

Original Article

Correlation Between Histological Evaluation and PCR-based Clonality Analysis of Gastric MALT Lymphomas Treated by Eradication of *Helicobacter pylori*

Sumie Takase Sakugawa¹⁾, Tadashi Yoshino¹⁾, Tomohiko Mannami¹⁾, Mitsuru Munemasa¹⁾, Koichi Ichimura¹⁾, Hiroyuki Okada²⁾, Motowo Mizuno²⁾, Mitsune Tanimoto³⁾, Mine Harada⁴⁾ and Tadaatsu Akagi¹⁾

Mucosa-associated lymphoid tissue lymphoma (MALT lymphoma) arises from *Helicobacter pylori* (*H. pylori*)-associated chronic gastritis, and eradication of *H. pylori* is an effective treatment in most cases. Using Wotherspoon and Isaacson's scoring system for the evaluation of gastric MALT lymphoma, in which grades up to 3 are considered reactive and grades 4 and 5 neoplastic, we assessed the histological findings of gastric biopsied specimens after eradication of *H. pylori*, and examined the relationship between the grade and the PCR-based monoclonality or the MIB-1 index. Expression of Ki-67 (MIB-1) antigen was examined immunohistochemically in 17 biopsied specimens from 3 patients, and 59 samples taken from these specimens were analyzed by PCR for immunoglobulin heavy chain gene rearrangement. Monoclonality was observed in 88%, 67%, and 17% of grade 5, 4 and 3 samples, respectively. No monoclonal band was detected in the samples of grades 1 and 2. The MIB-1 indices (mean \pm SD) in the interfollicular areas of grade 1 to 5 were 0.9 \pm 0.7, 0.9 \pm 0.2, 1.1 \pm 0.6, 1.5 \pm 0.5, and 2.9 \pm 1.6%, respectively. It is concluded that there is a good correlation between histological scoring and PCR-based monoclonality as well as MIB-1 indices.

Key words MALT lymphoma, post-*Helicobacter pylori* eradication, PCR-based clonality, histological scoring, MIB-1 index

INTRODUCTION

Gastric mucosa-associated lymphoid tissue (MALT) lymphoma is a unique disease; it arises from *Helicobacter pylori* (*H. pylori*)-associated chronic gastritis, and eradication of *H. pylori* is an effective treatment in most cases¹⁻⁴. Furthermore, a number of MALT lymphoma cells proliferate

depending upon other stimuli such as cytokines produced by *H. pylori*-specific T cells^{5,6}. Therefore, neoplastic and reactive stages are not clearly delineated. Wotherspoon *et al.* proposed a scoring system for histological evaluation of gastric MALT lymphoma². They divided *H. pylori*-associated gastric lesions into 6 grades ranging from a reactive state to a neoplastic state: grade 0 is defined as the state of no or scanty infiltration of lymphoid cells; grade 1 shows small aggregates of mature lymphocytes without lymphoid follicles; grade 2 is chronic active gastritis accompanied by lymphoid follicles; grade 3 is defined as probably benign, but shows focal infiltration of dense lymphoid cells into the epithelium; grade 4 is defined as being probably malignant with infiltration of lymphoid cells showing mild atypia and lymphoepithelial lesion (LEL)-like changes in small groups; grade

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Departments of ¹⁾Pathology, ²⁾Medicine and Medical Science, and ³⁾Hematology, Oncology and Respiratory Medicine, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan; and ⁴⁾Medicine and Biosystemic Science, Internal Medicine, Medicine and Surgery, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan

Address correspondence and reprint request to Tadashi Yoshino, Department of Pathology, Okayama University Graduate School of Medicine and Dentistry, Shikata-cho 2-5-1, Okayama 700-8558, Japan

5 is typical and definite low-grade MALT lymphoma with dense infiltration of centrocyte-like (CCL) cells and prominent LELs; grades 3 and 4 are regarded as the gray zone.

There have been reports on the relationship between the histological scoring and clonality of infiltrating lymphoid cells⁷⁻⁹. However, the validity of this scoring system remains to be evaluated. In this report, polymerase chain reaction (PCR)-based clonality analysis was conducted in consecutively biopsied specimens from 3 cases of gastric MALT lymphoma in the course of antibiotic therapy. This study was undertaken with the expectation to provide a precise evaluation of the scoring system regarding monoclonality. Furthermore, expression of Ki-67 antigen was examined as a marker of cell proliferation that may reinforce the scoring system.

MATERIALS AND METHODS

Patients

We examined three patients with gastric low-grade MALT lymphoma diagnosed at the Department of Pathology, Okayama University Graduate School of Medicine and Dentistry (Table 1). The diagnosis of MALT lymphoma was based on characteristic histological features

such as LELs. Informed consent for the investigation was obtained from the patients.

Case 1: A 46-year-old female subject suffering from abdominal pain. Endoscopic examination revealed multiple ulcers of the stomach. Biopsied specimens showed proliferation of CCL cells and LELs, which are characteristic of MALT lymphoma. PCR analysis confirmed the clonality of the lymphoma cells. One month after eradication therapy for *H. pylori*, the lesion improved as observed endoscopically; however, a small number of lymphoma cells remained infiltrated the epithelium (grade 4). The lymphoma cells disappeared from the biopsied specimen 4 months after eradication therapy.

Case 2: A 61-year-old female subject underwent endoscopic examination of the stomach, and specimens were taken from the rough surface of the body mucosa. The lesion was histologically diagnosed as gastric MALT lymphoma. She received eradication therapy for *H. pylori*, resulting in marked reduction of the lesion, 9 months later.

Case 3: The patient was a 40-year-old male subject. An irregularly-shaped ulcer of the stomach was detected endoscopically. The biopsied specimens had the features of MALT lymphoma. Eradication therapy for *H. pylori* lasting 4 months reduced the lesion, and the lymphoma

Table 1. Histological scoring, PCR clonality, and MIB-1 indices of the specimens sequentially biopsied after eradication of *H. pylori*

Case	Biopsy	Months after eradication	*Grade	No. of PCR samples	No. of monoclonal samples	MIB-1 index (%)
1	A	0	5	2	2	2.1
	B	0	5	6	6	2.4
	C	1	4	ND	ND	ND
	D	4	2	4	0	0.7
	E	8	2	6	0	1
	F	14	1	6	0	1.4
2	A	0	5	3	2	0.8
	B	1	5	5	4	4.8
	C	3	4	3	1	ND
	D	9	3	3	1	0.7
	E	16	1	3	0	0.4
3	A	0	5	—	—	4.3
	B	4	2	3	0	0.9
	C	7	3	3	0	1.5
	D	13	4	6	4	ND
	E	26	4	3	2	1.1
	F	27	4	3	3	1.8

*, evaluated by Wotherspoon's scoring system; ND, not done; —, not available

cells disappeared histologically (grade 2). However, the biopsy specimens taken 13 months after eradication showed infiltration of CCL cells (grade 4), suggesting the recurrence of MALT lymphoma.

Immunohistology

To determine the cellular characteristics, immunohistological examination was performed by the indirect immunoperoxidase method, using a dextran polymer-conjugated secondary antibody labeled with peroxidase (Envision⁺, DAKO Japan, Kyoto, Japan), or by the labeled streptavidin-biotin (LSAB) method, using a biotinylated secondary antibody and peroxidase-labeled streptavidin (ScyTek Lab, Logan, Utah, USA). Briefly, approximately 3 μ m-thick paraffin sections were prepared and deparaffinized. Immunostaining was carried out according to the manufacturer's instructions using the Envision⁺ immunostaining system or the biotin-streptavidin system kit. The following primary monoclonal antibodies (MAbs) were used: MAbs to CD10, CD5, and CD23 from Novocastra Lab Co. Ltd (Newcastle, U. K), MAbs to BCL-2, CD20, CD79a, CD45RO, CD3 ϵ and Ki-67 antigen from DAKO Japan, MAb to cytokeratin AE1/AE3 from Roche Diagnostics (Tokyo, Japan), and MAb to cyclin D1 from Zymed Co. Ltd. (So-SF, CA, USA). For the double immunostaining of the Ki-67 antigen and the T (CD3 ϵ) or B (CD79a)-cell marker, the LSAB method using alkaline phosphatase-labeled streptavidin was applied to detect Ki-67 antigen.

PCR for the detection of immunoglobulin gene rearrangement

Two to six DNA samples were extracted from each of the 17 paraffin sections according to the method described by Wright and Manos¹⁰. To detect the monoclonal rearrangement of immunoglobulin heavy chain genes, each DNA sample was subjected to the two-step PCR method described by Ramasamy *et al.* to amplify the FR2A-LJH and the FR2A-VLJH regions¹¹. A sample from reactive lymph node hyperplasia and a sample lacking the DNA template were amplified in a similar way to serve as negative controls. DNA extracted from the lymph node of diffuse large B-cell lymphoma, whose mono-

clonality was confirmed by Southern blot analysis, was used as a positive control. The amplified products were subjected to electrophoresis on a 4% agarose gel. The specimen was evaluated as being monoclonal when two or more samples from each paraffin section showed identical bands. Monoclonality of the samples after *H. pylori* eradication was attributed only when the bands were identical before and after the eradication.

Estimation of Ki-67 antigen expression in lymphoid cells

Expression of Ki-67 antigen in the 17 specimens from the 3 patients was examined immunohistochemically using MIB-1 MAb as described previously^{12,13}. The presence of the Ki-67 antigen was evaluated on photographs taken at high magnification, and the MIB-1 index (number of Ki-67-positive cells/number of total lymphoid cells x 100) was calculated.

Statistical analysis

The χ^2 test was used to analyze the correlation between the rate of monoclonality and histological grades. The MIB-1 indices of the samples were compared by the Student's *t*-test.

RESULTS

Immunostaining

The histological diagnosis of MALT lymphoma was confirmed by positive immunostaining of lymphoma cells for CD20, CD79a, and BCL-2 and negative immunostaining for cytoplasmic CD3 ϵ CD45RO, CD10, CD5, CD23, and cyclinD1.

Histological scoring and frequency of monoclonality

Monoclonality of the biopsied specimens was examined before and after eradication of *H. pylori* (Table 1). Monoclonal B-cell proliferation was detected as a single band by PCR analysis of the immunoglobulin heavy chain genes (Fig. 1). No monoclonal band was detected in the samples lacking DNA or in samples from reactive lymph node hyperplasia. Generally, B-cell mono-

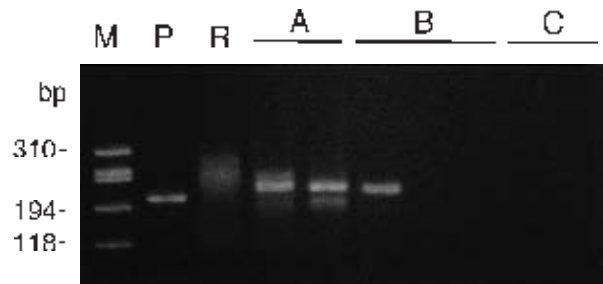


Fig. 1. PCR analysis of immunoglobulin heavy chain genes for case 2
 A, grade 5 (before eradication); B, grade 3 (9 months after eradication); C, grade 1 (16 months after eradication). P: positive control, R: reactive lymph node hyperplasia. A clonal band is present in the PCR products from both grade 5 samples and from one of the three grade 3 samples. There is no clonal band in grade 1 samples.

clonality detected by PCR method was in good correlation with histological scoring. Monoclonal proliferation was observed in 14 out of 16 grade 5 samples (88%), and 10 out of 15 grade 4 samples examined (67%). In contrast, no monoclonal band was detected in the 9 grade 1 samples and the 13 grade 2 samples. The difference of frequency of monoclonality between grades 4-5 and grades 1-3 was statistically significant ($p < 0.01$). In the grade 3, 1 out of 6 samples (17%) showed monoclonal proliferation.

Expression of Ki-67 antigen and histological scoring

The interfollicular areas with a considerable amount of lymphoid cell aggregates were selected as the fields for estimation of MIB-1 indices (Fig. 2A-C). The reactive germinal centers and the lesions of follicular colonization were not estimated for comparison because these areas showed extremely high MIB-1 indices (Fig. 2D). The immunoreactive cells were rather lar-

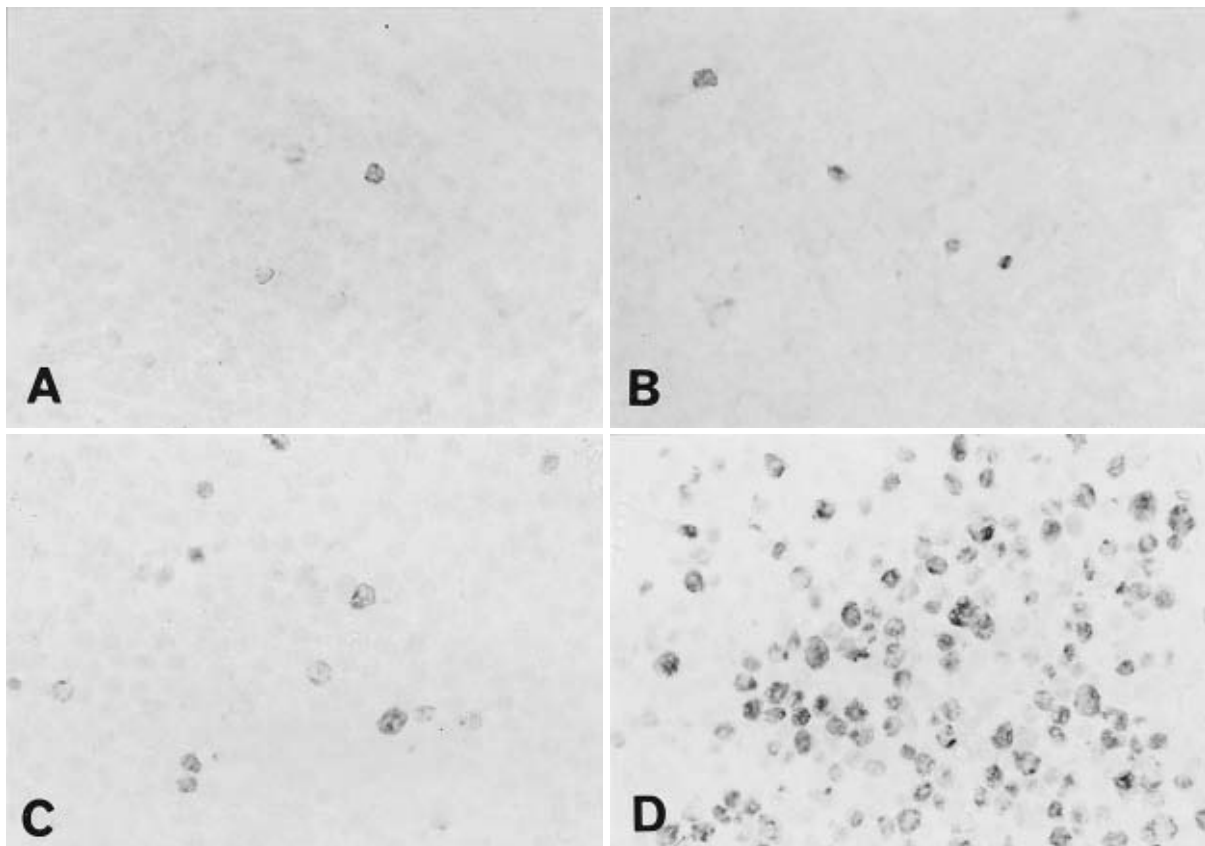


Fig. 2. Expression of Ki-67 antigen in MALT lymphomas
 A, interfollicular area of grade 2; B, interfollicular area of grade 4; C, interfollicular area of grade 5; D, follicular colonization of grade 5.

ger than the nonreactive cells. MIB-1 indices (mean \pm SD) in the interfollicular areas of grades 1-5 were 0.9 ± 0.7 , 0.9 ± 0.2 , 1.1 ± 0.6 , 1.5 ± 0.5 , and $2.9\pm 1.6\%$, respectively. The average MIB-1 index for grade 5 samples was significantly higher than that of grades 1-3 ($p=0.02$). The average MIB-1 index of follicular colonization was $38.2\pm 5.5\%$. Double immunostaining of MALT lymphoma tissues revealed that these MIB-1-positive cells in the samples were exclusively B cells. (data not shown).

DISCUSSION

In the present study, we found a good correlation between histological scoring and PCR-based clonality in gastric MALT lymphomas. In grades 4 and 5, 77% of the specimens were monoclonal, whereas no monoclonal band was found in the cases of grades 1 and 2. Not all B-cell lymphoma samples were shown to be monoclonal by the PCR analysis used in this study. The sensitivity of this method is reported to be about 66-100%^{11,14}. The frequencies of monoclonality for grades 4 and 5 in the present study were compatible with these reports. In the biopsy A of case 3, we could not detect any monoclonal band (Table 1). This DNA sample was not amplified with PCR primers for β -globin; therefore this specimen seems to be inadequate for DNA analysis. Interestingly, samples of grade 1 or 2 occasionally showed faint clonal bands; however, this clonality was not reproducible, and a second trial resulted in smears or clonal bands of different size. According to our experience, this phenomenon does not happen in nodal lymphomas such as diffuse large B-cell lymphoma or follicular lymphoma (data not shown). The precise reason for this phenomenon has not been clarified yet. There may be several expanding clones in these samples, some of which might be detected rather at random. According to these findings, we would like to emphasize the importance of reexamination of the same samples. A number of authors described that PCR monoclonality was sustained for a considerably long period after eradication of *H. pylori*, even without MALT lymphoma histology^{15,16}. We suspect that some of these findings might be derived from the "transient" clonal bands.

Although the usefulness of the histological

scoring appeared convincing, its application to pretreated and post-eradication specimens was not identical. In the cases where eradication was effective, lymphoma cells in the lamina propria and LELs disappeared rapidly after the treatment, compared with lymphoma cells in deeper regions and cellular atypia^{7,17}. In some of the post-treated cases, LELs were very few in spite of rather prominent cellular atypia. These cases were evaluated as grade 5. Evaluation of grade 3 was also somewhat difficult. In our study, B-cell clonality was detected in one grade 3 specimen when sample was taken after the eradication of *H. pylori*. In contrast, no clear monoclonality was detected in grade 3 specimens before the treatment (data not shown). These findings strongly suggest that histological scoring should be carefully applied especially after the eradication therapy. It may be necessary to devise modified scoring system for such cases.

Nakamura *et al.* reported that the MIB-1 index of low grade MALT lymphoma was 3.1-17.7%¹⁸. In the present study, the mean MIB-1 index for grade 5 (2.9%) was lower than the reported values, although it was significantly higher than the average MIB-1 index for grades 1-3. This discrepancy may reflect the difference of estimation methods between the two studies. Follicular colonization showing the high MIB-1 index might be involved in their estimation. The average MIB-1 index for grade 4 was between that of grades 1-3 and of grade 5, but this difference was not statistically evaluated because not enough specimens suitable for estimation was available for grade 4. The grade 4 samples showed monoclonality at a rate comparable to grade 5. However, the size of lymphoid cells was smaller, and the density of LELs was lower in grade 4. MIB-1 positivity seemed to be related to the cell size. After the eradication therapy succeeded, the sizes of lymphoma cells decreased dramatically. Proliferation of MALT lymphoma cells is thought to be helped by T-cells, which react with *H. pylori*^{5,6}. Eradication of *H. pylori* may abolish the involvement of T cells and diminish the size and proliferating activity of activated B cells.

In conclusion, both PCR-based clonality and MIB-1 index in MALT lymphomas had a good correlation with histological scoring. These methods are relatively easy to perform; therefore, they may be of important help for the diagnosis, follow up and remission decision of MALT

lymphomas.

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