ORIGINAL ARTICLE

Improved clonality assessment in germinal centre/post-germinal centre non-Hodgkin's lymphomas with high rates of somatic hypermutation

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Background: PCR detects clonal rearrangements of the Ig gene in lymphoproliferative disorders. False negativity occurs in germinal centre/post-germinal centre lymphomas (GC/PGCLs) as they display a high rate of somatic hypermutation (SHM), which causes primer mismatching when detecting Ig rearrangements by PCR.

Aims: To investigate the degree of SHM in a group of GC/PGCLs and assess the rate of false negativity when using BIOMED-2 PCR when compared with previously published strategies.

Methods: DNA was isolated from snap-frozen tissue from 49 patients with GC/PGCL (23 diffuse large B cell lymphomas (DLBCLs), 26 follicular lymphomas (FLs)) and PCR-amplified for complete (VDJH), incomplete (DJH) and Igκ/λ rearrangements using the BIOMED-2 protocols, and compared with previously published methods using consensus primers. Germinal centre phenotype was defined by immunohistochemistry based on CD10, Bcl-6 and MUM-1.

Results: Clonality detection by amplifying Ig rearrangements using BIOMED-2 family-specific primers was considerably higher than that found using consensus primers (74% DLBCL and 96% FL vs 69% DLBCL and 73% FL). Addition of BIOMED-2 DJH rearrangements increased detection of clonality by 22% in DLBCL. SHM was present in VDJH rearrangements from all patients with DLBCL (median (range) 5.7% (2.5–13.5)) and FL (median (range) 5.3% (2.3–11.9)) with a clonal rearrangement.

Conclusions: Use of BIOMED-2 primers has significantly reduced the false negative rate associated with GC/PGCL when compared with consensus primers, and the inclusion of DJH rearrangements represents a potential complementary target for clonality assessment, as SHM is thought not to occur in these types of rearrangements.

The detection of clonality using Ig gene rearrangements by PCR in B cell lymphoproliferative disorders aids diagnosis and detection of minimal residual disease, especially when morphology and immunophenotype are equivocal.¹ Evaluating the rearranged Ig by PCR in B cell tumours has several limitations, the most relevant being that of its well-recognised false negativity rate, with up to 30% of cases lacking a clonal result depending on the PCR strategy and the lymphoma subtype.^{2–6} Diss *et al*⁴ have proposed several explanations for the high rates of false negativity, with a major factor being the degree of somatic hypermutation (SHM) that exists in certain B cell malignancies (germinal centre/post-germinal centre malignancies (GC/PGCL)). The presence of somatic mutations in the variable region of the immunoglobulin genes is a marker of germinal centre origin, as pregerminal centre lymphocytes harbour unmutated Ig genes. Malignancies of germinal centre or post-germinal centre B cells, such as follicular lymphomas (FLs) and diffuse large B cell lymphomas (DLBCLs), contain somatically mutated Ig genes.^{7–8} While somatic mutations are introduced into the complementarity-determining regions of the Ig gene to allow affinity maturation of the antibodies to their target antigens, leading to B cell selection, they also occur in the framework (FR) regions, where most consensus and family primers are designed.^{9–11} This can result in suboptimal primer binding, either from a lack of consensus target sequences or from target site alterations.^{3–6} The use of consensus and family primers is generally optimal for a part of the relevant gene segment, but they show a lower homology to other gene segments. The introduction of the BIOMED-2

Concerted Action BMH4-CT98-3936 study has addressed these issues by designing primers for each of the three FR regions of the seven VH families and for the seven diversity region (DH) families, and the Igκ and λ families. Incomplete DJH rearrangements represent a potential complementary target for clonality assessment as SHM does not seem to be very frequent in these rearrangements.¹²

There is only a small number of papers in the literature reporting the use of the BIOMED-2 approach to clonality assessment in GC/PGCL. Therefore, the aim of this study was to investigate the degree of SHM in a group of GC/PGCL and assess the detection rate of false negativity when using BIOMED-2 compared with previously developed consensus PCR strategies.

MATERIALS AND METHODS

Samples were obtained after written informed consent for clinical investigation according to the Declaration of Helsinki.

Case selection

A panel of 49 well-characterised fresh tissue specimens consisting of 23 DLBCL, 26 FL and 15 reactive lymphoid proliferations were included in this study. All samples were from clinically diagnosed cases, and were classified according to the World Health Organization classification of lymphoid

Abbreviations: DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; FR, framework; GC/PGCL, germinal centre/post-germinal centre lymphoma; SHM, somatic hypermutation

Table 1 Complementarity of different multiplex PCR targets for clonality detection in 49 germinal centre/post-germinal centre malignancies

Multiplex PCR tubes	DLBCL (%)	FL (%)
IGH VH-JH FR1	5/23 (22)	14/26 (54)
IGH VH-JH FR2	13/23 (56)	22/26 (84)
IGH VH-JH FR3	12/23 (52)	14/26 (54)
IGH VH-JH (FR 1-3)	17/23 (74)	25/26 (96)
IGH DH-JH	8/23 (35)	6/26 (23)
IGH VH-JH (FR 1-3) +DH-JH	22/23 (96)	26/26 (100)
IGκ	17/23 (74)	24/26 (92)
IGλ	3/23 (13)	4/26 (15)
IGH VH-JH (FR 1-3) +DH-JH+IGκ+IGλ	23/23 (100)	26/26 (100)
t(14;18)	9/23 (39)	17/26 (65)
IGH VH-JH (FR 1-3) +DH-JH+IGκ+t(14;18)	23/23 (100)	26/26 (100)

DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; FR, framework.

neoplasms, based on clinical, histological, immunohistological and molecular genetic criteria.¹³

DNA isolation

High molecular weight genomic DNA from fresh tissue samples was obtained by standard proteinase K digestion, phenol-chloroform extraction and ethanol precipitation.

BIOMED-2 PCR

DNA was PCR-amplified for complete VDJH rearrangements, using three different sets of family-specific primers covering the three FR regions according to the BIOMED-2 multiplex approach to clonality analysis.¹² Amplification of incomplete DJH rearrangements was performed as described previously. Amplification of the IGκ and IGλ locus and t(14;18) was done as described previously.¹² Subsequently, the PCR products were analysed by heteroduplex and GeneScan analysis to determine whether samples were monoclonal or polyclonal.

Ig consensus PCR

PCR amplification of rearrangements at the Ig locus was performed using consensus primers termed Fr2A, Fr2B and Fr3A as described previously.^{11 14}

Sequencing analysis of PCR products

All clonal PCR products were eluted from polyacrylamide gels after heteroduplex analysis and sequenced directly using an automated ABI 3100 capillary sequencer using Big-Dye terminators (Applied Biosystems, Warrington, Cheshire, UK). The sequences were aligned to VH sequences from GenBank and IMGT databases. VH gene sequences containing >2% deviation from the germline sequence were considered as somatically mutated rather than as genomic polymorphisms.

Immunohistochemical analysis

Immunohistochemical analysis was performed on 4 µm thick sections cut from paraffin wax-embedded tissue blocks onto aminopropyltriethoxysilane-coated slides (Sigma, Poole, Dorset, UK), and a Nexes automated immunostainer (Ventana Medical Systems, Strasbourg, France) was used according to the manufacturer's instructions.

Monoclonal antibodies to the following antigens were used: CD20 (Dako, Ely, Cambridgeshire, UK; dilution 1:400), CD3 (LabVision, Fremont, California, USA; dilution 1:50), CD10 (Novocastra, Newcastle-upon-Tyne, UK; dilution 1:5), CD5 (Novocastra; dilution 1:5), cyclin D1 (LabVision; dilution 1:50), bcl-2 (Dako; dilution 1:5), bcl-6 (Novocastra; dilution 1:5), MUM-1 (Dako; dilution 1:50) and MIB-1 (Dako; dilution 1:100). Immunostaining was performed using appropriate positive and negative controls. For negative controls, the primary antibody was omitted and replaced with normal rabbit serum (Dako).

RESULTS

Evaluation of germinal centre origin by immunohistochemical results

The immunohistochemical studies were performed on formaldehyde-fixed, paraffin wax-embedded tissue samples. All neoplastic B cells from FL expressed bcl-2 and bcl-6 proteins

Table 2 Complementarity of PCR targets for clonality detection in 49 germinal centre/post-germinal centre malignancies

Multiplex PCR tubes	DLBCL (%)	FL (%)
Fr2A	14/23 (60)	15/26 (57)
Fr2B	12/23 (52)	13/26 (50)
Fr3A	13/23 (56)	13/26 (50)
Fr2 and Fr3	16/23 (69)	19/26 (73)

DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma.

Figure 1 Examples of PCR-based GeneScan analysis of IGH VH-FR1, VH-FR2, VH-FR3 and IGκ (IGκ and KDE) rearrangements from patients with diffuse large B cell lymphomas and follicular lymphoma. Arrows indicate clonal peaks within the expected size range.

Table 3 VH, DH and JH usage and percentage of mutations in VDJH and DJH rearrangements in 49 lymphomas

Patient	Disorder	V _H	% SHM	D _H	J _H	D _H	% SHM	J _H
1	FL	V _H 3-74	4.5	D _H 1-26	J _H 5	D _H 1-26	0	J _H 4
2	FL	V _H 3-48	4.2	D _H 2-8	J _H 6			
3	FL	V _H 2-2	7.5	D _H 2-15	J _H 5			
4	FL	V _H 3-30	11.8	D _H 3-3	J _H 3			
5	FL	—	—	—	—	D _H 2-8	0	J _H 4
6	FL	V _H 3-53	8.7	D _H 5-12	J _H 3			
7	FL	V _H 4-39	4.5	D _H 3-9	J _H 4	D _H 3-10	0	J _H 6
8	FL	V _H 1-2	2.6	D _H 3-3	J _H 1			
9	FL	V _H 3-11	8.2	D _H 3-22	J _H 3	D _H 1-07	0	J _H 5
10	FL	V _H 3-23	11.5	D _H 3-22	J _H 1			
11	FL	V _H 5-51	2.6	D _H 2-2	J _H 6	D _H 5-12	0	J _H 4
12	FL	V _H 1-3	3.1	D _H 6-19	J _H 4			
13	FL	V _H 1-18	2.3	—	J _H 4			
14	FL	V _H 1-18	5.3	D _H 3-3	J _H 2			
15	FL	V _H 4-34	4.7	D _H 3-3	J _H 4	D _H 4-23	0	J _H 4
16	FL	V _H 1-18	9.6	D _H 2-2	J _H 5			
17	FL	V _H 1-18	2.4	D _H 1-26	J _H 4			
18	FL	V _H 3-38	2.8	D _H 5-12	J _H 6			
19	FL	V _H 3-48	7.4	D _H 3-9	J _H 6	D _H 4-23	0	J _H 4
20	FL	V _H 3-48	10.1	D _H 3-9	J _H 5			
21	FL	V _H 4-39	8.2	—	J _H 4			
22	FL	V _H 1-69	4.3	D _H 2-2	J _H 6			
23	FL	V _H 1-18	5	D _H 5-12	J _H 4	D _H 1-26	0	J _H 4
24	FL	V _H 3-53	11.9	D _H 2-15	J _H 6			
25	FL	V _H 1-2	6.7	D _H 2-21	J _H 4			
26	FL	V _H 3-21	7.9	D _H 3-9	J _H 5			
27	DLBCL	V _H 4-4	4.7	—	J _H 1	D _H 3-10	0	J _H 6
28	DLBCL	V _H 3-11	9.3	D _H 2-02	J _H 6			
29	DLBCL	V _H 4-61	11.1	D _H 1-20	J _H 3			
30	DLBCL	V _H 3-30	9.2	D _H 3-10	J _H 1			
31	DLBCL	—	—	—	—	D _H 3-10	0	J _H 4
32	DLBCL	—	—	—	—			
33	DLBCL	V _H 1-2	3.6	D _H 1-26	J _H 6	D _H 3-23	1	J _H 5
34	DLBCL	V _H 5-51	2.6	D _H 6-6	J _H 3			
35	DLBCL	V _H 4-34	13.5	D _H 4-17	J _H 3	D _H 3-22	0	J _H 4
36	DLBCL	V _H 3-48	9.4	D _H 3-16	J _H 3			
37	DLBCL	—	—	—	—			
38	DLBCL	V _H 1-69	3.8	D _H 1-26	J _H 6			
39	DLBCL	V _H 4-39	8	—	J _H 5	D _H 3-22	0	J _H 4
40	DLBCL	—	—	—	—			
41	DLBCL	V _H 3-21	2.5	D _H 2-15	J _H 2			
42	DLBCL	V _H 1-18	8.5	D _H 2-2	J _H 3			
43	DLBCL	V _H 1-8	2.6	D _H 2-21	J _H 3	D _H 2-21	0	J _H 6
44	DLBCL	V _H 2-26	9.2	D _H 5-18	J _H 4			
45	DLBCL	V _H 6-1	5.7	D _H 1-26	J _H 2			
46	DLBCL	—	—	—	—			
47	DLBCL	—	—	—	—	D _H 2-15	0.5	J _H 6
48	DLBCL	V _H 1-2	3.2	D _H 3-3	—			
49	DLBCL	V _H 2-26	4.5	D _H 3-22	J _H 4			

DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; SHM, somatic hypermutation.
A dash indicates that no clonal allele was detected.

in follicular and diffuse components, in keeping with the germinal centre origin of tumour. Neoplastic B cells from DLBCL were classified as either GC or PGCL, on the basis of expression of CD10, bcl-6 and MUM-1.¹⁵ In all, 13 cases were classified as GC lymphomas and 6 were classified as PGCL, with germinal centre status not available in 4 cases.

Analysis of BIOMED-2 Ig rearrangements

All reactive lymphoid proliferations failed to produce a clonal Ig rearrangement. Among 49 patients, a monoclonal Ig rearrangement was detected in 100% of patients with DLBCL and among 100% of patients with FL (table 1; fig 1).

Analysis of consensus Ig rearrangements

All reactive lymphoid proliferations failed to produce a clonal Ig rearrangement.

Among 49 patients, a monoclonal Ig rearrangement was detected in 69% of patients with DLBCL and in 73% of patients with FL when using Ig consensus PCR (table 2).

Sequence data of BIOMED-2 PCR for determination of VH, DH and JH gene usage

Table 3 shows the VH, DH and JH gene segment usage, and the rate of SHM for each patient with DLBCL and FL.

The VH genes used by the DLBCL cases were derived from 6 of the 7 human VH gene families in the following distribution: VH1, 29%; VH2, 11%; VH3, 24%; VH4, 24%; VH5, 6%; and VH6, 6%. The VH genes used by the FL cases were derived from 5 of the 7 human VH gene families in the following distribution: VH1, 36%; VH2, 4%; VH3, 44%; VH4, 12%; and VH5, 4%.

The most frequently encountered genes in FL were VH1-18 (n = 5, 20%) and VH3-48 (n = 3, 12%). The most frequently encountered gene in DLBCL was VH1-2 (n = 2, 12%).

Somatic hypermutation rates in VDJH rearrangements

SHM was present in VDJH rearrangements from all patients with DLBCL (median (range) 5.7% (2.5–13.5)) and FL (median (range) 5.3% (2.3–11.9)), in which a clonal allele was detected

(table 3). None of the incomplete DJH rearrangements presented with >2% deviation from the germline sequence.

DISCUSSION

Historically, the evaluation of monoclonal Ig gene rearrangements in GC/PGCL by PCR exhibits a high degree of false negativity, with rates reported up to 30% depending on the PCR strategy used and lymphoma subtype studied. Diss *et al*⁴ have proposed several explanations for the false negativity associated with PCR. Firstly, DNA may not be amplified owing to partial rearrangements or chromosomal translocations, as up to 30% of DLBCL and the vast majority of FL contain the translocation t(14;18), which can render one allele unsuitable for VDJ amplification.¹⁶ Also, the presence of increased numbers of diluting polyclonal B cells in FL can compete for consensus primers and hamper clonality assessment. However, the major factor is thought to be the degree of SHM that exists in these mature malignancies. Recently, an extensive primer set has been developed by a European collaborative study (BIOMED-2), the goal of which was to optimise sensitivity and specificity when standardising molecular investigations of lymphoma. Our study assessed the role of the BIOMED-2 primers in detecting clonality in a group of GC/PGCL and compared this with previously published methods using consensus primers.

This paper has demonstrated the efficacy of BIOMED-2 protocols in detecting a monoclonal rearrangement in 100% of patients with DLBCL and 100% of patients with FL when utilising all BIOMED-2 targets (table 1). When analysing only the BIOMED-2 IGH (FR1,2,3), clonal rearrangements were detected in 74% of DLBCL and 96% of FL cases (table 1). For comparison, the same samples were analysed by the method described by Ramasamy *et al*,¹¹ and the clonal rearrangements were detected in 69% of DLBCL and 73% of FL cases (table 2). This represents a considerable difference in detection rates in the FL group and a small difference in the DLBCL group. This is most likely due to the degree of SHM, which exists in these mature malignancies, which alters the sequence of the region amplified by the consensus primer so that hybridisation is suboptimal.⁵ This is circumvented in the BIOMED-2 group as the primers are designed to each individual VH family.

The 100% sensitivity in both DLBCL and FL was achieved by the use of seven multiplex PCR combinations, although certain targets were not very informative. Analysis of IG λ failed to produce a significant number of rearrangements. Also, the VH-FR1 combination failed to detect a significant number of rearrangements in DLBCL but was more successful in FL samples.

However, we did detect a high rate of clonal Ig rearrangements when using only three BIOMED-2 multiplex combinations (FR2, FR3 and IG κ). This detected clonal rearrangements in 21/23 DLBCL and 26/26 FL cases (table 1). The use of IG κ gene rearrangements as an additional marker of B cell clonality has been demonstrated previously^{17,18} and, in this study, was particularly useful in the group of DLBCL, as this increased the detection rate from 74% (17/23) to 91% (21/23). Interestingly, the six DLBCLs that produced polyclonal results by VH (FR1, FR2 and FR3) PCR were all post-germinal centre lymphomas as assessed by immunohistochemistry. SHM was present in VDJH rearrangements from all the patients with DLBCL (median (range) 5.7% (2.5–13.5)) and FL (median (range) 5.3% (2.3–11.9)) in which a clonal allele was detected. This is also evident as the inclusion of incomplete DJH rearrangements increased the detection rate from 74% (17/23) to 96% (22/23) in DLBCL and from 96% (25/26) to 100% (26/26) in FL. While the occurrence of incomplete DJH rearrangements is well recognised in immature malignancies,¹⁹ reports also exist for the existence of DJH rearrangements in mature malignancies.^{20,21} DJH rearrangements therefore represent an important

Take-home messages

- Detection of clonal Ig rearrangements has been achieved in 100% of germinal centre/post-germinal centre lymphomas.
- Germinal centre lymphomas exhibit a high degree of somatic hypermutation.
- Analysis of both Ig and IG κ rearrangements is necessary to achieve a high degree of sensitivity.

complementary target for clonality analysis as it is proposed that incomplete rearrangements in the Ig locus will not contain SHM. As we have demonstrated in GC/PGCL, where SHM are frequent, the DJH rearrangements represent a suitable target to detect a B cell clone.

Our data confirm that the BIOMED-2 approach to clonality analysis has a higher detection rate than that of the previous method using consensus-based primers in a group of mature B cell malignancies. Our data suggest that, for the efficient detection of clonal Ig gene rearrangements in GC/PGCL, samples should first be subjected to the analysis of VH-FR3 and VH-FR2. All negative cases should then be characterised using IG κ or DH depending on the sample type. IG κ PCR is particularly suited to DNA generated from paraffin wax-embedded tissue, which is highly degraded, whereas incomplete DJH rearrangements have not been assessed from archival tissues.

In conclusion, BIOMED-2 primers for the detection of clonal Ig gene rearrangements in GC/PGCL achieved 100% sensitivity in this study. To achieve this level of sensitivity, analysis of both Ig and IG κ rearrangements must take place, with the inclusion of DJH rearrangements representing a complementary target for clonality assessment.

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