Original paper

Single-step next-generation sequencing of *immunoglobulin heavy chain* genes to detect clonality in classical Hodgkin lymphoma

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Classical Hodgkin lymphoma (cHL) diagnosis occasionally requires clonality assessment. However, the paucity of neoplastic Hodgkin and Reed-Sternberg cells among abundant reactive elements dramatically reduces the sensitivity of standard polymerase chain reaction (PCR)-based BIOMED-2/EuroClonality protocols, which fail to detect clonality in approximately one-third of cases. Next-generation sequencing (NGS)-based approaches have been proposed to overcome this limitation, but their performance in routine diagnostics for cHL remains insufficiently evaluated.

We analyzed seven cHL cases and five follicular lymphoid hyperplasia (FLH) controls, all showing polyclonal patterns by standard PCR/capillary electrophoresis. DNA extracted from formalin-fixed paraffin-embedded lymph node biopsies was subjected to *immunoglobulin heavy chain* (*IGH*) gene rearrangement analysis using the commercially available LymphoTrack® *IGH* assay on the MiSeq platform.

Next-generation sequencing identified clonal *IGH* rearrangements in 3 of 7 (43%) cHL cases previously undetectable by conventional methods. Clonal cases exhibited a dominant rearrangement representing > 2.5% of total reads, with the second most frequent rearrangement being less than half of the first. The remaining cHL cases and all FLH controls displayed polyclonal patterns.

The LymphoTrack® *IGH* NGS assay demonstrated superior sensitivity over standard PCR-based protocols for detecting clonality in cHL, supporting its implementation in routine diagnostic workflows for challenging cases.

Key words: clonality, *immunoglobulin heavy chain*, BIOMED2, Hodgkin lymphoma, diagnostic accuracy, PCR, next-generation sequencing, LymphoTrack® *IGH* Assay.

Introduction

Hodgkin lymphoma (HL) is the commonest B-cell malignancy in young adults in Western countries [1]. Differently from the vast majority of lymphomas, the cellular population composing this neoplasm is mainly represented by non-neoplastic, reactive elements, including B and T lymphocytes, granulocytes, macrophages and dendritic cells [1]. Clonality analysis in classic Hodgkin lymphoma (cHL) is of added value for correctly diagnosing patients with atypical presentation or histology characteristic of T-cell lymphoma, and for establishing the clonal relationship in patients with recurrent disease. However, such analysis has been hampered by the sparsity of malignant Hodgkin and Reed-Sternberg (HRS) cells in a background of reactive immune cells. Indeed, the diagnostic, neoplastic cells (Reed Sternberg, Hodgkin, and variants) constitute less than 10% of the entire population, making the diagnosis extremely complicated. The causes of HL are not clearly defined, and it seems to be associated with family factors, viral exposure and immune suppression. In fact, it has been seen that same-sex siblings of a patient with HL have a 10-fold higher risk of developing disease, as in the case of monozygotic twins, where the risk of developing the disease is higher than for dizygotic twin siblings [2]. In addition to genetic causes, there is also an abnormal response to infection in the pathogenesis of HL; and this is the reason why there is an association with human immunodeficiency virus (HIV) infection, and HIV-infected patients have a significantly increased risk of developing HL. Furthermore, some epidemiologic and serologic studies have found a connection between Epstein-Barr virus (EBV) and the manifestation of HL disease, as the EBV genome has been detected in tumour specimens of patients with HL [2]. Although the pathological diagnosis of HL is more often based on morphology and immunophenotyping, in a percentage of cases, molecular testing and particularly clonality assessment is required.

Clonality studies of lymphoid malignancies are currently centered on standardized protocols developed by the BIOMED2 consortium based on polymerase chain reaction (PCR) assays followed by capillary electrophoresis (CE) and/or Sanger sequencing [3] in order to detect *immunoglobulin heavy chain* (*IGH*) and × *light chain* (*IGK*) gene rearrangements, followed by fragment analysis using GeneScan or heteroduplex analysis. Such protocols guarantee high sensitivity and specificity even in routine formalin-fixed paraffin-embedded (FFPE) material [3]. However, the sensitivity, i.e. the ability to identify clonal rearrangements, is dramatically affected in HL due to the paucity of neoplastic cells, allowing detection only in two-thirds of cases [4]. To overcome this limitation, next-generation sequencing (NGS)-based approaches have recently been proposed [5, 6], indeed providing evidence of their powerfulness also in HL clonality detection [7]. Furthermore, despite the insufficient concentration of DNA due to the low tumour population of the tissues analyzed by biopsy, circulating tumour DNA (ctDNA) coupled with NGS has emerged as a promising approach for the diagnosis and monitoring of patients with cHL [8]. In fact, it was seen that it could improve the diagnosis, risk stratification and follow-up procedures for patients with this disease. Another advantage of NGS is the immediate sequence availability of the identified clonotypes, both of the malignant clone as well as the non-neoplastic background B-cells. This will provide more reliable information to detect minor clones in a background of polyclonal B-cells and assessing the clonal relationship of multiple B-cell malignancies within the same patient. However, they have not been tested so far in the real life routine diagnostics in these settings. In fact, the achievement of an international consensus on the design of personalized or targeted NGS panels constitutes a discussed topic that is still too heterogeneous, and different laboratories have used their own protocols. In particular, the necessity for a consensus in studies of mature B-cell neoplasms with NGS has been highlighted by the French LYSA (Lymphoma Study Association) and the Groupe de Biologistes Moléculaires des Hemopathies Malignes [8].

In this study, we aimed to test the sensitivity of a commercially available kit for NGS analysis (Invivoscribe Technologies' LymphoTrack® *IGH*) in a series of cHL cases in which the PCR-based approach had already failed to detect clonality.

Material and methods

We analyzed 7 cHL cases for which DNA was extracted from lymph-node biopsies by QIAamp DNA kit (Qiagen, Limburg, Netherlands) as previously reported [9] and analyzed it for purity and concentration using Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). Five cases of follicular lymphoid hyperplasia (FLH) were also studied.

Immunoglobulin heavy chain clonality analysis by PCR/CE was performed according to the BIOMED2 protocols and EuroClonality guidelines (http://www.euroclonality.org/) [3, 5, 10]. All cases of both cHL and FLH showed polyclonal patterns at PCR/CE.

Library preparation and sequencing on the MiSeq platform (Illumina, San Diego, CA, USA) were performed as recently described, starting from 1 μg of DNA, using the LymphoTrack® *IGH* [11].

The positive control consisted of DNA from a B-cell line with a well-characterized *IGH* rearrangement; the negative control consisted on tonsil

Table I. Results of LymphoTrack® next-generation sequencing assays

Case number	DNA concentration [ng/ l]	V-gene	J-gene	Percentage of total reads
195	257	*IGHV3-21_02*	*IGHJ5_02*	12.43
196	545	*IGHV3-33_01*	*IGHJ6_02*	0.89
197	414	*IGHV3-23_04*	*IGHJ5_02*	1.37
198	529	*IGHV4-39_01*	*IGHJ4_02*	8.97
199	309	*IGHV1-3_01*	*IGHJ6_02*	1.30
200	292	*IGHV3-21_02*	None	0.56
201	64	*IGHV3-21_02*	*IGHJ6_02*	6.21

The cases showing a clonal rearrangement are indicated in grey.

DNA characterized by no sequence with a frequency higher than 1%.

Results

Following NGS analysis, we observed that 3 out of 7 cases presented with a clonal rearrangement (i.e. representing more than 2.5% of all reads, the second commonest being less than half of the first) (Table I). Bearing in mind that the neoplastic cells can be really scant in cHL, we then investigated whether rearrangement with a frequency below 2.5% (conventionally accepted for clonality assessment with this assay) could also be indicative of a clonal rearrangement. To achieve this, we evaluated the top 10 most represented rearrangements to assess whether the most represented rearrangements were anyway at least twice as much as the second ones. Indeed, this condition was observed only in the three cases for which clonality was already established. In the remaining 4 cases, the difference among the top 10 rearrangements was really minimal in terms of frequency (Figure 1). As expected, all cases of FLH presented polyclonal patterns, as previously reported [6].

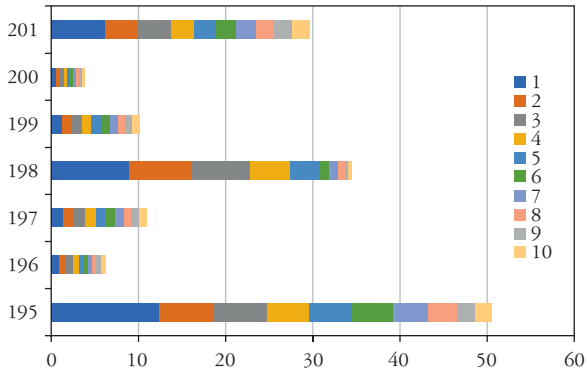

Figure 1. Top 10 rearrangements detected in the 7 HL cases (percentage of total reads on x axis)

Case number (195–201) is indicated on y axis.

Discussion

In a subsequent study [12], it was shown that NGS performance was better than conventional BIOMED-2/EuroClonality analysis to detect clonal gene rearrangements in 16 well-characterized primary cHL cases within the *IGH* and *IGK* loci. This was most obvious in FFPE tissue specimens, where three times more clonal cases were detected with IG-NGS (9 cases) compared to BIOMED-2 (3 cases). In total, almost four times more clonal rearrangements were detected in FFPE with IG-NGS ($n = 23$) as compared to BIOMED-2/EuroClonality ($n = 6$).

The most recent literature showed an efficiency of 20–65% in the use of PCR-based methods for the detection of *IGH* clonality [4]. Of note, the addition of the light chains loci (*IGK*/*IGL*) may dramatically increase the sensitivity [13]. However, the latter results as well as those obtained by semi-nested PCR [4] need to be confirmed in independent studies and proper diagnostic accuracy studies. Conversely, NGS-based approach was proved to be able to detect clonal rearrangements in HL cases even from peripheral blood [7] and, by direct comparison of NGS-based clonality assessment with the conventional BIOMED-2/GeneScan analysis to detect gene rearrangements in undissected cHL-tumour tissue, our study demonstrated a significantly improved performance of the NGS assay [12].

It must also be stressed that the use of NGS in clonality detection of cHL, compared to conventional PCR methods, has proved to permit a better and more objective resolution and interpretation of the results, bettering the characterization, classification, and stratification of these neoplasms; furthermore, NGS enables the analysis of archival samples with particularly degraded DNA and is thus more suitable for retrospective studies [14, 15].

Moreover, in a perspective of optimization and standardization, EuroClonality-NGS Working group has developed protocols, primers, and bioinformatics pipelines for a reliable NGS-based clonality analysis of *IGH* and *IGK* loci in B-cell lymphoproliferative

disorders, like cHL [16]. The use of this technique allows to collect information regarding different clonotypes in the same tumour sample, highlighting the clonal diversity and showing more unrelated clonal IG clonal gene rearrangements in the same cHL cases and involving both the *IGH* and *IGK* genes [10, 17]. The clonotypes detected by NGS were shown to be of neoplastic cell origin, based on the identification of identical IG gene rearrangements in HRS-enriched cell fractions as compared to whole tissue specimens. Furthermore, data from the 16-case study revealed evidence of biclonality in two cases of chronic lymphoid lymphoma (cHL), confirmed by single-cell analysis in one of the cases [12]. This new standardized assay has been validated as an improvement in efficiency of clonality detection in different mature B-cell lymphoma subtypes as compared to the conventional BIOMED-2 analysis [12, 18].

In addition, several studies about NGS-based analyses also demonstrated a significant increase of reliability and feasibility as regards the minimal residual disease (MRD) monitoring of these malignancies, virtually extending it to all patients, and, therefore, significantly enhancing the monitoring of the disease progression and treatment response [19, 20]. In fact, thanks to sequencing, it will be possible to biologically classify lymphoma, which has three distinct subtypes, and identify and monitor patients at a very high risk of recurrence within these subtypes [21]. This is extremely important considering the percentage of recurrence and relapse with this pathology; in fact, approximately 20–30% of patients with cHL relapse after achieving a complete metabolic response and if first-line therapy fails, high doses of chemotherapy and autologous hemopoietic stem cell transplantation are curative in only half of patients [8, 20]. Therefore, the early recognition of high-risk cHL patients and the monitoring of MRD in order to plan therapy remains one of the main objectives of research. This is very relevant especially considering lymphoid malignancies like HL in which, as mentioned, the neoplastic population can be meager. Eventually, the combined use of tissue NGS and ctDNA represents a promising strategy for initial molecular identification and MRD monitoring. Recent studies on large cohorts demonstrate that the majority of cHL patients have detectable ctDNA at baseline and that ctDNA profiling can provide prognostic and predictive information comparable to that of tumour tissue. The integration of NGS clonality calling with ctDNA analysis could therefore extend the possibility of molecular monitoring to patients with low tumour tissue representativeness [21].

The LymphoTrack® *IGH* was recently shown to be highly effective in detecting clonality in both lymphomas and multiple myeloma patients [11]. Based on this, we explored, for the first time, its potential role in challenging (i.e. polyclonal with conventional methods) cHL cases. We ended up with 3/7 (43%) of clonality detection, which is remarkable if compared with the 0/7 obtained in the same series by PCR. However, it should be considered that while the positive predictive value turned out to be 100%, a negative predictive value of 56% should be improved. In the real practice this might be allowed by the addition of *IGK*/*IGL* testing which, however, implies a further cost increase.

Therefore, the limitations of the present study include the small size of the cohort and the lack of systematic analysis of light chains; prospective studies on larger cohorts, preferably multicenter and with standardized NGS pipelines (EuroClonality), are needed to define diagnostic thresholds and to clinically validate the routine use of NGS and ctDNA in the diagnostic and MRD workup of cHL [18].

Bearing this in mind, we believe that adequate phase III and IV diagnostic accuracy studies should be planned comparing the most promising PCR-based techniques (e.g. the recently described semi-nested PCR [4] and NGS, including both heavy and light chain loci. This would eventually allow applying and standardizing evidence-based strategies.

Conclusions

Next-generation sequencing-based LymphoTrack *IGH* assay turned out to be more sensitive than conventional PCR for clonality detection in difficult cHL cases. Additionally, this new testing will be an ideal tool in clinical diagnostics for recurrent disease, since clonal comparison based on nucleotide sequences and assigned clonotypes is more reliable than comparing fragment lengths. However, further studies are needed to set NGS as a possible new standard in clonality testing.

Disclosures

1. Institutional review board statement: The study was developed and conducted in accordance with the Helsinki Declaration; informed consent was obtained by all participants and this study was approved by the local Ethics Committee of the AVEC (approval decision no: Protocol number 1/2011/U/Tess, dated: January 2011).
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4. Conflicts of interest: None.

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