B-Cell Gene Rearrangement in Benign and Malignant Lymphoid Proliferations of Mucosa-Associated Lymphoid Tissue and Lymph Nodes

EMINA TORLAKOVIC, MD, DAVID L. CHERWITZ, MD, JOSE JESSURUN, MD, JOHN SCHOLES, MD, AND RONALD McGLENNEN, MD

The polymerase chain reaction (PCR) with polyacrylamide gel electrophoresis was used to study patterns of immunoglobulin heavy chain (IgH) gene rearrangement (GR) in formalin-fixed, paraffin-embedded specimens of lymphomas and reactive conditions of mucosa-associated lymphoid tissue (MALT) and lymph nodes. DNA amplification was performed directly on sections obtained from paraffin blocks. Five patterns of PCR products were observed: a single band, two or more discrete bands, smearing, a single band overlying a smear, and two or more bands over a smear. A pure polyclonal pattern (smear) was observed in all of the reactive lymph nodes but in only 15% of cases of Helicobacter pylori (HP) gastritis with lymphoid hyperplasia, 25% of cases of HP gastritis without lymphoid hyperplasia, and 37% of colonic specimens of various types. Patterns consisting of multiple bands with or without background smearing were common in gastritis, colitis, and gastric lymphomas. Single bands or dominant bands were present in all lymph node and salivary gland lymphomas, 12 of 14 cases of gastric lymphoma, and 17 of 20 cases of HP gastritis with lymphoid hyperplasia. These bands were reproducible in deeper sections from the same paraffin block or similar areas sampled in different blocks in all of the lymph node and salivary gland lymphomas, 11 of 12 gastric lymphomas, but only 1 of 17 cases of HP gastritis with lymphoid hyperplasia. Bands were also found in 3 of 20 cases of HP gastritis without lymphoid hyperplasia and 17 of 38 colonic specimens, but these were not reproducible. The complexity of patterns of IgH GR in acquired MALT compared with lymph nodes may be the result of a relative paucity of B-cell clones or preferential proliferation of B-cell clones with a limited area of distribution. HUM PATHOL 28:166--173. This is a US government work. There are no restrictions on its use.

Key words: immunoglobulin gene rearrangement, PCR, MALT, colitis, gastritis, gastric lymphoma.

Abbreviations: PCR, polymerase chain reaction; GR, gene rearrangement; IgH, immunoglobulin heavy chain; MALT, mucosa-associated lymphoid tissue; PAGE, polyacrylamide gel electrophoresis; P, polyclonal proliferation; M, monoclonal proliferation; O, oligoclonal proliferation; HP, Helicobacter pylori.

Use of the polymerase chain reaction (PCR) for the detection of the B- and T-lymphocyte gene rearrangement (GR) is a relatively new method for the study of lymphoid neoplasms. This method has many advantages when compared with the Southern blot technique. It is a faster, relatively simple, and inexpensive technique that avoids the use of radioactive materials and requires smaller amounts of less intact genomic DNA. DNA obtained from formalin-fixed, paraffin-embedded tissue has been shown to be a suitable substrate for PCR.

The sensitivity of PCR detection of a clonal population is dependent on several factors: (1) the ability of the primers to detect a specific GR; (2) the amplification technique; (3) the size homogeneity of the amplified product; (4) the relative numbers of monoclonal and polyclonal B- and T-lymphocytes present in the tissue being studied; and (5) the method of DNA extraction. PCR-based detection of GR has been reported to be capable of detecting one malignant cell in 100,000 cells with the use of case-specific primers, or two in 10,000 cells with a seminested PCR assay. In contrast, the typical sensitivity of Southern blot detection of GR is approximately 1 to 2 in 100 cells, to 1 in 500 in an optimized procedure.

Detection of immunoglobulin heavy chain (IgH) GR is not specific for the neoplastic DNA. Both benign and malignant lymphocytes undergo GR early in their maturation, which can be shown by PCR amplification. In most lymphomas, there are variable numbers of benign lymphocytes in addition to the malignant clone. Although these are not detected by Southern blot, they may produce background smearing on PCR-based detection of GR. Polyclonal patterns and combinations of a dominant clone and a polyclonal background have been described in reactive lymph nodes and in lymphomas with a significant benign lymphocytic background, respectively.

This phenomenon has also been reported in studies of formalin-fixed, paraffin-embedded specimens. However, benign conditions, particularly those of mucosa-associated lymphoid tissue (MALT), have only recently been investigated with PCR-based GR detection.

This study investigates patterns of IgH GR in benign and malignant lymphoid proliferations of MALT and compares these with benign and malignant lymphoid proliferations in lymph nodes. Also, the efficacy of a PCR-based technique using formalin-fixed, paraffin-embedded tissue as an aid in the diagnosis of gastric lymphoma is assessed.
Lymph node were classified according to the criteria for MALT lymphoma

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<td>Reactive hyperplasia</td>
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<td>Stomach</td>
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<td>0</td>
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<td>0</td>
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<td>Total</td>
<td>59</td>
<td>32</td>
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* Previously evaluated for immunoglobulin gene rearrangement by Southern blot and PCR performed on DNA extracted from fresh and frozen tissue.

**Materials and Methods**

**Specimen Selection**

Specimens used in this study were obtained from the surgical pathology files of the University of Minnesota Hospital and Clinics, the VA Medical Center (Minneapolis, MN), and New York University Medical Center. All tissues were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. The paraffin blocks contained either small biopsy specimens (stomach and colon) or larger sections of resected specimens (lymph node, colon, stomach, and salivary gland). Hematoxylin and eosin–stained sections were examined by four surgical pathologists to confirm the diagnosis (E.T., D.L.C., J.J., and J.S.). The lymphomas of the stomach were classified according to the criteria for MALT lymphoma of the European Association of Hematology either as low-grade (n = 9) or high-grade (n = 5) B-cell lymphomas. The tissues and corresponding diagnoses are listed in Table 1.

**Tissue Preparation for PCR**

All PCR reactions were performed on formalin-fixed, paraffin-embedded tissues without pretreatment. Representative paraffin blocks were cut at 4 to 6 μm using a clean disposable microtome blade for each block. Before sectioning, the excess paraffin was trimmed from the block. The first and the last section from each ribbon were mounted on a glass slide, stained with hematoxylin and eosin, and reviewed by light microscopy to ensure representative sampling. The paraffin sections were allowed to roll and then transferred directly into the PCR reaction tubes (discussed later). In cases with high cellularity in large portions of tissue (eg, lymph nodes, salivary gland lymphomas), a 5-mm³ representative area of the embedded tissue was selected for sectioning. The number of paraffin sections per reaction varied from one for resection specimens to three or four per tube for small biopsy specimens from the stomach and colon (discussed later).

**PCR Protocol**

The reaction mixture of 100 μL included 56 pmol of each primer, 200 μmol of each dNTP; 5 mmol/L magnesium chloride, and standard 10× buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl (pH 9.0), 1% Triton X-100). The primer pair for the detection of IgH GR has been previously described. These consist of a universal VH primer based on an oligonucleotide sequence located at the 3′ end of the FRIII region (5′GTG TCG ACA CGG CCG TGT ATT AGC G3′) and a consensus JH primer (5′AAC TGC AGA GGA GAC GGT GAC C3′). After a 10-minute incubation at 100°C (“hot start”), the temperature was lowered to 80°C, and 2.5 U of Taq-polymerase was added to each tube. The PCR cycles consisted of denaturation at 98°C for 1 minute, annealing of the primers at 64°C for 1 minute, then extension of the DNA at 73°C for 1 minute. A total of 40 cycles was used for each reaction. Twenty-microliter aliquots of the reaction mixture were then run on a 6% polyacrylamide gel at 100 volts for 1 hour (PAGE). Selected samples were also run on 10% and 12% polyacrylamide gel at 135 volts for comparison of banding resolution. The gels were stained with ethidium bromide and photographed under ultraviolet light.

**Controls**

Four lymph-node–based follicular lymphomas and one salivary gland lymphoma that had been previously tested for immunoglobulin heavy and light chain GR by Southern blot and for IgH GR by PCR using fresh tissue were included in the study. Tissue from these cases, as well as an additional 10 reactive lymph nodes, were used as controls for monoclonal and polyclonal proliferations. Each run of gastric and colonic biopsy specimens contained at least one positive control of each type. Negative controls consisted of a PCR tube containing the reaction mixture but no tissue. One negative control was generally included for five test reactions. These were used to detect contamination by paraffin fragments, which could potentially occur at the time sections were cut from the paraffin block or in the thermal cycler while adding Taq polymerase to the reaction tubes. To avoid this type of contamination, the paraffin sections were placed directly into tubes already containing the reaction mixture, and the tubes were widely spaced in the thermal cycler.

Several sets of internal comparisons were performed to determine the optimum number of paraffin sections required to obtain interpretable PCR product. This was accomplished by evaluating the electrophoretic patterns of reaction products produced using 1 to 20 paraffin sections from the same paraffin block per reaction tube. In addition, a comparison of the electrophoretic patterns was performed to evaluate the reproducibility of results over time for the same patient and for different areas from the same specimen. These included a comparison of the IgH GR patterns of a lymph node–based B-cell lymphoma (follicular, small cleaved) sampled at different times (1978 and 1993) and a comparison of the electrophoretic patterns of reaction products produced by paraffin sections from different levels of the same paraffin block or from histologically comparable areas of different paraffin blocks from the same specimen.
TABLE 2. Electrophoretic Patterns of PCR-Based Immunoglobulin Heavy Chain Gene Rearrangement From Formalin-Fixed, Paraffin-Embedded Tissues

<table>
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<tr>
<th>Tissue/Diagnosis</th>
<th>n</th>
<th>P</th>
<th>M</th>
<th>M+P</th>
<th>O(2)</th>
<th>O(&gt;2)</th>
<th>O+P</th>
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<tr>
<td>Follicular center cell lymphoma</td>
<td>4</td>
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<tr>
<td>Reactive hyperplasia</td>
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<td>10</td>
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Abbreviations: n, number of cases; P, polyclonal smear without bands; M, single band without background smearing; M+P, single band with background smearing; O(2), 2 bands; O(>2), more than 2 bands; O+P, two or more bands with background smearing; nr, no detectable PCR product.
* Eleven cases had reproducible bands.
† One case had reproducible bands.

RESULTS

Eighty-three percent of PCR reactions with formalin-fixed, paraffin-embedded tissues produced detectable product. Five patterns of the PCR product were observed after gel electrophoresis and staining with ethidium bromide: (1) diffuse smearing consistent with a polyclonal proliferation (P); (2) a single band consistent with a monoclonal proliferation (M); (3) two or more bands consistent with an oligoclonal proliferation (O); (4) a single band superimposed over a diffuse smear consistent with a monoclonal proliferation with a polyclonal background (M+P); and (5) two or more bands over a smear consistent with an oligoclonal proliferation with a polyclonal background (O+P). No significant difference in band resolution was observed among 6%, 10%, and 12% polyacrylamide gels.

Controls

Comparison of PCR reaction products from fresh or frozen tissue with formalin-fixed, paraffin-embedded tissue from lymph node and salivary gland lymphomas showed no difference in GR patterns. In addition, all 10 reactive lymph nodes produced intense, diffuse smearing in the expected molecular weight range, indicative of a polyclonal population. These results are shown in Table 2.

A comparison using different numbers of sections from the same area of the paraffin block was performed for 20 cases (3 salivary gland lymphomas, 5 gastric lymphomas, 2 cases of collagenous colitis, 2 cases of Crohn's disease, 2 cases of ulcerative colitis, and 6 cases of Helicobacter pylori-related [HP] gastritis with lymphoid hyperplasia). Reaction product was observed in only 50% of cases when one paraffin section from small biopsy specimens was used. Conversely, diffuse smearing throughout the lane on PAGE was observed in 43% of reactions with 6 to 10 paraffin sections and 76% of reactions with more than 10 paraffin sections. This pattern of smearing was different from that observed with a polyclonal proliferation in reactive lymph nodes and is likely to be PCR long product, a result of DNA template overload.

When small biopsy specimens from stomach or colon were tested, all reactions with two to five paraffin sections produced some product in the expected molecular weight range. Also, a single 4-μm thick section of a 5-mm² area from cellular resection specimens consistently produced some product on PAGE. In most cases, no change in the electrophoretic pattern was observed over this range of sections, although there was a tendency for smearing in the expected molecular weight range to become wider and more intense as more paraffin sections were used. Using either a smaller (one to three paraffin sections) or larger (six or more paraffin
sections) amount of tissue in the reaction tubes did not increase the number of bands detected. Based on these findings, we determined that it was optimal to use three or four paraffin sections per reaction from small biopsy specimens, and a single section of a 5-mm² area from lymph node, salivary gland, gastric, and colonic resection specimens.

Case Studies

The results of PCR GR by site and diagnosis are summarized in Table 2. Each of the 14 reactions with lymph node tissue produced detectable product, as did the four salivary gland lymphomas. All four nodal lymphomas produced a single (monoclonal) band, with three of these overlying a polyclonal background. A follicular small cleaved cell lymphoma in lymph node biopsy specimens from the same patient in 1978 and 1993 had an identical GR pattern in both biopsy specimens. All 10 lymph nodes with reactive hyperplasia produced a polyclonal pattern without identifiable bands. Three salivary gland lymphomas produced a pure monoclonal pattern. The fourth showed only a polyclonal smear without a detectable band.

Compared with the lymph node lesions, gastric lymphomas and inflammatory conditions of the gastrointestinal tract produced more complex patterns (oligoclonal and the combination of oligoclonal and polyclonal). Such patterns were found in 50% (7 of 14) of gastric lymphomas (Figs 1 to 3), whereas a monoclonal band (with or without background smearing) was detected in only 36% (5 of 14). Fourteen percent (2 of 14) of gastric lymphomas diagnosed by light microscopic criteria had a polyclonal pattern by PCR GR. HP gastritis with lymphoid hyperplasia showed patterns similar to those of gastric lymphoma (Figs 4, 5), except none of 20 cases tested produced a monoclonal band in any of the multiple PCR reactions performed on these specimens. This was also true for other inflammatory conditions of the gastrointestinal tract (Fig 5). However, only 28% (12 of 43) of cases of gastritis or colitis produced a pure polyclonal pattern, compared with 100% (10 of 10) of reactive lymph nodes.

Evaluation of the effects of sampling either within a single paraffin block or from separate blocks of histologically comparable areas of the same lesion was performed on all cases of colitis, one salivary gland lymphoma, all gastric lymphomas, and all cases of HP gastritis. The PCR reactions were repeated from 2 to 10 times (at different times or in parallel). No significant change in the type of pattern was observed. However, different or additional bands appeared in 88% of reac-
FIGURE 4. Ethidium bromide-stained/UV-illuminated gel containing amplified products of PCR gene rearrangement from a case of HP gastritis (right) with lymphoid hyperplasia. (Hematoxylin and eosin; original magnification ×150.) Lane 1, DNA bp size marker (Phi X 174 RF DNA/Hae III digest); Lanes 2, 3 and 4, different levels of a single paraffin block. Note the oligoclonal pattern and that the bands are not reproducible in different levels from the paraffin block. The bands below 72 bp represent primer dimers.

Irreproducible Bands

A significant finding in this study is the complexity of patterns in both inflammatory and neoplastic proliferations of acquired MALT in the gastrointestinal tract compared with similar lesions in lymph nodes. Because of the implications this has both for the potential use of PCR GR in formalin-fixed, paraffin-embedded gastrointestinal biopsy specimens and for our understanding of MALT, it is important to determine whether this finding is simply an artifact produced by PCR amplification of this type of specimen or reflects an actual difference between lymphoid proliferations of MALT and other sites.

“Nonspecific” bands in PCR-based IgH GR were described by Wan et al. They found that samples with a low DNA concentration or samples with very diluted B-cell clones may produce one or more bands after electrophoresis on agarose gels. However, bands of different molecular weight were obtained from duplicate samples. They also found that increasing the PCR cycle number often increased the number of nonspecific bands.

Although DNA derived from formalin-fixed, paraffin-embedded tissue is usually markedly degraded and of low quantity, it is not entirely clear whether undigested paraffin sections used as a solid-phase template can be considered a “low-DNA concentration” source. A number of our findings suggest that these factors may not completely explain the banding patterns seen in specimens of MALT. First, our samples, particularly from the colon, were selected in part on the basis of high cellularity and then optimized to prevent DNA overload. As previously mentioned, samples with more than six paraffin sections per PCR reaction frequently

DISCUSSION

The results of this study confirm the efficacy of PCR-based evaluation of IgH GR in formalin-fixed, paraffin-embedded tissue. Lymph node–based lymphomas and salivary gland lymphomas that had been thoroughly characterized using both Southern blot and PCR methods on fresh tissue showed no differences in results when studied using formalin-fixed, paraffin-embedded material without pretreatment. This is in concordance with a study by Slack et al showing that the purity of DNA is not a significant factor in the success of PCR amplification. The “hot start” used in our protocol melts the paraffin, which then combines with oil on the surface. Sectioning at 4 to 6 μm can be expected to cut through a sufficient number of nuclei to make an adequate amount of DNA available for PCR. The use of the “hot start” technique with the addition of paraffin wax or petroleum jelly has been recommended for PCR reactions and may increase the sensitivity and specificity of the amplification. The paraffin in the tissue sections appears to have a similar effect of preventing nonspecific reactions before the melting temperature is reached.
resulted in an electrophoretic pattern characteristic of DNA overload. Second, we did not observe similar complex and irreproducible electrophoretic patterns in benign and malignant proliferations of lymph nodes with comparable sample sizes. Furthermore, in 10 of 14 cases of gastric lymphoma, single bands (pattern M) and dominant bands (in patterns M+P, O, and O+P) were reproducible with tissue sections from deeper levels of the paraffin block or from other blocks, a finding that differs from those of Wan et al. Third, multiple irreproducible bands were not frequent in samples of histologically normal colon, polypos without inflammation, or chronic active gastritis without lymphoid hyperplasia. These samples had the smallest amount of lymphoid tissue of any of our cases and thus would be expected to have the lowest concentration of amplifiable DNA. Finally, we have observed that irreproducible bands were more common when a polyclonal background was present. The exceptions to this were HP gastritis with lymphoid hyperplasia and Crohn’s disease.

The appearance of irreproducible bands in our study may be the result of relative paucity of B-cell clones available for amplification, either because of the actual small number of B-cells in the specimen or a true selective proliferation of B-cell clones, preferential amplification attributable to primer characteristics, or a combination of these factors. Although the small number of B-cells in colonic biopsy specimens may be a possible explanation for the appearance of irreproducible bands, this is less likely to be the case in HP-related gastritis because specimens with lymphoid hyperplasia tended to produce bands more frequently than those without lymphoid hyperplasia (85% vs 15%). This is the opposite of what would be expected if the actual number of B cells was responsible for this phenomenon. Hsi et al. similarly found that specimens containing lymphoid aggregates or germinal centers with many B cells produced clonal patterns. It is thus more likely that these bands are caused by selective proliferation of a relatively small number of B-cell clones in acquired MALT. This conclusion is in concordance with the findings of Kossakowska et al. They used the Southern blot technique in a study of immunoglobulin and T-cell receptor GR in various inflammatory and other lesions in acquired MALT and found that in various sites, small clonal proliferations of B and T cells are frequent.

PCR GENE REARRANGEMENT (Torlakovic et al)

In our study, complex patterns (M+P, O, and O+P) were common to gastritis, colitis, and gastric lymphoma. However, only lymphomas produced a pure monoclonal pattern. This pattern had 100% specificity for malignant lymphoma in our study. The low sensitivity of a pure monoclonal pattern (3 of 14 cases) in gastric lymphomas was most likely the result of the simultaneous amplification of other clones or the polyclonal background. A comparison of HP gastritis without lymphoid hyperplasia and HP gastritis with lymphoid hyperplasia showed that when lymphoid follicles are not present, PCR product is unlikely to be obtained (60% of HP gastritis without lymphoid hyperplasia showed no PCR product). However, when lymphoid aggregates were present, the PCR patterns were similar to those of gastric lymphoma.

It has been suggested that in low-grade MALT lymphomas, infiltrating T cells that recognize specific strains of H pylori potentiate the proliferation of neoplastic B cells. If the patterns obtained in our study are representative of B-cell clones present in gastritis with lymphoid hyperplasia and gastric lymphoma, we speculate that there are limited numbers of B-cell clones available for this proliferation or that there is a selective proliferation of B-cell clones in response to H pylori. MALT-type lymphomas are typically preceded by accumulation of MALT in the stomach during H pylori infection, in the salivary gland with Sjögren’s syndrome, and in the thyroid with Hashimoto’s thyroiditis. Two previous studies reported a clonal expansion of B-cell lymphocytes in salivary gland tissue involved by Sjögren’s syndrome that were evaluated by the Southern blot technique for GR. It is possible that the GR patterns seen in our study represent a similar clonal expansion in gastric mucosa. In support of this hypothesis, Pan et al. showed the presence of nonreproducible bands (a “ladder” with variable numbers of bands) in both tumor follicles and reactive follicles. Also, Ott et al. showed that in gastric lymphoma of MALT-type, tumor-free mucosa (diagnosed as chronic gastritis associated with H pylori) may contain small clonal proliferations, some of which share DNA sequences in their clone-specific CDR3-regions with the main lymphoma, as well as rare additional clonal B-cell populations not related to the lymphoma. These additional B-cell...
clones may be similar to those detected in our study in chronic gastritis with lymphoid hyperplasia. Regardless of the explanation, our findings suggest that for practical purposes detection of a single and reproducible band strongly supports the presence of a malignant clone in gastric mucosa. If complex patterns are detected, dominant band(s) are highly suggestive of malignancy if they can be reproduced using tissue from other areas of the lesion. Therefore, if only formalin-fixed, paraffin-embedded tissue is available, we recommend that PCR-based GR studies be run in two or more reactions using additional sections from the same paraffin block or from histologically comparable areas sampled from different paraffin blocks. The reproducibility of clonal patterns was also emphasized by Pan et al. The sensitivity of this method in detecting a malignant clone in gastric biopsy specimens is similar to a previously reported sensitivity of 75% for PCR detection of clonal GR in gastric lymphoma in formalin-fixed, paraffin-embedded biopsy specimens and other studies that used a single primer pair. Inagaki et al. used tissue microdissected from atypical areas in three serial paraffin sections followed by protein digestion as the DNA template. The three serial paraffin sections used for a single PCR reaction in their study is comparable to the two to five serial sections in our study.

Inagaki et al. also studied 12 cases of chronic gastritis, all of which had a simple polyclonal pattern (smearing in the expected molecular weight range without bands). In our study, only 15% (3 of 20) of cases of HP gastritis with lymphoid hyperplasia had a simple polyclonal pattern. Additionally, 25% (5 of 20) of cases of HP gastritis without lymphoid hyperplasia had a pure polyclonal pattern. Also, HP gastritis with lymphoid hyperplasia was much more likely than HP gastritis without lymphoid hyperplasia to have detectable PCR product on PAGE (100% v 40%). The differences between our findings and those of Inagaki et al. may thus be attributable to the selection of cases. From a practical point of view, biopsy specimens of chronic gastritis with prominent lymphoid hyperplasia are more likely to create the diagnostic problem of distinguishing florid lymphoid hyperplasia from lymphoma. It is therefore of significance that PCR GR may also produce misleading results if the reproducibility of dominant bands is not adequately evaluated.

**SUMMARY**

PCR-based detection of IgH GR in formalin-fixed, paraffin-embedded tissue may produce several patterns (bands, smears, and their combination). A single band indicates the presence of a dominant monoclonal population, whereas smearing indicates the presence of a polyclonal population. These or a combination of the two patterns are typically observed in lymph nodes and can usually distinguish benign and malignant lymphoid proliferations, even in the presence of a background component of benign lymphocytes in a malignant lymphoma. More complex patterns are characteristic of inflammatory conditions of stomach and colon, as well as gastric lymphoma of MALT type. The practical consequence is the potential difficulty in distinguishing benign and malignant lymphoid proliferations. PCR-based detection of IgH GR in MALT supports the histological diagnosis of lymphoma only when single or dominant bands on PAGE can be consistently reproduced either from deeper levels of the same paraffin block or from sections of histologically similar areas in different paraffin blocks. Furthermore, a diagnosis of lymphoma should never be made on the basis of a clonal band on PAGE, without histopathological correlation.

**REFERENCES**


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