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Original Article

Use of IGK gene rearrangement analysis for clonality assessment of lymphoid malignancies: a single center experience

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Abstract: Diagnosis of B-non Hodgkin lymphomas (NHLs) is based on clinical, morphological and immunohistochemical features. However, in up to 10-15% of cases, analysis of immunoglobulin heavy (IGH) or light (IGK/IGL) chains genes is required to discriminate between malignant and reactive lymphoid proliferations. In this study, we evaluated the feasibility and efficiency of IGK analysis in the routine diagnostic of B-cell lymphoproliferative disorders (B-LD) when applied to formalin-fixed paraffin-embedded (FFPE) tissues. Clonality patterns were studied in 59 B-LD using the BIOMED-2 protocol for IGK assays, after failure of the IGH assay. PCR products were evaluated by both heteroduplex and GeneScan analysis. IGK analysis was technically successful in all cases. Overall, it supported the histopathological suspicion in 52/59 cases (88%), the sensitivity and specificity being 83% and 80%, respectively. Further, positive and negative predictive values were 95% and 50%, respectively. Interestingly, among various lymphoma subtypes, marginal zone lymphoma and follicular lymphoma most frequently required IGK analysis. In conclusion, IGK study according to the BIOMED-2 protocol resulted feasible and extremely useful in supporting challenging diagnosis of B-LD even if applied on FFPE samples. Accordingly, when NHL is suspected, negative results at IGH analysis should not be considered as conclusive and further investigation of IGK is appropriate.

Keywords: BIOMED-2, molecular diagnostic, IGK, non Hodgkin lymphoma, PCR

Introduction

Non Hodgkin's lymphomas (NHLs) represent a heterogeneous group of tumors derived from and resembling normal lymphoid elements at different stages of differentiation. They account for 5% of all human cancers, being at present, the fifth commonest tumor and their incidence being progressively increasing [1-3].

According to the WHO classification, [4] the diagnosis of B-NHLs, is based on morphology, immuno-phenotyping, and recognition of specific genetic lesion or clinical characteristics. However, in up to 10-15% of cases, histomorphology, cytomorphology and immunophenotyping cannot successfully discriminate between malignant and reactive lymphoproliferations. Therefore, for cases with atypical features, defective phenotype or a reactive background masking the neoplastic population, analysis of specific immunoglobulin chains genes rearrangements is extremely useful. These tests, in fact, allow to detect with high sensitivity and specificity even small monoclonal or oligoclonal proliferations [5], representing 1-5% of the examined population. In particular, clonality assessments are more frequently performed by studying the IGH genes, whose rearrangement is the first event in the differentiation of B cells, and that present with a detectable clonal rearrangement in around 82% of lymphoid tumors [5]. On the other hand, in a portion of B cell malignancies IGH amplification can fail due to the loss of target sequences or the structural alteration resulting from somatic hypermuta-

tions (SH) [5-7]. In these cases, the amplification of the light chain of immunoglobulin (IGK/IGL) can be studied; in fact, in the vast majority of mature B-NHLs, a single IG light chain expression supports the clonal origin of the tumor [5, 8]. Furthermore, IGK/IGL are rarely somatically mutated, thus preventing false negative results caused by inappropriate primers annealing [9, 10]. Overall, by combining IGH and IGK/IGL analyses, molecular assessment of clonal rearrangements with standardized protocols as the one prepared by the BIOMED-2 can reach a sensitivity up to 98-100% [5]. However, so far, the reported data on sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were mainly obtained on fresh/frozen cases, while little is know about the capability of such tests on formalin fixed, paraffin embedded (FFPE) samples. Indeed, the latter represent the vast majority of available specimens in the clinical practice. Therefore, diagnostic tests have to be extensively validated in this setting as well.

In this study, we analyzed our more recent experience on molecular diagnostics of lymphoproliferative processes aiming to evaluate the feasibility and efficiency of IGK rearrangement analysis for the assessment of clonality in FFPE tissues.

Materials and methods

Case series

From October 2009 to January 2011, a total of 59 B-lymphoproliferative disorders were studied for clonality assessment by IGK analysis, the initial IGH evaluation being not decisive in the diagnosis work up. In such instances, basing on morphology, immunophenotype, FISH, molecular and clinical information, the diagnoses were: follicular lymphoma (FL, N=22), marginal zone lymphoma, (MZL, N=8), diffuse large B-cell lymphoma (DLBCL, N=5), classical Hodgkin lymphoma (HL, N=2), plasmablastic lymphoma (PBL, N=2), B-non Hodgkin lymphoma not otherwise specified (B-NHL, NOS; N=5), and reactive lymphoid hyperplasia (RLH, N=15). The histological and immunophenotypical analyses were carried on as previously reported [11] and cases were interpreted according to the WHO classification of Tumors of the Hematopoietic and Lymphoid Tissues [4]. All the patients were treated (or not) according to the final diagnosis and followed up after that.

DNA extraction

Section of 5 μm thick were prepared from paraffin embedded biopsy specimens. Samples were first deparaffinized in hystoclear and then rehydrated in ethanol. Tissue digestion was carried out in 200 microliter of a solution containing 20 microliter of proteinase K in 180 microliter ATL Buffer (Qiagen) subsequent, the material was incubated at 56 °C overnight. Finally, DNA purification was performed by using a Qiagen DNA mini kit according to the manufacturer's instructions. DNA sample concentration and quality were assessed by spectrophotometry (260/280 nm using the NanoDrop). Only case with a 260/280 nm ratio between 1,8-2 and 260/230 ratio of about 2.2 were considered evaluable.

Genomic sequences amplification by polymerase chain reaction (PCR)

Amplification of a 300-bp segment of PLZF gene (forward primer, 5' TGC GAT GTG GTC ATC ATG GTG 3' and reverse primer 5' CGT GTC ATT GTC GTC TGA GGC 3') was performed in every samples as an internal control to verify the DNA integrity. IGH and IGL gene rearrangement were analyzed by multiplex PCR. Family-specific VH, Vk primers were used in combination respectively with one JH consensus and a set of two Jk primers (Jk1–4, covering the first four J segments and Jk5 covering the fifth) or a Kde primer. For the analysis of Kde rearrangements an additional forward primer recognizing a sequence upstream of the intronRSS was made. (**Table 1**) (BIOMED-2 Concerted Action) [5]. Amplification reactions were performed in an automated thermocycler (mastercycler eppendorf) according to the BIOMED-2 multliplex PCR protocol [5]. Each 50-microliter PCR reaction included 100 ng of DNA, 10 pm of each primers, 0,2 mmol/L dNTP, 5 microliter of 10X Gold buffer, 1.5 mmol/L of MgCl2 and 1 U of AmpliTaq Gold polymerase (Applied Biosystems). The cycling parameters were as follows: pre activation for 7 minutes at 95°C, 35 cycles of denaturation (95°C for 30 sec), an annealing step (60°C for 30 sec), an extension step (72°C for 60 sec), finally 10 minutes of extension at 72°C. Each sample was evaluated using Heteroduplex analysis and GeneScanning to determine polyclonal or monoclonal character.

Table 1. Sequence of primers used in the DNA amplification protocol

Gene Target	Primer Sequence (5' 3')
Biomed 2 Tube A sense	
VH1-FR1	5' GGCCTCAGTGAAGGTCTCCTGCAAG 3'
VH2-FR1	5' GTCTGGTCCTACGCTGGTGAAACCC 3'
VH3-FR1	5' CTGGGGGGTCCCTGAGACTCTCCTG 3'
VH4-FR1	5' CTTCGGAGACCCTGTCCCTCACCTG 3'
VH5-FR1	5' CGGGGAGTCTCTGAAGATCTCCTGT 3'
VH6-FR1	5' TCGCAGACCCTCTCACTCACCTGTG 3'
VH1-FR2	5' CTGGGTGCGACAGGCCCCTGGACAA 3'
Biomed 2Tube B sense	
VH2-FR2	5' TGGATCCGTCAGCCCCCAGGGAAGG 3'
VH3-FR2	5' GGTCCGCCAGGCTCCAGGGAA 3'
VH4-FR2	5' TGGATCCGCCAGCCCCCAGGGAAGG 3'
VH5-FR2	5' GGGTGCGCCAGATGCCCGGGAAAGG 3'
VH6-FR2	5' TTGGGTGCGACAGGCCCCTGGACAA 3'
VH7-FR2	5' TGGATCAGGCAGTCCCCATCGAGAG 3'
Biomed 2 Tube C sense	
VH1-FR3	5' TGGAGCTGAGCAGCCTGAGATCTGA 3'
VH2-FR3	5' CAATGACCAACATGGACCCTGTGGA 3'
VH3-FR3	5' TCTGCAAATGAACAGCCTGAGAGCC 3'
VH4-FR3	5' GAGCTCTGTGACCGCCGCGGACACG 3'
VH5-FR3	5' CAGCACCGCCTACCTGCAGTGGAGC 3'
VH6-FR3	5' GTTCTCCCTGCAGCTGAACTCTGTG 3'
VH7-FR3	5' CAGCACGGCATATCTGCAGATCAG 3'
Biomed 2 Tube A, B, C anti	
JH	5' CTT ACC TGA GGA GAC GGT GAC C 3'
Biomed 2 Tube A, B sense	
VK1F/6	5' TCAAGGTTCAGCGGCAGTGGATCTG 3'
VK2F	5' GGCCTCCATCTCCTGCAGGTCTAGTC 3'
VK3F	5' CCCAGGCTCCTCATCTATGATGCATCC 3'
VK4	5' CAACTGCAAGTCCAGCCAGAGTGTTTT 3'
VK7	5' GACCGATTTCACCCTCACAATTAATCC 3'
VK5	5' CCTGCAAAGCCAGCCAAGACATTGAT 3'
Biomed 2 Tube A anti	
IGK JK1-4	5' CTTACGTTTGATCTCCACCTTGGTCCC 3'
IGK JK5	5' CTTACGTTTAATCTCCAGTCGTGTCCC 3'
Biomed 2 Tube B anti	
IGK KDE	5' CGTGGCACCGCGAGCTGTAGAC 3'
IGK INTR	5' CCTCAGAGGTCAGAGCAGGTTGTCCTA 3'

Heteroduplex analysis

In order to allow the spontaneous formation of homoduplex or heteroduplex from DNA fragments, amplicons were denaturated by heating (94 °C for 5 min) and cooled at low temperature (4°C for 1 hour). PCR products were then visualized by electrophoresis on polyacrylamide gels being separated according to their length and conformation. According to BIOMED-2 protocols, samples were defined as monoclonal when a single band was identified within a predictable size range, and polyclonal when only a smear was detected [5]. This technique has a well recognized detection limit of ~5% due to the frequency of polyclonal/reactive lymphocytes present in the tissue.

Genescanning analysis

After amplification, 1 μl of PCR product with 0.5 μl of a standard molecular weight product (LIZ

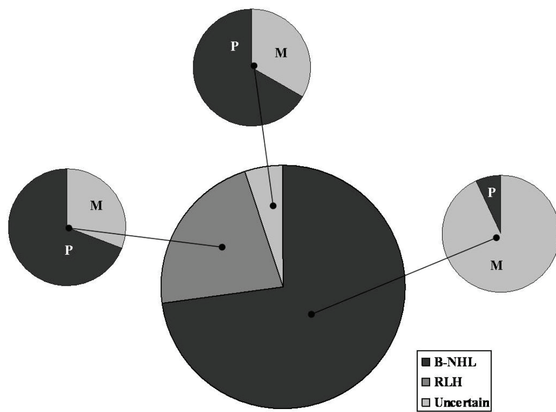

Figure 1. Cases series according to the diagnosis made after the blinded review and relation with the molecular pattern (P= polyclonal; M= monoclonal).

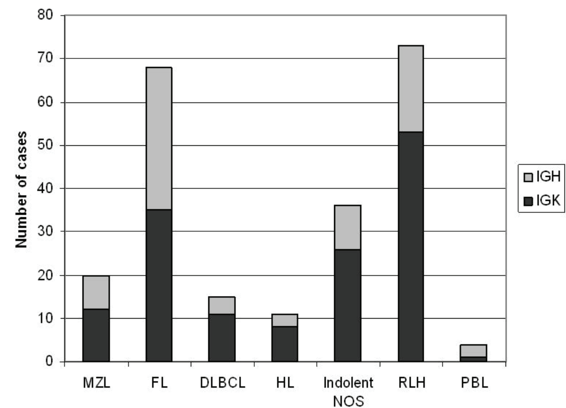

Figure 2. Cases for which a molecular test was requested at our Unit in the considered period. IGH analysis was performed in all instances, while IGK evaluation was then required in selected instances.

Applied Biosystems) were mixed with 12 μl of formamide to induce denaturation into single DNA strands (1 minute to 95C°). Subsequently, they were separated through a polymer capillary electrophoresis system and automatically detected by fluorescence reading with a laser system in automatic DNA sequencer (ABI Prism 310 Applied Biosystems).

Samples were considered as monoclonal if 1 or 2 peaks of amplified products PCR in question were obtained; detection of 3 to 5 peaks was counted as an oligoclonal result, while a Gaussian distribution of peaks was referred to polyclonal populations. (**Figure 1**)

Statistical analysis

Chi square test was used to calculate differences in frequency by which IGK analysis was requested in different NHL subtypes. In addition, Fisher exact test was used for comparing the ability of IGH and IGK in this series to detect clones. The limit of significance for all analyses was defined as $P<0.05$; two-sided tests were used in all calculations.

Results

Accuracy of IGK analysis in the detection of lymphomas vs. reactive non-neoplastic conditions

Among of 1839 suspect B-cell lymphoproliferative disorders diagnosed at our Institute during the considered period, clonality detection by molecular analysis was requested in 218/1839 (12%). First IGH status was evaluated 159/218 (72%) being sufficient to complete the diagnostic work up. Conversely, a secondary study of IGK was requested by the hematopathologists in 59/218 (28%), in order to complete the diagnostic work up due to the global difficulty in the cases. In 2/59 (3%) cases IGH genes were not amplified, while in 9/59 (15%) it was only possible the evaluation of the framework region 3 (FR3). This was probably due to the high SH rate that impedes a correct primers annealing to the sequence target. In such cases, conversely, IGK PCR assay was always technically successful (and then evaluable). In addition, in all instances, an internal control gene (PLZF, 300 bp) was productively amplified. Thus, such 59 cases were selected for the present study.

In order to assess the sensitivity (ST) and specificity (SP) of the assay in our panel, we considered the pathological diagnosis as the diagnostic gold standard. Thus, the cases were blindly reviewed by two expert hematopathologists and diagnoses were made only basing an morphology, phenotype and ISH/FISH, whereas molecular results were not made available. The cases were then diagnosed as either B-NHL (N=46), or reactive lymphoid hyperplasia (RLH, N=10), or uncertain (N=3) (when the diagnosis could not be defined). The latter cases were then excluded from the calculation of ST, SP, PPV, and NPV.

Subsequently, we evaluated the consistency of molecular results and histopathological diagno-

Table 2. Accuracy of IGK analysis in the detection of lymphomas vs. reactive non neoplastic conditions

		Blinded Histopathology Review			
		B-NHL	RLH		
IGK	Monoclonal	38	2	0.95	PPV
	Polyclonal	8	8	0.5	NPV
		82.61%	80.00%		
		ST	SP		

ST= Sensitivity; SP= Specificity; PPV= Positive predictive value; NPV= Negative predictive value; B-NHL= B-non Hodgkin lymphoma; RLH= Reactive lymphoid hyperplasia.

ses. We found that basing on heteroduplex (HD) and/or genescan (GS) IGK evaluation, evidence of clonality was observed in 38 of 46 cases of lymphoma, the ST thus being 83%. On the other hand, the molecular analysis documented a polyclonal pattern in 8/10 cases finally diagnosed as RLH, the SP thus being 80%. Accordingly, the PPV and NPV were calculated as 95% and 50%, respectively (Table 2). Overall, the IGK analysis was consistent with the histopathological diagnosis in 46 out of 59 instances (78%).

Impact of IGK analysis on the final diagnosis

Three out of fifty-nine cases (5%) could not be diagnosed without the molecular test and were interpreted as “uncertain” after the blinded review. However, after making available the IGK analysis results, they were diagnosed as either B-NHL (N=1) or RLH (N=2). Importantly, it should be underlined that the diagnosis was not exclusively based on the molecular results, but rather on the integration of the latter with the other elements, which, by their selves, were initially not sufficient.

Heteroduplex analysis by gel electrophoresis vs. genescan

In this study we also compared the efficiency of HD and genescan in detecting clonal population. Specifically, in 14/59 cases both the techniques identified a polyclonal pattern (Figure 3A), while in 41/59 both identified a clonal rearrangement (Figure 3B). Conversely, in 4/59 (7%) cases we found discordant results. In particular, a band consistent with monoclonal rearrangement was observed by HD analysis, while a clear polyclonal pattern was detected by genescan (Figure 3C). The latter, according to the international guidelines was considered as valuable, confirming the importance of such tool in the clonality assessment of lymphoid proliferations.

IGK analysis request by NHL type

Finally, we investigated possible differences among different pathological entities as far as the requirement of molecular investigations was concerned. We found that the diagnosis of FL and MZL was slightly more frequently associated with necessity of molecular studies and, specially, of IGK analysis, though the differences were not significant (chi-square test, p=0.45). Specifically, by considering all the cases diagnosed at our Institution from October 2009 to January 2011, molecular tests were requested in 20/165 MZL, 68/264 FL, 15/409 DLBCL, 11/520 HL, 73/213 RLH, 36/240 indolent B-NHL NOS, and 4/8 PBL. Moreover, among cases for which a molecular test was required, IGK analysis was then requested in 8/20 MZL, 33/68 FL, 4/15 DLBCL, 3/11 HL, 20/73 RFH, 10/36 indolent B-NHL NOS, and 3/4 PBL (Figure 2). In general, though tumors carrying higher loads of SH (i.e. FL and MZL) more frequently requested the IGK analysis, the difference was not significant as the global complexity of the diagnostic work up was probably a more relevant factor.

Discussion

The diagnosis of lymphoproliferative disorders is complex and generally based on the integration of morphological, immunophenotypic, genetic and clinical data. Importantly, in a significant proportion of cases (assessed around 10-15% in most series), the distinction between reactive vs. malignant lesion is indeed complicated and require additional methods to confirm or generate a final diagnosis and clonality tests are necessary to improve the diagnostic accuracy. In

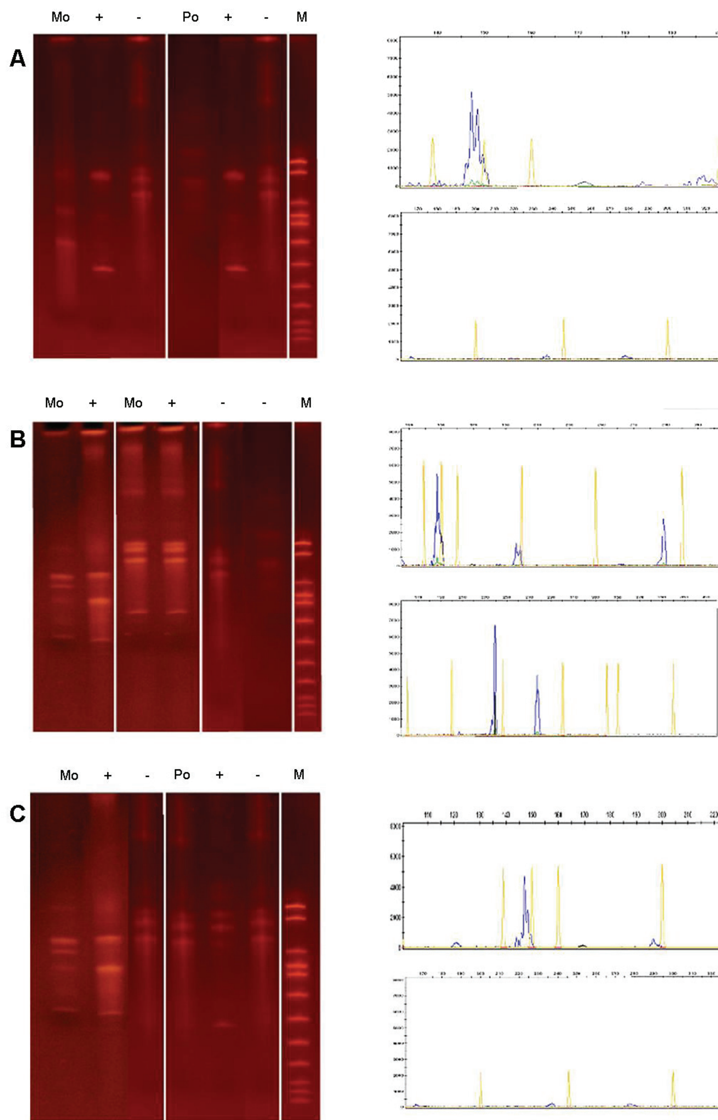

Figure 3. Clonality analysis of IGK using BIOMED-2 protocol. A) polyclonal sample identified both polyacrylamide gel and Gene Scan. B) monoclonal population identified both methods C) example of discordant case (monoclonal pattern by polyacrylamide gel and polyclonal pattern by Gene Scan) . M= marker; Mo=Monoclonal sample; P=Polyclonal sample; +=positive control; -= negative control.

particular, the IGH genes rearrangements are most commonly studied for detecting B-cell clonality in the routine laboratory setting [5]. However, as the overall sensitivity of this approach does not exceed 80% [12, 13], further attempts are sometimes required.

In this study, we evaluated the consistency and the possible impact of IGK genes rearrangements analysis in the routinary clinical practice, which is based on FFPE material. To address this issue, the cases were first blindly reviewed excluding the molecular information; therefore, the diagnosis was based on the other conventional parameters, which constituted the gold standard.

First, we could assess that this method was definitely feasible in unselected FFPE samples as it was technically successful in all instances, including those cases in which IGH analysis failed or was results amplified the only FR3 region. It is well known, in fact, that a sensible percentage of cases cannot be correctly diagnosed with this only technique; in particular, in many neoplastic B cells, the high rate of SH, occurring in the VH segments during the germinal centre (GC) reaction, is responsible for possible errors in primers hybridization, thus inducing false negative results [10, 14]. On the other hand, IGK genes do not undergo the SH process, thus largely preventing the presence of false negatives. In addition, importantly, several studies have also shown that IGK rearrangements can be detected virtually in all mature B-cell tumors, irrespectively of their light chain restriction (Igk vs. Igλ) [5].

Noteworthy, in the majority of cases the molecular tests was consistent with the pathological diagnosis. Particularly, the recognition of a monoclonal pattern was associated with a final diagnosis of malignancy in the vast majority of cases, this conferring to the test a remarkably PPV highlighting the efficiency of the assay in FFPE tissues. Indeed, the recognition of clonal population after that a lymphoid malignancy is suspected based on histopathological analysis was strictly associated with a diagnosis of lymphoma.

On the other hand, apparently, the NPV of the test was not definitely brilliant (50%). In fact,

8/16 cases with a polyclonal pattern were finally defined as lymphoma. This is not, however, completely surprising by considering the characteristics of the studies series which included many cases in which the proportion of reactive elements overvalued the neoplastic cells. In addition, the relatively low NPV which we obtained, intimately depended on the low number of RLH included in the study, representing the ratio between “false positive” (i.e. lymphomas without evidence of clonality) and “true positive” (i.e. RLH without evidence of clonality) cases (Table 2).

On the other hand, remarkably, in 3 cases, which were considered not concluded often revision of morphology and IHC only, the molecular test was decisive in leading the diagnosis.

The efficiency of IGK analysis in FFPE samples have been described so far only in a few papers. Pai et al.[15] reported an increase of sensitivity from 81% with IGH PCR alone to 90% with combined IGK/IGH PCR while Gong et al. reported a improved of sensitivity from 66% to 85%. They concluded that IGK PCR is an approach particularly useful in the characterization of B-cell lymphoproliferative disorder in material with poor preservation of genomic DNA. Melotti et al.[16] reported in their study, that sensitivity could vary from 20% to more than 90%, by combining IGH, IGK, and IGLV assays. On the other hand, a direct comparison with the original BIOMED-2 studies was not possible, as they mainly referred to fresh/frozen samples [5].

In addition, our study compared for the first time HD and genescan usage in this setting, confirming the need for the latter in order to avoid puzzling results and specially, according to our experience, false positive detections.

Of note, a potential pitfall of IGK PCR techniques, is the relatively large size range of expected rearranged IGK product that implies long runs for genescan. In addition, the complexity of multiple rearrangement in the IGK locus together with a low level of cross-annealing of Vk primers may results in patterns with multiple bands or peaks resembling oligoclonality and be difficult to interpret . For this reasons, it is currently recommended to limit the IGK genes analysis only in those cases in which it has impossible to demonstrate the presence of a monoclonal B-cell population after IGH genes test [5, 17].

Finally, we investigated whether the diagnosis of different pathological entities was differently associated to the requirement of specific molecular tests. Indeed, though tumors carrying higher loads of SH (i.e. FL and MZL) more frequently requested the IGK analysis, the differences were not significant. This fact possibly reflected the higher failure rate of IGH tests in case of high prevalence of IGH SH, but also indicated the relevance of the global complexity of the diagnosis for requiring an ancillary test.

In conclusion, our results showed that IGK rearrangement analysis is feasible and definitely effective in routine diagnostic work up of suspected B-cell lymphoproliferative disorders, irrespectively of the entity type. Thus, negative results at IGH analysis should not be considered as conclusive, and further investigation of IGK may be appropriate.

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