IMPLEMENTATION OF MICROFLUIDIC CHIP ELECTROPHORESIS FOR THE DETECTION OF B-CELL CLONALITY

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Abstract

Introduction: A clonal population of B-cells is defined as those cells arising from the mitotic division of a single somatic cell with the same rearrangement of immunoglobulin genes. This gives rise to DNA markers for each individual lymphoid cell and its progenies and enables us to study clonality in different B-cell malignancies using multiplex polymerase chain reaction - PCR. The BIOMED-2 protocol has been implemented for clonality detection in lymphoproliferative diseases and exploits multiplex PCR reaction, subsequently analyzed by heteroduplex analysis (HDA) using polyacrylamide gel electrophoresis (PAGE). With the advent of miniaturization and automation of molecular biology methods, lab-on-chip technologies were developed and replace partially the conventional approaches. We tested device for microfluidic chip, which is used for B-cells clonality analysis, using a PCR reaction for three subregions called frameworks (FR) of the immunoglobulin heavy locus (IGH) gene.

Material and Methods: For the implementation and comparison of the two methods we analyzed three unknown B-cell chronic lymphocytic leukemia (B-CLL) samples. As positive control (PK) we used one formalin-fixed, paraffin-embedded (FFPE) sample from B-CLL lymph node. The DNA was extracted from FFPE sections and multiplex PCR was used to amplify IGH gene segments. After PCR, the HDA was performed, the DNA fragments were evaluated on the PAGE and the microfluidic chip electrophoresis as well, and the results were compared.

Results: Using HDA with subsequent PAGE, we were able to confirm the clonality of the positive control and the tested samples. The same results were obtained by the Bioanalyzer 2100. The microfluidic chip electrophoresis was persuasive in all tested samples.

Conclusion: The implementation of microfluidic chip electrophoresis for detection of B-cell clonality by BIOMED-2 protocol on the device Agilent 2100 Bioanalyzer was successful and yielded the same results as the HDA - PAGE. Moreover, chip electrophoresis system is faster for preparation and less laborious than the conventional HDA - PAGE method.

Keywords: B-cell clonality, protocol BIOMED-2, HDA-PAGE, microfluidic chip electrophoresis,

INTRODUCTION

The detection of monoclonality in lymphoid populations is one of the most important successes in molecular pathology diagnostics and there exist a lot of different methods which are routinely used in many laboratories. One of the standard method for defining monoclonality in B-cell neoplasms is the demonstration of a single light chain isotype on the cell surface of neoplastic lymphoid cells. However, procedures which are based on PCR are welcomed additionally, because they are relatively quick, easy and they can be carried out on formalin-fixed, paraffin-embedded samples (FFPE) [1]. Moreover, there is no analogous system of surface markers indicating clonality in T-cell tumors.

PCR methods for the detection of immunoglobulin clonality are based on the detection of the DNA rearrangement of the immunoglobulin heavy locus (IGH) gene during the somatic recombination from variable (V_H), diversity (D_H), and junction (J_H) gene segments. In all, 46-52 functional V_H segments (depending on the individual haplotype) have been identified, which can be grouped according to their homology in six or seven V_H subgroups. In addition, approximately 30 nonfunctional V_H segments have been described. Furthermore, 27 functional D_H segments and six functional J_H segments have been consistently found [2].

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V(D)J recombination is essential for functional expression of immunoglobulin receptor (IgR) and is critical for generating antigen recognition. Further variability is introduced by random insertion of nontemplated nucleotides at the V-J and D-J junctions and somatic hypermutation. These rearrangements can be exploited as markers for B and T-cell clonality and can distinguish a reactive (polyclonal) from neoplastic (monoclonal) proliferation.

To be able to work with highly degraded DNA extracted from FFPE sections, the method exploits the properties of V_H segments which contain three framework (FR) and two complementarity-determining regions (CDRs). The FRs are characterized by their similarity among the various V_H segments, whereas the CDRs are highly different even within the same V_H family. Furthermore, the CDRs represent the preferred target sequences for somatic hypermutations in the course of the germinal center reaction, which increase the variability within those regions. FR regions are usually less affected by somatic mutations and therefore, they are the targets for primer annealing. PCR-based strategies for the detection of clonal B-cell populations in histological sections and cell suspensions have already been established in the early 1990s. However, the initial PCR protocols used single VH and JH consensus primers, which were able to bind to one of the three FR regions. Such consensus primers were not suitable to amplify all VH segments with the same efficiency leading to nondetectability of a significant number of clonal rearrangements. The BIOMED-2 protocol is original in its comprehensiveness of primer sets [3], which have the unprecedentedly high detection rate. This is mainly based on the complementarity of the various combinations concerning the primers. In particular, combined application of IGH (VH-JH and DH-JH), IGK and IGL primers in separate tubes can detect virtually all clonal B-cell proliferations, even in B-cell malignancies with high levels of somatic mutations. The analytical phase after PCR includes heteroduplex analysis (HDA) consisting of denaturation and subsequent reannealing of PCR fragments followed by PAGE [3].

The aim of lab-on-a-chip or microfluidics technology is to shrink processes, in this case chemical and analytical, to very small dimensions, thus allowing to handle very little sample volumes. The Agilent 2100 Bioanalyzer, which was developed in collaboration with Caliper Technologies (Mountain View, USA), is the first fully commercialized implementation of microfluidics technology to date [4, 5]. The method uses smaller volumes of PCR products than HDA-PAGE and avoids laborious casting of gels and working with toxic liquid acrylamide.

In our approach, we have been comparing the results of microfluidic chip electrophoresis to HDA-PAGE in order to find an alternative, which can be offered by a miniaturized electrophoresis technique for rapid and automated analysis of DNA on a chip.

MATERIAL AND METHODS

Patient samples

Patient s samples were obtained at the Department of Pathological Anatomy, University Hospital in Martin, Slovakia. We used three FFPE samples (samples 1, 2 and 3) of unknown clonality obtained from patients with chronic lymphocytic leukemia (CLL) and one FFPE sample (positive control, PK) from lymph node of patients with CLL previously confirmed as clonal by immunohistochemistry.

DNA extraction

DNA was extracted by using commercial available DNA isolation kit Wizard DNA purification kit (Promega, USA) according to the manufacturer s instructions. After DNA isolation, quality and quantity of DNA was assessed. DNA quantity was measured using spectrophotometer. Quality was measured visually by standard agarose gel electrophoresis.

Multiplex PCR protocol and heteroduplex analysis

PCR was prepared according to standardized BIOMED-2 protocol [3]. Each sample was analyzed in three multiplex PCR reactions. The first PCR reaction consists of seven primers

from the FR1 binding to all seven V_H sequence families. The second PCR reaction consists of 7 forward primers from the FR2 and the third 7 primers from the FR3, all binding to all seven V_H sequence families. All PCRs had one consensus reverse primer binding to J segment $J_{cons.}$ The PCR was performed in 50 l. For the preparation of reaction mix were used nuclease free water (35.6 µl), 200µM dNTP (1 µl), 10x buffer without Mg^{2+} (5 µl), 25mM $MgCl_2$ (5 µl), 10 pmol VHFR1-3 primers, 10 pmol Jcons, and 2 U Taq polymerase (0.4 µl) and 100 ng of template DNA. The PCR conditions were as follows: denaturation at 95°C, 10 min., 35 times cycling at 95°C, 45 sec., 60°C, 1 min. 72°C, 1:30 min. The last extension was at 72°C for 10 min. Immediately after PCR, the HDA was performed by denaturation at 95°C, 5 min. and reannealing at 4°C, 60 min. The samples were loaded on 6% PAGE and electrophoresis was running typically 2 hours at 180-200V. Finally, the gel was stained with ethidium bromide for visualization of individual PCR products and evaluated on UV transilluminator. The sample was considered as positive when at least 2 frameworks showed positive signals: IGH FR1 within ranges of 310-360bp, IGH FR2 250-295bp, and IGH FR3, 100-170bp). As molecular weight standard, 50 bp ladder (Fermentas, Germany) was used.

DNA chip analysis on Agilent 2100 Bioanalyzer

For DNA chip preparation was used Agilent DNA 1000 kit (Agilent technologies, Germany) following instructions of the manufacturer. Briefly, Gel-Dye Mix has been prepared by mixing DNA dye concentrate and DNA gel matrix, which was loaded into the DNA 1000 chip. Kit marker has been loaded into the all 12 sample wells and ladder well. Finally, 1µl PCR product of each tested sample was loaded on chip Agilent DNA1000 chip kit. Prepared DNA 1000 chip was vortexed and had to run on Agilent 2100 bioanalyzer for 30 min.

Data were analyzed using the software Agilent 2100 Expert (Agilent technologies, Germany) which automatically provides the size of peaks and their quantification. The sample was considered positive if at least two peaks were identified in one sample within the expected range (IGH FR1, 310-360bp; IGH FR2, 250-295bp; IGH FR3, 100-170bp).

RESULTS

Clonality detection by PAGE

Using HDA with subsequent PAGE, we were able to confirm the clonality of the positive control (Fig. 1, line PK). Moreover, all samples from the CLL patients were shown to be clonally positive in all frameworks tested (Fig. 1, lines 1, 2 and 3). The PCR products achieved

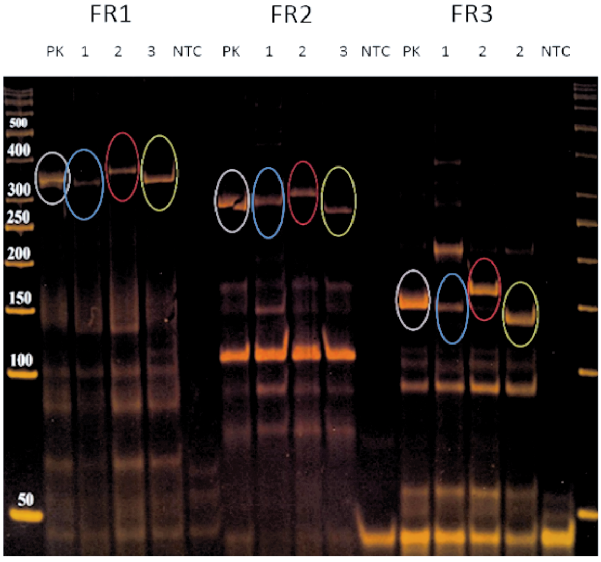

Fig. 1 6% PAGE of samples 1, 2 and 3 showed positivity for clonality testing in all three tested frameworks with expected sizes of the PCR products (FR1 - 310-360bp, FR2 - 250-295bp, FR3 - 100-170bp) shown in color circles (blue sample 1, red sample 2, yellow sample 3), PK - positive control, NTC – negative template control.

for FR1, FR2 and FR3 in the expected sizes defined by the position of primers which means 360-310bp, 295-250bp and 170-100 bp, respectively.

Detection of clonality by microfluidic chip electrophoresis

Gel-like images of the microfluidic chip electrophoresis have shown clonal products in all three tested cases and in all frameworks tested with expected sizes (Fig. 2). However, the samples in line 1 have certain polyclonal features and seems to be boundary. The software of the Agilent 2100 Bioanalyzer enables the imaging in two different forms, as a standard gel-like image with PCR products as bands (Fig. 2) and the PCR products depicted as peaks as well (Fig. 3) which is helpful for the final evaluation.

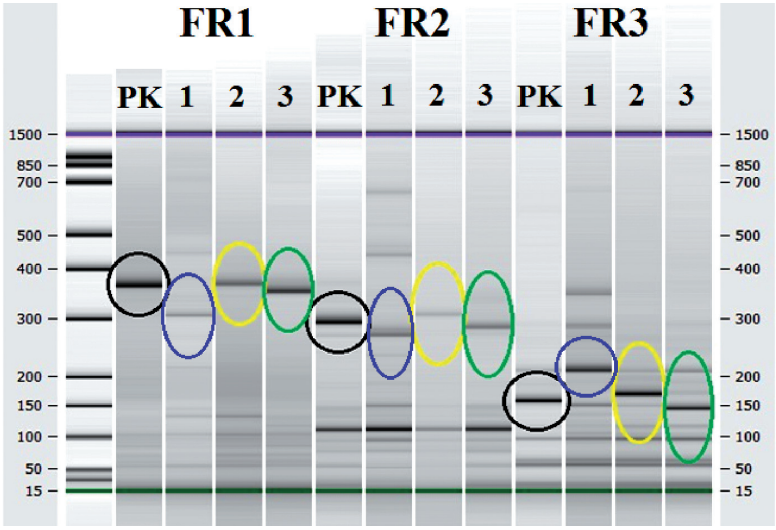

Fig. 2 Gel-like image from the microfluidic electrophoresis of the tested samples. Positive control (PK) in black circle, samples 1, 2 and 3 in blue yellow and green circles, respectively.

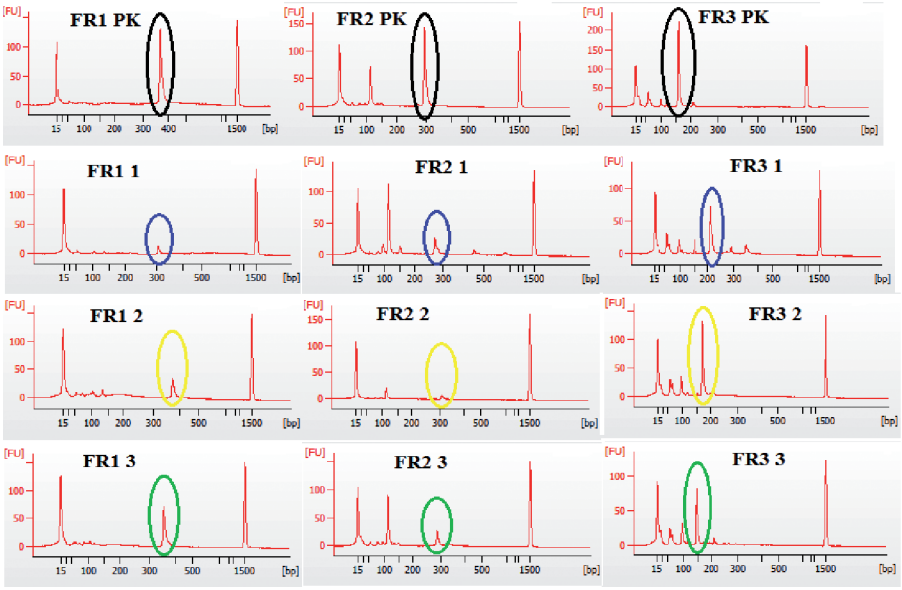

Fig. 3 Electropherograms showing the samples and positive control as peaks comprising all 3 frameworks, visualized by Agilent 2100 Expert Software.

DISCUSSION

In our work, we implemented the microfluidic chip based electrophoresis for the detection of IGH clonality which has proven to be reliable method for clonality detection compared to the original protocol BIOMED-2 that has been using HDA with subsequent PAGE. When we compared this two methods, all our samples were clonal, however, one sample from the chip electrophoresis exhibits polyclonal features. The reason can be the higher sensitivity of the microfluidic approach. The advantages of the microfluidic chip electrophoresis and thus the 2100 Bioanalyzer system are the minimal amount of the used sample and less time-consumption compared to the PAGE. The microfluidic chip-based gel-like images and electropherograms are easy to interpret, however, we have to test more samples, to see also samples with low-intensity bands. The IGK and IGL testing will be involved in the future as well. Very useful advantage of this system is the automatized analysis process, easy evaluation, comparing and archiving of digitally stored data. The user has access to primary data for further manual or automatized processing either in terms of electropherogram (peaks representation) (Fig. 1C and D) or in terms of gel-like imaging (Fig. 1B). Comparison of the results between chips is also possible by the software. In conclusion it can be noted that the method implemented on the device for microfluidic chip electrophoresis is reliable for testing cases with dominant PCR products and gives the same results as the HDA-PAGE method. Therefore, we can say that both of methods are sufficient for clonality detection, however, in microfluidic chip electrophoresis is quality of detection directly proportional to price and inversely to preparation time and run of analysis itself. Vice versa, in case of HDA-PAGE is price lower and time is several times higher. The sensitivity of the chip electrophoresis remains to be validated.

Here we implemented the lab-on-chip technology for clonality testing. In the future, we plan to validate this methods on more samples and test its sensitivity compared to HDA-PAGE so that this method could become routine in clinical settings.

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