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ORIGINAL ARTICLE

Capillary electrophoresis single-strand conformation analysis (CE-SSCA) for clonality detection in lymphoproliferative disorders

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Abstract Clonality analysis is a critical tool for the diagnosis of suspect lymphoproliferative disorders. Amplification of the immunoglobulin and T cell receptor genes on genomic DNA from the suspect samples is followed by analysis of the PCR products to distinguish between polyclonal and clonal rearrangements. These analyses are based on electrophoresis in polyacrylamide gels after heteroduplex formation, or more frequently, GeneScan analysis is performed by capillary electrophoresis in automated DNA analysers, providing higher resolution and sensitivity. An alternative method for clonality analysis is the use of single-strand conformation analysis; however, this usually required labour intensive work with polyacrylamide gels and radioactive labelling. Within the Euro-Clonality group, we have developed a non-radioactive automated analysis based on capillary electrophoresis of single-strand PCR products that combines some of the benefits of heteroduplex and GeneScan analysis. This new method could be particularly suitable for challenging cases and could be implemented as an alternative to the more laborious heteroduplex analysis in standard gels in some instances.

Keywords Clonality analysis · Lymphoproliferative disorders · Single-strand conformation analysis

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Introduction

The diagnostic pathway for suspect lymphoproliferative disorders involves a multidisciplinary approach combining morphological assessment with immunophenotyping or immunohistochemistry data in most cases. The diagnosis of clonality is normally based on the expression of aberrant markers in the cell surface, and in the case of B cell disorders, this can be aided by the immunoglobulin light chain restriction of the clonal population [1]. However, in a number of cases—varying between 5% to 10%, depending on the type of disease—confirmation of clonality is required by molecular analysis of the immunoglobulin heavy (*IGH*) and light chains (kappa –*IGK*- and lambda –*IGL*-) rearrangements or the T cell receptor rearrangements (*TCR*) [2]. Several methods have been described for the amplification of the immunoglobulin and TCR gene rearrangements, including the one developed by a European concerted action within the BIOMED-2 programme [3–6]. Electrophoresis in agarose gels is only useful to address

whether amplification of the target genes has occurred, but cannot distinguish between polyclonal and clonal amplifications. In order to analyse the clonality of the rearranged genes, there are currently two options. First, heteroduplex analysis can be performed by denaturation of the PCR products followed by slow renaturation at low temperatures and analysis by non-denaturing polyacrylamide gel electrophoresis (PAGE) [7–9]. In this case, the clonal products reanneal as homoduplexes, with the same DNA sequence, while the polyclonal products will form a combination of heteroduplexes that migrate at lower speed on PAGE, allowing the distinction between clonal and polyclonal products (Fig. 1a). The second method is high-resolution polyacrylamide analysis by capillary electrophoresis or "GeneScan", where fluorescently labelled PCR products are completely denatured in formamide and run in denaturing conditions on an automated sequencing analyser [6, 10–12]. Here, the resolution is as high as 1–2 bp allowing the distinction of polyclonal products by the presence of a Gaussian distribution of peaks, as opposed to clonal products that produce a distinct single peak of a particular size (Fig. 1b). GeneScan analysis has the advantages of being automated, less labour intensive, and has a higher limit of sensitivity for detection of low number of clonal cells. In contrast, heteroduplex analysis requires manual set up of PAGE, followed by staining of the DNA (e.g. ethidium bromide), and has a reduced limit of sensitivity compared to GeneScan. On the other hand, in cases where the target rearrangements do not provide a widespread distribution of the polyclonal Gaussian curve, due to the limited gene pool and diversity of rearrangements, heteroduplex is a very useful tool for clonality assessment. This is particularly important in the analysis of targets such as *IGK*, *IGL*, *TCRD*, and, to a lesser extent, *TCRG* [3–5, 13].

An ideal combination of both methodologies could be achieved by performing heteroduplex analysis on automated capillary electrophoresis (CE-HDA), therefore making the most of the ability to tease out the clonal products of the former with the enhanced sensitivity and automation of the latter. In order to achieve this, PCR products need to be subjected to heteroduplex formation followed by CE analysis with non-denaturing polymer at low temperatures (20°C to 30°C) [14–17]. Alternatively, PCR products can be subjected to full denaturation in formamide to obtain single-strand DNA products that can also be run on CE with non-denaturing polymer at low temperatures (20°C to 30°C). In this latter strategy, called CE-single-strand conformation analysis (CE-SSCA), the PCR products run exclusively based on conformation (i.e. DNA sequence) [18, 19]. Therefore, the polyclonal background containing multiple gene rearrangements of different sequences is dispersed across the electrophoretic range, leaving clonal PCR products to be detected as distinct peaks of variable size, depending on their conformation.

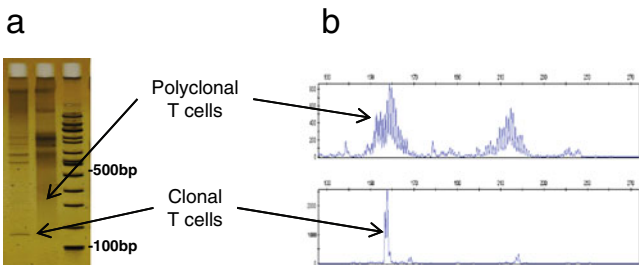

Fig. 1 Heteroduplex and GeneScan analysis for clonality assessment. **a** Heteroduplex analysis in PAGE with silver staining of a clonal (single discrete band) and a polyclonal sample (smear) amplified with the BIOMED-2 TCRGA tube. **b** GeneScan analysis of fluorescent fragments for the same samples, with a clear discrete peak on the clonal sample and a Gaussian distribution of polyclonal TCRG rearrangements on the polyclonal sample

Here, we outline the protocols and methodology for CE-HDA and CE-SSCA of amplified rearrangements of the immunoglobulin and T cell receptor genes. This study is intended as proof of principle, with the initial aim of establishing whether there is a potential role for CE-HDA or CE-SSCA as a new tool for clonality analysis. With this objective in mind, we provide a few examples of different cases of variable complexity and also highlight specificity and sensitivity issues and discuss the potential advantages and disadvantages of these methodologies for routine diagnostic use.

Material and methods

Clonality analysis was performed for all targets according to the EuroClonality (BIOMED-2) protocol [4] using FAM- or HEX-labelled primers to allow for detection by capillary electrophoresis. Heteroduplex analysis was performed using commercial, ready-to-use polyacrylamide gels (GeneGel Excell 12,5/24 Kit) run on GenePhor (Pharmacia Biotech) Electrophoresis subunit. Duplexes were visualized by silver staining, using the PlusOne DNA Silver Staining kit (GE Healthcare).

To perform CE-SSCA, an automated sequencer capable of using non-denaturing polymer and running at low temperatures is needed. For all our experiments, we used a 3130xl (LifeTechnologies, Foster City, US) using non-denaturing polymer mix consisting of 5% CAP polymer (LifeTechnologies, Foster City, US), 3730 running buffer, and 10% glycerol. One microlitre of the PCR products was mixed with 10 μL of deionized formamide and 0.5 μL of LIZ600 size standard (LifeTechnologies, Foster City, US) and then run on the 3130xL. All samples were run with buffer containing 10% glycerol at 20°C. For CE-HDA, the same approach was used with the exception that 1 μL of the

PCR products was mixed with 20 μL of ddH2O followed by heteroduplex formation as per BIOMED-2 protocol [4].

In non-denaturing conditions, the size standard (LIZ600 in our experiments) runs based on conformation rather than size; therefore, an adjustment on the size parameters is required. This is done by running the size standard under the same non-denaturing conditions and upon analysis in GeneMapper software assigning an arbitrary size (in bp) based on the scan number that the discrete peaks produced divided by 10. This allows for the correct alignment of all PCR products, although it obviously does not provide "real" sizing data.

Results and discussion

Evaluation of heteroduplexes vs single-stranded molecules on capillary electrophoresis

Figure 2a shows an example of CE-HDA for a clonal *IGH* (FR1, FR2) and *IGKDE* rearrangement in a clonal B cell lymphoproliferative disorder. The clonal peaks closer to the left on each panel (in blue) represent the homoduplexes, while the peaks at the far right of the panels represent heteroduplex products. The pattern of homoduplexes and heteroduplexes is very similar to the patterns found when running heteroduplex analysis on PAGE (Fig. 2b). Heteroduplex analysis by capillary electrophoresis has the following advantages over PAGE: no need for casting gels or the use of toxic staining (i.e. ethidium bromide), loading of samples is performed automatically, no need to take pictures of the gels, and sizing of the peaks is accurate, facilitating monitoring of the clonal populations over time or in different samples. However, similar to heteroduplex analysis on PAGE, there is a decrease in sensitivity compared to GeneScan analysis due to the formation of heteroduplexes, and this may be especially prominent in cases with high polyclonal background, where clonal rearrangements can be masked by heteroduplex formation.

An alternative to this approach is to use CE-SSCA, whereby the PCR products run according to conformation, thus avoiding loss of sensitivity caused by the formation of heteroduplexes. Figure 3 shows a comparison between conventional GeneScan analysis, CE-HDA and CE-SSCA in a clonal sample with and without polyclonal background and a polyclonal sample. The polyclonal and clonal samples are very easy to interpret by all three methodologies; however, as expected, it seemed that the analysis by CE-SSCA was slightly more sensitive than CE-HDA. Therefore, for the purpose of this review, we focused our efforts on the analysis of clonal and polyclonal products by CE-SSCA.

Detection of clonal Ig and TCR gene rearrangements by CE-SSCA

In order to assess the suitability of CE-SSCA as an alternative method for clonality analysis, we compared a series of samples amplified according to the BIOMED-2 protocol using GeneScan and CE-SSCA. These samples included clearly polyclonal and clonal specimens as well as more challenging samples. Figure 4 shows a series of polyclonal and clonal samples for a series of *IGH*, *IGK* and TCR targets, analysed by conventional GeneScan and CE-SSCA. As expected, the Gaussian distribution of the peaks in the polyclonal samples by GeneScan is not detected after

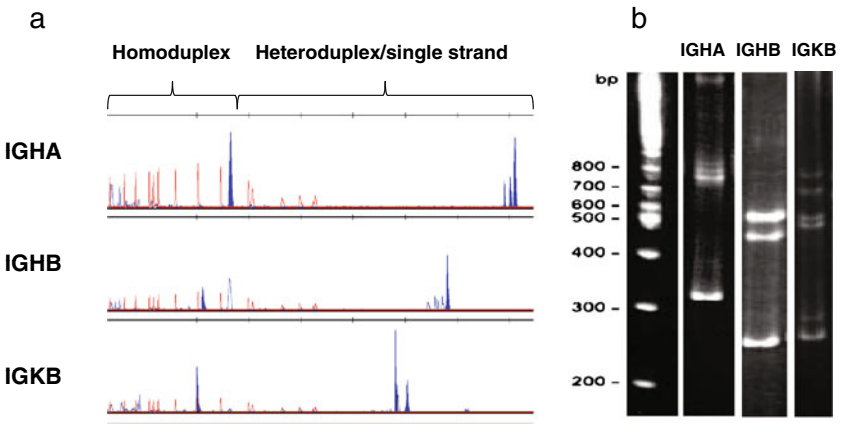

Fig. 2 Heteroduplex analysis in capillary electrophoresis and PAGE. **a** An example of CE-HDA for BIOMED2 IGHA, IGHB and IGKB tubes in a clonal B cell lymphoproliferative disorder. The *red peaks* represent the internal size standard while the *blue peaks* represent the clonal rearrangements. The size areas of expected homoduplex and heteroduplex (or single-strand) products are shown. **b** An example of a clonal B cell lymphoproliferative disorder analysed by heteroduplex analysis in PAGE (adapted from the original manuscript in *Leukemia* 17:2257–2317, 2003)

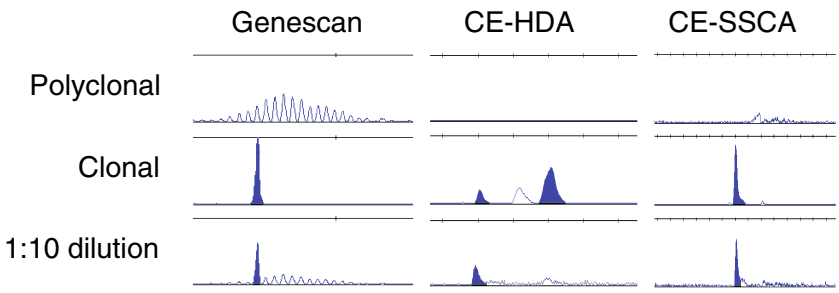

Fig. 3 BIOMED2 IGHA analysis by conventional GeneScan, CE-HDA and CE-SSCA. Comparison between a polyclonal and a clonal sample as well as a 1 in 10 dilution of the clonal DNA in polyclonal DNA (diluted prior to PCR amplification) using GeneScan, CE-HDA and CE-SSCA. The characteristic Gaussian distribution of the polyclonal rearrangements is lost by CE-HDA and CE-SSCCA analysis as polyclonal rearrangements represent different PCR products resulting in different conformations. The clonal peak by GeneScan analysis can result in more than one peak by any of the conformation analysis methods, as more than one stable conformer can co-exist at a given temperature in some instances (see text)

CE-SSCA due to the fact that polyclonal rearrangements represent a collection of multiple fragments that are different not only in size but also in sequence, therefore leading to a wide range of individual conformations of the PCR products which under CE-SSCA conditions run with different mobility. In contrast, the clonal samples are easily detected by both methods for all the targets tested. Interestingly, in some cases where only one clonal peak is present on GeneScan, CE-SSCA shows two or more peaks (e.g. Fig. 4, tubes TCRBB and TCRGA). This artefact is due to the formation of multiple stable conformers at any given temperature in some instances, depending on the sequence of the clonal product. This pattern cannot be predicted beforehand, and therefore, the presence of multiple peaks by CE-SSCA cannot be interpreted as oligoclonality unless confirmed by GeneScan.

Next, we wanted to test the performance of CE-SSCA in more challenging samples which had shown complex patterns by conventional GeneScan analysis. Figure 5 shows a few examples of samples with rearrangement patterns that are not straightforward to analyse. The first example (case 1) shows a case with a very weak clonal TCRBB rearrangement in the presence of a polyclonal background. The weak clonal peak can hardly be seen (indicated by the arrow) by GeneScan analysis; however, it becomes clearer by CE-SSCA. It is important to note that these challenging cases can be further analysed by GeneScan using different "window size" parameters in GeneMapper which in some instances (like in case 3, Fig. 5) can lead to a better resolution of the weak clonal peaks. In the second example (case 2), an irregular polyclonal TCRGB pattern is seen by GeneScan, and this pattern becomes more difficult to interpret by CE-SSCA,

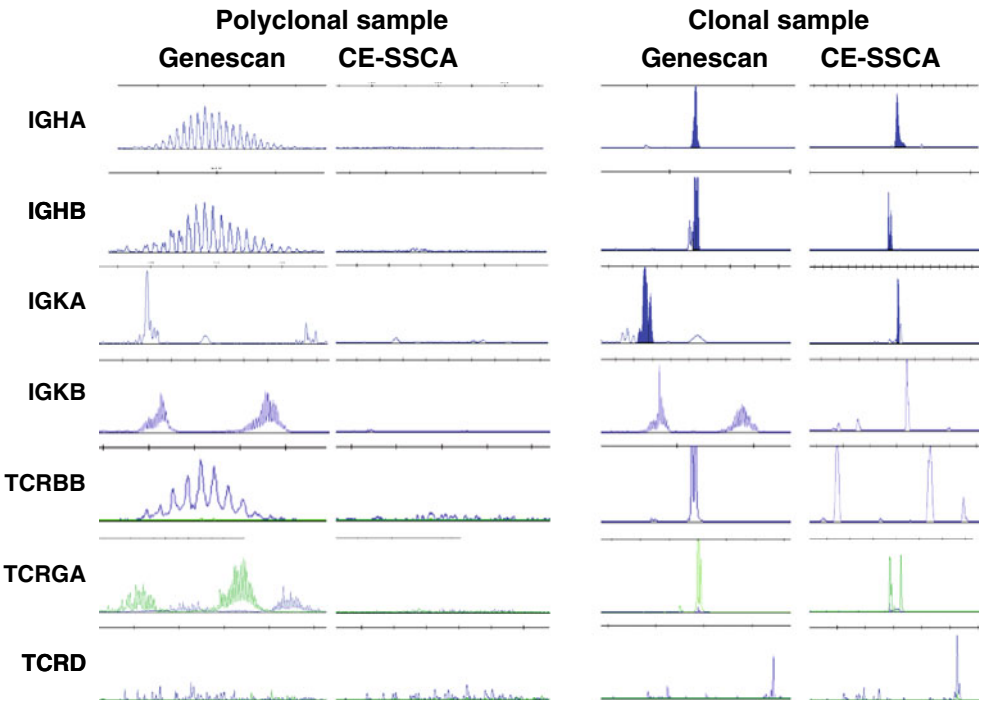

Fig. 4 Comparison between GeneScan and CE-SSCA in a series of clonal and polyclonal samples for different IGH, IGK and TCR targets. The comparison shows agreement between the two methods for all samples shown. The polyclonal Gaussian distribution seen in GeneScan is lost by CE-SSCA while the clonal peaks detected by GeneScan are still detected by CE-SSCA. The detection of single clonal rearrangements in tubes TCRBB and TCRGA is clear, even though the pattern obtained by CE-SSCA shows 2 peaks, resulting from two different stable conformers in these cases

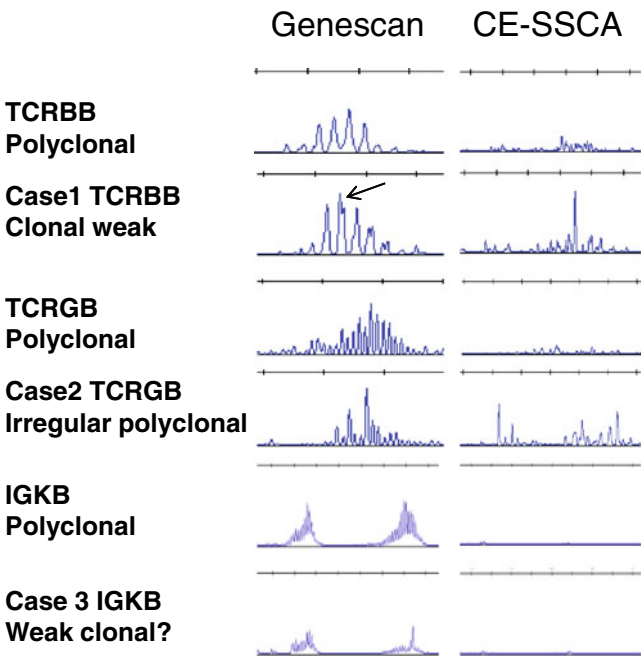

Fig. 5 Comparison between GeneScan and CE-SSCA in a series of samples with rearrangement patterns difficult to interpret by GeneScan. Case 1 represents a small clonal TCRBB rearrangement that is masked by the polyclonal background on GeneScan and becomes clearer upon CE-SSCA. Case 2 shows an example of an irregular polyclonal pattern in TCRGB tube that results in multiple weak clonal peaks on CE-SSCA. Case 3 shows the analysis of IGKB tube where there appears to be a weak clonal rearrangement within a polyclonal background in GeneScan analysis that is not detectable by CE-SSCA

where multiple peaks are seen. Finally, in case 3, GeneScan analysis shows a potentially clonal peak (weak) in IGKB (Kde) that is not seen after CE-SSCA. This discrepancy could be due to either the clonal peak being below the limit of sensitivity of CE-SSCA or, alternatively, due to the peak being part of the polyclonal profile in GeneScan.

Sensitivity of CE-SSCA

To address the sensitivity of CE-SSCA, we compared GeneScan analysis and CE-SSCA in serial dilutions of clonal DNA samples in polyclonal DNA samples. In particular, we analysed DNA obtained from two cell lines with clonal rearrangements (Jurkat for T cell rearrangements and SUDHL for B cell rearrangements) which were serially diluted in polyclonal DNA obtained from reactive tonsil samples. The dilutions analysed included 50%, 20%, 10%, 5%, and 1% of clonal DNA in polyclonal DNA. Figure 6 shows the results of these dilution series in a selection of clonality targets. The limit of detection of CE-SSCA is comparable to conventional GeneScan, with some cases showing slightly better resolution due to the lack of a polyclonal background (IGHA, IGKA, and TCRBA). The figure shows clearly how a clonal peak by GeneScan can result in multiple clonal peaks by CE-SSCA (seen for all targets except for IGHA), most likely due to the presence of multiple stable conformers for that particular sequence,

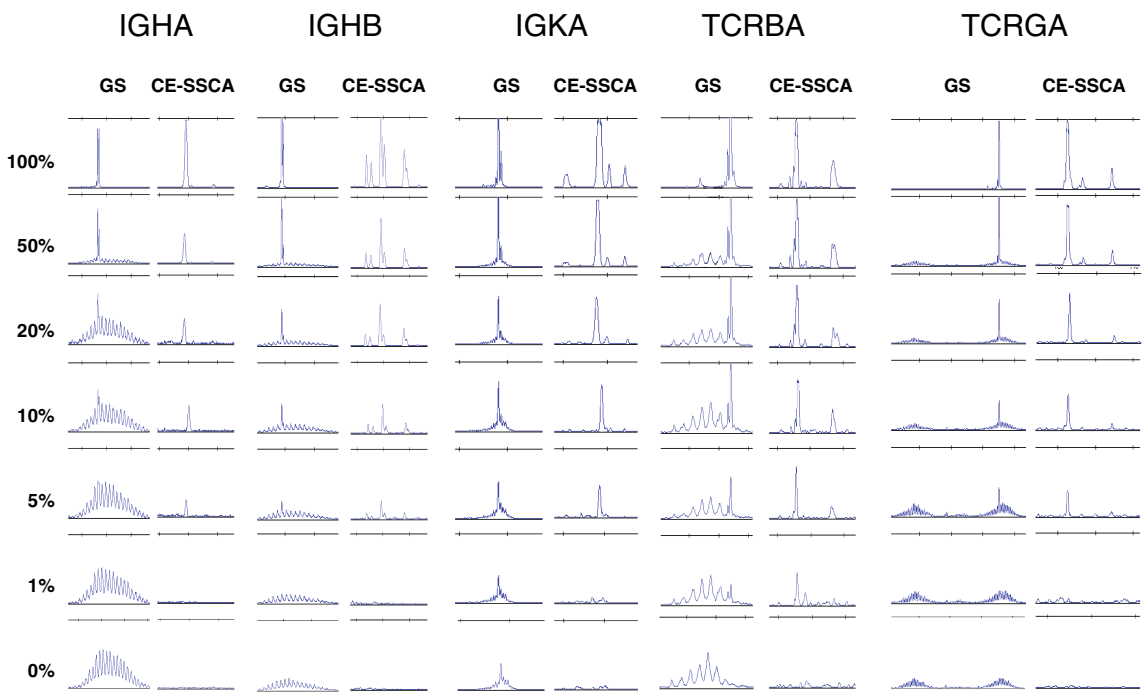

Fig. 6 Dilution series of clonal and polyclonal DNA analysed by GeneScan and CE-SSCA. The clonal cell lines used were Jurkat (T cell) and SUDHL (B cell). The percentage on the *left side* of the figure indicates percentage of clonal DNA in polyclonal DNA, with 100% being only clonal DNA and 0% being polyclonal DNA. Note that in some cases where GeneScan clearly shows only one peak (*IGHB*, *IGKB*, *TCRBA* and *TCRGA*), multiple peaks are detected by CE-SSCA

although theoretically, it can also result from the presence of slightly different sequences within the clonal product (i.e. intraclonal variation due to somatic mutations). It is important to note that these subtle differences in sensitivity are not target specific but rather sequence specific, as some particular rearrangements may have less stable conformers on CE-SSCA than others. In any case, these results show that CE-SSCA could be particularly useful for clonality assessment in samples with a high polyclonal background.

Advantages and disadvantages of the various approaches

CE-SSCA provides a significant advantage over conventional heteroduplex analysis on the basis of reduced labour and increased automation, with potentially higher limits of sensitivity. On the other hand, CE-SSCA requires a home-made polymer, which in some cases can be difficult to implement (i.e. where core facilities are used for GeneScan). Heteroduplex analysis has in turn the advantage of estimating approximate sizes, allowing for easier interpretation of the clonal patterns. In order to facilitate the use of these methods, commercially available pre-cast PAGE gels and silver staining methods can be employed for conventional heteroduplex, as well as ready-made non-denaturing polymers for use on capillary electrophoresis, when they become commercially available [20].

When compared to GeneScan, the main advantage of CE-SSCA is that it provides resolution of the clonal peaks based on sequence rather than size, therefore allowing for discrimination of weak clonal peaks embedded in a polyclonal background. It can also be a useful tool in difficult cases where the pattern of rearrangements is difficult to interpret, abolishing the need for more time-consuming PAGE-based heteroduplex analysis. However, the main disadvantage of CE-SSCA for clonality assessment is the inability to distinguish between polyclonal patterns and lack of PCR amplification, which can hamper the interpretation of clonality results. As explained before, some cases with a single clonal peak by GeneScan analysis can produce two or more peaks in CE-SSCA, due to the possibility of several stable conformers coexisting for any given DNA sequence. This can be particularly important in mature lymphoma cases where ongoing hypermutation processes can lead to the presence of several dominant clones with a clonal rearrangement of the same size (single peak by GeneScan) but with slightly different sequences that would result in different peaks by CE-SSCA. Therefore, using CE-SSCA for detection of clonality without GeneScan analysis in a routine setting may as such complicate the assessment. Furthermore, due to the lack of appropriate sizing, it can make the interpretation of the type of rearrangement difficult, especially when different samples from the same patient are compared (i.e. follow-up studies or samples from different tumour sites in the case of lymphomas).

Conclusion

This proof of principle study presented here is aimed to address whether CE-SSCA could be of benefit for clonality analysis in the clinical setting. In general, CE-SSCA appears to be a promising strategy to be used as a complementary method to conventional GeneScan or heteroduplex analysis, rather than as an alternative. There are clear advantages compared to PAGE-based heteroduplex analysis, mainly in relation to automation of the process and increased sensitivity. However, the interpretation of the results is hampered by the lack of accurate sizing and the presence of multiple clonal peaks in some cases with single rearrangements. In addition, it is not possible to distinguish between a polyclonal sample and a sample with no amplification of clonality targets, which would make it difficult to distinguish between reactive lymphoid proliferations and paucity of B or T cells. Thus, it would be recommended to perform CE-SSCA in combination with conventional GeneScan, to be able to distinguish between these two scenarios. For CE-SSCA, the availability of a commercial off-the-shelf non-denaturing polymer would be desirable to increase reproducibility and ease of use, as it has potential in some particular areas. One particular benefit of CE-SSCA is the sequence-specific conformational patterns produced which could be helpful in assessing ongoing hypermutations between biopsies taken from different sites or at different times in some lymphoma types given that the slight differences in sequence which cannot be detected by GeneScan could be easily distinguished by the different conformations of the individual sequences in CE-SSCA. Nonetheless, based on our current data, additional assessment of the applicability of CE-SSCA is currently needed before a final conclusion can be reached. A collaborative study comparing the performance of standard PAGE-HDA and CE-SSCA in a large series of patients is necessary prior to considering implementation in routine clinical use.

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Conflicts of interest The authors declare that they have no conflict of interest.

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