Original article

Analysis of immunoglobulin light-chain mRNA in gastric malignant lymphoma using new highly sensitive in situ hybridization method

YOSHIKI SUMIYOSHI¹, MASAHIRO KIKUCHI², YUICHI YAMASHITA¹, TAKAFUMI MAEKAWA¹, and TAKAYUKI SHIRAKUSA¹

¹Second Department of Surgery, Fukuoka University School of Medicine, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan

²First Department of Pathology, Fukuoka University School of Medicine, Fukuoka, Japan

Abstract:

Background. To analyze B-cell monoclonality, we need fresh materials for the Southern blotting method. In situ hybridization (ISH) for the detection of immunoglobulin light-chain mRNA can be used in formalin-fixed and paraffin-embedded tissues. However, it is difficult to detect low levels of mRNA copies. So we must develop new highly sensitive in situ hybridization methods to diagnose gastric malignant lymphoma.

Methods. We analyzed materials from 15 patients with gastric malignant lymphoma, using a new highly sensitive ISH method, and compared the results with those of a conventional method.

Results. With this new method, 13 of 15 cases were shown to have monotypic cytoplasmic signals, and monoclonality was detected in 3 of 4 cases of mucosa-associated lymphoid tissue (MALT) lymphoma.

Conclusions. The accuracy of endoscopic biopsies is not satisfactory. However, the detection of immunoglobulin light chain mRNA, using the new highly sensitive ISH method with a non-radioactive isotope oligonucleotide probe, can be used to diagnose gastric malignant lymphoma even when conventional immunoglobulin light-chain mRNA or proteins alone cannot demonstrate the clonality.

Key words: in situ hybridization, gastric malignant lymphoma, immunoglobulin light-chain mRNA, MALT, ImmunoMax

Introduction

Malignant gastric lymphomas constitute approximately 1%–7% of malignant gastric neoplasms and are the most common non-epithelial malignancies of the

stomach [1–3]. The endoscopic appearance of gastric lymphomas varies widely, and it is often difficult to distinguish between a benign lymphoproliferative disorder and a malignant lymphoma, even with histologic evaluation. Since more than 90% of primary gastric lymphomas are of B-cell origin, detection of immunoglobulin gene rearrangement could provide information for distinguishing malignant neoplastic from reactive diseases [4,5]. Southern blotting is limited for diagnostic work because it is time-consuming and complex, and requires the use of radioactive isotopes and large amounts of DNA. In addition, it is not well suited to the study of formalin-fixed and paraffin-embedded material because of the consequent DNA degeneration [6–9]. Although immunohistologic studies for the detection of immunoglobulin light chains have been widely employed to determine monotypic restriction, the results have not been consistently successful, particularly with formalin-fixed and paraffin-embedded sections [10,11]. Recently, new techniques for the detection of immunoglobulin light chain mRNA have been reported [12,13]. However, these new methods of in situ hybridization (ISH) present some problems that must be solved. To address these issues, we developed a new highly sensitive ISH method that is an adaptation of the new maximized immunohistological method (ImmunoMax) [14]. To prove the clonality of gastric malignant lymphoma in formalin-fixed and paraffin-embedded tissue, we performed ISH for immunoglobulin light-chain mRNA and compared the results with those of immunohistological staining and a conventional ISH method.

Materials and methods

Tissue and immunohistochemical study

Formalin-fixed paraffin-embedded specimens from the surgically resected gastric malignant lymphomas of 15

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patients were obtained from our files accrued from 1978 to 1996. The histological diagnoses of all 15 specimens were evaluated and classified by pathologists according to the Lymphoma Study Group (LSG) classification [15]. All specimens were fixed in 10% formalin, embedded in paraffin, cut into 3- μ m-thick slices, and mounted on poly-L-lysine coated slides. All slides were stained with hematoxylin-eosin, Giemsa, periodic acid-Schiff, and silver impregnation for reticulin fibers. Additionally, immunohistological studies were performed by the labeled-streptavidin-biotin method (LSAB), using the monoclonal antibodies UCHL-1 (CD45RO; Dakopatts, Glostrup, Denmark), MT-1 (CD43; BioScience, Emmenbrucke, Switzerland); L-26, (CD20, Dakopatts), Anti bcl-2 (Dakopatts), CD5 (Becton-Dickinson, Mountain View, CA, USA), immunoglobulin kappa light chain (Dakopatts), and immunoglobulin lambda light chain (Dakopatts).

Conventional in situ hybridization

Conventional ISH, based on the method of Pringle et al. [12], was conducted using cocktails of fluorescein isothiocyanate (FITC)-labeled oligoprobes, which were specific for human kappa and lambda immunoglobulin light chain mRNA (Dakopatts). Hybridization products were detected using a monoclonal mouse anti-FITC antibody, biotinylated horse anti-mouse immunoglobulin, and streptavidin biotin complex-alkaline phosphatase, and visualized with the new fuchsin.

To confirm the specificity of this ISH method, we used negative control slides which were submitted to RNase treatment at 37°C for 1 h.

New highly sensitive in situ hybridization

The same probes used for the conventional method described above were used for the new highly sensitive ISH method. For prehybridization treatment, the slides were put in 0.2 N HCl for 10 min and then in 50 μ g/ml proteinase K solution for 10 min, and finally in 1.5% H₂O₂ for 10 min at room temperature. After dehydration, the slides were air-dried. Next, 100 μ l of FITC-labeled oligoprobe cocktails was added to the slides and they were incubated at 37°C for 3 h. The slides were washed three times in Tris buffered saline (TBS) and left at room temperature for 5 min. Diluted mouse monoclonal anti-FITC antibody was prepared in 20% normal horse serum/2% casein TBS, added to the slides, and the mixture incubated overnight at 4°C. Next, biotinylated horse anti-mouse immunoglobulin was added and incubated at room temperature for 30 min. Then streptavidin-biotin complex-horseradish peroxidase (Dako kit) was added and the mixture left at room temperature for 30 min. Next, diluted biotinylated

tyramine (Dupont, Wilmington, DE, USA) was prepared in 0.035% H₂O₂/TBS and added to each slide at room temperature (10 min). Finally, streptavidin-biotin complex-alkaline phosphatase (Dako kit) was added at room temperature (30 min). A staining solution was prepared with the new fuchsin substrate and incubated for 10 min at room temperature to give an insoluble red color-reaction product. Finally, counter-staining with hematoxylin was performed for approximately 5 min.

For negative control slides we used the same procedure as that for the conventional ISH method.

Results

In this series, we used formalin-fixed and paraffin-embedded tissues from 15 patients with surgically resected, gastric malignant lymphoma. The clinical findings of these patients are summarized in Table 1, and the results of immunohistochemical study, conventional ISH, and the new highly sensitive ISH are summarized in Table 2. The patients' ages ranged from 36 to 76 years (mean, 53.0 years). The ratio of men to women was 8:7. According to the LSG classification, 7 patients had diffuse large type; 3, diffuse mixed type; 4, diffuse medium type; and 1, diffuse and follicular large type. In 4 patients whose disease was identified as diffuse medium type by the LSG classification, mucosa-associated lymphoid tissue (MALT) lymphoma was identified histologically.

Immunohistological study

With the monoclonal antibodies against immunoglobulin light-chains, monotypic restriction was observed in 8 of the 15 cases (53.3%). We did not observe monoclonality in the MALT lymphoma cases. Other immunohistological studies are summarized in Table 3.

Conventional in situ hybridization

Monoclonal restriction was shown in 7 of the 15 cases (46.7%), compared with 8 of 15 in the immunohistological results. However, 1 case of monoclonality was identified among the MALT lymphoma cases.

New highly sensitive in situ hybridization

Monotypic cytoplasmic mRNA signals were recognized in 13 of the 15 cases (86.7%). Among the 4 MALT lymphoma cases, we identified monoclonal restriction of the immunoglobulin light chain in 3 cases. (Fig. 1A, B)

Table 1. Summary of clinical characteristics

Patient no.	Age (years)	Sex	Surgery date; year/month/day	Surgery (type of gastrectomy)	Histology
1	36	F	94. 04. 06	Total	Diffuse mixed
2	41	F	98. 06. 01	Distal	Diffuse large
3	44	F	87. 07. 01	Total	Diffuse large
4	47	F	78. 03. 29	Distal	Diffuse medium
5	48	F	84. 04. 02	Total	Diffuse medium
6	50	F	96. 03. 11	Distal	Diffuse large
7	57	F	89. 07. 03	Total	Diffuse medium
8	61	M	90. 09. 26	Total	Diffuse large
9	62	M	93. 06. 28	Total	Diffuse mixed
10	62	M	87. 12. 26	Distal	Diffuse medium
11	63	M	90. 08. 08	Total	Diffuse large
12	66	M	80. 11. 19	Total	Diffuse large
13	72	M	86. 04. 25	Total	Diffuse large
14	72	M	82. 12. 04	Distal	Diffuse mixed
15	76	M	86. 03. 12	Distal	Diffuse and follicular large

Table 2. Comparison of phenotype results

Patient no.	Histology	Immunohistology	Conventional ISH	New highly sensitive ISH
1	Diffuse mixed	Lambda	Lambda	Lambda
2	Diffuse large	Kappa	Kappa	Kappa
3	Diffuse large	Unknown	Lambda	Lambda
4	Diffuse medium	Unknown	Unknown	Unknown
5	Diffuse medium	Unknown	Unknown	Kappa
6	Diffuse large	Lambda	Unknown	Lambda
7	Diffuse medium	Unknown	Kappa	Kappa
8	Diffuse large	Kappa	Kappa	Kappa
9	Diffuse mixed	Unknown	Unknown	Lambda
10	Diffuse medium	Unknown	Unknown	Kappa
11	Diffuse large	Kappa	Unknown	Unknown
12	Diffuse large	Lambda	Lambda	Lambda
13	Diffuse large	Lambda	Unknown	Lambda
14	Diffuse mixed	Unknown	Kappa	Kappa
15	D and F Large	Kappa	Unknown	Kappa

D and F, Diffuse and follicular; ISH, in situ hybridization

Table 3. Results of immunohistological study

Patient no.	Monoclonal antibodies				
	UCLH-1	MT-1	L-26	Anti-bcl-2	CD5
1	—	—	+	—	—
2	—	—	+	—	—
3	—	—	+	—	—
4	—	—	+	—	—
5	—	—	+	—	—
6	—	—	+	—	—
7	—	—	+	—	—
8	—	—	+	—	—
9	—	—	+	—	—
10	—	—	+	—	—
11	—	—	+	—	—
12	—	—	+	—	—
13	—	—	+	—	—
14	—	—	+	—	—
15	—	—	+	+	—

Discussion

The diagnosis of lymphoma can often be difficult when only routine morphologic examination is used. Immunohistochemical study has been used to detect immunoglobulin light-chain restriction, which facilitates diagnosis because almost all primary malignant gastric lymphomas are of B-cell origin [5,7,10,16]. However, as cell membrane immunoglobulins can be optimally immunostained only in cryostat sections of fresh tissue, the advantages of using immunohistochemistry with routinely processed sections are limited. Furthermore, difficulties in interpretation have arisen because of the frequent absence of staining and the presence of extracellular immunoglobulins, which obscure staining patterns [17].

Fig. 1A,B. In situ hybridization (ISH) with gastric malignant lymphoma (mucosa-associated lymphoid tissue [MALT] lymphoma) using fluorescein isothiocyanate-labeled oligonucleotide cocktail probe for **A** Lambda and **B** Kappa light-chain mRNAs. Almost all lymphoma cells expressed positive signals for Kappa immunoglobulin light chain mRNA

Some authors have reported that the analysis of immunoglobulin heavy chain rearrangement by a polymerase chain reaction (PCR) method is valuable and that this is the most sensitive method for the diagnosis of gastric malignant lymphoma [18,19]. However, the ISH method is useful, even if the sensitivity for detection of monoclonality for immunoglobulin is less than that with PCR. The greatest advantage of ISH over PCR could be the ability to detect signals with cellular accuracy. Secondly, although a “negative” result with PCR does not always mean that the lesion is polyclonal, ISH can determine polyclonal proliferation of lympho-

Fig. 2A,B. ISH detection of **A** Kappa and **B** Lambda light-chain mRNA in large cell-type gastric malignant lymphoma, intermingled with normal small lymphocytes. The lymphoma cells and normal small lymphocytes are negative for Kappa immunoglobulin light chain mRNA, although the only normal plasma cells are strongly positive (**A**). However, only the tumor cells are strongly positive for Lambda immunoglobulin light-chain mRNA (**B**)

cytes as well as monoclonal involvement of a lymphoid lesion (Fig. 2A,B).

ISH methods enable the detection of monotypic immunoglobulin light-chain mRNA in formalin-fixed, paraffin-embedded sections. The oligonucleotide probe, in particular, can be adapted to ISH, especially in paraffin sections [12,13]. Because of their small size, oligonucleotide probes readily penetrate paraffin-embedded sections, and mRNA can be detected readily if adequate amounts of mRNA transcripts are present [20,21]. However, the sensitivity of an oligonucleotide probe

tends to be lower than that of other probes, such as DNA or RNA probes. Although radioactively labeled probes are extremely sensitive for ISH studies, especially when mRNA transcripts are present at low levels, the routine laboratory use of radioactive materials presents obvious problems. In addition, results from a radioactively labeled probe are not available for approximately 2 weeks, whereas results from a non-radioactive isotope (RI) probe are obtained within 2 or 3 days. Accordingly, we need a new method to increase the sensitivity of ISH using a non-RI oligonucleotide probe. In the ImmunoMax method established by Merz et al. [14], combination of the antigen retrieval steps with the biotinylated tyramine enhancement step resulted in a 100- to 10000-fold boost in sensitivity without loss of specificity. We have used this method (ImmunoMax) in combination with ISH for detecting low quantities of mRNA with a non-RI oligonucleotide probe.

Other studies have reported that the monoclonality of gastric malignant lymphoma can be detected better with an ISH study than with an immunohistochemical study. However, in our results, the conventional ISH method showed a lower detection rate than the immunohistochemical method. The reason for the poor ability of the ISH method to demonstrate monoclonality may be related to the following factors: (1) The immunoglobulin-light chain mRNA may be destroyed due to formalin fixation and the paraffin embedding procedure, because the RNA is easily destroyed by ubiquitous RNase and may degenerate over the course of routine processing of specimens and during storage of tissue blocks. (2) Radioactively labeled probes were not used. (3) The new fuchsin, which is less sensitive than nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP), was used.

In our study, there were only four cases of MALT lymphoma and they were categorized as diffuse medium cell type according to the LSG classification [15]. We know that the marginal zone cells and MALT lymphoma cells show a faint reaction for immunohistological staining and ISH of immunoglobulin light chains [22,23]. Perhaps the MALT lymphomas could not be proven to demonstrate clonality for immunoglobulin light chains due to the low copy number of mRNA in the MALT lymphoma cells; this low number is because the MALT lymphoma cells are in an early differentiation phase, in which immunoglobulin light-chain production has either not yet begun or has just started [24,25]. Conclusive proof will require examination of more MALT cases. However, using our new highly sensitive ISH method, we were able to demonstrate clonality by immunoglobulin light-chain restriction, and we believe that this method will aid in the diagnosis of gastric malignant lymphoma, particularly MALT

lymphoma, in formalin-fixed, paraffin-embedded materials.

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