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Antigen Retrieval in Paraffin-Immunohistochemistry Detecting Monoclonality of lmmunoglobulin Light Chain in B-cell Malignant Lymphomas: Comparison with ln-situ-hybridization

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Introduction

lt is sometimes difficult to detect monoclonality in neoplastic cells of B-cell malignant lymphomas (B-MLs) by using conventional paraffin-immunohistochemistry (pIHC), although monoclonal antibodies such as MB-1, L26, Mx-pan B, LN-1, LN-2 and LN-3 can characterize phenotype of neoplastic cells in B-MLs [1]. Polyclonal antibodies against immunoglobulin kappa or lambda type light chain can detect monoclonality of lymphoma cells only in the less than 50% cases of B-MLs, detecting cytoplasmic immunoglobulin. Recently surface immunoglobulin of lymphoma cells in B-cell chronic lymphocytic leukemia was reported to be detected by pIHC with antigen retrieval pretreatment by microwave (microwave pretreatment) [2]. By in-situ-hybridization (ISH) of mRNA of immunoglobulin kappa or lambda type light chain gene it becomes possible to see whether the lymphoma cells produce monoclonal immunoglobulin or not.

We compared three methods, pIHC with and without microwave pretreatment and ISH, in order to see which method is the best to detect monoclonality in neoplastic cells of B-MLs.

Materials and methods

Materials were paraffin sections of 49 B-MLs diagnosed by pIHC employing a panel of antibodies, MT-1, UCHL-1, MB-1, Mx-pan B, L26, LN-1, LN-2, LN-3, LeuMl and anti-S 100 protein monoclonal antibodies and anti-immunoglobulin kappa and lambda type polyclonal antibodies [1]. The B-MLs were categorized according to the updated Kiel classification. Especially in mantle cell 1ymphoma, mantle-zone lymphoma and centrocytic lymphoma were subcategorized.

Table 1. Histochemical detection of monoclonality of B-cell malignant lymphoma cells, observing monoclonal immunoglobulin light chain or signals of its mRNA

Subtype		conventional pIHC	pIHC with M.W.P	ISH
B-cell chronic lymphocytic leukemia	B-CLL	0/4	2/4	0/4
Mantle cell lymphoma				
Mantle-zone lymphoma	MzML	0/3	3/3	1/3
Centrocytic	CC	0/4	4/4	4/4
Mucosa-associated lymphatic tissue type	MALT type	2/4	4/4	3/4
Centroblastic/centrocytic	CB/CC	0/3	3/3	2/3
Centroblastic lymphoma	CB			
Centrocytoid		0/5	4/5	1/5
Monomorphous		0/9	4/9	0/9
Polymorphous		4/8	7/8	0/8
B-cell immunobalstic	IB	2/2	2/2	0/2
Immunocytoma	IC	7/7	7/7	7/7
Total		15/49 (31%)	40/49 (82%)	18/49 (37%)

pIHC: Paraffin-immunohistochemistry
M.W.P.: Microwave pretreatment in 4M urea for 5 min. after boiling in a home electronic range
ISH: In-situ-hybridization of mRNA of immunoglobulin light chain gene

pIHC of immunoglobulin light chain with or without microwave pretreatment

. The method. of pIHC without microwave pretreatment (conventional pIHC) of immunoglobulin was reported in the previous paper [1].

Microwave pretreatment of dewaxed sections was performed as following. The sections were set in 4M urea solution and were heated by a home electric range for 5 min. after the solution came to the boil [2]. The power setting of the electric range was high. The sections were cooled rapidly by rinse in deionized water at room temperature and were stocked in 0.01M phosphate-buffered saline (PBS). The sections were incubated with anti-immunoglobulin kappa or lambda type polyclonal antibody at 4°C overnight and were processed by ABC method employing elite ABC solution (Vector Laboratories, Inc). Nuclear counterstain was performed with methy1-green.

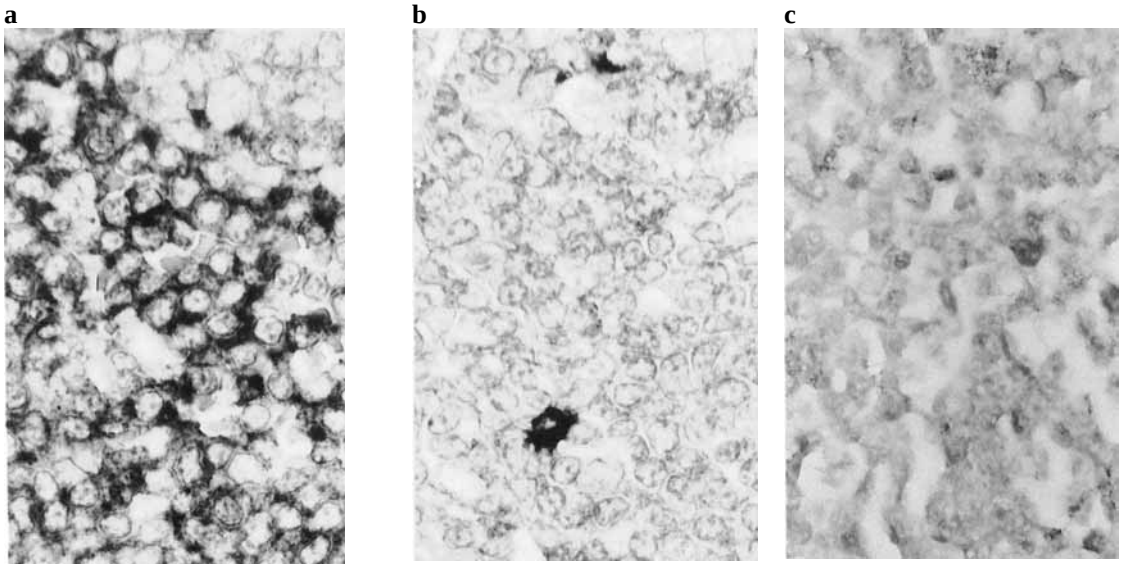
ISH of mRNA of immunoglobulin light chain

Sections were dewaxed, digested by proteinase K (10 μg/ml in diethylpyrocarbonate (DEPC)-treated 0.05M Tris/HCl , pH 7.6) for 30 min. at 37°C, post-fixed in 0.4% paraformaldehyde/DEPC-treated PBS for 20 min. at 4°C and dried through l00% ethanol and in vacuum chamber. Hybridization was performed at 37°C overnight with a cocktail of 15 or 11 biotinylated oligonucleotides of 30bp length for immunoglobulin kappa or lambda type light chain gene (DAKO In Situ Hybridization Education Kit, No. K003). After hybridization, the sections were washed 2 times in 2x Standard saline citrate (SSC) for 15 min. at 37°C and 2 times in 0.1xSSC for 15 min. at 37°C. The hybridized biotinylated-oligonucleotides were visualized by means of streptavidine-biotinylated alkaline phosphatase system and new fucshin system (DAKO). Nuclear counterstain was done with methyl-green.

Figure I. Parffin-imnunohistochemistry (pIHC) of immunoglobulin light chain with microwave pretreatment in 4M urca for 5 min. and in-situ-hybridization (ISH) of mRNA of immunoglobulin light chain gene in one case of mantle-zone lymphoma subtype of mantle cell lymphoma

a) pIHC of anti-kappa type antibody. b) pIHC of anti-lambda type antibody. Because of obviously positive stain of lymphoma cells in pIHC of anti-kappa type antibody kappa type monoclonality of lymphoma cells was evaluated. The positive stain looked to be of cell-surface but a small amount of cytoplasm was stained.

c) ISH of a cocktail of 15 oligonucleotides for immunoglobulin kappa type light chain gene. Only a few lymphoma cells showed weak signals. It was difficult to determine monoclonal activation of immunoglobulin light chain gene because of the weak signals in a small number of lymphoma cells.



Result

As shown in table 1, the conventional pIHC could detect cytoplasmic monoclonal immunoglobulin kappa or lambda type light chain in lymphoma cells of 15 cases B-MLs comprising 2 MALT type and 4 centroblastic lymphoma polymorphous type, 2 B-immunoblastic lymphomas and 7 immunocytomas. In most cases of the B-MLs lymphoma cells showed differentiation into plasma light chain in 40 (82%) cases of B-MLs examined. In some cases of B-MLs revealing no obvious differentiation into plasma cell, cell-surface and cytoplasmic monoclonal immunoglobulin was detected (Fig. 1). But in the other cases surface immunoglobulin of B-ML cells could not be detected even in cells morphologically.

The pIHC with microwave pretreatment could detect monoclonal immunoglobulin kappa or lambda type

Figure 2. Paraffin-immunohistochemistry (pIHC) of immunoglobulin light chain with microwave pretreatment in 4M urea for 5 min. and in-situ-hybridization (ISH) of mRNA of imnunoglobulin light chain gene in one case of lynphoplasmacytic immunocytoma

a) pIHC of anti-kappa type antibody. b) pIHC of anti-lambda type antibody. Because of obviously positive cytoplasmic stain of some lymphoma cells in pIHC of anti-lambda type antibody, lambda type monoclonality of lymphoma cells was evaluated. The positive cytoplasmic stain was seen in lymphoma cells having relatively rich cytoplasm. Cell-surface immunoglobulin could not be shown.

c) ISH of a cocktail of 11 oligonucleotides for immunoglobulin lambda type light chain gene. Relatively many lymphoma cells showed fine signals. Monoclonal activation of immunoglobulin lambda type light chin gene was shown.

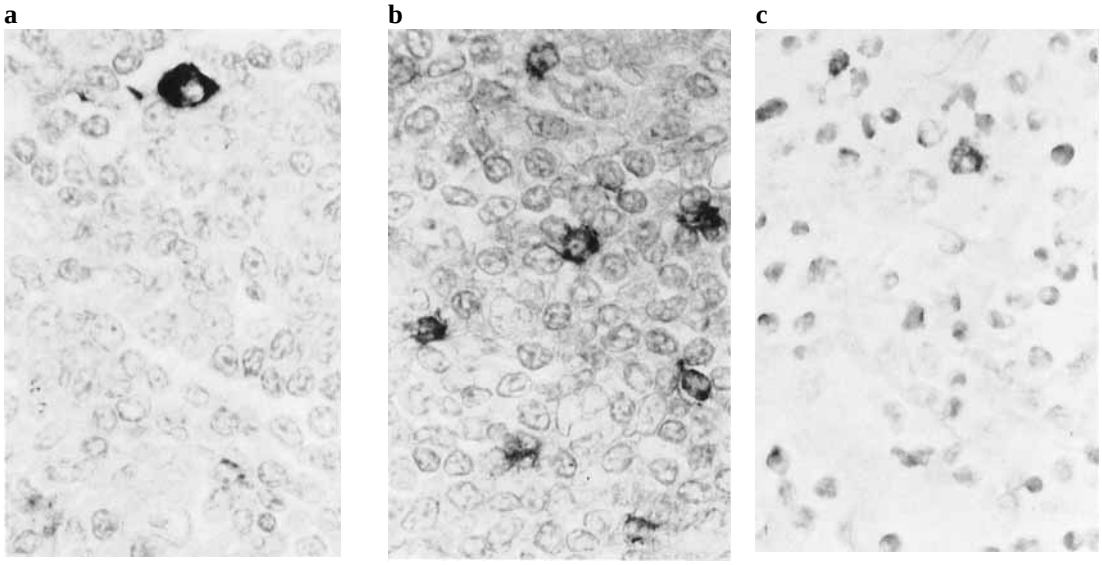


Table 2. Antigen retrieval in paraffin-immunohistochemistry by means of heating, reviewing papers.

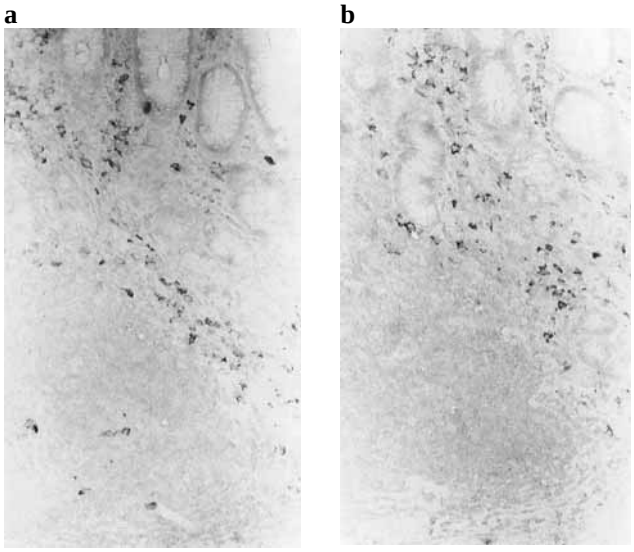
Antigen/Antibody	Method	Buffer	Reference
Ki-67(MIB1)	M.W.	Glycin HCl/Citrate	Taylor CR et al.
PCNA	M.W.	Water	Haerslev T et al.
	M.W.	H. Metal	Greenwell A et al.
	M.W.	Glycin HCl/Citrate	Taylor CR et al.
	Simple (90℃)	Citrate	Kawai K et al
Bcl-1/Cycline D1	M.W.	-	Yang WI et al.
p53	Autoclave/M.W.	Water	Igarashi H et al.
	M.W.	Glycin HCl/Citrate	Taylor CR et al.
	Simple (90℃)	PBS	Kawai K et al.
Androgen receptor	M.W.	Glycin HCl/Citrate	Taylor CR et al.
Progesterone receptor	M.W.	Glycin HCl/Citrate	Taylor CR et al.
c-erb B-2	Autoclave/M.W.	Water	Igarashi H et al.
CA125	Autoclave/M.W.	Water	Igarashi H et al.
HHF35	Simple	Water	Igarashi H et al.
CGA7	Simple	Water	Igarashi H et al.
Immunoglobulin	M.W.	H. Metal/Urea	Merz H et al.
Cytokeratin	M.W.	H. Metal/Urea	Cattoretti G et al.
Vimentin	M.W.	H. Metal/Urea	Cattoretti G et al.
T/B-cell markers	M.W.	H. Metal/Urea	Cattoretti G et al.
20 Abs react Ags only in frozen section			
21 Abs react Ags in paraffin sections			
39 Abs (No retrieval)	M.W.	Citrate	Cuevas EC et al.

M.W.: Microwave pretreatment
Simple: Heating in water bath
H. Metal: Heavy metal salt solution

Figure 3. An experiment of antigen retrieval of immunoglobulin in paraffin-immunohistochemistry, heating dewaxed sections in 4M urea in 2 ways

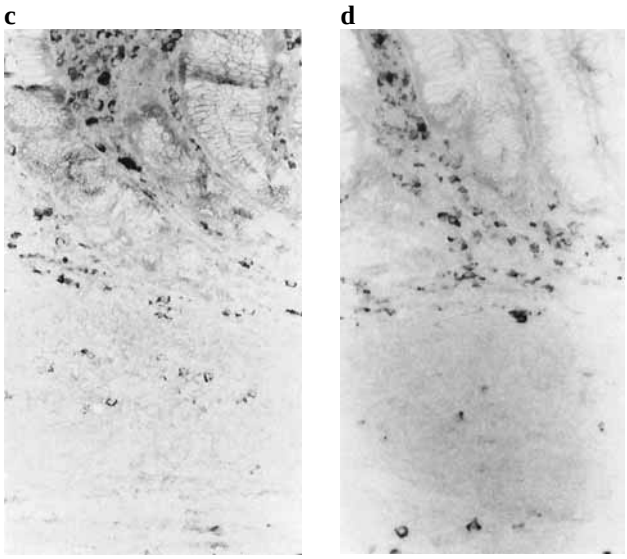
Heating sections in 4M urea by a home electric range for 5 min. after the 4M urea came to the boil

pIHC of anti-kappa type antibody (a)
and of anti-lambda type antiboidy (b)



Heating sections in 4M urea in a high pressure cookpot for 12 min.

pIHC of anti-kappa type antibody (c)
and of anti-lambda type antitxxly (d)



The comparison was shown in table 3.

Table 3. Antigen retrieval in paraffin-immunohistochemistry (pIHC) of immunoglobulin light chain by means of heating dewaxed sections in 4M urea by a home electric range (5 min.) and in a high pressure cookpot (12 min.)

Reactive lymphoid tissue around ulcer in stomach	Conventional pIHC	pIHC with antigen retrieval in 4M urea by the range	pIHC with antigen retrieval in 4M urea in the cookpot
Plasma cells	+++	+++	+++
Lymphocytes	-	+	+++
small	-	+/-	++
large	+/-	+	++
Mantle cells	-	+/-	+
Germinal center cells	+/-	++	+++
Preservation of cell morphology	+++	+++	+

immunocytoma (Fig. 2). In the centroblastic lymphoma (CB), cell-surface immunoglobulin could not be detected, although a small number of lymphoma cells had cytoplasmic monoclonal immunoglobulin.

In the ISH of mRNA of immunoglobulin light chain gene non-neoplastic and neoplastic plasma cells showed strong signals. But in some of the B-MLs without differentiation into plasma cells, lymphoma cells did not show signals enough to be evaluated as monoclonal activation of immunoglobulin light chain gene (Fig. 1c). Immunocytoma showed not strong but visible signals in many lymphoma cells (Fig. 2c). The ISH could detect monoclonal mRNA of immunoglobulin light chain gene in 18 cases (37%) of the B-MLs. Signals could not be recognized in lymphoma cells of the centroblastic lymphomas and those of the B-immunoblastic lymphomas.

Discussion

Antigen retrieval method in pIHC by heating dewaxed sections has been developing. Reviewing recent papers concerning the antigen retrieval method in pIHC, various heating ways and buffers were applied as shown in table 2. As for heating of the sections, at least 3 methods have been recommended, simple heating in water bath (less than 100°C), boiling in microwave and in autoclave (121°C, 2 atmospheric pressure). As for buffers, glycine HCl or citrate were often applied to the antigen retrieval of intranuclear antigens such as Ki-67, PCNA and hormones. Heavy metal salts or urea were applied to the antigen retrieval of cell-surface and cytoplasmic antigens such as immunoglobulin and T/B-cell markers. The antigen retrieval method in pIHC is an epockmaking technique in immunohistochemistry but it must be considered that only a half of antibodies were reported to show antigen retrieval [3].

We performed pIHC of immunoglobulin light chain with microwave pretreatment in 4M urea according to Merz H et al [2]. But the pIHC detected a low concentration of monoclonal immunoglobulin in cytoplasm rather than in cell surface.

One of the reasons why cell-surface immunoglobulin could not be detected in the most cases of the B-MLs may be difference in the quantum efficiency of microwave. Examining 3 buffers, 4M urea, 0.1M citrate buffer pH 6.0 and 0. 1M citrate pH 6.0 and 2 heating methods, heating sections in a home electric range for 5 min. after the incubation buffer came to the boil (microwave pretreatment) and in a high pressure cookpot (lower than 121°C, higher than 100 C) for 12 min. (high pressure cookpot pretreatment) and followed rapid cooling in water, it was determined which combination of buffer and heating method could get the best quality in the staining of infiltrating lymphocytes and lymphofollicles in the gastric mucosa. In the combination of 4M urea with the microwave pretreatment (Fig. 3, a and b) and in the combination of 4M urea (Fig. 3, c and d) and 0.1M citrate with the high pressure cookpot pretreatment, antigen retrieval was achieved. Only in the combination of 4M urea and the both heating methods cell-surface immunoglobulin in mantle lymphocytes could be visualized and the stain was stronger in the high pressure cookpot pretreatment than in the microwave pretreatment (Fig. 3, table 3). But the preservation of tissue morphology was poor in the high pressure cookpot pretreatment. Further improvement in heating procedure of the antigen retrieval pretreatment will make possible for pIHC to detect cell-surface immunoglobulin of lymphoma cells of B-MLs.

This high pressure cookpot pretreatment seemed to be the best heating method. The heating condition is expected to be stable at the temperature higher than 100°C but lower than 121°C and at the high pressure less than 2 atmorpheric pressure. We thought it very useful in practice that the handling of the sections heated in the high pressure cookpot to cool them was not dangerous in comparison with the handling of the sections heated in an autoclave.

Comparing the pIHC of immunoglobulin light chain with and without microwave pretreatment and the ISH of mRNA of immunoglobulin light chain gene in this study, the conventional pIHC and the ISH had almost the same competence for detecting monoclonality in lymphoma cells of B-MLs. The pIHC with microwave pretreatment was shown to be the best method to detect histochemically monoclonality of lymphoma cells in B-MLs. It was also shown that the ISH could detect monoclonality in lymphoma cells in some cases of B-MLs without obvious differentiation into plasma cell but the judgment under microscope needed careful observation and comparison. There may be methods to make the ISH more sensitive. A procedure of heating sections in 70% formamide 2xSSC at higher than 70°C and followed rapid cooling is one of the methods to denature DNA in sections in DNA-DNA ISH but the possible pretreatment to make DNA-RNA ISH more sensitive.

On the other hand, rearrangement of immunoglobulin heavy chain gene in combination with oncogenes such as myc and bcl-2 was reported to be observed in some B-MLs. The low rate of detecting monoclonality of immunoglobulin light chain in the centroblastic lymphoma by the ISH and the pIHC with microwave pretreatment in this study suggests the rearrangment of immunoglobulin heavy chain gene. In such cases of B-MLs monoclonality of lymphoma cells may be detcetd by the pIHC of immunoglobulin heavy chain.

In such cases of B-MLs that lymphoma cells have down-regulated and rearranged immunoglobulin gene, the detection of the rearranged immunoglobulin gene may be possible by means of polymerase chain reaction in DNA extracted from paraffin sections of the lymphoma tissue.

Consequently, it is no exaggeration to say that the pIHC with antigen retrieval pretreatment of immunoglobulin can detect monoclonality in lymphoma cells of B-MLs except rare cases mentioned above.

Summary

ln order to see which is the best histochemical method to detect monoclonality in immunoglobulin light chain, three methods; paraffin-immunohistochemistry (pIHC) with and without microwave pretreatment in 4M urea for antigen retrieval and in-situ-hybridization (ISH) of mRNA of immunoglobulin light chain gene were compared in analyzing 49 cases of B-MLs. The pIHC with and without microwave pretreatment and the ISH could detect monoclonality of immunoglobulin in 40 (82%), 15 (31%) and 18 (37%) cases of the B-MLs respectively. The pIHC with microwave pretreatment could detect monoclonality of cytoplasmic immunoglobulin in many B-MLs and that of cell-surface immunoglobulin in some B-MLs. It was difficult in some B-MLs without differentiation into plasma cell to see monoclonal signals of immunoglobulin light chain gene in lymphoma cells under microscopic observation. Consequently, it was shown that the pIHC of immunoglobulin with the antigen retrieval pretreatment is quite useful in surgical pathology of B-MLs to detect monoclonality of lymphoma cells.

Key words: Paraffin-immunohistochemistry, antigen retrieval, immunoglobulin light chain, microwave, in-situ-hybridization, B-cell malignant lymphoma.

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