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# Rapid Diagnosis of Gastric Malignant Lymphoma from Biopsy Specimens: Detection of Immunoglobulin Heavy Chain Rearrangement by Polymerase Chain Reaction

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**The endoscopic appearances of the gastrointestinal lymphomas differ widely, and it is often difficult to make the distinction between a benign lymphoproliferative disorder and a malignant lymphoma even with a histologic evaluation. Since almost all primary malignant lymphomas of the gastrointestinal tract are of B-cell origin, the confirmation of monoclonality in immunoglobulin (Ig) is helpful for differential diagnosis. Rearrangements of the Ig heavy chain gene were examined by polymerase chain reaction (PCR) analysis in frozen biopsy specimens of human stomach. The sensitivity of the analysis was sufficient to detect even a 5% clonal B-cell proliferation and results could be obtained within 17 h. In a clinical investigation, seven of eight cases (88%) of primary gastric malignant lymphoma showed a single band in polyacrylamide gel electrophoresis (PAGE) after PCR, suggesting a monoclonal proliferation of B-cell lineage. By contrast, all seven cases of reactive lymphoreticular hyperplasias showed a broad smear pattern in PAGE, which is thought to reflect polyclonal proliferation. None of the lymphocytes infiltrating around gastritis (7 cases), gastric ulcers (12 cases) and gastric carcinomas (15 cases) showed a monoclonal proliferation pattern. These findings suggest that detection of monoclonality in Ig heavy gene rearrangement by PCR is useful for the differential diagnosis of B-cell lymphoproliferative diseases in the gastrointestinal tract.**

Key words: Gastric malignant lymphoma — Reactive lymphoreticular hyperplasia — Immunoglobulin gene — PCR

Primary gastric lymphomas constitute about 1–7% of malignant neoplasms of the stomach and are the most common disease in the category of malignant non-epithelial tumors.[1,2] Since there is considerable overlap in clinical and gross features of reactive lymphoreticular hyperplasia (RLH) and malignant lymphoma, particularly in the early stage,[3] the diagnosis still rests firmly on histologic findings aided to some extent by immunohistochemical study. The accuracy of endoscopic biopsies is not satisfactory, ranging from 52 to 88%.[4] Sampling may be problematic where there is a considerable reactive lymphoid infiltration adjoining the lymphoma. Errors may also occur in differentiating lymphoma from poorly differentiated carcinoma.[4]

During B-cell differentiation, the genes coding for immunoglobulin heavy (IgH) and light chains rearrange to determine the structure of the Ig produced by the cell. This occurs through processes of deletion and splicing of DNA segments from the variable (V), diversity (D), joining (J) and constant regions of the gene to form a unique coding sequence.[5,6] Since more than 90% of primary gastric lymphomas are of B-cell origin, detection of the Ig gene rearrangement would provide information for distinguishing neoplastic from reactive diseases.[7,8] Southern blotting is a standard method for detection of monoclonality in gene arrangement, but a small biopsy specimen is not enough for this assay.

The rearranged Ig gene has been shown to contain three hypervariable regions surrounded by framework regions which are well concerved.[9] These regions are called complementarity determining regions (CDR) I, II and III.[10,11] The DNA sequence of CDR-III is unique to each IgH and useful to identify the clonality of B-cell lineage.

Recently, Trainor *et al.* have reported that rearrangement on the IgH gene and monoclonality in hematopoietic malignancies can be simply detected by using a probe for CDR-III in the polymerase chain reaction (PCR).[12]

Herein, we report that analysis of IgH rearrangement by the PCR method is valuable for the diagnosis of gastric malignant lymphoma.

## MATERIALS AND METHODS

**Human leukemia lines and peripheral blood lymphocytes** B cell lines (P32/ISH, BALL-1, U266) and T cell lines (MOLT3, MOLT4B, P12/ISH, RPMI8402) were

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grown in RPMI1640 media supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a water-saturated atmosphere of 95% air-5% carbon dioxide at 37°C. Polyclonal peripheral blood lymphocytes were obtained from a healthy male volunteer using a Ficoll-Isopaque gradient centrifugation technique as described elsewhere.[13]

**Gastric biopsy specimens** One or two biopsy specimens were endoscopically collected from 8 cases of primary gastric malignant lymphomas (finally diagnosed from surgically resected specimens), 7 cases of RLHs (diagnosed from biopsy specimens), 15 cases of gastric cancers and 19 cases of benign lesions. All the lymphomas proved to be of B cell lineage on morphological and immunological examinations after operation. Some of them were also shown to have IgH rearrangement by restriction enzyme analysis with Southern blotting analysis.

**DNA preparation** DNA was extracted by proteolysis and phenol-chloroform extraction as previously described.[14] Briefly, biopsy specimens (about 5 mg) were resuspended in phosphate-buffered saline (PBS) containing 1% sodium dodecyl sulfate (SDS), and 1 mg/ml Proteinase K, and incubated for 12 h at 37°C in a shaking water bath.

**PCR reaction conditions**[15] All reactions were set up on ice. Amplification with *Taq* polymerase was performed in 100 μl reaction mixtures containing 5 μl (1 μg) of template DNA, 2.0 unit of *Taq* polymerase (Amplitaq; Perkin Elmer-Cetus, Norwalk, CT), deoxyribonucleoside triphosphates (dATP, dTTP, dCTP, and dGTP) at 100 μmol/liter each, 100 ng of each primer, 67 mmol/liter Tris-HCl, pH 8.8, 10 mmol/liter 2-mercaptoethanol, 200 μg/ml gelatin, and 2.0 mmol/liter $MgCl_2$. Each PCR round was preceded by 2 min of denaturation at 94°C, 1 min of annealing at 55°C and 2 min of extension at 72°C. The primers were synthesized on an Applied Biosystems 381A DNA synthesizer as described[12, 16, 17] and their sequences were: for V region, 5′ACACGGC(C/T)-(G/C)TGTATTACTGT3′ (termed FR3A); for the J region, 5′TGAGGAGACGGTGACC3′ (termed LJH); or 5′GTGACCAGGGTNCCTTGGCCCCAG3′ (termed VLJH). For experiments in which a single PCR was performed, this round comprised 30 cycles with primers LJH and FR3A. When a second PCR was employed, a 1:2500 dilution of the first PCR product was used as the template, and 20 cycles were carried out with primers VLJH and FR3A. Five microliters of the reaction product was electrophoresed through 12% polyacrylamide gel in TBE buffer at 20 mA for 1 h. The gel was stained with ethidium bromide and photographed under ultraviolet light. A 123bp DNA ladder was purchased as a size marker from GIBCO BRL Life Technologies, Tokyo.

**Determination of sensitivity of PCR analysis** DNA from a cultured B-cell line (P32/ISH) and DNA from normal lymphocytes were mixed in various proportions. One microgram of the mixtures was subjected to polyacrylamide gel electrophoresis (PAGE) after PCR.

## RESULTS

**Immunoglobulin heavy chain rearrangement in T and B cell lines** IgH rearrangement was analyzed using DNAs extracted from three B-cell lines and four T-cell lines by PCR. A single band, 100 base pairs to 200 base pairs in length, was detected in all B lymphoma cell lines after the first PCR analysis (Fig. 1). None of four T lymphoma cell lines showed the rearrangement band of IgH (data not shown).

**Sensitivity of PCR analysis of heavy chain rearrangement** In order to estimate the sensitivity of this assay, 1 μg of a mixture of DNA extracted from normal lymphocytes and B cell leukemia (P32/ISH) was subjected to PCR analysis. Depending on the proportion of DNA content from B-cell lymphoma, the single rearrangement band of monoclonal IgH gene became thicker (Fig. 2). The sensitivity of the analysis was satisfactory and only 5% of the tumor DNA in total was detected.

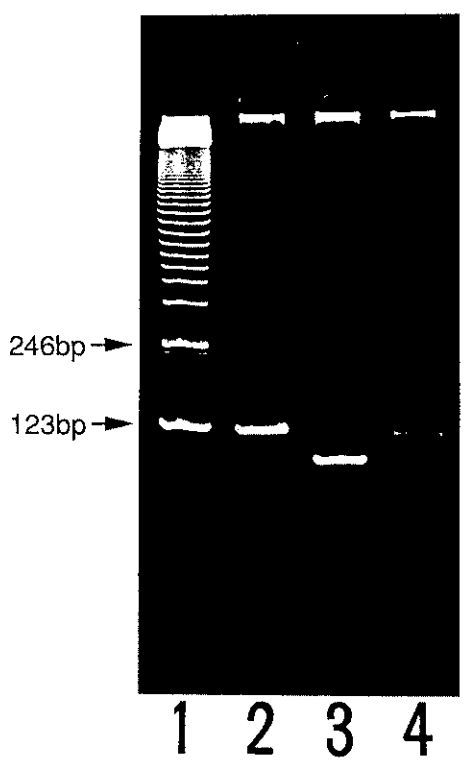


Fig. 1. Amplification of CDR-III region of 3 cases of B-cell leukemia cell lines by PCR. Lane 1 is 123-bp marker DNA. Lanes 2, 3 and 4 are B-cell lines, P32/ISH, BALL-1, and U-266, respectively, which show a single rearrangement band of monoclonal IgH gene.

**PCR amplification of Ig gene of gastric biopsy specimen** As can be seen in Fig. 3, the cases of gastric lymphoma showed a single rearrangement band of 100–200 base pairs (lanes 2 and 3). However, the cases of RLH demonstrated a broad smear (lanes 4 and 5), suggesting polyclonality in DNA fragment length. No visible bands were observed in the cases of gastric cancer or ulcer. It took only 17 h (12 h for DNA extraction, 3 h for PCR and 2 h for PAGE) to obtain results.

Table I summarizes the clinical and molecular details of the samples. Seven of eight cases (88%) of lymphomas showed monoclonality in IgH chain rearrangement by

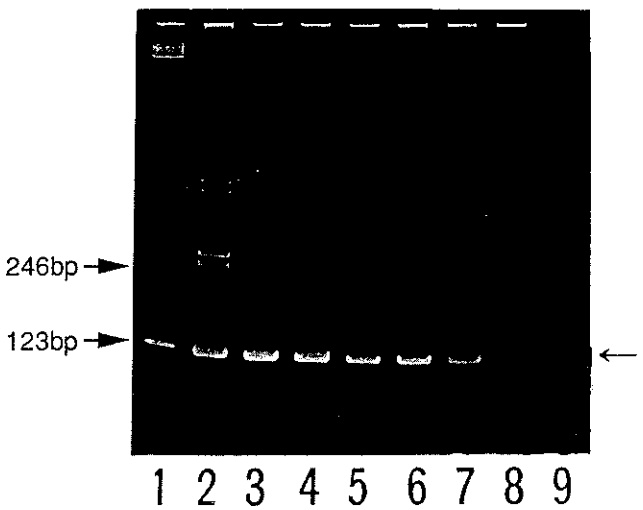


Fig. 2. Polyacrylamide gel electrophoresis after PCR of DNA mixture from a normal lymphocyte and a B-cell leukemia cell (P32/ISH). Two kinds of DNA were mixed in various proportions and 1 $\mu$g was subjected to PCR. Lane 1 is marker DNA. Lanes 2 to 8 correspond to 100%, 50%, 30%, 20%, 10%, 5% and 0% tumor DNA, respectively. Lane 9 is the negative control. The arrow on the right indicates the rearrangement band of IgH chain gene.

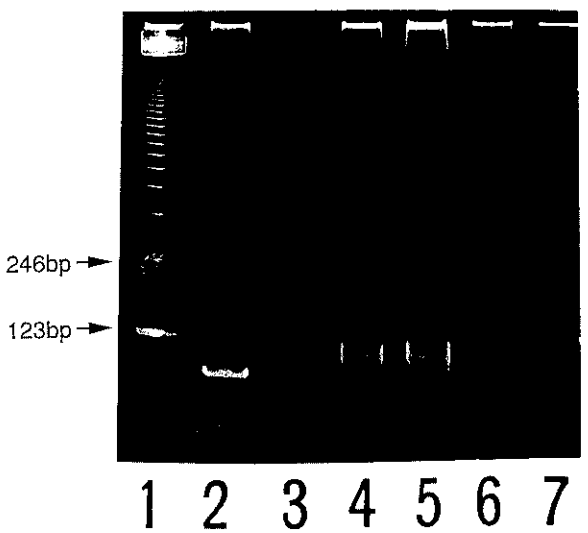


Fig. 3. Polyacrylamide gel electrophoresis after PCR of DNA from various gastric diseases. Lane 1 is 123-bp marker DNA. Lanes 2 and 3 are primary gastric lymphoma (case 2 and case 4 in Table I, respectively), showing a single band of IgH gene. Lanes 4 and 5 (case 9 and case 10 in Table I, respectively) are RLH, showing broad smear patterns. Lanes 7 and 8 are gastric cancer and ulcer, respectively.

Table I. Molecular Biological Diagnosis for Gastric Lymphoproliferative Disorders Compared with Endoscopic and Histologic Diagnosis

| Case number | Endoscopic diagnosis | Histological diagnosis (biopsy) | IgH rearrangement | Post-operation diagnosis |
|---|---|---|---|---|
| 1 | ML | ML | + | ML |
| 2 | ML | ML | + | ML |
| 3 | ML | ML | + | ML |
| 4 | ML | ML | + | ML |
| 5 | RLH | RLH | + | ML |
| 6 | ML | ML | + | ML |
| 7 | ML | ML | − | ML |
| 8 | ML | RLH | + | ML |
| 9 | RLH or ML | RLH | − | not operated |
| 10 | RLH or ML | RLH | − | not operated |
| 11 | RLH | RLH | − | not operated |
| 12 | RLH | RLH | − | not operated |
| 13 | RLH | RLH | − | not operated |
| 14 | RLH | RLH | − | not operated |
| 15 | RLH | RLH | − | not operated |

ML, malignant lymphoma; RLH, reactive lymphoreticular hyperplasia.

PCR analysis. Case 5[18] was initially diagnosed as an early gastric cancer combined with RLH endoscopically and histopathologically. However, the PCR analysis by biopsy specimens indicated a clonal B-cell proliferation, highly suggestive of malignant lymphoma. When the patient underwent surgery for early cancer on the cardia, the coexisting RLH lesion was also resected. Final diagnosis revealed a malignant lymphoma of diffuse medium-sized B-cell type, from immunohistochemical evaluation. In Case 8, a lymphoma was suspected from the endoscopic appearance of massive elevated tumors, but histological and cytological examinations in the biopsy specimen demonstrated RLH. A monoclonal IgH rearrangement was detected in the same biopsy specimen using PCR and the postoperative diagnosis was lymphoma. On the other hand, none of the RLH, which were followed for more than a year in the out-patient clinic, showed a monoclonal proliferation of B-cells. No monoclonal arrangement was seen in 15 gastric cancers, 12 gastric ulcers or 7 cases of gastritis.

## DISCUSSION

IgH rearrangement is a marker of commitment of the B-cell lineage. It is generally accepted that lymphocytes in RLH, gastritis, ulcer and carcinoma are characterized by a polyclonal population of B-cells, representing many different IgH rearrangements. By contrast, B-cell lymphomas are monoclonal, containing one or, most commonly, two rearranged alleles. Clonal Ig gene rearrangement can be identified by Southern blot analysis. However, since it requires a large amount of material (usually 30 biopsy specimens or more) and the use of redioactive materials, Southern blot analysis is unsuitable for routine diagnosis. Very recently, several reports have demonstrated that there are sufficient conserved sequences within the V, D and J regions to enable the construction of consensus oligonucleotide primers.[12, 16, 17] Therefore, we investigated the clinical application of PCR analysis for diagnosis of gastric lymphomas. Preliminary examinations revealed that one biopsy specimen (about 3 mg) could provide enough DNA for the analysis and that sensitivity was as high as 5% of the lymphoma cells in polyclonal lymphocytes (Fig. 2). Mohri[19] reported that reactive lymphoid cell hyperplasia was found in 39.8% of gastric malignant lymphoma cases and Ishido *et al.*[20] also demonstrated a high ferquency of reactive lymphoid infiltration around the lymphoma. In view of this characteristic of lymphoma, PCR analysis will be valuable to detect a small number of tumor cells in the adjacent reactive lymphoid infiltration. Furthermore, it is advantageous that results can be obtained within a day by PCR analysis compared with several days by Southern blot analysis.

Another point of interest is genotypic analysis of hyperplastic lymphoid lesions. As mentioned above, RLH (pseudolymphomas) was hypothesized to have a polyclonal lymphoid population in contrast to a monoclonal population in malignant lymphoma. However, several recent reports[21, 22] showed the presence of monoclonality in RLH and this finding has led some authors to suggest that there exist monoclonal-type pseudolymphomas, actually low-grade malignancies.[23, 24] In the present study, all the cases of RLH, which were diagnosed endoscopically and histopathologically, showed a polyclonal B-cell proliferation by the PCR analysis. Therefore, so-called RLH may consist of a benign polyclonal hyperplasia and a low-grade lymphoma. Molecular biological examination for RLH using a PCR method is necessary to solve this problem. In addition, it should be noted that some cases of pseudolymphomas preceding and being associated with true gastric lymphoma have been reported.[25, 26] The true entity of RLH has yet to be elucidated.[27]

One case of lymphoma (Case 7) was negative in IgH rearrangement in PCR analysis. An operation specimen was carefully examined by immunohistochemical staining and Southern blot analysis. The final diagnosis was malignant lymphoma of diffuse B-cell type with immunoglobulin rearrangement (data not shown). Since it has been reported that this primer has about 85% homology with published DNA sequences of variable region by database analysis,[17] we speculated that the primers may not bind to the consensus regions due to mismatch in sequences. Therefore, several different primer sets may be needed for decreasing the rate of detection failure. To increase the accuracy rate of the diagnosis for malignant lymphoma, genetic examinations should be considered. Further investigations of the PCR method are required to increase the sensitivity and to allow the use of formalin-fixed paraffin-embedded samples for a routine examination.[28]

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