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Immunoglobulin/T-cell receptor clonality diagnostics

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Clonality testing in lymphoid malignancies has become technically relatively easy to perform in routine laboratories using standardized multiplex polymerase chain reaction protocols for Ig/T-cell receptor (TCR) gene analysis. Expertise with clonality diagnostics and knowledge about the biology of Ig/TCR recombination are essential for correct interpretation of the Ig/TCR clonality data. Several immunobiologic and technical pitfalls that should be taken into account to avoid misinterpretation of data are addressed in this review. Furthermore, the need to integrate the molecular data with that from (hemato-)pathology, and preferably also flowcytometric immunophenotyping for appropriate interpretation, is discussed. Such an interactive, multidisciplinary diagnostic model guarantees integration of all available data to reach the most reliable diagnosis.

Keywords: clonality, GeneScan, heteroduplex, Ig, PCR, TCR

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1. Introduction

Cancer cells are the progeny of a single malignantly transformed cell and consequently these cells are clonally related. Hence, monoclonality is a key feature of malignant tumor cell populations, which enables discrimination from oligoclonal or polyclonal, reactive processes. Clonality assessment is thus an important tool in the diagnosis of tumors. Establishment of the clonal relationship between multiple lesions at distinct locations or over time is another useful adjunct in cancer diagnostics. Detection of clonality is possible via several technical approaches, including the study of chromosomes, DNA markers, (tumor-)specific proteins and patterns of proteins, also known as the tumor phenotype. In lymphoid malignancies, clonality detection is relatively straightforward due to the ample availability of highly polymorphic DNA markers: the rearrangements in the genes encoding the antigen receptors in B and T lymphocytes, the Ig and T-cell receptor (TCR) genes, respectively [1-4]. In this review, several immunobiologic aspects related to Ig/TCR clonality and its detection in lymphoid malignancies are addressed, with special emphasis on correct interpretation and potential pitfalls.

2. Ig/T-cell receptor clonality detection

During early lymphoid differentiation, genes encoding the Ig and TCR molecules are formed by the stepwise rearrangement of V, D, and J gene segments. This process is referred to as V(D)J recombination [3,4]. During this recombination process, nucleotides are deleted and randomly inserted at the joining sites, resulting in an enormous diversity of unique antigen receptors. The result is the formation of a so-called junctional region, which is sometimes also referred to as complementarity-determining region 3 (CDR3). Junctional regions or CDR3 motifs form one of the actual antigen-binding parts of the antibody, and they are

highly variable in both size and nucleotide composition. As Ig/TCR gene rearrangements occur sequentially in the earliest stages of lymphoid differentiation they are present in almost all immature and mature lymphoid cells [5,6]. As lymphomas and leukemias are derived from a single malignantly transformed lymphoid cell, the tumor cells of virtually all lymphoid malignancies contain one or several identical (clonal) Ig and/or TCR gene rearrangements. Heterogeneity in Ig/TCR rearrangements thus identifies the presence of polyclonally activated cells, as opposed to identically rearranged Ig or TCR genes that reflect a monoclonal lymphoid cell population.

Before the era of polymerase chain reaction (PCR) techniques, Ig/TCR clonality testing was usually performed by means of Southern blot analysis, based on size detection of restriction fragments (i.e., germline bands, rearranged clonal bands or polyclonal smears of multiple bands) [2,7-9]. Although time demanding and cumbersome, the Southern blot approach has been very reliable with a high specificity and a fair sensitivity (detection limit: clone size of ≥ 10%) [2]. However, a major disadvantage is the need for relatively large amounts of high molecular weight DNA, thus complicating routine analysis of small biopsies and/or paraffin-embedded material. For this reason, the PCR technology has been considered a better option and consequently many different PCR strategies for Ig [10-18] and TCR [19-25] clonality testing have been described. No matter how valuable in the beginning, virtually all PCR strategies suffered from two major drawbacks: i) false negativity through improper primer annealing; and ii) false positivity due to poor discrimination between polyclonal and monoclonal Ig/TCR rearrangements.

3. Standardization of polymerase chain reaction-based clonality testing

In the late 1990s, a European consortium of ~ 45 laboratories (BIOMED-2 Concerted Action BMH4-CT98-3936) was initiated with the aim to establish a highly reliable standard in PCR-based clonality testing. The issue of false negativity was addressed at several levels: i) design of complete sets of primers to cover all possible V-J rearrangements of Ig/TCR loci; ii) inclusion of incomplete rearrangements as additional targets (e.g., DH-JH and Dβ-Jβ); and iii) inclusion of multiple Ig targets (*IGK* and *IGL* next to *IGH*) and multiple TCR targets (*TCRB* and *TCRD* next to *TCRG*). This concept of complementarity of targets was only feasible for routine testing by designing multiplex PCR reaction mixtures consisting of multiple primers. The other challenge was to prevent false positivity, which was achieved by introducing standardized, reliable methods for evaluation of PCR products: heteroduplex analysis [26-28] and GeneScan™ (Applied Biosystems Foster City, CA, USA) fragment analysis (**Figure 1**) [29,30]. Following its technical evaluation [31], the multiplex protocol was successfully applied to ~ 600 different well-defined WHO lymphoma entities with unprecedented high frequencies of malignant cases showing clonality [32-38]. In the B-cell malignancies that were tested, high *IGH* clonality detection rates could be found in series from well-defined entities of both pregerminal center (mantle cell lymphoma [MCL]) and (post)-germinal center (chronic lymphocytic leukemia [CLL], marginal zone B-cell lymphoma, follicular lymphoma [FL], diffuse large B-cell lymphoma) origin (**Table 1**); a further increase in the sensitivity of the assays can be seen by adding *IGK* as a target, especially in the (post)-germinal center malignancies. *IGL* genes do not show clear added value. Analysis of TCR targets in these B-cell entities also showed clonality, albeit to different degrees, being derived from either accompanying (oligo-)clonal T lymphocytes or from parallel monoclonal TCR rearrangements in the transformed B cells [33]. Various T-cell malignancies (T-cell prolymphocytic leukemia, T-cell large granular lymphocytic leukemia, peripheral T-cell lymphoma not otherwise specified, angioimmunoblastic T-cell lymphoma [AILT], anaplastic large cell lymphoma [ALCL]) showed high levels of clonality upon both *TCRB* and *TCRG* analysis, with clear complementarity when both targets were evaluated; *TCRD* was of little added value (**Table 1**). Interestingly, lower detection rates were seen in ALCL, which can be explained by null-type ALCL that is known not to harbor any TCR rearrangements, and AILT, which is due to the generally small tumor clones in this entity [34]. Moreover, Ig clonality was also identified, especially in AILT, which reflects the known pathophysiologic occurrence of B-cell clones in AILT; Ig clonality in the other entities was generally rather limited [34]. The above-mentioned BIOMED-2 multiplex PCR approach has now become a world standard [39-43].

4. Pitfalls and recommendations

Now that PCR-based clonality testing and assessment by GeneScan and/or heteroduplex analysis has technically become relatively easy to perform, knowledge of and experience with Ig/TCR rearrangement analysis are required more than ever to avoid misinterpretation of data. Molecular biologists, pathologists and clinicians should be aware of several technical and immunobiologic pitfalls that should be considered when interpreting Ig/TCR clonality findings (**Table 2**).

4.1 Technical pitfalls

With respect to expected size ranges of PCR products that are mentioned in protocols for a given Ig/TCR target, it is important to realize that those represent the 5 – 95 percentiles of the natural heterogeneity of the CDR3 region [31]. Hence, bands or peaks just outside this size range can be interpreted as true rearrangement products, even without formal proof via sequencing. When the products are considerably smaller (undersized) or larger (oversized), they still can represent rearrangements, but sequencing

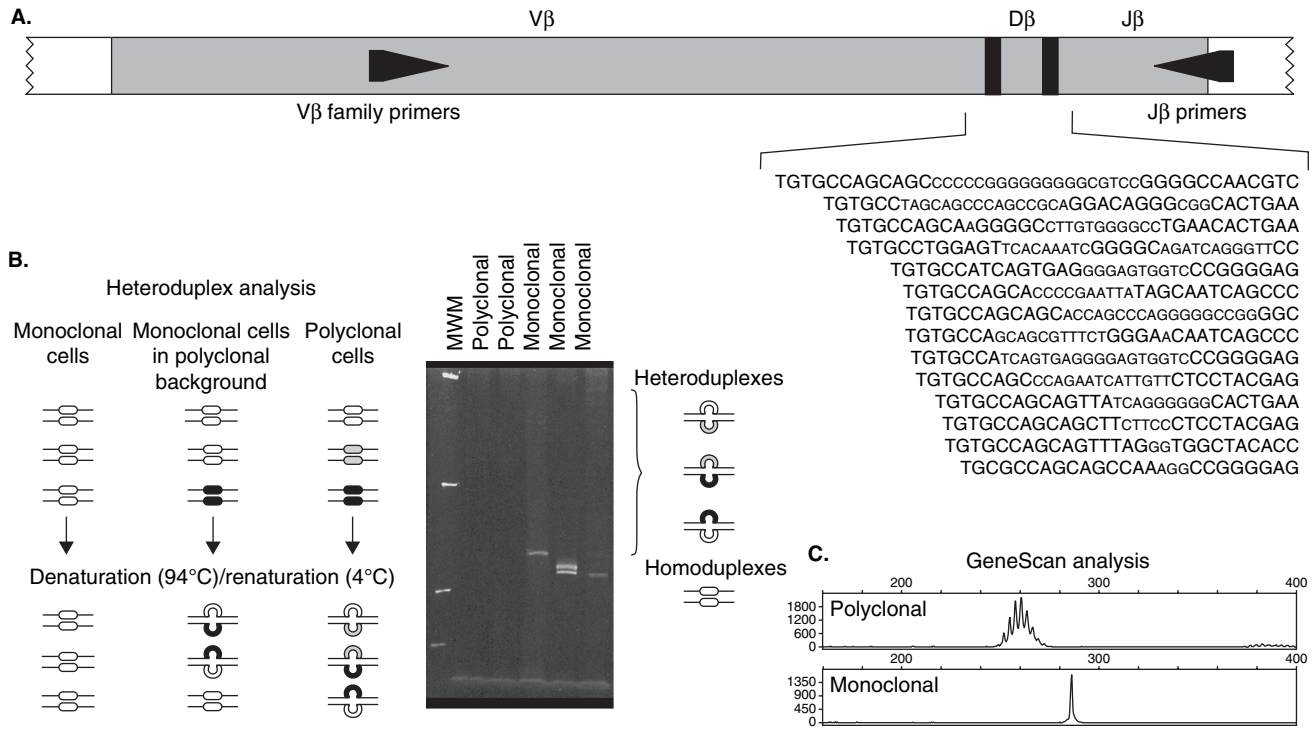

Figure 1. Schematic diagram of heteroduplex analysis and GeneScan fragment analysis of PCR products from Ig/TCR gene rearrangements. A. Rearranged Ig/TCR genes (here *TCRB* rearrangements are shown as example) show heterogeneous junctional regions (also known as CDR3 motifs) that differ in size and nucleotide composition. V, D and J germline nucleotides are shown in large capitals, and randomly inserted nucleotides in small capitals. Junctional heterogeneity can be exploited to discriminate between polyclonal and monoclonal PCR products based on differences in size and composition (heteroduplex analysis) or size only (GeneScan fragment analysis). **B.** In heteroduplex analysis, PCR products are denatured (5′, 94°C) and reannealed (rapid cooling at 4°C for 60 min) [18,27]. Monoclonal PCR products (in this example derived from a case of T-cell large granular lymphocyte leukemia) give rise to homoduplexes, whereas polyclonal PCR products (derived from an activated T-cell proliferation during viral infection) mainly form heteroduplexes, resulting in a smear of slow-migrating fragments. **C.** In GeneScan fragment analysis, fluorochrome-labeled PCR products are denatured for high-resolution fragment analysis of the single-stranded fragments [27,31]. Monoclonal PCR products of identical size, as in the case of the T-cell leukemia, give rise to a peak, whereas polyclonal PCR products show a Gaussian size distribution.
PCR: Polymerase chain reaction; TCR: T-cell receptor.

analysis is then warranted for confirmation. Undersized peaks or bands might be caused by (small) deletions within, for example, the V gene segment, as can sometimes be seen in (post-)germinal center proliferations harboring somatic hypermutations [44]. If such deletions occur within the primer annealing region (e.g., the FR3 region) these rearrangement products would be missed completely using a FR3 primer, stressing once more the importance of the concept of complementarity of targets [33,34]. The opposite situation with oversized peaks/bands is to be considered in *IGH* and *IGK* analysis, in which amplification from a downstream J gene segment might occur when the actual rearranged J segment is, for example, somatically mutated, leading to suboptimal primer annealing [45]. Similar clustering of J gene segments with relatively small (a few hundred bp) intergenic distances is also apparent in the *TCRB* locus, sometimes giving rise to two differently sized PCR products within the same reaction mixture due to efficient primer annealing to both the rearranged and the neighbouring downstream Jβ segment (**Figure 2**) [45]. Due to the composition of the BIOMED-2 *TCRB* multiplex tubes with two V-J reaction mixtures harboring mutually exclusive sets of Jβ primers, the situation can look even more complex. Thus, in particular cases the same Vβ-Jβ rearrangement can be identified with both mixtures, with the second mixture giving rise to an 'oversized' product due to primer annealing to the neighbouring downstream Jβ segment that is recognized by the primer specific for that downstream Jβ segment in the second mixture. A complex interpretation pitfall concerns the question of whether detection of multiple clonal signals is equivalent to biclonality. Although biclonal malignancies do occur, several immunobiologic and technical explanations for multiple clonal signals should first be considered. First, as B and T lymphocytes carry two independently rearranging chromosomes, biallelic rearrangements are more common than biclonality. Second, as mentioned above, extended products in especially the *IGH*, *IGK* and *TCRB* loci might give rise to additional 'clonal' signals derived

Table 1. PCR-based Ig/TCR profiles in particular lymphomas and chronic leukemias of B- and T-cell origin.

Entity	n	*IGH %*	*IGK %*	*IGH + IGK %*	*IGL %*	*TCRB %*	*TCRG %*	*TCRB + TCRG %*	*TCRD %*
MCL	54	100	100	100	44	9	11	NE	4
CLL/SLL	56	100	100	100	30	25	18	NE	12
FL	109	86	84	100	21	6	2	NE	5
MZL*	41	95	83	100	29	24	15	NE	12
DLBCL	109	85	80	98	28	21	15	NE	14
T-PLL	33	9	3	NE	3	100	94	100	6
T-LGL	28	0	4	NE	4	96	96	100	29
PTCL-NOS	47	9	2	NE	0	98	94	100	15
AIT	37	30	19	NE	5	89	92	95	35
ALCL‡	43	2	0	NE	0	74‡	74‡	79‡	9‡

Percentage positive cases are based on published data in [33,34].

*MZL comprises both extranodal (31) and nodal (10) cases.

‡The lower percentages in ALCL are partly caused by a series of nine null-type ALCL without any TCR gene rearrangements.

AILT: Angioimmunoblastic T-cell lymphoma; ALCL: Anaplastic large cell lymphoma; B-CLL: B-cell chronic lymphocytic leukemia; DLBCL: Diffuse large B-cell lymphoma; FL: Follicular lymphoma; MCL: Mantle cell lymphoma; MZL: Marginal zone B-cell lymphoma; NE: Not evaluated; PCR: Polymerase chain reaction; PTCL-U: Peripheral T-cell lymphoma, unspecified; TCR: T-cell receptor; T-LGL: T-cell large granular lymphocytic leukemia; T-PLL: T-cell prolymphocytic leukemia.

Table 2. Pitfalls in Ig/TCR clonality testing.

Pitfall	Phenomenon	Solution/action
Bands/peaks just outside size range	CDR3 regions/junctions outside 5 – 95% size range interval	Accept as true rearrangement product; in case of doubt, sequence for confirmation
Undersized bands/peaks	Internal deletion in, for example V segment	Potential rearrangement product; confirm by sequencing
Oversized bands/peaks	Extended amplification from downstream J (due to, e.g., SHM in rearranged J segment)	Potential rearrangement product; confirm by sequencing
Multiple clonal signals	Bi-allelic rearrangements; multiple rearrangements per allele (*TCRB*, *IGK*), or biclonality	Consider the number of potential rearrangements per allele and per locus and judge whether this fits with clonality or biclonality
Lack of clonal signal and lack of polyclonal Gaussian curve	i) Few T/B cells in sample ii) Poor DNA quality iii) Clonal signal that is not detected due to SHM in malignant cells	i) Check T/B cell content by histology or flow cytometry ii) Check DNA quality in control PCR iii) Evaluate other FR or Ig target
Selective amplification and pseudoclonality, due to low level of specific template	Few T/B cells in sample	Repeat PCR in multiplicates (same tissue, second independent DNA isolation, and/or related tissue) → compare patterns for consistency
Oligoclonal T-/B-cell repertoire in PB of especially elderly individuals	Incomplete immune system, for example due to immunosenescence	Repeat PCR in multiplicates (same tissue, second independent DNA isolation, and/or related tissue) → compare patterns for consistency and compare with primary process (in case of staging)
Oligo-/monoclonality in histologically reactive lesion	Exaggerated immune response with dominant specificity, presence of large germinal centers	i) Repeat PCR in multiplicates (same tissue, second independent DNA isolation, and/or related tissue) → compare patterns for consistency ii) (Re-)evaluate histopathology

CDR: Complementarity-determining region; FR: Framework region; PB: Peripheral blood; PCR: Polymerase chain reaction; SHM: Somatic hypermutation.

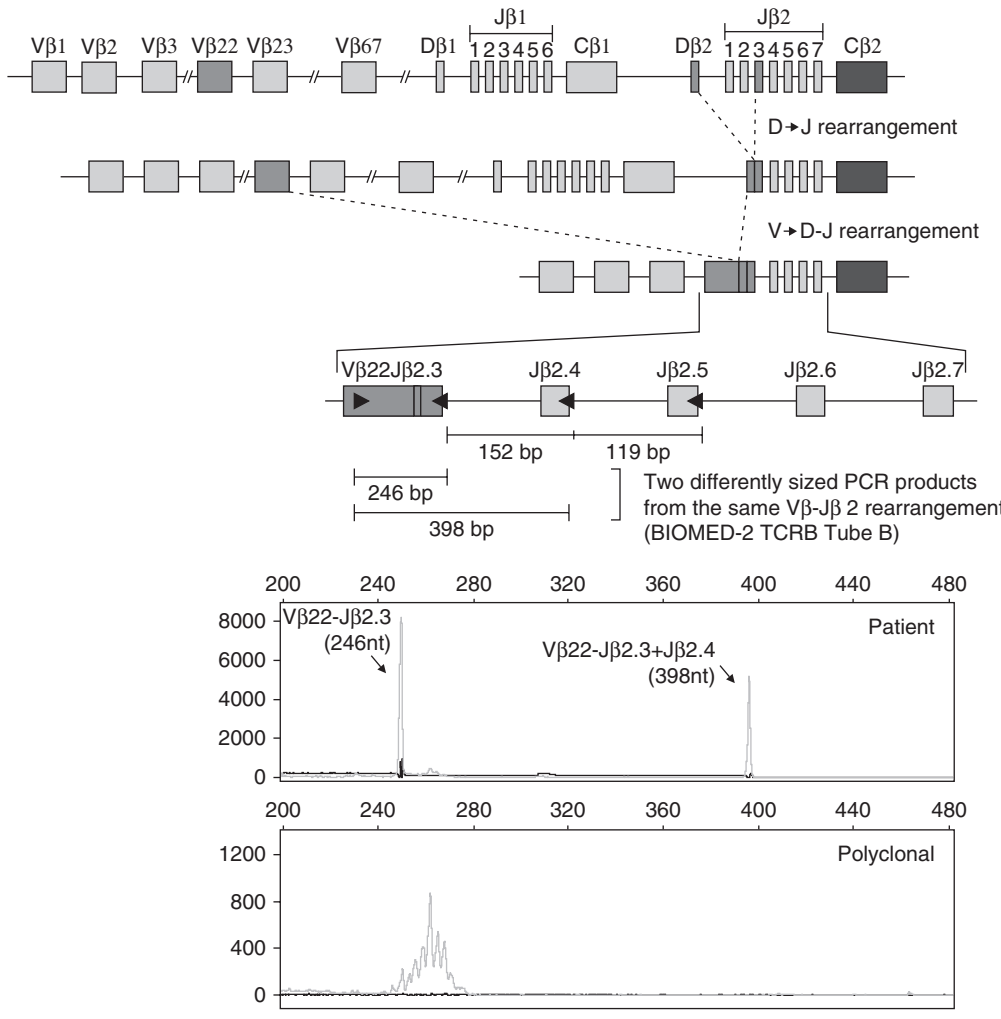

Figure 2. Two differently sized PCR products arising from the same *TCRB* rearrangement. Schematic representation of the *TCRB* locus. Shown is an ALCL sample (case 2005-082) in which the Vβ22-Jβ2.3 rearrangement is amplified with the Jβ2.3 primer from BIOMED-2 *TCRB* mixture B, and the same rearrangement is also amplified as an 'oversized' product with the Jβ2.4 primer from this mixture B.
ALCL: Anaplastic large cell lymphoma; PCR: Polymerase chain reaction; TCR: T-cell receptor.

from the same rearrangement. Third, some Ig/TCR loci have a more complex configuration in that two rearrangements occur simultaneously on the same allele, mounting up to a total of four rearrangements per B- or T-cell clone. This holds for the *IGK* locus where Vκ-Jκ rearrangements can reside on the same allele in combination with an intron-Kde inactivating rearrangement (**Figure 3**) [46]; in the *TCRB* locus V-Jβ1 or Dβ1-Jβ1 rearrangements can be followed by a Dβ2-Jβ2 rearrangement on the very same allele [8]. Taken together, all of these phenomena illustrate that in case of multiple clonal signals, biclonality is more the exception than the rule.

4.2 Immunobiologic pitfalls

Several other pitfalls have a more immunobiologic basis. First, the lack of both monoclonal and polyclonal signals in a given sample might be explained by a very low number of B or T lymphocytes, which should be checked by histologic examination of the same tissue material that was used for DNA extraction (hematoxylin and eosin staining of the slides directly prior to and following the DNA extraction slides) and/or flowcytometry (also discussed in Section 5). In addition, the quality of the isolated DNA might be poor, such that Ig or TCR targets are not efficiently amplified; DNA quality of such cases should, therefore, always be checked in a control PCR protocol in which a range of differently sized PCR products from non-polymorphic genes are amplified [31]. However, in some cases, the lack of signal might be explained by a biologic phenomenon. The presence of a large B-cell clone that is heavily somatically mutated and would remain undetected when analyzing only one or a few Ig targets, is one possibility. For this reason, the authors strongly advise to check multiple Ig targets to assess clonality [33]. On the other hand, low numbers of B

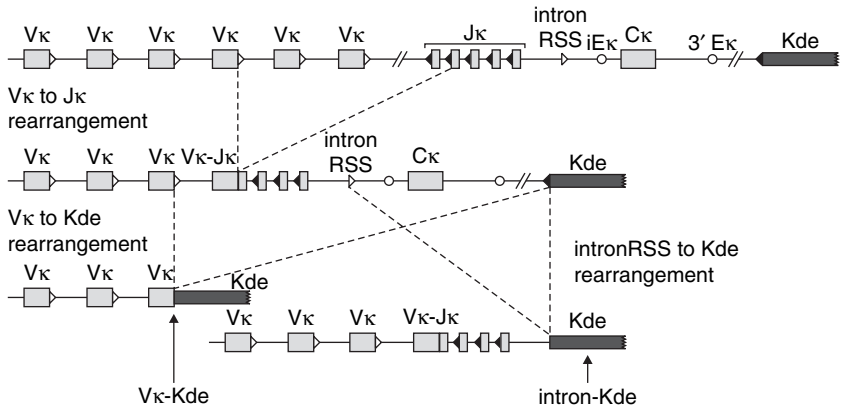

Figure 3. Examples of recombinations in the human *IGK* locus. Schematic overview of classical recombinations in the human *IGK* locus. *IGK* recombination mostly starts with a Vκ-Jκ recombination, the functionality of which can be disrupted by recombination of Kde to Vκ gene segments (Vκ-Kde, deleting the entire Jκ-Cκ region) or to an isolated heptamer in the intron between the Jκ and Cκ segments (intron-Kde, resulting in Cκ deletion). In the latter case the intron-Kde rearrangements resides on the same chromosome allele as the original Vκ-Jκ recombination.

Table 3. Applications of Ig/TCR clonality testing.

Application	Value
Discrimination of tumor versus reactive versus normal	++
Staging: evaluation of dissemination/localization (note: limited sensitivity level of 1 – 10%)	+/++
Evaluation of clonal relationship between multiple lesions at same time	++
Evaluation of clonal relationship between diagnosis and disease recurrence (relapse)	++
Lineage determination (T versus B versus NK)	+
Monitoring/evaluation of treatment effectiveness (note: limited sensitivity level of 1 – 10%)	+

NK: Natural killer; TCR: T-cell receptor.

and/or T lymphocytes might also create another pitfall: pseudoclonality or selective amplification due to a low level of specific Ig/TCR gene template. To avoid misinterpretation of the clonality data in such cases, repeated analyses on the same or a related tissue sample are essential to check for consistency of the pattern; alternatively, a second independent DNA isolation from the same sample might prove useful. In all of these, only a consistent clonality pattern can be interpreted as sign of a true monoclonal cell population. Another pitfall concerns detection of oligoclonality or even (low level) monoclonality of T or B lymphocytes under certain immunobiologic conditions, such as infections and inflammatory conditions [47-53]. Detection of an oligoclonal T-cell repertoire in elderly individuals should be considered as a potential sign of an incomplete immune system due to ageing of the system (immunosenescence) [54]; the same might apply to the B-cell repertoire, though possibly to a lesser extent. In addition, repeated analyses, using the same sample, a second independent DNA isolation from the same sample and/or a related sample need to be evaluated for consistency of the results. In the case of disease staging, it is important to include a DNA sample from the primary location for the purpose of comparison. Histologically reactive lesions represent a broad spectrum of lesions ranging from heterogeneous, polyclonal lymphocytes in true reactive lymphoproliferations, to proliferations containing (oligo-)clonally activated lymphoid cell populations or even a monoclonal component [35,55,56]. Hence, oligoclonality or monoclonality in such cases often reflects an exaggerated immune response with a dominant immunospecificity. Apart from repeated analyses on the sample, on independent second DNA isolations and/or on a related sample, a careful (re)evaluation of histopathologic and molecular findings is needed for reaching a correct interpretation of the oligo-/monoclonality results.

4.3 Control samples in clonality testing

Finally, irrespective of the above pitfalls, the authors advise for any clonality testing to include not only monoclonal cell lines [31,38], but also samples such as peripheral blood mononuclear cells or tonsillar cells showing the typical polyclonal pattern of Ig/TCR products with a heterogeneous CDR3 size distribution. This acts as control for primer quality as well. In addition, samples without rearranged Ig/TCR genes (e.g., the HeLa epithelial cell line) can be informative in identifying non-specific bands/peaks that are sometimes found in multiplex assays [31]. These three types of control samples facilitate the interpretation of the patterns in the clinical samples.

5. Interactive interpretation model

No matter how important the knowledge about technical and immunobiologic pitfalls of Ig/TCR rearrangement

Table 4. Ig/TCR translocations in lymphomas of B- and T-cell origin.

Entity	Translocation	Involved genes	Frequency %	Detection rate in routine PCR
BL	t(8;14)	*IGH, MYC*	> 95	Low, due to scattered breakpoints
MCL	t(11;14)	*IGH, CCDN1*	> 95	Maximum 30 – 40%
FL	t(14;18)	*IGH, BCL2*	> 90	~ 90%
DLBCL	t(14;18) t(8;14)	*IGH, BCL2* *IGH, MYC*	25 10	~ 90% Low, due to scattered breakpoints
MM	t(11;14) t(4;14)	*IGH, CCDN1* *IGH, FGFR*	15 – 20 10	Low, due to scattered breakpoints Low, due to scattered breakpoints
T-PLL	t(14;14)/inv(14)	*TCRA/D, TCL1*	85 – 90	Low, due to scattered breakpoints

BL: Burkitt lymphoma; DLBCL: Diffuse large B-cell lymphoma; FL: Follicular lymphoma; MCL: Mantle cell lymphoma; MM: Multiple myeloma; PCR: Polymerase chain reaction; TCR: T-cell receptor; T-PLL: T-cell prolymphocytic leukemia.

analysis is, it does not suffice in all cases. For appropriate interpretation of the molecular data it is absolutely essential to integrate these with data from (hemato-)pathology, and preferably also with the results from flowcytometric immunophenotyping. Such an interactive interpretation model with regular contacts between molecular biologists, pathologists, hematologists and immunologists guarantees integration of all available data to reach the most reliable diagnosis. Especially, the percentage of suspected cells and the percentages of normal (reactive) B and T lymphocytes, as available from histopathology and/or flow cytometry, are important parameters to be considered for estimating the relevance of the Ig/TCR clonality findings. Thus, the finding of weak clonal signals (in one or more targets) in a background of polyclonal signals is hardly compatible with a large suspect cell population and, hence, such Ig/TCR findings should be interpreted with caution. On the other hand, the absence of both monoclonal and polyclonal *IGH* patterns in a sample showing a large B-cell infiltrate is a finding that is not logical, and that should lead to further analysis of other Ig targets before definitive conclusions can be drawn. Finally, the true meaning of clonality can also be dependent on the histologic context. The presence of a clonal pattern in a case with a diffuse infiltrate is different from a clonal signal in a case with large germinal centers in histopathology, in which the clonality most likely reflects an exaggerated immune response with a dominant specificity (see Section 4.2 also) [35]. Regular discussion of cases in, for example, (bi-)weekly multidisciplinary patient meetings should avoid the above-mentioned misinterpretations.

6. Applications

Although multiple applications of Ig/TCR clonality testing can be defined (**Table 3**) [57], discriminating tumor cells from reactive or normal lymphocytes is beyond doubt the most important and most valuable application. The current protocols have shown to be very reliable and useful for this purpose, although clonality in cases with histopathologically reactive lesions should be reviewed and discussed more extensively, as discussed in Section 5. In ambiguous cases, parallel analysis of Ig-/TCR-associated chromosome aberrations might be considered as alternative or additional PCR clonality testing approach, as in some entities (especially FL and, to a lesser extent, MCL) reasonable to high detection rates of such specific aberrant Ig/TCR rearrangements can be expected (**Table 4**). Ig/TCR clonality testing is also very useful in establishing the clonal relationship between multiple lesions at the same time (one tumor versus two different tumors) or in case of recurrence of the disease (true relapse versus secondary malignancy). In such cases, the samples are best evaluated in the same experiment for the most direct and accurate comparison. Evaluation of dissemination/extranodal localization and monitoring of treatment effectiveness can also be performed via clonality testing, albeit the added value is influenced by the detection limits of the assays, which are ~ 1 – 10% depending on the Ig/TCR target [31]. When more sensitive approaches are required, real-time PCR-based strategies with (patient-)specific primers and probes should be applied [58]. Finally, lineage determination via Ig/TCR assays is an option, but here the value is limited by the fact that Ig and TCR rearrangements are not exclusive for B and T lymphocytes, respectively, and can occur as cross-lineage rearrangements [59].

7. Improvements and perspectives

Even though highly standardized protocols are now available, further improvements are still needed in PCR-based Ig/TCR clonality testing. This includes, for example, the reliable use of fixed and paraffin-embedded tissue for molecular clonality testing. Due to the use of different protocols for formalin fixation and paraffin embedding of tissue, clonality assessment from paraffin-embedded tissues is far from optimal. Methods for tissue fixation and tissue processing need adaptation and standardization to improve DNA quality and to increase the rate of clonality detection. Following standardization, a further perspective of the current protocols is their routine use in clonality testing of paraffin-embedded tissue biopsies. Application of the current protocols for Ig/TCR repertoire

studies is another interesting application. The latter concerns the use of family-specific primers in 'simplex' reactions to study skewing of V usage between families and/or CDR3 sizes within families, which is relevant in inflammatory or immunocompromised conditions (e.g., in patients with a primary or secondary immunodeficiency).

8. Conclusion

The introduction of the standardized BIOMED-2 multiplex PCR protocols for analysis of Ig/TCR gene rearrangements has greatly increased the reliability of clonality testing in suspected lymphoproliferations [31,32]. This has resulted in a worldwide use of the BIOMED-2 protocols. Therefore, correct interpretation of the Ig/TCR clonality data becomes more and more important. In this context, knowledge on the biology of Ig/TCR recombination and ample expertise with clonality testing are essential. In this review several immunobiologic pitfalls that should be taken into account to avoid misinterpretation of data were addressed: pseudoclonality due to low numbers of T and B lymphocytes, oligoclonality during immunosenescence, monoclonality in reactive lesions showing an immunodominant repertoire, multiple rearrangements per allele. In addition, technical pitfalls need attention, such as poor DNA quality, and 'undersized' and 'oversized' PCR products. Finally, an interactive model in which interpretation of the molecular data are optimally integrated with data from (hemato-)pathology, and preferably also flowcytometric immunophenotyping, was discussed. Close interaction between the involved scientists (molecular biologists, pathologists, hematologists, immunologists) should guarantee integration of all available data to reach the most reliable diagnosis.

9. Expert opinion

Nowadays, reliable Ig/TCR clonality testing in specialized laboratories has become a realistic option. It has been convincingly shown that PCR-based clonality testing can reliably replace Southern blot-based analysis and, hence, could now be considered the standard strategy for the coming years, thus overcoming the difficulties and practical problems of Southern blotting. In clonality testing, PCR-based strategies will not readily be replaced by other methodologies, such as array-based strategies; at best flowcytometric immunophenotyping of T-cell proliferations using a panel of antibodies directed against the V domains of TCR chains can have a role as screening approach [60,61]. Although multiple PCR protocols exist that might all serve the same purpose, the most optimal strategy would have to combine full coverage (complementarity of Ig/TCR targets) with ease of use (multiplexing of primer combinations), such as in the BIOMED-2 multiplex Ig/TCR strategy, which has become a world standard.

With the method being standardized to a high level, several aspects of internal and external quality control should now be considered. This not only includes exchange of samples in quality control rounds, but certainly also refers to correct interpretation of the results based on immunobiologic insight in recombination and knowledge about pitfalls. In addition, standardization and evaluation of pre-analytical procedures for tissue fixation and processing is needed to improve DNA quality and thereby molecular clonality analysis. Taken together, this implies that Ig/TCR clonality testing should be performed in a laboratory that has a reasonable weekly throughput of samples to have a critical volume of analyses to build and sustain expertise. Furthermore, such a laboratory would also have to function in a multidisciplinary team having regular contacts with other scientists on histopathology and/or immunophenotyping results. Scientists should learn from each other's specific diagnostic possibilities, but also from the limitations and pitfalls of the assays. Educational workshops at local, regional, national or international level play an important role in this respect.

Finally, all of the above considerations also apply to other types of tumors in which clonality testing is of importance in the diagnostic process. Development of standardized protocols is a first step to improve the procedure and to make it valuable and reliable test. Quality control rounds need to be introduced to increase the average level of skills and interpretation, and results need to be discussed in multidisciplinary teams.

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