B-Cell Clonality Determination Using an Immunoglobulin κ Light Chain Polymerase Chain Reaction Method

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To augment the detection of clonality in B-cell malignancies, we designed a consensus primer κ light chain gene (Ig κ) polymerase chain reaction (PCR) assay in combination with a consensus primer immunoglobulin heavy chain gene (IgH) PCR assay. Its efficacy was then evaluated in a series of 86 paraffin tissue samples comprising neoplastic and reactive lymphoproliferations. Analysis after PCR was accomplished by 10% native polyacrylamide gel electrophoresis after heteroduplex pretreatment of PCR products and by a post-PCR chip-based capillary electrophoresis analytic method. Overall, 49 of 68 (72%) of mature B-cell neoplasms yielded discrete Ig κ gel bands within the predicted size range with no clonotypic Ig κ products observed among reactive lymphoid or T-cell proliferations. The application of Ig κ PCR improved overall sensitivity from 81% with IgH PCR alone to 90% with combined Ig κ /IgH PCR, with this effect being most notable in germinal center-related lymphomas. Sequencing of positive Ig κ rearrangements revealed that most rearrangements involved members of the V κ 1 (40%) and V κ 2 (34%) gene families along with J κ 1 (26%), J κ 2 (23%), and J κ 4 (51%) gene segments. Involvement of V κ pseudogenes was identified in 24% of cases with V κ -KDE rearrangements. Our results demonstrate the efficacy of Ig κ PCR in improving the detection rate of clonality in B-cell neoplasms and further introduce a novel post-PCR chip-based capillary electrophoresis analytic method for rapid PCR fragment size evaluation. (*J Mol Diagn* 2005, 7:300–307)

methods have the advantage of rapidity and the requirement for minimal sample material (compared to genomic Southern blot analysis), the potential false-negative rate is a considerable shortcoming. A significant proportion of B-cell lymphomas do not demonstrate clonotypic IgH amplification mainly due to suboptimal primer binding, either from a lack of consensus target sequences or target site alteration as a result of somatic hypermutation. Alternative strategies for detecting IgH clonality using consensus primers directed against IgH framework region 2 (FR2) or framework region 1 (FR1) have been described,^{5–7} but these approaches are also subject to pitfalls. For example, IgH PCR using FR2 consensus primers may be subject to false-negative amplifications due to the lack of highly conserved sequences among many VH-FR2 regions, necessitating the use of highly degenerate consensus primers. The use of multiple IgH FR1 family-specific consensus primers can achieve a high clonal detection rate, but results in larger sized PCR products that are often not well amplified from paraffin-embedded tissue samples.^{8,9} In light of these issues, the immunoglobulin light chain genes can present attractive alternative targets for B-cell clonality determination.

During normal B-cell differentiation, IgH gene rearrangements precede immunoglobulin κ light chain (Ig κ) gene rearrangements, which in turn occur before immunoglobulin λ light chain (Ig λ) gene rearrangements.¹⁰ For a particular allele, if the Ig κ gene rearrangement produces a nonfunctional V κ -J κ product, the locus may undergo segmental deletion via rearrangement with the downstream κ -deleting element (KDE).^{10–12} In fact, the vast majority of phenotypic λ -expressing B cells and a subset of κ -expressing B cells have rearrangements involving the KDE. Similar to V κ -J κ joinings, KDE-mediated Ig κ gene rearrangements occur via recombination signal sequences located in either the J κ -C κ intron (ie, intron recombination signal sequences) or immediately 3' to the V κ gene segments, and these often exhibit junctional diversity with the addition of nontemplated (N) nucleotides. Both V κ -J κ and KDE rearrangements thus offer additional targets for detection of B-cell clonality by PCR.

Consensus primer polymerase chain reaction (PCR) amplification of the immunoglobulin heavy chain (IgH) gene complementary-determining region 3 (CDR3) is a routine ancillary diagnostic technique for evaluating clonality in B-cell lymphoproliferative disorders.^{1–4} Although PCR

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To further augment the ability to detect clonal B-cell populations in formalin-fixed, paraffin-embedded diagnostic tissue biopsies, we developed and applied a multiplex PCR approach to detect V κ -J κ and V κ -KDE rearrangements in 68 cases of diverse types of mature B-cell neoplasms, as well as 18 other lymphoid proliferations. We demonstrate that a comprehensive Ig κ PCR approach to identify both V κ -J κ and V κ -KDE gene rearrangements significantly augments consensus primer IgH PCR for the detection of B-cell clonality and is suitable for paraffin-embedded tissue sources. This study further provides preliminary data regarding the utility of a post-PCR chip-based capillary electrophoresis (CBCE) analytic method that is capable of superior resolution for amplicon detection and sizing compared to standard gel analysis.

Materials and Methods

Patients and Samples

Paraffin blocks from 68 cases of B-cell neoplasms (25 diffuse large B-cell lymphomas, 13 follicular lymphomas, 10 small lymphocytic lymphomas, 9 mantle cell lymphomas, 8 marginal zone lymphomas, and 3 multiple myelomas), along with 7 Hodgkin lymphomas, 2 peripheral T-cell lymphomas, and 9 reactive lymphoid proliferations were retrieved from the Pathology Departments of the University of New Mexico and William Beaumont Hospitals. All of the samples were clinical diagnostic cases obtained between 2001 to 2003 and were classified in accordance with the World Health Organization classification of hematopoietic neoplasms.¹³ The histological diagnosis was reviewed in each case by two of the investigators (R.P., D.V.), along with details of available flow cytometry data. This study was approved by the University of New Mexico Human Research Review Committee.

DNA Preparation and Polymerase Chain Reaction Analysis

Genomic DNA from all cases was extracted from formalin-fixed, paraffin-embedded tissue sections, according to the manufacturer's directions (DNEasy kit; Qiagen, Santa Clarita, CA) and quantitated by UV absorbance spectrophotometry. Three consensus family-specific V κ FR3 region primers (designed to target the V κ 1 through V κ 6 gene families of Ig κ), one consensus J κ primer, and one KDE primer were used based on slight modifications of reported V κ -FR3, J κ , and KDE sequences.^{14–16} Less degeneracy was used in the family-specific V κ -FR3 and J κ primers at the 3' end compared to previous studies.^{14–16} The three forward V κ primers were designated as follows: V κ I/VI targeting the V κ I and V κ VI gene families; V κ III/IV targeting the V κ III and V κ IV gene families; and V κ II targeting the V κ II gene family. One consensus J κ reverse primer was designed to target J κ I, J κ II, J κ III, and J κ IV genes, and one reverse primer was de-

Tube 1

V κ I/VI primer: 5'-TTCAG(T/C)GGCAGTGGATCTGG-3'

V κ II primer: 5'-TTCAGTGGCAGTGGGTCAGG-3'

J κ primer: 5'-GTTTGATCTCCACCTTGGTCCC-3'

Tube 2

V κ III/IV primer: 5'-TTCAGTGGCAG(T/C)GGGTCTGG-3'

J κ primer: 5'-GTTTGATCTCCACCTTGGTCCC-3'

Tube 3

V κ I/VI primer: 5'-TTCAG(T/C)GGCAGTGGATCTGG-3'

V κ III/IV primer: 5'-TTCAGTGGCAG(T/C)GGGTCTGG-3'

KDE primer: 5'-CCCTTCATAGACCTTCAGGCAC-3'

Figure 1. Schematic diagram of PCR strategy to detect V κ -J κ and V κ -KDE rearrangements of the Ig κ gene. Primer set combinations for each of the three PCR tubes are as indicated: **tubes 1 and 2** identify V κ -J κ gene rearrangements and **tube 3** amplifies V κ -KDE gene rearrangements. Nucleotide bases in **parentheses** indicate degenerate sites in the primers. Use of a touchdown PCR method promotes amplification of dominant rearrangement(s) despite some consensus primer degeneracy or minor mismatching.

signed to target the KDE locus. Nucleotide sequences for V κ , J κ , and KDE primers, as well as the schematics of the Ig κ PCR strategy, are depicted in Figure 1.

The Ig κ PCR approach consisted of three different reaction tubes per sample (Figure 1). In tube 1, two V κ primers (V κ I/VI at 10 pmol/reaction and V κ II at 20 pmol/reaction) were combined with the J κ primer (at 10 pmol/reaction). In tube 2, one V κ primer (V κ III/IV at 30 pmol/reaction) was combined with the J κ primer (at 20 pmol/reaction). In tube 3, two V κ primers (V κ I/VI at 10 pmol/reaction and V κ III/IV at 30 pmol/reaction) were combined with the KDE primer (at 10 pmol/reaction). The primer combinations were arrived at based on preliminary experiments to optimize amplification efficiency and minimize primer interference and nonspecific product generation.

For the Ig κ PCR tubes, 200 ng of genomic DNA were subjected to amplification in a reaction mixture containing 1 $\times$ GeneAmp PCR buffer (Applied Biosystems, Foster City, CA), 1.5 mmol/L MgCl₂, 200 μ mol/L of each dNTP, 2.5 U of *Taq* polymerase (Applied Biosystems), and the V κ , J κ , and KDE primer combinations as designated above, and shown in Figure 1. A touchdown PCR was used involving a denaturing period of 95°C for 5 minutes followed by eight cycles of 95°C for 30 seconds, 1 minute at 64°C, then decreasing the annealing temperature by 0.5°C each subsequent cycle, and 1 minute extension at 72°C. This was followed by 32 cycles of 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute, with a final primer extension at 72°C for 5 minutes.

Immunoglobulin heavy chain (IgH) gene rearrangements were amplified by PCR, using consensus oligonucleotide primers for VH-FR3 and JH regions, as previously described.¹⁷ For IgH PCR, 400 ng of genomic DNA were subjected to amplification in reaction mixtures con-

taining 1× GeneAmp PCR buffer (Applied Biosystems), 1.5 mmol/L MgCl₂, 200 μmol/L of each dNTP, 10 pmol of each primer, and 2.5 U of *Taq* polymerase (Applied Biosystems). PCR amplification was performed by denaturation at 95°C for 5 minutes followed by 40 cycles of 95°C for 30 seconds, 1 minute at 58°C, and 1 minute at 72°C. This was followed by a final primer extension at 72°C for 5 minutes. In all PCR experiments, genomic DNA from known monoclonal neoplastic B cells and reactive tonsil tissue were included as positive and negative controls, respectively. In addition, no DNA template (blank) controls were included with each sample run to exclude potential contamination. Amplification of a 165-bp segment of the β -globin gene (forward primer, 5'ACA-CAACTGTGTTCACTAGC3'; and reverse primer, 5'TG-GTCTCCTTAAACCTGTCTTG3') was performed in every sample as an internal control for DNA integrity. An additional larger fragment size β -globin gene amplification (325 bp) was subsequently performed (same forward primer with reverse primer 5'ATCAGGAGT GGACA-GATCC3') in a subset of cases found to be negative for V κ -KDE amplification, to verify DNA integrity in this larger amplicon range. For Ig κ PCR assay sensitivity determination, serial 10-fold dilutions of DNA from a patient sample positive for an Ig κ V κ -J κ clonal rearrangement were made into tonsil DNA (polyclonal B-cell background). Each dilution was kept at 200 ng of total DNA. PCR amplification was performed as described for standard Ig κ PCR.

Gel Electrophoresis of PCR Products

All Ig κ PCR products were subjected to a heteroduplex (HDX) procedure, which involved heating the reaction for 5 minutes at 94°C followed by immediate immersion on ice for 60 minutes.¹⁸ Ten μl of the Ig κ PCR products were then analyzed by 10% nondenaturing polyacrylamide gel electrophoresis (PAGE) in 1× TBE buffer for 40 minutes (Bio-Rad Mini-Protean II system; Bio-Rad, Hercules CA) at room temperature, stained with ethidium bromide, and visualized under UV light. The V κ -J κ PCR is predicted to amplify rearranged products of ~130 to 150 bp, whereas the V κ -KDE PCR was expected to amplify products of ~250 to 300 bp. IgH PCR products (predicted size range ~70 to 140 bp) were analyzed by 10% PAGE, but without HDX pretreatment. Samples with an unequivocal dominant band in the expected size ranges were interpreted as monoclonal, and those with a minimal smear pattern or absence of products were interpreted as polyclonal or negative, respectively. β -Globin controls were assessed concurrently with Ig κ and IgH PCR product gel electrophoresis.

Sequencing Analysis of Ig κ PCR Products

To confirm the specificity of amplified Ig κ gene rearrangements and determine patterns of gene segment usage, the clonal bands from all positive Ig κ PCR cases were sequenced. PCR products were gel purified from 4% agarose gels using the Qiagen gel extraction kit, (Qiagen). The extracted PCR products were directly se-

quenced in one or both directions with V κ , J κ , or KDE primers, using the dye terminator method on an ABI 3100 capillary sequencer (Applied Biosystems). The derived sequences were compared to known germline DNA sequences of the Ig κ V- and J- regions using the V-BASE (CPE Cambridge, UK; <http://www.mrc-cpe.cam.ac.uk/vbase-ok>) and BLAST (NCBI/GenBank; <http://www.ncbi.nlm.nih.gov/blast>) computer programs. Individual positive cases, particularly with similar V κ region use, were further compared to each other by pair-wise BLAST nucleotide alignment, to ensure unique sequence identity. From the nucleotide sequence information, data regarding V κ and J κ gene segment usage in each Ig κ -positive case were obtained. These results were compared to established germline frequencies for V κ and J κ gene segments using χ^2 statistical analysis.

Product Size Determination by CBCE

Analysis after PCR was also performed by a CBCE analytic method in 29 cases comprising a subset of V κ -J κ (21 cases) and V κ -KDE (8 cases) clonal-positive cases, as well as several PCR-negative cases. For CBCE, the previously heteroduplexed PCR products were analyzed on a model 2100 bioanalyzer using DNA 500 chips (Agilent Technologies, Palo Alto, CA). The 16-well chips were prepared for analysis according to the manufacturer's directions and using 1 μl of PCR product per sample. A run time of ~40 minutes was required for one complete chip (12 samples total), with real-time software rendering and display of the data. A clonal population was defined in this study as a single peak of greater than 15 relative fluorescence units on the y axis (for a 1-μl PCR product amount), falling in the expected product size range for Ig κ amplicons. A low-intensity bell-shaped pattern or lack of products indicated polyclonal or negative results, respectively. The CBCE criteria were derived from our preliminary experience with this platform.¹⁹ The fragment size and relative intensity of each Ig κ amplicon was compared with HDX-PAGE results.

Results

Evaluation of Patient Samples with Ig κ and IgH PCR

The Ig κ PCR approach consisted of three different reaction tubes (Figure 1) to detect both V κ -J κ and V κ -KDE rearrangements. Using this PCR strategy and post-PCR HDX-PAGE analysis, Ig κ clonal products could be detected in 72% of mature B-cell non-Hodgkin lymphomas (Table 1). In each positive (clonal) case, a discrete band was identified by HDX-PAGE within the expected size range of 130 to 150 bp for V κ -J κ rearrangements and 250 to 300 bp for V κ -KDE rearrangements (Figure 2). Larger sized, nonspecific HDX bands were also noted in the V κ -J κ PCR.

For Ig κ PCR, the highest detection rates were found in diffuse large B-cell lymphomas (76%), small lymphocytic lymphomas (80%), and marginal zone lymphomas (88%).

Table 1. Frequencies of IgH and Ig κ Rearrangements in B-Cell Neoplasms

Lymphoma type	IgH PCR	V κ -J κ PCR	V κ -KDE PCR	Ig κ PCR* overall	IgH/Ig κ overall
Diffuse large B-cell lymphoma	21/25 (84%)	15/25 (60%)	6/25 (24%)	19/25 (76%)	25/25 (100%)
Follicular lymphoma	6/13 (46%)	4/13 (31%)	4/13 (31%)	8/13 (62%)	8/13 (62%)
Small lymphocytic lymphoma	10/10 (100%)	7/10 (70%)	1/10 (10%)	8/10 (80%)	10/10 (100%)
Mantle cell lymphoma	9/9 (100%)	6/9 (67%)	2/9 (22%)	6/9 (67%)	9/9 (100%)
Marginal zone lymphoma	7/8 (88%)	6/8 (75%)	3/8 (38%)	7/8 (88%)	7/8 (88%)
Multiple myeloma	2/3 (67%)	0/3 (0%)	1/3 (33%)	1/3 (33%)	2/3 (67%)
Hodgkin lymphoma	1/7 (14%)	0/7 (0%)	2/7 (29%)	2/7 (29%)	2/7 (29%) [†]
Reactive hyperplasias and T-cell neoplasms	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)
Overall B-cell non-Hodgkin lymphoma cases	55/68 (81%)	38/68 (56%)	17/68 (25%)	49/68 (72%)	61/68 (90%)

*A case is defined as being positive for Ig κ PCR overall if either a V κ -J κ or V κ -KDE rearrangement was identified.

[†]Positive Hodgkin lymphoma cases with syncytial histology.

Figure 2. HDX-PAGE detection of Ig κ gene rearrangement with V κ -J κ and V κ -KDE PCR. **A:** Representative set of various B-cell lymphoma cases with positive (monoclonal) V κ -J κ Ig κ gene rearrangements (lanes 1 to 7). White arrow indicates expected size region for positive bands (~130 to 150 bp). Note slight variation in PCR product sizes for each sample. Some higher molecular size, nonspecific amplicons are present in most cases. Lanes 8 and 9 represent reactive (polyclonal) B-cell and water-only (no template) controls, respectively. L indicates the 100-bp size ladder. **B:** Illustration of V κ -J κ and V κ -KDE PCR in combination with IgH PCR for a representative set of cases as follows: diffuse large B-cell lymphoma (lane 1, IgH; lane 2, V κ -J κ ; lane 3, V κ -KDE), follicular lymphoma (lane 4, IgH; lane 5, V κ -J κ ; lane 6, V κ -KDE), extranodal marginal zone lymphoma (lane 7, IgH; lane 8, V κ -J κ ; lane 9, V κ -KDE), and Hodgkin lymphoma, syncytial nodular sclerosis type (lane 10, IgH; lane 11, V κ -J κ ; lane 12, V κ -KDE). Expected size ranges for PCR products: IgH, ~70 to 140 bp; V κ -J κ Ig κ , ~130 to 150 bp; V κ -KDE Ig κ , ~250 to 300 bp. L indicates the 100-bp size ladder. For all samples, DNA integrity was confirmed by amplification of a segment of the β -globin gene (not shown).

The lowest detection rates were obtained in follicular lymphomas (62%), mantle cell lymphomas (67%), and myelomas (33%) (Table 1). The addition of Ig κ PCR significantly increased the overall detection of clonality in mature B-cell tumors from 81% (IgH PCR alone) to 90% (combined Ig κ /IgH PCR). This effect was most notable in the germinal center-associated lymphomas. For diffuse large B-cell lymphomas, the additional analysis of Ig κ rearrangements improved the overall detection rate from 84% with IgH PCR alone to 100% with combined IgH/Ig κ PCR (Tables 1 and 2). For follicular lymphomas, the additional analysis of Ig κ rearrangements improved the overall detection of clonality from 46% with IgH PCR alone to 62% with combined IgH/Ig κ PCR (Tables 1 and 2). For small lymphocytic lymphoma/chronic lymphocytic leukemia, marginal zone lymphoma, and mantle cell lymphoma, additional analysis by Ig κ PCR did not significantly impact the overall detection rate of clonality. Ig κ PCR also detected clonal rearrangements in two of seven cases of classical Hodgkin lymphoma, with one of these cases also demonstrating monoclonality by IgH PCR (Table 1); these cases were notable for syncytial histological features. No clonal Ig κ products were observed in any of the 11 reactive lymphoid proliferations or T-cell lymphomas. The dilutional sensitivity of Ig κ PCR was found to be 10%, using DNA from a V κ -J κ clonal lymphoma case diluted into a polyclonal B-cell background (Figure 3).

Examination of the positive Ig κ cases revealed that V κ -J κ rearrangements generally predominated over V κ -KDE joinings, with the exception of an equal V κ -KDE frequency in follicular lymphomas, and only V κ -KDE use in the positive Hodgkin lymphoma and myeloma samples (Table 1). The surface light chain expression of 36 mature B-cell lymphomas was known by prior flow cytometric analysis. Of the 23 κ -expressing B-cell lymphomas, 10 cases (44%) had only a V κ -J κ rearrangement, 4 cases

Table 2. Comparison of IgH and Ig κ PCR for Diffuse Large B-Cell Lymphoma and Follicular Lymphoma

IgH/Ig κ	DLBCL (n = 25)	FL (n = 13)
IgH+/Ig κ +	15	6
IgH-/Ig κ +	4	2
IgH+/Ig κ -	6	0
IgH-/Ig κ -	0	5

Figure 3. Dilutional sensitivity of the Igκ PCR assay. Genomic DNA from a B-cell lymphoma with a known monoclonal Igκ rearrangement was serially diluted into DNA from reactive tonsil tissue and analyzed by multiplex Vκ-Jκ PCR and HDX-PAGE. Dilutions are as indicated: **lanes 1 to 5:** 200 ng, 20 ng, 2 ng, 0.2 ng, and 0.02 ng of target DNA, respectively; **lane 6:** tonsil DNA alone; **lane 7:** water-only (no template) control. L denotes a 100-bp ladder. A clonal PCR amplicon could be detected in the dilution containing 20 ng of sample DNA, representing detection of a 10% dilution (indicated by **white arrow**).

(17%) had a Vκ-KDE rearrangement only, 1 case (4%) had both Vκ-Jκ and Vκ-KDE rearrangements, and 8 cases (35%) had no rearrangements detected by Igκ PCR. Of the 13 λ-expressing B-cell lymphomas, 5 cases (39%) had only a Vκ-Jκ rearrangement, 3 cases (23%) had a Vκ-KDE rearrangement only, 3 cases (23%) had both Vκ-Jκ and Vκ-KDE rearrangements, and 2 cases (15%) had no rearrangements detected by Igκ PCR. These findings are summarized in Table 3. Of note, DNA samples from Vκ-KDE-negative cases were successfully reamplified with primers encompassing a larger segment of the β-globin gene (325 bp), to ensure that insufficient DNA integrity was not the cause for the negative PCR results (data not shown).

Sequence Data of Igκ PCR for Determination of Vκ and Jκ Gene Usage

A total of 56 Igκ PCR clonotypic alleles were sequenced. Vκ and Jκ gene use was assigned by comparison with known germline sequences using the V-BASE and BLAST computer programs. The prevalence of Vκ gene families in Vκ-Jκ gene rearrangements generally reflected the number of available germline Vκ gene segments within each family (Figure 4). Most Vκ-Jκ rear-

Table 3. Igκ Rearrangement Frequencies by Light-Chain Expression

Igκ rearrangement type	κ (n = 23)	λ (n = 13)
Vκ-Jκ only	10	5
Vκ-KDE only	4	3
Both Vκ-Jκ and Vκ-KDE	1	3
None	8	2

Figure 4. Observed relative frequencies of Vκ gene segments in clonal Vκ-Jκ and Vκ-KDE rearrangements. The relative frequencies of Vκ-Jκ rearrangements (**black bars**) and Vκ-KDE rearrangements (**gray bars**) were derived from sequence analysis of Vκ-Jκ and Vκ-KDE PCR amplicons. Note that Vκ pseudogene use was not encountered with Vκ-Jκ rearrangements, but was observed in occasional Vκ-KDE rearrangements.

rangements involved members of the Vκ1 (40%) and Vκ2 (34%) gene families. There was a significant relative overrepresentation of Vκ4 gene family usage (14%) when compared to the number of available germline Vκ4 genes using χ^2 statistical analysis ($P = 0.015$, $\chi^2 = 5.92$). Rearrangements involving Vκ pseudogenes were not identified in any of the Vκ-Jκ rearrangements. For Vκ-KDE rearrangements, members of the Vκ1 (35%) and Vκ2 (29%) gene families were most often used (Figure 4). Rearrangements involving Vκ pseudogenes were identified in 4 of 17 (24%) of the Vκ-KDE-positive cases and primarily involved Vκ2 and Vκ7 pseudogenes. Concerning the Jκ region, Jκ1 (26%), Jκ2 (23%), and Jκ4 (51%) gene segments were used most frequently (Figure 5). However, no involvement of the Jκ3 segment was observed.

CBCE Evaluation of Igκ PCR Products

CBCE provided highly accurate PCR amplicon sizing in 29 clonal Igκ-positive samples tested, with representative results shown in Figure 6, A to C. By CBCE, the size range for Vκ-Jκ rearrangements was between 120 to 145 bp. An additional nonspecific peak was sometimes iden-

Figure 5. Observed relative frequencies of Jκ gene segments in clonal Vκ-Jκ rearrangements. The relative frequencies were derived from sequence analysis of Vκ-Jκ PCR amplicons. Jκ3 segments were not detected among these cases.

Figure 6. Analysis after PCR by CBCE. One μ l of heteroduplexed PCR products was analyzed on a 2100 Bioanalyzer (Agilent Technologies) with real-time generation of electropherogram data. Peaks labeled with an asterisk at far left and right ends of the electropherogram images represent 15-bp and 600-bp size standards, respectively. The x axis parameter is sample run time in seconds, however the software detects and calculates sample peaks in bp size relative to the standards. The y axis represents relative fluorescence units (RFU). A clonal population was defined as a single peak of greater than 15 RFU. The electropherograms are as shown: **A:** diffuse large B-cell lymphoma positive for a V κ -J κ clonal rearrangement with a PCR amplicon size of 139 bp; **B:** follicular lymphoma positive for a V κ -KDE clonal rearrangement with a PCR amplicon size of 267 bp; **C:** polyclonal (negative) PCR result; **D** through **F:** dilution sensitivity assay with PCR products from same Ig κ -positive lymphoma sample as in Figure 3 (**D:** 200 ng tumor DNA; **E:** 20 ng tumor DNA; **F:** 2 ng of tumor DNA). Note that CBCE produces highly accurate fragment sizing for the positive cases, however, the dilutional sensitivity of this platform with a consensus Ig κ PCR primer method is essentially similar to PAGE results (ie, 20 ng of target template or 10%; Figure 3). Peaks immediately to the right of the 15-bp size standard in electropherograms represent primer dimers.

tified with V κ -J κ PCR at $\sim$ 210 bp. By CBCE, the size range for V κ -KDE rearrangements was between 260 to 290 bp. PCR-negative samples showed either a minimal polyclonal pattern in the expected size ranges, or no deviation from baseline. The data from CBCE agreed well with HDX-PAGE results. CBCE was also more rapid and provided highly resolved histogram data for clonal results. Dilutional sensitivity of CBCE using the same clonal V κ -J κ lymphoma sample as in Figure 3 was comparable to HDX-PAGE at 10% detection in a polyclonal B-cell background (Figure 6; D to F).

Discussion

The recent publication of the European BIOMED-2 consortium underscores the value of using comprehensive PCR primers and multiple antigen receptor gene targets to establish the presence of a clonal lymphoid proliferation.²⁰ Ig κ gene rearrangements have been previously studied as additional markers of B-cell clonality.^{14–16,21} Although some investigators have thoroughly characterized the specific nature of V κ -J κ and V κ -KDE rearrange-

ments for use in minimal residual disease detection in B-lineage acute leukemias,^{14,21} relatively few efforts have evaluated the potential of using Ig κ PCR to augment IgH PCR for B-cell clonality determination in the routine molecular hematopathology laboratory setting.^{15,16} Our study is complementary to work from other groups and enhances previous data by investigating a broad range of mature B-cell neoplasms, using a simplified Ig κ PCR strategy.

This study is unique in also evaluating the frequency and nature of the KDE (specifically V κ -KDE) locus gene rearrangements in a series of commonly encountered mature B-cell malignancies. The addition of V κ -KDE analysis is particularly helpful in PCR clonality detection of both κ - and λ -expressing B-cell lymphomas. In our group of 68 well-characterized mature B-cell neoplasms, application of Ig κ PCR alone resulted in the demonstration of clonality in 72% of cases. Significantly, by combining the Ig κ and IgH PCR methods, the detection rate for clonality in B-cell lymphomas increased from 81 to 90%, with this effect being most evident among follicular lymphomas and diffuse large B-cell lymphomas. We attribute the relatively high overall rate of IgH clonality observed in this series to selection bias, as well as the inclusion of a relatively pronounced number of small B-lymphocyte neoplasms, which are typically positive in IgH PCR analysis using FR3-JH consensus primers. Both V κ -J κ and V κ -KDE rearrangements were observed in Ig κ PCR-positive cases, indicating the utility of the latter locus for clonality determination. Furthermore, comparison of V κ -J κ and V κ -KDE by surface light chain immunoglobulin type demonstrated κ locus clonal rearrangements in 85% of λ -expressing B-cell lymphomas, emphasizing the broad applicability of this approach. Of note, V κ -KDE clonal bands were also detected in two of seven cases of Hodgkin lymphoma, one of which also harbored a clonal IgH rearrangement. Pathological review of these two cases revealed syncytial histology, suggesting that sampling of these tissue areas may have enriched for detection of neoplastic B-lineage Reed-Sternberg cells. Among 18 cases of reactive B-cell proliferations and T-cell lymphomas, none yielded discrete bands with the Ig κ PCR. The dilutional sensitivity for this Ig κ PCR assay was found to be 10%; although better sensitivity is generally expected with PCR-based methods, this finding is in keeping with the experience of other investigators,²⁰ particularly when using a polyclonal B-cell background diluent. Nonetheless, this level of sensitivity should be considered when analyzing tissues only partially involved by a B-cell lymphoma using Ig κ PCR.

Despite substantially improving the overall clonal detection in B-cell neoplasms (when combined with IgH PCR), our Ig κ PCR assay is nevertheless associated with a false-negative rate. A number of factors account for this issue and the extensive work of the BIOMED-2 Concerted Action group is also instructive in this regard.²⁰ Firstly, the primer strategy used in our approach was relatively limited compared to the BIOMED-2 protocol for Ig κ PCR.²⁰ However, we opted to use a simplified set of primers targeting only V κ FR3 regions and fewer total primers, to reduce the complexity inherent in multiprimer

PCR techniques. Second, the single consensus J κ primer sequence used in our assays was slightly more degenerate, or mismatched against the J κ 3 gene segment, which may account for the absence of J κ 3 among the V κ -J κ gene rearrangements in Ig κ -positive cases. This effect, however, should have been mitigated in part by the use of the touch down PCR method as described. Similarly, the consensus J κ primer was not homologous with the J κ 5 gene segment, although this segment is infrequently used overall in V κ -J κ joinings. Third, we did not use an intron recombination signal sequence region primer to detect a subset of intron recombination signal sequence-KDE κ rearrangements known to occur at least in chronic B-cell leukemias.²¹ Lastly, the Ig κ loci may be germline in some λ -expressing mature B cells due to rare reversals of the usual, orderly gene rearrangement hierarchy, such that successful λ light chain gene rearrangements precede κ light chain rearrangements.^{10,22} As a technical footnote, the upper limit of the PCR product size range for V κ -KDE amplicons approaches 300 bp, which may prove problematic when amplifying this target in samples with excessive DNA degradation, although this situation was not encountered in our study. In short, our Ig κ PCR primer strategy sought to minimize complexity in favor of robust and unequivocal amplification results, while accepting a modest compromise in overall detection rate. As is evident from the data in this series of B-cell neoplasms, combined Ig κ and IgH PCR substantially improves the overall clonal detection rate, particularly for germinal center-related subcategories of lymphoma. In the cases of follicular lymphoma, the combined overall results, although improved, indicate the need for yet additional evaluation of BCL2/IgH rearrangements to further augment the clonality detection rate.

We assessed 56 cases by direct sequencing to determine the distribution of V κ and J κ gene families in V κ -J κ and V κ -KDE rearrangements and found the frequency of gene segment use generally reflected their representations in the genome. Most Ig κ rearrangements involved members of the V κ 1 and V κ 2 gene families, which account for 44% and 39% of functional germline V κ genes, respectively.^{23–25} In our analysis, there was a statistically significant relative overrepresentation of V κ 4 gene family usage (14%), which only accounts for ~2% of germline V κ genes. In keeping with the previous observations of others, V κ pseudogenes were not involved in any of the V κ -J κ rearrangements.²³ The distribution of V κ gene usage demonstrated in this study is also similar to other published series involving both normal and malignant B cells, with the exception of a comparatively greater degree of V κ 2 gene prevalence seen in our series.^{23,26–28} The V κ 2 gene family has been previously shown to be used more frequently than other V κ gene segments in nonproductive V κ -J κ rearrangements.²⁷ The relative prominence of V κ 2 gene usage seen in our series may thus in part be the result of nonproductive V κ -J κ rearrangements that favored the more frequent use of the V κ 2 region. Among these mature B-cell neoplasms, the distribution of J κ genes in V κ -J κ gene rearrangements was similar to other published series involving both normal and malignant B cells,^{22,23,26,27} with the J κ 1, J κ 2, and

J κ 4 gene segments used most frequently. The J κ 3 segment was not identified in any of our cases; however, at least in normal B cells, this J κ gene is only present in ~5% of V κ -J κ gene rearrangements.²⁷ Nevertheless, we cannot be completely certain that the inability to detect J κ 3 segment rearrangements in our cases represents a statistical artifact of sample size, or inefficient PCR detection due to compromised primer-template homology. Notably, no significant association was identified between subtypes of non-Hodgkin B-cell lymphoma and preferential use of κ gene families, although a larger study would likely be needed to adequately address this issue.

The distribution of V κ genes in V κ -KDE gene rearrangements in mature B-cell neoplasms was also approximately representative of their presence in the genome. Of interest, we documented occasional rearrangements involving V κ pseudogene segments (primarily V κ 2 and V κ 7 families) in the V κ -KDE-positive cases. As noted, the use of pseudogenes does not appear to occur in V κ -J κ rearrangements in either κ - or λ -positive B cells, but may occur in V κ -KDE joinings, perhaps as a mechanism to exclude unwanted or nonfunctional rearrangements.^{23,27} Regardless, the presence of such rearrangements remains a valuable clonotypic marker that can be detected with the PCR primers described in this study.

Finally, although PAGE was used in our cases to evaluate heteroduplexed PCR products, we compared standard PAGE analysis to a CBCE platform, to determine the utility of the latter method in detecting V κ -J κ and V κ -KDE rearrangements. CBCE analysis resulted in superior resolution for amplicon detection and permitted more accurate and rapid molecular fragment sizing than PAGE, with comparable dilutional sensitivity. In conclusion, this study demonstrates that the combined approach of IgH and Ig κ gene rearrangement analysis can significantly increase the detection rate of B-cell clonality in mature B-cell lymphomas. Multiplex Ig κ PCR followed by HDX-PAGE or CBCE is thus a useful ancillary diagnostic tool for detection of B-cell clonality in formalin-fixed paraffin-embedded tissues.

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