contains three baculovirus repeat inhibitor of apoptosis (*IAP*) domains, one caspase recruitment domain (BIR) (CARD), and one RING finger domain.²⁴ The common domain of the inhibitor of the apoptosis family is the BIR motif, which has been shown to fulfill an essential anti-apoptotic function.²⁵ Although the function of the protein encoded by *MALT1* gene remains unknown, it shows homologies with the immunoglobulin-like domain of CD22 β , the laminin-5 α 3b subunit, and F22D3.6 of *Caenorhabditis elegans*.¹⁹ Our previously published data suggest that truncation of the *API2* gene distal to its three BIR domains and fusion of this truncated gene with the carboxy-terminal region of *MALT1* may lead to an increased inhibition of apoptosis and thereby help MALT lymphomas to survive.²⁰

The detection of chimeric *API2-MALT1* transcripts is important for exploration of the pathogenesis of MALT lymphoma as well as of clinicopathological significance because it represents direct evidence of the clonal expansion of lymphoma cells and may be relevant for prognosis. So far, reverse transcriptase-polymerase chain reaction (RT-PCR) has been the major tool for the detection of *API2-MALT1* fusion transcript;^{20–22} because there are at least four breakpoints in each *API2* and *MALT1* genes, the detection of this fusion transcript requires high quality RNA so that frozen tumor materials are mandatory. Because the advantages of being able to use routinely prepared paraffin samples far outweigh any demerits, we were interested in developing a detection assay that can be used for archival specimens. Here we describe our efforts to establish a multiplex RT-PCR assay to detect various *API2-MALT1* fusion transcripts using archival formalin-fixed, paraffin-embedded lymphoma tissues.

Materials and Methods

API2-MALT1 Fusion Transcript-Positive Controls

Previous analyses of *API2-MALT1* fusion transcripts in MALT-type lymphomas^{18–22} have detected four breakpoints in *API2* at 1,203 bp, 1,446 bp, 1,701 bp, and 1,743 bp (accession no. L49432), that were designated in this study as A1203, A1446, A1701, and A1743, respectively (Figure 1). Four breakpoints have been identified for *MALT1* at 541 bp, 814 bp, 1,123 bp, and 1,150 bp (accession no. AF130356), and they were similarly designated as M541, M814, M1123, and M1150, respectively (Figure 1). The *API2-MALT1* fusion transcripts are more important for the pathogenesis of MALT lymphoma than the reciprocal transcript, *MALT1-API2*, because the 5' region of the *MALT1* gene is frequently deleted.^{19,20} Although 16 different fusion variants of the *API2-MALT1* fusion may theoretically be present, eight variants have been reported to date: A1446-M814, A1446-M1123,

Figure 1. Locations of *API2* and *MALT1* breakpoints and primers for multiplex RT-PCR assay. The arrows indicate the positions of *API2* and *MALT1* breakpoints previously reported. Numbering is in accordance with accession number L49432 for *API2* and accession number AF130356 for *MALT1*. Primers PA1, PA3, PA5, PM2, PM4, and PM6 are used for the first-round multiplex RT-PCR. Next, three parallel multiplex nested PCRs are performed, namely, second PCR-A using primer set PA2, PM1, PM3, and PM5; second PCR-B using primer set PA4, PM1, PM3, and PM5; and second PCR-C using primer set PA6, PM1, PM3, and PM5.

A1446-M1150, A1701-M1123, A1203-M814, A1446-M541, A1743-M814, and A1743-M1123.^{18–22} We have recently reported on the first four variants, which were confirmed by sequencing of RT-PCR products generated from frozen tumor materials [reported as cases 7, 8, 2, and 3, respectively, by Motegi et al²⁰; the latter two cases were cytogenetically positive for t(11;18)(q21;q21)²⁰]. For the study presented here, we used, for positive controls, formalin-fixed, paraffin-embedded tumor sections containing these four fusion variants (A1446-M814, A1446-M1123, A1446-M1150, and A1701-M1123).

In addition, we synthesized *in vitro* four other variant transcripts (A1203-M814, A1446-M541, A1743-M814, and A1743-M1123) from *API2* and *MALT1* cDNA in our laboratory. As shown in Figure 2, an *API2*-side fragment of an objective chimeric *API2-MALT1* gene was PCR amplified by using a sense primer (P1, 20 mer) and an

Figure 2. Artificial generation of *API2-MALT1* fusion cDNA from *API2* and *MALT1* cDNA. The P2 primer (40 mer) for *API2* cDNA amplification has 20 mer of the *MALT1* sequence on the 3' side, and the P3 primer (40 mer) for *MALT1* cDNA contains 20 mer of the *API2* sequence on the 5' side. After amplification of *API2* gene with primer pair P1-P2 and of *MALT1* gene with primer pair P3-P4, both PCR products are mixed together, and the fusion gene is amplified by PCR using a primer pair P1-P4.

Table 1. Oligonucleotide Sequences of Primers

Primer	Sequence
PA1	ttcatccgtcaagtccaagc
PA2	agccagttaccctcatctac
PA3	ttactcaatgcagaagatga
PA4	gaaataagggaaggaggag
PA5	caagagaactgattgatacg
PA6	attgcagccactgtattcag
PM1	cagccaagactgcctttgac
PM2	ttgaacaaaaggatgccag
PM3	ggcatcagcttttggaagt
PM4	actgtaaaaccaatgtgctg
PM5	aaaggctggtcagttgttg
PM6	ttcctatcaaaaggcaacc
AC1	gagcaagagagggcatcct
AC2	cagtgttacggccagagg
AC3	tggagaaaatctggcaccac
AC4	gaggcgtacagggatagcac

PA1–6, AC1, and AC3, sense primers; PM1–6, AC2, and AC4, antisense primers.

antisense primer (P2, 40 mer) containing both *API2* (20 mer) and *MALT1* (20 mer) sequences. Similarly, a *MALT1* side fragment of the chimeric cDNA was amplified by using a sense primer (P3, 40 mer) containing both 20-mer *API2* and 20-mer *MALT1* sequences and an antisense primer (P4, 20 mer). The *API2*-side and *MALT1*-side PCR products were diluted to 1:1,000, then admixed at a ratio of 1:1, and subjected to PCR using P1 sense and P4 antisense primers. The objective chimeric PCR product thus produced was subcloned into a pGEM-T Easy Vector (Promega, Madison, WI). After confirmation of the sequence and direction of the chimeric PCR product, the vector DNA was treated with the *Sa*I restriction enzyme, followed by *in vitro* synthesis of the objective transcript with the aid of the T7 RNA polymerase (RNA Transcription Kit; Stratagene, La Jolla, CA) according to the manufacturer's recommendations. The transcript was treated with RNase-free DNase I (Roche Diagnostics, Tokyo, Japan) for 3 hours, then extracted in phenol/chloroform and resuspended in RNase-free water. The quantity of the transcript was estimated by means of electrophoresis and a spectrophotometer (Ultrospec III; Pharmacia Biotech, Uppsala, Sweden). Subsequently, the transcript was diluted to 1 ng/ μ l in RNase-free water. After this strategy, four variant fusion transcripts were synthesized and then used as positive controls. The P1-P4 primer pair (Figure 2) used to generate transcripts, A1203-M814, A1446-M541, A1743-M814, and A1743-M1123 were PA1-PM4, PA3-PM2, PA5-PM4, and PA5-PM6, respectively (Figure 1 and Table 1).

Negative Controls

Various negative controls were included to confirm the specificity of the RT-PCR assay. A negative control without a sample RNA was included in every experiment. Also included were a negative control without reverse transcriptase and one treated with DNase-free RNase A (Roche Diagnostics). RNA extracted from paraffin sections of nonspecific chronic lymphadenitis and chronic gastritis was also used.

Clinical Samples

Extranodal non-Hodgkin's lymphomas were selected from the files of the Department of Pathology, Nagoya City University Medical School, Nagoya, Japan. All cases were carefully reviewed, and diagnoses were made according to the criteria of the REAL classification.² Briefly, extranodal low-grade MZBLs of MALT type ($n = 16$) were composed of small- to medium-sized tumor cells with a characteristic diffuse and/or marginal zone-based infiltration pattern and the formation of lymphoepithelial lesions. Four cases were of pulmonary origin, nine cases originated in the stomach, and three cases in the colon. The immunophenotype of the tumor cells was CD20+, CD79a+, cyclin D1–, CD5–, CD10–, CD3–, CD45RO–, and CD56–. Twenty-four extranodal high-grade B-cell lymphomas (gastric origin, 17; ileum, 4; and colon, 3) contained sheets of blasts with a cytological spectrum ranging from centroblasts to immunoblasts and plasmablasts. Four of the 17 high-grade gastric cases simultaneously exhibited a low-grade component. Monoclonality was detected in all of the low- and high-grade cases by molecular and/or immunophenotypic techniques as previously described.²⁶ Twenty-four specimens were obtained by biopsy and 16 by surgical resection.

Multiplex RT-PCR Strategy

To detect various types of *API2-MALT1* fusion transcripts and to achieve maximal sensitivity, we designed a multiplex one-tube RT-PCR (the first round) followed by three parallel multiplex nested PCRs (the second round). The primers were designed so that various fusion types could be distinguished by the size of the second round PCR products. To verify the results, positive PCR products of the second round amplification were directly sequenced. Primers were designed to allow identical conditions for all RT-PCR and PCR reactions. We tested the constructed primer pairs in amplifying reactions before and after combining the different primer sets into the multiplex PCR reaction. If they did not function properly, the primers were redesigned. To minimize the risk of cross-contamination and product carry-over, strict precautions were taken including the use of filter tips, different locations in the laboratory, and indigenous pipettes for sample processing, RNA extraction, RNA amplification, and electrophoresis. All samples were tested in at least two separate experiments.

RNA Extraction from Paraffin Sections

The lymphoma samples had been fixed in 10% formalin, embedded in paraffin, and stored at room temperature for 0.5 to 15 years. Total RNA was extracted from the paraffin sections as previously described.²⁷ Briefly, the tumor sections (3- μ m thick) were deparaffinized and air-dried. With a serial hematoxylin and eosin section as a guide, lymphoma cells were scraped off with a knife and collected in a tube, then incubated at 56°C overnight in 200 μ l of digestion buffer (20 mmol/L Tris, pH 8.0, 20

Table 2. Size (bp) of the Second-Round Multiplex PCR Products

Second round multiplex PCR	Primers for second round PCR	API2 breakpoint	MALT1 breakpoint			
			M541	M814	M1123	M1150
Second PCR-A	PA2, PM1, PM3, PM5	A1203	122 bp	147 bp*	135 bp	108 bp
Second PCR-B	PA4, PM1, PM3, PM5	A1446	94 bp*	119 bp*	107 bp*	80 bp*
Second PCR-C	PA6, PM1, PM3, PM5	A1701 or A1743	112 bp or 154 bp	137 bp [†] or 179 bp*	125 bp* or 167 bp*	98 bp or 140 bp [‡]

Most of the fusion types could be easily distinguished on an 8% polyacrylamide gels by the size of the second round PCR products because the primers were designed to generate more than 12-bp difference in size between the PCR products.

*Previously reported fusion variants are all included in our study as positive controls.

The size of fusion A1701-M814[†] is close to that of A1743-M1150[‡] although the two can be distinguished by digestion with restriction enzymes: the former possesses restriction sites for *Bb*II and *Hin*II, and the latter for *Mbo*II. bp, base pair.

mmol/L ethylenediaminetetraacetic acid, 2% sodium dodecyl sulfate, and 400 μ g/ml proteinase K). Total RNA was extracted with the aid of concentrated phenol/guanidine isothiocyanate (Trizol LS; Life Technologies, Tokyo, Japan), followed by RNase-free DNase I (Roche Diagnostics) treatment, and final resuspension in 50 μ l of RNase-free water.

Multiplex One-Tube RT-PCR (First Round)

Five μ l of RNA extracted from the paraffin sections and positive RNA controls synthesized *in vitro* were heated to 70°C and then placed on ice. The RT-PCR mixture was then added, and the final mixture of 25 μ l/tube contained 8.25 U of RNase inhibitor (Toyobo, Osaka, Japan), 50 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies), 20 pmol/L of each primer, 200 μ mol/L of each of the four deoxynucleotides, 1 U of TaqGOLD DNA polymerase (Applied Biosystems, Foster City CA), 1 $\times$ Taq buffer containing 1.5 mmol/L MgCl₂, and template RNA. Three different primer pairs (PA1-PM2, PA3-PM4, and PA5-PM6 for the API2-MALT1 fusion transcripts) were added to the reaction mixture. The locations and sequences of the primers are shown in Figure 1 and Table 1. The thermocycler was programmed first to incubate the samples for 30 minutes at 37°C for the initial RT step, and next for 10 minutes at 95°C for inactivation of the reverse transcriptase as well as for activation of the DNA polymerase, and then to carry out 35 cycles of PCR at 95°C for 30 seconds, at 50°C for 30 seconds, and at 72°C for 45 seconds.

Multiplex Nested PCR (Second Round)

The first round RT-PCR product diluted with water to 1:1,000 was subjected to three parallel second-round multiplex nested PCRs using TaqGOLD DNA polymerase and 1.5 mmol/L MgCl₂. To detect fusion genes possessing an API2 breakpoint at bp 1,203, primers PA2, PM1, PM3, and PM5 were included in the second-round multiplex PCR (second PCR-A). Similarly, to detect fusion genes possessing an API2 breakpoints at bp 1,446 and bp 1,701 or bp 1,743, the second round PCR used two primer sets, ie, PA4, PM1, PM3, and PM5 (second PCR-B) for bp 1,446 and PA6, PM1, PM3, and PM5 (second PCR-C) for bp 1,701 or 1743. The amplification conditions consisted of 35 cycles at 95°C for 15 seconds, at 55°C for 30 seconds, and at 72°C for 45 seconds. The

primers used for the second round PCR were designed so that more than 12-bp size difference among the PCR products was generated in each second round PCR (Table 2, second PCR-A, -B, and -C). The PCR products were detected by ethidium bromide staining on 8% polyacrylamide gels. The band sizes of the fusion variants are listed in Table 2.

DNA Direct Sequencing

The breakpoints of the fusion transcripts were confirmed by direct sequencing. Fragments obtained in the second round PCR were separated by electrophoresis on a low melting point gel and purified. The fragments were directly sequenced by means of cycle sequencing with dye-labeled terminators (BigDye Terminators, Applied Biosystems) and analyzed on a DNA sequencer (Model 310, Applied Biosystems). API2 primers required for the second round PCR were used as sequencing primers.

Internal Positive Control

Because false-negative results because of varying RNA quality and/or handling errors are an inherent problem in RT-PCR assays, we amplified the ubiquitously expressed β -actin mRNA as an internal positive control. β -actin transcript amplified in the first-round one-tube RT-PCR by using an outer primer pair, AC1 and AC2, was further amplified in the nested PCR by using an internal primer pair, AC3 and AC4 (Table 1). The size of the amplified β -actin fragment was 190 bp.

Results

Positive Controls

Four breakpoints each in API2 and MALT1 cDNA, and eight API2-MALT1 fusion variants have been reported.^{18–22} As positive controls, we prepared all eight fusion variants either from clinical samples or by artificial generation with *in vitro* transcription. As shown in Figure 3, all eight fusion variants were successfully detected in our assay consisting of multiplex one-tube RT-PCR (first round) and the subsequent three parallel multiplex nested PCRs (second round, second PCR-A, -B, and -C, respectively). Because the primers were designed to

Figure 3. Detection of various API2-MALT1 fusion transcripts, positive controls. Positive RNA controls are first amplified with multiplex one-tube RT-PCR. The products are then subjected to three parallel multiplex nested PCRs (second PCR, A–C). **Lane 1**, fusion transcript A1203-M814; **lane 2**, A1446-M541; **lane 3**, A1446-M814; **lane 4**, A1446-M1123; **lane 5**, A1446-M1150; **lane 6**, A1701-M1123; **lane 7**, A1743-M814; **lane 8**, A1743-M1123; and N, negative control without template RNA. **A**, **B**, and **C**: Shown is the 8% polyacrylamide electrophoresis of the second round PCR including primers PA2, PM1, PM3, and PM5 (second PCR-A) (**A**); PCR including primers PA4, PM1, PM3, and PM5 (second PCR-B) (**B**); and PCR including primers PA6, PM1, PM3, and PM5 (second PCR-C) (**C**).

generate more than 12-bp size difference among the PCR products in each second round PCR, fusion types could be determined with ease by the size of the amplified products run on 8% polyacrylamide gels (Figure 3, A–C). Amplified fragments in the second round PCR were directly sequenced, and the sequence data were identical with the fusion variants determined by electrophoresis. Nonspecific bands were rarely observed throughout the experiments. β -actin transcript was amplified in all four clinical samples used as positive controls. Negative controls (no template inclusion, no reverse transcriptase inclusion, pretreatment with RNase A, and lymphadenitis and gastritis samples) produced no visible bands.

In the A1446-M1150 control whose RNA was extracted from a clinical specimen, a 351-bp PCR band was amplified in the second PCR-A in addition to an expected

80-bp band in the second PCR-B. This 351-bp band was the A1446-M1150 fragment amplified with a primer pair, PA2 and PM5, as confirmed by direct sequencing. No such amplification was observed for any other positive controls.

Clinical Samples

β -actin transcript used as an internal control was amplified in 35 of 40 cases (88%); four gastric and one ileal specimens were negative for β -actin transcript. RNA preservation was not associated with the size, length of storage, or sampling method (biopsy or resection) of the paraffin-embedded specimens. Subsequently, 15 MALT lymphomas and 20 extranodal high-grade B-cell lymphomas were considered to be suitable for detection of API2-MALT1 fusion transcripts. The clinicopathological features of these 35 cases are shown in Table 3. Of the MALT lymphomas, five cases (33%) were positive for API2-MALT1 fusion (Figure 4): three of four in the lung (fusion types A1203-M814, A1446-M541, and A1446-M1150), one of eight in the stomach (A1446-M541), and one of three in the colon (A1446-M541). The case harboring the A1446-M1150 fusion showed a 351-bp band in the second PCR-A (Figure 4A, case 2) in addition to an 80-bp band in the second PCR-B (Figure 4B, case 2) as was also observed for the corresponding positive control. The sequencing analyses confirmed the results. All chimeric transcripts detected were fused in-frame, and none of the positive cases showed atypical transcripts such as an insertion or a deletion. No chimeric transcript was detected in any of 20 extranodal high-grade lymphomas including four cases with a concomitant low-grade component.

Discussion

There were several difficulties in detecting the API2-MALT1 fusion transcript when using archival materials: a small amount of considerably degraded RNA in archival materials, inherent risks of contamination during the course of RNA processing and PCR amplifying procedures, many breakpoints in both API2 and MALT1 genes, and erroneous interpretation of many kinds of fusion breakpoint combinations. To overcome these difficulties, we first designed the primers so that the amplified RNA fragments were less than 250 bases in length. To increase sensitivity, we used a combination of one-tube RT-PCR and nested PCR.²⁷ One-tube PCR is a highly sensitive and simple method: use of specific primers can improve RT sensitivity 25- to 125-fold compared to that of random hexamer primers,²⁸ and the RT reaction can directly proceed to the PCR amplification without the need for RT product purification. Another advantage of one-tube RT-PCR is that it can minimize contamination risks as a result of a reduction in manipulation steps. The subsequent nested PCR can then further increase the detection sensitivity. To detect various types of API2-MALT1 fusion transcripts, we have chosen a multiplex approach^{28–30} for both first round and second round

Table 3. Clinicopathological Features of Lymphoma Patients

Case	Age (yrs)	Sex	Origin	Histology	Stage	API2-MALT1 fusion	Fusion type	Outcome
1	43	F	Lung	LG	II	+	A1446-M1150	AW 1.5 yr
2	73	F	Lung	LG	II	+	A1446-M541	AW10 mo
3	77	F	Lung	LG	I	+	A1203-M814	AW 1.5 yr
4	61	F	Lung	LG	I	—		AW 4 yr
5	52	M	Stomach	LG	I	+	A1446-M541	AW 1.5 yr
6	43	F	Stomach	LG	I	—		AW13.5 yr
7	48	F	Stomach	LG	I	—		AW11 yr
8	31	F	Stomach	LG	I	—		AW 8.5 yr
9	41	M	Stomach	LG	I	—		AW 6 mo
10	23	M	Stomach	LG	I	—		AW13 yr
11	60	F	Stomach	LG	I	—		AW 1 yr
12	65	M	Stomach	LG	I	—		AW 1.5 yr
13	60	M	Colon	LG	II	+	A1446-M541	AW 2 yr
14	62	F	Colon	LG	I	—		AW 9 mo
15	66	M	Colon	LG	II	—		AW 3 yr
16	47	F	Stomach	HG+L	I	—		AW 7 yr
17	51	M	Stomach	HG+L	I	—		AW 3 yr
18	53	M	Stomach	HG+L	I	—		AW 7 mo
19	64	F	Stomach	HG+L	I	—		AW 2.5 yr
20	83	F	Stomach	HG	II	—		AW 2 yr
21	54	M	Stomach	HG	I	—		AW 7 yr
22	60	M	Stomach	HG	III	—		DOD 6 yr
23	60	F	Stomach	HG	II	—		AW 6 mo
24	55	F	Stomach	HG	II	—		DOD 2 mo
25	34	M	Stomach	HG	I	—		AW12.5 yr
26	62	M	Stomach	HG	II	—		AW 5 yr
27	85	M	Stomach	HG	II	—		DOD 3 mo
28	71	F	Stomach	HG	III	—		DOD 2 mo
29	80	F	Stomach	HG	III	—		AW 1 yr
30	66	M	Ileum	HG	II	—		DOD 4 mo
31	64	M	Ileum	HG	III	—		DOD 7 mo
32	65	M	Ileum	HG	II	—		AW 7.5 yr
33	62	F	Colon	HG	II	—		AW 2.5 yr
34	41	M	Colon	HG	III	—		AW 6 yr
35	45	F	Colon	HG	II	—		AW 3.5 yr

LG, low-grade; HG, high-grade; HG+L, high-grade with residual low grade component; AW, alive and well; DOD, died of disease.

PCRs, although multiplex RT-PCR has been rarely used for archival materials. When considering the many breakpoints present in each *API2* and *MALT1* gene, a large number of separate PCR reactions are needed for the standard screening of a lesion, which is labor intensive and very costly, and therefore probably not practically feasible. Using a multiplex approach, we could detect the chimeric transcripts in only two rounds of PCR amplification. The type of *API2-MALT1* fusion could be directly determined by ethidium bromide staining on 8% polyacrylamide gels because the primers were designed to generate a more than 12-bp size difference among PCR products of each second round amplification. Direct sequencing confirmed the results. In our assay, we could successfully detect various *API2-MALT1* fusion transcripts and determine their breakpoints by using RNA extracted from formalin-fixed, paraffin-embedded samples.

Different fusion products generated by cytologically identical chromosomal translocation can have major clinical correlates. In hematological malignancies possessing *BCR-ABL* rearrangement, the position of the breakpoint within the *BCR* gene determines which *BCR* exons are included in the encoded chimeric tyrosine kinase, thereby leading to either chronic myeloid leukemia or acute lymphoblastic leukemia.³¹ In some malignant soft

tissue tumors, the type of fusion created by different breakpoints has been shown to be prognostically relevant.^{32–34} Therefore, determination of *API2* and *MALT1* breakpoints by means of RT-PCR, which might be difficult when fluorescent *in situ* hybridization or immunohistochemistry is used, may have clinicopathological significance. RT-PCR is also useful for monitoring the minimal residual disease, particularly in MALT lymphomas, where histological identification of individual lymphoma cells in the heterogeneous cell populations of the lesions is difficult. The use of paraffin sections as the RNA source facilitates a direct comparison of the tumor histopathology with the relevant molecular data.

It is generally thought that RNA is easily destroyed by ubiquitous RNase and may be degraded during the course of tissue processing and storage of specimens. RNA samples extracted from archival specimens are rarely used for study purposes. Nevertheless, under the right conditions, RNA can be preserved for years in archival specimens, as evidenced by the successful RT-PCR results in our study, with some tumor specimens having been stored for more than 10 years. β -actin transcript is a good internal control for RNA quality assessment of not only frozen but also cytological and histological materials.²⁷ In our archival lymphoma specimens,

Figure 4. Detection of various API2-MALT1 fusion transcripts, clinical samples. Total RNA extracted from archival samples of MALT lymphoma are subjected to the multiplex nested RT-PCR assay. **A:** Second PCR-A. **B:** Second PCR-B. **C:** Second PCR-C; **lanes 1, 3, and 5** (cases 5, 2, and 13, respectively, Table 3), MALT lymphomas each showing a 94-bp band in the second PCR-B, indicating that A1446-M541 type fusion is present (Table 2); **lane 2** (case 1), MALT lymphoma showing an 80-bp band in the second PCR-B and a 351-bp band in the second PCR-A, which is interpreted as indicating possession of a A1446-M1150 type fusion transcript (Table 2); **lane 4** (case 3), MALT lymphoma showing a 147-bp band in the second PCR-A harbors A1203-M814 type fusion (Table 2); **lanes 6 and 7** (cases 6 and 7, respectively), MALT lymphomas in which API2-MALT1 fusion is not detected. **D:** RT-PCR using primers for β -actin gene is performed to confirm that samples possess RNA of an adequate quality.

β -actin transcripts were well preserved in paraffin-embedded specimens (35 of 40, 88%), regardless of their size, storage period, or sampling method (biopsy or resection). This suggests that a fragment of small biopsy, even after it is routinely processed, could be used for our assay.

In our study, the API2-MALT1 fusion was detected only in MZBLs of MALT type, and in none of the primary high-grade or secondary high-grade lymphomas. A similar observation has been repeatedly reported in recent studies.^{18–22,35} MALT lymphomas harboring the API2-

MALT1 fusion do not seem to show a greater likelihood of transforming to large cell lymphomas. However, genetic evidence for a clonal link between low- and high-grade components has been reported for a limited number of gastric MALT lymphomas.³⁶ Therefore, MALT lymphomas without the API2-MALT1 translocation are expected to become the subject of future investigations.

Using RNA extracted from archival samples, we were able to identify five new cases harboring the API2-MALT1 fusion transcripts out of 15 MALT lymphomas without high-grade components. This incidence (33%) is comparable with those (21 to 48%) so far reported for frozen materials.^{20–22} With respect to the primary site of MALT lymphomas, API2-MALT1 fusion was positive in three of four cases of pulmonary origin, but it was detected in one of eight cases of gastric origin. As we previously pointed out,²⁰ the lung seems to be the preferential anatomical site of origin for MALT lymphomas containing the API2-MALT1 fusion. Remstein and colleagues²² recently reported that six of 11 (55%) pulmonary MALT lymphomas were positive for API2-MALT1 fusion. Varying frequencies of API2-MALT1 fusion in gastric MALT lymphomas have been reported by different researchers, ranging from 7 to 48% of cases.^{20–22} Rosenwald and colleagues,³⁵ using fluorescent *in situ* hybridization, detected the t(11;18) translocation in seven of 21 (33%) gastric MALT lymphomas. The reason for this variation is not clear, but we speculate that *H. pylori* infection in the stomach may be an important factor because the infection rate varies among the geographic regions investigated.^{37,38} A large-scale study will be needed to clarify the prevalence of API2-MALT1 fusion cases in various anatomical sites in relation to underlying chronic inflammation.

To summarize, we have established a multiplex RT-PCR assay for the detection of API2-MALT1 fusion transcripts using formalin-fixed, paraffin-embedded materials. The rate of detection with this assay of the API2-MALT1 gene alteration in MALT lymphomas was 33%, and this percentage is comparable with the results obtained with previously reported RT-PCR assays using frozen materials. Our multiplex RT-PCR assay can be expected to become an important tool not only for diagnosis of MALT lymphoma but also for facilitating further investigation of the clinicopathological significance of API-MALT1 fusion.

References

1. Isaacson PG, Wright DH: Malignant lymphoma of mucosa-associated lymphoid tissue: a distinctive type of B-cell lymphoma. *Cancer* 1983; 52:1410–1416
2. Harris NL, Jaffe ES, Stein H, Banks PM, Chan JKC, Cleary ML, Delsol G, De Wolf-Peeters C, Falini B, Gatter KC, Grogan TM, Isaacson PG, Knowles DM, Mason DY, Müller-Hermelink HK, Pileri SA, Piris MA, Ralfkiaer E, Warnke RA: A revised European-American classification of lymphoid neoplasms: a proposal from the international Lymphoma Study Group. *Blood* 1994; 84:1361–1392
3. Isaacson PG, Spencer J: Malignant lymphoma of mucosa-associated lymphoid tissue. *Histopathology* 1987; 11:445–462
4. Wotherspoon AC, Ortiz-Hidalgo C, Falzon MR, Isaacson PG: Helicobacter pylori-associated gastritis, and primary B-cell gastric lymphoma. *Lancet* 1991; 338:1175–1176

5. Parsonnet J, Hansen S, Rodriguez L, Gelb AB, Warnke RA, Jellum E, Orentreich N, Vogelstein JH, Friedman GD: *Helicobacter pylori* infection, and gastric lymphomas. *N Engl J Med* 1994, 330:1267-1271
6. Isaacson PG: Mucosa-associated lymphoid tissue lymphoma. *Semin Hematol* 1999, 36:139-147
7. Greiner A, Knorr C, Qin Y, Sebald W, Schimpl A, Banchereau J, Müller-Hermelink HK: Low-grade B cell lymphomas of mucosa-associated lymphoid tissue (MALT-type) require CD40-mediated signaling, and Th2-type cytokines for in vitro growth and differentiation. *Am J Pathol* 1997, 150:1583-1593
8. Wotherspoon AC, Dogliani C, Diss TC, Pan L, Moschini A, de Boni M, Isaacson PG: Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of *Helicobacter pylori*. *Lancet* 1993, 342:575-577
9. Levine E, Arthur D, Machnicke J, Frizzera G, Hurd D, Peterson B, Gajl-Peczalska K, Bloomfield C: Four new recurring translocations in non-Hodgkin's lymphoma. *Blood* 1989, 74:1796-1800
10. Griffin C, Zehnbauser B, Beschoner W, Ambinder R, Mann R: t(11;18)(q21;q21) is a recurrent chromosome abnormality in small lymphocytic lymphoma. *Genes Chromosom Cancer* 1992, 4:153-157
11. Horsman D, Gascoyne R, Klasa R, Coupland R: t(11;18)(q21;q21): a recurring translocation in lymphomas of mucosa-associated lymphoid tissue (MALT)? *Genes Chromosom Cancer* 1992, 4:183-187
12. Leroux D, Sieite P, Hillion J, LeMarc'hadour F, Pegourie-Bandelier B, Jacob M, Larsen C, Sotto J: t(11;18)(q21;q21) may delineate a spectrum of diffuse small B-cell lymphoma with extranodal involvement. *Genes Chromosom Cancer* 1993, 7:54-56
13. Auer I, Gascoyne R, Connors J, Cotter F, Greiner T, Sanger W, Horsman D: t(11;18)(q21;q21) is the most common translocation in MALT lymphomas. *Ann Oncol* 1997, 8:979-985
14. Dalla-Favera R, Bregni M, Erikson J, Patterson D, Gallo RC, Croce CM: Human c-myc oncogene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. *Proc Natl Acad Sci USA* 1982, 79:7824-7827
15. Seto M, Jaeger U, Hockett RD, Graninger WB, Bennett S, Goldman P, Korsmeyer SJ: Alternative promoters and exons, somatic mutation and deregulation of the Bcl-2-Ig fusion gene in lymphoma. *EMBO J* 1988, 7:123-131
16. Rosenburg CL, Wong E, Petty EM, Bale AE, Tsujimoto Y, Harris NL, Arnold A: PRAD1, a candidate BCL1 oncogene: mapping and expression in centrocytic lymphoma. *Proc Natl Acad Sci USA* 1991, 88:9638-9642
17. Seto M, Yamamoto K, Iida S, Akao Y, Utsumi K, Kubonishi I, Miyoshi I, Ohtsuki T, Yawata Y, Namba M, Motokura T, Arnold A, Takahashi T, Ueda R: Gene rearrangement and overexpression of PRAD1 in lymphoid malignancy with t(11;14)(q13;q32) translocation. *Oncogene* 1992, 7:1401-1406
18. Dierlamm J, Baens M, Wlodarska I, Stefanova-Ouzounova M, Hernandez JM, Hossfeld DK, De Wolf-Peeters C, Hagemeijer A, Van den Berghe H, Marynen P: The apoptosis inhibitor gene API2 and a novel 18q gene, MLT, are recurrently rearranged in the t(11;18)(q21;q21) associated with mucosa-associated lymphoid tissue lymphomas. *Blood* 1999, 93:3601-3609
19. Akagi T, Tamura A, Motegi M, Suzuki R, Hosokawa Y, Nakamura S, Morishima Y, Seto M: A novel gene, MALT1 at 18q21, is involved in t(11;18)(q21;q21) found in low-grade B-cell lymphoma of mucosa-associated lymphoid tissue. *Oncogene* 1999, 18:5785-5794
20. Motegi M, Yonezumi M, Suzuki H, Suzuki R, Hosokawa Y, Hosaka S, Kodaera Y, Morishima Y, Nakamura S, Seto M: API2-MALT1 chimeric transcripts involved in mucosa-associated lymphoid tissue type lymphoma predict heterogeneous products. *Am J Pathol* 2000, 156:807-812
21. Baens M, Maes B, Steyls A, Geboes K, Marynen P, De Wolf-Peeters C: The product of the t(11;18), an API2-MLT fusion, marks nearly half of gastric MALT type lymphomas without large cell proliferation. *Am J Pathol* 2000, 156:1433-1439
22. Remstein ED, James CD, Kurtin PJ: Incidence and subtype specificity of API2-MALT1 fusion translocations in extranodal, nodal, and splenic marginal zone lymphomas. *Am J Pathol* 2000, 156:1183-1188
23. Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV: The TNFR2-TRAF signaling complex contains two novel proteins related to vacuolar inhibitor of apoptosis proteins. *Cell* 1995, 83:1243-1252
24. LaCasse EC, Baird S, Korneluk RG, MacKenzie AE: The inhibitors of apoptosis (IAPs) and their emerging role in cancer. *Oncogene* 1998, 17:3247-3259
25. Birnbaum MJ, Clem RJ, Miller LK: An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. *J Virol* 1994, 68:2521-2528
26. Inagaki H, Nonaka M, Nagaya S, Tateyama H, Sasaki M, Eimoto T: Monoclonality in gastric lymphoma detected in formalin-fixed, paraffin-embedded endoscopic biopsy specimens using immunohistochemistry, in situ hybridization, and polymerase chain reaction. *Diag Mol Pathol* 1995, 4:32-38
27. Inagaki H, Murase T, Otsuka T, Eimoto T: Detection of SYT-SSX fusion transcript in synovial sarcoma using archival cytologic specimens. *Am J Clin Pathol* 1999, 111:528-533
28. Pallisgaard N, Hokland P, Riishoj DC, Pedersen B, Jørgensen P: Multiplex reverse transcription-polymerase chain reaction for simultaneous screening of 29 translocations and chromosomal aberrations in acute leukemia. *Blood* 1998, 92:574-588
29. Downing JR, Khandekar A, Shurtleff SA, Head DR, Parham DM, Webber BL, Pappo AS, Hulshof MG, Conn WP, Shapiro DN: Multiplex RT-PCR assay for the differential diagnosis of alveolar rhabdomyosarcoma and Ewing's sarcoma. *Am J Pathol* 1995, 146:626-634
30. Chong SS, Boehm CD, Higgs DR, Cutting GR: Single-tube multiplex-PCR screen for common deletional determinants of α -thalassemia. *Blood* 2000, 95:360-362
31. Melo JV: The diversity of BCL-ABL fusion proteins and their relationship to leukemia phenotype. *Blood* 1996, 88:2375-2384
32. de Alava E, Kawai A, Healey JH, Fligman I, Meyers PA, Juvos AG, Gerald WL, Jhanwar SC, Argani P, Antonescu CR, Pardo-Mindan FJ, Ginsberg J, Womer R, Lawlor ER, Wunder J, Andrulis I, Sorensen PHB, Barr FG, Ladanyi M: EWS-FLI1 fusion transcript structure is an independent determinant of prognosis in Ewing's sarcoma. *J Clin Oncol* 1998, 16:1248-1255
33. Kawai A, Woodruff J, Healey JH, Brennean MF, Antonescu CR, Ladanyi M: SYT-SSX gene fusion as a determinant of morphology and prognosis in synovial sarcoma. *N Engl J Med* 1998, 338:153-160
34. Inagaki H, Nagasaka T, Otsuka T, Sugiura E, Nakashima N, Eimoto T: Association of SYT-SSX fusion types with proliferative activity and prognosis in synovial sarcoma. *Mod Pathol* 2000, 13:482-488
35. Rosenwald A, Ott G, Stilgenbauer S, Kalla J, Bredt M, Katzenberger T, Greiner A, Ott MM, Gawin B, Dojner H, Konrad H, Müller-Hermelink HK: Exclusive detection of the t(11;18)(q21;q21) in extranodal marginal zone B cell lymphomas (MZBL) of MALT type in contrast to other MZBL and extranodal large B cell lymphomas. *Am J Pathol* 1999, 155:1817-1821
36. Peng H, Du M, Diss TC, Isaacson PG, Pan L: Genetic evidence for a clonal link between low and high-grade components in gastric MALT B-cell lymphoma. *Histopathology* 1997, 30:425-429
37. Nakamura T, Nakamura S, Yonezumi M, Suzuki T, Matsuura A, Yatabe Y, Yokoi T, Ohshi K, Seto M: *Helicobacter pylori* and the t(11;18)(q21;q21) translocation in gastric low-grade B-cell lymphoma of mucosa-associated lymphoid tissue type. *Jpn J Cancer Res* 2000, 91:301-309
38. Alpen B, Neubauer A, Dierlamm J, Marynen P, Thiede C, Bayerdörffer E, Stolte M: Translocation t(11;18) absent in early gastric marginal zone B-cell lymphoma of MALT type responding to eradication of *Helicobacter pylori* infection. *Blood* 2000, 95:4014-4015