

Kappa and Lambda Determination by Single Colored Immunohistochemistry and Chromogenic in Situ Hybridization Techniques in B-Cell Malignancies

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ABSTRACT

Background: kappa and lambda light chains detection in bone marrow trephine sections help in the determination of B-cell clonality through evaluation of light chain restriction.

Aim of the Work: was to compare the efficacy of single color detection-based immunohistochemistry (IHC) and chromogenic in situ hybridization (CISH) in evaluating kappa/lambda expression in tissues harboring B-lymphoid lesions. **Patients and Methods:** Forty patients were enrolled in this study. They were divided into three groups chronic lymphocytic leukemia (CLL/SLL) group I (n=13), non-Hodgkin lymphoma (NHL) group II (n=24) and hairy cell leukemia (HCL) group III (n=3). The 24 NHL cases comprised of (11 diffuse large B-cell lymphoma, 6 mantle cell lymphomas, 3 marginal zone lymphoma, 2 lymphoplasmacytic lymphoma, 1 follicular lymphomas and 1 Burkitt's lymphoma). Kappa and lambda light chains were detected in their bone marrow trephine sections using single colored immunohistochemistry, chromogenic in situ hybridization and the results were compared to the flowcytometry as reference method.

Results: Light chain restriction (LCR) was detected by FCM in 100% of the cases followed by CISH (52.1%; 12/23) of the cases and finally IHC (43%; 18/40).

Conclusion: Both conventional CISH and IHC are effective in determining monoclonality in cases of mature B- cell neoplasm that has plasmacytic differentiation and with high amount of cytoplasmic Ig light chains such as MZL and LP. However, they are not effective in determining monoclonality in cases with low amount of Ig light chain such as cases of pregerminal and germinal center lymphoma. Yet, CISH is more informative than IHC due to the lack of background staining which allowed for greater discrimination between absence and presence of monoclonality.

Keywords: kappa and lambda, chromogenic in situ hybridization, B-cell malignancies, light chain restriction.

INTRODUCTION

The discrimination between benign and malignant lymphoid infiltrates in bone marrow trephine sections is one of the major dilemmas that confront hematopathologist⁽¹⁾.

Several morphological criteria have been suggested to resolve this issue such as: the size and number of the lymphoid aggregates, the presence of cellular atypia, the histotopography which is the localization of the lymphoid aggregates within the bone marrow space, relation to the surrounding tissue (margination or interstitial spillage of lymphoid cells), and the presence of reticulin fibres, fat cells and surrounding large sinuses. However, their application is often problematic and multiple exceptions to the general rules have been described⁽²⁻⁴⁾. The phenotypic data using CD2 and CD3 might help to distinguish between benign and malignant aggregates as homogeneous staining for CD20 favors bone marrow involvement by B cell lymphoma, whereas a mixed

infiltrate of CD20 and CD3 suggests a reactive lymphoproliferative process⁽⁵⁾. Also, distribution of B and T lymphocytes within lymphoid aggregates may serve as a useful criterion as aggregates which consist of a central core of T cells surrounded by a rim of B cells, are more likely to be benign. On the other hand, an increased likelihood of malignancy occurs when aggregates consist of a central core of B cells surrounded by a rim of T cells^(2,3).

Since there is no definitive numerical and morphological "cutoff" between lymphoid aggregates/nodular lymphoid hyperplasia (NLH), and malignant lymphoid aggregates in malignant lymphoma the study of the usefulness of whether monoclonality is definite proof of malignancy has emerged⁽⁶⁾.

In hematopathology laboratory, the determination of kappa or lambda light chain restriction can be done using flowcytometric immunophenotyping (FCI) but

this methodology requires fresh tissue which is occasionally unavailable⁽⁷⁾. Also, it can be detected using single color detection based immunohistochemistry (IHC) which is the most common mean to assess for light chain restriction in histological sections. Unfortunately, single color detection based IHC is complicated by high background staining, poor sensitivity in non-plasma cell populations, and difficulty identifying particular cell populations in adjacent sections⁽⁸⁾.

Chromogenic in situ hybridization is a new cytogenetic technique that combines the chromogenic signal detection method of immunohistochemistry with in situ hybridization using oligonucleotide probes with specificity for kappa and lambda light chain mRNA can be used. It has the advantage that it lacks the background tissue staining typically seen using IHC methods. Moreover, some studies have demonstrated higher sensitivity for CISH in establishing monoclonality, even in B cell non-Hodgkin lymphomas with lower levels of light chain expression than is generally seen in plasma cell neoplasia⁽⁹⁾.

The aim of the current study was to compare the efficacy of Single color detection-based immunohistochemistry (IHC) and Chromogenic in situ hybridization (CISH) in evaluating kappa/lambda expression in tissues harboring B-lymphoid lesions.

PATIENTS AND METHODS

The present study was carried out on 40 newly diagnosed patients with mature B-cell neoplasms with bone marrow trephine biopsy infiltration. T-cell lymphoid infiltration was excluded from the study by assessing CD3 antibody on bone marrow trephine sections. **The study was approved by the Ethics Board of Ain Shams University.**

The patients were attending to the Hematology/Oncology Unit of Ain Shams University Hospitals (from Jan 2014 to Jan 2016). A written informed consent and ethical committee approval were obtained. Patients were recruited to the study on the basis of clinical, laboratory, bone marrow aspirate, trephine biopsy findings and immunophenotypic criteria of mature B-cell neoplasms. In some cases, lymph nodes biopsy results were provided to confirm the diagnosis, which was made according to the WHO classification (2008).

Their ages ranged from 25 to 75 years, with a mean age of 51.2 years. They were 22 males and 18 females with a male to female ratio of 1.2:1.

The 40 patients were divided into 3 groups: chronic lymphocytic leukemia (CLL/SLL) group I (13 patients), non-Hodgkin lymphoma (NHL) group II (24 patients) and hairy cell leukemia (HCL) group III (3 patients). The 24 NHL cases comprised of (11 diffuse large B-cell lymphoma, 6 mantle cell lymphoma, 3 marginal zone lymphoma, 2 lymphoplasmacytic lymphoma, 1 follicular lymphomas and 1 Burkitt's lymphoma).

Patients were subjected to full history taking, clinical examination laying stress on the presence of splenomegaly, hepatomegaly, and lymphadenopathy, complete blood count with examination of peripheral blood smears stained with Leishman stain, bone marrow aspiration, and examination, bone marrow trephine biopsy, flowcytometric immunophenotyping was performed on bone marrow or peripheral blood samples, using a panel of monoclonal antibodies for the different lymphoproliferative disorders (CD19, CD5, CD10, CD11c, CD20, CD22, CD23, CD38, CD79b, FMC7, CD103, CD25, kappa and lambda), histopathological evaluation of formalin-fixed paraffin-embedded BM trephine biopsy stained by Leishman stain and Giemsa for further assessment of marrow cellularity, cell distribution, morphology and infiltration. Immunohistochemical staining of bone marrow trephine sections by CD3 and CD20, kappa and lambda light chain. Kappa and lambda light chain detection on bone marrow trephine using chromogenic in situ hybridization.

Specimens

Two milliliters peripheral blood samples were collected in a sterile ethylene-diamine-tetra-acetic acid (EDTA) containing vacutainers for CBC. Bone marrow (BM) aspiration was withdrawn; the first few drops were spread on glass slides for morphological examination and 1 mL into sterile EDTA containing vacutainers for IPT. BM trephine core biopsy was obtained and transferred immediately in a sterile plastic cup containing 10% formalin neutral buffer as a fixative for histopathological evaluation, immunohistochemical staining and chromogenic in situ hybridization evaluation of paraffin-embedded core biopsy.

Immunohistochemistry

Fixation was performed for 24 hours then decalcification of the core was done using disodium EDTA for 48 hours. This was followed by passing the core in serial concentrations of ethyl alcohol (50%, 70%, 85%, 90%, and 100%) ending with xylene then

followed by paraffin embedding. Serial 3- μ m sections were cut from the paraffin block, mounted on positively charged slides and dried overnight in a 60°C oven. Deparaffinization in xylene for 24 hours followed by hydration in descending grades of alcohol; 100%, 90%, 85% and 70% was done. Antigen unmasking was done by heat induced epitope retrieval (HIER) method using antigen retrieval solution, Tris EDTA Buffer (pH 9) for kappa and lambda light chain, citrate buffer, (pH 6) for CD20 and CD3 detection for a period of 10 min in a microwave at 800 Watt.

Endogenous peroxidase activity was blocked by incubation of the tissue section with 3% hydrogen peroxide in water for 10-15 min followed by incubation with the primary antibody (Genemed, USA). The mouse/rabbit polydetector HRP/DAB system is a non-biotin, 2-step polymeric detection system was used as a detection kit (Poly HRP DAB Kit Genemed, USA). The sections were then counterstained in Meyer's hematoxylin, cover-slipped by DPX mount media and examined under light microscope. The positivity of the immunostaining was detected by percentage of positive cells, intensity, and pattern of staining (Table 1). According to percentage of positive cells: four grades were identified; 0: no reaction, 1+: <5%, 2+: 5–9%, 3+: 10–20% and 4+: \geq 20%. Monoclonality was confirmed by either κ/λ ratios of more than 3 (κ clone) or less than 0.4 (λ clone) (10).

Table (1): Interpretation of results (10).

Primary antibody	Pattern of staining	Grade of positivity
CD20	Brown membranous	4+
CD3	Brown membranous /cytoplasmic	3+
Kappa light chain	Brown membranous /cytoplasmic	2+
Lambda light chain	Brown membranous /cytoplasmic	2+

Chromogenic in situ hybridization

We used the same protocol used in immunohistochemistry for sample preparation. Deparaffinization in xylene twice for 15 min followed by hydration in descending grades of ethanol; 100%, 96%, 70 followed by incubation 10 min in 3% H₂O₂. Proteolysis step was done using 100 μ l of pepsin solution for 90 min. Add 10 μ l of digoxigenin-labeled

oligonucleotides which target Ig-kappa (κ) mRNA and biotin-labeled oligonucleotides which target Ig-lambda (λ) mRNA (Zytovision Germany). Duplex formation of the digoxigenin-labeled probe can be visualized indirectly using HRP-conjugated anti-digoxigenin antibody. The enzymatic reaction of AEC (3-amino-9-ethylcarbazole) leads to the formation of strong red signals. Duplex formation of the biotin-labeled probe can be visualized using AP-conjugated streptavidin. The enzymatic reaction of NBT/BCIP (Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate) leads to the formation of strong blue-violet signals that can be visualized by light microscopy at a 10-20x dry lens.

STATISTICAL ANALYSIS

Data were analyzed statistically using SPSS Version 21.0 (International Business Machines Corporation, New York, 2012). The following tests were done: descriptive statistics including quantitative data (mean \pm standard deviation) for parametric results and in the form of median and range in non-parametric ones) and qualitative data (number and percentage), analytical statistics including Chi-Square, Wilcoxon Rank-sum test (Z-value) and Kruskal Wallis Test “one way ANOVA” (P-value).

RESULTS

In the CLL/SLL group, BM aspirate lymphocytes percentage ranged from 42% to 85% with a mean of 67% \pm 12.4. In the NHL group, the BM aspirate lymphocytes ranged from 5% to 75% with a mean of 21.4% \pm 17.7. Bone marrow trephine biopsy showed lymphocytic infiltration in all patients. In the CLL/SLL group, nine cases (69.2%) showed patchy infiltration and four (30.7%) cases showed a diffuse pattern of infiltration with packed marrow. In the NHL group, the predominant pattern of infiltration was the patchy type (11/24, 45.8%), followed by the diffuse pattern (8/24, 33.3%), the focal pattern (3/24, 12.5%) then the mixed pattern (2/24, 8.3%). The three HCL cases showed an interstitial pattern of infiltration.

Immunohistochemical staining

CD20 was positive in all patients among different studied groups. Concerning CD3 positivity, it was detected on normal T-cell in the background of the infiltrating B-cell in a very small percentage approximately (1-5%). Higher CD3 positivity was detected in 5 out of 24 B-NHL cases ranging from 10% to 20%; two cases of which were T-cell rich DLBCL

and the other three cases showed a mixed pattern of CD20 and CD3 expression. However, there was a central core of B cells (CD20+ cells) and peripheral rim of T cells (CD3+ cells) favoring the diagnosis of B-NHL.

In CLL/SLL group, 7.6 % of the cases was kappa restricted, while none of the cases showed lambda light chain restriction. In B-NHL group, 41.6 % of the cases were kappa restricted, of which 4 were DLBCL, 3

MCL, 2 LP and 1 MZL. However, lambda restriction was observed in 25 % of cases. Which consists of 3 DLBCL, 2 MZL, and one MCL. All cases of B-NHL group showed weak to moderate staining intensity (Figure 1). In HCL group, only one case out of three 33.3% was kappa restricted and none of the cases showed lambda restriction. On comparing LCR among the three studied groups, there was significant difference being higher in B-NHL group (P = 0.002) (Table2).

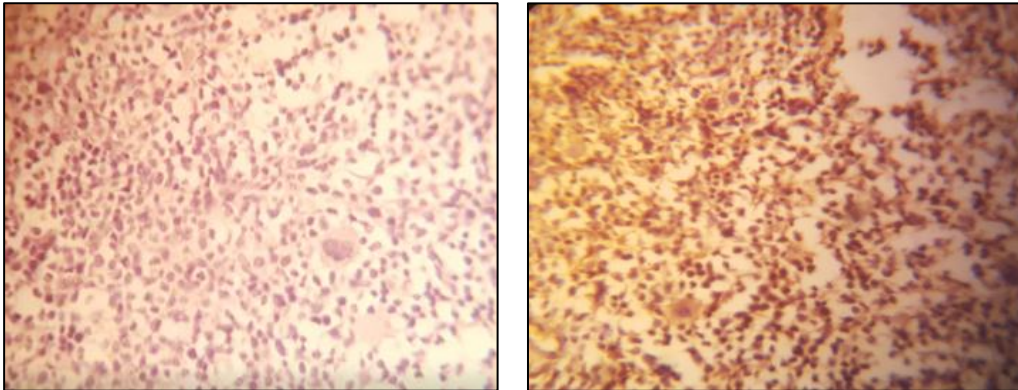


Figure (1): A case of MCL with kappa light chain restriction of moderate intensity.

Table (2): HC positive cases of Kappa light chain, Lambda light chain, and LCR of the studied groups.

Variance	Groups			Total (n=40)	X2	P	Sig
	Group I (CLL/SLL (n=13)	Group II (B-NHL) (n=24)	Group III (HCL) (n=3)				
Kappa	1 (7.6%)	10 (41.6%)	1 (33.3%)	12(30%)	4.65	0.098	NS
Lambda	0 (0%)	6 (25%)	0 (0%)	6(15%)	4.7	0.095	NS
LCR	1 (7.6%)	16 (66 %)	1(33.3%)	18 (43.1%)	12.0	0.002	S

Chromogenic in situ hybridization

LCR was detected in 52.1% of the cases. These cases considered to have sufficient mRNA integrity to be reliably interpreted. It was found that dual CISH was effective in determining light chain restriction in all cases of MZL and LPL but less effective in cases of SLL/CLL, MCL, FL, and DLBCL (Figure 2, table3).

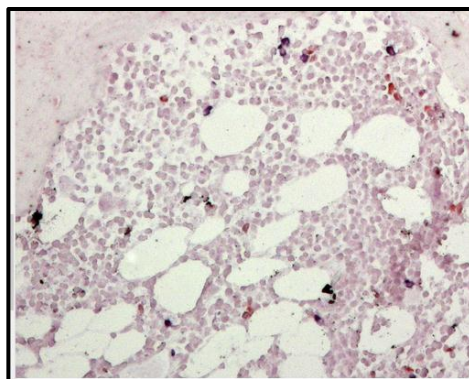


Figure (2): A case of MZL showing lambda light chain restriction by CISH.

Table (3): LCR Positive cases in different cases by CISH.

Variance	LCR POSITIVE CASES				
	NO of applicable cases	Positive Cases			%Positivity
		k	l	T	
CLL/SLL	7/13	2	0	2	28.6%
DLBCL	5/11	1	0	1	20%
MCL	4/6	4	0	4	80%
MZL	3/3	1	2	3	100%
LP	2/2	2	0	2	100%
HCL	1/3	0	0	0	0%
FL	0/1	0	0	0	0%
Burkit	1/1	0	0	0	0%

Flow cytometric detection of kappa and lambda

Out of 40 cases, 24 cases had enough P.B and bone marrow for further assessment of kappa and lambda light chain restriction. 16 cases were kappa restricted and 8 were lambda restricted. LCR was detected in 24/24 (100%) of the cases. Concordant results of light chain restriction were detected in all kappa and lambda positive cases between FCM and either IHC or CISH. Comparing our results with FCM findings, no cases were erroneously classified as the incorrect light chain restriction.

Evaluation of the % positivity of LCR, higher percentage of LCR detection was detected by FCM

followed by CISH and finally IHC (Figure 3). Comparing immunohistochemistry to CISH results. Out of 18 positive cases by IHC, 9 cases were excluded from CISH results due to the technical difficulty we faced during protocol establishment until we obtained satisfactory results. The remaining 9 cases were all detected by CISH with concordant results in both kappa and lambda restriction. However, CISH singly detected 3 cases which were negative by IHC. Although LCR was detected in a higher number of cases than IHC, the detection level was still unsatisfactory.

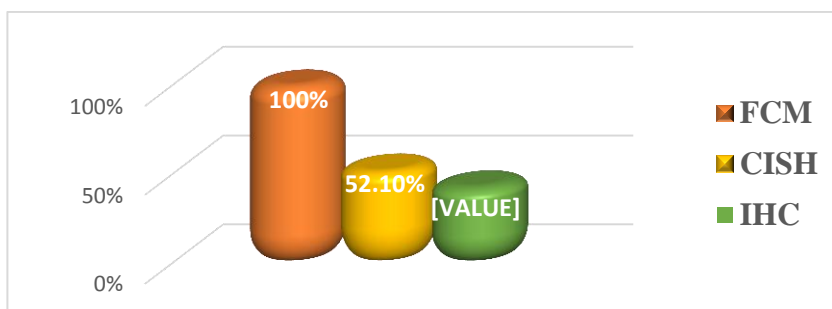


Figure (3): Positivity % of light chain restriction by different methods.

DISCUSSION

clonal expansion of B-lymphocytes harboring immunoglobulin (Ig) gene rearrangements is the hallmark of B-cell malignancy and it is commonly manifested as a restricted expression of immunoglobulin light chain RNA and protein (either κ or λ). Clinical laboratory detection of Ig light chain restriction (LCR) is a helpful ancillary tool in the differential diagnosis between lymphoid hyperplasia and B-cell neoplasia. LCR can be readily detected as an abnormal kappa/lambda immunoglobulin ratio by lots of methods⁽¹¹⁾.

It can be demonstrated by flow cytometric immunophenotyping or frozen tissue immunohistochemistry. However, both of these methods require fresh unfixed or frozen tissue, which may not be available in a given case. Moreover, correlation with morphologic findings is difficult using either technique. In contrast, formalin-fixed, paraffin-embedded (FFPE) tissues are widely available for immunohistochemical analysis⁽¹¹⁾.

Therefore, in hematopathology laboratory when only formalin-fixed, paraffin embedded (FFPE) tissue is available for evaluation, as it is often the case in many clinical settings, immunohistochemistry (IHC) and conventional chromogenic in situ hybridization (CISH) techniques are feasible.

Single colored Immunohistochemical detection of light chain protein is currently the most frequently used method to detect Ig light chain restriction in histologic sections. It has been used routinely to evaluate the presence of monoclonality in PC dyscrasia and more variable in cases of B cell non-Hodgkin lymphoma. This technique provides accurate detection of

cytoplasmic Ig when expression levels are high, as they are in more differentiated B cells⁽¹²⁾.

However, the utility of IHC in the detection of light chains is hampered by nonspecific staining of adsorbed Ig in surrounding stroma⁽¹³⁾ and of damaged or degenerating tissue⁽¹⁴⁾. Several apparently successful methodology have been published. Yet, the success of these protocols has been difficult to replicate⁽¹²⁾.

Due to the vast drawbacks that IHC technique faced, attention was drawn towards ISH technique, where assessment of mRNA avoids the

issue of background staining and can detect a low level of Ig⁽¹⁵⁾.

Given the advantages of ISH (suitability for fixed tissues, the absence of background) as well as technological developments in the field, a new methodology known as dual chromogenic *in situ* hybridization has appeared (CISH). CISH uses nucleic acid probes to detect target mRNA accompanied by colorimetric detection systems, which are convenient formats for pathologists along with avoidance of nonspecific background staining that occurs in IHC detection⁽¹¹⁾.

The aim of our study was to compare single colored immunohistochemistry and chromogenic in situ hybridization techniques in detection of kappa and lambda light chains in assessing B cell monoclonality in B cell neoplasms.

In the current study, we were able to detect LCR in 43% (18/40) of the cases by immunohistochemical technique. This is in accordance with results obtained by *Ashton-Key*⁽¹⁶⁾, *Weiss*⁽¹²⁾, and *Afaf and her colleagues*⁽¹⁷⁾ who detected LCR in a range of 30%-58%.

IHC may not be as reliable when used on formalin fixed, paraffin-embedded tissue due to alteration of Ig epitopes during the fixation process while IHC done on frozen section, lacks the fixation step by formaldehyde that masks the antigenic epitope and makes it less sensitive⁽¹⁸⁾. Thus, higher results were observed by *Kurtin et al.*⁽¹⁹⁾ who demonstrated LCR in 64 of 84 (76%) of B-cell lymphomas. However, *Warnke and Rouse*⁽¹³⁾ noted that use of frozen lymphoid tissue results in higher levels of background staining due to interstitial Ig.

higher results were obtained by *Marshall-Taylor*⁽⁸⁾ that demonstrated monotypic light chain expression in 81% (91 of 113) cases and *Biesemier et al.*⁽²⁰⁾ who detected LCR in (91%)52/57, despite they both used HIER technique as antigen retrieval as used in this study. The reason for their higher results that their studies were done on soft tissues (lymph nodes). Immunostaining of immunoglobulin light chains is technically challenging in all tissues but presents particular problems in bone marrow, in which the interstitium is rich in tissue fluid containing abundant immunoglobulins. High background staining is therefore almost always encountered, making specific staining of lymphoid cell membranes and plasma cell cytoplasm difficult to assess. In addition to the decalcification process, which can ruin antigenicity of tissue, producing a weak reaction⁽²¹⁾.

Similarly, *Ashton-Key et al.*⁽¹⁶⁾ demonstrated the greatest sensitivity of light chain restriction (91%) of the cases were restricted. Most probably this difference as they used the automated system.

In our study, we were able to detect LCR accurately in all cases that have a higher level of cytoplasmic Ig expression and plasmacytic differentiation such as LPL and MZL. This is in accordance with results obtained *Beck et al.*⁽⁹⁾ who detected LCR in 100% of the cases of LPL and 70% of cases of MZL.

Moreover, we were faced by nonspecific mild to moderate background staining which was observed for both antibodies and typically more pronounced at the tissue edges. This is due to the presence of normal physiologic interstitial Ig⁽²¹⁾. The uptake of polytypic immunoglobulin by dead or damaged cells. All these complicate the interpretation of the specific staining of immunoglobulin-producing cells⁽¹²⁾. Nonspecific staining increased in cases of HCL which showed excessive deposition of fibrous tissue, this is in accordance to study done by⁽²²⁾ who faced the same problem due to collagen deposition.

Using CISH technique, we detected LCR in (52.1%). Similar to IHC, we were able to detect clonality in 100% of cases that have plasmacytic differentiation i.e. MZL and LP lymphoma. Moreover, CISH was not sensitive enough to detect light chain mRNA in cases of CLL/SLL, MCL, DLBCL, follicular lymphoma and Burkitt's lymphoma, which is consistent with data indicating that light chain mRNA expression is detectable by manual CISH only in more differentiated B cells⁽²³⁾.

Slightly higher results were obtained by *Beck et al.*⁽⁹⁾ who detected monoclonality in (68%) 9 of 13 cases of B cell non-Hodgkin lymphoma. They also concluded that CISH was effective in determining light chain restriction in all cases of MZL and LPL but less effective for cases of SLL and MCL and this is in agreement with our results. Higher results may be due to the automated system they used, yet we were using conventional manual CISH.

In contrary, *Rimsza et al.*⁽¹¹⁾ reported much higher percentages than our study. They found that (88.6%) 70/79 cases had either kappa or lambda light chain restriction. This is due to multiple factors. First, their study was performed on Ventana Benchmark XT automated system which is able to optimize all the conditions to give a high yield of mRNA. Moreover, decalcified samples from bone marrow trephines were

not included in their study. They were working only on soft tissue sample e.g. lymph node. Therefore, the mRNA was not affected by the decalcification methods. Furthermore, they used a new detection system which is highly sensitive, efficient, quick and good option for detecting low copies of nucleic acids. It depends on amplification of the signal after in situ hybridization to increase the sensitivity of the test.

Moreover, *Minca et al.*⁽²³⁾ reported higher results 96% using CISH with novel branched-chain technology (BRISH) that highlights individual mRNA transcripts within lymphocytes and offers a high level of sensitivity by amplifying the mRNA signal. It is achieved via a sequential hybridization, using amplifier molecules which hybridize to the oligonucleotides z shaped probe. It was performed using the (*RN Ascope, Advanced Cell Diagnostics, and Hayward, CA*)⁽²³⁾.

We concluded that both conventional CISH and IHC is effective in determining monoclonality in cases of mature B- cell neoplasms that have plasmacytic differentiation and with a high amount of cytoplasmic Ig light chains such as MZL and LP. However, they are not effective in determining monoclonality in cases with low amount of Ig light chain such as cases of pre-germinal and germinal center lymphoma. Yet, CISH is more informative than IHC due to the lack of background staining which allowed for greater discrimination between absence and presence of monoclonality.

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