bjh research paper

BIOMED-2 PCR assays for *IGK* gene rearrangements are essential for B-cell clonality analysis in follicular lymphoma

Karen Payne,[1] Penny Wright,[1] John W. Grant,[1] Yuanxue Huang,[2] Rifat Hamoudi,[2] Chris M. Bacon,[3] Ming-Qing Du[2] and Hongxiang Liu[1,4]

[1]Department of Histopathology, Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust, Cambridge, [2]Department of Pathology, University of Cambridge, Cambridge, [3]Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne, and [4]Molecular Malignancy Laboratory, Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK

Received 17 March 2011; accepted for publication 8 June 2011

Correspondence: Dr Hongxiang Liu, Molecular Malignancy Laboratory and Department of Histopathology, Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust, Level 3 Lab Block, Box 231, Hills Road, Cambridge CB2 0QQ, UK.
E-mail: hl291@cam.ac.uk

Summary

B-cell clonality analysis is commonly performed by polymerase chain reaction (PCR) targeting the *IGH* genes although a high false-negative rate is recognized for germinal centre/post-germinal centre B-cell malignancies, especially follicular lymphoma. We assessed the diagnostic value of BIOMED-2 *IGK* assays and investigated the cause of *IGH* PCR failure in 77 patients with follicular lymphoma. Using the full set of BIOMED-2 reactions, clonal immunoglobulin gene rearrangements were detected in 74 (96%) cases. The clonality detection rate was 86% by two *IGK* reactions but only 68% by five *IGH* reactions ($P < 0{\cdot}001$). Sequencing of the clonal PCR products showed significantly fewer somatic mutations in the rearranged *IGKV* (9/27 cases, 33%, mean mutation rate 0·5%) than *IGHV* (17/17 cases, 100%, rate 11·0%) ($P < 0{\cdot}01$). All *IGHV-IGHJ* PCR failures occurred in cases with at least one mutation at the corresponding *IGHV* primer binding sites. t(14:18)(q32:q21)/*IGH-BCL2* was detected in 50 of 71 (70%) cases and the presence of the translocation was not associated with the poor performance of *IGH* assays. Our results showed that BIOMED-2 *IGK* assays are significantly more sensitive than *IGH* assays in follicular lymphoma due to the fact that the rearranged *IGKV* is less frequently targeted by somatic hypermutation than *IGHV*, and therefore, are essential in routine clonality analysis of these lymphomas.

Keywords: clonality analysis, BIOMED-2 PCR assays, *IGH*, *IGK*, follicular lymphoma.

The immunoglobulin genes, which contain many different variable (V), diversity (D) and joining (J) gene segments, rearrange sequentially during B-cell development, with rearrangement of *IGH* first, followed by *IGK*. If the rearranged *IGK* genes are not functional and IgH/Igκ is not expressed, *IGKJ-IGKC* or *IGKC* gene segments are deleted via recombination of *IGKV* segments or the *IGKJ-IGKC* intron with the downstream *IGK* deletion element (K_{de}), and *IGL* rearrangement proceeds, resulting in potential expression of IgH/Igλ (Alt *et al*, 1986; Langerak & van Dongen, 2006). This process implies that the *IGK* loci are rearranged in essentially all differentiated B-cells. Using Southern blotting (Arnold *et al*, 1983; Cleary *et al*, 1984; Williams *et al*, 1987; Spencer *et al*, 1989) and polymerase chain reactions (PCR) (Bourguin *et al*, 1990; McCarthy *et al*, 1990; Trainor *et al*, 1990; Kuppers *et al*, 1993), it has been shown that both the rearranged *IGH* and *IGK* are highly sensitive and complementary markers in the detection of monoclonal B-cell proliferations. However, in routine practice *IGH* is more commonly used than *IGK* for PCR-based B-cell clonality analysis, despite being known to give a high false negative rate in germinal/post-germinal centre B-cell malignancies (Gong *et al*, 1999; Diss *et al*, 2002; Pai *et al*, 2005; Amara *et al*, 2006).

To improve clonality analysis, the European BIOMED-2 study consortium optimised and standardized the PCR-based analysis of rearranged immunoglobulin and T-cell receptor genes (van Dongen *et al*, 2003). One of the most significant advances made in this study was the targeting of multiple complementary gene loci. For example, B-cell clonality assays include analysis of complete *IGH* V-D-J and incomplete *IGH* D-J rearrangements, *IGK* V-J and K_{de}-involved rearrangements, and *IGL* V-J rearrangements. Based on frozen tissues, the sensitivity of the *IGK* assays was equivalent to that of the

First published online 26 July 2011
doi:10.1111/j.1365-2141.2011.08803.x

IGH assays irrespective of lymphoma subtype and combined analysis of both loci produced an unprecedented efficacy (van Dongen *et al*, 2003; Evans *et al*, 2007). By applying these assays to a large series of routine diagnostic specimens, we have also demonstrated their high sensitivity and specificity in paraffin-embedded specimens (Liu *et al*, 2007). Nevertheless, achievement of a high clonality detection rate in germinal centre/post-germinal centre B-cell lymphomas, particularly follicular lymphoma, required testing both the *IGH* and *IGK* rearrangements. Among the 32 follicular lymphoma cases examined, clonality was detected in 30 cases (94%) by the full set of B-cell clonality assays. However, the clonality detection rate was only 57% by all five *IGH* V-J and D-J reactions but 80% by two reactions for *IGK* V-J and K_{de}-involved rearrangements.

Follicular lymphoma is the second most frequent non-Hodgkin lymphoma and clonality analysis may be required to assist in its diagnosis. However, the best strategy for application of the BIOMED-2 assays to routine clonality analysis in follicular lymphoma remains to be established. It is also unknown whether the clonality detection rate by various BIOMED-2 assays is affected by histological grade of the lymphoma. Furthermore, the cause for the high false negative rate of *IGH* PCR in follicular lymphoma is unclear. Although somatic hypermutation of the rearranged *IGH* genes and involvement of the *IGH* in translocations have been thought to account for the failure of *IGH* PCR, this has never been directly investigated (Kosmas *et al*, 1998; van Dongen *et al*, 2003; Kuppers, 2003; Bagg, 2008). To address these issues, we assessed the BIOMED-2 PCR assays in a large cohort of follicular lymphomas and analysed somatic hypermutations of the *IGHV* and *IGKV* regions and the presence of t(14:18)(q32:q21)/*IGH-BCL2*. We then investigated the association of the mutations and translocation with the corresponding PCR failures.

Materials and methods

Patients and specimens

Formalin-fixed and paraffin-embedded specimens from 77 patients diagnosed with follicular lymphoma at the Department of Histopathology, Addenbrooke's Hospital between 2003 and 2009 were studied. The patients consisted of 35 males (median age 65 years, range 33–89 years) and 42 females (median age 63 years, range 26–94 years) ($P > 0{\cdot}05$). The specimens were mostly from lymph nodes (57, 74%) and the remaining samples were from skin (6%, 8%), gastrointestinal tract (4%, 5%), breast (3%, 4%) and one each from the spleen, pancreas, omentum, pleura, salivary gland, ocular adnexa and bone. Follicular lymphoma was diagnosed by specialist Haematopathologists based on the World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues (Jaffe *et al*, 2001; Swerdlow *et al*, 2008). Three cases originating from the skin were diagnosed as primary cutaneous follicle centre lymphoma. In 56 cases the tumours could be graded and were classified as grade 1–2 in 45 cases (80%) and grade 3 in 11 cases (20%). Twenty-seven cases had been analysed for clonality in a previous study (Liu *et al*, 2007). The use of these pathological specimens for research was approved by the local research ethics committee.

B-cell clonality analysis

DNA was prepared from paraffin-embedded specimens by proteinase K digestion as described previously (Liu *et al*, 2007). The quality of DNA samples was assessed using the BIOMED-2 specimen control reaction and only samples yielding control gene PCR products ≥300 bp were included in the study (van Dongen *et al*, 2003). B-cell clonality was determined in duplicate with the full set of BIOMED-2 assays (InVivoScribe Technologies, San Diego, CA, USA), which include five reactions targeting *IGH* [IGH_A: FR1 (variable region framework 1)-J; IGH_B: FR2-J; IGH_C: FR3-J; IGH_D: D_{1-6}-J; IGH_E: D_7-J], two reactions targeting *IGK* (IGK_A: V-J; IGK_B: V-K_{de} and JC intron-K_{de}) and one reaction for *IGL* (V-J) (van Dongen *et al*, 2003). The PCR products were heteroduplex-treated followed by electrophoresis on polyacrylamide gels (Liu *et al*, 2007).

Analysis of somatic hypermutation of the clonal rearranged immunoglobulin genes

Clonal PCR products of *IGH* V-J and *IGK* V-J reactions were sequenced to compare the level of somatic mutations of the rearranged *IGHV* and *IGKV* regions. For mutation analysis of the *IGHV* region, clonal products of the IGH_A reaction were directly sequenced. Where the IGH_A failed to give a clonal product, a multiplex PCR using family-specific leader part primers (*IGHV* Leader) and J primers (Leader-J) was performed and the clonal PCR products were sequenced (Campbell *et al*, 1992). For cases in which both IGH_A and *IGHV* Leader-J failed to give a clonal product but IGH_B was clonal, the IGH_B products were sequenced.

The IGK_A reaction contains six family specific *IGKV* primers, which are positioned at different *IGKV* framework regions, resulting in different sizes of the PCR products (van Dongen *et al*, 2003). The *IGKV2f*, *IGKV4* and *IGKV5* primers are positioned at the FR1 region and clonal products amplified with these primers are directly sequenced. The *IGKV3f* primer is positioned at the FR2 region and *IGKV1f/6* and *IGKV7* primers are positioned at the FR3 region such that the resulting PCR products contain very limited *IGKV* sequences and are inadequate for mutation analysis. To overcome this problem, new family-specific primers were designed at the FR1 region of *IGKV1f*, *IGKV3f*, *IGKV6* and *IGKV7* families (Table S1). All cases that were clonal by BIOMED-2 *IGK V3f*, *V1f/6* or *V7* primers were re-amplified using the newly designed primers and the resulting PCR products were directly sequenced.

The clonal PCR products were extracted from the polyacrylamide gels using the QIAquick® Gel Extraction kit (Qiagen, West Sussex, UK) and sequenced using first the consensus *IGHJ* or *IGKJ* primers and then family specific *IGHV* or *IGKV* primers on an ABI 377 Automated DNA Sequencer (Applied Biosystems Inc., Foster City, CA, USA). The sequences were verified and aligned to the germline sequences of the *IGHV* or *IGKV* families and somatic mutation was analysed using the International ImMunoGeneTics (IMGT) and GenBank databases.

Analysis of t(14:18)(q32:q21)/IGH-BCL2

t(14:18)(q32:q21)/*IGH-BCL2* was examined by BIOMED-2 PCR assays (van Dongen *et al*, 2003) and/or fluorescence *in situ* hybridization (FISH) in all of the cases studied. With the exception of six cases where there was insufficient tissue, all PCR-negative cases were examined by FISH as the PCR assays are known to miss approximately 40% of translocations in paraffin-embedded materials (Evans *et al*, 2007). FISH was performed using the Vysis LSI *IGH* and *BCL2* dual colour break-apart probes and *IGH-BCL2* dual colour dual fusion translocation probes (Abbott Molecular, Maidenhead Berkshire, UK) (Ye *et al*, 2003; Bacon *et al*, 2009).

Statistical analysis

Where applicable, Fisher's exact test was performed to compare categorical variables and the one-way analysis of variance (ANOVA) test was used for analysis of quantitative variables. Differences were considered significant when the resulting P value was $< 0{\cdot}05$.

Results

Detection of clonal immunoglobulin gene rearrangements

Using the full set of BIOMED-2 reactions for B-cell clonality analysis, clonal immunoglobulin gene rearrangements were detected in 74 of 77 (96%) cases studied. Three cases (one cutaneous and two nodal) did not show a clonal immunoglobulin gene rearrangement by any BIOMED-2 reactions although all were positive for *IGH-BCL2* translocation.

Among the 74 cases showing clonal immunoglobulin gene rearrangements, clonality was detected by IGK_A and IGK_B in 65% and 62% of cases, respectively, which is much higher than by any of the *IGH* and *IGL* reactions ($P < 0{\cdot}003$, Fig 1 and Table S2). The two *IGK* reactions combined achieved a detection rate of 86%, whereas three *IGH* V-J reactions (IGH_{A+B+C}) and all five *IGH* reactions ($IGH_{A+B+C+D+E}$) achieved a detection rate of only 54% and 68%, respectively (all $P < 0{\cdot}01$). Combined use of *IGK* and *IGH* reactions significantly improved the detection rate, with addition of IGH_B, or both IGH_B and IGH_D to the two *IGK* reactions yielding a detection rate of 93% or 99%, respectively. Maximum sensitivity required combined use of all reactions, although the contribution of IGH_E and *IGL* reactions was minimal.

The effect of DNA quality on clonality detection was examined. IGH_A, IGH_B, IGK_B and *IGL* showed a lower rate of clonality detection in cases with control gene PCR products of 300 bp in size ($n = 21$) than in cases with control gene PCR products ≥400 bp ($n = 53$) although none of the differences was statistically significant (all $P > 0{\cdot}18$, Table S2). Overall, *IGH* reactions performed worse in the group with lower DNA quality (43% for three *IGH* V-J reactions and 53% for all five *IGH*

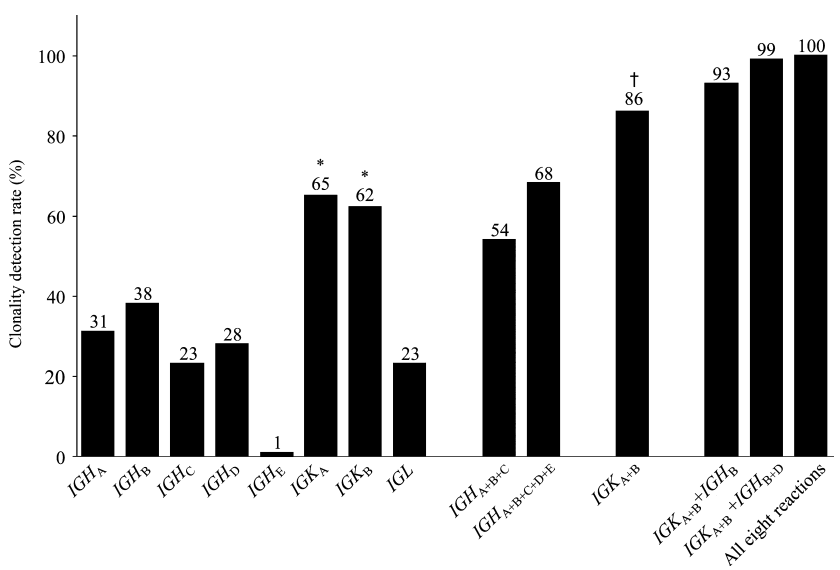


Fig 1. Rate of detection of clonal immunoglobulin gene rearrangements by individual and combined BIOMED-2 reactions in follicular lymphoma. IGH_A: *IGH* FR1-J; IGH_B: *IGH* FR2-J; IGH_C: *IGH* FR3-J; IGH_D: *IGH* D_{1-6}-J; IGH_E: *IGH* D_7-J; IGK_A: *IGK* V-J; IGK_B: *IGK* V-K_{de} and JC intron-K_{de}; *IGL*: *IGL* V-J. *$P < 0{\cdot}003$, IGK_A or IGK_B *versus* any other single reaction; †$P < 0{\cdot}001$, two *IGK* reactions *versus* three *IGH* V-J or all five *IGH* reactions.

reactions) than in the group with higher DNA quality (59% and 74%, respectively, all $P > 0{\cdot}1$). In contrast, the combined *IGK* reactions performed significantly better than the combined *IGH* reactions in both lower (95%) and higher (83%) DNA quality groups (all $P < 0{\cdot}01$, IGK_{A+B} *versus* IGH_{A+B+C}).

Detection of clonality was correlated with the histological grade of the follicular lymphomas (Table S2). Whilst the detection rate of *IGH* D-J, *IGK* and *IGL* reactions was not affected by the tumour grades (all $P > 0{\cdot}05$), each of the three *IGH* V-J reactions performed worse in grade 3 than in grade 1–2 tumours and combined use of these three reactions showed a much lower detection rate in the former (18%) than in the latter group (65%) ($P = 0{\cdot}007$).

Mutation of clonal rearranged IGHV and IGKV regions

Clonal *IGH* V-J and *IGK* V-J products were successfully sequenced in 17 and 27 cases, respectively, including two cases amplified using *IGHV* Leader-J primers and five cases amplified using in-house *IGKV* primers (Table I). Excluding primer sites, an average of 205 bp *IGHV* segment and 144 bp *IGKV* segment were analysed for somatic mutations. The *IGHV* region was mutated in each of the 17 cases analysed and the mean mutation rate was 11·0% (median 10·4%, range 3·8–20·3%). The *IGKV* gene was mutated in nine of 27 (33%) cases analysed and the mean mutation rate was 0·5% (median 0%, range 0–3·2%), markedly lower than that of the *IGHV* gene ($P < 0{\cdot}001$). Eleven cases had both the *IGHV* and *IGKV* sequenced and the mean mutation rate was respectively 10·6% and 0·3% ($P < 0{\cdot}001$). The mutation frequency of the *IGHV* and *IGKV* was not associated with their family gene usage. The association with grades of the tumour could not be assessed as only one grade 3 tumour was successfully sequenced for the *IGHV* gene (Case 13, mutation rate 7·3%).

Distribution of mutation on the *IGHV* genes was also analysed. There was no apparent difference in the incidence of mutations among the FR1 (12·9%, $n = 11$), CDR1 (13·5%, $n = 13$), FR2 (9·2%, $n = 15$), CDR2 (12·7%, $n = 16$) and FR3 (9·9%, $n = 17$) regions of the *IGHV* genes ($P = 0{\cdot}32$). The numbers of mutations at the primer binding sites are detailed in Table I. Remarkably, mutations were found frequently at the FR1 (one of three cases), FR2 (11 of 15 cases) and FR3 (16 of 17 cases) primer sites. The number of mutations at the *IGHV* primer sites ranged from zero to 6 and was significantly associated with failure of the corresponding PCR ($P = 0{\cdot}03$) (Table I and Fig 2). Among the 12 PCRs with primer binding sites having ≥3 mutations, 11 (92%) failed ($P < 0{\cdot}01$); of the 16 PCRs with primer binding sites carrying 1 or 2 mutations, 3 (19%) failed; and finally, none of the six PCRs without mutation at the primer binding site showed a corresponding PCR failure.

Detection of t(14:18)(q32:q21)/IGH-BCL2

IGH-BCL2 translocation was detected in 50 of 71 (70%) cases. All translocation-negative cases were clonal for immunoglobulin gene rearrangements and two of them showed a gain of an extra copy of the *BCL2* locus. The frequency of the translocation was not associated with the site (nodal: 37/52, 71%; extranodal: 13/19, 68%) or grade (grade 1–2: 33/44, 70%; grade 3: 8/11, 73%) of the tumours (all $P > 0{\cdot}05$). The translocation was also not associated with the overall rate of clonality detection by *IGH* or *IGK* PCR, nor with the mutation frequency of the *IGHV* region (all $P > 0{\cdot}05$). However, the detection rate of *IGH* D-J PCR was higher in *IGH-BCL2* negative cases (62%) than in *IGH-BCL2* positive cases (17%) ($P = 0{\cdot}001$, Table S2).

Discussion

The present study confirmed our previous finding from a small series of cases that the BIOMED-2 *IGK* assays are far more sensitive than *IGH* assays for clonality detection in paraffin-embedded specimens of follicular lymphoma (Liu *et al*, 2007). Among the 74 cases showing clonal immunoglobulin gene rearrangements, the clonality detection rate was more than 60% by each of the two *IGK* reactions and was 86% by combined use of both reactions, and these rates did not differ between the grade 1–2 and grade 3 follicular lymphomas. In contrast, the clonality detection rate was only 54% by three *IGH* V-J reactions and 68% by all five *IGH* V-J and D-J reactions, and these rates were even lower in grade 3 lymphomas (18% and 36%, respectively). Therefore, exclusion of *IGK* assays would have resulted in a failure to detect clonality in one-third of all cases and in the majority of grade 3 follicular lymphomas studied.

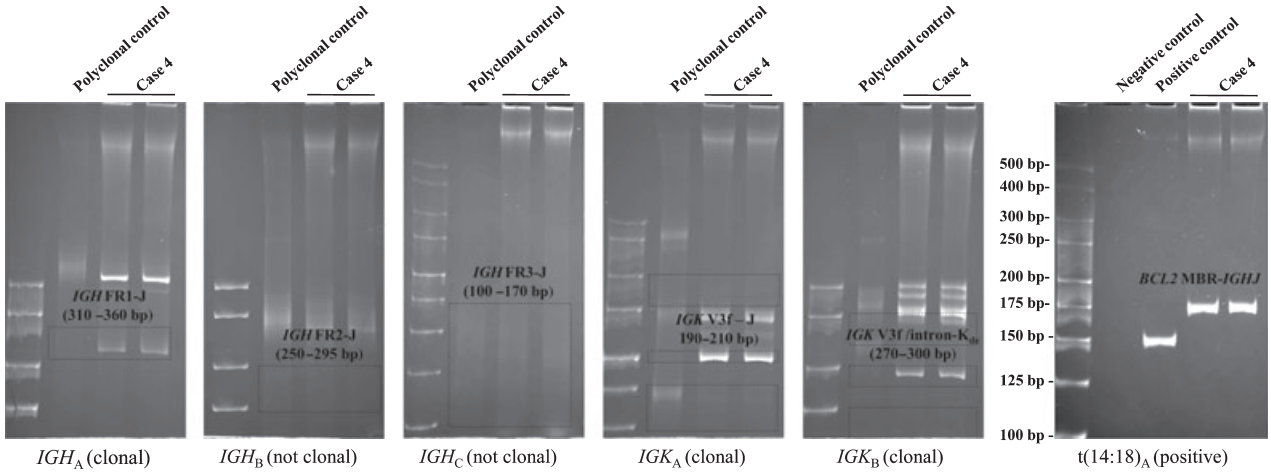
Our results are consistent with those reported by others on paraffin-embedded follicular lymphoma specimens (Amara *et al*, 2006; Halldorsdottir *et al*, 2007; Berget *et al*, 2011). In particular, Halldorsdottir *et al* (2007) analysed 40 paraffin-embedded follicular lymphoma specimens from 19 patients using BIOMED-2 assays and showed a clonality detection rate of 35% by three *IGH* V-J reactions and 93% by two *IGK* reactions. The superior performance of *IGK* PCR in B-cell clonality analysis has also been demonstrated using non-BIOMED-2 assays (Gong *et al*, 1999; Diss *et al*, 2002; Pai *et al*, 2005; Amara *et al*, 2006). However, based on frozen materials, the BIOMED-2 group showed a similar sensitivity between the *IGH* (86%) and *IGK* (84%) assays in follicular lymphomas (van Dongen *et al*, 2003; Sandberg *et al*, 2005; McClure *et al*, 2006; Catherwood *et al*, 2007; Evans *et al*, 2007). Paraffin-embedded tissues are less optimal than fresh/frozen tissues for clonality analysis and the suboptimal quality of DNA might adversely affect the sensitivity of *IGH* assays. Although all the cases included in this study had control gene PCR products of at least 300 bp, which is thought to be suitable for reliable clonality analysis using BIOMED-2 assays, (van Dongen *et al*, 2003) the reactions with larger amplicon size (IGH_A: 310–360 bp, IGH_B: 250–295 bp, IGK_B: 210–390 bp) tended to perform worse in the group with lower DNA quality (control gene PCR products = 300 bp) than that with higher DNA

Table I. Mutation of the clonal rearranged *IGHV* and *IGKV* gene fragments in follicular lymphoma.

	IGHV gene fragment								*IGKV* gene fragment			
			Base pairs of nucleotide†		No. of mutation at primer site (−: PCR negative)						Base pairs of nucleotide†	
Case	PCR reaction used for sequencing	*IGHV* gene usage	Sequenced	Mutated (%)	FR1	FR2	FR3	PCR reaction used for sequencing	*IGKV* gene usage	Sequenced	Mutated (%)
1	*IGHV* Leader-J	*IGHV3-30-3**01	288	30 (10·4)	0	1	6 (−)	IGK_A	*IGKV2-28**01	168	0 (0)
2	IGH_A	*IGHV1-2**04	219	32 (14·6)		4	4 (−)	IGK_A	*IGKV1-33**01	62	0 (0)
3	IGH_A	*IGHV3-7**02	223	22 (9·9)		0	1	IGK_A	*IGKV2-30**01	200	0 (0)
4	IGH_A	*IGHV1-2**02	219	26 (11·9)		1 (−)	4 (−)	*IGK* V3-J‡	*IGKV3D-15**01	195	2 (0)
5	IGH_A	*IGHV4-34**01	220	15 (6·8)		2	2	IGK_A	*IGKV1D-43**01	62	0 (0)
6	IGH_A	*IGHV3-23**01	223	22 (9·9)		3 (−)	2	*IGK* V1-J‡	*IGKV1-33**01	159	0 (0)
7	IGH_A	*IGHV1-3**01	219	9 (4·1)		1	1	IGK_A	*IGKV1-39**02	62	0 (0)
8	IGH_A	*IGHV4-59**01	219	34 (15·5)		2	4 (−)	IGK_A	*IGKV1-39**02	62	1 (1·6)
9	IGH_A	*IGHV2-5**01	215	30 (14·0)		0	2	*IGK* V3-J‡	*IGKV3D-20**01	198	0 (0)
10	IGH_A	*IGHV3-15**02	203	21 (10·3)		1	5 (−)	IGK_A	*IGKV5-2**01	200	1 (0·5)
11	IGH_A	*IGHV3-53**02	185	17 (9·2)		0	4 (−)	IGK_A	*IGKV4-1**01	193	0 (0)
12	IGH_A	*IGHV4-34**01	220	30 (13·6)		3 (−)	2 (−)				
13	IGH_A	*IGHV1-2**02	219	16 (7·3)		2	2				
14	*IGHV* Leader-J	*IGHV3-15**01	295	35 (11·9)	2 (−)	0	3 (−)				
15	IGH_B	*IGHV3-23**04	130	5 (3·8)			1				
16	IGH_A	*IGHV3-21**01	74	15 (20·3)		4 (−)	0				
17	IGH_B	*IGHV3-48**02	114	16 (14·0)			6 (−)				
18								*IGK* V3-J‡	*IGKV3-11**01	169	1 (0)
19								*IGK* V3-J‡	*IGKV3-20**01	198	0 (0)
20								IGK_A	*IGKV2-30**01	127	0 (0)
21								IGK_A	*IGKV1-39**01	62	2 (3·2)
22								IGK_A	*IGKV2-28**01	62	0 (0)
23								IGK_A	*IGKV2-28**01	200	1 (0·5)
24								IGK_A	*IGKV1-16**01	62	0 (0)
25								IGK_A	*IGKV1-17**01	62	0 (0)
26								IGK_A	*IGKV2D-29**02	201	5 (2·5)
27								IGK_A	*IGKV3-11**01	108	1 (0·9)
28								IGK_A	*IGKV4-1**01	193	0 (0)
29								IGK_A	*IGKV3-11**01	62	1 (1·6)
30								IGK_A	*IGKV1-33**01	62	0 (0)
31								IGK_A	*IGKV1-39**01	62	0 (0)
32								IGK_A	*IGKV1-9**01	62	0 (0)
33								IGK_A	*IGKV3D-20**01	108	0 (0)
Mean			205	(11·0)						142	(0·5)

†Primer sequences were not included.

‡PCR performed using in house primers.




```
                <---------------------------------- FR1-IMGT ------------------------------->_____________ CDR1-IMGT ___________<----
Case 4          ..................................................ggcctcagtgaaggtctcctgcaaggcttctggatacaccttcagcgactattat............ttaca
IGHV1-2*02      caggtgcagctggtgcagtctggggct...gaggtgaagaagcctgg|----------------------|------------------c--g---c---............a-g--

                ---------------- FR2-IMGT ------------------->___________ CDR2-IMGT ________<----------------------------------------
Case 4          ctgggtgcgacaggcccctggactaggccttgaatggatgggatgtatcaaccctaatagtggtggcaca......aactatggacagaaatttcag...ggcagggtcaccatgacca
IGHV1-2*02      |----------------------|a--g-----g----------g----------c----------......-------c------g------...--------------------

                ---------- FR3-IMGT ----------------------------------------------------> CDR3-IMGT
Case 4          gggacacgtcctccaagacaaccctcatggagatgagccggctgacgtctgacgacacggccatttattattgtgccaaaaattcgtcttcattcggggacttattagactgggccggg
IGHV1-2*02      ----------at--gc---g--ta--|-----c-----a------ga---->----------g-g-----c-----g-g-g-
```

Fig 2. Effect of somatic mutations of *IGHV* gene on *IGH V*-J PCR in a representative case of follicular lymphoma (Case 4 in Table I). DNA extracted from paraffin embedded specimens was analysed for B-cell clonality using the BIOMED-2 PCR assays and heteroduplex gel electrophoresis. A clonal band (within dotted squares on the gel images which indicate the expected size ranges of PCR products for each reaction) was detected by IGH_A, IGK_A and IGK_B but not by IGH_B or IGH_C (shown), or IGH_D, IGH_E and *IGL* (not shown). The sample was positive for *IGH-BCL2* by BIOMED-2 *BCL2* MBR-J_H primers. The clonal band from IGH_A was sequenced and the nucleotide sequence of the *IGHV* region matches the germline gene *IGHV1-2**2. Sequence identity is indicated by a dash, base differences are shown by letters and BIOMED-2 *IGHV1* FR1, FR2 and FR3 primer sites are indicated by open arrows. Among the 219 nucleotides (excluding FR1 primer sequences) of the *IGHV* region sequenced, 26 nucleotides (12%) are mutated. The mutations included one at the 3′ end of the *IGHV* FR2 primer site and four at the FR3 primer site, which were most likely responsible for the failure of the corresponding PCRs. The clonal *IGKV* gene region (*IGKV3D-15**01, Table I) sequenced from the same case showed no mutation (sequences not shown).

quality (control gene PCR products ≥ 400 bp), whereas those with smaller amplicon size (IGH_C: 100–170 bp, IGK_A: 120–300 bp) showed no difference between the two groups. It was also shown that the combined *IGH* reactions performed worse in the group with lower DNA quality whereas the combined *IGK* reactions performed superbly in both DNA groups. These findings, although statistically not significant, did indicate that suboptimal DNA quality affects the *IGH* assay more than the *IGK* assay and the *IGK* assay is more suited to paraffin-embedded tissue samples. Small PCR products (140–160 bp) of the most commonly rearranged *IGKV* family genes (*IGKV*1, used in >50% of normal and malignant B-cells) may have contributed to the superior performance of *IGK* assay with DNA of suboptimal quality (van Dongen *et al*, 2003). The same explanation, however, could not apply to the IGH_C reaction, which also amplifies small PCR products. Therefore, DNA quality is not the only factor accounting for the low sensitivity of the *IGH* assay observed. Similarly, limited studies including direct comparison using paired frozen and paraffin-embedded materials show that the effect of DNA quality on the rate of clonality detection using the BIOMED-2 assays is small if appropriate quality control is put in place (McClure *et al*, 2006; Halldorsdottir *et al*, 2007).

The heteroduplex analysis of PCR products used in this study might also affect the sensitivity of *IGH* assays as it is known to be less sensitive than GeneScan. However, poor performance of *IGH* assays has been found using both heteroduplex analysis and GeneScan analysis (Lukowsky *et al*, 2006; Halldorsdottir *et al*, 2007; Liu *et al*, 2007). Indeed, heteroduplex gel electrophoresis is the preferred method for analysis of *IGK* PCR products due to limited junction diversity of the rearranged *IGK* (van Dongen *et al*, 2003; Berget *et al*, 2011).

Nonetheless, studies on both paraffin embedded and frozen materials consistently show that the maximum detection of clonality in follicular lymphoma requires combined use of both *IGK* and *IGH* assays (McClure *et al*, 2006; Evans *et al*,

2007; Halldorsdottir *et al*, 2007; Berget *et al*, 2011). Based on our data, addition of IGH_B and/or IGH_D to the two *IGK* reactions can improve the clonality detection rate to over 93% and performing the remaining reactions is only needed for a small number of clonal cases. Such a stepwise and combined use of the BIOMED-2 reactions, which had been proposed in our previous study, (Liu *et al*, 2007) should be the most sensitive and cost-efficient strategy for routine clonality analysis in suspected follicular lymphomas.

For the first time, the present study compared the level of somatic hypermutation of the clonally rearranged *IGH* and *IGK* and examined the effect of mutation on the success of their amplification by PCR. Sequencing of clonal PCR products showed that the *IGHV* genes were mutated in each of the 17 cases analysed with a mean mutation rate of 11·0%, whereas the *IGKV* genes were mutated in only nine of the 27 cases analysed and the mutation rate in these nine cases was much lower (1·4%). Our findings compare well with those of Stamatopoulos *et al* (1997) who reported a mean mutation rate of 9·3% (range 3·7–12·6%) for *IGHV* and 4·7% (0·4–9·1%) for *IGKV* in ten cases of follicular lymphoma. Together, these findings are consistent with the notion that the rearranged *IGKV* regions are much less frequently mutated than the rearranged *IGHV* regions in follicular lymphoma.(Chapman *et al*, 1996; Klein *et al*, 1998; Riboldi *et al*, 2003) More importantly, we have shown direct evidence that the failure of *IGH* V-J PCR is the consequence of somatic hypermutation of the *IGHV* region because PCR failure was significantly associated with the number of mutations (≥3) at the primer binding site.

As *IGH-BCL2* translocation potentially renders only one allele of *IGH* but two alleles of *IGK* amenable to clonality analysis, the presence of the translocation in follicular lymphoma has been thought to account for the difference in clonality detection rate between *IGH* and *IGK* PCR (Halldorsdottir *et al*, 2007; Bagg, 2008) Our results however do not support this notion as the presence or absence of the translocation had no effect on detection of clonal *IGH* V-D-J gene rearrangements. The findings of near 100% detection of clonal *IGH* V-D-J gene rearrangements in mantle cell lymphomas, a pre-germinal centre B-cell neoplasm characterized by the presence of t(11:14)/*BCL1-IGH* translocation and infrequent somatic mutation in their *IGHV*, also argue against any impact of *IGH*-involved translocation on the clonality detection (Pai *et al*, 2005; McClure *et al*, 2006; Evans *et al*, 2007; Liu *et al*, 2007). However, the detection rate of incomplete *IGH* D-J rearrangements was significantly higher in *IGH-BCL2* negative cases (62%) than in *IGH-BCL2* positive cases (17%), which is in line with previous findings that incomplete *IGH* D-J rearrangements are found much less frequently in mature B-cell neoplasms with *IGH*-involved translocations than those without (Evans *et al*, 2007). Interestingly, both an *IGH* V-D-J rearrangement and an *IGH* D-J rearrangement were identified in five cases with *IGH-BCL2* translocation, suggesting that *BCL2* was juxtaposed to an *IGH* allele that had already undergone rearrangement. Indeed, translocation of the *BCL2* gene into the *IGH* locus can occur during *IGH* V-D-J recombination, class switching or somatic hypermutation and both un-rearranged and rearranged *IGH* can be involved, with non-functionally rearranged *IGH* alleles, often those with incomplete D-J rearrangement, being the predominant target (Jager *et al*, 2000; Kuppers & Dalla-Favera, 2001).

In summary, we demonstrate that BIOMED-2 *IGK* assays are far more sensitive than *IGH* assays for clonality analysis in follicular lymphomas because the rearranged *IGKV* region is much less frequently targeted by somatic hypermutation than the *IGHV* region. Nevertheless, the *IGK* assays are not 100% sensitive and maximal detection of clonality requires testing of both *IGH* and *IGK* rearrangements. A stepwise and combined use of the BIOMED-2 reactions, as proposed in our previous study, is the most sensitive and cost efficient strategy for routine clonality analysis in follicular lymphoma (Liu *et al*, 2007).

Acknowledgements

The authors gratefully acknowledge all Consultant Histopathologists at Addenbrooke's Hospital and colleagues in the Molecular Malignancy Laboratory of Addenbrooke's Hospital and in the Division of Molecular Histopathology, Department of Pathology, University of Cambridge, who contributed to this study. This study is supported by the Cambridge University Hospitals NHS Foundation Trust, UK, the NIHR Cambridge Biomedical Research Centre, UK and Leukaemia and Lymphoma Research.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table I. Family-specific IGKV gene PCR primers.

Table II. Detection of clonal immunoglobulin gene rearrangements by individual and combined BIOMED-2 reactions in follicular lymphoma.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

Alt, F.W., Blackwell, T.K., DePinho, R.A., Reth, M.G. & Yancopoulos, G.D. (1986) Regulation of genome rearrangement events during lymphocyte differentiation. *Immunology Review*, **89,** 5–30.

Amara, K., Trimeche, M., Ziadi, S., Sriha, B., Mokni, M. & Korbi, S. (2006) PCR-based clonality analysis of B-cell lymphomas in paraffin-embedded tissues: diagnostic value of immunoglobulin kappa and lambda light chain gene

rearrangement investigation. *Pathology, Research and Practice*, **202,** 425–431.

Arnold, A., Cossman, J., Bakhshi, A., Jaffe, E.S., Waldmann, T.A. & Korsmeyer, S.J. (1983) Immunoglobulin-gene rearrangements as unique clonal markers in human lymphoid neoplasms. *New England Journal of Medicine*, **309,** 1593–1599.

Bacon, C.M., Diss, T.C., Ye, H., Liu, H., Goatly, A., Hamoudi, R., Wotherspoon, A., Gascoyne, R.D., Dogan, A., Du, M.Q. & Isaacson, P.G. (2009) Follicular lymphoma of the thyroid gland. *American Journal of Surgical Pathology*, **33,** 22–34.

Bagg, A. (2008) Malleable immunoglobulin genes and hematopathology – the good, the bad, and the ugly: a paper from the 2007 William Beaumont hospital symposium on molecular pathology. *Journal of Molecular Diagnostic*, **10,** 396–410.

Berget, E., Helgeland, L., Molven, A. & Vintermyr, O.K. (2011) Detection of clonality in follicular lymphoma using formalin-fixed, paraffin-embedded tissue samples and BIOMED-2 immunoglobulin primers. *Journal of Clinical Pathology*, **64,** 37–41.

Bourguin, A., Tung, R., Galili, N. & Sklar, J. (1990) Rapid, nonradioactive detection of clonal T-cell receptor gene rearrangements in lymphoid neoplasms. *Proceedings of the National Academy of Sciences of the United States of America*, **87,** 8536–8540.

Campbell, M.J., Zelenetz, A.D., Levy, S. & Levy, R. (1992) Use of family specific leader region primers for PCR amplification of the human heavy chain variable region gene repertoire. *Molecular Immunology*, **29,** 193–203.

Catherwood, M.A., Gonzalez, D., Patton, C., Dobbin, E., Venkatraman, L. & Alexander, H.D. (2007) Improved clonality assessment in germinal centre/post germinal centre non-Hodgkin's lymphomas with high rates of somatic hypermutation. *Journal of Clinical Pathology*, **60,** 524–528.

Chapman, C.J., Zhou, J.X., Gregory, C., Rickinson, A.B. & Stevenson, F.K. (1996) VH and VL gene analysis in sporadic Burkitt's lymphoma shows somatic hypermutation, intraclonal heterogeneity, and a role for antigen selection. *Blood*, **88,** 3562–3568.

Cleary, M.L., Chao, J., Warnke, R. & Sklar, J. (1984) Immunoglobulin gene rearrangement as a diagnostic criterion of B-cell lymphoma. *Proceedings of the National Academy of Sciences of the United States of America*, **81,** 593–597.

Diss, T.C., Liu, H.X., Du, M.Q. & Isaacson, P.G. (2002) Improvements to B cell clonality analysis using PCR amplification of immunoglobulin light chain genes. *Molecular Pathology*, **55,** 98–101.

van Dongen, J.J., Langerak, A.W., Bruggemann, M., Evans, P.A., Hummel, M., Lavender, F.L., Delabesse, E., Davi, F., Schuuring, E., Garcia-Sanz, R., van Krieken, J.H., Droese, J., Gonzalez, D., Bastard, C., White, H.E., Spaargaren, M., Gonzalez, M., Parreira, A., Smith, J.L., Morgan, G.J., Kneba, M. & Macintyre, E.A. (2003) Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia*, **17,** 2257–2317.

Evans, P.A., Pott, C., Groenen, P.J., Salles, G., Davi, F., Berger, F., Garcia, J.F., van Krieken, J.H., Pals, S., Kluin, P., Schuuring, E., Spaargaren, M., Boone, E., Gonzalez, D., Martinez, B., Villuendas, R., Gameiro, P., Diss, T.C., Mills, K., Morgan, G.J., Carter, G.I., Milner, B.J., Pearson, D., Hummel, M., Jung, W., Ott, M., Canioni, D., Beldjord, K., Bastard, C., Delfau-Larue, M.H., van Dongen, J.J., Molina, T.J. & Cabecadas, J. (2007) Significantly improved PCR-based clonality testing in B-cell malignancies by use of multiple immunoglobulin gene targets. Report of the BIOMED-2 Concerted Action BHM4-CT98-3936. *Leukemia*, **21,** 207–214.

Gong, J.Z., Zheng, S., Chiarle, R., Wolf-Peeters, C., Palestro, G., Frizzera, G. & Inghirami, G. (1999) Detection of immunoglobulin kappa light chain rearrangements by polymerase chain reaction. An improved method for detecting clonal B-cell lymphoproliferative disorders. *American Journal of Pathology*, **155,** 355–363.

Halldorsdottir, A.M., Zehnbauer, B.A. & Burack, W.R. (2007) Application of BIOMED-2 clonality assays to formalin-fixed paraffin embedded follicular lymphoma specimens: superior performance of the *IGK* assays compared to IGH for suboptimal specimens. *Leukemia and Lymphoma*, **48,** 1338–1343.

Jaffe, E.S., Harris, N.L., Stein, H. & Vardiman, J.W. (2001) *World Health Organization Classification of Tumors: Pathology and Genetics of Tumors of Hematopoietic and Lymphoid Tissues*. IARC Press, Lyon, France.

Jager, U., Bocskor, S., Le, T., Mitterbauer, G., Bolz, I., Chott, A., Kneba, M., Mannhalter, C. & Nadel, B. (2000) Follicular lymphomas' BCL-2/IgH junctions contain templated nucleotide insertions: novel insights into the mechanism of t(14;18) translocation. *Blood*, **95,** 3520–3529.

Klein, U., Rajewsky, K. & Kuppers, R. (1998) Human immunoglobulin *(Ig)M+IgD+* peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *Journal of Experimental Medicine*, **188,** 1679–1689.

Kosmas, C., Stamatopoulos, K., Papadaki, T., Belessi, C., Yataganas, X., Anagnostou, D. & Loukopoulos, D. (1998) Somatic hypermutation of immunoglobulin variable region genes: focus on follicular lymphoma and multiple myeloma. *Immunology Review*, **162,** 281–292.

Kuppers, R. (2003) Somatic hypermutation and B cell receptor selection in normal and transformed human B cells. *Annals of the New York Academy of Sciences*, **987,** 173–179.

Kuppers, R. & Dalla-Favera, R. (2001) Mechanisms of chromosomal translocations in B cell lymphomas. *Oncogene*, **20,** 5580–5594.

Kuppers, R., Zhao, M., Rajewsky, K. & Hansmann, M.L. (1993) Detection of clonal B cell populations in paraffin-embedded tissues by polymerase chain reaction. *American Journal of Pathology*, **143,** 230–239.

Langerak, A.W. & van Dongen, J.J. (2006) Recombination in the human *IGK* locus. *Critical Reviews in Immunology*, **26,** 23–42.

Liu, H., Bench, A.J., Bacon, C.M., Payne, K., Huang, Y., Scott, M.A., Erber, W.N., Grant, J.W. & Du, M.Q. (2007) A practical strategy for the routine use of BIOMED-2 PCR assays for detection of B- and T-cell clonality in diagnostic haematopathology. *British Journal of Haematology*, **138,** 31–43.

Lukowsky, A., Marchwat, M., Sterry, W. & Gellrich, S. (2006) Evaluation of B-cell clonality in archival skin biopsy samples of cutaneous B-cell lymphoma by immunoglobulin heavy chain gene polymerase chain reaction. *Leukemia Lymphoma*, **47,** 487–493.

McCarthy, K.P., Sloane, J.P. & Wiedemann, L.M. (1990) Rapid method for distinguishing clonal from polyclonal B cell populations in surgical biopsy specimens. *Journal of Clinical Pathology*, **43,** 429–432.

McClure, R.F., Kaur, P., Pagel, E., Ouillette, P.D., Holtegaard, C.E., Treptow, C.L. & Kurtin, P.J. (2006) Validation of immunoglobulin gene rearrangement detection by PCR using commercially available BIOMED-2 primers. *Leukemia*, **20,** 176–179.

Pai, R.K., Chakerian, A.E., Binder, J.M., Amin, M. & Viswanatha, D.S. (2005) B-cell clonality determination using an immunoglobulin kappa light chain polymerase chain reaction method. *Journal of Molecular Diagnostics*, **7,** 300–307.

Riboldi, P., Ikematsu, W., Brambilla, B., Caprani, C., Gerosa, M. & Casali, P. (2003) Diversity and somatic hypermutation of the *Ig* VHDJH, V kappa J kappa, and V lambda J lambda gene segments in lymphoma B cells: relevance to the origin of the neoplastic B cell clone. *Human Immunology*, **64,** 69–81.

Sandberg, Y., Gastel-Mol, E.J., Verhaaf, B., Lam, K.H., van Dongen, J.J. & Langerak, A.W. (2005) BIOMED-2 multiplex immunoglobulin/T-cell receptor polymerase chain reaction protocols can reliably replace Southern blot analysis in routine clonality diagnostics. *Journal of Molecular Diagnostics*, **7,** 495–503.

Spencer, J., Diss, T.C. & Isaacson, P.G. (1989) Primary B cell gastric lymphoma. A genotypic analysis. *American Journal of Pathology*, **135,** 557–564.

Stamatopoulos, K., Kosmas, C., Papadaki, T., Pouliou, E., Belessi, C., Afendaki, S., Anagnostou, D. & Loukopoulos, D. (1997) Follicular lymphoma immunoglobulin kappa light chains are affected by the antigen selection process, but to a lesser degree than their partner heavy chains. *British Journal of Haematology*, **96,** 132–146.

Swerdlow, S.H., Campo, E., Harris, N.L., Jaffe, E.S., Pileri, S.A., Stein, H., Thiele, J. & Vardiman, J.W. (2008) *WHO Classification of Tumors of Hema-*

topoietic and Lymphoid Tissues. IARC, Lyon, France.

Trainor, K.J., Brisco, M.J., Story, C.J. & Morley, A.A. (1990) Monocloality in B lymphproliferative disorders detected at the DNA level. *Blood*, **75,** 2220–2222.

Williams, M.E., Innes, D.J.J., Borowitz, M.J., Lovell, M.A., Swerdlow, S.H., Hurtubise, P.E., Brynes, R.K., Chan, W.C., Byrne, G.E.J., Whitcomb, C.C., Christopher, Y. & Thomas, I.V. (1987) Immunoglobulin and T cell receptor gene rearrangements in human lymphoma and leukemia. *Blood*, **69,** 79–86.

Ye, H., Liu, H., Attygalle, A., Wotherspoon, A.C., Nicholson, A.G., Charlotte, F., Leblond, V., Speight, P., Goodlad, J., Lavergne-Slove, A., Martin-Subero, J.I., Siebert, R., Dogan, A., Isaacson, P.G. & Du, M.Q. (2003) Variable frequencies of t(11;18)(q21;q21) in MALT lymphomas of different sites: significant association with CagA strains of H pylori in gastric MALT lymphoma. *Blood*, **102,** 1012–1018.