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Pushing the boundaries of *in situ* hybridisation for mRNA demonstration: demonstration of kappa and lambda light chain restriction in follicular lymphoma

A Warford[a], M Rahman[a], JA Hughes[b], G Gerrard[c] and DA Ribeiro[a]

[a]Life sciences, University of Westminster, London, UK; [b]Advanced Diagnostics, University College London, London, UK; [c]Sarah Cannon Molecular Diagnostics, HCA Healthcare, London, UK

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The demonstration of clonality is important in differentiating non-Hodgkin lymphomas (NHL) from reactive lymphoid conditions that can have similar morphological characteristics. For B-cell NHL, this can be done using immunocytochemistry (ICC) on formalin fixed paraffin embedded (FFPE) sections or by molecular methods applied to tissue homogenates [1,2]. ICC is quicker to undertake and is widely used as part of an antibody panel approach to assist in subclassifying NHL [3]. For the determination of clonality, kappa and lambda light chain immunoglobulins are demonstrated. In reactive conditions the cellular kappa/lambda ratio is around 6/4. However, in B-cell NHL this is perturbed with one of the light chains predominating, typically resulting in a 9/1 ratio. When this is observed clonality is strongly suggested and the term 'light chain restriction' is used to define this. In many instances the interpretation of ICC needs to be carefully undertaken as immunoglobulin secreted from B-cells is also demonstrated in the interstitial areas of the tissue resulting in a non-specific background reaction [3].

As the precursor to protein, mRNA is present in all B-cells expressing kappa and lambda immunoglobulin. mRNA levels reflect the B-cell maturation. Accordingly, in naïve and memory cells of this lineage levels are very low compared with B-cells that are synthesising immunoglobulin in response to antigen challenge. Cells in the latter category include centrocytes and centroblasts that contain moderate amounts of mRNA and plasma cells that contain an abundance of mRNA. Unlike immunoglobulin protein, mRNA is never secreted and, accordingly, its demonstration should be free of non-specific background. This indeed has been shown to be so in tissue sections in which kappa and lambda light chain mRNA has been demonstrated using *in situ* hybridisation (ISH) [4,5]. A drawback of the use of ISH has been its lack of sensitivity in comparison with ICC due to the lower copy number and lability of mRNA in B-cell NHLs that are not synthesising immunoglobulin. Follicular lymphomas are representative of such a situation and while successful demonstration of mRNA light chain restriction has been reported [6], the method has not been routinely adopted. Potentially the branched DNA ISH (BDISH) method, that combines specificity via couplet oligonucleotide hybridisation and employs an ultra-sensitive detection procedure, could address this issue [7]. Accordingly, the purpose of this study was to establish if the BDISH method could demonstrate light chain restriction in follicular lymphoma and to compare the results with ICC.

Sections cut at 4µm, mounted on SuperFrost plus slides, and dried for 1 h at 61 °C, from 13 samples of neutral buffered formalin (NBF) fixed FFPE samples were used. 10 of these had been diagnosed as follicular lymphoma with five being core biopsies and five being larger resection samples. All samples had been immersed in NBF without prior dissection and the duration of fixation ranged from 24 to 72 h (Table 1). The remaining three cases, included as controls, were reactive tonsils with unknown fixation time. The sections were obtained from the University College London Cancer Institute, Biobank and Pathology Core Facility Service, London and transferred into the University of Westminster Biobank in compliance with Human Tissue Authority regulations.

BDISH for mRNA demonstration was undertaken using RNAscope® materials (Advanced Cell Diagnostics, a Biotechne Company, USA). These comprised: HybEZ hybridisation system together with associated consumables; version 2.0 and 2.5 RNAscope® reagents that included pre-treatment solutions, ready to use hybridisation probes for peptidylprolyl isomerase B (PPIB) and Ig kappa, Ig lambda for mRNA demonstration together with Reagent Kit Brown detection kit. BDISH was undertaken on

CONTACT A Warford a.warford@westminster.ac.uk Life sciences, University of Westminster, London, United Kingdom

Table 1. Summary of light chain restriction for mRNA and protein.

Sample type	Fixation time	mRNA restriction only	mRNA and protein restriction	Protein restriction only	No restriction demonstrated
Core biopsy (5)	12–24 h (4) >24 h (1)	1	3	0	1
Resection (5)	24–72 h (5)	1	0	3	1

Numbers in parenthesis refer to sample numbers
Note: due to suboptimal PPIB results only two of the five resection samples were hybridised with the Ig light chain probes for mRNA. All resection samples were subject to ICC staining for kappa and lambda light chain proteins.

dewaxed sections in semi-manual fashion in strict accordance with version instructions. Briefly, the procedure involved pre-treatment of sections to block endogenous peroxidase activity followed by immersion in Target Retrieval solution at 100 °C for between 10 and 20 mins. Sections were then air dried and held at ambient temperature overnight. Unmasking of mRNA was completed using the Protease Plus solution at 40 °C for 15 to 30 mins. Hybridisation and detection were then undertaken using the HybEZ system as specified. Sections were counterstained with haematoxylin, dehydrated, cleared and permanently mounted.

The demonstration of mRNA was undertaken in two stages. In the first, a minimum of two different pre-treatment times were employed using the Target Retrieval and Protease Plus solutions followed by hybridisation with the PPIB probe. This moderately and ubiquitously expressed target was used to determine whether mRNA could be demonstrated and, if so, to determine optimal pre-treatment conditions for subsequent hybridisation with the Ig kappa/lambda probes. Control of the boiling of sections in the Target Retrieval solution required constant monitoring and slight variations could affect results considerably. No standard time in either pre-treatment solution could be determined for the samples as a group and, accordingly, each one was individually optimised. Optimal demonstration of PPIB mRNA presented as several to many individual cytoplasmic 'dots' in cells. In those undertaking extensive mRNA synthesis the staining merged into a dense granular cytoplasmic staining, for example in plasma cells and centroblasts. Optimal PPIB staining was observed in two of the three tonsil samples and in seven of the follicular lymphoma samples (five core biopsy and two resection samples). Only these samples were taken forward for hybridisation with the Ig kappa and lambda probes.

Hybridisation for kappa and lambda mRNA was undertaken using the individually optimised pre-treatment conditions. As with PPIB, granular cytoplasmic staining indicated the presence of the target mRNA. One of the two tonsil cases gave appropriate distribution of plasma cells and centroblasts that exhibited intense uniform staining. In germinal centres many additional cells contained several cytoplasmic 'dots' while in surrounding mantle zones fewer 'dots' per cell were observed. These results were entirely consistent with expected levels of light chain mRNA synthesis. In the other tonsil sample hybridised with the Ig light chain probes a suboptimal result was recorded with only plasma cell and centrocyte/centroblast staining being observed. In the follicular lymphoma samples light chain mRNA restriction was demonstrated in four of five core biopsy samples and in one of the two resection samples hybridised with the Ig kappa and lambda probes. The criteria used for this was alteration of kappa/lambda ratio of staining in the malignant follicles (Figure 1 and Table 1). As expected, non-specific background staining was not present in the BDISH samples.

The results demonstrate that it is possible to demonstrate low copy mRNA for light chain immunoglobulin and restriction using the BDISH method. A caveat is that individual samples required 'pre-screening' using the moderately expressed PPIB probe to establish that they contained sufficient mRNA and to optimise pre-treatment conditions. Thus, of the 13 tonsil and follicular lymphoma samples nine were suitable for hybridisation with the Ig light chain probes and of this subset six gave expected results. Importantly, of the seven follicular lymphoma samples hybridised with the Ig kappa and lambda probes, light chain restriction was demonstrated in five of these. Within this group, light chain restriction was recorded in four of the five core biopsy samples, but only in one of the resection samples.

ICC for protein demonstration was undertaken using the automated Leica Bond III platform according to a standard diagnostic protocol. Dewaxed sections were pre-treated with an antigen retrieval solution at pH 6.0 (ER1, Leica Biosystems, UK) for 30 mins before being incubated at ambient temperature for 15 mins with kappa (A019102-2) or lambda (A019302-2) polyclonal antibodies supplied by Dako Agilent Pathology Solutions, UK. For core biopsy samples the antibodies were pre-diluted in 1/4000 and 1/6000, respectively, for incubation. For resection samples the antibodies were pre-diluted 1/2000 and 1/3000, respectively, for incubation. The Bond Polymer Refine Detection kit (DS9800, Leica Biosystems, UK) was used to demonstrate the reaction of the antibodies providing a peroxidase/diaminobenzidene based end product. Sections were counterstained with haematoxylin, dehydrated, cleared and permanently mounted. Light chain immunoglobulin restriction at protein level was demonstrated in three core biopsy and three resection samples (Figure 1 and Table 1).

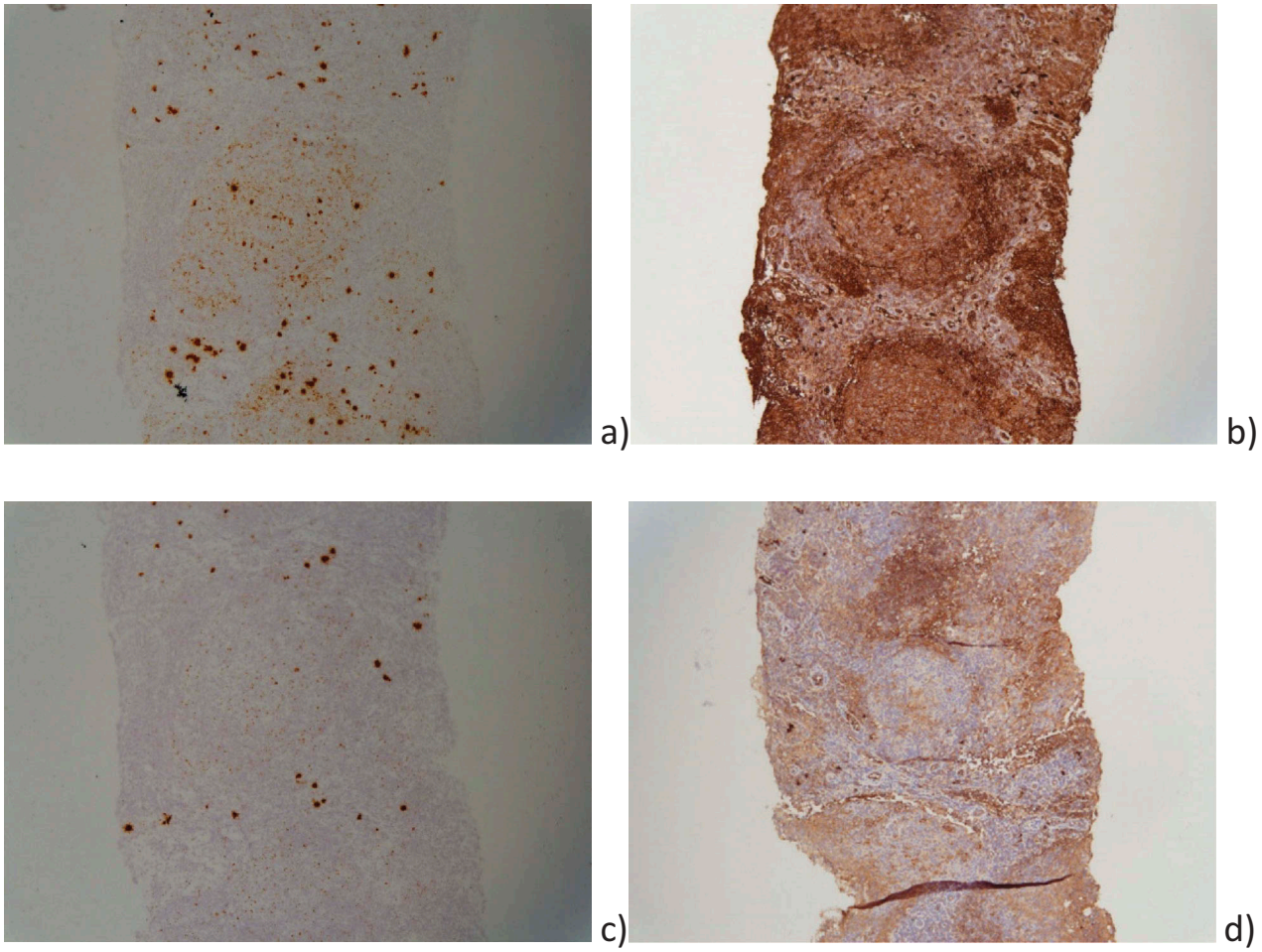

Figure 1. Demonstration of light chain restriction in a core biopsy of follicular lymphoma.

a) Ig kappa mRNA, b) kappa light chain protein, c) Ig lambda mRNA, d) lambda light chain protein (x100 magnification). Note: kappa reaction in follicles for mRNA and protein, but absence of lambda reaction. Non-specific interstitial staining is present in the protein preparations (b and d).

In several samples interstitial staining of tissue was observed (Figure 1(b,d)), while in others, although the intensity light chain staining differed in the malignant follicles between kappa and lambda, no clear restriction was demonstrated. In three core biopsy samples, light chain restriction was demonstrated at mRNA and protein level, but in the resection samples there was no overlap in results

The use of BDISH to demonstrate light chain restriction in FFPE samples of follicular lymphoma has been reported in three studies [8–10]. Information on sample handling and duration of fixation time was not included in these investigations. In these investigations Immunoglobulin Lambda Like Polypeptide 5 (IGLL5) was included as a hybridisation probe as it shares partial homology with Ig lambda light chain sequence and can lead to difficulty in interpretation of light chain restriction. In the samples included in the present study, there was no evidence of this homology causing interpretative difficulties, though in a larger sample set inclusion of this hybridisation probe would need to be considered.

In the study, comparing BDISH with flow cytometry (FC) for identification of light chain restriction Tubbs, et al [8] demonstrated concordance of results in 16/17 of the follicular lymphoma cases studied in a larger cohort set of B-cell lymphomas. In a similar study, Guo, et al [9] demonstrated light chain restriction in 14/15 follicular lymphomas using BDISH compared with 13/15 samples analysed by FC. Arora, et al [10] reported BDISH light chain restriction in 6/6 follicular lymphomas, but were unable to demonstrate this by ICC in matched biopsy tissue. In addition, they investigated the application of BDISH to fine needle aspirate samples and compared results with FC. Seven cases with a final diagnosis of follicular lymphoma were included in this series. Using both techniques, light chain restriction was demonstrated in five cases using either method, but concordance between the methods was demonstrated in only four of these.

The suitability of BDISH for the demonstration of low copy number mRNA is substantiated by the present investigation and emphasises that it can provide complementary diagnostic information to ICC. The differential success in the demonstration of light chain restriction between the methods in core biopsy and resection samples points to the need for further investigations using a larger sample set. Potential explanations for the present results could relate to

the differential effects of cold ischemia, the penetration of NBF into the larger resection samples and/or the longer fixation time used for these samples on the differential preservation of mRNA and protein. NBF, as the most benign of the formalin-based fixatives is recommended for ISH. However, it does progressively degrade nucleic acids [11] and this may also account for the suboptimal results obtained with resection samples. Ideally investigation of a larger sample set would be supplemented by inclusion of model experiments using appropriate cell lines and tissue to investigate the potential effects of these parameters. Once this has been completed and clear parameters established for the successful demonstration of Ig light chain mRNA in FFPE samples then clinical use could be contemplated.

This work represents an advance in biomedical science in demonstrating that low copy mRNA can be demonstrated in FFPE samples using the BDISH method and has the potential to be used alongside ICC for the demonstration of light chain restriction in follicular lymphomas.

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ORCID

A Warford http://orcid.org/0000-0003-3979-4963
G Gerrard http://orcid.org/0000-0002-4614-227X

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