The Use of Immunoglobulin Gene Rearrangement Polymerase Chain Reaction Assays for Detection of B-Cell Clonality for Plasma Cell Neoplasms Using Novel PCR Primers

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Abstract

We have designed a standardized protocol with multiplex-primer sets capable of detecting majority IG kappa (IGK) and IG lambda (IGL) light chain rearrangements in plasma cell neoplasms (PCN). Thirty primers were combined in three multiplexed PCR reactions to target IGK, KDE and IGL rearrangements. Variable region (V) primers were designed to prevent “primer dimers”, provide matching melting temperatures (Tm), minimize amplicon size, and optimize sequencing time. Amplicons were subjected to capillary gel electrophoresis for analysis. In a discovery series, we tested 37 plasma cells neoplasms PCN (28 PCNs at diagnosis and 9 PCNs post-treatment). The assay investigated an additional 52 prospective PCN cases in the validation series. Results were compared to bone marrow morphology, immunohistochemical (IHC), flow cytometry data, and standard IGH FR III gene rearrangement assay. In the discovery series, the following sensitivities/specificities were obtained for mature B-cell neoplasms: IGH FR III: 29.7%/100%, IGK: 80.4%/100%, KDE: 25.0%/100%, and IGL: 35.1%/96.8%. The combination of IGH FR III, IGK, and KDE detected 83.6% (31/37) vs 67.3% (35/52) in the discovery vs validation series, respectively, for the PCN population. Interestingly, 21.2% (11/52) of the validation samples positive by IG clonality, were negative by IHC and flow cytometry. In IHC/flow cytometry positive cases with a PCN representing a tumor burden of >50%, 10% to 50%, 1% to 10%, 0% to 1% of cells, the combined sensitivity of the IG clonality assay was 100% (20/20), 72% (23/32), 53% (10/19) and 20% (1/5) respectively. This IGK/IGL clonality assay has good sensitivity at diagnosis.

Keywords: Immunoglobulin; B-cell receptor; Clonality; Lymphoproliferative disorder; Lymphoma; PCR clonality analysis; Hematopathology; Haematopathology

Introduction

The use of polymerase chain reaction (PCR) plays a critical role in the diagnosis and monitoring of multiple myeloma (MM) population [1]. Traditional methods of detection like serum free-light chains with kappa and lambda ratios, bone marrow morphology, FISH, and flow cytometry have been used to characterize B-cell neoplasms [2,3]. However, PCR based assay to monitor therapeutic response is becoming widely accepted in the routine clinical practice. The European collaborative study group created BIOMED-2 which is a comprehensive set of primers and standardized PCR protocols for the diagnostic clonality analysis of immunoglobulin (IG) gene rearrangements [4,5]. The combination of the PCR primer libraries demonstrates the ability to detect the vast majority of IG rearranged in both B and T cell lymphoid leukemia [1]. Other groups have designed consensus primers for IGK to accompany IGH primers to improve the detection of clonality in B-cell neoplasms using capillary electrophoresis [6]. These PCR designs demonstrate unbiased multiplexed detection of IG rearranged clones utilized for disease monitoring for MM population.

The use of PCR based detection of B-cell clones confers added benefit with uses in minimal residual disease (MRD) monitoring [7,8]. The presence of MRD correlates with poor outcomes in both high and low risk cytogenetic cohorts [3]. Next generation sequencing (NGS) has the potential to provide a universal method to examine multiple genes simultaneously to elucidate causative mutations that will allow for use of targeted therapies [9]. More specifically, a proprietary NGS assay has demonstrated usefulness in monitoring MM patients with high correlation with overall survival and longer time to tumor progression when molecular MRD is negative [10]. A requirement of NGS is a small and uniform amplicon size (<150 bp) and the BIOMED2 light chain primers do not meet this requirement [11]. Undersized amplicons have been shown to be representative of monoclonal lymphoproliferative disorder [12]. Smaller amplicons offer an advantage with higher percentage amplification during thermal cycling step in PCR and requiring less time on the sequencer with consumption of less lab technician time and materials.

This study was initiated to develop a PCR-based multiplexed, labs-developed assay for lymphoid leukemia with the goal of creating shorter amplicons without sacrificing detection rates. We have designed a standardized protocol and multiplex primer sets capable of detecting almost all possible IG kappa (IGK) and IG lambda (IGL) light chain rearrangements. A discovery series was used to determine the validity of the PCR assay, followed by a validation series of 52 consecutive PCN specimens that were subjected to clonality analysis during a 2-year period.

The purpose of this study was for the following:

i) determine the clinical and analytical specificity/sensitivity for clonality for this PCR assay,

ii) determine the utility for routine clinical evaluation of MM,

iii) to develop a practical strategy for application of B-cell clonality testing which can be applicable to other hematopoietic diseases,

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iv) to evaluate the efficacy of each individual PCR reaction and determine the best combination,
v) and to correlate tumor burden and B-cell clonality detection by this PCR assay.

Material and Methods

Specimens

A total of 172 specimens were submitted for clonality analysis for IG gene rearrangements during a period from 2010 to 2014. 120 B-cell neoplasm specimens were involved in the discovery series and 52 PCN specimens were involved in the validation series. The discovery testing of the cohort of 120 patients was performed retrospectively. 3/120 were from peripheral blood, 59/120 were from bone marrow aspirates, 1/120 from cytology cell block, 52/120 FFPE tissue blocks, 5/120 specimens from fresh tissue. 22/120 cases were large B-cell lymphomas, 5/120 B-ALL, 2/120 T-cell lymphoma, 37/120 plasma cell neoplasms (28 PCN at diagnosis, 9 PCN post-treatment), 3/120 CLL/SLL, 5/120 marginal zone lymphoma, 3/120 follicular lymphoma, 2/120 mantle cell lymphoma, 23/120 normal control tonsils, 1/120 colonic mucosa with lymphoid aggregates, 1/120 hairy cell leukemia, 5/120 myelodysplastic syndrome, 11/120 unclassified. The sample sources were 66 bone marrow specimens, 3 peripheral blood, 11 lymph nodes, 23 tonsils, 11 soft tissue tumors, 5 skins, and 1 FNA sample. Although, in the discovery series, a variety of B-cell lymphoproliferative neoplasms were tested using the lab-developed IG clonality primers, we narrowed the scope of the analysis to compare specifically plasma cell myeloma population. Some of the discovery specimens were sent to Mayo clinic and Clarient Inc. for detection confirmation with BIOMED-2 primers. No difference was observed between paraffin and fresh/frozen specimens as long as DNA quality was adequate for analysis.

In the subsequent validation series from November 2012 until March 2014, a total of 52 consecutive specimens from 43 patients with PCN were submitted for IG rearrangement clonality analysis. These validation cases represent prospective clinical cases. All of the specimens were obtained from fresh from bone marrow (BM) aspirates 51/52, with the exception of 1/52 specimen was obtained from a kidney biopsy. The patients who were treated either with chemotherapy and/or stem cell transplant constituted 62% (32/52) while the IG clonality testing at diagnosis with no prior treatment constituted 36.5% (19/52) with 1.9% (1/52) with an unknown clinical history. The further classifications of plasma cell neoplasms included the in-validation series are listed in the order of frequency: MM 86.5% (45/52), MGUS 7.6% (4/52), Waldenstrom’s macroglobulinemia 3.8% (2/52), plasmacytoma 1.9% (1/52). In the validation series when specimens that were grossly positive on light microscopy and flow cytometry, IG clonality testing was not performed because it was deemed medically unnecessary due to obvious tumor involvement, thus were excluded from the study.

DNA extraction

DNA preparation from paraffin specimens used 7μm sections. The areas of interest were identified on hematoxylin–eosin stained slides along with the corresponding unainted paraffin sections. The tumor areas were microdissected from the slide into a micro-centrifuge tube. The tissue was deparaffinized with xylene and with ethanol. The DNA sample was suspended in ATl buffer and Proteinase K was used to digest the milieu of the DNA extraction. The sample was placed in 56°C overnight to aid with digestion. The sample was placed on the QiaCube (Qiagen, Valencia, CA) for further DNA extraction/purification following standard protocol from the manufacturer.

| Gene Name (s)  | Tm  | Self-dimer G (kcal/mol) |
|----------------|-----|------------------------|
| IGK1           | 53.7| -6.3                   |
| IGK2           | 53.8| -6.3                   |
| IGK3           | 51.1| -6.3                   |
| IGK4           | 53.6| -6.3                   |
| IGK5           | 54.3| -6.3                   |
| KDE            | 54.8| -6.2                   |
| IGKV 1-5 and 1D-42 | < 54 | -3.55                  |
| IGKV 1-6, 8, 9, 13, 16, 17 | 54 | -3.55                  |
| IGKV 1 D-8, 13, 16, 17, 43 | 54 | -3.55                  |
| IGKV 1-12 and 1D-12, 39 | 52.5 | -3.55                 |
| IGKV 1-22, 1D-22 | 52.7 | -7.05                  |
| IGKV 1-27, 37, 39 and 1D-27, 37 | 52.6 | -3.54                   |
| IGKV 1-32 and 1D-32 | 56.7 | -3.91                   |
| IGKV 1-32 and 1D-32 | 50.6 | -3.55                   |
| IGKV 1-35 and 1D-35 | < 52.6 | -3.54                   |
| IGKV 2-4, 10, 14 and 2D-10, 14 | 56.4 | -5.14                   |
| IGKV 2-18, 19, 23, 24, 26, 29, 30 | 53.8 | -1.94                   |
| IGKV 2D-18, 19, 23, 24, 29, 30 | 53.8 | -1.94                   |
| IGKV 2-26, 40 and 2D-26, 40 | 51.7 | -2.94                   |
| IGKV 2-36, 2D-36 | 48.7 | -4.5                    |
| IGKV 2-38, 2D-38 | 54.7 | -6.76                   |
| IGKV 3-7, 11, 15 and 3D-7, 15, 52 | 52.2 | -7.05                   |
| IGKV 3-20 and 2D-20 | 53.7 | -7.05                   |
| IGKV 3-25 and 3D-25 | 55.2 | -7.05                   |
| IGKV 3-31 and 3D-31 | 53.9 | -7.05                   |
| IGKV 3-34 and 3D-34 | 52.4 | -5.36                   |
| IGKV 4-1 | < 52.2 | -7.05                   |
| IGKV 5-2 | 50.2 | -4.65                   |
| IGKV 6D-21 | < 53.7 | -7.05                   |
| IGKV 6-21 | < 53.7 | -7.05                   |
| IGKV 6D-41 | < 50.6 | -3.91                   |
| IGKV 7-3 | 49.6 | -7.05                   |
| IGLJ1*01 | 56.1 | -6.3                   |
| IGLJ2 | 56.7 | -6.3                   |
| IGLJ3 | 56.7 | -6.3                   |
| IGLJ4*0RF | < 56.7 | -3.6                   |
| IGLJ6*0TORF | 56.5 | -3.6                   |
| IGLJ7 | 55.9 | -3.6                   |
| IGLV 1-36, 40, 44, 47, 50, 2-8, 11, 4-60 | 52.7 | -3.14                   |
| IGLV 1-41, 11-55 | 52.8 | -3.61                   |
| IGLV 1-41*02 | 43.5 | -3.61                   |
| IGLV 1-51 | 50.5 | -3.61                   |
| IGLV 2-5 | 54.8 | -3.61                   |
| IGLV 2-51, 2-14, 18, 23, 34 | 54.8 | -3.61                   |
| IGLV 2-33 | 50.1 | -3.53                   |
| IGLV 3-1 | 52.2 | -3.14                   |
| IGLV 3-9, 12 | 50.52-52.2 | -3.14                   |
| IGLV 3-10*01 | 51.6 | -3.14                   |
| IGLV 3-10*02 | < 51.6 | -3.14                   |
| IGLV 3-13 | 42 | -3.61                   |
| IGLV 3-16, 22, 25, 31 | 52.2 | -3.61                   |
| IGLV 3-19, 25, 5-39 | 50 | -3.14                   |
| IGLV 3-21 | 50 | -3.14                   |
| IGLV 3-27, 4-68, 5-37, 45, 48, 52 | 53.4 | -3.14                   |
| IGLV 3-32 | - | -3.14                   |
| IGLV 4-3 | 46.9 | -3.14                   |
| IGLV 6-57 | 54.1 | -3.14                   |
| IGLV 7-46 | 54.3 | -3.9                   |
| IGLV 7-43 | 52.4 | -3.9                   |
| IGLV 8-61 | 46.9 | -4.99                   |
| IGLV 9-49 | 56.2 | -4.89                   |
| IGLV 10-54 | 56.4 | -3.61                   |

Table 1: IGK and IGLR primer binding properties: The binding Tm is within 6°C for greater than 90% of the V and J gene segments.
The fresh aspirates was obtained from the bone marrow was collected in EDTA tube. Saline, AL buffer, and proteinase K were added to the tube. The tube was heated to 56°C for 10 mins then the sample was centrifuged. The pellet was isolated and the sample was run on the QiaCube using the QIAamp DNA blood mini protocol. Severely hemolyzed whole blood and/or clotted or frozen whole blood/bone marrow specimens were rejected.

The DNA quantitation was performed using NanoDrop (ThermoScientific, Wilmington, DE) using the manufacturer’s standard protocol and the DNA concentration and purity were recorded.

**PCR**

The PCR amplifications were performed on the commercial Bio-Rad real-time PCR thermal cycler. The PCR master mixes and controls used standardized PCR BIOMED-2 protocol [4]. Each PCR reactions contained between 50 ng to 200 ng of DNA and each run was in duplicates. Thirty primers were combined in three multiplexed PCR reactions to target IGK, KDE and IGL rearrangements. The full set of IG gene arrangements included the primers set to target IGK (18 VK, 2 JK), KDE (18 VK, 1 KDE) and IGL (7 VL, 2 JL) rearrangements [13] (Table 1). BIOMED-2 variable region (V) primers were used for the IGH framework III consensus region. Mutations were introduced to primer sequences to prevent “primer dimers” and obtain matching melting temperatures (T_m).

**Capillary gel electrophoresis**

Amplicons were subjected to capillary gel electrophoresis on an ABI 3500 (Applied Biosystems by Life Technologies, New York) [14]. The migration pattern of duplicate PCR products were reviewed for reproducibility and the fragment pattern compared to a negative polyclonal control (Figure 1). The rearrangements in a clonal specimen demonstrated discrete peak(s) with a distinct size range with or without a polyclonal background. Homoduplex bands and oligoclonal bands were compared with baseline testing. In cases with equivocal or weak bands, the amplifications were reported as such. The sample base pairs (BP) sizes of the PCR amplicons were performed with baseline testing at diagnosis compared to subsequent testing. The amplicons were considered identical clones to the baseline testing if the sizes were within 1 BP.

**Correlation with orthogonal methods**

Results of the light chain gene rearrangement assay were correlated with bone marrow morphology, immunohistochemical (IHC) profile, flow cytometry data and a standard IG heavy (IGH FRIII) chain assay. The final diagnosis was made based on clinical, morphological, immunophenotype and molecular genetic classification based on the WHO classification of tumors of hematopoietic and lymphoid tissues. A board-certified hematopathologist was involved with the interpretation of the diagnosis in each case. The 52 clinical cases represented positive MM cases by flow cytometry, IHC, and/or IG rearrangement B-cell clonality study, during the 2 years of this study. Bone marrow plasmacytosis was determined to be positive with >10% plasma cell percentage in the marrow determined by morphology and IHC using H&E staining, CD138 staining and kappa and lambda staining. The flow cytometric immunophenotyping was used to determine the presence of a plasma cell neoplasm with the looking an aberrant cluster of cells that were CD19 negative, CD20 positive, cytoplasmic CD138 positive with kappa or lambda restriction, and looking for aberrant immunophenotype in this cell population. The estimations of tumor burdens were determined from percentage of plasma cells from the H&E/IHC and/or flow cytometry.

**Results**

Detection of the clonal IG rearrangement in the discovery series demonstrated the following sensitivities/specificities for the mature B-cell lymphoproliferative disorders: IGH FRIII-29.7%/100%, IGK-80.4%/100%, KDE-25.0%/100%, and IGL-35.1%/96.8%. In the subpopulation of PCNs, the combination of IGH FRIII, IGK, and KDE detected 83.8% (31/37) of the PCNs and the combination of IGK, KDE, and IGL detected 86.5% (32/37) (Table 2). Clonal IG rearrangement was not detected in 13.5% (5/37) of the diagnostic specimens in the discovery series despite a morphological and flow diagnosis of plasma cell neoplasm.
be a true positive based on the electrophoretic mobility of the baseline clonal amplicons. One case (1/11) did not have baseline testing, thus confirmation of a true positive result was not possible. One case (1/11) demonstrated a different clone than found on previous baseline studies. Of the 52 clinical cases, FISH was not performed on 4 cases, but of the cases tested, FISH was able to detect an abnormality in 30/48 (62.5%) of the MM cases with only 2/48 (4.2%) being label equivocal. The serum free light chain with kappa/lambda ratios were performed in 50 of the 52 cases within 2 week prior to the BM biopsy and was able to detect 28/50 (56.0%) cases where the ratio cut off of >1.65.

The detection rates of B-cell clonality correlated directly with tumor burden of the PCN specimens. The data from both the discovery and validation series can be seen in Table 3. The results of in IHC flow cytometry positive cases with a PCN representing a tumor burden of >50%, 10% to 50%, 1% to 10%, 0% to 1% of cells, the combined sensitivity of the IG clonality assay was 100% (20/20), 72% (23/32), 53% (10/19) and 20% (1/5) respectively (Figure 2). The tumor burden rates were obtained from the reports generated from flow cytometry and estimation from IHC reporting. There was a strong correlation between tumor burden and detection rates using PCR based B-cell clonality testing. In rates over >50%, the IG clonality assay detected 100% of the positive cases, while only 20% was detected if tumor burden was <1%.

### Discussion

A number of studies have used PCR-based B-cell clonality testing for investigations of IG and TCR gene rearrangements in lymphoproliferative disorders [6,15-17]. Majority of the testing was using BIOMED-2 primers and the published results are promising, and with the different combination of multiplex assays can detect up to 95% of MM [1,15,16]. The best combination of the BIOMED-2 primers, was shown to be IGK, with either IGH or IGK to generate the best two reaction combinations with the detection rate of 79% using IGH FRIII+IGK VJ+IGK KDE (Table 2). Our results in the discovery series indicate that the detection rates are 83.8% of PCN using our lab-developed PCR assay using the same multiplex combination. Our detection rates are for B-cell clonality testing in the MM population are non-inferior using BIOMED2 primers. The advantage our primer library confers is that they are shorter and target a different region of the IG rearrangement. Thus, our amplicons are unique and shorter. However, in clinical practice, the detection rate is at 67.3% using the combination (IGHFRIII+IGK VJ+IGK KDE) which is closer to the real rate of detection. The clinical detection of PCN using the B-cell clonality PCR assay will be lower than the discovery series because of the selection bias in the cases tested with higher tumor burdens (Table 3). The discovery series selected more cases with tumor burdens greater than 50% with the validation series included more MRD cases and with cases with <1% tumor burdens. This is important as the real detection rates will be lower than the discovery series as in clinical practice, a wide variety of samples from post-chemotherapy ablated marrow and post-stem cell transplant will be part of the sample mixture.

There were 32.7% (17/52) number of cases, where the B-cell PCR based clonality testing did not detect PCN even though it was detected by microscopy/IHC and flow cytometry. The PCR primers may have targeted areas in the IG genes near regions of somatic hypermutation which prevents the annealing of the IG primers, leading to inefficient amplifications of the clonal IG rearrangement. In addition, the another possible explanation is the MM clones may have cytogenetic anomalies like FGFR3-IGH rearrangement of (t(4,14)p16.3, q32) which does not amplify in the multiplex PCR reaction, especially if the region of the translocation is targeted by the PCR primer library.
The amplicons vary based on the primer position and the expected extent of the insertion at the junctions. The majority of PCR products in BIOMED2 above 300 BP [15,18]. Our average amplicons for amplicon extent of the insertion at the junctions. The majority of PCR products in our lab along with the appropriate pathways for other suspected B-cell clonality in MM for fresh and fixed specimen has been defined as the tumor burden percentage requires a microscopic or flow cytometry assessment. This group can represent an independent subclone present at low concentration at diagnosis that survived treatment or can represent a newly acquired mutation from the previous clone. Clonality testing helps detect a small but significant percentage of MM cases that otherwise will not be identified using traditional methods.

A sensitive combination of three or more reactions included IGH FR3, IGK VJ and IGK VDE. From a practical point of view, the use of additional primers for IGL did not add significantly more coverage to the clonality screening. A strategy for the routine use of PCR assay for B-cell clonality in MM for fresh and fixed specimen has been defined in our lab along with the appropriate pathways for other suspected B-cell PCNs. Currently, experimentation with one-tube multiplex combination has proven to be promising (Figure 3). Although not validated or routinely performed, IGK VJ and IGK KDE primer combinations have been designed to produce the individual clonal peaks on capillary electrophoresis. This confers benefits as smaller samples can be interrogated for clonality if one tube assay can be performed. Other lymphoproliferative disorders have been assessed using this PCR-based assay not reported in this study. Currently, TCR-γ is currently not being utilized but plans are in the works for its development for looking at T-cell clonality.

Once PCR clonality testing is coupled to next generation sequencing (NGS), the detection rates of MM will be much more sensitive. Martinez-Lopez et al. has determined that NGS of amplicons have detection rates of I in 100,000 cells [10]. The high throughout methods used in the study used lymphoSIGHT™ (Sequenta Inc., San Francisco, CA) method for acquisition of B-cell clonality amplicons sequencing for MRD testing. The strategy is for NGS baseline testing at diagnosis to provide the clonal sequence information like finger print of the tumor. Subsequent NGS testing can look for that specific clone with the exact sequence determined at baseline testing to determine if the sample has minimal residual disease. This method demonstrates MRD assessment using deep sequencing is a useful technique for determination of patient risk stratification for clinical vs molecular remission. Challenges in MRD testing will be the standard criteria for molecular remission and demonstrate unexplored territory. Furthermore, additional subclone mutations can be followed if necessary as many MM can confer resistance to chemotherapy. NGS will allow for more sensitive MRD testing of MM and many other diseases and represent the future of cancer detection and follow-up.

In summary, this study demonstrates that the novel PCR primer sets in a lab-developed stage is non-inferior to BIOMED2 and has the added benefit of shorter amplicon read-time once coupled with NGS. The detection rate of 83.8% is comparable to the reported highest detection rates of 95% for BIOMED2. The best selected combination uses a stepwise multiplexed reaction for routine assessment of B-cell clonality in the MM population. This can be applicable to both fresh and fixed tissue.

We are willing to license these amplicons to academic institutions who are interested in creating an in-house B-cell clonality assessment for lymphoproliferative disorders.

**Conclusion**

This IGK/IGL clonality assay has good sensitivity at diagnosis. Currently, the assay lacks the analytical sensitivity needed for MRD testing. This shortcoming can be overcome with the adoption of deep sequencing, which our IG PCR primers are designed to accommodate. The discrepancy between detection rate in the discovery and validation series can be attributed to case selection bias.

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