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Multiple Immunoglobulin κ Gene Rearrangements within a Single Clone Unraveled by Next-Generation Sequencing—Based Clonality Assessment

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Clonality assessment of the Ig heavy- and light-chain genes (IGH and IGK) using GeneScan analysis is an important supplemental assay in diagnostic testing for lymphoma. Occasionally cases with an IGK rearrangement pattern that cannot readily be assigned to a monoclonal lymphoma are encountered, whereas the occurrence of biclonal lymphomas is rare, and the result of the IGH locus of these cases is in line with monoclonality. Three such ambiguous cases were assessed for clonality using next-generation sequencing. Information on the sequences of the rearrangements, combined with knowledge of the complex organization of the IGK locus, pointed to two explanations that can attribute seemingly biclonal IGK rearrangements to a single clone. In two cases, this explanation involved inversion rearrangements on the IGK locus, whereas in the third case, the cross-reactivity of primers generated an additional clonal product. In conclusion, next-generation sequencing—based clonality assessment allows for the detection of both inversion rearrangements and the cross-reactivity of primers, and can therefore facilitate the interpretation of cases of lymphoma with complex IGK rearrangement patterns. *(J Mol Diagn 2021, 23: 1097—1104; https://doi.org/10.1016/j.jmoldx.2021.05.002)*

Given that lymphomas are derived from a single malignantly transformed lymphoid cell, the tumor cells of virtually all cases of B-cell non-Hodgkin lymphoma contain clonal Ig heavy- and light-chain gene (IGH and IGK) rearrangements. Clonality assessment of these gene rearrangements is an important supplemental assay in the diagnosis of B-cell non-Hodgkin lymphoma.[1] The introduction of the standardized BIOMED-2 multiplex PCR protocols (now called *EuroClonality protocols*) has greatly increased the reliability of clonality testing in lymphoproliferations[2] and has resulted in their worldwide use. The accurate interpretation of IG clonality data is important for supporting diagnostics, and includes determining the number of B-cell clones present.[3] IG loci can rearrange on both alleles; in particular, if the rearrangement on the first allele is not productive (ie, does not result in an open reading frame), this rearrangement will be inactivated and the other allele will rearrange as well. Therefore, the presence of two clonal products per target is relatively common and most likely reflects the occurrence of biallelic rearrangements in a single clone rather than biclonality.[4] The interpretation of rearrangement patterns of the light-chain IGK locus can be especially challenging: Due to the specific configuration of the IGK locus,[5] the inactivation of this locus via an intron κ—deleting element (Kde) rearrangement does not delete the original IGKV-IGKJ rearrangement, resulting in two IGK rearrangements on the same allele. If this inactivation event

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occurs on both alleles, a total of four detectable rearrangements can be attributed to a single clone, a common observation in the IGK locus.[6,7]

In the setting of routine diagnostics, some GeneScan results obtained by the BIOMED-2 protocol have shown more than four clonal IGK products, or a combination of *IGK* rearrangements that cannot readily be assigned to a monoclonal lymphoproliferation. On the other hand, the occurrence of biclonal lymphomas is rare (ie, approximately <5% of all cases of lymphoma).[4] Biclonality mainly occurs in cases in which the morphology and/or the immunophenotype of the lymphoma also suggests a collision lymphoma. The evaluation of cases with multiple IGK rearrangements is difficult and may result in the inaccurate identification of a case as bi- or oligoclonal, which can result in confusion in diagnosis.

This article presents three cases of lymphoma that showed complex IGK-rearrangement patterns on analysis by GeneScan. The application of next-generation sequencing (NGS)-based assessment of clonality provided the sequences of the IG gene rearrangements. Their annotation and analysis using ARResT/Interrogate[8,9] pointed to two explanations of these puzzling results.

Materials and Methods

Three samples of lymphoma (cases 1, 2, and 3) were retrieved from the local archive of the Department of Pathology, Radboud University Medical Centre (Nijmegen, the Netherlands), and were collected in accordance with the

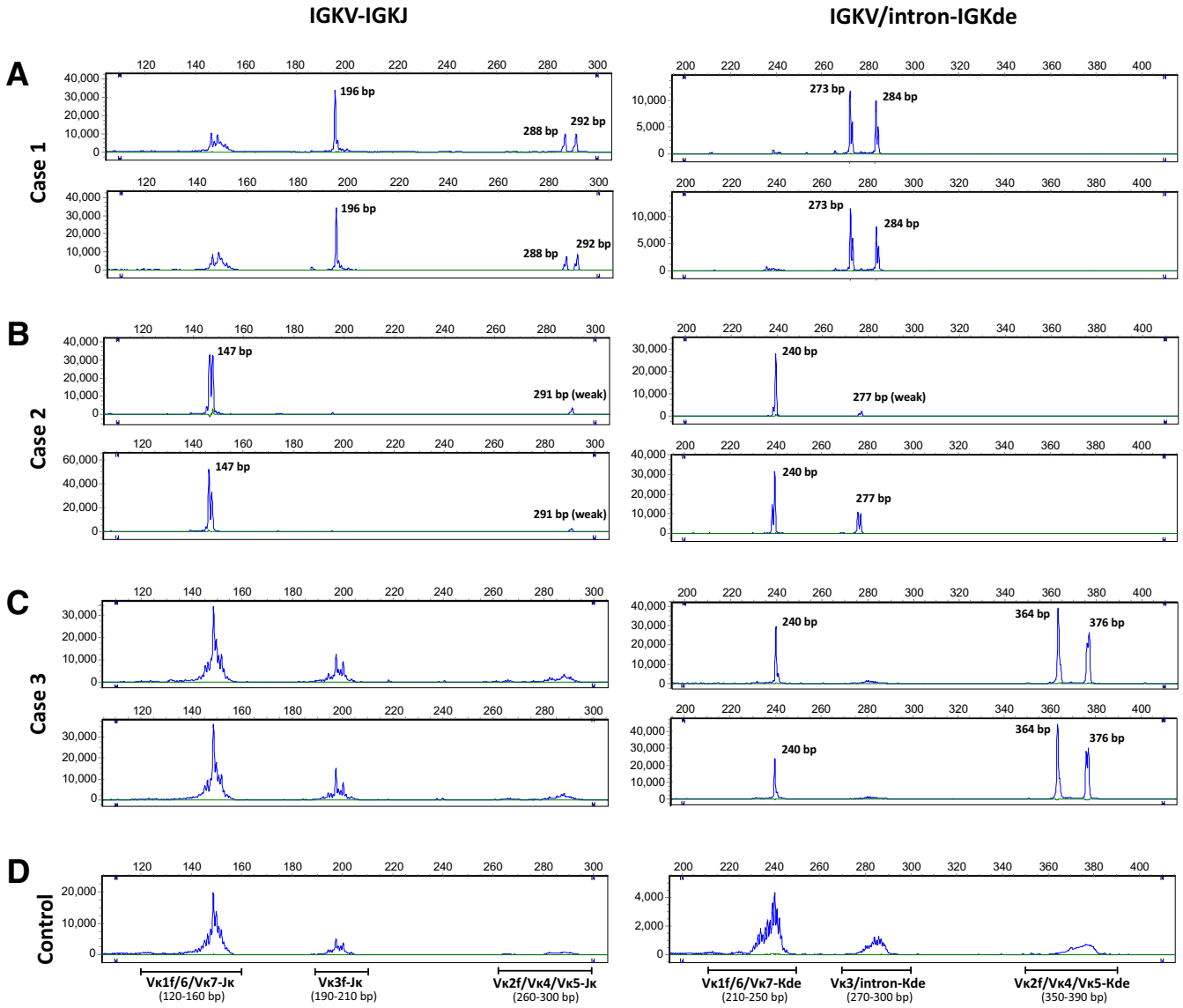


Figure 1 GeneScan results on the IGK locus of cases 1, 2, and 3 and a polyclonal control. All samples were analyzed in duplicate. **A:** Case 1, a dominant clonal IGKV-IGKJ product (196 bp) and two less-dominant clonal rearrangements (288 and 292 bp) are detected. Also, two clonal IGKV/intron-Kde rearrangements (273 and 284 bp) are detected. **B:** Case 2, a clonal IGKV-IGKJ product (147 bp), as well as an additional very weak rearrangement (291 bp), is observed. In addition, a clonal IGKV-Kde rearrangement (240 bp) and a clonal IGKV/intron-Kde rearrangement (277 bp) are detected. **C:** Case 3, three clonal IGKV-Kde rearrangements (240, 364, and 376 bp) are observed. **D:** A polyclonal control sample (tonsil); the size-window of the polyclonal control is indicated.

principles set forth in the Declaration of Helsinki. DNA samples were extracted from formalin-fixed, paraffin-embedded (FFPE) tissues according to standard procedures. Size-ladder PCR and the BIOMED-2 multiplex PCR for the analysis of IGH and IGK using GeneScan were performed according to standard protocols[1] using 20 and 40 ng of DNA (determined by Qubit fluorometer 3, Invitrogen, Carlsbad, CA) in 25 μL of PCR mixture. The FFPE tissue blocks yielded degraded DNA that allowed for the amplification of a 200-, 300-, and 400-bp product (cases 1, 2, and 3, respectively) as the largest product of the size-ladder PCR. The GeneScan results of the BIOMED-2 assays were scored according to a guideline on conventional BIOMED-2 clonality assays.[3]

IG clonality was detected using NGS as previously described.[8] In short, multiplex PCR was performed in 25 μL of PCR mixture using 40 ng of DNA as input, followed by library preparation and Ion Torrent sequencing (Ion PGM template OT2 200 kit, Ion Chef, and Ion OneTouch 2 or Ion S5 XL system; Thermo Fisher Scientific, Waltham, CA). Sequencing data were analyzed, annotated, and visualized with the ARResT/Interrogate software platform version 1.20.253,1.[9,10]

The PCR design of NGS-based clonality assessment is different from that of the conventional BIOMED-2/EuroClonality assays and results in smaller PCR fragments that are better suited to amplification using degraded DNA from FFPE material. The primers used in NGS-based IGKV-IGKJ and IGKV/intron IGKde assessment yield products in the size range of approximately 110 to 205 bp. In conventional PCR using GeneScan, the IGKV1f/6/V7-IGKJ/IGKde, IGKV3-IGKJ, IGKV3/intron-IGKde, and *IGK*2f/V4/V5-IGKJ/*IGK*de yield products in different size-windows. These size-windows vary from 120 to 160 bp, to 350 to 390 bp.[1] The differences in primer design between NGS-based clonality assays and the conventional BIOMED-2 clonality assays and GeneScan, as well as the suboptimal quality of the DNA in these three cases, explain why the frequencies of the rearrangements observed with NGS-based clonality detection did not fully correlate with the results obtained on conventional assay.

Results

Case 1 was a patient with follicular lymphoma. Clonality testing was performed to confirm the localization of the lymphoma in the bone marrow. In this lymphoma, three clonal IGKV-IGKJ rearrangements and two clonal Kde rearrangements were observed with GeneScan (Figure 1A), as compared to the polyclonal control with the typical Gaussian curves (Fig. 1D). The IGH locus demonstrated one clonal IGHV-IGHJ rearrangement and polyclonal IGHD-IGHJ rearrangements (Table 1). The final molecular interpretation of the clonality status is based on the integration of the rearrangements of both the

Table 1 Results of Conventional BIOMED-2 Clonality Assays and EuroClonality NGS–Based Clonality

		GeneScan results		NGS clonality results		
Case	*IG* locus	Peak size	Size-window	Clonotype	Frequency, %	Productive/unproductive
1	IGHV-IGHJ	C125 bp	n.a.	V3-23(D) -2/25/-6 J4	82	Productive
	IGHD-IGHJ	Polyclonal	n.a.	Polyclonal	n.a.	n.a.
	IGKV-IGKJ	C196 bp	VK3f-JK	V3D-11 -1/0/-4 J5	28	Unproductive
		C288 bp	VK2f/VK4/VK5-JK	V2(D)-30 -7/2/-0 J4	31	Unproductive
		C292 bp	VK2f/VK4/VK5-JK	V2D-29 -0/0/-0 J1	28	Productive
	IGKV/intron-Kde	C273 bp	IGKV/intron-Kde	Intron -1/1/-13 Kde	56	n.a.
		C284 bp	IGKV/intron-Kde	Intron -0/0/-1 Kde	34	n.a.
2	IGHV-IGHJ	No specific product	n.a.	V1-18 -4/17/-5 J5	95	Productive
	IGHD-IGHJ	No specific product	n.a.	No specific product	n.a.	n.a.
	IGKV-IGKJ	C147 bp	VK1f/6/VK7-JK	V1(D)-27 -1/0/-4 J5	55	Unproductive
		Cw291 bp	VK2f/VK4/VK5-JK	V2-24 -1/0/-0 J5	40	Unproductive
	IGKV/intron-Kde	C240 bp	VK1f/6/VK7-Kde	V1(D)-27 -3/0/-1 Kde	55	n.a.
		Cw277 bp	IGKV/intron-Kde	Intron -1/0/-8 Kde	43	n.a.
3	IGHV-IGHJ	C146 bp	n.a.	V3 -6/46/-2 J4	52	Productive
	IGHD-IGHJ	Polyclonal	n.a.	Polyclonal	n.a.	n.a.
	IGKV-IGKJ	Polyclonal	n.a.	Polyclonal	n.a.	n.a.
	IGKV/intron-Kde	C240 bp*	VK1f/6/VK7-Kde			
		C364 bp	VK2f/VK4/VK5-Kde	V4-1 -2/0/-8 Kde	63	n.a.
		C376 bp	VK2f/VK4/VK5-Kde	V(D)2-28 -4/2/-0 Kde	28	n.a.

Note: IGK V2D-29 gene (case 1) can be assigned as distal by ARResT/Interrogate version 1.20.253,1.

n.a. = not applicable, as: i) the IGHV-IGHJ rearrangements are centered in one size-window, or ii) this is not applicable for polyclonal or no specific product scorings, or iii) an IGKV/intron-Kde rearrangement cannot be productive or unproductive; C, clonal; Cw, clonal weak; IGKV(D), the gene is either a proximal or a distal (D) IGKV gene that cannot be distinguished based on the sequence information.

*Product in GeneScan due to cross-annealing of primers.

Table 2 Sequence Information of the Clonal *IG* Rearrangements

Case	Clonal rearrangement	Sequence	Reads
1	IGH V3-23D -2/25/-6 J4	5′-TCCAGAGACAATTCCAGGAACACACTGTATCTGCA AATGAACAGCCTGAGAGCCGAGGACACGGCCGTATACTACTGT GCGAAAAATGAAACCCAACCAGGGGGGGCCCTTGACTTCTGGG GCCAGGGAACCCT-3′	9622
	IGK V3D-11 -1/0/-4 J5	5′-GGCCTGGGACAGACTTCACTCTCACCATCAGCAGC CTAGAGCCTGAAGATTTTGCAGTTTATTACTGTCAGC AGCGTAGCAACTGGCATCACCTTCGGCCAA-3′	62,721
	IGK V2D-30 -7/2/-0 J4	5′-GGTCAGGCACTGATTTCACACTGAAAATCAGCAGGGT GGAGGCTGAGGATGTTGGGGTTTATTACTGCATG CAAGGTACACACTAGGCTCACTTTCGGCGGA-3′	70,921
	IGK V2D-29 -0/0/-0 J1	5′-GCAGCGGGTCAGGGACAGATTTCACACTGAAAATC AGCCGGGTGGAGGCTGAGGATGTTGGGGTTTATTACT GCATGCAAAGTATACAGCTTCCTCCGTGGACGTTCGGCCAA-3′	63,696
	IGK intron -1/1/-13 Kde	5′-CACCGCGCTCTTGGGGCAGCCGCCTTGCCGCTAGTGGCCGTGGCCA CCCTGTGTCTGCCCGATTGATGCTGCCGTAGCCAGCTTTCCTGATCCAG CCCAGGGCGACTCCTCATGAGTCTGCAGCTGC-3′	23,725
	IGK intron -0/0/-1 Kde	5′-CACCGCGCTCTTGGGGCAGCCGCCTTGCCGCTAGTGG CCGTGGCCACCCTGTGTCTGCCCGATTAATGCTGCCGTAGCCAGCTT TCCTGATGGAGCCCTAGTGGCAGCCCAGGGCGACTCCTCATGAGTC TGCAGCTGC-3′	14,311
2	IGH V1-18 -4/17/-5 J5	5′-CATGACCACAGACACATCCACGAACACGGCCTACATGGAC CTGAGGAGCCTGAGATCTGACGACACGGCCATATATTAT TGTGCGACAAATAATAGTGGTACTTGGTTCGACCCCTGGG GCCAGGGAACCCT-3′	7775
	IGK V1-27 -1/0/-4 J5	5′-TGGATCTGGGACAGATTTCACTCTCACCATCAGCAGCCTGC AGCCTGAAGATGTTGCAACTTATTACTGTCAAAAGT ATAACAGTGCCCCTCACCTTCGGCCAA-3′	68,220
	IGK V2-24 -1/0/-0 J5	5′-GGGCAGGGACAGATTTCACACTGAAAATCAGCAGG GTGGAAGCTGAGGATGTCGGGGTTTATTACTGCATG CAAGCTACACAATTTCCTCGATCACCTGCGGCCA AGGGACACGACTGGAGATTAAACGT-3′	49,671
	IGK V1-27 -3/0/-1 Kde	5′-TGGATCTGGGACAGATTTCACTCTCACCATCAGCAG CCTGCAGCCTGAAGATGTTGCAACTTATTACTGTCAAAGT ATAACAGTGCCCCGAGCCCTAGTGGCAGCCCAGGGCGAC TCCTCATGAGTCTGCAGCTGC-3′	51,329
	IGK intron -1/0/-8 Kde	5′-CACCGCGCTCTTGGGGCAGCCGCCTTGCCGCTA GTGGCCGTGGCCACCCTGTGTCTGCCCGATTGATGCT GCCGTAGGCAGCTTTCCTGATAGTGGCAGCCCAGGGCG ACTCCTCATGAGTCTGCAGCTGC-3′	41,637
3	IGH V3 -6/46/-2 J4	5′-TCCAGAGACAATTCCAAGAACACACTGTATCTCCAAAT AAACAACCTGAGAGCCGAGGACACGGCTGTATATTAT TGTGCACGAGATAATGAGAGTTGTATATATGGTGT CTGTAAGATGAAATATTACTTTGACTACTGGGGCCAGGGAACTCT-3′	29,131
	IGK V4-1 -2/0/-8 Kde	5′-GCGGGTCTGGGACAGATTTCACTCTCACCATCAGCAG CCTGCAGGCTGAAGATGTGGCAGTTTATTACTGTCAG CAATATTATAGTACTCCTAGTGGCAGCCCAGGGCGAC TCCTCATGAGTCTGCAGCTGC-3′	56,287
	IGK V2D-28 -4/2/-0 Kde	5′-GATCAGGCACAGATTTTACACTGAAAATCAGCAGAGTGGA GGCTGAGGATGTTGGGGTTTATTACTGCATGCAAG CTCTACAAACTCTTGGAGCCCTAGTGGCAGCCCAGGGC GACTCCTCATGAGTCTGCAGCTGC-3′	24,872

IGH and IGK loci. In this case, the rearrangement of the IGH locus was in line with a monoclonal cell population; however, the presence of three clonal IGKV-IGKJ rearrangements as well as two Kde rearrangements did not fit with the expected rearrangement pattern of a monoclonal lymphoma, or even with a biallelic IGK-

rearranged lymphoma. This rearrangement pattern necessitated closer inspection for assignment as either monoclonal or biclonal.

With NGS-based clonality analysis using ARResT/Interrogate, a single clonal rearrangement on the IGH locus and five clonal rearrangements on the IGK locus were found (Tables 1 and 2). The bone marrow biopsy sample from this patient demonstrated the same three IGKV-IGKJ and two intron-Kde clonotypes in a similar distribution, as well as the same IGHV-IGHJ clonotype as in the primary lymphoma.

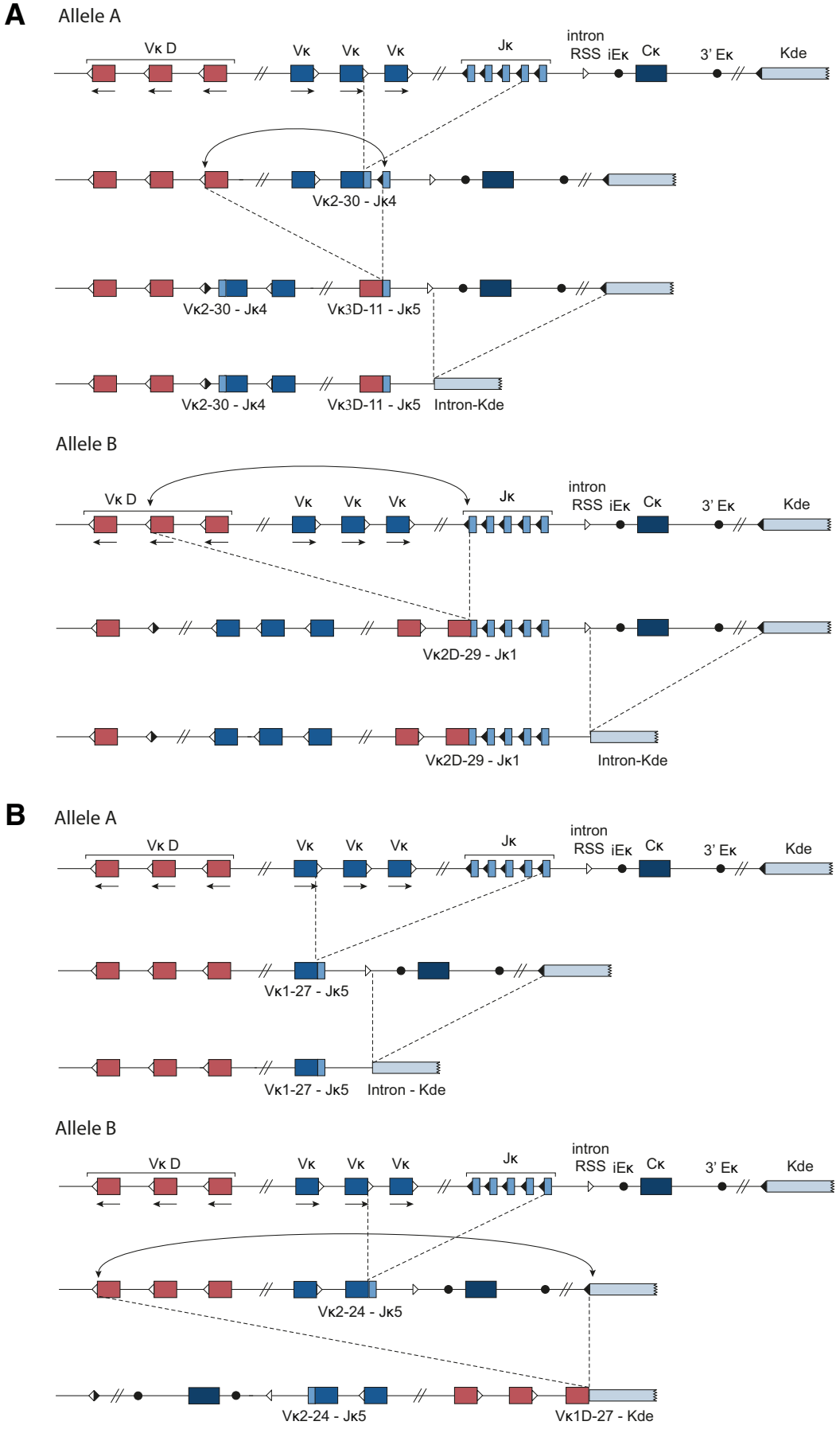
Annotation of the IGK genes with ARResT/Interrogate showed that two and possibly three of the clonal IGKV-IGKJ rearrangements involved genes from the distal IGKV(D) cluster. This cluster is located approximately 800 kb upstream of the proximal IGKV cluster in the opposite chromosomal orientation. Given that the IGKV(D) cluster was duplicated from the IGKV cluster during evolution, the IGKV(D) and IGKV gene sequences are very similar and often cannot be distinguished. More importantly, because the IGKV(D) cluster has the genome orientation opposite that of the IGKJ cluster, an IGKV(D)-to-IGKJ recombination event results in an inversion of the intervening DNA rather than a deletion, which is the general process of *IG* rearrangements. Consequently, when the intervening DNA is maintained in the genome, any previous IGKV-IGKJ rearrangement on the same allele can theoretically still be detected.[5,7] It was hypothesized that such an inversion event took place, in addition to an unproductive clonal IGKV-IGKJ rearrangement on one of the alleles. This inversion event explains the presence of a third IGKV-IGKJ rearrangement in case 1, and therefore makes it possible to assign the observed rearrangement pattern to a biallelically rearranged monoclonal B-cell population. The proposed consecutive rearrangements in case 1 are shown in Figure 2A. Notably, the proposed rearrangement pattern does not have to be the exact order in which the rearrangements occurred, but it is an example of how an inversion event could explain the observed rearrangements in this case.

Case 2 was a patient with a history of follicular lymphoma. Seven years later, this patient developed a low-grade B-cell lymphoma with morphologic features similar to those of marginal zone lymphoma. The detection of a *BCL2* translocation confirmed the recurrence of the follicular lymphoma, now with marginal zone differentiation. The lymphoma showed a prominent clonal IGKV-IGKJ rearrangement plus a weak IGKV-IGKJ rearrangement (Figure 1B) on conventional clonality analysis using GeneScan in comparison to the polyclonal control (Figure 1D) . In addition, a clonal IGKV-Kde rearrangement and a clonal IGKV/intron-Kde rearrangement were observed. The presence of an IGKV-Kde rearrangement would normally imply that any previous IGKV-IGKJ rearrangement on the same allele, as well as the entire IGKJ cluster, has been deleted, disabling further rearrangements on the same allele.[7] On the other allele, again an IGKV-IGKJ rearrangement in combination with an intron-Kde rearrangement can potentially occur. However, the second IGKV-IGKJ rearrangement observed in this case would not fully fit with the presence of a single clone. Finally, no clonal IGHV-IGHJ and IGHD-IGHJ rearrangements were detected, further hampering assignment as clonal. Given that there was no evidence of the presence of two lymphomas on morphology or immunophenotyping, the IGK rearrangements needed closer inspection.

NGS-based analysis of clonality in case 2 again showed a possible involvement of genes from the IGKV(D) cluster (Tables 1 and 2). An inversion event resulting from the recombination of an IGKV(D) gene with the Kde would preserve the original IGKV-IGKJ rearrangement on the genome. This inversion event explains how the observed combination of IGK rearrangements in case 2 can belong to a biallelically rearranged monoclonal B-cell population. The proposed rearrangement pattern in case 2 is presented in Figure 2B. Unfortunately, the DNA of the primary lymphoma in this patient was severely degraded, disallowing NGS-based detection of immunoglobulin clonality. The assessment of a bone marrow biopsy sample from the same year as the lymph-node biopsy of the recurrent lymphoma showed the same two IGKV-IGKJ clonotypes, the same IGKV-Kde and intron-Kde clonotypes in a similar distribution, as well as the same IGHV-IGHJ clonotype as in the lymphoma.

Case 3 was a consultation case, with a fine-needle aspirate sample of an abdominal process with a possible low-grade B-cell lymphoma, not otherwise specified. Clonality testing was performed and supported a diagnosis of a B-cell lymphoma. Three clonal IGKV-Kde rearrangements were observed with GeneScan (Figure 1C) as compared to the polyclonal control (Figure 1D). Given that each allele of the IGK locus has only one Kde, the presence of more than two clonal Kde rearrangements suggests that more than a single B-cell clone is involved. However, IGH demonstrated a single clonal IGHV-IGHJ rearrangement and no clonal IGHD-IGHJ rearrangement.

In contrast to the three IGKV-Kde rearrangements detected on conventional clonality analysis using GeneScan, NGS-based clonality testing in case 3 detected only two IGKV-Kde rearrangements (Tables 1 and 2), which fit a single clone. Closer inspection of the sequences of these rearrangements and the primer sequences used for the BIOMED-2 clonality assay (GeneScan) and the EuroClonality NGS assay, provided insight into the underlying mechanism. It was hypothesized that cross-reactivity, or cross-annealing, of one of the BIOMED-2 primers to a highly similar IGKV gene might have taken place in the multiplex PCR, resulting in an additional PCR product for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one of the IGKV-Kde rearrangements. As indicated in Table 1, the rearrangement at 364 bp corresponded to IGKV-Kde [V4-1 -2/0/-8 Kde], and the rearrangement at 376 bp is the same as IGKV-Kde [V2(D)-28 -4/2/-0 Kde] detected with NGS. The rearrangement at 240 bp was not detected with NGS. A rearrangement of 240 bp would start 136 bp downstream from the used BIOMED-2 primer (376 − 136 = 240 bp) for IGKV*2-28*. The sequence at this location is highly similar to the IGKV1f/6 BIOMED-2 primer binding site. It is therefore very likely that cross-annealing of the IGKV1f/6 BIOMED-2 primer to the clonal IGKV*2-28-IGKJ* rearrangement resulted in the detection of a third IGKV-Kde clonal peak at 240 bp on GeneScan analysis, next to the correct IGKV-Kde rearrangements.

Discussion

In the three cases described in this study, the rearrangement patterns of the IGK locus observed on GeneScan analysis could not readily be assigned to a single clone, whereas the number of rearrangements of the IGH locus would be compatible with a monoclonal cell population, or hampered the assignment as clonal when no clonal IGH rearrangement was detected. Similar to the IGK pattern found on GeneScan, NGS-based clonality assessment also demonstrated multiple IGK rearrangements in these three cases (Table 1). However, the nucleotide sequences obtained by NGS, as well as the assignment of the IG genes in ARResT/Interrogate, were useful in unraveling the IGK-recombination profile. The three cases described were encountered in routine diagnostic testing for lymphoma. Because the caseload, the frequency of testing, and the specific indications for clonality testing differ by laboratory, it is difficult to provide the frequency of cases with an aberrant IGK-rearrangement pattern. In the Department of Pathology, Radboud University Medical Centre, with a high percentage of consultation cases, the estimated frequency of cases with an aberrant IGK-rearrangement pattern is approximately 3% to 5% of the B-cell cases of lymphoma in which clonality is assessed.

The existence of IGK inversions has been suggested due to the complex configuration of the IGK locus[5,7]; however, this is the first report of rearrangements that may have resulted from IGK inversions detected in lymphoma specimens. An inversion rearrangement is one of the causes of lymphomas with an aberrant IGK-rearrangement pattern. Unfortunately, additional experimental evidence of such inversion events in cases 1 and 2 could not be provided, as both lymphoma specimens were from FFPE tissue blocks that yielded fragmented DNA that did not allow for whole-genome sequencing. In addition, aberrant IGK rearrangement patterns may be caused by artefacts from the cross-reactivity of primers, as illustrated in case 3. Both the inversion rearrangements as well as the cross-reactivity of primers can be explained and confirmed using NGS-based clonality assessment.

It is important that these inversions and cross-reactivity events are taken into account in the assessment of the IGK locus in the discrimination between monoclonal and biclonal lymphoproliferations. NGS-based clonality assessment can therefore facilitate the interpretation of complicated cases in diagnostic testing for lymphoma.

Author Contributions

A.M.L., L.I.K., and P.J.T.A.G. analyzed the data and wrote the manuscript. M.v.d.B. and P.J.T.A.G. designed and supervised the study. A.M.L., J.R., and J.L. performed experiments. L.I.K. and A.W.L. generated the figures. K.M.H. provided additional pathology data and reviewed the cases. N.D. supported the ARResT/Interrogate analysis. All co-authors critically revised the manuscript for important intellectual content. P.J.T.A.G. is the guarantor of this work and, as such, had full access to all of the data in the study

Figure 2 Schematic representation of the proposed consecutive rearrangements on the IGK locus of cases 1 and 2. **A:** Case 1, it is assumed that on allele A, the IGKV-IGKJ (V2-30 -7/2/-0 J4) rearrangement takes place first, resulting in the deletion of the intervening DNA. Subsequently, the IGKV(D)-IGKJ (V3D-11 -1/0/-4 J5) rearrangement occurs, during which the intervening DNA is inverted rather than deleted, so that the original IGKV-IGKJ rearrangement is preserved in the DNA. Lastly, the IGK locus on this chromosome is inactivated by an intron-Kde rearrangement, which removes the IGKC region and the enhancers (iEκ and 3′Eκ). On allele B, the IGKV-IGKJ (V2D-29 -0/0/0 J1) rearrangement (inversion event) occurs first and is followed by an inactivating intron-Kde rearrangement. **B:** Case 2, it is proposed that on allele A, recombination starts with the IGKV-IGKJ (V1-27 -1/0/-4 J5) rearrangement and removal of the intervening DNA. Next, the IGK locus on this chromosome is inactivated via an intron-Kde (intron-1/0/-8 Kde) rearrangement. On allele B, the IGKV-IGKJ (V2-24 -1/0/-0 J5) rearrangement takes places first, and causes the deletion of the intervening DNA. Subsequently, the IGKV(D)-IGKJ (V1D-27 -3/0/-1 Kde) rearrangement (inversion event) inactivates the allele. Because this recombination event involves a gene from the IGKV(D) cluster, the intervening DNA is inverted rather than deleted, therefore preserving the initial IGKV-IGKJ rearrangement on this allele. This scenario explains how inversion rearrangements could result in the observed rearrangements in these cases, although the option of a different order in which the rearrangements have occurred cannot be excluded. In line with the inactivation of the κ locus on both alleles in cases 1 and 2, λ light-chain restriction was demonstrated by immunofluorescence staining (case 1) and by IHC analysis (case 2). No IHC analysis/fluorescence or flowcytometry was performed for the light chains of case 3, given that this was cytological material and a consultation case.

and takes responsibility for the integration of the data and the accuracy of the data analysis.

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