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Original Article

Rapid in-situ hybridization and immunohistochemistry: a pilot comparative study of two rapid diagnostic techniques for establishing monoclonality in plasma cell dyscrasias

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ABSTRACT

Background: Light chain restriction needs to be established on the paraffin embedded tissue in certain types of plasma cell dyscrasias when serum levels of monoclonal immunoglobulins and light chain assays in the urine and serum may be normal. Rapid-in-situ-hybridisation (RISH) is thought to be a superior to immunohistochemistry (IHC) for kappa and lambda staining due to brighter and crisp staining without any background.

Methods: Fifty cases were included in this pilot study. Serum light chain restriction status of the case was taken as gold standard. The results of standard IHC for kappa and lambda immunoglobulins on the bone marrow biopsy of these cases was compared with RISH performed by the two commercially available kits. The results of the two methods were compared for sensitivity, need to repeat the test and background staining.

Results: The study found that in IHC first run sensitivity was 58% which improved to 88% after the second run. For RISH the sensitivity was 100%.

Conclusion: Rapid-in-situ-hybridisation (RISH) is a superior technique to IHC for detecting kappa and lambda light chain in plasma cells. The test is as labour intensive and time consuming as the routine IHC but has no background staining with more bright and crisp staining quality.

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Introduction

The plasma cell dyscrasias are a group of clinically and biochemically diverse proliferative disorders (plasma cell

neoplasms) characterized by the proliferation of a clone of plasma cells in the bone marrow and by the production of monoclonal immunoglobulins in the serum and urine that bear structural and electrophoretic homogeneity. Normally $\leq 5\%$ of bone marrow cells are plasma cells. In plasma cell

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dyscrasias, their levels are elevated. Plasma cell dyscrasias include the following disorders¹: monoclonal gammopathy of undetermined significance (MGUS), smouldering multiple myeloma (also referred to as asymptomatic multiple myeloma), multiple myeloma, Waldenström's macroglobulinemia, solitary plasmacytoma, systemic AL amyloidosis and POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal protein, skin changes). Assessment of plasma cell content is used to diagnose plasma cell dyscrasias as well as to follow-up and determine minimal residual disease in the patient.^{2,3} CD138 has been used as an immunohistochemistry (IHC) marker for semiquantitative assessment of percentage of plasma cell infiltrates in formalin-fixed, paraffin embedded material. The presence of a clear-cut light chain restriction with a kappa/lambda ratio of more than 10:1 or the reverse confirms the monoclonality of these diseases.⁴ The disease can either be kappa restricted or lambda restricted. Usually IHC for kappa and lambda is used to establish monoclonality (light-chain restriction) in biopsy specimens. The need to establish monoclonality on biopsy is essential in case of certain types of plasma cell dyscrasias such as plasmacytomas, residual disease in multiple myeloma post therapy, smouldering myeloma and light-chain only secretion, and monoclonal gammopathy of undetermined significance. In these situations, the serum levels of monoclonal immunoglobulins and light-chain assays in the urine and serum may be normal.

Keeping this in mind, the present study was planned to compare two rapid diagnostic modalities—the routinely used IHC and new evolving rapid in-situ hybridization (RISH) in establishing monoclonality of plasma cells (light chain restriction). Whereas, IHC detects the protein (immunoglobulin) and RISH detects the messenger RNA (mRNA). IHC for kappa and lambda is notorious for background staining. This leads to errors in interpretation and also resorting to repeated testing. The effect of decalcification on bone marrow biopsy often weakens the immunohistochemical staining of kappa and lambda immunoglobulins. The background staining results due to uptake of polyclonal immunoglobulin by dead or damaged cells and bone marrow stromal cells and extracellular immunoglobulin,^{5,6} which obscures patterns of cellular staining. These disadvantages are supposed to be overcome in RISH. The present study will use bone marrow biopsies of cases of multiple myeloma. However, its application in practice will be in situations as discussed above where serum markers are not available for establishing monoclonality.

Material and methods

The study was conducted at a tertiary-care center. Fifty retrospective and prospective (01 January 2014 to 31 December 2016) bone marrow biopsies done in cases diagnosed as multiple myeloma, based on established diagnostic criteria, were included in this pilot study. The serum-free light-chain restriction status was noted from the hematology departmental records of all these cases. Serum-free light-chain ratio (kappa to lambda) was less than 0.8 and greater than 1.6 in lambda and kappa restricted cases,

respectively. This was taken as the gold standard for the kappa/lambda restriction status of the case and subsequent comparison with the results of IHC and RISH. The demographic profile (age and sex) of the selected cases was noted. Cases were divided arbitrarily into two groups less than 50 and more than 50 years. The paraffin section of the bone marrow biopsy was used. Histomorphological detection of increased number of plasma cells in hematoxylin and eosin stained section and their pattern of infiltration was noted. CD138 immunohistochemical confirmation was done before selecting the cases. Standard IHC technique was used with Path Situ Biotechnology ready to use CD138 Rabbit monoclonal, clone EP 201, (Rabbit IgG isotype), incubation of 30 min. Membranous positivity was noted. The cases were divided into semiquantitative counts based on CD138 positive cells, of less than or equal to 20%, 21–50%, and greater than 50%. The methodology of the study was to compare the results of detecting monoclonality for kappa and lambda using IHC and rapid in-situ hybridization and then compare the results of these two diagnostic protocols to serum light-chain free assay (gold standard for type of light-chain restriction) results in the cases. In cases where background staining in either kappa or lambda was observed, the staining was repeated with reducing the incubation time from 30 to 20 min to get interpretable results for both kappa and lambda for the case. RISH protocol was performed on each of these biopsy sections by two commercially available kits which are most commonly used for diagnostic purpose. Biocare Cat No. R10004T/R10005T and Zytovision kits (Zytofast plus) were used. Both kits essentially work on the same principle of detecting messenger RNA by chromogenic in-situ hybridization (CISH) in formalin-fixed paraffin embedded tissues. The kits use digoxigenin-labelled oligonucleotide DNA probes, which are detected using primary antibodies. These primary antibodies are detected using polymerized enzyme-conjugated secondary antibodies. The enzymatic reaction of chromogenic substrates (DAB) leads to formation of strong color precipitates that can be visualized by light microscopy. Both the kits essentially had steps of deparaffinization, protein digestion, and retrieval, probe hybridization, post hybridization wash, and detection of probe. The diagnosis of all these cases of multiple myeloma had already been made as per guidelines, and the serum light-chain restriction status of each case was also known and was not based on the result of IHC or RISH. The protocol followed for kits was as per kit literature and standardized at our laboratory. This study design was intended to work toward validating an evolving diagnostic protocol of RISH/CISH for monoclonality of kappa and lambda light chains in plasma cells in formalin-fixed paraffin-embedded tissue and compare it with the traditionally used IHC for the same purpose.

The results of both these rapid techniques RISH and IHC for kappa/lambda staining will be evaluated and compared to check which one is a superior technique. The results were compared for sensitivity and presence or absence of background staining and the need to repeat the protocol. Control will be tonsil tissue (polyclonal plasma cells). It will be a laboratory diagnostic study. Clearance was taken from the institutional ethics committee.

Table 1 – Comparison of serum-free light-chain restriction status with results of n = 50.

Restriction status	Serum-free light-chain restriction status	IHC				Freq by Biocare Medical RISH Kit	Freq by Zytovision plus RISH Kit
		IHC 1 st run		IHC 2 nd run			
		C*	I**	C*	I	—	—
Kappa restriction	34	21	13	30	4	34	34
Lambda restriction	16	8	8	14	2	16	16
Total	50	29 (58%)	21 (42%)	44 (88%)	6 (12%)	50	50
IHC, immunohistochemistry; RISH, rapid <i>in-situ</i> hybridization.							

IHC, immunohistochemistry; RISH, rapid in-situ hybridization.

Results

The present study evaluated formalin-fixed paraffin embedded bone marrow biopsies in patients diagnosed with multiple myeloma between 01 January 2014 and 31 December 2016. The age of the patients included in the study ranged from 39 years to 85 years with a mean of 61.2 years and median of 62.5 years. Seven patients (14%) were aged less than 50 years, while 43 patients (86%) were aged greater than 50 years. Males outnumbered females in our study with 40 males (80%) and 10 females (20%). 25 cases (50%), 12 (24%), 8 (16%), and 5 (10%) showed diffuse (massive infiltration by plasma cells with effacement of bone marrow architecture), interstitial (interstitial infiltration of plasma cells with some preservation of hematopoietic tissue, normal bone marrow fat spaces and architecture), and mixed (nodular and interstitial and nodular preserved hematopoietic tissue, normal bone marrow fat spaces, and architecture with nodules of plasma cells scattered without interstitial infiltration) pattern of infiltration, respectively. Semi-quantitatively for count of plasma cells using CD 138 IHC staining, the cases were categorized into $\leq 20\%$, 21%–50%, and $>50\%$ infiltration in 9 (18%), 10 (20%), and 31 (62%) cases, respectively. Table 1 compares the serum-free light-chain restriction status of the cases with results of IHC after 1st and 2nd run and also by the two kits used for RISH. 34/50 (68%) and 16/50 (32%) were kappa and lambda restricted, respectively, as per the serum-free light-chain restriction. In these 50 cases after the 1st run of IHC, out of 34 cases of kappa restriction only, 21 could be confirmed, while 13 were indeterminate. But after the 2nd run of IHC on these cases, 30 could be confirmed, while 4 still remained indeterminate. While for lambda-restricted cases after 1st run of IHC, 8 cases were confirmed and 8 remained indeterminate. Thus, overall 21 (13 + 8) (42%) cases after 1st round of IHC staining were indeterminate and had to be repeated in an effort to conclude at kappa or lambda restriction because of difficulty in interpretation of results. There was excessive background

staining in these 21 cases. In repeat staining with 20 min primary antibody incubation instead of 30 min, the background staining was reduced, but it also gave a faded/lighter stain to the kappa- and lambda-positive plasma cells. The results however were interpretable in 15 out of these 21 cases. Finally, despite two repeats in 6 (4 + 2) (12%) cases (case no. 5, 15, 19, 21, 24, and 29), results were non-interpretable because of persistence of background staining even in the repeat staining. Fig. 1a–c show background staining for kappa and lambda antibody respectively in case no. 5, 15, and 21. Fig. 2a–c show interpretable IHC staining for kappa and lambda without any background staining in cases 7, 18, and 48.

Table 1 also shows results of RISH by the two kits used in the study. Biocare medical and Zytovision plus RISH DNA probe for detecting kappa and lambda mRNA. There was no background staining in any case for both kappa and lambda once the protocol on each case had been standardized on the control sections of the tonsils and bone-marrow biopsy. In two cases, the run had to be repeated since even the internal control did not give result, most likely because of inadequate antigen retrieval. Neither was there any case where the results were indeterminate. Fig. 3a–d show results of RISH staining using the two kits used in the present study in case no. 15, 11, 30, and 21, respectively. The results in all these cases matched with serum-free light-chain restriction for the case.

Statistical data

Immunohistochemistry

In the first run, 21 (42%) out of 50 cases had indeterminate results which reduced to 6 (12%) even after the second run. So, sensitivity of IHC for kappa and lambda restriction in the first run was 29/50 (58%) only, since 21 cases were indeterminate. This improved to 44/50 (88%) after the second run.

Fig. 1 – Background staining in IHC. (a) kappa 400x (case no. 5), (b) kappa 400x (case no. 15), (c) lambda 400x (case no. 21). IHC, immunohistochemistry.

Fig. 2 – Interpretable staining in IHC: (a) lambda 400x (case no. 7), (b) kappa 400x (case no. 18), (c) lambda 100x (case no. 48). IHC, immunohistochemistry.

Fig. 3 – RISH, Crisp, bright staining with no background: (a) kappa 100 × (case no.15) (Biocare Medical Kit), (b) lambda 400 × (case no. 11) (Zytovision Plus Kit), (c) lambda 100 × (case no. 30) (Biocare Medical Kit), (d) lambda 40 × (case no. 21) (Zytovision Plus Kit). RISH, rapid in-situ hybridization.

Rapid in-situ hybridization

As far as RISH was concerned, in 48/50 cases, the kappa or lambda restriction could be identified correctly in the first run. In two cases, it was found that the internal control also did not give results. For these two cases, the repeat run for RISH could establish the correct restriction and therefore having a sensitivity of 100%.

Discussion

Plasma cell dyscrasias are a group of clinically and biochemically diverse proliferative disorders (plasma cell neoplasms) characterized by the proliferation of clone of plasma cells in the bone marrow and by the production of monoclonal

immunoglobulins in the serum and urine that bear structural and electrophoretic homogeneity.

We included a total of 50 cases diagnosed as plasma cell dyscrasias at our center over a period of 2 years from January 2014 to August 2016, both retrospectively as well as prospectively.

The serum-free light-chain restriction status of each of these cases was noted from the patient records. The bone-marrow biopsies were processed and stained with hematoxylin and eosin to determine the patterns of bone-marrow infiltration by the plasma cells, IHC for CD138 to semi-quantify the percentage of plasma cells, and kappa/lambda was done to determine the type of restriction by both IHC and RISH. The results of the two diagnostic protocols were compared. The age of the patients included in our study ranged from 39 years to 85 years with a mean of 61.2 years and median of 62.5 years.

In a study done on 55 patients by Subramanian et al.,⁷ the median age of the patients was 55 years, which is less as compared with our study. Maximum cases were above 50 years of age (86%). Males outnumbered females in our study (80% males and 20% females) (4:1). In a study done on 55 patients by Subramanian et al.,⁷ the male preponderance was seen with a male to female ratio of 1.9:1. Another study done by Bai et al.,⁸ of the 24 cases studied, the male to female ratio was 3:1. This is in concordance with our study, ratio of 4:1.

The patterns of bone marrow infiltration by plasma cells on H&E were nodular-5 (10%), interstitial-12 (24%), mixed-8 (16%), and diffuse-25 (50%). Thus, maximum cases in our study showed diffuse pattern of infiltration. Study by Subramanian et al.⁷ on 55 cases also revealed maximum number to be of diffuse pattern of infiltration—35 cases (64%). In this study, the other patterns seen were interstitial-15 (27%), mixed-4 (7%) and nodular- 1 (2%) respectively. 31 cases (62%) showed high (>50%) CD138 positivity which correlated with the results of Subramanian et al.⁷ of 39 cases (71%). Maximum cases in our study showed kappa restriction (68%). The ratio of kappa: lambda in the cases studied was 2.1:1. Results are based on serum light-chain restriction status and RISH. Boo et al.⁹ studied the immune-histological properties in 10 cases, shown in Table 1. In the latter out of 10 cases, 7 were kappa (70%), 2 were lambda (20%), and 1 was inconclusive. Thus, the result of our study correlated with that of Boo et al.⁹ Very few studies have been done in the past comparing results of IHC for kappa and lambda. The drawbacks of IHC for kappa and lambda have been brought in several studies in the past.^{6,10} In our study, in the initial run of IHC 21 (42%) out 50 cases, we got indeterminate results because of excessive background staining, which did not allow the interpretation and quantification of kappa and lambda positive plasma cells. Thereby, it did not allow us to arrive at a conclusion regarding the kappa/lambda restriction. Indeterminate results were as high as 42%. This drawback of IHC leads to delay in reporting out of cases. On repeating the IHC with 20 min of primary antibody incubation time instead of 30 min in these 21 cases, it did reduce the background staining but allowed us to interpret the results, though the staining of kappa and lambda positive plasma cells appeared faded/lighter. Even on repeat testing, 6 out of these 21 cases still remained indeterminate as the background staining significantly persisted. As compared with IHC in RISH after standardizing the protocols with the two commonly used commercially available kits in the market on control sections of tonsils for kappa and lambda, we could achieve interpretable results in 48 out 50 cases in the first run. For two cases, because the internal controls also did not give results (likely due to inadequate antigen retrieval) along with cases, the run had to be repeated, and interpretable results were achieved. Beck et al.¹⁰ in their study on plasma cell dyscrasias and non-Hodgkin lymphomas found IHC to have given indeterminate results, 4 (10.5%) out of 38 cases of plasma cell dyscrasias (multiple myelomas and plasmacytomas) as compared with CISH, where it was only in 1 case out of 38. Whereas in non-Hodgkin B-cell lymphoma, IHC could not detect monoclonality in 7 (54%) out of 13 cases; whereas for CISH, it was 4 (31%) out of 13 cases. For levels of kappa and lambda light-chain mRNA being significantly

lower in non-Hodgkin lymphoma, the conclusion of monoclonality is that much difficult. This study too found CISH to be a superior technique because of lack of background staining and easier interpretation of results. Rimsza et al.¹¹ in their study published in 2014 concluded that CISH detected lower levels of mRNA, making clonality assessment in formalin-fixed biopsies for mature B-cell neoplasms superior as compared with flow cytometry and IHC. Hence, CISH/RISH can be extended very effectively as diagnostic protocol for plasmacytomas, MGUS, post-therapy residual plasma cells in bone marrow in cases of multiple myeloma, especially since serum-free light chains may be absent in such cases.

Conclusion

Rapid in-situ hybridization(RISH)/CISH is a superior technique to IHC for detecting kappa and lambda light chain in plasma cells. The test is as labor intensive and time consuming as the routine IHC but has no background staining with more bright and crisp staining quality making interpretation easier than IHC. It also avoids repeat testing and thus delaying the sign out of report. Sensitivity in RISH in our study was 100% as compared with IHC, where it was 58% in the first run and improved to 88% after the second run.

Conflicts of interest

The authors have none to declare.

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